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# Salt stress responses in pear and quince: physiological and molecular aspects

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*To people I love most:*
*Stefano, mum and dad*

*"……..For almost all of us, one of the reasons that we liked science in high school and college is that we were good at it. That can't be the only reason - fascination with understanding the physical world and an emotional need to discover new things has to enter into it too. But high-school and college science means taking courses, and doing well in courses means getting the right answers on tests. If you know those answers, you do well and get to feel smart.*

*A Ph.D., in which you have to do a research project, is a whole different thing. For me, it was a daunting task. How could J possibly frame the questions that would lead to significant discoveries; design and interpret an experiment so that the conclusions were absolutely convincing: foresee difficulties and see ways around them, or, failing that, solve them when they occurred? My Ph.D. project was somewhat interdisciplinary and, for a while, whenever I ran into a problem, I pestered the faculty in my department who were experts in the various disciplines that l needed. I remember the day when Henry Taube (who won the Nobel Prize two years later) told me he didn't know how to solve the problem I was having in his area. I was a third-year graduate student and I figured that Taube knew about 1000 times more than I did (conservative estimate). If he didn't have the answer, nobody did.*

*That's when it hit me: nobody did. That's why it was a research problem. And being my research problem, it was up to me to solve. Once I faced that fact, I solved the problem in a couple of days. (It wasn't really very hard; I just had to try a few things). The crucial lesson was that the scope of things I didn't know wasn't merely vast; it was, for all practical purposes, infinite. That realization, instead of being discouraging, was liberating. If our ignorance is infinite, the only possible course or action is to muddle through as best we can.*

*[…..] we don't do a good enough job of teaching our students how to be productively stupid –that is, if we don't feel stupid it means we're not really trying. [….] Science involves confronting our "absolute stupidity".*

*Productive stupidity means being ignorant by choice. Focusing on important questions puts us in the awkward position of being ignorant. One of the beautiful things about science is that it allows us to bumble along, getting it wrong time after time, and feel perfectly fine as long as we learn something each time. No doubt, this can be difficult for students who are accustomed to getting the answers right No doubt, reasonable levels of confident and emotional resilience help, but I think scientific education might do more to ease what is a very big transition: from learning what other people once discovered to making your own discoveries. The more comfortable we become with being stupid, the deeper we will wade into the unknown and the more likely we are to make big discoveries."*

*From "The importance of stupidity in scientific research"*
*by Martin A. Schwartz*
*Journal of Cell Science, 121, 2008.*

# GENERAL INDEX

## Chapter 4: MATERIALS AND METHODS *A LONG-TERM EXPERIMENT*



## Chapter 5: RESULTS AND DISCUSSION *A SHORT-TERM EXPERIMENT*

# *ABSTRACT*

*The productivity of agricultural crops is seriously limited by salinity. This problem is rapidly increasing, particularly in irrigated lands. Like almost all the fruit tree species, Pyrus communis is generally considered a salt sensitive species, but only little information is available on its behavior under saline conditions.*

*Previous studies, carried out in the Department of Fruit Tree and Woody Plant Science (University of Bologna), focused their attention on pear and quince salt stress responses to understand which rootstock would be the most suitable for pear in order to tolerate a salt stress condition. It has been reported that pear and quince have different ability in the uptake, translocation and accumulation of chloride ($Cl^-$) and sodium ($Na^+$) ions, when plants were irrigated for one season with saline water (5 dS/m).*

*The aim of the present work was to deepen these aspects and investigate salt stress responses in pear and quince. Two different experiments have been performed: a "short-term" trial in a growth chamber and a "long-term" experiment in the open field.*

*In the short-term experiment, three different genotypes usually adopted as pear rootstocks (MC, BA29 and Farold®40) and the pear variety Abbé Fétel own rooted have been compared under salt stress conditions. The trial was performed in a hydroponic culture system, applying a 90 mM NaCl stress to half of the plants, after five weeks of normal growth in Hoagland's solution. During the three-weeks of salt stress treatment, physiological, mineral and molecular analyses were performed in order to monitor, for each genotype, the development of the salt stress responses in comparison with the corresponding "unstressed" plants. Farold®40 and Abbé Fétel own rooted showed the onset of leaf necrosis, due to salt toxicity, one week before quinces. Moreover, quinces displayed a significant delay in premature senescence of old leaves, while pears emerged for their ability to regenerate new leaves from apparently dead foliage with the salt stress still running. Physiological measurements, such as shoots length, chlorophyll (Chl) content, and photosynthesis, have been carried out and revealed that pears exhibited a significant reduction in water content and a wilting aspect, while for quinces a decrease in Chl content and a growth slowdown were observed.*

*At the end of the trial, all plants were collected and organs separated for dry weight estimation and mineral analyses (Cu, Fe, Mn, Zn Mg, Ca, K, Na and Cl). Mineral contents have been affected by salinity; same macro/micro nutrients were altered in some organs or relocated within the plant. This plant response could have partially contributed to face the salt stress. Leaves and roots have been harvested for molecular analyses at four different times during stress conditions.*

*Molecular analyses consisted of the gene expression study of three main ion transporters, well known in Arabidopsis thaliana as salt-tolerance determinants in the "SOS" pathway: NHX1 (tonoplast $Na^+/H^+$ antiporter), SOS1 (plasmalemma $Na^+/H^+$ antiporter) and HKT1 ($K^+$ high-affinity and $Na^+$ low-affinity transporter). These studies showed that two quince rootstocks adopted different responsive mechanisms to NaCl stress. BA29 increased its $Na^+$ sequestration activity into leaf vacuoles, while MC enhanced temporarily the same ability, but in roots. Farold®40, instead, exhibited increases in SOS1 and HKT1 expression mainly at leaf level in the attempt to retrieve $Na^+$ from xylem, while Abbé Fétel differently altered the expression of these genes in roots. Finally, each genotype showed a peculiar response to salt stress that was the sum of its ability in $Na^+$ exclusion, osmotic tolerance and tissue tolerance.*

*In the long-term experiment, potted trees of the pear variety Abbé Fétel grafted on different rootstocks (MC, BA29 and Farold®40), or own rooted and also rootstocks only*

*were subjected to a salt stress through saline water irrigation with an electrical conductivity of 5 dS/m for two years. The purposes of this study were to evaluate salinity effects on physiological (shoot length, number of buds, photosynthesis, etc.) and yield parameters of cultivar Abbé Fétel in the different combinations and to determine the salt amount that pear is able to tolerate over the years. With this work, we confirmed the previous hypothesis that pear, despite being classified as a salt-sensitive fruit tree, can be cultivated for two years under saline water irrigation, without showing any salt toxicity symptoms or severe drawbacks on plant development and production. Among different combinations, Abbé Fétel grafted on MC resulted interesting for its peculiar behaviors under salt stress conditions.*

*In the near future, further investigations on physiological and molecular aspects will be necessary to enrich and broaden the knowledge of salt stress responses in pear.*

# Chapter 1: INTRODUCTION

## 1.0 Pear and quince: origin and taxonomy

The genus *Pyrus* found its origin between 65 and 2 millions of years ago in the mountainous land where nowadays is located the west part of China. From that site, this genus spread out in all directions, adapting to different climatic and soil conditions. In this way genus *Pyrus* differentiated in all known present species.

The original centers of *Pyrus* were: China (*Pyrus pyrifolia, Pyrus ussuriensis* and *Pyrus calleryana*), Middle East (around Caucasus region, main location for *Pyrus communis*) and central Asia (*Pyrus communis* crossed with *Pyrus heterophylla* and *Pyrus communis x Pyrus bretschneideri*).

The *Pyrus* genus is placed in the Rose family (Rosaceae), subfamily Pomoideae along with apple (*Malus domestica*) and quince (*Cydonia oblonga*). Pomoideae have 17 chromosomes and all species belonged to genus *Pyrus* are diploids (2n=34) and only some cultivars of *P. communis* are known to be polyploids (Fideghelli, 2007).

The genus *Pyrus* is composed of about 22 species, found in Asia, Europe, and northern Africa.

Two major species are commercially cultivated:

- Asian pears: the most famous species with commercial importance belonging to this group is *Pyrus pyrifolia*, also called "Japanese" or "Oriental" pear, or "Nashi". Grown mostly in the Orient, this fruit, more similar to apple, has been increasing its popularity also in USA. Asian pears are the sweet-fleshed fruits of *Pyrus pyrifolia, P. bretschneiderii*, and *P. ussurienses* and they are round, aromatic, and crisp-fleshed.
- European pears: the most representative of this group is *Pyrus communis*, this species possibly derives from two wild species *P. caucasia and P. nivalis*. This is the most important commercial pear in Europe. The European pear was described by Homer as one of the fruits in the garden of Alcinöus as a “gift of the gods”. Pomona, goodness of fruit, was a member of Roman Pantheon and Roman farmers and some agricultural writers: Cato, Varro, and especially, Pliny documented many different types of pears, pear growing and grafting techniques

(http://www.usapears.com/pears/history.asp; http://www.uga.edu/fruit/pear.html; Janick, 2005; Bellini and Nin, 2007).

Later in the history, pears reached also the new world with the early colonists on the east coast and then they were spread in west direction with pioneers in 1800's. Systematic breeding of European pear started in 1800's mainly in France, Belgium and England; in fact the first early promoter of breeding in plant was Jean Baptiste Van Mons (1765-1842). He systematically collected clones of pear, planted seed of the best material and made selections for eight generations; at the end he noised about over 400 cultivars. Sexual hybridization to produce new pear cultivars (as well as apple, cherry, strawberry, plum, and nectarine) was first attempted by Thomas Andrew Knight (1759-1838) that was not so famous but for some topics the found the same result of Mendel but his papers, with experimental results, disappeared after his death (Janick, 2005).

*Pyrus communis* in its evolution crossed with several Asiatic and European species: *P. elaeagrifoglia, P. salicifolia, P. syriaca, P. nivalis, P. caucasia.* Between *Pyrus communis* species exists also a variety called pyraster (wild pear); it is present in south Italy and historically it was retained (by Linneo) as a promising parent for future evolution of cultivars. It is used as rootstock for *Pyrus communis* for its high affinity and good adaptability (Fideghelli, 2007).

*P. communis* is a vigorous tree that can be tall until 20 m, but tree size is heavily dependent on rootstock and training system adopted. The trees require an annual period of chilling to break dormancy for proper resuming growth, and the seeds require a period of cold stratification before they will germinate. *P. communis* has elliptic-ovate leaves with acute tip, with finely serrate margin and green bright color. Its inflorescence is corymbose, containing 4-12 white flowers with longer pedicels than apple flowers. The genus *Pyrus* is characterized by a high self incompatibility, so most cultivars require cross pollination to obtain commercial fruits; honey bees are the main natural pollinator. Its fruits are defined "pome", like for apples and the fleshy edible part is derived from hypanthium tissue (false fruit). In addition, fruit can be medium or big and the shape is quite different depending on the cultivar, but generally all European pears are pyriform, while Asian pears are round. The flash can be from crisp to melting, color cream-white with some

exceptions and it frequently contains grit cells (called brachysclereids) that are lignified cells that confer the European pear flesh texture.

The genus *Cydonia* encloses only one member: the species *oblonga* (quince) that belongs to Rose family (Rosaceae) along with apple and pear and it was originally cultivated for its fruits. *Cydonia oblonga* is one of the oldest fruit tree known, already 4,000 years ago by Babylonian people, for its fragrant fruit and attractive pink flowers. Greeks considered quince as the holy fruit for Venus and some Roman authors mentioned it in some writings. *Cydonia oblonga* found its origins in Middle East and in Caucasus region and nowadays it is spread in Mediterranean area and in China. It is still an important fruit crop in its native region and in South America (Argentina produces 20,000 tons per year). However, quinces are more essential as rootstocks than as fruiting plants, in fact *Cydonia oblonga* is the primary dwarfing rootstock adopted for pear, mostly in Europe. Since quince is incompatible with many pear scions, a compatible interstock must be placed between the scion and a quince rootstock. Because it is not cold-hardy, quince is a suitable rootstock for pears only where winter injury is not so easy to occur (www.uga.edu/fruit/index.html; http://it.wikipedia.org).

### 1.0.1 Pear production and principal varieties

The world pear production is growing and in 2004, it reached 17.9 million of tons. FAO statistics show how China is the main producer with 10 million of tons in 2004 equals to 57% of global production. In the world there are 3 principal areas where pears are mostly cultivated: China for the east part, Italy for the Europe and USA for the new world (Fig. 1-1). Some countries, belonging to these main producer areas, assign almost the total pear productivity to export and in this way they became giants in the world market (for instance Chile and Argentina).

European pear production is maintained stable around 2.5 millions of tons, even if in 2008 a decrease can be expected. In Europe, importations from extra UE are almost double in comparison with exportations which amount only 6.5% of total tons produced. And in particular, Italy, even if it is the main pear producer in European Community, finds difficult to export 16,000 t; that are equal to 17% of total national production. Italian exportations are mainly addressed to other

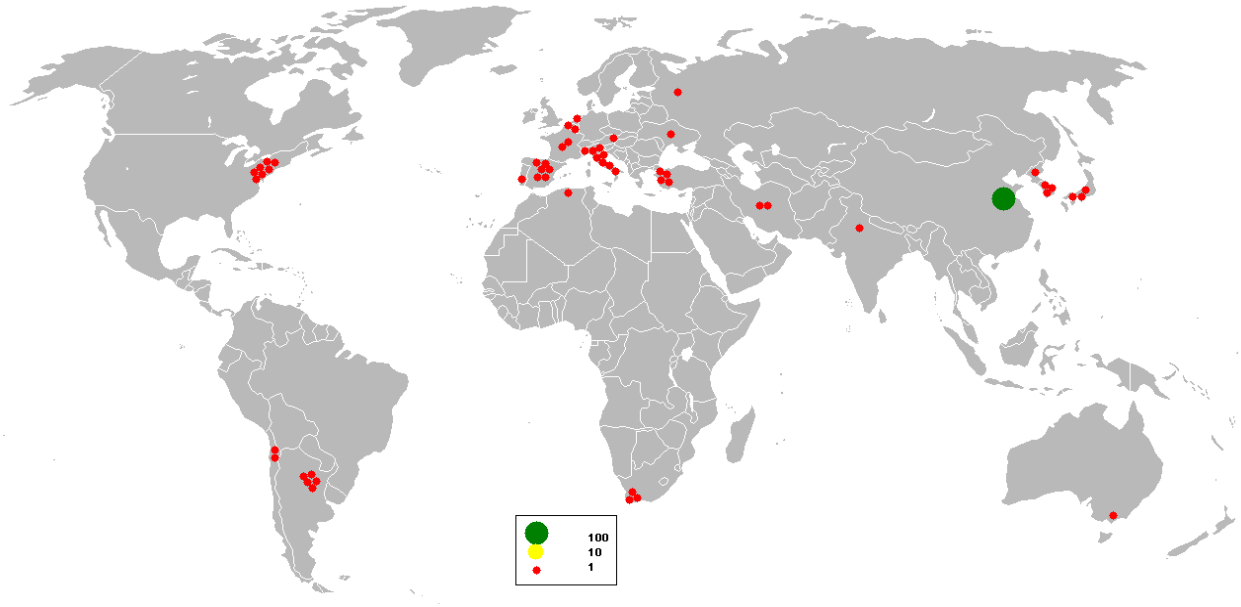



**Fig. 1-1: Main locations in the world of pear and quince production in 2005 (shown as percentage of the top producer; China 1,153,700 tons).**

European countries (92%) and only 7% to extra UE countries. (Miotto, 2005; Trentini et al., 2008).

In the past, European pears varieties were numerous (about 100), but nowadays only few of them are accomplished and well distinguished. In Europe, only eight varieties, and related mutants represent about 80% of the entire European yield: Conference has just over 30%, William and its red mutants have 14%, Abbé Fétel 12% (Fig. 1-2).

And the main UE countries where pear is cultivated are: Italy, which by itself corresponds to a quarter of the entire European pear dedicated area; Spain,

| | Europa % | | | Italia % | | |
|---|---|---|---|---|---|---|
| | 99-00 | 03-04 | 06-07 | 99-00 | 03-04 | 06-07 |
| Conference | 24,5 | 30,5 | 31,6 | 16,2 | 14,8 | 13,6 |
| Bartlett (William) | 12,3 | 13,1 | 13,1 | 20,5 | 20,8 | 21,0 |
| Max Red Bartlett | 1,3 | 1,2 | 1,2 | 3,5 | 3,2 | 3,1 |
| Abate Fétel | 9,7 | 10,9 | 12,9 | 27,3 | 30,8 | 34,6 |
| Blanquilla - Spadona E. | 9,6 | 7,8 | 5,4 | - | - | - |
| Decana del Comizio | 5,1 | 4,9 | 5,1 | 7,0 | 6,1 | 5,6 |
| Coscia - Ercolini | 4,8 | 4,2 | 4,2 | 6,4 | 6,3 | 6,7 |
| Dr. J. Guyot | 5,1 | 4,5 | 4,2 | 0,7 | 0,5 | 0,3 |
| Kaiser (B. Bosc) | 2,2 | 2,5 | 2,4 | 6,1 | 6,8 | 6,5 |
| Passa Crassana | 1,6 | 1,5 | 1,2 | 2,3 | 1,8 | 0,9 |
| Altre | 23,9 | 19,1 | 18,7 | 9,9 | 8,9 | 7,8 |
| **Totale (000 t)** | **2351,0** | **2301,0** | **2471,5** | **831,0** | **829,5** | **922,0** |

Fonte: Eurofel 2007

**Fig. 1-2: Pear yield trend in Europe (15 countries) and separately Spain and Italy (modified from Sansavini and Ancarani, 2008).**

Belgium, France and Holland and, as last, Portugal (Sansavini, 2006; Sansavini and Ancarani, 2008). From data of seven years (period 2000-2007) on European pears' trends, it is clear how William has been stable; Conference has increased as Abbé Fétel that is mainly produced in Italy (300,000 tons).

The Italian pear varieties selection is divided in two main groups based on maturation time of fruits, in:

- Summer cultivars: Coscia, Santa Maria, William and Red William (also known as Max Red Barlett).
- Autumn/winter cultivars: Conference, Abbé Fétel, Decana del Comizio, Kaiser and Passa Crassana (Bellini and Natarelli, 2007).

Nowadays, in Italy, the pear crop is concentrated for 75% in Emilia-Romagna, Veneto and Lombardia regions, because this species finds there suitable climatic and soil conditions and, additionally, the presence of proper structures and services for fruit conservation and commercialization. In particular, Emilia-Romagna, leading pear area in Europe, produces more than 600,000 tons yearly (2005) with an average yield of 24 t/h (Musacchi, 2008) and equal to 68% of national yield (Musacchi et al, 2006b).

Abbé Fétel is a valuable cultivar that increased its importance in the last 5 years; this surge is due to the rise of new plantings (peaks of over 40 t/h with new high density plantings), but also to the decrease of productive potential of other varieties such as: Kaiser, Decana, Max Red Barlett, Passa Cressana and Conference (Fig. 1-3; Mazzotti and Miotto, 2006).

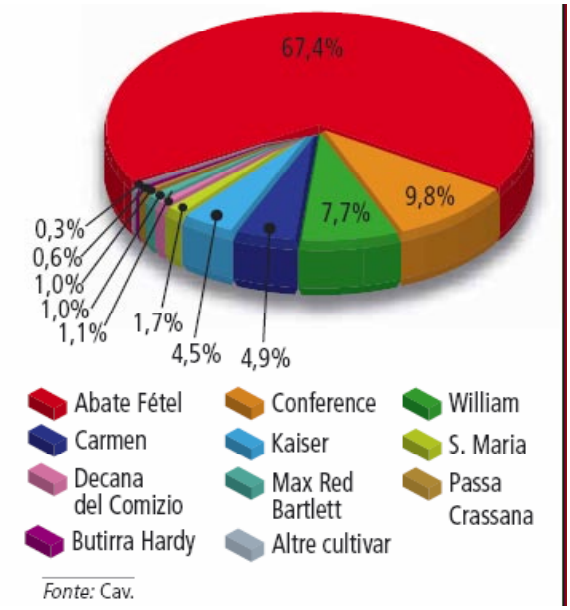



**Fig. 1-3: Production of certified plants in Emilia-Romagna (2007) divided for varieties (from Musacchi, 2007a).**

Abbé Fétel was identified in France by the homonym monk Abbé Fétel and spread in 1876. This tree is quite vigorous with elevated yield and, being self incompatible, needs to be pollinated by cvs. Coscia, Butirra Hardy, Kaiser and Passa Crassana.

Another recent trend, mostly connected with Abbé Fétel choice, is the progress towards intensive plantings with densities of 10-13000 trees/ha; and for this purpose Abbé Fétel is generally grafted on quinces (Sydo, BA29 and MC). In the particular case of Abbé Fétel on MC the use of intermediate Butirra Hardy has been suggested to avoid any possibility of graft disaffinity (Musacchi, 2007a; Musacchi, 2008).

The commercial harvest time for Abbé Fétel is usually the first ten days of September, and, at that time, fruits are not physiologically ripen yet, but in this way can they be conserved for several months (8-10). The fruit is quite big (about 250 g), with a characteristic oblong shape (Fig. 1-4), elongated basal portion and a bulbous end; the skin is green-yellowish with russet areas and sometimes a bit red colour only in fruits best exposed to light. The flesh is white, melting, very juicy, sugary and aromatic with fine texture and a good taste very appreciated in Europe (Bellini and Natarelli, 2007).

**Fig. 1-4: Pear Abbé Fétel**

### 1.0.2 Overview on graft

The origin of grafting technique can be found in ancient times thanks to Chinese people (Liu, 2006). Grafting is an asexual propagation technique, widely adopted for fruit trees, that aims to encourage two different parts of plant to become a unique individual. The new plant is composed by an upper part known as “scion” that will provide the foliage and a lower part termed “rootstock” chosen for its performance as roots system. The grafting point constitutes the physically weakest point of the new plant, because sometimes it happens that vascular tissues do not fuse together and in that position plant may break. Sometimes in order to bypass the onset of incompatibility between the two parts of the graft, a third biont (interstem or interstock) is placed in-between the others two (Baldini, 1986).

Nowadays, grafting technique is largely adopted in most of the fruit trees species in order to obtain defined agronomic purposes and/or economical advantages.

For *Pyrus*, nowadays over 95% of trees are grafted (Musacchi, 2007b).

Some of the most important goals for grafting are reported in succession:

- The possibility to keep under control the vigor of the tree, because graft shifts plant equilibrium from vegetative to reproductive, providing an early bearing with a increase in yield.
- The need to contain the vigor of the tree adopting dwarfing rootstocks; for peculiar traits of these stocks, the volume of the scion is reduced.
- The presence of different environmental or soil conditions that may represent a problem for some crops.
- To reduce the juvenile period that characterizes seedlings fruit trees.
- To improve fruit quality of the chosen cultivar (Baldini, 1986).

In general, the probability of grafting success is proportional to the botanic similarity between the two bionts, but there are some exceptions like pear that prefers quince as rootstock instead of apple (Baldini, 1986, Musacchi's phD thesis, 1996). Many different kinds of grafting techniques exist and the choice among them depends on: the type of scion material available to use and its developmental stage (young or lignified), the time of grafting (spring or winter) and the purpose.

### 1.0.3 Pear rootstocks

Three main species dominate the pear-rootstock scene:

A. *Pyrus calleryana* shows a good anchorage to soil and is resistant to fire blight, but is sensible to cold and lime-chlorosis; but overall it increases production efficiency and is compatible with European pears and Nashi.
B. *Pyrus communis* is rustic and vigorous or semi-vigorous and so less suitable for intensive pear orchards; instead clonal *Pyrus* rootstocks are less vigorous.
C. *Cydonia oblonga* induces less vigor than pear rootstocks, being more suitable for high density plantings, but the main risk is the onset of graft incompatibility, because not all pear cultivars are compatible with quince. Additionally some other drawbacks are the limited winter hardiness and the susceptibility to lime-chlorosis in the case of calcareous soils, which renders plant unprofitable without any treatments (Campbell, 2003).

### 1.0.3.1 Quinces

The modern Italian pear crop found in quince the main rootstock to adopt in medium-high density plantings (from 3,000 to 13,000 trees/ha), because it is able to reduce the size of trees (< 3-4 m height), promote the earlier bearing and improve the pear quality in comparison with fruits obtained by plants grafted on seedlings, more diffused in the past in Italy. Quince clonal rootstocks were selected mainly in England and France and, nowadays, are available several clonal lines with peculiar characteristics that render a quince suitable for a particular soil or environmental conditions or for a specific training system (Wertheim, 1998; Campbell, 2003; Sansavini 2007; Musacchi, 2008).

In Europe, quinces are chosen as rootstock for 92%, on the contrary in North America are most used seedling, pear hybrids and some Asiatic cultivars as *P. calleryana.* The most adopted quinces rootstocks (also chosen for our experiments) are reported following in a decreasing order related to the plant vigor induced by the rootstock:

- BA29

  It is a French selection (INRA-1963) that had a great success in Italy in '80-'90s for its rusticity and easy propagation way; it shows a good adaptability to different and poor soils (clay or not so fertile) and a lower susceptibility to chlorosis (Fig. 1-5). In France, plants grafted on BA29 showed a bigger vigor than on Sydo and MA (not the same in Italy) and were more productive also because this rootstock does not show any symptoms of graft incompatibility. In addition it has been recommended for naturally weak-growing and precocious cultivars as Passa Crassana (Jackson, 2003). Some drawbacks of BA29 are: too vigorous in fertile soil, lack of precocity, low tolerance to infective diseases such as viruses and phytoplasms and mildly sensible to fire blight. In 2007, nursery industry in Emilia-Romagna produced about 583000 BA29 trees and it represents the second most required rootstock only after Sydo (Musacchi, 2007a; Sansavini, 2007; Colombo and Bolognesi, 2008). Actually, BA29 is adopted with cv. William with interstock and it has been suggested for low-medium density plantings, in particular with Abbé Fétel, trained to the slender spindle or palmette or Bibaum® systems. Abbé Fétel

grafted on BA29 produces fruits with an average weight bigger than with others combinations. In fact this combination, such as Abbé Fétel/Sydo, provides fruit defined "type A" that means they respect the standard features of Abbé Fétel (see paragraph 1.0.1) (Bolognesi and Colombo, 2008).

- MC

  It was selected and produced in virus-free form at East Malling (England) and it is the most dwarfing quince (Fig. 1-5) among all commercial ones and for this reason is the favorite for high density plantings trained to the hedgerow or double-hedge V-shape or Bibaum® systems (3,000-5,000 trees/ha). Plants grafted on MC have 20-40% vigor less than MA and they do not reach 2.5 m in height. It is characterized by a superficial root system that is threatened by soil managements and excess of temperatures (cold and hot), for this reason it is recommended to adopt a fertigation plan. It induces a really high yield, but sometimes the pear size decreases. With MC it is easier that incompatibility symptoms appear before than in other combinations and this can be a cause of the shorter "life" of an orchard grafted on MC. The great advantage linked to MC is that orchard management costs less than usual, because the tree size is smaller. Cultivars as Decana del Comizio and Conference can be directly grafted on MC without any problem; instead William and Kaiser needs an interstock in combination with MC. For Abbé Fétel the direct graft with MC is appropriate, but one possibility to improve the yield efficiency is to interpose, also in this case, the Butirra Hardy interstock. In general, during 2007, nursery industry in Emilia-Romagna produced about 417,000 MC trees (Musacchi, 2007a; Sansavini, 2007).

### 1.0.3.2 Pear clonal rootstocks

Pear clonal rootstocks sometimes are preferred to quinces, because they have a good tolerance to iron chlorosis; but in comparison they present a slow initial bearing and have some problems with propagation and vigor (Musacchi, 2008).

Some different series belong to pear clonal rootstocks:

- OHxF series

  The most successful pear clonal rootstocks in the world are the American hybrids OHxF produced in '60-'70 by crossing Old Home x Farmingdale both resistant to fire blight. In this group, in particular OHF (Farold®) 40, 69 and 87 are interesting:

  I. Farold®40 shows vigor higher than BA29 and Farold®69; it presents a slow initial bearing and a good yield with fruits of good size (Fig. 1-5). In addition it is resistant to *Erwinia amylovora* and tolerant to pear decline. It finds its application, in particular with William and Abbé Fétel in medium density plantings. In Emilia-Romagna it is the fifth kind of rootstocks more produced by nursery in 2007 with 68,500 trees.

  II. Farold®69 shows vigor similar to pear seedlings, good resistance to cold and fire blight, but yield efficiency lower than quince or pear seedlings.

  III. Farold®87 is the most vigorous between its series and its behavior is more similar to pear seedling. It is interesting in poor soils in combination with William and Abbé Fétel (Sansavini, 2007; Colombo and Bolognesi, 2008).

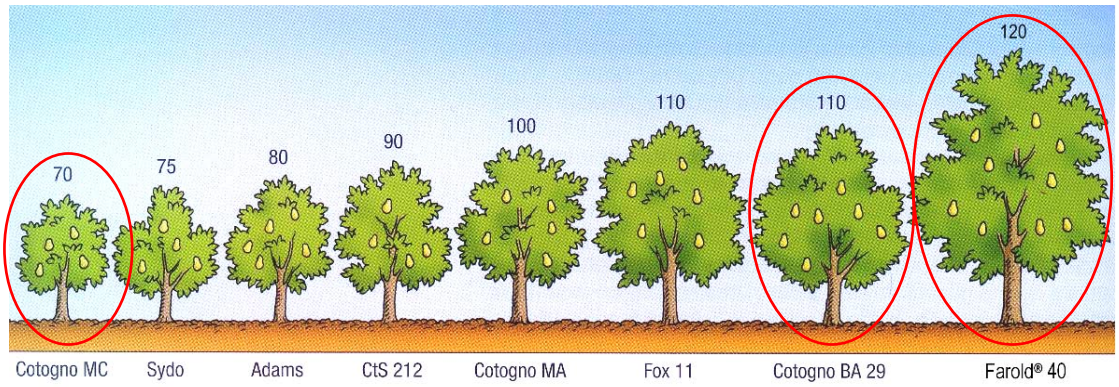



**Fig. 1-5: Plant vigour induced by the different rootstocks (quinces and pear clonal rootstocks). With red circles are underlined rootstocks chosen for this experiment. On the top level of vigor.**

#### 1.0.3.3 Own-rooted plants

The own-rooted plants are trees that are planted in the orchard without any grafting; they are produced "*in vitro*" via micropropagation. The choice of using these trees is connected to some advantages, such as: rusticity, tolerance to lime, absence of incompatibility and high productivity (not true for Decana del Comizio). There are otherwise some disadvantages like: the big size of the plant that implies a more expensive cost of management, and the long phase of juvenility. In general, during 2007, nursery industry in Emilia-Romagna produced about 82,700 Abbé Fétel own-rooted trees which are suitable for low density planting (< 2000 tree/ha) trained to palmette systems (Colombo, 2003).

## 1.1 Salinity in the modern agriculture

Salinity is one of the most severe environmental factors limiting the productivity of agricultural crops. The term *salt-affected* refers to soils that are saline or sodic, and these cover over 400 million hectares, spread in all the five continents, but mostly in dry areas. Desertification and salinization are growing rapidly worldwide with consequent reduction of more than 50% of the average productivity of major crops (Bray et al., 2000; Pitman and Läuchli, 2002).

About one fifth of the cultivated areas (19.5%), corresponding to 6% of the world lands, are affected by salinity, and this problem, which involves as well the irrigated areas, is gradually increasing; in fact other estimates are considerably higher and indicate that up to 50% of irrigated lands may be salt-affected. About 10 million hectares of farmland are abandoned each year as a result of salinisation and sodication and water saturation. Furthermore there is a deterioration of about 1% of agricultural lands due to salinity each year (Mahajan et al., 2008).

Sodicity is a derived consequence of salinity in clay soils, where leaching, through either natural or human-induced processes, has washed soluble salts into the subsoil, and left sodium bound to the negative charges of the clay (Pitman and Läuchli, 2002). Most of the soil salinity is of natural origin (primary salinity), but also human action should be considered as adding part in salinization process (secondary salinity; Tab. 1-1).

**Table 1-1: Global estimate of secondary salinisation in the world's irrigated lands.**

| Country | Total land area cropped Mha | Area irrigated | | Area of irrigated land that is salt-affected | |
|---|---|---|---|---|---|
| | | Mha | % | Mha | % |
| China | 97 | 45 | 46 | 6.7 | 15 |
| India | 169 | 42 | 25 | 7.0 | 17 |
| Soviet Union | 233 | 21 | 9 | 3.7 | 18 |
| United States | 190 | 18 | 10 | 4.2 | 23 |
| Pakistan | 21 | 16 | 78 | 4.2 | 26 |
| Iran | 15 | 6 | 39 | 1.7 | 30 |
| Thailand | 20 | 4 | 20 | 0.4 | 10 |
| Egypt | 3 | 3 | 100 | 0.9 | 33 |
| Australia | 47 | 2 | 4 | 0.2 | 9 |
| Argentina | 36 | 2 | 5 | 0.6 | 34 |
| South Africa | 13 | 1 | 9 | 0.1 | 9 |
| Subtotal | 843 | 159 | 19 | 29.6 | 20 |
| World | 1,474 | 227 | 15 | 45.4 | 20 |

Source: Ghassemi et al. (1995) compiled from FAO data for 1987
http://www.plantstress.com/Articles/index.asp

Primary Salinity

Primary or natural salinity arises as the accumulation of salts over long periods of time, through natural processes, in the soil or groundwater. It is the result of two natural processes:

- the weathering processes break down rocks and release soluble salts like sodium chloride (the most soluble salt), calcium, magnesium and minimally sulphates and carbonates.
- The deposition of oceanic salt carried in wind and rain; these salts are termed “cyclic salts” because they come from sea and are deposed in land by weather factors (Tab. 1-2). The rainwater decreases its salts content with the distance from the coast.

  The content of salts in the soil depends also on the soil type and composition, for instance sandy soils retain low amount of salts, and instead clay soils trap a high percentage, as mentioned above (Munns, 2005).

**Table 1-2: Concentration of salts in rain and seawater.**
The composition of rainwater from a northern hemisphere source (Encyclopaedia Britannica) and the composition of seawater is uniform around the globe.

| Ion | rainwater (local) | | seawater (global) | |
|---|---|---|---|---|
| | mg/kg (ppm) | (µmol/L) µM | g/kg (ppt) | (mmol/L) mM |
| Sodium ($Na^+$) | 2.0 | 86 | 10.8 | 470 |
| Chloride ($Cl^-$) | 3.8 | 107 | 19.4 | 547 |
| Sulfate ($SO_4^{2-}$) | 0.6 | 6 | 2.7 | 28 |
| Magnesium ($Mg^{2+}$) | 0.3 | 11 | 1.3 | 53 |
| Calcium ($Ca^{2+}$) | 0.1 | 2 | 0.4 | 10 |
| Potassium ($K^+$) | 0.3 | 8 | 0.4 | 10 |
| Total | 7.0 | | 35.0 | |

Secondary Salinity

The secondary or human-induced salinity is due to wrong cultural practices; in particular an irrational management of irrigation can increase the accumulation of salts in soil, making it less productive. In fact human activities alter the hydrologic balance of the soil between water from rainfall and water utilized by crops. The most frequent causes of this unbalance are:

- Land clearing and substitution of perennial crops (deep rooted) with annual ones (shallow-rooted).
- Irrigation scheme with insufficient drainage or/and using poor quality irrigation water.

Land clearing and irrigation change the natural water balance causing an excess of water that cannot be used by crops. This surplus of water makes the water table higher and this implies a consequent rise by capillarity of salts, formerly stored in subsoil, in the areas surrounding roots apparatus.

In this condition, crops use water trying to leave salts in the soil until the salt concentration become too high for plants to uptake water. In addition, water table continue to rise and water moves away from the ground for transpiration and evaporation (rather than normal percolation) and this happens especially in arid or semi-arid regions where the high temperatures facilitate this process and the scarcity of rainfall contributes to concentrate salts for lack of leaching. Consequently salts accumulate, resulting in the formation of a “salt scald” on the land surface. In some cases when the crop is unable to use all the applied water,

and the weather is not extremely dry, waterlogging can occur. In Australia, one of the most salt- affected countries in the world, 2 Mha of land have been damaged by rising watertables due to land clearing and other 15 Mha are subjected to salinization risk in the next years (Flowers and Yeo, 1995; Caliandro et al., 2000; Pitman and Läuchli, 2002; Tester and Davenport., 2003; Munns 2005).

Moreover, in many countries around the world, especially in areas with arid weather, with high rate of population growth, urbanization and industrialization, water is becoming a scarce resource, and this affects its supply for irrigation farming. Fresh water available to the world is 2.5% of the total and in the last years, the demand has been increased with the rise of population, so the water has become a more and more limited resource. The quantity of water available for agriculture is decreasing, so water of poor quality started to be used, such as saline waters, those that contain toxic elements and sediments. Good quality irrigation water has a concentration of NaCl less than 2 mM, otherwise this amount may increase significantly in the waters of low quality. The use of marginal quality waters can lead in the long term to a decrease in production and to soil deterioration, because it adds appreciable amounts of salts. It is calculated that in one year crops require 6,000-10,000 $m^3$ of water per hectare and using a 500 mg/L (salt) water, this can imply an addition of 3-5 tons of salt/ha. Moreover, solid fertilizers used in fertigation are highly dissociable salts; this practice generates an increase of electrical conductivity of soil solution (Shiklomanov, 1993; Levy and Syvertsen, 2004; Munns, 2005).

### 1.1.1 Salinity in Italy

In Italy, the most salt-affected soils are spotted around in semi-arid regions particularly in Sicily and in general in the southern part of Italy, and amounted to 450,000 ha, including potentially salt-affected soils. Irrigation with saline waters is applied in Sicily in many areas where these “poor” waters represent the only source of water available for irrigation (only 300 million $m^3$ of good quality water is available against the need of 1600 million $m^3$), causing secondary salinization and sodication. In semi-arid and dry sub-humid areas of southern Italy salinity is a result

of seawater intrusion as well as poor soil and water management practices in these areas (http://www.fao.org/ag/agl/agll/spush/topic2.htm 2000).

### 1.1.2 The effect of salinity on plants

The first effect of salinity on plants is the inhibition of growth (Tester and Davenport, 2003). It is caused by two factors:

1. The presence of salts in high concentrations in the soil leads to difficulties in the water uptake by roots for osmotic reasons, although water is not limiting. This aspect implies a reduction in growth rate (osmotic effect of salinity).
2. The excessive amount of salts enters into the transpiration stream and cause damage to the transpirating leaves, limiting their growth (ion-excess effect of salinity).

Since plants grown in saline soils suffer as in drought stress condition, because they cannot satisfy their water needs, also their mineral nutrition is strongly affected by the presence of salts in high concentrations. These salts interfere with the uptake of other important ions for the plant (i.e. $Ca^{2+}$ and $K^{+}$) for its efficient functioning. Salinity strongly interferes with normal physiological processes of the plant, causing an osmotic damage, linked to the decrease in the water potential and consequently the decrease of cell turgor, which leads to dehydration of the tissues until the death of the plant (Caliandro et al., 2000; Buchanan et al., 2003).

Another important consequence of the presence of high concentrations of $Na^{+}$ and $Cl^{-}$ in the land is the unbalance of ionic homeostasis within the plant, which absorbs through the roots, the largest salts concentrations, altering the normal functioning of cells. Adsorbed salts can lead to toxic damage for cells because if they are not compartmentalized into vacuoles, when free in the cytosol, are able to disrupt the cellular metabolism. The compartmentalization and adjustment of osmolites in the cytoplasm inside the plant cell are critical for achieving tolerance to osmotic stress, such as salt stress (Pitman and Läuchli, 2002; Zhu et al., 2002).

There are also secondary effects of salinity in plants that cannot be neglected, as:

- Disturbance of $K^{+}$ acquisition.

  Potassium is an essential element for plants and $Na^{+}$ competes with it for uptake, in particular when the external condition is high salt concentrated.

The perturbation of potassium uptake can influence several plant processes, because it is involved in maintaining water status, cell turgor pressure, controlling opening and closing of stomata and consequently is connected with photosynthetic activity. $K^+$ is also necessary for accumulation and translocation of synthesized carbohydrates as well as in cellulose synthesis (Mills and Benton Jones, 1997).

- Generation of reactive oxygen species (ROS).

  One aspect directly linked to salt stress in plants is the induction of ROS formation, such as superoxide radical ($O_2^-$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical (OH •). The ROS arise during the stress from an alteration in the metabolism of mitochondria and chloroplasts, as they are formed by a break in one step of $O_2$ reduction (Breusegem et al., 2001; Apse and Blumwald, 2002; Wang et al., 2003).
- Membrane dysfunction.
- Weakening of photosynthesis and other biochemical processes.
- Programmed cell death (PCD).

To face these effects on crop growth and physiology, there are two main strategies that can contribute to enhance yield stability of crops in saline soils. The first strategy, to be adopted, that needs to be supported by continuous research, is remediation of salinized soil. The remediation of these soils can be carried out by implementing large engineering scheme for irrigation and drainage, planting perennials to lower water tables, but also using plant growth-promoting bacteria which may demonstrate to provide a significant benefit to the plants to facilitate their growth in saline soils (Mayak et al., 2004; Botella et al., 2005).

The second approach can be complementary to the first one and it is based on increase salt tolerance of crop plants. This goal can be achieved with several approaches such as: classic breeding methods, traditional mutagenesis, molecular marker-assisted selection techniques and biotechnology. The limitation of the breeding approach is due to the fact that domestication has selected against the salt adaptation capacity, which is present in wild species or primitive cultivars of some current crops, but it is going to be lost in modern crops and, in addition, salt tolerance determinants seemed to be negatively linked to loci responsible for high

yield. The strategy to enhance salt tolerance is strictly connected with a deep comprehension of salt determinants and their cross-talks and interactions in salt stress response in plant (Hasegawa et al., 2000; Botella et al., 2005).

In conclusion, it is important to keep in mind that salinity tolerance is a complex phenomenon that involves several physiological processes that are under a multi-genic control. On the basis of many studies, there is sufficient evidence to be confident that salt tolerance is a multigenic trait determined by a number of sub-traits any of which can be set by several genes (Lovelock and Ball, 2002; Flowers, 2004).

### 1.1.3 Photosynthesis and salinity

The most evident response of plant to salinity is a dramatic decrease in stomatal conductance. This is due to the disorder in plant water status that occurs during drought such as in salt or cold stress (Verslues et al., 2006). Stomatal responses are for sure linked to the osmotic effect of salinity or drought outside the roots; in fact water deficiency implies partial stomatal closure, loss of cell turgor in the mesophyll with salt accumulation in the apoplast or its evident effect of toxicity. In addition the osmotic effect of salinity stimulates abscisic acid (ABA) production that can trigger a decrease in stomatal conductance, chlorophyll content and Rubisco activity (Ashraf, 2004). Salinity level and leaf area are usually inversely proportional and during rising salinity, water loss per plant decreases by transpiration also for this reason (Marschner, 1997).

The net $CO_2$ fixation decreases with the rise of salinity (equal to a decrease in osmotic potential) and also it is related to the $Na^+$ concentration in leaves, while at the same time the dark respiration increases. The reduction in carbon allocation is reflected on decline in long-term growth; in fact salt taken up by roots does not directly affect growth of young new leaves, because meristematic tissues are fed by phloematic stream where salts are excluded, so that in a first moment only older leaves are involved in salt income which enhances their senescence. The rate of leaf death on new leaf produced is fundamental for survival of plant; if the leaf death is predominant on new production, it means that plant may not survive to this stress because it does not own enough expanded photosynthetic area to support ongoing

growth (Marschner, 1997; Munns, 2005). However, it is still an unsolved aspect if a reduced rate of photosynthesis is the cause of a growth reduction or the opposite. Another consequence of salinity on plant is the decline in cell elongation, so leaves are smaller and thicker, this can imply an increase in stored unused carbohydrates in growing tissue that gives feedback signals to down-regulate photosynthesis (Ashraf, 2004; Munns and Tester, 2008). A salinity tolerance behavior can be shown as ability to maintain a net photosynthetic rate, stomatal conductance and high chlorophyll content (Ashraf, 2004). Many research studies investigated on the relationship between photosynthesis and growth of plants exposed to salt stress; there are several works that report little or absent relation between these two parameters and others that show positive correlation between photosynthesis and crop yield under salinity (Ashraf, 2004; Tab. 1-3).

Therefore it is clear that genetic differences in rate of photosynthesis under salinity exist among species and also among cultivars, so this physiological parameter can be considered as a selection criterion for those species where a relationship between photosynthesis and growth exists (Ashraf, 2004).

Additionally, salts can build up in different locations inside the plant, as in the apoplast causing dehydration of the cell and later of the entire tissue, in the cytoplasm interfering with many enzymes and, at the end, they can accumulate in chloroplasts with consequent toxic effect on all the photosynthetic process (Munns, 2005). Excess salt amount in photosynthetic tissues can cause shrinkage of thylakoids, $K^+$ deficiency inside chloroplasts and degeneration of PSII (Ashraf, 2004).

It is well known that any environmental situation or stresses that cause a decrease in photosynthesis - while plant is in high light condition - can allow an excessive production of ROS, normally produced in chloroplasts and mitochondria for basic metabolism (Munns, 2005). However there is evidence suggesting that when NaCl is removed, photosynthesis recovers much more quickly than leaf salt concentrations fall (Munns and Termaat, 1986).

In saline conditions it has been reported an increase in roots respiration rate, this is due to their need of carbohydrate to maintain respiration in saline substrates. This need probably comes from the numerous activities carried out in roots during salt

stress such as managing ions (extrusion or compartmentalization) and repairing cellular damage (Marschner, 1997).

**Table 1-3: Relationship of photosynthesis with growth of plants exposed to salinity (Ashraf, 2004).**

| Plant species | Photosynthesis increased or decreased due to salinity | Relationship of photosynthesis with growth | Reference |
|---|---|---|---|
| *Hibiscus cannabinus* | Decreased | No | Curtis & Läuchli (1986) |
| *Hordeum vulgare* | Decreased | No | Rawson et al. (1988) |
| *Trifolium repens* | Decreased | No | Rogers & Noble (1992) |
| *Triticum aestivum* | Decreased | No | Hawkins & Lewis (1993), Ashraf & O'Leary (1996b) |
| *Brassica juncea* | Decreased | Positive | Burman et al. (2003) |
| *Zea mays* | Decreased | Positive | Crosbie & Pearce (1982) |
| *Spinacia oleracea* | Decreased | Positive | Robinson et al. (1983) |
| *Phaseolus vulgaris* | Decreased | Positive | Seemann & Critchley (1985) |
| *Vigna mungo* | Decreased | Positive | Chandra Babu et al. (1985) |
| *Gossypium hirsutum* | Decreased | Positive | Pettigrew & Meredith (1994) |
| Six diploid and amphiploid species of *Brassica* | Decreased | Positive | Ashraf (2001) |
| *Beta vulgaris* and *Solanum melongena* | Increased at mild salinity | No | Heuer & Plaut (1981) |
| *Spinacia oleracea* | Unaffected at chloroplast level | No | Downton et al. (1985) |
| *Helianthus annuus* | Unaffected at mild salinity | No | Steduto et al. (2000) |

### 1.1.4 Variation in salt tolerance among species

In general, according to the reactions of plants to salt stress, they can be divided in two main groups:

- Halophytes

  The halophytes are plants that can grow and multiply in the presence of salts, have highly enhanced the ability to partition salt and/or to exclude it, and some of them have developed adaptive specific morphological structures, such as *Mesembryanthemum crystallinum* (ice plant) that accumulates salt in salt glands located on the surface of leaves and acting as salt sinks in order to release it, later, as crystals (Adams et al., 1998).

  Among halophytes, the plant with more potential for the study of salt tolerance is *Thellungiella halophila,* salt cress, typical of saline soils in China and Eastern Europe and closely related to *Arabidopsis thaliana* (Fig. 1-6), there is in fact 90% of cDNA identity between the two species and the same transformation method (Floral dipping method) is efficient for both (Zhu, 2001).


**Fig. 1-6: The two main plant models for the study of salinity stress compared: *Arabidopsis thaliana* and *Thellugiella halophila* (Vinocur and Altman, 2005).**

- Glycophytes

  In this group are gathered plants relatively sensitive to salinity, whose growth is inhibited by high concentrations of salt. Most fruit trees are sensitive to salinity including *Malus domestica, Prunus armeniaca, Pyrus spp., Prunus domestica, Prunus persica*, and *Citrus spp; Olea europaea* and

*Ficus spp*. are moderately tolerant but *Phoenix dactylifera* is very tolerant (Gucci et al., 1997; Kozlowski, 1997).

In this group are also listed: *Lycopersicon esculentum,* a widely distributed annual vegetable crop recently used as model and *Arabidopsis thaliana*, the main model species for molecular biology with completely sequenced genome (at the end of 2000). Since *Arabidopsis thaliana* is a sensitive plant, it can provide information on mechanism of salinity tolerance only comparing its behavior with *Thellungiella halophila'*s one; the differences between the two species are clear by their responses to 100 mM NaCl in high transpiration conditions, in fact *Arabidopsis* cannot finish its life cycle, while *Thellungiella* shows only a growth rate reduction (Munns and Tester, 2008). Many of the genes involved in salinity stress tolerance in plants were found in glycophytes as well, they are also present in halophytes, and this supports the hypothesis of acclimatization as a mechanism to achieve tolerance and suggests that difference in the response of these plants is not in the genome, but in the fine-tuned regulation of genes involved (Zhu, 2001a). Other studies established that glycophytes could adapt to high levels of salinity, providing salt stress gradually in moderate increments (Botella et al., 2005; Verslues et al., 2006).

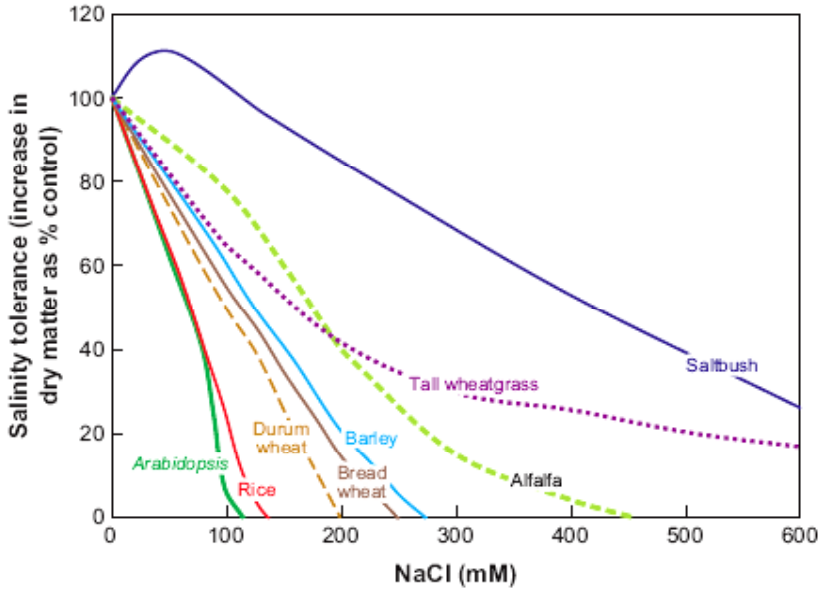



**Fig. 1-7: Diversity in salt tolerance of different species exposed to NaCl stress, shown as increases in dry matter (%) respect to control (Munns and Tester, 2008).**

Plants differ greatly in salt tolerance and another criterion to distinguish crops for their tolerance is looking at their increase in shoot dry matter (%) after grown in a solution or sand culture containing NaCl for at least 3 weeks, relative to plant growth in the absence of NaCl (Fig. 1-7).

The variation in salinity tolerance in dicotyledonous species is even greater than in monocotyledonous species. Some legumes are more sensitive than rice, suffering a 50% reduction in biomass at about 75 mM NaCl, Alfalfa is contrarily very tolerant and as extreme example there is Saltbush (*Atriplex* spp.) that is able to grow also in salt concentrations greater than that of seawater. It is important to underline that wheat is one of the more salt-tolerant crop species; in particular bread wheat more than durum wheat and Tall wheatgrass is one halophytic relative that continues to grow also at concentration normally unsuitable for crops (Munns, 2005).

According to a USDA Salinity Laboratory's study, 81 crops were divided in four categories: sensitive, moderately sensitive, moderately tolerant and tolerant, on the base of their reduction in yield in response to a range of salinities expressed as EC value (Fig. 1-8). $EC_e$ is the traditional soil salinity measurement with units of decisiemens per meter (1 dS/m = 1 mmho/cm).

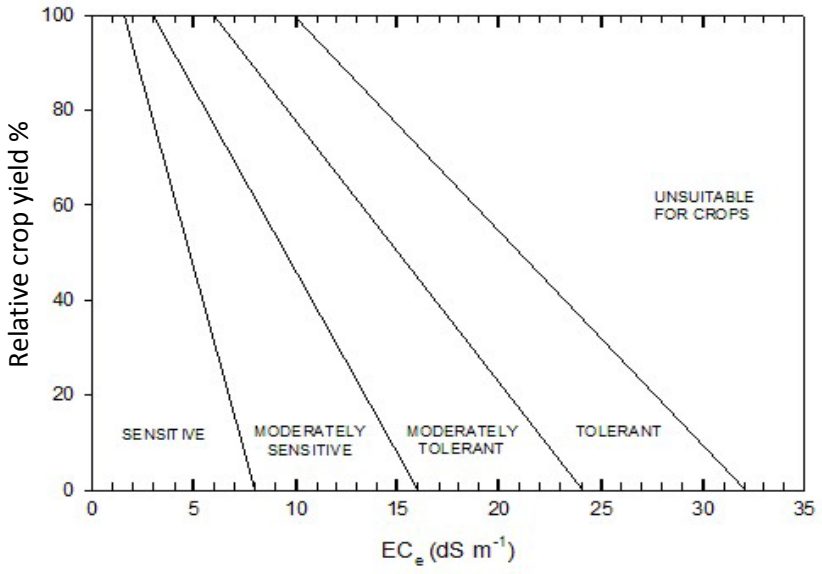



**Fig. 1-8: Categories for classifying crop tolerance to salinity according to the USDA Salinity Lab.**

For soil salinities exceeding the threshold of any given crop, relative yield ($Y_r$) can be estimated with the following equation:

$$Y_r = 100 - b(EC_e - a)$$

where $a$ = the salinity threshold expressed in dS/m; $b$ = the slope expressed in percent per dS/m; and $EC_e$ = the mean electrical conductivity of a saturated paste taken from the root zone (Tanji and Kielen, 2002).

### 1.1.5 The state of art about salt stress experiments in plants

#### 1.1.5.1 Horticultural crops

Most horticultural crops are glycophytes and have evolved in controlled conditions where salinity was kept low, but as mentioned before, modern agricultural is tightly linked to irrigation and in particular, for horticultural crops grown in a protected environment, irrigation is fundamental and the most serious drawback of this is the secondary salinization (Flowers, 1999). In saline condition, the performance of crops is not comparable with their behavior in normal conditions, because several physiological aspects are altered and this situation can drive to a reduction in crop yield or in a decrease of product quality (Grattan et al., 1999).

In general, salinity adds a new level of complexity to the comprehension of physiological mechanisms in plants.

Nowadays, research is focused on several aspects of salinity and approaches are really varied and wide; from field experiments, to evaluate more salt stress tolerant cultivars irrigated with saline water or grown in saline soil, to extremely advanced techniques as the genetic introgression of some salt-tolerant determinants in sensitive genotypes in order to gather resistance and yield in one unique commercial cultivar (Grattan et al., 1999; Botella et al., 2005).

Afterwards, it will be discussed the research situation and progress in salt stress applied to some important plants, chosen as models.

1. Tomato

   Tomato is a widely distributed annual crop which is consumes in several, different processing forms from fresh to canned; it shows a good adaptation to a variety of climates, but it prefers warm weather typical of Mediterranean areas and California. Where climatic condition is optimal for tomato, often also salinity is present. Tomato showed a 50% yield reduction if irrigated with saline water around 9 dS/m and a tomato crop is not commercially profitable if yield decreases between 10-15%. It has been

reported that salinity causes in tomato a growing modification of plants with height reduction, increase in dry matter and chlorophyll content (Caliandro et al., 2000). In 1964, Strotogonov underlined that plant stress tolerance could be enhanced by a NaCl treatment on seed before sowing. In tomato this "seed priming" leads to an early germination, emergence and faster seedling growth. This last aspect seemed to be due to a better capacity of osmotic adjustment; in fact primed plants had higher sugars and organic acid contents in leaves in comparison with untreated seeds (Cuartero et al., 1999). About tomato breeding, most works on improving salt tolerance in this crop used wild tomato cultivars that appeared more tolerant and selected them for speed in germination. Several other characteristics suitable for use in breeding have emerged and should be found in a donor to try to transmit to commercial crop, such as: fruit size, vigor and absence of stress symptoms. From a study carried out by Sacher et al., 1985 it was possible to observe the osmoregulation mechanism in tomato, in fact different cultivars were subjected to 100 mM NaCl in hydroponic culture and tolerant ones showed an increase in myo-inositol content in leaves that remained high for all the salt stress imposition (Sacher and Staples, 1985). Another important aspect of growing tomato in salt stress condition is that these plants seem to possess fruits of higher quality; total soluble solids (TSS) in ripe fruits increased with salinity and this result can suggest the use of moderately saline water in order to improve fruit quality (Cuartero et al., 1999). It has been seen that grafting technique provides an alternative way to enhance salt tolerance, limiting the transport of $Na^+$ and $Cl^-$ to shoot with a consequent good fruit yield, in some combinations better than in commercial hybrids with their own rootstocks (Estan et al., 2005).

An additional source of variability, besides wild cultivars, could come from other organisms and be introduced by genetic engineering, as tomato can be transformed via *Agrobacterium*. An example about this kind of experiment can be the one performed by Rus and co-workers (2001); they transformed *L. esculentum* with the yeast HLA1 gene that facilitates intracellular $K^+$ accumulation and decreases intracellular $Na^+$. They observed that transgenic

lines were more tolerant to stress showing a minimized reduction in fruit production (Rus et al., 2001). Tomato is used as a model system as it is relatively easy to transform and produces large fruits with well-defined ripening characteristics; it can be used as a model fruit system to investigate gene function (Atkinson et al., 1998).

2. *Arabidopsis*

   Since years, *Arabidopsis thaliana*, a small annual flowering plant also known as “thale cress”, has been chosen as model plant for molecular biology and genetics. Although it has little direct importance for agriculture, nevertheless it has several traits that make it a useful model for understanding the genetic, cellular, and molecular biology of flowering plants; such as:

   - Small size and simple growth conditions suitable for laboratory experiments;
   - Diploid plant, self-compatible, it produces thousands of seeds per plant therefore obtaining progeny from mutant or transgenic lines is rapid;
   - Short life cycle (8 weeks);
   - Small and dense genome, completely sequenced;
   - Easy to transform by Floral dipping method;
   - Many "knock out" mutants are available (silencing of the gene of interest to study the phenotype) and chips for microarray analysis (Somerville and Koornneef, 2002).

*Arabidopsis* has played a key role in the salt stress experiments, because it was chosen as model also to determine which genes were essential for salt tolerance in higher plants. In 1996, Wu and co-workers were the first to identify four *Arabidopsis* mutants hypersensitive to salt (*sos1-1, sos1-2, sos1-3, sos1-4*) using the "root-bending assay” (test of root curvature) on agar plates containing NaCl. Growth inhibition of *Arabidopsis* seedlings, determined by salt, can be seen as a root reduction in elongation, as well as the cotyledons and leaves expansion. This root-bending assay (Fig. 1-9) aimed to identify the inhibition of elongation of the seedlings roots. Initially, seedlings derived from seeds were grown uniformly in absence of salt and then were transferred, with roots upside down, onto vertical agar plates

containing appropriate levels of NaCl as the stress agent. Continued growth on NaCl plates leads to roots bending for gravitropism; instead the lack of radical curvature was clear sign of growth inhibition by salt (Wu et al., 1996; Verslues et al. 2006). Through this test it was possible to screen about 5,000 seeds derived from EMS mutagenesis and then placed on agar plates with 50 mM NaCl. From here they could identify putative *sos* mutants that have been subsequently re-analyzed using the same test, showing that the *sos1-1* mutant exhibited a normal curvature radical in a control plate, while in plates added with salt, the growth was stopped. Through this fundamental experiment, twenty-one putative mutants were identified, but only five maintained hypersensitivity to salt in the third generation; they were called "salt overly sensitive (sos) mutants" (Wu et al., 1996). Later allelism tests, by pair-wise crosses between the mutants, revealed that the mutants fall into five complementation groups that gave the name to genes involved in tolerance to salt: SOS1, SOS2, SOS3, SOS4, SOS5 (Zhu, 2000).

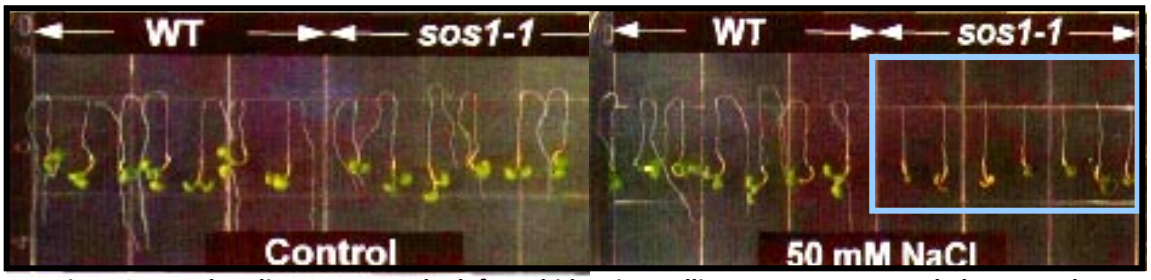



**Fig. 1-9: Root bending assay, on the left Arabidopsis seedlings grown on control plates, on the right selection of *sos1-1* mutants with inhibition of growth (Wu et al., 1996).**

Genetic approaches and yeast complementation assays have found out individual elements of sodium homeostasis, but only the systematic genetic strategy carried out on *Arabidopsis* by Zhu and colleagues have led to the identification of a salt-stress induced $Ca^{2+}$-regulated pathway (SOS pathway) involved in $Na^{+}$ homeostasis (Bartels and Sunkar, 2005).

The discovery of SOS genes (Salt Overly Sensitive) triggered the understanding of the pathway which determines the ionic homeostasis and tolerance to salt stress (Chinnusamy et al., 2004). A more detailed description of each SOS gene involved in this pathway will be done in next paragraphs, because their functions are really important to better understand the signal transduction mechanism in response to salt stress in *Arabidopsis thaliana*.

### 1.1.5.2 Woody plants

Woody plants, in general are classified as glycophytes; in origin in fact they were grown in non-saline areas, but the development of agriculture led to use more salinized soils or, as mentioned before, to utilize poor quality water that can lead to secondary salinization. The salt sensibility of some crops varies largely between species and among them also cultivars show different behavior. Salt–induced responses of woody perennials are a bit different to those of herbaceous species, because the behavior of perennials is influenced by the residual effect of previous growing conditions (additional effect). For fruit trees, the choice of the more suitable rootstocks for saline condition is particularly important because the salt resistance in most glycophytes is associated with the restriction of $Na^+$ and/or $Cl^-$ transport from the root to the shoot (Gucci and Tattini, 1997; Storey and Walker, 1999). Afterwards, it will be summarized the performance of some fruit/woody plants in salt stress conditions. Because of the cost and time required to obtain fruit yields, tolerances of several crops have been determined for vegetative growth only. In contrast to other crop groups, most woody fruit and nut crops tend to be salt sensitive, even in the absence of specific-ion effects. Only date-palm is relatively salt tolerant, whereas olive and a few others are believed to be moderately tolerant (Tanji and Kielen*,* 2002*).*

1. Citrus

   Citrus is an important world product, second only to bananas in terms of volume of world trade. Citrus species are among trees the most sensitive to soil salinity, but their ability to tolerate salt stress depends on genotype and rootstock (Maas, 1993).

   All commercial citrus trees are grafted on rootstocks which can regulate the amount of $Cl^-$ and/or $Na^+$ in leaves. Previous studies underlined that the rootstock Cleopatra mandarin (salt tolerant) is considered to be a $Cl^-$ excluder, while Carrizo citrange (salt sensitive) is retained a $Na^+$ excluder, but a $Cl^-$ accumulator; in fact in Citrus the content of chloride is a major limitation for growth and yield respect to sodium amount. Briefly, the ability of citrus plant to tolerate salinity seems to depend on its capacity in chloride exclusion. (Hassan and Galal, 1989; Garcia-Sanchez et al., 2006). An

additional consideration is about leaf injury: it did not seem to be related with $Na^+$ content, in fact plant exhibiting leaf burn have less $Na^+$ accumulation than no-injured ones.

Recently, it has been found out that the use of an interstock (i.e. Salustiano orange) in citriculture can increase growth and salt resistance of orange trees; in fact the decrease in transpiration rate and the reduction of ion transport led to accumulate half of $Na^+/Cl^-$ content in comparison with the same combination without an interstock (Verna lemon/sour orange). This application can imply a large increase in orange tree yield (Camara Zapata et al., 2004). Some tetraploid rootstocks of Citrus were compared with their respective diploid rootstock to investigate plant growth, leaf fall and ion content during salt stress; these results suggested that citrus tetraploid rootstocks were more tolerant to salinity (Saleh et al., 2008).

On the other hand, it has been reported that the yield reduction in salt stress conditions occurred in Citrus without excessive accumulation of the two main ions and with no toxic effect, this lead to think about an osmotic effect like dominant consequence of salinity. But this hypothesis is weakened by some other results showing that the lack of $Cl^-$ exclusion ability in some rootstocks is responsible for decrease in orange yields (Maas, 1993).

Overall, Citrus responses to salinity of the soil solution varied comparing different experiments and this renders the comprehension of its physiology under salt stress more and more complicate. This variation in responses is due to several factors listed below:

- Salinity is generally more detrimental to tree growth in semi-arid climates than in subtropical ones.
- The response varies in relation to duration of exposure to salt stress.
- Both rootstocks and scions show difference in salt tolerance and in their ability to limit accumulation of $Na^+$ and $Cl^-$ inside the plant (Prior et al., 2007).

Grieve and co-workers (2007) reported that saline irrigation water reduced growth of “Valencia” orange trees and effects of stress increased with duration of salinization. Salt stress exhibited minor effect on fruit quality, in

fact salt increased juice sugar content, but diminished fruit size, juice content and TSS yield (Grieve et al., 2007).

Another variable to take in consideration in analysing effect of salinity is the $Na^+/Ca^{2+}$ ratio of the saline treatment solutions; in fact it has been noticed that the addition of calcium to a saline solution greatly reduced the transport of $Na^+$ and $Cl^-$ to leaves of scions on Cleopatra mandarin such as Troyer citrange (Maas, 1993). But not all the effects of salinity are negative, in fact, as moderate osmotic stress, it reduces growth and physiological activity allowing citrus seedlings to overtake a cold stress and a short-term salt stress can determine an increase in flowering after stress release (Levy and Syvertsen, 2004).

Some years ago, citrus research on salinity embarked on somatic cell cultures in order to select cell lines for high salt tolerance "*in vitro*"; but this project resulted in a non resistance of the whole plant derived from NaCl resistant cell lines, largely because salt resistance in Citrus is, like in most plants, a multigenic trait (Storey and Walker, 1999). From a genetical point of view, in fact, salt stress tolerance often showed the characteristics of a multigenic trait, with quantitative trait loci (QTLs), associated with tolerance and with ion transport under saline conditions, which were identified in barley, citrus and rice. In Citrus inter-specific crosses, chloride accumulation showed continuous variation among progenies, suggesting that this is a polygenic trait, although with a strong heritable basis (Flowers 2004).

2. Olive

Between fruit trees, generally retained sensitive to salt stress, olive such as pistachio and pomegranate are exceptions (Levy and Syvertsen, 2004). Olive is more salt and drought tolerant than other temperate fruit trees, despite being classified amore glycophytes. For this reason, olive can be considered a model species for woody plant in order to study its higher tolerance and its variability between genotypes in responses to salt stress (Gucci and Tattini, 1997). Olive is one of the major Mediterranean crops that are being expanded in irrigated lands; its productivity is reduced only by 10% when the

electrical conductivity (EC) of soil solution is 4-6 dS/m or can be 6-8 dS/m in soils of high calcium status (Therios and Misopolinos, 1988).

Typical main symptoms of salt stress in olive are: reduced growth, leaf burn tip, leaf chlorosis, leaf rolling; some other effects of salinity can involve reproduction with the decrease in pollen germinability and viability, and the oil quality, in fact it has been reported that salt stress increases content of aliphatic and triterpenic alcohol and linoleic-linolenic acid ratio.

Previous works disclosed that the olive salt tolerant "Frantoio" cultivar showed high photosynthesis rate after five weeks of 100 mM NaCl stress and a significant reduction of this fundamental activity was registered starting from 200 mM NaCl; in addition this cultivar proved to be able to recover growth and photosynthesis after stress mitigation (Tattini et al., 1997). On the other hand another cultivar "Leccino" was evaluated as representative of olive sensitive cultivars; the comparison between these two cultivars showed that at root levels the $Na^+$ content was comparable, instead in leaves and stem it was higher in the salt sensitive cultivar. From these observations, it was possible to deduce that the main mechanism, that drives the accumulation of salts inside the plant, is the reduction of sodium translocation rather than exclusion of sodium uptake. Unlike Citrus, where the $Cl^-$ exclusion seems to be the key aspect to reach the tolerance, in olive the $Cl^-$ content in leaves is three times bigger than in roots after five weeks of stress (Gucci and Tattini, 1997). In this species, the ability to regulate the ions distribution is also linked to free sterols and phospholipids; in fact "Frantoio" cultivar showed a higher ratio of these two components than in "Leccino", that means a lower permeability of the membranes. Also the accumulation of mannitol and glucose was reported as consequence of salinity in plants for their role in osmoregulation (this aspect will be deeper discussed in next paragraph 1.2.2.1); in olive cultivars (Frantoio and Leccino) the increase in mannitol was similar in sensitive cultivar and in tolerant one, and this rise did not result sufficient to face the increasing $Na^+$ content (Tattini et al., 1996).

In addition it has been reported the role of proline and polyamines as well as others ormoregulators in osmotic adjustment in plants under saline condition (Greenway and Munns 1980). In fact plant acts to maintain leaf turgor when the osmotic potential decreases because of the salt stress by synthesizing new compatible solutes. But in olive leaves, it was observed that proline and polyamines contents did not vary significantly in saline condition (Tattini et al., 1993). Other mechanisms conferring salt tolerance to olive, beyond salt exclusion and osmoregulation are: stomatal closure, leaf dehydration and leaf abscission.

Other traits that distinguish olive from other fruit plants are:

- High hydraulic resistance of the stem that allows plant to continue uptake during salt stress, in parallel with stomatal closure and therefore decrease in transpiration.
- Slow growing that implies a lower energy demand than other crops.

For all these considerations, olive can be a good solution for salinized soils instead of planting salt tolerant herbaceous crops. In comparison with other woody plants, about olive and salinity a lot is known, but anyway research is focused on improving salt tolerance of some olive rootstocks and varieties (Gucci and Tattini, 1997).

#### 1.1.5.3 Pear

**European pear**

According to the classification of crops for their tolerance to salinity listed by Tanji and Kielen, 2000 (FAO data), pear belongs to sensitive crop and really few information is reported about it. During salt stress both ions $Na^+$ and $Cl^-$ are taken up by roots and are transported to shoots, chloride more rapidly than $Na^+$ and in higher concentration; for this reason $Cl^-$ seems to be the main responsible for the onset of first symptoms of toxicity (Boland et al., 1997). Generally, sodium tends to be stored in roots, trunk and branches for several years, in order to save leaves from high concentration of this ion. So wood represents a sink for salts, but after some years the "wood capacity of $Na^+$ storage" ends and this ion starts to accumulate in leaves. This observation was done by Myers and co-workers in 1995;

they evaluated long-term (nine years) effects of saline irrigation on yield and growth of mature (40-years-old) Williams pear trees. The salinity treatments at 2.1 dS/m led, during the seventh year, to a reduction of lateral shoots growth, leaves and fruits were smaller, the yield reduced and leaf fall happened earlier in treated trees compared with the control ones. In the ninth year, the yield decreased of 50%, and also 40% of trees died (Myers et al., 1995). In this experiment, pear also has demonstrated the restriction of $Na^+$ transport and accumulation in the trunk wood. This observation lets think about the existence of a $Na^+$ threshold in the trunk that, if is exceeded, it triggers the onset of salt damage. This threshold of $Na^+$ level cannot be standardized for all woody plants, in fact it varies between species and at different tree ages (Boland et al., 1997).

Mainly responsible for the sodium uptake/exclusion is the rootstock, in fact a suitable choice of rootstock can ameliorate the detrimental effect of salinity on pear cultivars (Okubo and Sakuratani, 2000). For this reason research in this field is focused on clarifying the behavior of different rootstocks in saline conditions (saline water or soil).

In a recent short-term experiment carried out in the Department of Fruit Tree and Woody Plant Science (University of Bologna), pear trees cultivar Abbé Fétel own-rooted and grafted on two clonal quince rootstocks (EMC and Sydo) and on clonal pear rootstock Farold®40, were grown and irrigated with saline water (conductivity equal to 5 dS/m) for 5 months. Irrigation with saline water led to:

- A reduction in shoot length during the growing season, as the graph shows

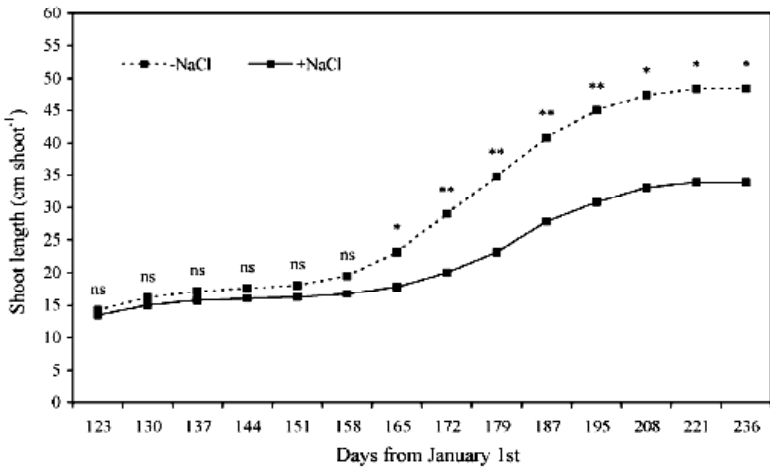



**Fig. 1-10: Pattern of the shoot length recoded during growing season in control (-NaCl) and treated (+NaCl) trees (Musacchi et al., 2006a).**

regardless the genotype (Fig. 1-10).

- An increase of $Na^+$ uptake in trees on both quinces, and Abbé Fétel/MC reported also a significant increase of $Cl^-$ not found in Abbé Fétel/Sydo (Fig. 1-11).

This study demonstrated therefore that trees grafted on quinces accumulate a significant amount of $Cl^-$ in the leaves, but were able to store most of the adsorbed $Na^+$ in their roots, this mechanism prevented xylem loading and transport to the leaves. On the contrary, pear roots adopted an ion exclusion strategy to avoid accumulation of $Na^+$ and $Cl^-$, therefore an high selectivity $K^+/Na^+$ mechanism could be proposed and utilized as a physiological marker for the ion component of salt stress response (Musacchi et al., 2006a).

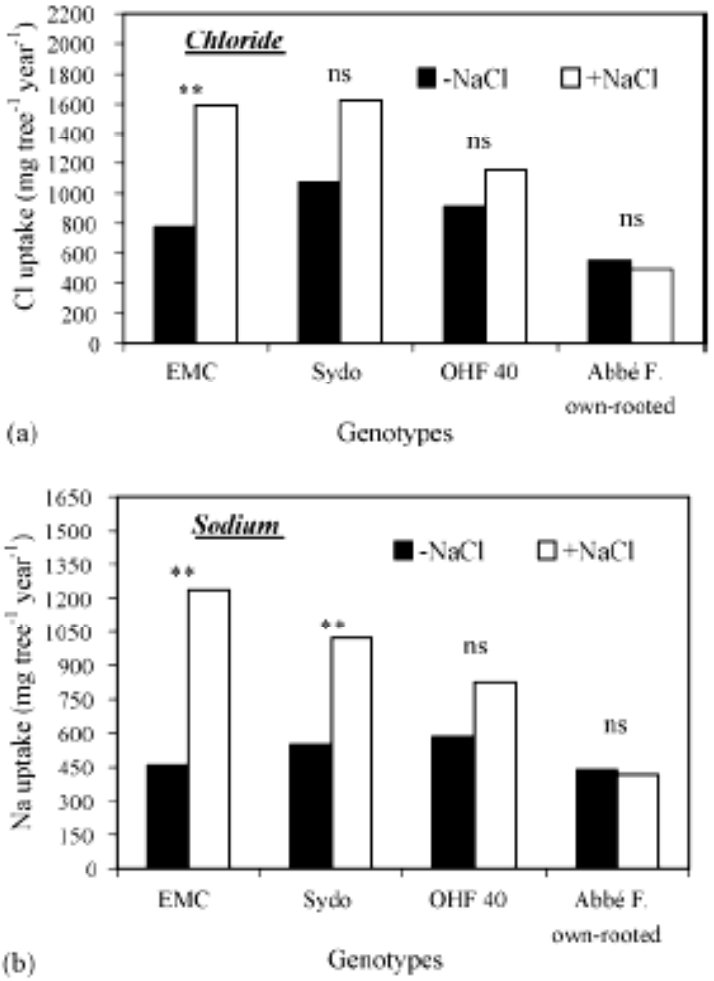



**Fig. 1-11: Effect of genotype and NaCl treatment on chloride (a) and sodium (b) uptake (mg tree$^{-1}$ year$^{-1}$) (Musacchi et al., 2006b).**

Another two-year-trial was planned using pear trees cultivar Abbé Fétel grafted on three quince rootstocks (MC, BA29 and Sydo) and planted in an area characterized by the presence of water with electrical conductibility greater than 5 dS/m (Mezzano-FE). This experimental orchard was compared with the same tree combinations located in an area with low saline water (Cadriano-BO). It has been observed that fruit yield obtained from trees in saline condition (Mezzano) was higher than production in low saline condition. Then fruits from Mezzano showed an increase in size and in some of their qualitative features such as higher firmness, sugar content, acidity and skin russet than "control" fruits in Cadriano (Fig. 1-12) (Caliandro et al., 2000; Musacchi et al., 2000).

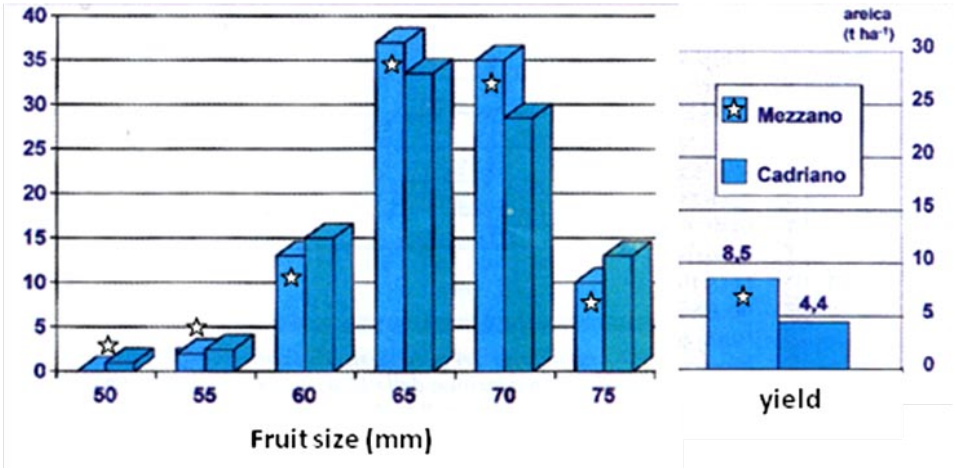



**Fig. 1-12: Fruit size for Abbé Fétel/MC located at Mezzano (FE, white star) and at Cadriano (BO), on the left. On the right, comparison of production (t ha$^{-1}$) in the two locations (modified from Caliandro et al., 2000).**

In conclusion, pear in field experiments seems to be salt tolerant in the short term, but its behavior looks different in the long-term salinity treatment.

An "*in-vitro*" approach was adopted with pear cultivars Conference and Abbé Fétel in order to generate NaCl-tolerant variants and to evaluate the possibility to add NaCl during regeneration to select for salt tolerance. This experiment was performed by Marino and Molendini (2005) using shoot apex cultures with media with modified NaCl. It has been reported that leaf explants exhibited high susceptibility to salt and in particular Abbé Fétel regeneration was totally prevented in media with 5 g/L NaCl, instead some shoots were formed at 3 g/L NaCl media, but they appeared so weak that did not survive to first subculture in elongation medium. This result seems not to agree with the behavior of the same cultivar on clonal pear rootstock or own-rooted reported in Musacchi et al., 2002 where no

effect of salinity on sodium leaf concentration was reported for combinations of Abbé Fétel on *Pyrus communis* roots; this can be explained by the different culture system ("*in vivo*" against "*in-vitro*") (Musacchi et al., 2002; Marino and Molendini, 2005). Another "*in-vitro*" culture experiment of the pear rootstock OHxF333 subjected to salt stress showed that increasing NaCl in the medium, Fe, Mn and Zn contents in shoots decreased. Salinity probably increases the demand of energy by cells in order to face the osmotic regulation and competition of ions; this can also explain the nutrient imbalances (Sotiropoulos et al., 2006).

**Asian pear**

Between Asian *Pyrus* species, it has been reported that the ornamental evergreen and *Erwinia amylovora* resistant (Fideghelli, 2007) pear *P. Kawakamii* is tolerant to salinity and it is also adopted in subtropical areas. Information about salt tolerance of other rootstocks is limited. Some experiments carried out by Okubo and co-workers aimed to clarify the behavior of some Asian pear rootstocks at different level of salinity, to investigate on survival, leaf burn and mineral uptake. Two-year-old pear rootstocks *Pyrus betulifolia* Bunge (BET) and *P. pyrifolia* Nakai (PYR) were potted and irrigated with increasing NaCl concentration (0, 25, 50, 100, 150, 200 mM, last concentration only for BET). This trial showed that BET appeared high tolerant with high survival rate and slight leaf injuries around 100 mM NaCl; contrarily PYR presented severe leaf burns at low concentration of salt and, in particular at 25 mM NaCl, several plants died. Furthermore mineral analyses revealed that leaf $Na^+$ amount in BET at 100 mM NaCl was similar to the concentration obtained in PYR plants at 25 mM NaCl (Okubo and Sakuratani, 2000). They also investigated the response of one Chinese, one Japanese and one European pear cultivars grafted on BET and PYR and irrigated with 20% Hoagland's solution added with 0, 25, 50 mM NaCl. All plants grafted on BET showed good performance at 50 mM NaCl while the others on PYR suffered seriously. Additionally, flower bud formation was promoted in all scion-rootstock combinations, whereas bloom date and number of flowers (for cluster) were not altered by salt irrigation. The good performance of BET rootstock under saline irrigation was explained by mineral analyses, that showed the rootstock ability to restrict or exclude the translocation of $Na^+$ and $Cl^-$ to foliage. In conclusion, BET

showed good potentialities as pear rootstock in areas affected by salinity and, being graft-compatible with both European and Asian pears varieties, could represent a potential resource (Okubo et al., 2000; Matsumoto et al., 2006a). All these considerations are related to short-term experiments, so the BET behavior as rootstock should be investigated also in long term experiments to have a wider opinion on its performance. The same species of Asian *Pyrus* rootstocks (but not grafted) were chosen by Matsumoto and co-workers to dig deeper into rootstocks behavior in salt stress, comparing them with other species such as: *P. calleryana* (CAL) and *P. dimorphophylla* (DIM). The highest tolerance was showed by BET until 200 mM NaCl, with no visible symptoms of burn. In contrast, PYR and CAL exhibited lower salt tolerance with severe injuries, defoliation and death (Matsumoto et al., 2006b).

Moreover, another comparison was performed among native Mediterranean *Pyrus* rootstocks (*P. amygdaliformis* [AMY] and *P. elaeagrifolia* [ELA]) and native Asian ones (BET and PYR); the Mediterranean species did not show any leaf injury during 30 day at two different salt concentrations (75 and 150 mM NaCl), instead Asian species developed leaf burns. Ion contents ($Na^+$ and $Cl^-$) were markedly higher in PYR and BET at both salt concentrations than those of native Mediterranean species. Also the photosynthetic rates of AMY and ELA under stress were higher than PYR and BET; this can be explained by the ability of Mediterranean species to limit salt movement in the shank, avoiding the accumulation of crucial ions in foliage and so preventing the photosynthetic rate decrease under salt stress (Matsumoto et al., 2006c). Nowadays, *P. amygdaliformis* and *P. elaeagrifolia* as pear rootstocks are limited to coastal areas surrounding Mediterranean and are characterized, respectively, by drought resistance and juicy-flesh fruits (Procopiou and Wallace, 2000; Matsumoto et al., 2006c; Fideghelli, 2007).

## 1.2 Stress concept and tolerance/susceptibility responses in plants

An important characteristic that distinguishes plants from other multicellular organisms is that plants are sessile, and therefore are forced to face environmental challenges commonly known as stresses (Zhu, 2001b). Stress is defined as the pressure of some adverse forces that tend to inhibit the normal functioning of a biological system (Short Oxford English Dictionary), then the term can mean "any external condition that affects growth, development and productivity of the plant" (Vernieri et al., 2006).

Because of the presence of stress many crops do not exceed 20% of their productive potential. Plants can be subjected to different stresses that are divided into: abiotic and biotic. The first type arises because of an excess or a deficiency linked to environmental, chemical and/or physical conditions, while the latter type is linked to the presence of another organism which can carry a disease.

In general, the abiotic stresses determine approximately 70% of the production losses of the main crops. Among abiotic stresses are included: drought, high/low temperature, nutrient deficiencies water and/or of soil salinity, excess/lack of light, heavy metals contaminations. Many of these, for their importance, are target of breeding in order to obtain plants that can cope with adverse conditions without suffering severe losses of production (Buchanan et al., 2003).

Plants react differently to different environmental stresses on the bases of their intensity, duration, number of exposures and the possible combined action of more stresses (multiple stress), but also the type of organ/tissue involved, the plant development stage and the genotype. Stresses trigger in plants a wide range of responses, alteration of gene expression and cellular metabolism, but also changes in growth rates and field production.

The two main types of plant response to environmental stresses are:

- Susceptibility that leads inevitably to the death of the plant that fails to cope the stress.
- Resistance that means that plant shows the ability to survive and grow although subjected to a stress.

Stress response may be direct, if directly triggered by stress (i.e. drought) or indirect if derived from a stress-induced damage (i.e. loss of membrane integrity). It is

important to understand deeply the mechanisms of plant response to stress in order to manipulate the plant to ensure a suitable productivity even in absence of ideal conditions (Buchanan et al., 2003).

The resistance mechanisms that a plant can show to face a stress are mainly three:

1. "avoidance" which prevent exposure to stress (i.e. plants phreatophytes with deep roots);
2. "tolerance" which permits the plant to withstand the stress;
3. “acclimation” which means the adjustment of individual organisms in response to changing environmental factors. During acclimation plant alters its homeostasis, in order to be able to adapt to adverse environmental conditions. A period of acclimation before the onset of a stress may confer resistance to a plant that would otherwise be vulnerable (Buchanan et al., 2003).

   This fact leads to hypothesize that the genetic program for tolerance is, at least to some extent, also present in non-tolerant plants (Bartels and Sunkar, 2005). These plants may need a gradual adjustment (adaptation) to achieve an adequate expression of genes responsible for the acquisition of tolerance (Zhu, 2001a).

### 1.2.1 Stress perception and signalling in response to abiotic stress

The plant response to stress begins when it senses the stress signals at the cellular level (Fig. 1-13). Once a stress has been recognized by plant, the signal transduction is triggered in the single cell and then in the entire plant. The signal transduction is the process by which plant cells receive and process the stress signals and transmit them to the cellular machinery to activate adaptive responses (Xiong and Zhu, 2001).

In general, almost all signals, that affect the plant, arrive to the nucleus inducing alteration of genes expression and therefore a modification of cellular metabolism, which will determine plant responses to stress (i.e. alteration of growth). A general way of signal transduction begins precisely with the perception of the signal by specific receptors, which after activation, initiate or suppress a cascade to transmit intracellularly the signal.

The signal perception triggers the generation of second messengers (i.e. inositol phosphates and ROS) that are easily diffusible molecules capable of conveying information from one extracellular source to the major enzymes present in the target cell. The second messengers are able to modulate levels of intracellular $Ca^{2+}$ often initiating a phosphorylation cascade, whose final target are proteins directly involved in cell protection, or transcription factors that control specific set of genes regulated by stress (Xiong et al., 2002).

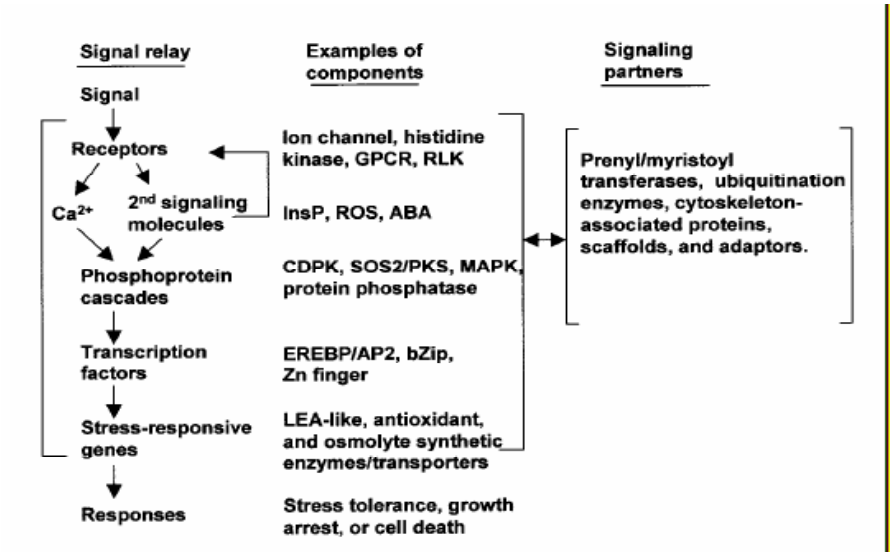



**Fig. 1-13: A generic pathway for the transduction of cold, drought, salt stress signals in plants (Xiong et al., 2002).**

Products of these genes may participate in the synthesis of regulatory molecules such as hormones: abscisic acid (ABA), ethylene and salicylic acid (SA). They in turn can initiate a second round of signalling that may follow the generic pathway, despite different components are involved (Xiong et al., 2002).

The signal transduction network is made up of relatively independent functional units called modules. They require a proper coordination of space and time, among these are included molecules involved in the editing, assembly and release of signalling components, but they do not transmit directly the signal; however they are not less important, and indeed have a key role in the signal transduction accuracy. Enzymes modifiers, scaffolds and adapters belong to this group of signalling components (Xiong and Zhu, 2001).

In conclusion, plants have evolved complex signalling pathways in response to several stresses as salt, drought, wounding, cold and have acquired plasticity in metabolic functions in order to face these stress conditions. All these abiotic

stresses have repercussions on plant development and growth, in fact cold, drought and salinity firstly limit plant growth, because of a strong osmotic effect that is common to them, and, secondarily, each stress implies other effect on plant, like for salt stress, the onset of ion toxicity. Therefore, it is well known that cross-talk between different stress signal transduction pathways exist in plants, meaning with the term "cross-talk" the possibility for different signalling pathways to share one or some components or have similar outputs effects (Zhu, 2002; Munns, 2002a; Chinnusamy et al., 2004; Ma et al., 2006).

### 1.2.2 Drought and salt stress signalling pathways

Many of the abiotic stresses are linked by the fact that at least part of their damaging effect on the "plant performance" is caused by a disorder in plant water status (Verslues et al., 2006). This may be caused by a reduction in the water availability during drought or by ion content modification and different water uptake in saline conditions. In fact, these two stresses involved in water availability are the most important and since ancient age they shaped plant evolution (Zhu, 2002). "Water stress" in broad sense encompasses both drought stress and salt stress; both modify plant physiology and metabolism and these changes occur mainly as clearly adaptive responses.

These responses can be grouped in four categories (Fig. 1-14):

1) Water/osmotic homeostasis; 2) Ionic homeostasis; 3) Detoxification: protection/damage repair; 4) Cell division and expansion regulation.

All these mechanisms challenge to restore the cellular ion and water content to levels similar to those present under unstressed conditions, to prevent or repair cellular damage, caused by the stress, and to reestablish the homeostasis that in a broad sense drives to the "water stress" tolerance. Homeostasis in general, is the property of a system, typically a living organism, which regulates its internal environment so as to maintain a stable, constant condition of equilibrium, while external conditions vary (Zhu, 2002; Verslues et al., 2006).

In parallel with the four water stress responses, also the salt and drought signalling can be partitioned in analogous four different signallings:

- Osmotic signalling;
- Ionic signalling;
- Detoxification signalling;
- Cell division and expansion signalling.

Osmotic and ionic signallings are both responsible for restoration of homeostasis and in particular the second one is mainly relevant in salt stress; all these four pathways are in some ways linked, because, for instance, once the cellular homeostasis is reestablished, stress damage and injury would be alleviated and vice versa. On the other hand, homeostasis and detoxification signallings lead to reduce the growth inhibition (Cell division and expansion signalling) (Zhu, 2002).

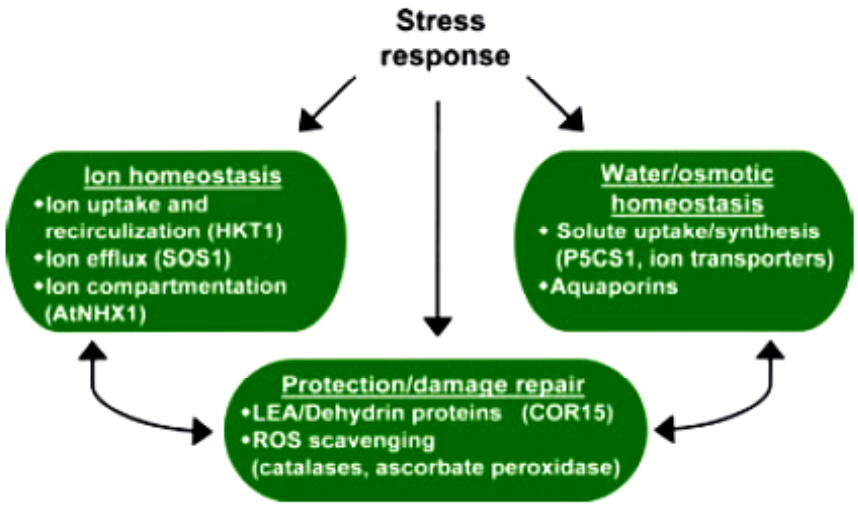



**Fig. 1-14: Homeostasis and protection/damage repair model of abiotic stress response in plant (Verslues et al., 2006).**

### 1.2.2.1 Osmotic signalling

Plants subjected to salt stress respond with the reduction of shoot growth and this occurs in two phases: the osmotic phase, caused by the rapid increase of external osmotic pressure, and an ionic phase, which is lower than the first one and is due to $Na^+$ accumulation in leaves. The osmotic phase and in parallel the osmotic signalling starts when the salt concentration around roots begins to increase (Munns and Tester, 2008). The salt stress, as well as drought, decreases the water potential outside the cell (Kramer and Boyer, 1995). According to the concept of osmosis (process energetically spontaneous), water moves from compartments with higher water potential to more negative ones, and in the case of radical absorption, the

water movement is possible only when the water potential of the root is more negative than that of the soil, if this condition is not satisfied the plant cannot extract water from the soil (Taiz and Zeiger 1996; Buchanan et al., 2003).

If a plant cell is put in a concentrated saline solution, its loss of turgor will be evident leading to the plasmolysis and the disconnecting of protoplast from the cell wall, because of the flowing out of the water. Contemporarily, the solutes inside the cell are becoming more concentrated.

If the cell was able to further reduce its internal water potential, acting on the concentration of solutes in it, the gradient could be restored so that the water would come within the cell to achieve a balance. Instead, the persistence of this plasmolized condition of the cell can quickly be lethal (Fig. 1-15; Buchanan et al., 2003).

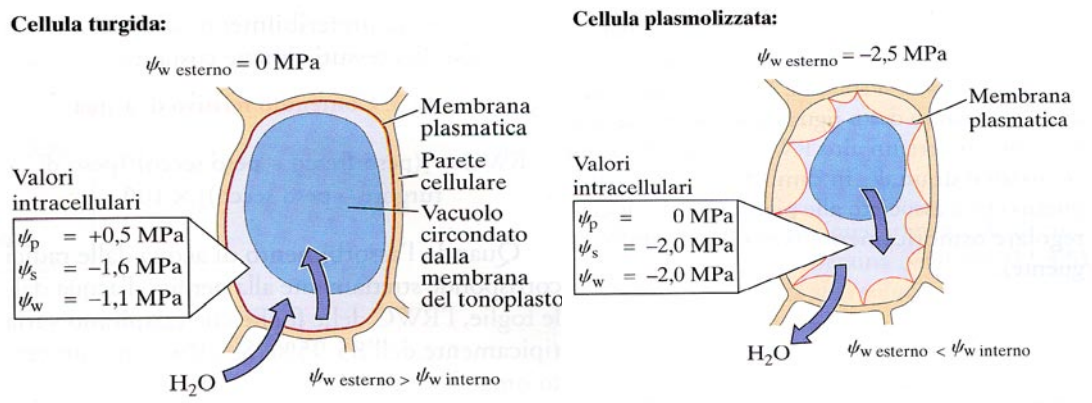



**Fig. 1-15: On the left a turgid cell in an unstressed external condition (ψw=0 MPa) able to uptake water; on the right the same cell plasmolyzed because exposed to a stress condition (salinity or drought) that confers an external negative water potential (ψw=-2.5 MPa).**

### 1.2.2.1 a Osmotic adjustment

One mechanism, that organisms can adopt to face salt stress and tolerate it, is the synthesis and accumulation of some compatible compounds or inorganic ions able in this way to decrease the ψw acting on ψs (Apse and Blumwald, 2002; Buchanan et al., 2003). This phenomenon that aims to support the water influx into cells is known as osmotic adjustment (OA). This mechanism is involved both in short-term and long-term drought or salt stress, because in the first case it acts avoiding the cell shrinking, while in the second it should guarantee the water uptake to allow plant to continue growing under continual water stress (Apse and Blumwald, 2002).

The compatible solutes are molecules with low molecular weight, non-toxic because they do not interfere with the primary metabolism and generally accumulate in the cytosol at high concentrations during osmotic stress. These osmolytes are defined compatible with the hydration shells of proteins, which means that they do not penetrate the shell, formed by water molecules, which surrounds the protein, whereas ions such as $Na^+$ and $Cl^-$ penetrate these shells interfering with strong interactions able to denaturize proteins. This is why salts in high concentrations within the cell adversely interfere with the plant metabolism (Buchanan et al., 2003). Usually these dangerous ions are mostly stored in vacuoles, while in the cytosol, compatible solutes and ions with harmless action such as $K^+$, work to maintain an osmotic balance with the vacuole.

For instance a mesophill salt stressed spinach cell (Fig. 1-16) concentrates $Na^+$ and $Cl^-$ inside the vacuole to preserve normal cell function and to balance this increase in concentration in the cytoplasm promotes the synthesis of osmolytes, in this particular case glycine-betaine (Buchanan et al., 2003).

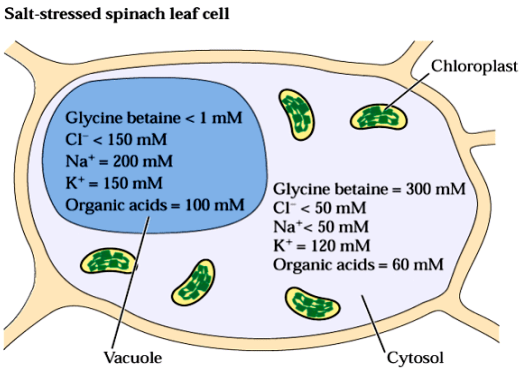



**Fig. 1-16: Salt-stressed spinach leaf cell during OA.**

The compatible osmolytes generally present in higher plants are: amino acids, amides, polyols, low molecular weight-sugars (mannitol, trehalose) and quaternary amines (glycine-betaine). Among the amino acids the most important in OA are: proline, arginine, alanine, glycine, serine, leucine, valine and additionally also non-protein amino acids such as citrulline and ornithine have a role in responses to salinity (Wang et al., 2003; Ashraf, 2004).

The main functions of these osmolytes are:

- To maintain the cell turgor
- To promote the gradient for the absorption of water
- As source of nitrogen for the stress recovery phase

- To act as free radicals scavengers or as molecular chaperons to stabilize membranes and native proteins replacing water at their surfaces (Bohnert and Shen, 1999; Hasegawa et al., 2000; Diamant et al., 2001; Serraj and Sinclair, 2002).

The osmotic adjustment is widely spread in plants, but it is extremely difficult to summarize a general behavior, in fact osmolytes vary between species, cultivars and also among different compartments within the same plant (Ashraf, 2004). Moreover osmolytes, that are necessary to achieve osmotic homeostasis, are synthesized from the plant and the most common are proline and glycine-betaine (Ashraf and Foolad, 2007). This increase in compatible solutes synthesis represents for the plant a great utilization of energy detrimental for growth and photosynthetic activity.

The high concentration of compatible solutes in drought or salt tolerant plants and in halophytes has provided strong evidence of their involvement in the process of osmotic adjustment or osmoprotection. So to increase stress tolerance in plant the idea of overexpressing compatible solutes in transgenic plants has been applied (Wang et al., 2003, Yamada et al., 2005). One of the first targets of metabolic engineering to enhance stress tolerance was the proline. In the proline biosynthetic pathway the first enzyme is bivalent and is called $\Delta^1$-pirroline-5- carboxylate synthase (P5CS), its role is the phosphorylation and transformation of L-glutamate to L-glutamate γ-semialdehyde (Fig. 1-17). It has been considered a good target for transformation and Kishor and co-workers overexpressed the p5cs gene in tobacco obtaining transgenic plants with 10- to 18- fold more proline than wild type plants; but this approach led to the upregulation of proline dehydrogenase that acted reducing proline levels. Later the experiment was performed by other researchers utilizing a proline dehydrogenase antisense that resulted in satisfactory proline content able to support better growth under salt stress (Kishor et al., 1995; Wang et al., 2003; Vinocur and Altman, 2005).

Also the external supply of proline was proposed in order to increase the tolerance of plant under salt stress, acting as osmoprotector and facilitating growth; but in other plants this application showed and adverse effect on plants as for *Arabidopsis* where chloroplasts and mitochondria were damaged.

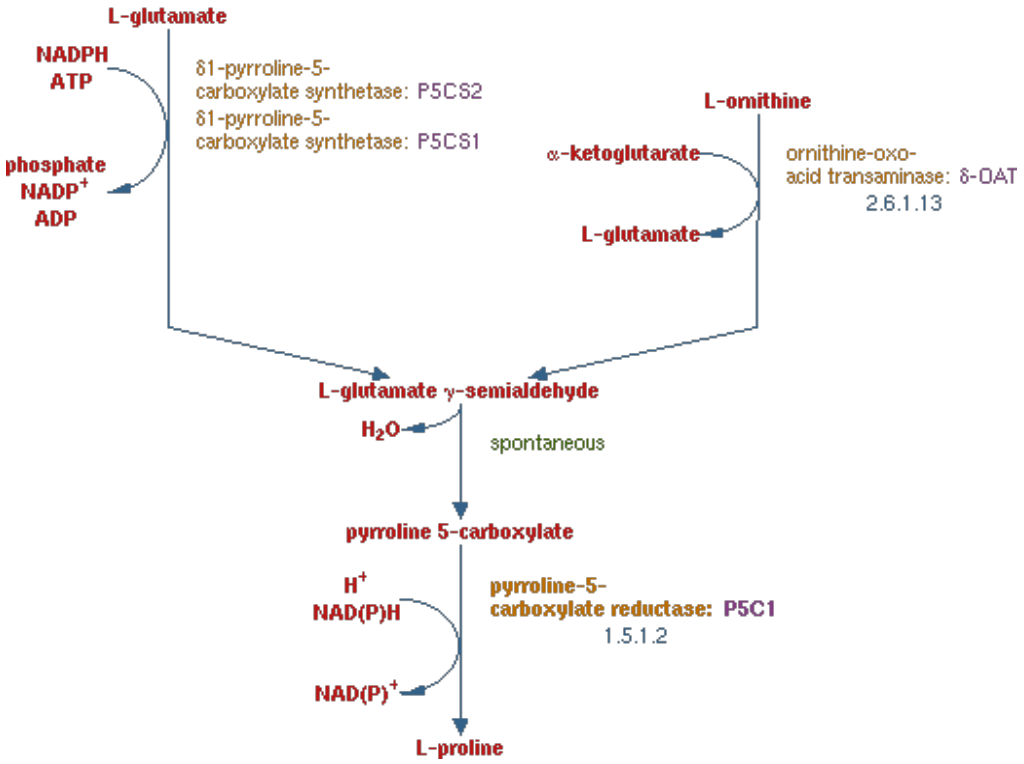



**Fig. 1-17: Proline biosynthesis in plant.**

Recently, a new debate about the possible toxicity of exogenous proline rose, in fact, some plants appeared to be negatively affected while the spread idea that proline is inert and positive is still alive; probably it should be defined the beneficial concentration to apply (Ashraf and Fooland, 2007; Verbruggen and Hermans, 2008). Similar works have been carried out for some key enzymes of the glycine-betaine biosynthetic pathway that starts from choline located in the chloroplast. Many important crops such as rice, potato and tomato, are not able to accumulate glycine-betaine so were good targets for transformations.

The main criticism that has been stated about plant transformation for the production of proline and glycine-betaine is that the amount of osmolytes produced is not really sufficient to mitigate the stress effects (Apse and Blumwald 2002).

#### 1.2.2.1 b MAPK pathway

The MAPKs (mitogen-activated protein kinases) are important enzymes that convert signals from different receptors/sensors activating a wide range of cellular responses in mammals, yeast and plants. In the last years, several components of MAPK cascades have been identified in the plant; through molecular and biochemical studies it was possible to figure out that plant MAPKs play an important role in response to a large range of abiotic stress (wound, temperature, drought, salinity) and biotic (infection by pathogens), but also in hormones signalling and cell division (Zhang et al., 2006).

The protein phosphorylation is one of the main mechanisms for the control of cellular functions in response to external signals. The phosphorylation is a process catalyzed by kinases, enzymes that transfer phosphate groups from high energetic donor molecules, such as ATP, to a specific target molecule (substrate). The purpose of phosphorylation is to activate a molecule, increasing its energy so that it could be able to participate in subsequent reactions. In general, MAPK cascades are characterized by the presence of three functionally related protein kinases. The activation of MAP kinase requires phosphorylation of tyrosine and threonine residues preserved in the so-called "TEY (Thr, Glu, Tyr) activation loop" by the MAPK kinase (MAPKK). In turn, the MAPKK is activated through phosphorylation of threonine residues of conserved threonine and or serine by a MAPKK kinase (MAPKKK) (Bartels and Sunkar, 2005). The activated MAPK can then migrate from the cytoplasm to the nucleus to directly activate transcription factors, or components of the signalling cascade for the regulation of gene expression, protein associated with the cytoskeleton or certain signal proteins for degradation (Xiong and Zhu, 2001). Different MAPK pathways may overlap and share common components, in fact MAPK cascades act as a cross-talk point in stress signalling (Zhang et al., 2006). The specificity is guaranteed by the protein scaffold or specific components of signal cascades (Xiong and Zhu, 2001).

### 1.2.2.1 c Phospholipid signalling

Several pathways are invoked as responses to osmotic stress; beyond MAPK pathway also a variety of phospholipid-based signalling pathways are implicated.

Most cell membranes are made up of two components equally present: lipids and glycoproteins. There are several classes of lipids involved in signalling, such as: phospholipids, glycerolipids, sphingolipids, fatty acids, oxylipins and sterols (Wang, 2004). These molecules have an important chemical and physical common characteristic: they are amphypatic, which means they are made of a hydrophilic portion, represented by the head that interacts with water and by a hydrophobic part, formed by tails. The double layer of membranes is made by interactions of lipid tails in the way to minimize contact with water. The most common lipids in membrane are phospholipids (Fig. 1-18) composed of a charged head (glycerol+ $PO_4^-$ + choline+ ethanolamine/serine) and two hydrocarbon tails that are fatty acids containing 14 to 24 carbon atoms and at least one double bond in *cis* configuration in either tail (Buchanan et al., 2003; Wang 2004).

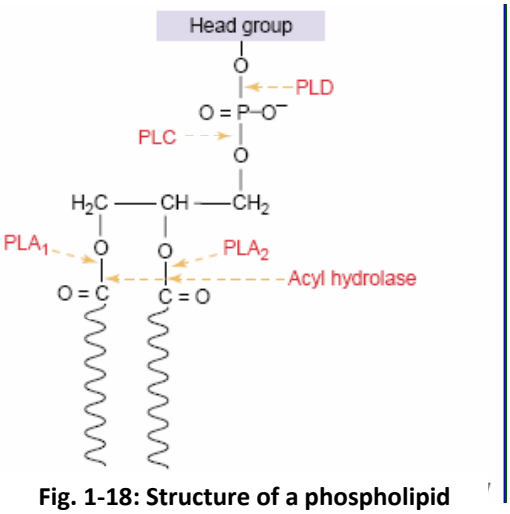



**Fig. 1-18: Structure of a phospholipid with its sites of phospholipid hydrolysis by PLD, PLC, PLA2, PLA1 (Wang, 2004).**

The plasma membrane plays a key role in the perception and transmission of environmental signals (Bartels and Sunkar, 2005). Osmotic stress often leads to a change in membrane fluidity and variations in phospholipids; these have recently been recognized as important events in the mediation of osmotic signals in plants (Munnik and Meijer, 2001).

In the membrane are located also receptors whose activation is often translated directly or indirectly (i.e., via G-proteins) into effector enzyme activity that converts lipids into signalling molecules (Munnik and Meijer, 2001). Phospholipids, in fact, are a dynamic system that generates through cleavages a multitude of signal molecules (IP3, PA, DAG). Phospholipid system, similar to the reactive oxygen species (ROS), is a double-edged sword because, as signal molecules in low

concentrations, the phospholipid messengers are able to activate adaptive responses; while high levels of hydrolysis products of phospholipids may mean stress damage or even be harmful for cells (Zhu, 2002).

The current hypothesis states that phospholipids are cut by enzymes called phospholipases (Fig. 1-19) acting producing secondary messengers. In plants, as well as in other organisms, there are four distinct classes of phospholipases that differ from the target of cutting: phospholipase C, D, A1 and A2 (Wang, 2002; Meijer and Munnik, 2003). Each enzyme (PLA, PLD, PLA1, PLA2) with its action triggers a cascade of signalling that takes its same name. For example, the first phospholipid-based signalling pathway in response to osmotic stress in plant is the PLC pathway. It is launched within minutes in both salt and water stresses and in this activation the "stress hormone" ABA plays an important role as it has been reported for leaf guard cells (Meijer and Munnik, 2003).

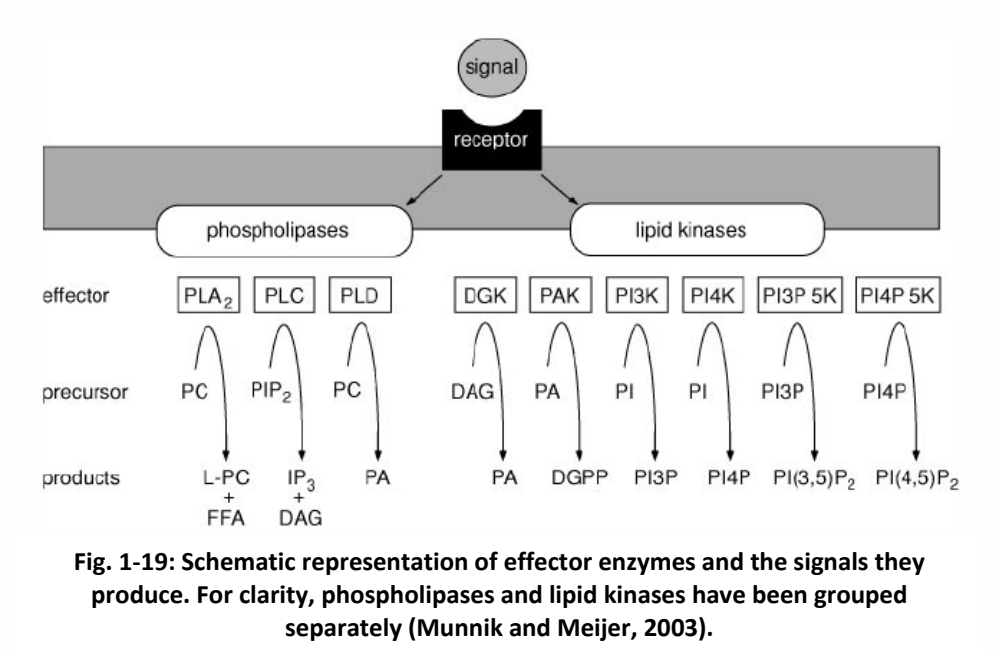



**Fig. 1-19: Schematic representation of effector enzymes and the signals they produce. For clarity, phospholipases and lipid kinases have been grouped separately (Munnik and Meijer, 2003).**

Once PLC pathway is activated, the precursor $PI(4,5)P_2$ is hydrolyzed by phospholipase C in two second messengers: DAG and $IP_3$.

DAG is rapidly converted, through a phosphorylation made by DAK, to phosphatidic acid (PA) which effectively is the real lipid signal. In various plant systems, $IP_3$ levels increase by hyperosmotic stress and this also implies a $Ca^{2+}$ release from intracellular stocks. In fact, IP3 spreads freely in the cytoplasm and binds to specific $Ca^{2+}$ channels in vacuoles and in rough endoplasmic reticulum (ER). The opening of

these channels allows the $Ca^{2+}$ release in the cytoplasm (Schroeder et al., 2001, Xiong and Zhu, 2001; Takahashi et al., 2001; Buchanan et al., 2003). This calcium release during water stresses leads to stomata closure, mechanism of "avoidance" in order to prevent dehydration due to excessive transpiration (Bartels and Sunkar, 2005). PLD has been proposed to participate in cellular events that lead to ABA responses (Sang et al., 2001). Many studies on different species have shown that stress leads to increased PLC transcripts (Bartels and Sunkar, 2005; Meijer and Munnik, 2003) In *Arabidopsis* and *C. plantagineum* (resurrection plant) a rapid activation of PLD in response to stress has been seen as consequence of dehydration. Two PLD genes have been cloned from *C. plantagineum*, a constitutively expressed and the other is induced by dehydration or by treatment with ABA. It was also found increased activity of PLD in cultivars susceptible to stress from drought, and this is inferred that the activation of PLD reflects the disintegration of the membranes during the injury-related stress (Zhu, 2002; Meijer and Munnik, 2003). As PLC, PLD is also involved in the stomata closure, in fact, the suppression by antisense of PLD leads to damage on this mechanism and an increase in the loss of water, while the overexpression leads to a reduction in those losses (Wang, 2002).

Even though the "phospholipid signalling" was included in the osmotic signalling, it has been classified also as part of the "detoxification signalling" because belongs to transmission events downstream the perception of ROS. In fact, PA and $Ca^{2+}$ are able to activate protein kinase OXI1 which in turn leads to induction of a cascade of MAP kinase downstream of which there is the induction of transcription factors that regulate both the "ROS-scavenging" and the "ROS-producing pathway" (Mittler et al., 2004).

Other several mechanisms could be discussed because involved in stress perceptions and lipid signalling, but no more details will be reported in this context.

#### 1.2.2.2 Detoxification signalling

Detoxification signalling is a functional category in the salt and water stress signalling that is focused on stress damage control, repair or detoxification. It includes all the changes that salt and drought stress induce and that are not already categorized as osmotic or ionic signalling. The input signal for the detoxification pathway is not an osmotic or ionic change, but the product of damage caused by stress, such as the formation of reactive oxygen species (ROS) or protein denaturation (Zhu, 2002). The signalling detoxification involves three main aspects: phospholipid signalling (already discussed above), activation of several enzymes involved in ROS scavenging and participation of protective proteins (chaperones) involved in adaptation.

#### 1.2.2.2 a ROS scavenging

The nature of damage that high salt concentrations inflict to plant is not entirely clear yet. It is well known that toxic effects of salinity can affect the integrity of cell membranes and the photosynthetic systems, can involve the activation of several enzymes and alter the nutrients (Zhu, 2001a). One aspect directly linked to salt stress in plants is the induction of ROS formation; a collective term to define all the species radical or not but all reactive originated by $O_2$, such as superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), hydroxyl radical (OH•), singlet oxygen and nitric oxide (NO). The first three elements are able to convert to one another (Quan et al., 2008).

ROS arise during stresses because of alterations in mitochondria and chloroplasts metabolisms. Plant mithocondria probably are the main compartments inside the cell involved in the production of $H_2O_2$, this because they are associated with physiological processes in aerobic conditions. But also chloroplasts are a major source of ROS, because of their intense rate of electron flow and oxygen in high concentration and in this compartment ROS detoxifying mechanisms are essential to protect photosystems from photoinhibition (Munns and Tester, 2008). In addition with peroxisomes, are responsible for the oxidative load inside cells during abiotic stresses (Breusegem et al., 2001; Apse and Blumwald, 2002; Wang, 2003; Parida and Das, 2005; Bartels and Sunkar, 2005; Quan et al., 2008).

Recently, it has been clarified that ROS can have a dual function as toxic compounds as well as regulators and signals for critical biological processes such as growth and development, programmed cell death, cell cycle and hormone signalling. This aspect on complex ROS role in plant processes leads to think that a fine regulation between ROS production and ROS scavenging is essential; in fact in *Arabidopsis* more than 150 genes are considered involved in this management, but knowledge of coordination and cross-talk between this network is still partial (Miller et al., 2008; Shao et al., 2008a; Munns and Tester, 2008). The dark side of ROS is linked to the high reactivity of free radicals that are able to oxidize and damage compounds essential for the cell like, lipid membranes, proteins and nucleic acids. To face this problem the plant exploits the action of antioxidant enzymes such as: superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione-S-transferase (GST) and glutathione peroxidase (GPX) to remove ROS from different cell compartments (Apse and Blumwald, 2002; Munns, 2005). In addition, also non-enzymatic antioxidants are important, for example tocopherols, ascorbic acid and glutathione (Quan et al., 2008). These antioxidants are activated in conditions as salinity, drought, but also during other stresses such as those related to extreme temperatures; they also function for limiting the photo-oxidative damage and detoxifying the excess of ROS during stress compared to those normally produced and necessary for the signal transduction and metabolism of plant (Parida and Das, 2005; Munns, 2005).

Among the protective molecules also the compatible osmolytes play a role, because at high concentrations they act in the osmotic adjustment as mentioned before, instead at low concentrations are involved in cellular protection against ROS and for this reason are termed “osmoprotectors” (Bartels and Sunkar, 2005; Munns, 2005). Transformation to produce plants overexpressing antioxidants can be adopted not only to get salt tolerance, but also to cold, freezing, heat and drought; all these stresses in fact determine oxidative stress with ROS production. From a certain point of view, engineering that has the detoxification as target is an appropriate way to obtain plants tolerant to multiple stresses, on the other hand certainly they lack of specificity for a particular stress (Zhu, 2001a, Wang et al., 2003).

#### 1.2.2.2 b Chaperone proteins

To cope with all the environmental stresses, plants also activate a broad set of genes that lead to the accumulation of specific proteins associated with stress; these are accumulated immediately after stress such as water, salt and high temperatures. As mentioned before, abiotic stresses in general lead to cellular dysfunction of enzymes and proteins. So in stress conditions it is particularly important to guarantee the maintenance of proteins in their functional conformations and preventing the aggregation of non-native proteins.

Two of the main types of proteins related to stress with particular importance as protectors during stress are:

- Heat-shock proteins;
- Late embryogenesis abundant (LEA)-type proteins (Wang et al., 2003).

In particular the "heat shock proteins" (Hsps) act as molecular chaperones (Fig. 1-20) whose function is to help structural and active proteins to fold back to their original and functional conformation previously lost by stress that leads to protein denaturation (Wang et al., 2004; Vinocur and Altman, 2005). Among the five families of Hsps, the small heat shock proteins (sHsps, 12 and 40 kDa) are ubiquitous in nature and are the most represented in the plant (Vierling, 1991; Buchanan et al., 2003).

In higher plants have been identified six nuclear gene families coding for sHsps which are then addressed to different cellular compartments, such as chloroplasts, cytosol, mitochondria and endoplasmic reticulum. This diversification in the location of sHsps is unique only for plants; in fact it was not found in any other eukaryotic organisms (Waters et al., 1996; Sun et al., 2002). Several studies have shown that sHsps are expressed not only in

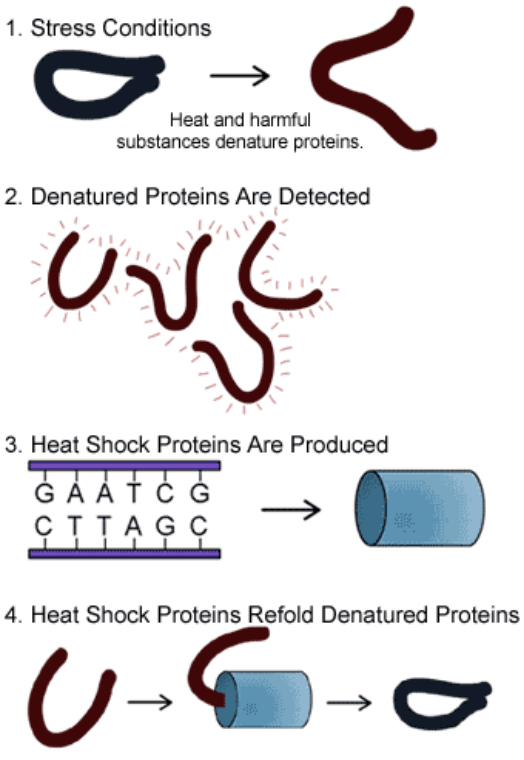



**Fig. 1-20: A heat shock protein in its role of molecular chaperone.**

response to heat stress, but also as a result of water, oxidative, cold and salt stress (Hamilton and Heckathorn, 2001; Wang et al., 2003-2004). Recently, Hamilton and Heckathorn (2001) demonstrated that small "heat shock proteins" act as antioxidants, protecting the complex I of electronic transport within mitochondria during salinity stress. A heat-shock protein with high molecular weight, Hsp70, belonged to a salt resistant cyanobacterium was used to transform tobacco plants; an increase in tolerance to salt was reported based on the observation that the rate of $CO_2$ fixation was approximately 85% compared to 40% of wild-type (Sugino et al., 1999).

The "late embryogenesis abundant (LEA)-type proteins" are another group of protective proteins expressed during the embryo maturation in all angiosperms, also they have been found in many plant species in response to drought, cold or osmotic stress (Wang et al., 2003; Bartels and Sunkar, 2005). LEAs have been identified for the first time in cotton seeds (Dure et al., 1981) and they represent the dominant protein in mature embryos. The accumulation of these proteins during embryogenesis is linked to increased levels of ABA and the acquisition of desiccation tolerance. LEAs are not expressed constitutively in plant tissues, but are induced by osmotic stress and by exogenous ABA. The classification, based on the molecular weight of these proteins, suggests five different groups, each with structural and functional different traits (Wang et al., 2003), but this organization in fact does not seem so clear and some groups might be considered part of others (Bies-Ethève et al., 2008).

Some LEAs characteristics common to all groups are:

- Secondary structure formed by α-helix random coil with repeated motifs,
- Strong hydrophilicity common to other proteins in response to osmotic stress,
- Stability to heat,
- Expression is regulated by ABA (Wang et al., 2003; Bartels and Sunkar, 2005).

Some studies have shown that certain specific LEA proteins are not responsible for desiccation tolerance, or that their individual presence is not sufficient to prevent the damage (Xu et al., 1996). Indeed it was noted that the multi-expression of several LEA proteins is related to stress tolerance so that it is assumed that these proteins work in synergy with others (Wang et al., 2003). Recently is has been

detected the entire *Arabidopsis* genome to find out LEA proteins genes using cotton LEAs as reference sequences; 50 genes were discovered and classified in 9 groups, this study confirms the variability and dynamicity of these LEA proteins and the presence of tandem duplication as well as modifications whose conservation suggests their beneficial effect on plants (Bies-Ethève et al., 2008; Dalal et al., 2009). Another study carried out by Dalal and co-workers (2009) identified in *Brassica napus* an abiotic stress and ABA-inducible LEA 4-1 gene as the highest expressed ABA-inducible genes in vegetative tissues. *Arabidopsis* overexpressing BnLEA 4-1 under constitutive or inducible promoters demonstrated enhanced tolerance to salt stress and drought (Dalal et al., 2009).

LEA proteins belonging to Group II are certainly the most famous and are known as dehydrins. They are characterized by some serine residues, a block rich in lysines and a conserved domain N-terminal and with those belonging to Group IV contribute to maintaining proteins and membranes structures (Bartels and Sunkar, 2005). Group II LEA was shown to confer stress tolerance to transgenic rice, *Arabidopsis* and potato (Dalal et al., 2009).

#### 1.2.2.3 Ionic signalling

The aim of the ionic signalling, as part of the big mechanism to achieve salt tolerance, is to re-establish and maintain an ion homeostasis (Zhu, 2002). The complexity of plant response to saline stress is explained by the fact that salinity, in addition to osmotic stress, implies also the salt toxicity (Bartels and Sunkar, 2005). To be precise, in this section the focused key ion will be $Na^+$, instead of $Cl^-$, even if in some species (citrus, grapevine and soybean) the second one is considered to be the more toxic ion (Munns and Tester, 2008). In addition, having the plant cells negative electrical potential, sodium can enter the cell passively while chloride cannot unless the cytosolic concentration is very low (Munns, 2005).

In a highly saline environment, $Na^+$ competes with $K^+$ and alters the normal potassium absorption resulting in toxicity risks for cells, because more than 50 enzymes are potassium-activated and this function can be replaced by sodium (Blumwald et al., 2000; Tester and Davenport, 2003). Besides an excess of sodium in the cell cytoplasm may lead to the inactivation of several enzymes essential for cell

metabolism, because of perturbation of protein structure (cytoplasmic toxicity); for this reason cell should prevent the accumulation of this ion inside the symplast. Mechanisms to achieve this purpose at cellular level are mainly three:

1) Restricting of sodium uptake across the plasma membrane,
2) Compartmentalizing the sodium into vacuoles,
3) Promoting the extrusion of this ion (Shi et al., 2002a; Ashraf, 2004; Bartels and Sunkar, 2005).

Moreover, thinking about the whole plant, others mechanisms to minimize the damage from high salinity can be added; so plant can also:

4) Minimize loading to the xylem (root to shoot) or maximize retrieval before reaching shoot,
5) Maximize recirculation by phloem of sodium out of shoots,
6) Exude salts on the leaf surface or allocate in particular part of shoots (old leaves or glands).

At the core of these mechanisms a key role is played by the ion transporters, which are crucial determinants in salt tolerance. Coming after will be discussed the sodium dynamics in cell (Fig. 1-21) such as its entrance, extrusion and compartmentalization (Tester and Davenport, 2003).

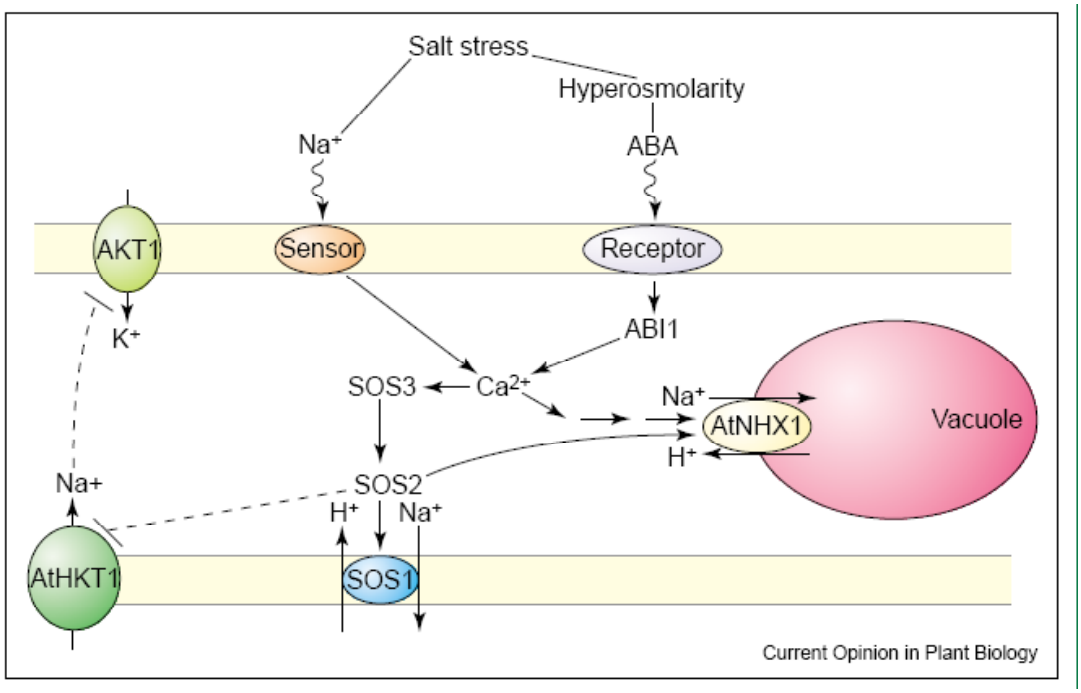



**Fig. 1-21: Sodium dynamics in cell such as its entrance, extrusion and compartmentalization and interaction in $Na^+$ homeostasis (Zhu, 2003).**

#### 1.2.2.3 a Sodium entrance into cell

Under normal physiological conditions, the plant cell maintains a high ratio potassium/sodium in the cytosol with relatively high concentrations of $K^+$ (100-200 mM) and low of $Na^+$ (1-10 mM). Because of the negative membrane potential (-140 mV) characteristic of the plasma membrane, an increase in extracellular $Na^+$ concentrations leads to a high electrochemical gradient that facilitates the passive transport of sodium from the outside into the cytosol (Blumwald et al ., 2000, Zhu, 2003). The restriction of sodium in root cells or into the xylematic flow is one of the ways to maintain an optimum in the $K^+/Na^+$ ratio in the cytosol during salt stress conditions (Chinnusamy et al., 2005). The uptake of sodium through the apoplastic pathway is conditioned by the radical development and deposition of silica in the cell wall (Zhu, 2003).

Under salt stress, sodium earns entry into the cytosol through high-affinity $K^+$ transporters (HKT), non-selective cation channels (NSCC) and low-affinity cation transporters (LCT1). In *Arabidopsis,* as well as eucalyptus and wheat, it was shown that high affinity $K^+$ transporters (HKT) act as low-affinity $Na^+$ carriers in saline conditions, so this demonstrate the reliability of the original hypothesis made by Epstein et al. (1963) that the potassium transporters in entrance (Fig. 1-21) are also able to carry in sodium (Horie and Schroeder, 2004; Botella et al., 2005; Rodriguez-Navarro and Rubio, 2006). It has been clarified that HKT transporters possess a bivalent operating mode: as $K^+/Na^+$ symporters and as $Na^+$ transporters, depending on the presence of a specific amino acid (Ser and Gly, respectively) in the "pore loop" (Horie and Schroeder, 2004).

A study carried out by Rus and co-workers (2001) on *Arabidopsis* demonstrated that a mutation of AtHKT eliminates hypersensitivity of *sos3* mutants; this means that the wild-type SOS3 is able to inhibit AtHKT activity as transporter of incoming sodium (Bartels and Sunkar, 2005). The inactivation of HKT through mutation or suppression of its expression by RNA antisense can significantly contribute to the achievement of salt tolerance in plants (Chinnusamy et al., 2005).

Moreover, in the root plasma membrane there are non-selective cationic channels (NSCC) that contribute to the entrance of sodium in the plant, although their molecular identity remains unknown (Bartels and Sunkar, 2005). The flows of

sodium mediated by NSCC are partially sensible to calcium and calmodulin, this could correlate with the inhibition of $Na^+$ entry into roots by calcium; it is uncertain if calcium regulation of NCSS activity is direct or indirect via intracellular regulatory proteins (Zhu, 2003).

The fact that in saline condition, sodium is necessary as vacuolar osmolyte may be the reason why the plants have not evolved transport systems that exclude $Na^+$ for advantaging $K^+$ (Hasegawa et al., 2000).

#### 1.2.2.3 b Sodium compartmentalization

In plants, the accumulation of ions and osmolytes in the vacuole represents a strategy to maintain the ion homeostasis. $Na^+$ compartmentalization in plants is an essential mechanism in salt tolerance; this is supported by evidences such as enzymes in halophytes plant are not more salt resistant than the corresponding enzymes in glycophytes (Munns and Tester, 2008).

Plant cell contain at least two types of vacuoles: storage and lytic they are classified according to their soluble proteins and the kind of aquaporins located in the membranes. Usually, compounds that damage plant metabolism are firstly removed by small vacuoles that possess higher surface-to-volume ratio and later transfer to the larger central vacuole (Martinoia et al., 2007). The vacuolar sequestration of sodium, not only decreases the concentration of this ion inside the cytosol, that when more of 100 mM disturbs the normal cell functioning, but also contributes to osmotic adjustment to ensure the water uptake of the plant in saline conditions as adaptative response. Also other organelles such as plastids and mitocondria can accumulate sodium and contribute in this way to the OA (Zhu, 2003; Botella et al., 2005).

The sodium transport into vacuoles is mediated by $Na^+/H^+$ antiporter, which in turn is driven by protons electrochemical gradient generated by enzymes as $H^+$-ATPase (V-ATPase) and $H^+$-PPiase (V-PPase). The ionic transport mediated by antiporters or symporters are active, because the ion to be transported must move against the electrochemical gradient; for achieving this, energy is necessary and it can be obtained mainly by ATP hydrolysis (Blumwald et al., 2000; Munns, 2005). This energy-dependent transport has, as direct consequence, the vacuolar alkalization

(Hasegawa et al., 2000). It has been reported that the main strategy of salt tolerance for *Sueda salsa* (halophyte) is the upregulation of V-ATPase activity to energize the vacuolar membrane, while V-PPases are less important (Parida and Das, 2005). On the other hand, in the glycophyte *Arabidopsis* overexpressing the vacuolar $H^+$-PPase (AVP1) a higher salt tolerance was confirmed probably thanks to the increased "driving force" (proton gradient) (Gaxiola et al., 2001).

A significant accumulation of sodium inside vacuoles can be possible only if a pH gradient is established through the tonoplast. In some species, the $Na^+/H^+$ antiporter is constitutive, while in others plants such as *Beta vulgaris* the antiporter constitutively active is also upregolated by high NaCl concentrations. The salt-sensitive plants rely mainly on sodium exclusion strategy at plasma membrane level, while the salt-tolerant species accumulate large amounts of sodium in the vacuoles. In support of this, the example of *Plantago* species that shows $Na^+/H^+$ antiporter activity only in salt-tolerant species (*Plantago maritima*), while in the sensitive one (*Plantago media*) any activity was not detected. It is important to note that the major tonoplast proton pumps ($H^+$-ATPases) increase their activity in response to sodium, while the $H^+$-PPiases are inhibited by high NaCl concentrations (Blumwald et al., 2000).

#### 1.2.2.3 c Sodium efflux

The exit of sodium from root cells prevents the build up of $Na^+$ to toxic levels in the cytosol and the transport of it to leaves. Molecular-genetic analyses of *sos* mutants of *Arabidopsis* have identified a $Na^+/H^+$ antiporter localized in the plasma membrane (SOS1) whose crucial role is to expel sodium from the cytosol of epidermal root cells under saline conditions (Chinnusamy et al., 2005; Bartels and Sunkar, 2005). The SOS1 activity is finely regulated by the SOS pathway and is assumed to be also a sodium sensor in plant; these aspects will be discussed later.

SOS1 was the first determinant in salt tolerance (SOS pathway) to be identified; this was performed by mapping the phenotype overly sensitive, *sos1-1* mutant identified by Wu and co-workers through the "root bending assay" (as described in paragraph 1.1.5.1-2). The secondary active $Na^+/H^+$ antiporter in the plasma membrane, similarly to that of tonoplast, functions thanks to the help of a protonic

pump ($H^+$/ATPase) because in higher plants sodium pumps ($Na^+$/ATPase) have not been found yet, while they are present in algae (Shi et al., 2000; Blumwald et al., 2000, Hasegawa et al., 2000; Apse and Blumwald, 2007). A comparison between $Na^+/H^+$ activity in plasma membrane vesicles extracted from *Arabidopsis* wild type roots against *sos1* mutant plants showed that this activity decreases in *sos1* mutants. This means that this mutant maintains a $N^+/H^+$ exchange activity due probably to the action of other possible exchangers. For example, in *Arabidopsis* a CHX family exists and is composed by 28 members of cation/proton exchangers that could be responsible for this sodium efflux activity in *sos1* mutant (Apse and Blumwald, 2007).

From SOS1 promoter-GUS gene fusion experiments in *Arabidopsis* the expression of this gene was clear; it is mainly expressed in epidermal cells at the root tip and in parenchyma cells at the xylem/symplast boundary of roots, stems and leaves (Fig. 1-22). This suggests that SOS1 has an important role in regulating the sodium transport in long distance, acting in loading/unloading xylem (Shi et al., 2002a). In next paragraphs it will be discussed SOS1 roles in whole plant and its function as key part of SOS pathway.


**Fig. 1-22: SOS1 promoter-GUS gene fusion experiment in *Arabidopsis*; SOS1 has an important role in regulating the sodium transport in long distance, acting in loading/unloading xylem. A) SOS1 is expressed in vascular system; K) detail of SOS1 expression in root tip (Shi et al, 2002a).**

#### 1.2.2.4 Whole plant: root to shoot $Na^+$ transport and xylem loading/unloading

Two mechanisms exist for $Na^+$ tolerance in plant:

1) the tolerance of a single cell to high NaCl concentration that involves all those activities mentioned before (compartmentalization, detoxification etc..);
2) the tolerance of the whole plant that involve mainly the regulation of long-distance transport and spots of sodium accumulation.

Both mechanisms are essential and, for the whole plant, their relative importance is related to species and conditions. This distinction is necessary because several examples prove that from salt-tolerant cells rarely it is possible to regenerate fertile salt-tolerant plants with hereditable salt tolerance; so it is clear that entire plant can also have a broad range of other mechanisms that imply the involvement of specific cell types.

These mechanisms manage the sodium uptake and its distribution inside the plant, through different steps that will be analyzed (Tester and Davenport, 2003; Botella et al., 2005).

a) Regulation of $Na^+$ transport to shoot

Some strategies linked to the acquiring of tolerance under salt stress are: maintaining low $Na^+$ content in shoots, because it was observed that growth and sodium amount in shoots are inversely correlated and also keeping $K^+$ at high levels; sometimes, in some species, it is more important the maintenance of a high $K^+/Na^+$ ratio. $Na^+$ uptake from soil happens spontaneously in root cortical cytoplasm because this ion is energetically favoured. High unidirectional influx of $Na^+$ occurs mostly through non-$Na^+$-selective mechanisms like an “accidental” process (Tester and Davenport, 2003).

An important concept is that the “net sodium accumulation” is the difference between passive influx and active efflux in plant. After several experiments it appeared clear that unidirectional influx of $Na^+$ occurs at high rates, but this does not imply a fast accumulation of $Na^+$. In fact the sodium content in roots remained quite constant during time and the accumulation of $Na^+$ happened slowly and this is the proof of the existence of $Na^+$ efflux (energy-dependent) through the plasma

membrane. Halophytes in comparison with glycophytes demonstrated to control the sodium transport better than the others, essentially regulating and preventing the initial $Na^+$ entry.

In plant, three pathways for $Na^+$ influx exist:

- $Ca^{2+}$ sensitive pathway

  It is noteworthy that addition of calcium in external solution can ameliorate the toxic effects of sodium; it acts at least partially reducing the $Na^+$ accumulation in the roots and shoots and supporting $K^+$ accumulation in the whole plant. The effect of $Ca^{2+}_{ext}$ on sodium and potassium transport was accredited to the activity of the SOS pathway (all the functioning of this pathway will be along discussed). It has been supposed and also argued about the possibility that the $Na^+$ influx $Ca^{2+}$-sensitive pathway was represented by non-selective-cation channels whose encoding genes are still uncertain. Two possible candidate genes for NSCC can be: the cyclic nucleotide-gated channels (CNGCs) and the putative glutamate-activated channels (Tester and Davenport, 2003; Munns and Tester, 2008).

- $Ca^{2+}$ insensitive pathway

  Since the $Na^+$ inhibition by calcium, above mentioned, was partial, evidence suggested that a possible $Ca^{2+}$ insensitive pathway could involve again NSCC. However other pathways are listed in this group, such as: HKT1, but also inward rectifying $K^+$ channels (KIRCs) and KUP/HAK family of $K^+/H^+$ symporters (Tester and Davenport, 2003; Botella et al., 2005).

- Bypass flow

  This third pathway seems to be due to a leakage in roots system that implies the circulation via apoplast. Experiments using apoplastic fluorescent dye in rice demonstrated that plant with high $Na^+$ amount in shoots also have high apoplastic water flow. So in this case most of the $Na^+$ does not pass through membranes, but instead enters by “leaks” in endodermis (particularly in Casparian barrier); this phenomenon occurs mainly in root branch points and root apices. It has been reported that halophytes have developed ways to

minimize the apoplastic entry like some anatomical modifications (i.e. Casparian band thickening) (Tester and Davenport, 2003; Botella et al., 2005).

$Na^+$ efflux out of the roots is determinant to estimate the net $Na^+$ accumulation into roots cortical cells and it is mainly performed by SOS1 antiporter activity (already mentioned before). This transport can work in both directions, so as efflux as well as influx, depending on the $Na^+$ concentration of the surrounding environmental; but mostly it acts to pump $Na^+$ out of cells (Munns and Tester, 2008).

It has been suggested the existence of a "set point" in roots able to regulate $Na^+$ content and net uptake in roots; so that roots can perceive $Na^+$ levels and control properly transporters. Roots can utilize a bidirectional $Na^+$ efflux: out from the roots to soil solution and into the xylem to be transported to shoots. The accumulation of sodium in the shoots really depends on the $Na^+$ influx at roots levels although it does not show effect on root sodium concentration. In conclusion, very small differences in transport activity can imply big differences in shoot $Na^+$ accumulation (Tester and Davenport, 2003).

b) Control of xylem loading

Sodium transport to shoots is still weakly understood (Davenport et al., 2007).

In order to prevent accumulation of $Na^+$ in shoots and to maintain low concentration of it in the xylem, it is possible to minimize the entrance of sodium into the xylem from the root symplast or to promote the retrieval back out of xylem before $Na^+$ reaches shoots. The net delivery of sodium to xylem can be distinguished into four steps:

1) $Na^+$ influx into cells in the outer part of the root (cortex);
2) $Na^+$ efflux back out from cortex cells to soil solution;
3) $Na^+$ efflux from the cells in the inner part of root (stele) to xylem;
4) $Na^+$ influx back from xylem to these cells in stele part, before $Na^+$ is delivered to shoots.

And in order to reduce the delivery of $Na^+$ to shoots, steps 1 and 3 should be diminished, while steps 2 and 4 must be maximized; and to support this purpose

plasma membrane transporters would need to work in the opposite direction (Tester and Davenport, 2003; Munns and Tester, 2008).

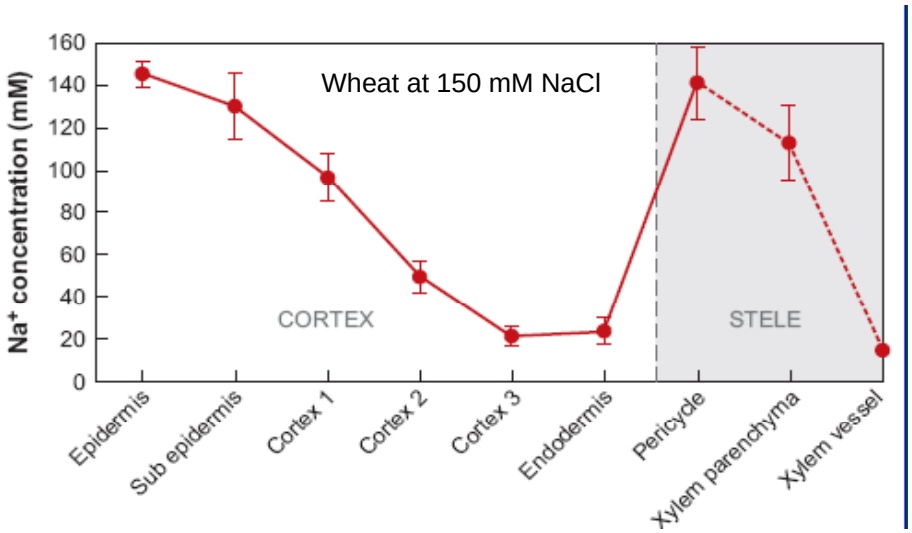



**Fig. 1-23: Graph shows that $Na^+$ content in vacuoles diminishes across the cortex root of wheat under 150 mM NaCl (Munns and Tester, 2008).**

At this point the $Na^+$ that remain in the root can be sequestered by vacuoles, like described above, or again translocated to shoots. Evidences reported that $Na^+$ content in vacuoles diminishes across the cortex root; in fact it is high in epidermis and sub epidermis and falls in endodermis (Fig. 1-23) (Munns and Tester, 2008).

The vacuolar compartmentalization suffers of passive $Na^+$ leakage back to cytoplasm, so it needs to be always active in order to keep $Na^+$ ions sequestrated.

SOS1 was reported to be expressed at the xylem symplast boundary of roots and to be involved in $Na^+$ efflux into xylem; but this aspect does not match with the observation that knocking-out this gene, $Na^+$ shoot level increased instead of decrease as expected. This can be explained still considering roots dividing in two parts; probably the knock-out of this gene function in the outer part of the root has more weight than the SOS1 knock-out in the inner part of it (Munns and Tester, 2008). To fully understand the SOS1 situation and this difficult aspect of xylem loading, also the retrieval from xylem should be considered (see below). Shi and colleagues (2002) proposed that under moderate salinity (25 mM), SOS1 could work in the direction of $Na^+$ loading into xylem; while at severe salt conditions could function in limiting net $Na^+$ uptake (Shi et al., 2002a; Botella et al., 2005). Another hypothesis suggests more easily that $Na^+$ loading into xylem can be active at low NaCl concentration, while can be passive at high level of salts. It is also important to

evaluate and know the energetics of $Na^+$ transport into xylem in order to understand if the movement is active or passive depending on pH of compartments, stoichiometry of $Na^+/H^+$ exchange and level of depolarization of membranes (Tester and Davenport, 2003).

For all these aspects to take in consideration, it is quite evident how the $Na^+$ loading into xylem is still an uncertain topic that needs to be further deepened to better reconcile all the knowledge on salt tolerance.

c) Retrieval from the xylem

Sodium retrieval from xylem is a possible mechanism that acts to limit the $Na^+$ accumulation into shoots. Shi and co-workers (2002) suggested that SOS1 can also work as $Na^+$-scavenging mechanism at the root xylem-symplast interface at high NaCl concentrations, but this situation seems to be extremely unlikely because it would imply a high concentration of $Na^+$ into xylem or a different stoichiometry of SOS1 exchange (Tester and Davenport, 2003; Munns and Tester, 2008).

Some others candidates have been proposed to play in the retrieval from xylem such as HKT family genes. In *Arabidopsis,* in fact, AtHKT1 is involved in this role, unloading sodium directly from xylem vessels to xylem parenchyma cells, before $Na^+$ reaches the shoot (Sunarpi et al, 2005). In particular, using radioactive tracers to detect $Na^+$ movements in *Arabidopsis*, it was possible to elucidate that AtHKT1;1 contributes to control of both $Na^+$ root accumulation and retrieval from xylem (Davenport et al., 2007).

An interesting aspect to investigate is where $Na^+$ goes after retrieval from xylem, and some answers can be: in vacuolated cells as in mature roots or at the stem base or along the stem in plants that have elongated stems. The possibility to remove $Na^+$ from the transpiration stream and accumulate in strategic points in plants can imply the protection of the growing tissues (Tester and Davenport, 2003).

d) Recirculation in the phloem

The recirculation of $Na^+$ trough phloem from shoots to roots has been reported yet in several species, such as lupine, sweet pepper and maize. The hypothesis is that the extent of recirculation can be directly proportional to plant tolerance to salinity;

this was reported in *Licopersicon pennelii* (salt-tolerant tomato wild species) comparing its recirculation with a cultivated tomato (Perez-Alfocea et al., 2000). Contrarily to the original view, recirculation via phloem (Fig. 1-24) can be a really important aspect in the whole plant system to achieve salt tolerance, but its mechanism and control need further studies (Tester and Davenport, 2003).

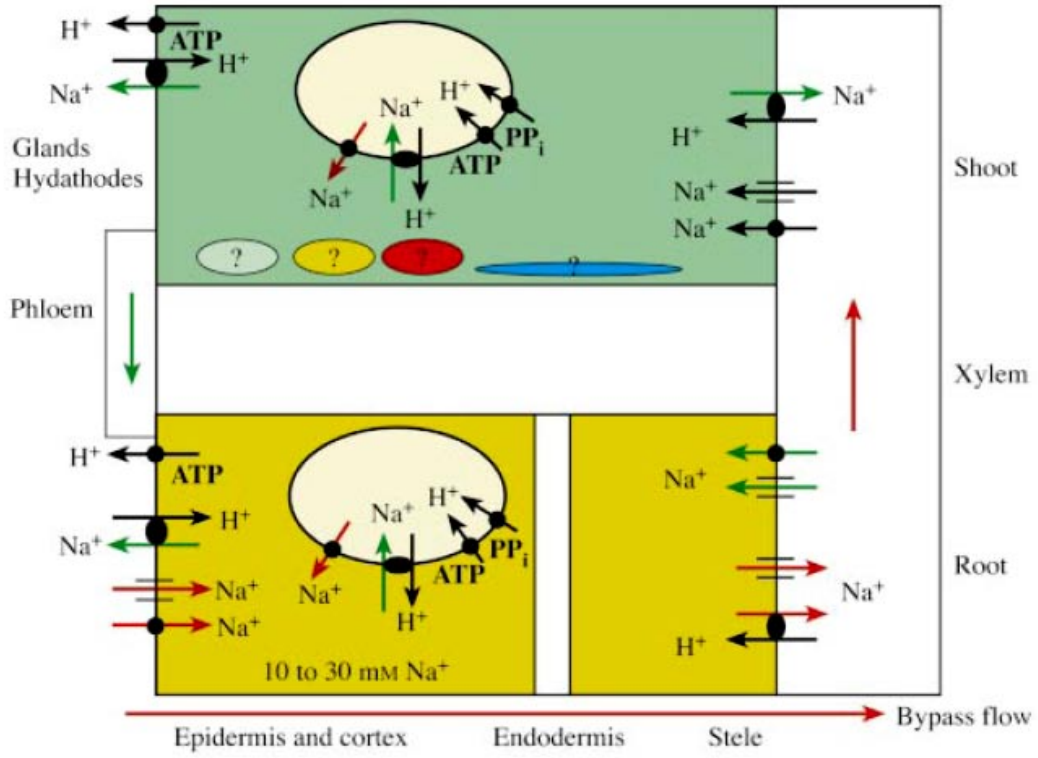



**Fig. 1-24: $Na^+$ transport processes influencing $Na^+$ tolerance in higher plants. Red arrows indicate $Na^+$ movement, the minimization of which would increase tolerance; green arrows represent $Na^+$ movements, the maximization of which would increase tolerance. The coloured shaped in the leaf represent chloroplasts (green), mitochondria (orange), peroxisomes (red) end endoplasmic reticulum (dark blue). $Na^+$ transport processes into and out of these organelles is unknown. Vacuoles are represented by light blue shapes (Tester and Davenpot, 2003).**

An example was proposed by Berthomieu and colleague (2003) that analysed the redistribution of $Na^+$ via phloem, finding out the HKT involvement by facilitating loading of $Na^+$ into phloem and unloading in the roots (Berthomieu et al., 2003; Botella et al., 2005). These hypotheses (Berthomieu et al., 2003; Sunarpi et al., 2005) about the role of AtHKT1;1 in $Na^+$ transport were established following results of tissue ion contents or of disruptive measurements of phloem and xylem contents of plant grown or in agar plates or in soil. These experimental differences make it complicate to evaluate results, so that Davenport and colleagues (2007) built up a new experiment with *Arabidopsis* plants in hydroponic condition in order to

standardize conditions. Moreover they used radioactive tracers to detect $Na^+$ movements in intact transpiring plants, coupled with mathematical modelling; this experiment concluded that AtHKT1;1 does not play in recirculation via phloem neither in $Na^+$ influx into roots, but it seems to manage $Na^+$ retrieval from xylem and root vacuolar loading (Davenport et al., 2007).

## 1.3 SOS pathway

The *sos* mutants are the origin of the discovery of the so termed "SOS pathway"; they showed a increased sensibility to NaCl and LiCl but not to mannitol, so that they appeared salt specific and not osmotic (Tester and Davenport, 2003). So the SOS genes characterization gave a great improvement to the knowledge about ion homeostasis and salt tolerance in plants. These genes in order to work together in the homonymous pathway need a fine regulation each other; as follow, each gene involved will be briefly discussed.

### SOS3

This gene encodes a protein binding $Ca^{2+}$, able to perceive the signal of cytosolic calcium elicited by salinity stress. SOS3 has a sequence homology with other calcium binding proteins with EF-hand motifs; such as: yeast B subunit of calcineurin (CNB) (Mendoza et al., 1994) and neuronal mammals' calcium sensors (NCS) (Mendoza et al., 1994; Zhu et al., 2002) and for this reason is also recently named as CBL4 (calcineurin B-like protein). A mutation in B subunit of calcineurin leads to the same reaction of *sos3* mutant, which is the hypersensitivity to $Na^+$ and $Li^+$. This information suggests that SOS3 may be the calcineurin in plants. SOS3 has four sites to bound calcium, each called "EF-hand" that is a helix-loop-helix structure that can accommodate $Ca^{2+}$ ions through a link involving 12 amino-acidic residues (Branden and Tooze, 2001; Sanchez-Barrena et al., 2005). Compared to other $Ca^{2+}$ sensors like calmodulin, SOS3 binds calcium with low affinity which can be a distinguishable feature of $Ca^{2+}$ sensors (Mahajan et al., 2008).

The deletion that characterized *sos3* mutant is located in a highly conserved region and most likely it disables the third site (EF3) of $Ca^{2+}$ binding (Liu and Zhu, 1998; Zhu, 2000, Bartels and Sunkar, 2005). A recent study carried out by Sanchez-Barrena

and colleagues (2005) was able to achieve the X-ray SOS3 protein structure (resolution 2.75 Å); and so protein appeared composed by two domains connected by a short linker, the protein dimerization depends on free-calcium-concentration. Another SOS3 characteristic is its N-term myristoylated motif; the myristoylation is a post-translational modification that links molecules of myristic acid to a glycine residue of the protein and plays a key role in membrane targeting, lipid metabolism and signal transduction in response to abiotic stress (Zhu et al., 2002). Ishitani and clleagues (2000) showed that the myristoylation is required for SOS2 activation, but not for the association to membranes in plant cells. For this particular function, SOS3 has 5 lysine residues that are sufficient for its anchorage to the membrane, perhaps the myristoylation assists for the proper protein positioning relative to the membrane, for instance nearby calcium channels in order to facilitate $Ca^{2+}$ signalling (Ishitani et al., 2000; Zhu, 2000; Sanchez-Barrena et al., 2005). Mutations affecting both myristoylation and calcium binding sites lead to obtain *Arabidopsis* plants hypersensitive to NaCl stress; it can be inferred that both SOS3 characteristics are essential for its tolerance to salt stress (Ishitani et al., 2000). The C-term of SOS3 instead is involved in physical interaction with SOS2 and its subsequent activation, this contact has been confirmed both "*in-vitro*" and by the "yeast two-hybrid system" (Young, 1998; Halfter et al., 2000). In conclusion, SOS3 is a small myristoylated $Ca^{2+}$ sensor without proper enzymatic activity, but it is essential for the activation of SOS2 and then of the complex SOS3-SOS2 with kinase activity; nevertheless it also negatively regulates the AtHKT1 activity (Zhu, 2003; Gong et al., 2004).

**SOS2**

SOS2 gene encodes a Serine/Threonine type protein kinase that was deeply studied in *Arabidopsis* and has got a central role in sodium homeostasis and salt tolerance. SOS2 is made of two domains, a N-term catalytic domain that acts as a kinase, the other at the C-term is a regulatory domain; both are essential for the functioning of SOS2 in salt tolerance (Liu et al., 2000). SOS3 works activating the kinase domain of SOS2 in a calcium-dependent manner; in fact also double-mutants-analysis of *sos2sos3* indicated that the two proteins operate within the same pathway.

SOS2 is in its steady-state inactive, but is activated by the specific link with SOS3 through the FISL motif that corresponds to a 21 amino acids sequence present in the SOS2 regulatory domain and takes its name from the most conserved amino acids in SOS2-like proteins (Guo et al., 2001; Gong et al., 2004).

Furthermore, the FISL motif is also involved in the link between regulatory domain and kinase domain, in fact they interact to maintain the kinase inactive in suitable environment for phosphorylation; likely preventing access of the substrate to the catalytic site (self-inhibition). So FISL has inhibitory function on self-phosphorilating activity of SOS2 kinase (Guo et al., 2001).

Studies carried out by Guo and collegues (2001) demonstrated that removing SOS2 regulatory domain, including FISL, protein kinase is constitutively active, underlining so that the self-inhibitory function of the motif where SOS3 is bound. SOS2 kinase domain contains also an "activation loop" that is a fragment delimitated by two conserved triplets of amino acids; and it needs phosphorylation by an upstream kinase in order that SOS2 gets activated. Mutation of a conserved threonine ($Thr^{168}$) in the activation loop with a residue of aspartate (Asp-D) leads SOS2 to be constitutively active; this amino acid substitution allows the overcoming of the inhibitory effect of the regulatory domain. This mutation in the activation loop and the deletion of self-inhibitory domain have a synergistic effect on SOS2 kinase activity and in addition, it is possible to create a "superactive" SOS2 if these two changes will occur together (Guo et al., 2001).

The presence of this kinase in SOS pathway (Fig. 1-25) allows to hypotise the involvement of protein phosphorylation in plant ionic homeostasis under salt stress (Liu et al., 2000). In fact calcium signalling often is coupled with protein phosphorylation, which is one of the most frequent and essential regulatory mechanisms in signal transduction (Guo et al., 2001).

SOS2 transcripts are present both in shoots than in roots, but are up-regulated in response to salt stress in roots; expression of this protein is very low in the absence of stress and slightly higher in response to stress. This low expression of SOS2, as well as that of SOS3, reflects their predominantly regulative function in the SOS pathway. Within the pathway, SOS3 forms a complex with SOS2 that is necessary for the phosphorylation and subsequent activation of SOS1, the plasma membrane

$Na^+/H^+$ antiporter (Bartels and Sunkar, 2005). Thanks to studies performed by Quintero and co-workers (2002) it has been noted that expressing in *S. cerevisiae* SOS3-SOS2, this complex was able to activate and phosphorylate SOS1 which determined a consequent increase of sodium efflux from the cell and therefore the salt tolerance in yeast, where, in this way, SOS pathway has been reconstructed (Quintero et al., 2002).

It can be said that SOS3 function can be bypassed by the presence of the activated form of SOS2 with its integral structure (without deletion of the FISL motif). Evidences suggest that the regulatory domain of SOS2 is necessary for salt tolerance (Guo et al., 2004). The expression of constitutively active SOS2 action under the CaMV 35S promoter in sos2 and sos3 mutants of *Arabidopsis* can partially rescue the salt hypersensitivity in shoots, but not in roots, this suggests that the SOS2 activity is sufficient for at least SOS pathway in shoots. A roots specific regulation of SOS2 may take place through its activation loop, and the Threonine/Aspartate mutation may interfere with such a regulation (Zhu 2002; Guo et al., 2004).

Another interesting observation about the SOS2 kinase is that if it is overexpressed in wild type-form in transgenic *Arabidopsis* plants, it does not confer any increase in salinity tolerance, but if it is modified in a constitutively activated state it leads to a significant increase in salt tolerance in these plants.

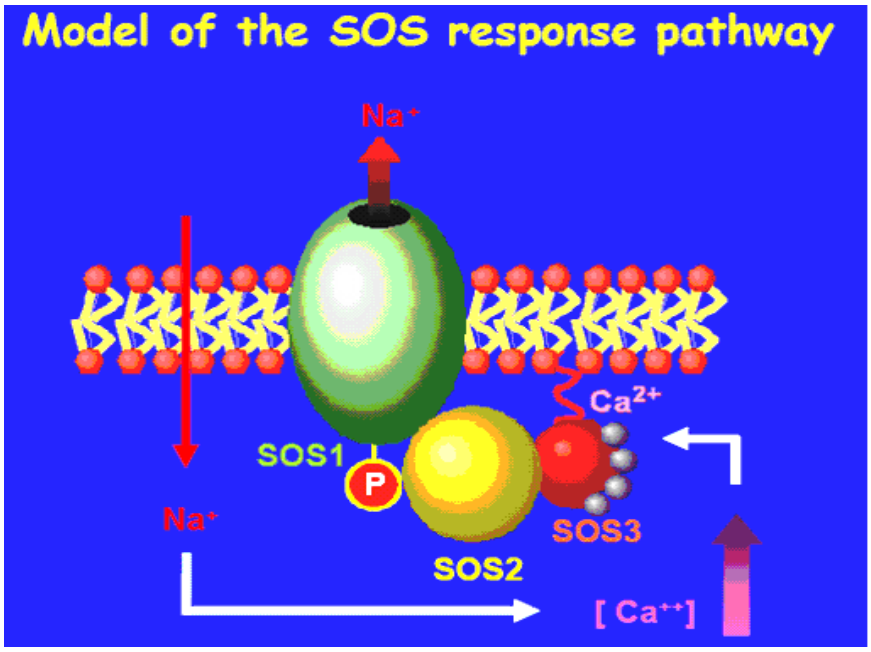



**Fig. 1-25: Scheme of interaction between SOS3, SOS2 and SOS1 in salt stress response.**

Finally, studies carried out by Qiu and colleagues (2004) have shown that the SOS pathway is also involved in the regulation of tonoplast $Na^+/H^+$ antiporter and SOS2 in particular, if added "*in-vitro*" to tonoplast vesicles (isolated from *sos2* mutants), leads to an increase of this vacuolar antiport activity (Qiu et al., 2004).

**SOS1**

SOS1 plays the most important role between the three SOS loci to impart salt stress tolerance; in fact *sos1* mutants resulted to be more sensitive to $Na^+$ and $Li^+$ respect to *sos2* and *sos3* mutants (Mahajan et al., 2008).

SOS1 gene encodes a putative $Na^+/H^+$ antiporter with a molecular weight of 127 kDa. After the cloning SOS1 protein structure was predicted; it was composed by 12 trans-membrane domains at the N-terminal and a long tail of about 700 hydrophilic amino acids at the C-term on the cytoplasm. Plasma membrane transporters that have long cytosolic tails are candidates to be possible sensors of the solute they transport (Zhu, 2002), but still little is known about sodium sensors in plants even if it can not be excluded that SOS1 acts both as antiporter and sodium sensor (Zhu, 2003). The presence of this tail makes of SOS1 the largest known $Na^+/H^+$ antiporter sequence and this particular domain was not found in other antiporters in Genbank (Mahajan et al., 2008).

The trans-membrane region of protein presents similarity in sequence with bacteria and fungi $Na^+/H^+$ antiporters. As mentioned before, activity and expression of SOS1 are both regulated by, SOS2 and SOS3, acting in the same SOS pathway for salt tolerance (Shi et al., 2000a). Between these three SOS genes, SOS1 plays the main role in salt tolerance in plant, because comparing three mutants *sos1, sos2, sos3* it is clear that *sos1* mutant is more affected by $Na^+$ and $Li^+$ stress (Zhu et al., 1998).

Phylogenetic analyses showed that SOS1 is more closely related to plasma membrane $Na^+/H^+$ antiporters of microorganisms rather than vacuolar plants or fungi, this confirmed that SOS1 is really the plasma membrane $Na^+/H^+$ antiporter in *Arabidopsis* (Shi et al., 2000a). A subsequent study carried out by Shi and colleagues (2002a) managed to precisely locate SOS1 protein in the plasma membrane, and this was possible thanks to the construction of a fusion protein produced by combining the SOS1 with GFP (green fluorescent protein), and then used to

transform *Arabidopsis* and, after that, protein was visualized at a confocal fluorescence microscope. Being a plasma membrane $Na^+/H^+$ antiporter, SOS1 has the function to export $Na^+$ from the cytosol to the extracellular space to avoid the rapid accumulation of this ion in the cytoplasm. In this regard, callus derived from *sos1* mutant plants accumulate more $Na^+$ than wild-type callus does; this emphasizes SOS1 role in $Na^+$ extrusion from the cell (Shi et al., 2002a).

Applying mutations that cause amino acid substitution in both protein tail and in its trans-membrane domains, it is clear that both components are essential for its functioning in salt tolerance, because these mutations of specific amino acids affect the functionality of this antiporter. SOS1 expression increases in response to salt stress, but not in response to drought, cold and ABA, this up-regulation reflects its unique role in salt tolerance. In previous studies conducted by Niu and co-workers (1993) it has been noted that stress leads to overexpression of genes coding for membrane $H^+$/ATPase, this probably helps to maintain homeostasis as $H^+$/ATPase pumps provide a greater proton-motive force necessary to ensure a high SOS1 activity (Niu et al., 1993).

Overexpression of SOS1 gene in *Arabidopsis* plants resulted in increased tolerance to salt in these plants, in addition to the discovery that SOS1, in absence of stress, is unstable and that stress causes a post-transcriptional stabilization of accumulated transcripts: an important gene regulation mechanism under stress (Shi et al., 2002b).

### 1.3.1 Digging deeper in HKT1

Phylogenetic trees of full-length HKT coding sequences present in databases showed that HKT family splits in two groups: the first subfamily includes HKT genes from dicotyledonous species (*Arabidopsis, Populus trichocarpa, Eucaliptus camaldulensis*, etc.), while the second group collect HKT genes from many graminaceous species; whereas rice HKT genes belong to both groups because they are quite different. Thanks to this phylogenetic tree it was possible to update the general HKT nomenclature, in fact the previous name AtHKT became AtHKT1;1 where the first number indicates the affiliation to one of the two subfamilies, and the second one differentiates genes within species. In both subfamilies HKT genes

contain two introns that are longer in the first group than in the second one; in addition members of subfamily 1 present a serine residue in the first pore loop of the protein instead members of subfamily 2 have a glycine in the same position (Platten et al., 2006).

*Arabidopsis* and rice are the two model plant species whose genomes have been completely sequenced and comparing them for the number of HKT genes, they result different. In fact rice has got eight HKT transporters (either low-affinity or high-affinity) and in roots the $Na^+$ uptake is rapid and high-affinity manner. Contrarily, *Arabidopsis* has only a single HKT homolog (AtHKT1;1) encoding a $Na^+$ low-affinity transporter. In these two model plants, HKT transporters play their role on the plasma membrane of different internal cells (xylem parenchyma, bundle sheath etc.), but only in rice the $Na^+$ uptake is mediated by low-affinity HKT transporters in root epidermal and cortical cells (Rodriguez-Navarro and Rubio, 2006). Studies on AtHKT1, SOS1 and $Na^+$ transport inside the plant underlined that the connection between $K^+$ nutrition and $Na^+$ homeostasis is more complicate than the basic assumption that sodium competes with potassium for its uptake. It appears that together, SOS pathway and AtHKT1, could be able to achieve a $Na^+$ and $K^+$ homeostasis, but if a dysfunction occurs in this system, the long-distance transport of $Na^+$ will be altered and therefore obtaining a salt-sensitive phenotypes (Pardo et al., 2006).

In a recent review written by Munns and Tester (2008), they dedicated a proper deepening on AtHKT1;1, titling it as “a case of confusion”, which is quite meaningful and clearly explain the updated situation of knowledge on AtHKT1;1 function. Summarizing, the last relevant experiment carried out by Davenport and colleagues (2007) clearly demonstrated that AtHKT1;1 is involved in $Na^+$ retrieval from the xylem. Even if AtHKT1;1 drives $Na^+$ influx into cells, its function at whole plant level seems to be reducing the net $Na^+$ influx into shoots; and its role in shoots is still unknown (Munns and Tester 2008).

### 1.3.2 Digging deeper in NHX1

In *Arabidopsis*, the $Na^+/H^+$ antiporter activity is carried out by a gene family. These six characterized genes located in the tonoplast are known as AtNHX1-6, but only for five of them the $Na^+/H^+$ antiporter activity was confirmed (Martinoia et al., 2007). AtNHX1 transcripts increase in response to NaCl, KCl, ABA and sorbitol (Yokoi et al., 2002), the same behaviour showed AtNHX2; it means that AtNHX1-2 are not specific to ionic stress, but they are responsive to hyperosmotic stress (Bartels and Sunkar, 2005). AtNHX1 is constitutively expressed and this let it think about a possible physiological role also under normal condition; in fact it seems to be involved in pH regulation and in the accumulation of $K^+$ into vacuoles (Bartel and Sunkar, 2005). This aspect was particularly evident in an AtNHX1 promoter-GUS analysis in transgenic *Arabidopsis* (Fig. 1-26) that showed a strong GUS expression in guard cells and in general AtNHX1 was present in all tissues except in root tips (Shi and Zhu, 2002).


**Fig. 1-26: AtNHX1 promoter-GUS analysis in transgenic Arabidopsis experiment; AtNHX1 has an important role in all tissues except in root tips (Shi and Zhu, 2002).**

Additionally, evidence suggests that other functions of AtNHX1 (besides the previous mentioned) are an involvement in intracellular trafficking and a transcriptional regulation (Martinoia et al., 2007).

It is well known that overexpressing ATNHX1 in *Arabidopsis* plants promoted tolerance to salinity up to 200 mM NaCl. Also transgenic tomato overexpressing this *Arabidopsis* gene showed a good growth and yield at 200 mM NaCl, with low content of sodium inside fruits (Apse et al., 1999; Wang et al., 2003).

The regulation of AtNHX is probably dependent on ABA via a SOS-indipendent ABA-dependent pathway, but it has been proved that also SOS pathway can regulate vacuolar $Na^+/H^+$ antiporters (see paragraph 1.3.0 on SOS2) in order to co-ordinate tonoplast and plasma membrane sodium regulation (Zhu, 2003;Martinoia et al., 2007).

## 1.4 Calcium and its role in salt stress

Calcium belongs to the essential nutrient for plant; in fact as divalent cation it is required for structural roles in cell walls and membranes and as intracellular messengers. This nutrient is taken up by roots and transported to shoots via xylem through two ways: across plasmodesmata (symplast) or into apoplastic space. Calcium deficiency is rare in nature, but it can happen when Ca is temporarily unavailable for growing tissues (White and Broadly, 2003).

In agriculture, to decrease $Na^+$ toxicity is common to make treatments with $Ca^{2+}$-containing compounds; this ameliorates salt toxicity symptoms in several plants, but the mechanism of action at cellular level is not still completely understood (Shabala et al., 2006).

Calcium has known as a regulatory molecule for a hundred years ago and in particular it is retained the most-ubiquitous cellular second messenger. In fact it is involved in almost all phases of plant life such as growth, development, reproduction, circadian rhythms, immunity, redox status, hormone biosynthesis, early signalling events and responses to biotic and abiotic stresses (Shao et al., 2008b). Fluctuations in $[Ca^{2+}]_{cyt}$ are responsible for closure of stomatal guard cells and also provide information about day length important for regulation of photosynthesis as well as other metabolic processes (Wheeler and Brownlee, 2008).

Oscillations in calcium concentration inside the cell were observed in plant cells treated with ABA, this hormone in fact activates a hyperpolarization-dependent $Ca^{2+}$-permeable channel in plasma membrane of *Arabidopsis* guard cells, resulting in the increase in $[Ca^{2+}]_{cyt}$ (Lecourieux et al., 2006). It was assumed that this transient increase of internal calcium preceded the stomatal closure; but recently it has been identified a $Ca^{2+}$ sensing receptor (CAS) localized in thylakoid membrane with essential role in stomatal closure (Nomura et al., 2008).

$Ca^{2+}$-dependent signalling is widely conserved among eukaryotes, but analysing *Arabidopsis* genome came out that calcium channels are very different between animals and plants and therefore probably also the ability to produce $Ca^{2+}$ signals , this can be due to the sessile existence that needs less very quick signalling mechanisms (Wheeler and Brownlee, 2008).

Calcium permeable channels can be considered the upstream elements in the $Ca^{2+}$-dependent signalling pathways, so their activation is quite important. These channels are placed in plasma membrane, tonoplast, as well as in ER (Buchanan et al., 2003) and their activity is coupled also with $Ca^{2+}$-ATPase whose main function is to maintain ion homeostasis pumping out calcium to terminate for instance a signalling event (Shao et al., 2008b). On plasma membrane also $Ca^{2+}$ voltage-dependent channels are present and their activation depend on depolarization of membrane. Elevation of $[Ca^{2+}]_{cyt}$ can be due also to $Ca^{2+}$ mobilization from organelles, event mediated by another second messenger, $IP_3$, that acts as ligand for calcium gated channels in vacuolar or ER membranes or by $Ca^{2+}$-antiporters (Buchanan et al., 2003; Lecourieux et al., 2006).

Moreover, it has been reported that the rise in $[Ca^{2+}]_{cyt}$ in plant seems to be provided by non selective cation channels (NSCCs) that allow the cations passage across membranes with limited discrimination between cations; they play also a crucial role in salinity stress (Dimidchik and Maathius, 2007). In fact it has been observed that elevated extracellular $[Ca^{2+}]$ inhibits plasma membrane NSCCs that mediate $Na^+$ influx, and proper this down regulation of $Na^+$ entrance can explain the ameliorative effect of $Ca^{2+}$ in salt conditions, because other $Na^+$ inward transporters are not sensible to calcium (Shabala et al., 2006). In addition, external $Ca^{2+}$ blocks also DA-NSCC (depolarization activated NSCC) to avoid $K^+$ efflux and maintain ion homeostasis (Fig. 1-27; Dimidchik and Maathius, 2007). This aspect was reported both for leaves and for roots; calcium always supports $K^+$ transport and prevent $K^+$ loss (Shabala et al., 2006).

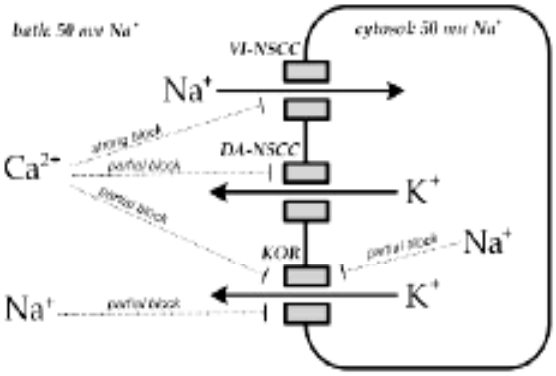



**Fig. 1-27: Calcium action to maintain ion homeostasis during salt stress.**

Normally, cytosolic calcium concentrations ($[Ca^{2+}]_{cyt}$) are maintained low around 100-200 nM respect to the vacuole and apoplast, that provide the possibility to ready import calcium inside the cell when it is necessary for signalling (Lecourieux et

al., 2006). In general, $Ca^{2+}$ influx across proper channels causes a quick rise in $[Ca^{2+}]_{cyt}$, this triggers the modulations of downstream effector proteins including calmoduline, $Ca^{2+}$-dependent kinases (CDPK) and calmodulin-dependent kinases (Wheeler and Brownlee, 2008). CDPKs were found from osmotically stressed plants, in fact a CDPK was reported to be expressed earlier and for longer in a drought tolerant species respect to the sensitive ones. In plant several isoforms of CDPKs exist and this can be explained with the need of fine tuning responses to diverse abiotic stresses (Shao et al., 2008b).

In plant, a common response to many stresses, salinity included, is the rise of cytosolic free $Ca^{2+}$. NaCl is able to increase $[Ca^{2+}]_{cyt}$ in few seconds, apparently $Na^{+}$ is able to activate the flux of calcium through both the plasma membrane and intracellular $Ca^{2+}$ permeable channels (Tracy et al., 2007; Munns and Tester, 2008).

The signal firstly activates PLC (see paragraph 1.2.2.1 c) that hydrolyzes PIP2 to generate $IP_3$ and DAG that causes an increase in intracellular calcium. This rise in concentration is perceived by calcium sensors with following activation of protein kinases. The calcium signalling proceeds with the "switching on" of multiple stress-responsive genes that implies a response to the stress, which can be growth inhibition, cell death or stress tolerance (Fig. 1-28; Mahajan et al., 2008).

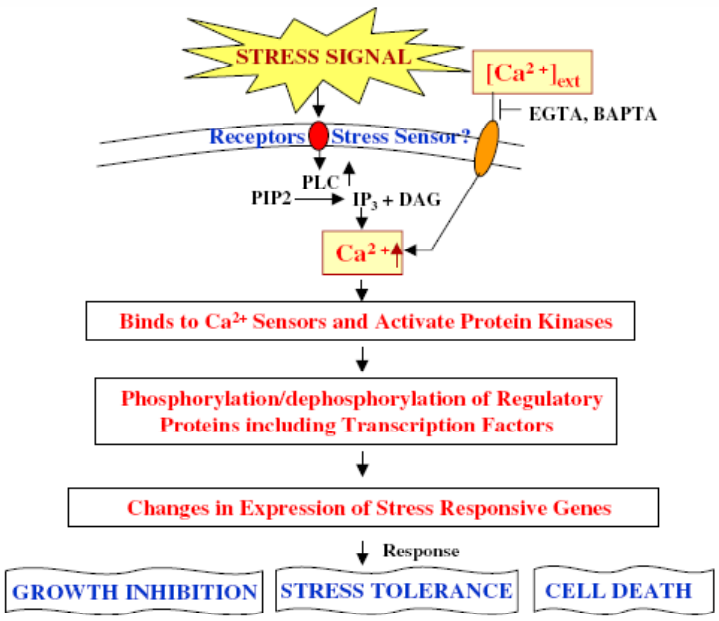



**Fig. 1-28: Calcium signalling cascade.**

The best studied signalling pathway that involves salt stress is the already mentioned $Ca^{2+}$-dependent-SOS pathway, completely defined in *Arabidopsis.* This

increase in calcium concentration inside the cytosol is perceived by the previously described SOS3 by its EF-hands; however the affinity of the binding is still indefinite, but the free calcium seems to induce the SOS3 protein dimerization and the connection with SOS2 to form the "core" complex of the pathway (Munns and Tester, 2008). The consequential activation of SOS1 leads to increase the $Na^+$ efflux and to restrict $Na^+$ influx thought HKT1, trying to maintain a $K^+/Na^+$ homeostasis (Shabala et al, 2006). The activity of SOS1 relies on $H^+$-ATPase action, in fact this loss of its function could determine the salt sensitivity in *Arabidopsis*.

Nowadays the most advanced technique adopted to study calcium signalling pathways on plant cells is the use of aequorin that is a $Ca^{2+}$-binding photoprotein found in jellyfish and utilized for transformation in plants in order to detect calcium based on bioluminescence (blue light emission). The transformation with aequorin is stable in plant and also allows following $Ca^{2+}$ targeting into organelles (Lecourieux et al., 2006). Through this technique it has been reported that NaCl-induced $[Ca^{2+}]_{cyt}$ increases in *Arabidopsis* were heterogeneous and limited to roots (Tracy et al., 2008).

## 1.5 ABA and its role in salt stress

Abscisic acid (ABA) is a plant hormone involved in several processes such as seed development, dormancy, and germination, but its principal role is to control plant water balance and osmotic stress tolerance. Mutant plants unable to produce ABA were useful in investigation of roles of this hormone. In fact *Arabidopsis* ABA-deficient mutants (*aba1, aba2, aba3*) under drought or salt stress appeared wilt and ready to die with the stress persistence (Zhu, 2002; Bartels and Sunkar, 2005). In *Arabidopsis* also ABA- insensitive mutants were discovered, these *abi1* and *abi2* plants show deregulation of stomata and wilty phenotype. ABI1 and ABI2 2 genes encode homologous serine/threonine protein phosphatises 2C and can have common function (Xiong and Zhu, 2001).

In drought and salt stress conditions, ABA plays a twofold role: in water balance via guard cell regulation and in cellular dehydration tolerance, activating particular responsive genes (ABA signalling) encoding for dehydration tolerance proteins (for example LEA) (Zhu, 2002; Bartels and Sunkar, 2005). The role of ABA in stomatal

closure is a signal of change in water balance for guard cells that decrease in turgor pressure and increase in free cytosolic calcium that regulate ion channels and control of cytoskeleton organization, with the consequence of stomatal closure (Bartels and Sunkar, 2005).

It has been reported that salt stress results in increased ABA levels which can ameliorate the inhibitory effect of NaCl on photosynthesis, growth and translocation of assimilates. This hormone in addition was able, in citrus, to reduce ethylene release and leaf abscission under salt tress most likely by limiting the accumulation of toxic $Cl^-$ in leaves. ABA also finds a role in salt stress acclimation in fact pre-treatments with ABA compounds provide increase to salt shock and this ABA-induced acclimation is rapid and is due to the expression of early salt induced (ESI) genes in roots (Parida and Das, 2005). Since osmotic stress leads to ABA accumulation, the ABA signalling is frequently mentioned in stress responses. In particular during two-hybrids screens on SOS pathway determinants, it was found that SOS2 interacts with ABI2, suggesting the probable connection between ABA signalling salt-stress responsive pathway with the well known SOS pathway (Xiong and Zhu, 2001). The proposed role of ABI2 is to dephosphorylate proteins phosphorylated by SOS2 in order to re-establish a homeostasis after a stress (Mahajan et al., 2008).

# Chapter 2: METHODOLOGICAL DEEPENINGS

## 2.0 Gas exchange measurements and LI-COR 6400

Nowadays gas exchange measurements on leaves are routinely carried out in physiological experiments. Since some years ago, this kind of measures were limited to laboratory systems, but they have been replaced by portable systems that are able to provide a real time measurement of $CO_2$ uptake (A), transpiration (E), leaf conductance (g1) and the intercellular $CO_2$ mole fraction (C1). Today over 95% of gas exchange measurements are obtained by using these portable systems that consent to the operator to collect values of all these parameters (A, E, g1…) only pushing some buttons in the instrument (Long and Bernacchi, 2003). This progress in miniaturizing of instruments made *in situ* field measurements possible (Jahnke and Pieruschka, 2006).

LICOR 6400 (Li-Cor Inc., Lincoln, NE, USA) belongs to the third generation of these portable gas exchange systems, it was developed in 1995 as an open system which means that photosynthesis and transpiration measurements are based on the differences in $CO_2$ and $H_2O$ in an air stream that is flowing through the leaf cuvette. A really useful improvement of the system is the integration of gas analyzer in the sensor head; in this way any variation in gas exchange can be detected as a change in $CO_2$ concentration.

LICOR 6400 portable system is composed by several parts:

1. CONSOLE

   Console is environmentally sealed and allows operator to double-check in real time the physiological status of plant and monitoring changes in parameters, like photosynthesis and record values pushing a single button. Thanks to the internal software (OPEN), every time gas exchange measurements are carried out, a new file can be opened and each measure is recorded in the internal computer memory as a “log” with a corresponding number.

   In the right side of the console there are sensor head connectors, battery components, and other connectors for the proper assembly of the

instrument. In the left side are placed $CO_2$ scrubber and dissecant tubes and also $CO_2$ cartridge holder.

In this experiment, 12 g $CO_2$ cartridges (Walther-Germany) have been used and placed in the proper holder; inside the reference $CO_2$ is contained and it enters in the flow system screwing it in the holder. When the cartridge is open it last for 8 hours of "in field" measurement.

2. SENSOR HEAD/IRGA

Sensor head includes leaf chamber (cuvette), typically less than 10 $cm^2$, often as small as 2 $cm^2$, with black neoprene gaskets that isolate an area of leaf where measure will be done and help to contain $CO_2$ diffusivity. Sensor head appears as a clasp, in fact the handle is squeezable to open and to close the leaf inside, the closure can be tighter, moving a screw that modifies distance between the two gaskets (Fig. 2-1). This so important part of the instrument includes also two Peltier thermoelectric coolers and sample and reference gas analyzers and light apparatus to measure PAR.

**Fig. 2-1: Detail of sensor head/IRGA with clamp closed on leaf**

Photosynthetic active radiation (PAR) is the most important environmental factor in photosynthesis and is not easy to be detected. LICOR 6400 is supplied with two sensors: a Gallium Arsenide Phosphide (GaAsP) PAR sensor and/or an external sensor; with the first one, it is possible to set a PAR and gas exchange measurement will be performed with a lamp that provides the desired PAR; instead using the external sensor, the real natural light is perceived. A thermocouple in the bottom of leaf chamber allows temperature measurements, touching the leaf when it is closed in the chamber.

3. CABLE ASSEMBLY

   Cable assembly includes two electrical cables and two air flow hoses and connectors to bond console to sensor head. These junctions are really delicate and for this reason are wrapped together with a flexible net.

4. CHEMICAL TUBES

   These tubes are important to remove $CO_2$ and water vapor from the incoming air stream. One tube contains a dissecant (Drierite) and the other soda lime for $CO_2$ trapping; both tubes have a valve on the top to partition the flow through the chemical inside them. During measurements made in this experiment valves were set completely scrubbed for $CO_2$ and bypassed for dissecant.

5. RECHARGEABLE BATTERIES

   LICOR 6400 works with two batteries contemporarily, they are rechargeable and when they are flat, the instrument emits a sound that advices operator to change batteries with a new charged pairs. This allows avoiding the switching off of the instrument during a measurement. They last about 1-2 hours each (Fig. 2-2).


**Fig. 2-2: LICOR 6400 Portable system**

The incoming air can be conditioned before entering the system, and the most important thing to be respected is that incoming concentration must be stable; this results crucial for $CO_2$, where the fluctuations can be huge. Since gas exchange measurements are based on reliving differentials, fluctuations of concentrations can be a real problem causing erratic interpretation of physiological situation.

In order to provide stable $CO_2$ concentration at the desired value (360 ppm in this case) 6400-01 $CO_2$ mixer (Fig. 2-3) was used to inject just enough $CO_2$ from the cartridge to maintain a constant concentration. To be more precise, all the incoming air is scrubbed by soda lime tube and the mixer adds whatever $CO_2$ the system need to keep it in constant concentration. After the application of the $CO_2$ source, it is better to wait 5-10 min for the stabilization of the internal system in particular for the $CO_2$ mixer.

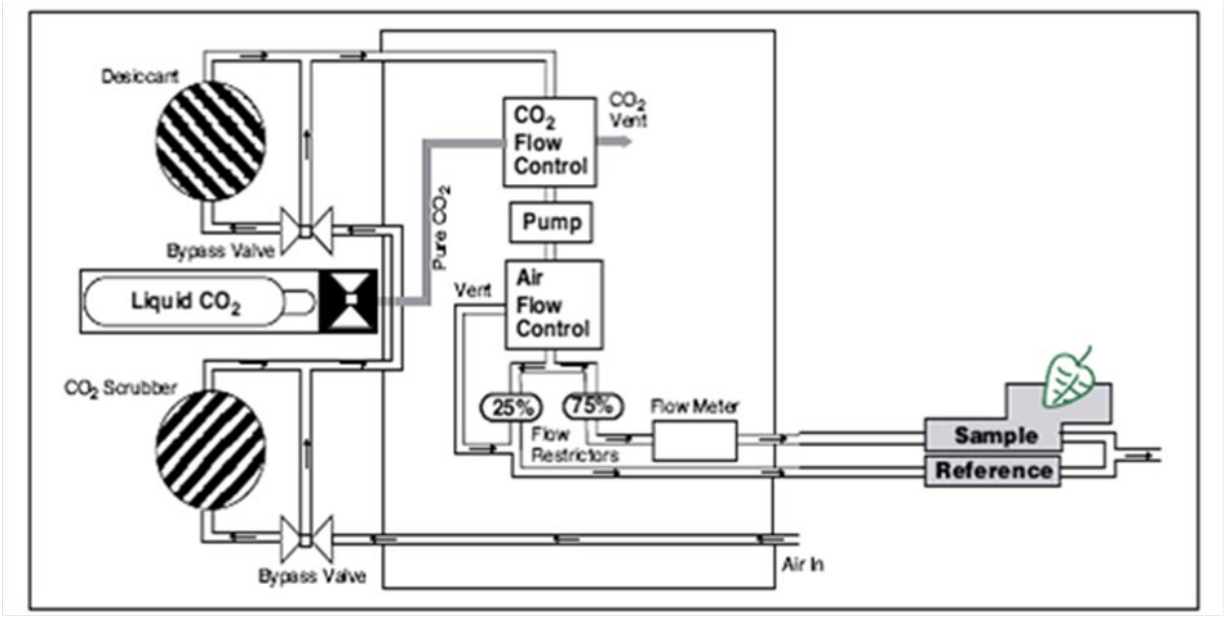



Fig. 2-3: LiCOR 6400 schematic flow with and without $CO_2$mixer

For humidity control in the leaf cuvette, the flow is regulated at 500 mol $s^{-1}$ and the flow is maintained thanks to a diverter valve present in the unit with $CO_2$ mixer.

The core of gas exchange measurements is the differences in concentration that are measured by two different IRGAs (Infra-red gas analyzer). IRGAs must be checked one against one other and this is possible with the “match mode” without disturbing leaf inside the chamber. With this procedure, sample IRGA is adjusted to match the reference IRGA; it should be done every time a new measurement

section starts and in particularly when $\Delta CO_2$ or $\Delta H_2O$ are really small and the un-matching could mean a significant error associated with the measure.

One of the configuration parameters of OPEN software involves specifying the light source (and indirectly, the sensor). So, with LICOR 6400 portable system is possible to carry out gas exchange measurement in real light condition using an external PAR sensor placed on the right side of the sensor head/IRGA or using an artificial light source of red and blue LEDs. The red only LED source provides a very suitable light source for photosynthetic studies, the addition of the blue LEDs in the lamp enlarges applications to the study of stomatal kinetics.

In this case for field measurement it has been used the lamp with 10% of blue LEDs with a setting PARin equal to 1300 µmol $m^{-2}$ $s^{-1}$ and for in growth chamber measurements the external PAR sensor has been adopted.

It could be interesting going deeper in LICOR 6400's calculations to obtain values for gas exchange parameters. The instrument includes in its software some equations that allow getting results for transpiration (E), photosynthesis (a) and leaf or stomatal conductance (g1); they correspond to those derived by von Caemmerer and Farquhar in 1981.

### 2.0.1 Transpiration

Transpiration is defined as the loss of water in form of vapor from a plant surface into external environmental and it is an important way that plants adopt in order to chill down. In plants, leaves are mainly involved in this physiological process and in particular stomata that have the function to allow gas exchange between internal leaf and atmosphere (Taiz and Zeiger, 1996).

In brief transpiration measured by LICOR 6400 can be expressed with this equation:

$$\mathbf{E = (flow * \Delta H_2O)/leaf\ area}$$

Both transpiration and photosynthesis change water and $CO_2$ concentration of air as it passes through the chamber, and E implicates that the out-coming flow rate from chamber ($u_o$) is greater than the in-coming one ($u_e$) as it is represented in the figure 2-4.

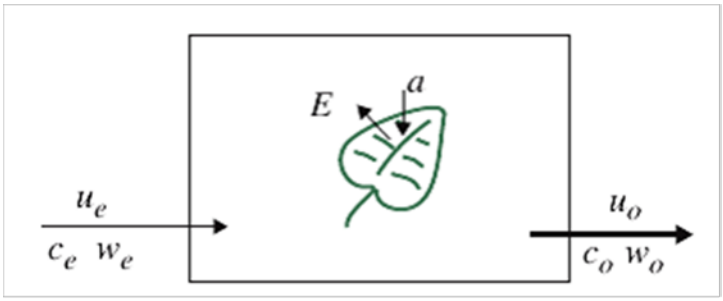



**Fig. 2-4: In and out-coming flow rate through leaf chamber.**

So the balance of water vapor in an open system is summarized in next equation (taken from original von Caemmerer and Farquhar's study):

$$\mathbf{sE = u_o w_o - u_e w_e}$$

Where: s= leaf area ($m^2$), e= transpiration rate (mol $m^{-2}s^{-1}$), $u_e$= in-coming flow rate (mol $s^{-1}$) and $u_o$= out-coming flow rate (mol $s^{-1}$) from the chamber, $w_e$=incoming water mole fractions (mol $H_2O$ (mol air)$^{-1}$) and $w_o$= out-coming water mole fractions (mol $H_2O$ mol $air^{-1}$). But $\mathbf{u_o = u_e + sE}$, so with some arrangements with previous equation, it changes in:

$$\mathbf{E = [u_e (w_o - w_e)]/[s(1-w_o)]}$$

From theory to real LICOR 6400 measures there are some parameters that need to be converted as follow: $u_e=F/10^6$; $w_e=W_r/10^3$; $w_o=W_s/10^3$; $s=S/10^4$

where F is air flow rate (µmol $s^{-1}$), $W_s$ and $W_r$ are sample and reference water mole fractions (mmol $H_2O$ (mol air)$^{-1}$), and S is leaf area ($cm^2$).

The equation used by LICOR 6400 for transpiration is thus:

$$\mathbf{E = [F (W_s - W_r)]/[100S(1000 - W_s)]}$$

### 2.0.2 Stomatal conductance

Air stream passing over the leaf inside the chamber can encounter a resistance; it will increase as the stomatal aperture size decreases. The stomatal conductance to water vapor decreases as the resistance increases (Swarthout, 2008). It is well known that stomata change in diameter depending on day time but also as reaction from abiotic stresses, like drought or salinity, so rates of photosynthesis and transpiration will vary because the pore size will provide a corresponding resistance

to the diffusion of $CO_2$ into and $H_2O$ out of the leaf. According to Ohm's law: conductance= 1/resistance and so it is possible to calculate conductance to these two gases across a leaf surface (Taiz and Zeiger, 1996).

The stomatal conductance $g_{sw}$ to water vapor (mol $H_2O$ $m^{-2}$ $s^{-1}$) is obtained from the total conductance by removing the contribution from the boundary layer and it is expressed as follow:

$$g_{sw} = \frac{1}{\frac{1}{g_{tw}} - \frac{k_f}{g_{bw}}}$$

Where $k_f$ is a factor based on the estimate K of the fraction of stomatal conductance of one side of the leaf to the other (termed stomatal ratio) and $g_{bw}$ is the boundary layer conductance to water vapor (mol $H_2O$ $m^{-2}$ $s^{-1}$) from one side of the leaf.

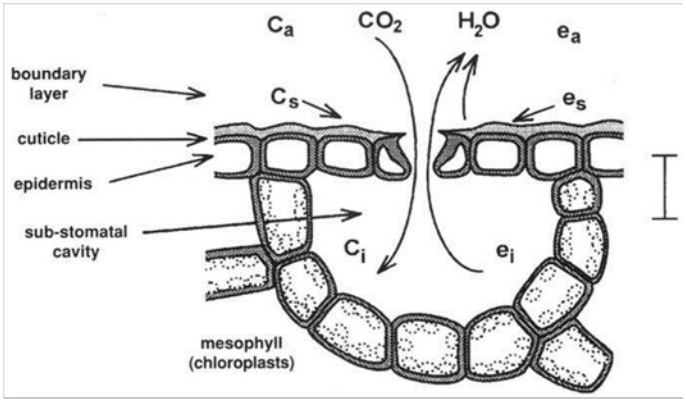



**Fig. 2-5: Stomata structure**

### 2.0.3 Photosynthesis

In parallel with the balance in water vapor for transpiration, balance of $CO_2$ is related with photosynthesis. In brief photosynthesis measured by LICOR 6400 can be expressed with this equation:

$$A = (flow * \Delta CO_2)/leaf\ area$$

And accordingly with what said before for transpiration, the balance of $CO_2$ in an open system is summarized by:

$$sa = u_e c_e - u_o c_o$$

Where $a$ is assimilation rate (mol $CO_2$ $m^{-2}s^{-1}$), $c_e$ and $c_o$ are entering and outgoing mole fractions (mol $CO_2$ mol $air^{-1}$) of carbon dioxide.

In this way it is possible to understand how many mol of $CO_2$ have been fixed by that leaf and to obtain the estimation of net photosynthesis calculated subtracting at total photosynthesis, the respiration rate.

So with some arrangements equation changes in this way:

$$sa = u_e c_e - (u_e + sE)c_o$$

$$a = [(u_e (c_e - c_o))/s] - Ec_o$$

From theory to real LICOR 6400 measures there are some parameters that need to be converted as follow: $c_e = C_r/10^6$; $c_o = C_s/10^6$; $a = A/10^6$.

Where $C_r$ and $C_s$ are sample and reference $CO_2$ concentrations (µmol $CO_2$ (mol $air^{-1}$), and A is net assimilation rate of $CO_2$ by the leaf (µmol $CO_2$ $m^{-2}$ $s^{-1}$).

At the end net photosynthesis is given by:

$$A = \{[F (C_r - C_s)]/100S\} - C_s E$$

From this equation is clear as water vapor added into the chamber from leaf transpiration influences other gases including $CO_2$ diluting them; for this reason the $C_s E$ part should be subtracted (Using the LI-6400, ver.6, LICOR Biosciences).

## 2.1 Leaf chlorophyll concentration and portable Chl meter SPAD-502

One of the most important aspects of photosynthesis in plant is the ability to absorb light; this can be possible thanks to photoreceptors present in leaves called chlorophylls. Chlorophyll molecule has a ring structure with an atom of Magnesium in the middle; its role is to maintain a rigid structure and avoid the dispersion of radiation that is used for the photosynthesis. Another distinctive feature of chlorophyll is the presence of *phytol*, a highly hydrophobic 20-carbon alcohol, esterified to an acid side chain (Fig. 2-6) that is important for the bound with the thylakoid membranes in the chloroplast (Berg et al., 2002).

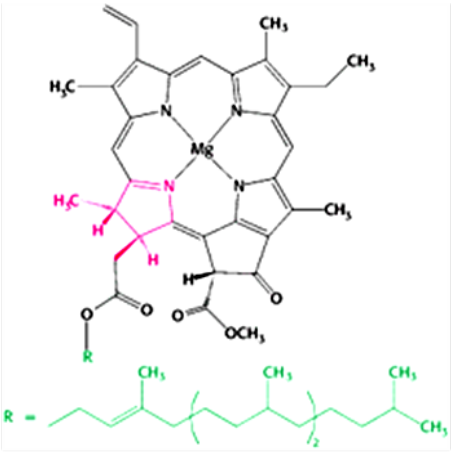



Fig. 2-6: Chlorophyll molecule structure.

Several kinds of chlorophylls exist in nature, the most spread form, among terrestrial plant, is chlorophyll a. Plant appears green because the wavelength of green is the less absorbed by chlorophyll. Chlorophylls in general absorb mostly in two areas: blue and red region (as figure 2-7), while the absorption is minimum in green - yellow region and in infrared area.

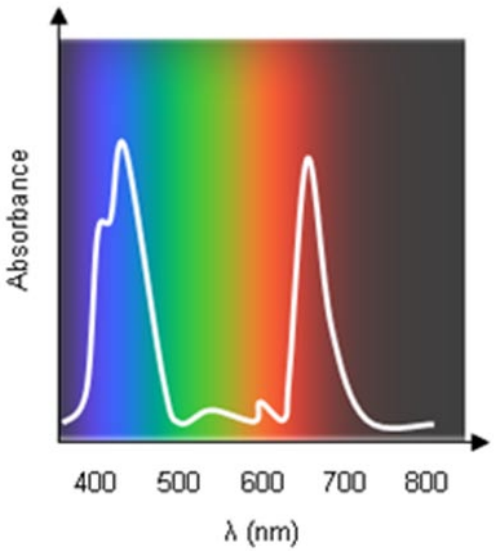


Fig. 2-7: Chlorophyll absorption spectrum.

Chlorophyll content is really important as marker of the healthy plant condition and can be used to determine when to add fertilizer to produce larger crop yields of higher quality. In fact it is well known the existing correlation between chlorophyll content and leaf N content. This fact is supported by the structure of the molecule that presents 4 N atoms linked directly to Mg (Fig. 2-6). So measuring Chl content can help in determining when plant is in N deficiency and therefore to recommend fertilizer application (Karele, 2001).

Low chlorophyll content may be induced in plants by subjecting them to certain nutrient deficiencies; a clear example of this can be the iron chlorosis that appears as a turning yellow of leaves. Since Shimshi (1967) in one of his study understood that lack of chlorophyll results in reduced stomatal aperture, it is possible to transfer this concept to abiotic stresses such as drought and salt stress. In fact, during these two abiotic stresses, a specific signalling to close stomata exists in order to temp to avoid dehydration for excessive transpiration (Bartels and Sunkar, 2005).

Also recent studies proved that salt stress condition decreases chlorophyll content and in particular that NaCl has effect on chlorophyll inhibiting synthesis of 5-aminolaevulinic acid, a precursor of chlorophyll. (Santos 2004; Eryılmaz 2006; Jaleel et al., 2008). For these reasons, in this experiment it has been decided to perform leaf chlorophyll content determination during the salt stress progress; this could be possible by an easy-to-use meter for measuring Chl content of plant leaves without damaging the plant. In the past for determining chlorophyll content it was

necessary to separate chloroplasts and then extract from them chlorophylls, this method implied the harvesting and destruction of several leaves to carry out analyses. Nowadays, the new instrument Chlorophyll Meter SPAD-502 (Minolta, Osaka, Japan) can be used to make quick and easy measurements of leaf greenness, which is positively related to leaf chlorophyll content (Minolta Manual). It is portable so in field measurements can be done easily also under the rain.

SPAD-502 has a compact shape and it is composed by:

- a "head" provided with a sample slot where leaf can be trapped and the illuminating system made of LEDs that emit red and infrared light.
- a display where values appear (Fig. 2-8).

Fig. 2-8: SPAD-502 Minolta.

The values measured by the Chlorophyll Meter SPAD-502 correspond to the quantity of chlorophyll present in the plant leaf. These values are obtained, related to the amount of light transmitted by the leaf, at two wavelengths where the absorption of chlorophyll is different. Based on the spectral absorption characteristics of chlorophyll mentioned before, the wavelengths ranges chosen to carry out measurement are the red area (high absorption and no influence of carotene) and the infrared (absorption really low). The light which passes thought the leaf, hits the receptor that converts the light in analog electrical signals.

## 2.2 Elemental determination and SpectrAA 200 atomic absorption spectrophotometry

Since early 1800s, plant analyses have developed and several elements were identified as essential for plant growth; later their content determination was useful as an index of available nutrient supply. An element is considered essential if the life cycle of the plant can't be terminated without it, can't be substituted by another one and performs a direct role in the plant (criteria decided by Arnon and Stout, 1939). These essential elements were sixteen and they were divided in two main categories: the macronutrients and the micronutrients (Fig. 2-9). The concentration requirements for the first category are from 10 to 5000 times greater than those of

the second one. Other elements have been found to be able to affect plant growth and development, but only Marschner (1986) include in the category "beneficial element" some as: aluminium (Al), selenium (Se), nickel (Ni), cobalt (Co), silicon (Si) and sodium (Na). The major nutrients are carbon, hydrogen and oxygen and they derive from air and water and they rotate around photosynthesis process. Instead the remaining essential nutrients come from soil or growing media or fertilizers (Mills and Benton Jones, 1997; Munson, 1998; Mengel and Kirkby, 2001).

| Sixteen nutrients needed by plants | | |
|---|---|---|
| Major nutrients from water and $CO_2$ | C | Carbon |
| | H | Hydrogen |
| | O | Oxygen |
| Primary Macronutrients | N | Nitrogen |
| | P | Phosphorus |
| | K | Potassium |
| Secondary Macronutrients | Ca | Calcium |
| | Mg | Magnesium |
| | S | Sulfur |
| Micronutrients | Fe | Iron |
| | Cu | Copper |
| | Mn | Manganese |
| | Mo | Molybdenum |
| | B | Boron |

Fig. 2-9: Classification of elements

Elemental determination can be carried out by atomic absorption spectrophotometry, prior plant tissue digestion, which allows obtaining a solution from the original material (Hanlon, 1998).

In general, atomic absorption spectrometry (AAS) consists on the generation of a free atoms cloud of the element to analyse by a nebulizer and make pass through this cloud a monochromatic radiation with a wavelength (nm) exactly equal to that one that excited atoms are able to emit. So that the adsorbing process happens and the amount of radiating energy is directly proportional to the number of atoms present in the fundamental state, following Lambert-Beer's law:

$$\mathbf{A = \varepsilon dc}$$

Where

A = absorbance

ε = molar extinction coefficient

d = path length in cm

c = molar concentration

Measure of amount of absorbed radiation by atoms at a defined λ is the absorbance (ABS) and it allows achieving element concentration to be investigated (Polesello et al., 1995). This direct proportionality mentioned before enables to plot a calibration curve measuring the absorbance of (five or more) standard solutions at known concentrations.

AAS is generally composed by five essential parts:

1. SOURCE OF RADIATION

   Usually as source of monochromatic radiation a hallow cathode lamp is used (HCL). This kind of lamp is made by a tube filled with an inert gas (Neon or Argon) and by two electrodes: anode prepared with tungsten and cathode made using the specific element for determination. This tube ends with a quartz circle. The cathode is cylindrical and hollow, in order to concentrate the emission of radiation intensity. The source of electromagnetic radiation emits with a characteristic spectrum for the element; spectrum is collimated to the optic system of the instrument.

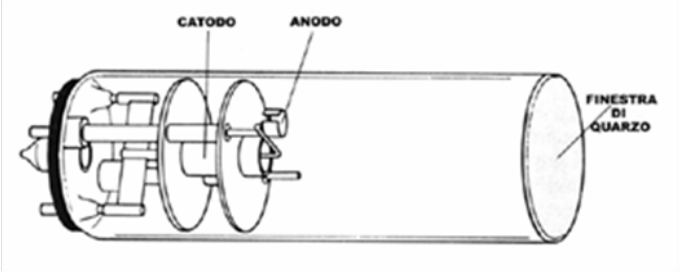


Fig. 2-10: a hallow cathode lamp

   The HLC lamps works thanks to a potential difference between electrodes that causes a partial ionization of the inert gas whose positive ions, accelerated by electric field, hit the cathode that releases atoms of the element to be analyzed in their fundamental status ($M^0$). This first step, which happens inside the lamp, is called "sputtering". After that, these atoms hit again with positive ions of gas and this triggers an excitation reaction ($M^*$).

   From this excited status, atoms decay quickly reverting into the fundamental status and releasing to the environment the energy that had been acquired before. This energy appears as light and the general mechanism is known as quenching (Fig. 2-11). This light has a particular spectrum known as line spectrum; it is defined (by Britannica encyclopaedia) as: "…..spectrum of incandescent gases, called line spectrum because only a few wavelengths are emitted. These wavelengths appear to be a series of parallel lines because a slit is used as the light-imaging device. Line spectra are characteristic of the elements that emit the radiation. Line spectra are also called atomic spectra because the lines represent wavelengths radiated from atoms when electrons change from one energy level to another".

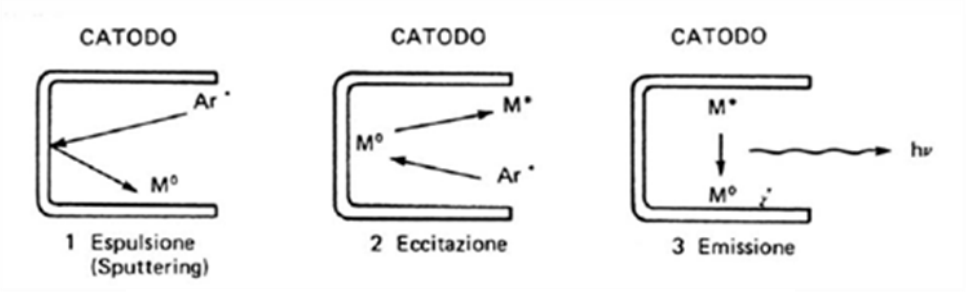



**Fig. 2-11: Ionization of the inert gas and "sputtering".**

For this purpose two kinds of lamps exist: a) specific lamp for single element and b) multi-elements lamps. The specific lamps have the cathode covered with only one element and for this they offer higher reliability and stability during measurements. On the other hand the multi-elements lamps give the opportunity to analyse more than one element without changing lamp; they are formed by more metals together inside the cathode.

In this experiment specific lamps have been used for Na, K, Ca, Zn, Mg, but a multi-elements lamp has been adopted to measure Fe, Cu and Mn.

These HLC lamps are really delicate and can be assembled in the instrument with proper holders; they are supplied with screws that allow an easy alignment of the lamps with the optical system. Before starting measurement the lamp referred to the element to be analyzed must be switched on 15 min before usage to warm it up. In addition before switching on the flame, it is important to check the linearity of the radiation ray from the source to the optical system. This is possible placing a small piece of cardboard on the flame burner guide rail and adjusting the light ray so that it hits the target printed on the cardboard.

2. ATOMIZATION SYSTEM

This second component of the AAS has main importance because its role is reducing the analyte solution into monatomic gas; this step is known as *atomization*. Some different atomizer systems exist, but the one used in this experiment is by the flame burner. This technique is well known since several years because it guarantees easily the excitation of many elements and it seems to be reliable also in the determination of low analyte concentration. The analyte solution must be warmed at a temperature that allows the solution to be dissociated in atoms. The necessary energy is

provided exactly by a flame. The flame is originated by the combustion between a burning gas and an oxidant; in our instrument they are respectively acetylene ($C_2H_2$) and air, this flame reaches temperature around 2300°C. The atomization system is provided with the instrument and is shared in two parts:

- Nebulizer (or atomizer)

  Sample to be analyzed is a solution that is introduced into the nebulizer by a fine pipe. The sample is sucked into the nebulizer by the oxidizing flow coming from a lateral Venturi's pipe. The solution is nebulised and the spray hits against an obstacle that works disrupting big drops in smaller ones. Sample drops should have an average diameter between 5 and 7 μm; only the 10% of the nebulised solution forms the cloud of fine drops that is directed by the air and mixed with burning gas and addressed to the flame burner (Fig. 2-12).

  The speed of the process depends on the shape of nebulisation chamber and on the pressure of the burning gas (acetylene). Every time a new batch of analyses has to be carried out, it is necessary to calibrate all the gas pipe system with the optimal pressure using pressure regulator knobs.

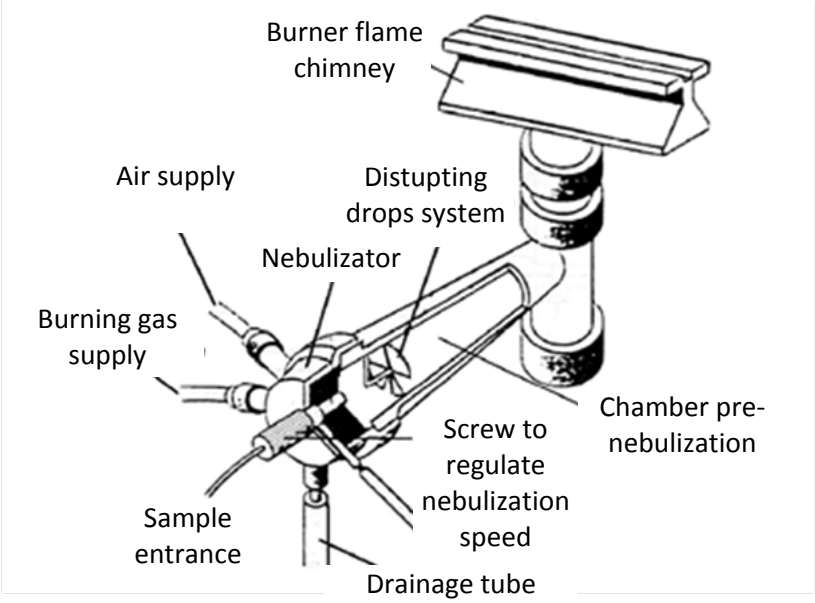



Fig. 2-12: Atomization system by flame burner.

- Flame burner

  The flame burner is made as a titanium rectangular chimney with a longitudinal slot in the middle of its upper surface (that appears as a guide rail). This structure allows flame to be laminar (Fig. 2-12). Also the position of the flame burner is adjustable by micrometric screws; it must be aligned with the source emission, the optical system and with the monochromator. From a general point of view, the flame also is important as a means of supporting the atoms in the light path. In fact, light from the source passes through the flame containing atoms of the element and they adsorb energy (photons). The degree of absorption is proportional to the concentration of the element in the flame as previously described with Lambert-Beer's law. Finally, to determine the concentration of the atomized sample solution, AAS measures the difference between the source light intensity and what passes through the flame.

3. OPTIC SYSTEM AND MONOCHROMATOR

   It is composed by lens and mirrors that collimate and address radiation coming from the source and exiting from the nebulised sample. The main role of the monochromator is to render the radiation as much monochromatic as possible, before addressing it to the detector. The radiation emitted by the HCL is analyzed by monochromator that select the wavelengths of the line spectrum of the source. An important thing to do, before switching on the flame, is selecting the wavelength to use to determine the analyte concentration. The monochromator usage is based on the diffraction properties of light and it is able to insulate a single chromatic component that corresponds to a precise wavelength. The instrument sensibility is strongly related to the ability of the monochromator to break the polychromatic radiation into a precise wavelength.

   The route of the radiation to the monochromator is addressed by the optical system composed by mirrors that can be named as single ray or double ray.

Usually in this optical system also a “chopper” is present; it is a rotating and reflecting mirror placed before the monochromator that allows the detector to discriminate between the radiation from the lamp and the other coming from the flame that can alter the signal and cause an error in the analyses (Fig.2-13).

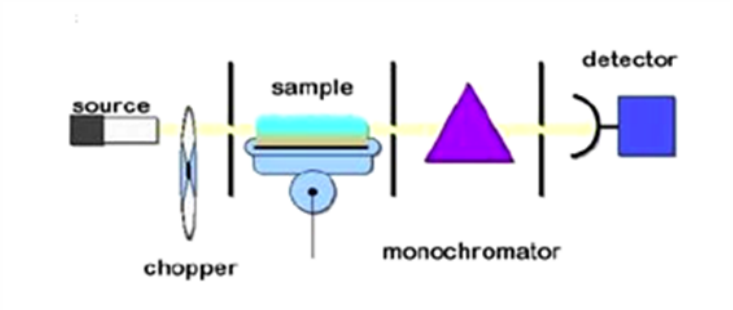



Fig. 2-13: Optic system inside an AAS.

4. DETECTOR

The detector is the sensorial component of the AAS; usually it is a photo-electrode that, using the particle theory of the light, highlights an incident radiation in an electrode by a potential difference. Briefly, it converts the light radiance into an electrical signal. Sometimes these analyses show really small differences that need to be amplified by a photo-multiplier in order to be detected.

5. DATA PROCESSING SYSTEM

Data processing system is usually a software made by instrument manufacturer that is fundamental to translate data, to calculate concentration and to save data. Nowadays all the atomic absorption spectrometers are connected with a PC that can support this software (Polesello et al., 1995; Hanlon 1998; http://www.chimicando.it/contributi/spettroscopiaAA.pdf.)

## 2.3 Quantitative Real Time PCR (qRT-PCR) and ABI PRISM 7000

Quantification of RNA levels by real time (RT) PCR has considerable potential for a variety of applications such as: medical diagnostic, neurosciences, developmental biology and plant biology (Perikles, 2003; Ramakers et al., 2003). Real time technology has recently extended the use of qRT-PCR, because it shows high sensibility, good reproducibility and a wide quantification range (Fleige and Pfaffl, 2006) and it is a fast way for mRNA quantification (Schefe et al., 2006). In addition qRT-PCR is suggested to be the best choice for low-abundant mRNA gene quantification (Fleige et al., 2006).

Real-time PCR measures PCR amplification as it occurs, allowing quantitative measurements to be made in the highly reproducible exponential phase of PCR.

Quantification occurs always in exponential phase of PCR, so that it can't be affected by any reaction components becoming limited instead in the plateau phase (Pfaffl, 2001). The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed.

Data from qRT-PCR are produced as sigmoid-shaped amplification plots in which fluorescence is plotted against the number of cycles in PCR reaction. Fluorescence first rises above a point known as threshold background fluorescence, a parameter known as threshold cycle ($C_t$). The more target there is in the starting material, the lower the $C_t$ (Ramakers et al., 2003; Nolan et al., 2006).

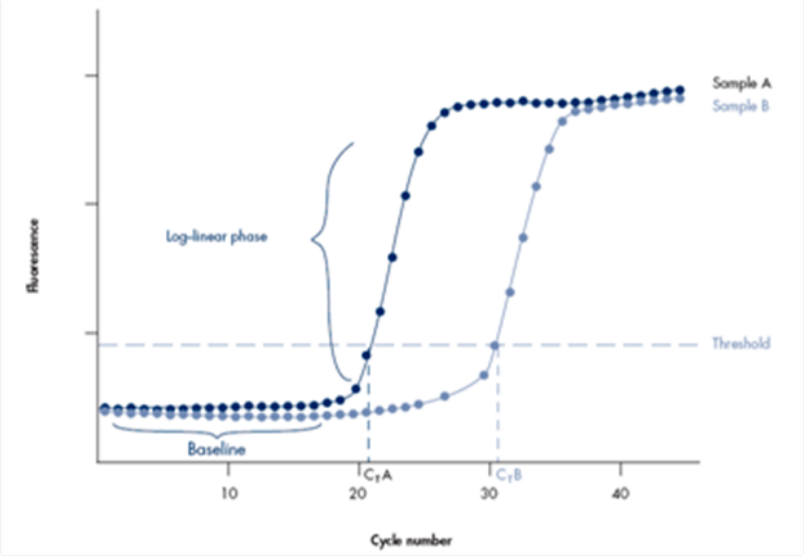



Fig. 2-14: Typical amplification plot of a qRT-PCR.

The $C_t$ serves as a tool for calculation of the starting template amount for each sample; in the figure it is clear as sample A contains a higher amount of starting template than sample B (Fig. 2-14). The $C_t$ remains the central value for quantification and in general not much effort has been put to standardize and optimize the determination of this parameter; in fact $C_t$ value is totally subjective as the threshold can be altered at will (Bustin et al., 2005; Pfaffl 2006). In this experiment threshold cycle was not adjusted, but the software default setting was maintained as suggested by specialists of this instrument. Most of RT platforms provide data already adjusted, doing an automatic background correction on $C_t$ known as "threshold cycle method" that assumes that all samples have the same DNA concentration at the threshold fluorescence.

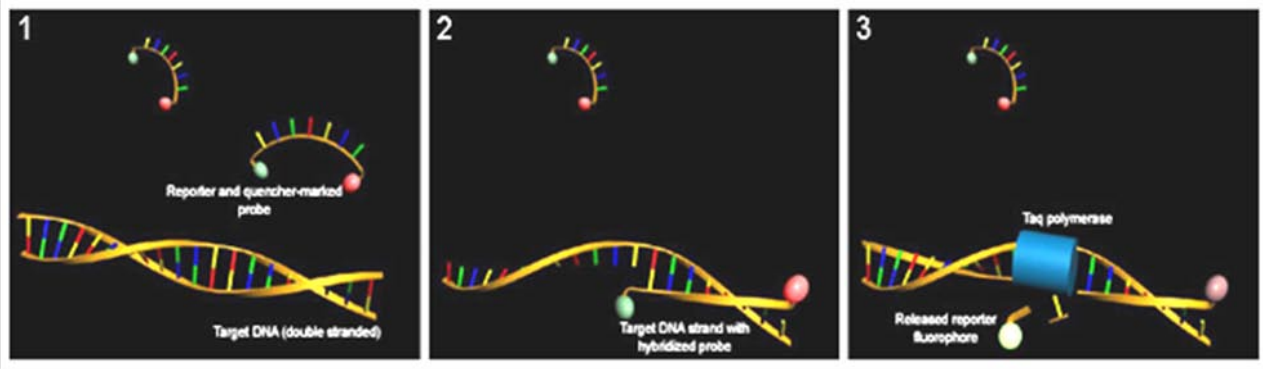



Fig. 2-15: Fluorescence emission during real time amplification.

Several types of chemistries have been developed to detect PCR product; the most known are: TaqMan and SYBER Green I chemistries (Fig. 2-15). TaqMan probe-based assay chemistry provides outstanding specificity and sensitivity, and the ability to multiplex reactions for real-time quantification and SNP genotyping assays.

Quantification of cDNA in RT-PCR in combination with the dsDNA specific dye SYBR Green I, that binds DNA in its minor groove, is based on the monitoring of the increasing fluorescence intensity after each PCR cycle (Pfaffl, 2001).

Two advantages related to the use of SYBR Green I Dye are: a) it can be used in quantifications of different targets without requiring sequence-specific fluorescent probes; b) its excitation and emission maxima are respectively 494 nm and 521 nm which are compatible with several real time cyclers (Bustin, 2002).

Fig. 2-16: ABI PRISM 7000 (Apply Bioscience).

ABI PRISM 7000 Sequence detection system (Fig. 2-16) is a sequence detection instrument capable of quantitative detection with fluorescent-based PCR chemistries and it has been used in this experiment for mRNA quantification. This instrument is able to perform quantitative real time analyses combining thermal cycling, fluorescence detection and application specific software (Relative quantification, guide for 7000 v.1.1). ABI PRISM 7000 appeared to replace the granddaddy of real-time instrument ABI PRISM 7700 that was based on a single excitation laser (488-514 nm) and some critics are moved about this because in comparison with newer instruments it is considered too narrow to excite efficiently the wide range of fluorophores available nowadays. The newer ABI PRISM 7000 replaces the laser with a tungsten-halogen lamp that is able to illuminate all samples in the plate simultaneously. Fluorescence emission is directed through four optical filters to a cooled CCD camera. This instrument has some advantages like: a) its compact size and reasonable price and b) its user-friendly-software already included that allows an easy exportation of raw data in Window (Bustin, 2002).

Using ABI PRISM 7000, 96 samples in one time can be amplified adopting proper well plates. For each gene a different master mix for PCR was prepared paying attention to keep all components on ice to defrost and to protect SYBR Green by direct light exposition. This dye could be stored after use at-20°C without any loss in fluorescence activity. In each well of a plate, a total volume of 20 µL (including cDNA, SYBR mix and specific primers) was pipetted in the bottom of it, trying to avoid dispersion of drops on the sides; in this case as first was dispensed cDNA in correct concentration by a micro pipette and secondarily master mix. The plate filling requires long time, because it is important to be as much precise as possible and to use regularly calibrated pipettes. Each reaction was performed in triplicates using RNA from the same extraction that was the result of four biological samples

pooling. In fact it is important to perform each PCR reaction in duplicate/triplicate for each sample to compensate for potential pipetting errors; this also enables calculation of standard deviations and means of expression.

ABI PRISM 7000 is compatible with heat-sealed film coverings that work very well in avoiding evaporation of the sample; when a plate is fill this sticker must be carefully placed on the top and the side parte removed before reaction start. On the covering a compression pad must be added as showed in the figure (Fig. 2-17).

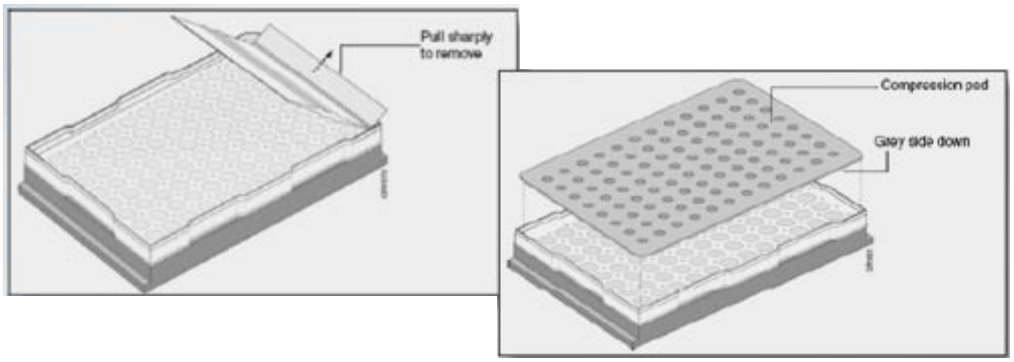



**Fig. 2-17: 96-well-plate for RT-PCR, covered film and compression pad.**

After that passage, it is important to put the plate in the thermal block in the correct way, it should fit well into it to ensure perfect PCR efficiency and uniformity between wells (Nolan et al., 2006). When plate has been positioned into the block, it is time to set parameters of qRT-PCR reaction using ABI PRISM software. It is possible to name all wells with labels in order to better order raw data when reaction is concluded. At the conclusion of the run, the data for each sample are displayed as a typical PCR curve (including linear phase of amplification and end "plateau-phase"). Melting curves can be checked for each sample in order to detect potential problems. The read-out is presented as the cycle number where the linear phase for each sample crosses the threshold level. Data can be exported in Window Excel format.

Scientific community claims that there are some doubts about qRT-PCR results and biological validity of quantification data, because this promising technique still needs universal agreement on basic issues such as quality/quantity control of RNA, assay design, guidelines for reporting results and standardization of protocols (reaction optimization to maximize sensitivity) (Bustin et al., 2005; Peters et al., 2004).

# Chapter 3: MATERIALS AND METHODS

## A SHORT-TERM EXPERIMENT

### 3.0. The aim of the experiment

A hydroponic culture experiment with pears and quinces genotypes was planned in order to keep under real time control leaves and roots during salt stress.

This system showed several advantages in comparison with an open field experiment, like: a) the possibility to easily access to the root apparatus to check health condition and to harvest material for biochemical/molecular analyses; b) the precise imposition of salt stress directly adding NaCl in the nutrient solution bypassing the presence of soil that usually exerts a “buffer” effect.

The aim of this trial was to highlight among quinces and pears rootstocks which of them was more tolerant to salt stress and can be used in saline soil or in particular dry areas. Therefore these genotypes were investigated both from a physiological aspect and from a molecular one. In particular, another purpose of this experiment was to focus on molecular analyses of some genes involved in the “SOS pathway” and well known as ion transporters, such as SOS1, NHX1, HKT1, determinants in salt tolerance.

Results obtained with this trial hopefully will clarify some aspects of physiological and molecular mechanisms that regulate sodium uptake, storage and/or transport in pear and quince rootstocks subjected to salt stress.

### 3.0.1 Plant material

For this hydroponic culture experiment one-year-old plants deriving from the nursery were used. Four different genotypes have been chosen, two pear as cv. Abbé Fétel own-rooted and Farold®40 and two quinces: BA29 and MC. Until the beginning of the experiment, plants were maintained in a cold room (4°C) to keep them dormant. Plants were briefly watered once a week in order to avoid their drying up. When trial started, each plant was trimmed at 40 cm (Fig. 3-1) from collar and if some big

**Fig. 3-1: One-year-old plant of own-rooted Abbé Fétel trimmer at 40 cm.**

roots were present a slight cutting was made to reduce their volume. Each root system was carefully washed to remove the entire soil residue and rinsed with distilled water.

### 3.0.2 Hydroponic culture of quinces and pears

The experiment started on April 10, 2007 and was set up in a growth chamber where two benches were set to contain tanks. Each bench could hold four 40 L plastic tanks, one for each genotype. Due to the space limitations only four plants per genotype were used and placed in the same tank. One bench was assigned to control plants (Fig. 3-2) and the other one to salted plants, but plants in both benches were likewise managed, apart from the salt treatment.

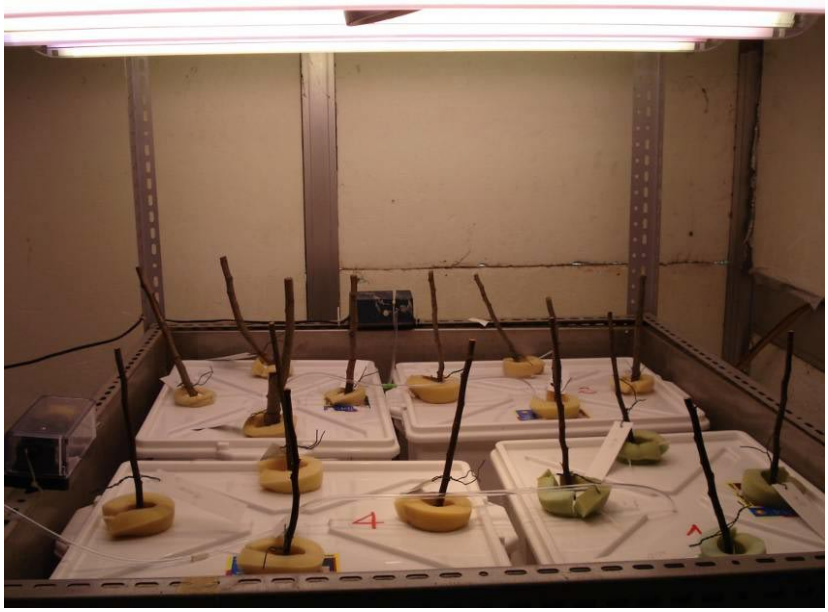


**Fig. 3-2: Bench containing four genotypes in trial dipped into nutrient solution, the first day of the experiment.**

The controlled growth room was set with a photoperiod of 16/8 h light/dark regime at 25/18°C and 50-80% relative humidity. Light was supplied by Grolux 36 W fluorescent lamps (Osama Sylvania, Washington DC, USA) and by an additional halogen lamp (12 V) covered with a glass and located in the middle of the others. Fluorescent lights are known to be softer and less damaging for tender young plants (Haughton, 2004). The light apparatus was positioned at 80 cm from the bench bottom and light intensity was measured at various positions on the benches with a hand-held luxmeter (Silimet s.r.l., Modena, Italy) and readings were expressed in µEinsteins per square meter per second ($\mu E/m^2/s$). Depending on the distance from

the lamps, light intensity measurements ranged from 60 to 100 µE/$m^2$/s; respectively at 20 cm from the tank lid (Fig. 3-3) and close to the lamps.

In comparison with other hydroponic culture experiments present in the literature, this light intensity was lower but enough to assure plant growth and avoid a photoxidation effect. To distribute position effect of light intensity on all plants, lids were 180° rotated weekly.

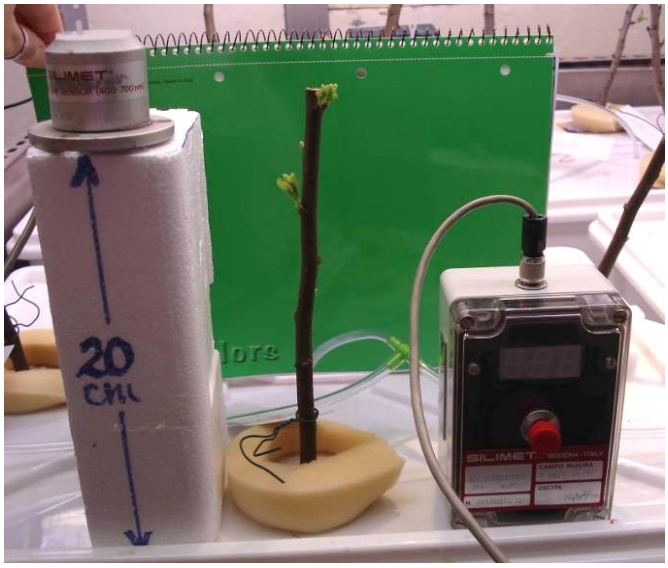



**Fig. 3-3: Measurement of light intensity by luxmeter at 20 cm from the tank lid, after 8 days of hydroponic culture.**

Each tank lid was modified by cutting four holes (Ø 6 cm) and a polystyrene circle was placed surrounding the trunk to support the plant; in this way plants were stable and out from the lid for 20 cm (Fig. 3-4 and 3-5). All tanks were initially filled with 30 L of 1/10 full strength Hoagland's solution that gradually was increased reaching the half strength concentration after five weeks of plant growing. The initial pH of Hoagland's solution (Table 3-1) was adjusted to 6.0 with 0.5 N KOH and monitored using a portable pH-meter, also before discharging the three-day-old solutions.

Using a portable EC-meter it was possible to control also the electrical conductivity of nutrient solution which ranged from 0.23 dS/m (1/10 Hoagland) to 0.85 dS/m (1/2 Hoagland) without any salt imposition.

The nutrient solution was continuously aerated by aquarium pumps (Schego pump 220 V, 5 W, 50 Hz, 350 L/h) connected by plastic pipes with porous chalks (Fig. 3-6).

**Fig. 3-4: Set up of the hydroponic culture in 30 L tanks; plants are fastened to the lid and the access to roots is easy.**

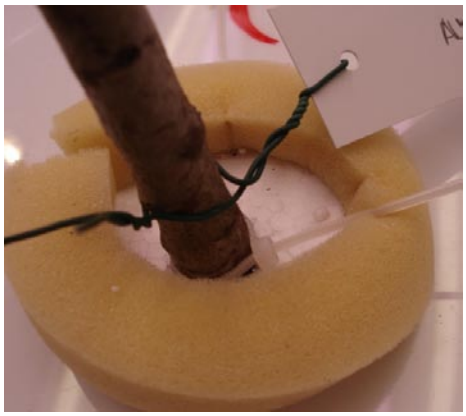


**Fig. 3-5: Detail of plant support system.**

**Fig. 3-6: Detail of porous chalks connected by plastic pipes with aquarium pump.**

**Fig. 3-7: Aquarium pump is connected by plastic pipes to provide aeration to two tanks simultaneously.**

One pump satisfied the aeration need of eight plants in two tanks (using two chalks per container) to prevent radical hypoxia. In the entire experimental set up, four pumps were used and regulated to bubble in the same way; in addition all tanks were covered with black plastic paper in order to prevent the formation of algae. Fresh solution was replaced every three days to avoid nutritional imbalance from evaporation and ion absorption by roots (Viti and Cinelli 1993).

After 34 days of hydroponic culture a Fe-EDTA-chelate foliar treatment with Anticlor Fe24® (3 ml/L) was performed on all plants to prevent chlorosis. Before doing this application, leaves and roots sampling was made and this material, collected in separated bags, was immediately frozen in liquid nitrogen for further molecular analyses. This first sampling was termed as T0 because it represented the vegetal material one day before salt stress imposition (May, 14$^{th}$).

**Table 3-1: 1/2 Hoagland nutrient solution**

| Components | final concentration | solution |
|---|---|---|
| $KNO_3$ | 2.5 mM | |
| $MgSO_4$ $7H_2O$ | 1.0 mM | 1 |
| $KH_2PO_4$ | 1.0 mM | |
| $Ca(NO_3)_2$ $4H_2O$ | 2.5 mM | 2 |
| $MnCl_2$ $4H_2O$ | 4.6 µM | |
| $H_3BO_3$ | 23.2 µM | |
| $Na_2MoO_4$ | 0.06 µM | 3 |
| $ZnSO_4$ $7H_2O$ | 0.4 µM | |
| $CuSO_4$ | 0.185 µM | |
| FeEDTA | 50 mM | 4 |
| Sol.1: 100 ml for 20 L<br>Sol.2: 50 ml for 20 L<br>Sol.3: 10 ml for 20 L<br>Sol.4: 10 ml for 20 L | | |

## 3.1 Physiological measurements

In about five weeks all plants in trial had developed a good foliage coverage and it was necessary to measure the shoot length and the number of shoots per plant before stress application; this measurement allowed to calculate the average shoot length for each genotype at T0. Shoot length was checked again after seven weeks from the beginning and at the end of the experiment (June 7$^{th}$) corresponding respectively to T2 and T4 timings.

Leaf chlorophyll concentration was also estimated the same day before the stress (T0) using a portable Chl meter (SPAD-502, Minolta, Osaka, Japan) on five fully expanded leaves on each of the four plants per tank. Leaf chlorophyll concentration measurement was repeated as previously reported after six weeks (May 22$^{nd}$) and after seven weeks from the beginning, corresponding to T1 and T3.

### 3.1.1 Salt stress imposition

The five-week plants grown (Fig. 3-8) in the bench assigned to “salted plants” were supplied with standard ½ Hoagland’s solution containing 70 mM NaCl and the electrical conductivity measured in each “salty” tank was around 6.74 dS/m. Fresh solution replacement was maintained twice a week also after the beginning of salt stress and the only difference between the two benches was the addition in the “salted one” of the calculated amount of NaCl dissolved in the nutrient solution. Plants and culture system (tanks and pipes) were rinsed with distilled water every time new nutrient solution was supplied, to prevent contaminations (fungi and/or algae) and also the electrical conductivity was double-checked in each tank with new solution.

In experiments for evaluating salt stress response like this one, an important consideration to think about is that plasmolisys should be avoided as much as possible; this can be achievable applying NaCl stress with gradual increase in steps of 50 mM or less, giving to the plants some time to adjust after each intensified application. On the contrary application of a salt shock can cause deleterious effects associated with plasmolysis of roots cells (Verslues et al., 2006; Munns, 2002b). For this reason, after two days NaCl concentration was increased to 80 mM (corresponding to 7.38 dS/m). After six days since the stress started, first necrosis

symptoms appeared in a couple of OHF plants and the 7$^{th}$ day also in Abbé Fétel own-rooted and in all the OHF plants. From previous field experiments on pear and quince plants (Musacchi et al., 2006a) it has been demonstrated that at 5 dS/m electrical conductibility, generated by irrigation with NaCl, these plants did not show any necrosis, but only a reduction in shoot length, regardless of the genotype. To our knowledge, hydroponic experiments like this one described on pears and quinces genotypes have not been performed before. So the salt peak threshold was established referring to the work made by Marino and Molendini (2005) where they consider NaCl levels above 5 g L$^{-1}$ (85.5 mM NaCl) as toxic for "*in-vitro*" cultured pear.

According with this result, after thirteen days, the salinity level was increased again until 90 mM NaCl (equivalent to about 9.5 dS/m) in order to clearly observe the difference in symptoms onset in all four genotypes, since at 80 mM NaCl quinces appeared still healthy. Once 90 mM NaCl concentration was reached, it was


**Fig. 3-8: Development of plants in hydroponic culture in 5 weeks without the imposition of the salt stress. A) MC plant at the starting day; B) MC plant after 1 week; C) MC plant after 3 weeks; D) MC plant after 5 weeks; E) and F) situation of general plant growth before salt stress imposition in both benches.**

maintained constant until the end of the trial.

During the salt stress, pictures were taken to follow up to necrosis progress for all genotypes.

### 3.1.2 Gas exchange measurements

Gas exchange measures were carried out twice during the entire experiment: the first one at T1 and the last one at T3, always in the morning using a portable gas exchange system LICOR 6400 (Bioscience-Nebraska USA, Fig. 3-8)).

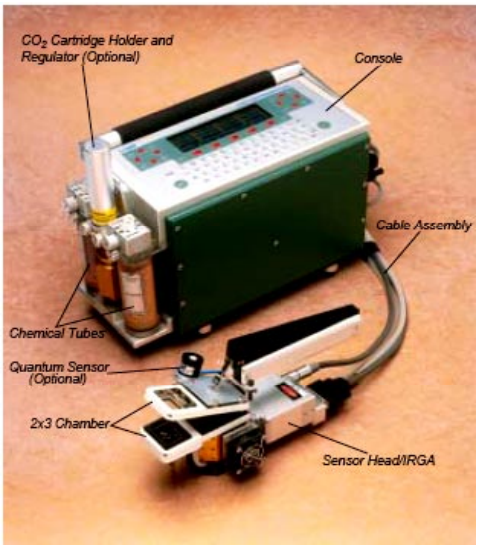



**Fig. 3-8: LiCOR 6400 apparatus**

This measure was made with an external quantum sensor (µmol $m^{-2}s^{-1}$) in order to evaluate photosynthesis with the real light of lamps presents in the growth chamber, instead of measuring it with an internal-chamber quantum sensor (µmol $m^{-2}$ $s^{-1}$) that need to be set with a determined PAR (for instance 1300 µmol $m^{-2}$ $s^{-1}$ as full field light conditions). For each plant, three fully expanded leaves exposed to light, and not in shadow, were chosen to perform the measure. During T1, gas exchange measure was performed on all four genotypes (controls and "salted"), instead in T3 necrosis was so expanded in both pear genotypes leaves that it was not possible to conduct the measures on them.

## 3.2 Plant material sampling for molecular analyses

During salt stress imposition, other leaves and roots samplings were made (besides T0) freezing immediately the vegetal samples in liquid nitrogen and storing them at -80°C, until following molecular analyses. In particular, T2, T3 and T4 samplings concerned both organs; instead for leaves an additional harvesting was made after one week of NaCl stress (named T1), but at that time it was not considered opportune to cut also fine roots because they were not so abundant for all genotypes and this could compromise the experiment. Later it was decided to not use the T1 for molecular analyses.

## 3.3 Plant destruction and disassembling phase

At the end of the eight weeks of experiment, all plants were collected and roots properly rinsed with distilled water to eliminate the salt excess on their surface.
Some pictures were taken to prove final condition of the four genotypes treated with NaCl, comparing with the corresponding control ones.
Then each plant was divided in organs: leaves (in any healthy condition), branches, trunk, coarse roots and fine roots (≤ 2 mm Ø). The fresh weight of the different plant parts was valued, then samples were dried in a ventilated oven at 60°C and dry weights were determined after 3-4 days for leaves and after one week for wooden parts. The difference between fresh and dry weights allows to evaluate the percentage in water content in all genotypes, comparing controls against NaCl treated samples. Dry samples were utilized in further steps for mineral analyses.

### 3.3.1 Mineral analyses

#### 3.3.1 a Mineralization

Representative subsamples of each dried organ were ground into powder using a Mill (Fritsch P14, Fritsch Germany) to pass a 0.2 mm mesh. Leaves were milled without removing the petiole.
From each sample, about 240-260 mg of powdered material was weighted and put into a Teflon vessel and 8 ml of 65% Nitric acid (Sigma) and 2 ml of $H_2O_2$ (Sigma) were added to the sample. The mineralization reaction was performed inside each vessel, properly closed, according to manufactures' instructions in a microwave oven "Ethos TC" Milestone. This instrument worked on set programs, depending on the kind of vegetal material mineralization can last longer. Mineralization cycle used for standard material, such as leaves, branches and trunk, was characterized by 2 steps at 800 W and 180 °C for 10 min, the first one to reach the conditions and the second one to hold them. Instead, the cycle used for roots had step 2 with same temperature and power, but long 15 min.

**Fig. 3-9: Mineralization cycle in progress visualized in the Ethos TC display.**

Only after vessels cooled down around 30°C, it was possible to recover digested material. The liquid vessel content appeared lightly yellow but clear; only for root samples siliceous residues could be present; in that case the liquid sample was filtrated through Whatmann paper #1. Otherwise, all the sample was poured into a 25 ml flask and vessel rinsed three times with double-distilled water to recover quantitatively the material. After having adjusted final volumes, all samples were collected in proper containers, resistant to acid until further analyses.


**Fig. 3-10: On the left: details of vessels incorporated in theirs supports immediately after the end of mineralization cycle and on the right the microwave Ethos TC, Milestone.**

### 3.3.1 b Macro-elements and micro-elements determination

Macro and micro-elements concentrations were determined by an atomic absorption spectrophotometer (SpectrAA200, Varian, Australia, Fig. 3-11).

For each element to be analyzed a calibration curve was done, preparing five solutions of the standard element at different concentrations starting from a 1000 ppm mother solution. For micro-elements analyses, original sample did not need any dilutions, instead for macro-elements samples were diluted 1:2 adding to final solution 4% (v/v) La and 1% (v/v) Cs. Micro-elements analyzed have been iron (Fe), copper (Cu), manganese (Mn) and zinc (Zn) using a calibration curve for each one with the following standard concentration solutions: 10, 5, 2.5, 1, 0.5 ppm. Macro-elements measured by AAS have been magnesium (Mg), potassium (K), calcium (Ca); Mg standard solutions were: 2.5, 5, 10, 20, 30 ppm, for K were: 5, 10, 50, 125, 250 ppm and for Ca: 20, 30, 40, 100, 200 ppm.

Sodium (Na) determination was carried out on original samples, but because of the NaCl treatment in the experiment, half of samples were really too much concentrated for this element; so that some samples were diluted 1:100 in order to stay in the calibration curve made by 1, 2.5, 5, 12.5 25 ppm Na solutions.

Each sample was made in triplicates and the instrument releases ppm value of that element expressed on dry weight of organ, mean of 3 repetitions.


**Fig. 3-11: Atomic absorption spectrophotometer (SpectrAA200, Varian).**

Chloride determination was carried out utilizing a method described in Liu (1998) for the Cl extraction (in water) and the method No.G-133-95 Rev.1 (multitest MT7/MT8) of AutoAnalyzer Application (AxFlow AA3 Bran+luebbe) for the quantification of the element. Chloride determination is based on a colorimetric assay that exploits the release of thiocyanate ions from mercuric thiocyanate by Cl ions in the sample. The liberated thiocyanate reacts with ferric iron to form a red colour complex of ferric thiocyanate. The colour of the resulting solution is stable and directly proportional to the original Cl concentration. The procedure seems very sensitive and the highly coloured plants extracts (obtained from the five organs) do not cause interferences because each sample goes through a dialysis membrane before entering into the colorimeter (Liu, 1998).

Statistical analysis was conducted on all data using double-way ANOVA with SAS software and means separation was performed by Student-Newman-Keuls (SNK) test (p=0.05).

### 3.4 Samplings and pooling of leaves and roots

All frozen samples for molecular analyses were the result of a complete random sampling; for example during salt stress, sampling was made picking up leaves with different level of necrosis and putting them in a same bag into liquid nitrogen in order to homogenise as much as possible the collected subsample representative of that date.

For roots, instead, no symptoms were evident in salinity condition; they appeared always alive also when the foliage showed high degree of necrosis caused by NaCl. So roots harvest was focused only on fine ones that included as older brown portion as white new tips. Coarse roots haven't been cut during sampling because this action could compromise the good plant condition.

For each timing (T0, T1, T2, T3, T4), vegetal material was picked up in the same quantity from foliage of each plant in the same tank in hydroponic culture. In this way each sampling counted 32 collected bags for leaves as for roots. Some shrewdnesses have been adopted in the sampling step; such as: each leaf was quickly dusted with slightly moist paper before putting in the bag and freezing and conversely, roots dipped in nutrient solution were cut and the excess of liquid absorbed with blotter paper then immediately chilled in liquid nitrogen.

Before starting with the RNA extraction, it was convenient organize samples and work; so that for each plant at each timing a pooling of material was carried out.

Pooling consisted in joining together four bags belonged to the same genotype in the same date (example: all leaf bags belonged to MC at T2) in order to reduce the effect of the single plant of a genotype and in this way to emphasize different behaviour of the four genotypes comparing them. Also in the literature some considerations have been made on pooling approach in experimental design, for example: "Pooling samples...increases precision by reducing the variability of the experimental material itself. When variability between individual samples is large and the units are not too costly, it may be worthwhile to pool samples."-Churchill, *Nature Genetics*, 2002.

Practically, pooling step of material was performed using a plastic container with a lot of liquid nitrogen and pouring inside it the content of each bag referred to same

genotype and same date. Leaves originally were entire and during this step they were ground roughly using a pestle but not till reaching powdered consistence.
The joined material was stored again at -80°C in labelled Falcon tubes. The same procedure was adopted for roots, crushing them to get smaller pieces to later grind for RNA extraction.

### 3.4.1 DNA extraction

DNA extraction was performed starting from leaves belonged to four genotypes of interest: Abbé Fétel, Farold®40 (OHF), MC and BA29. Leaves collected from trial were in a healthy condition and they were immediately frozen in liquid nitrogen and later lyophilized.

For each sample 0.05 g of leaves were weighted and put in a 2 ml eppendorf tube with the addition of a spatula of silicon carbide. DNA was extracted in two repetitions for each genotype. Tubes were loaded into a Mixer Mill MM301 (Retsch, USA) set at 29 shots/sec for 3 minutes (Fig. 3-12). This grinding step was repeated inverting tubes support inside the mill to better perform this procedure.

**Fig. 3-12: Mixer Mill MM301**

Pre-warmed (60°C) extraction buffer (Table 3-2) was added to each tube in a volume of 900 µL, and then samples were put at 60°C for 30 min with occasionally inversions. Then an equal volume of dichloromethane-isoamylic alcohol (24:1) was poured and the mixture emulsionated for 10 min. A centrifugation with Coulter Allegra X-22 centrifuge (Beckman) was done for 5 min at 5000 rpm at room temperature. Collected supernatants were placed in new tubes and 10 µL RNase enzyme put in all samples that then were incubated for 30 min at 37°C. Extraction proceeded with adding an equal volume of dichloromethane-isoamylic alcohol (24:1), centrifuging and taking upper phases as previously described.
Then DNA was precipitated with 550 µL of cold isopropanol inverting tubes several times; later samples were centrifuged 5 min at 5000 rpm. Pellets were washed in 500 µL of washing buffer (Table 3-3) which was later discarded. Pellets were kept

under sterile hood to make them dry and later they were resuspended in 300 µL of TE buffer (Table 3-4).

When pellets were well solubilized, 200 µL of 5 M NaCl was incorporated and all the mixture precipitated with addition of 2 volumes of 100% ethanol. To collect DNA at the bottom of tubes it was necessary to centrifuge again for 5 min at 2800 rpm and discard supernatant.

When new pellets obtained were dried again; they were dissolved in a small volume of sterile distilled $H_20$ (100-200 µL).

To measure DNA concentration obtained from extraction, 1 µL of each sample was utilized at Nanodrop ND-1000 UV-Vis spectrophotometer. DNA concentration is expressed in ng/µL and $A_{260}/A_{230}$ and $A_{260}/A_{280}$ ratios are useful to evaluate DNA purity from contaminants (proteins, phenols and polysaccharides).

**Table 3-2: DNA extraction buffer with CTAB**

| Reagents | Final concentration/amount in extraction buffer |
|---|---|
| CTAB (Sigma) | 2% |
| NaCl | 1.4 M |
| EDTA | 20 mM |
| Tris pH 8 | 100 mM |
| β-mercaptoethanol | 1% v/v |
| PVP-40 | 2% w/v |

**Table 3-3: Washing buffer**

| Reagents | Concentration/amount |
|---|---|
| Ethanol | 76 % |
| Sodium acetate | 0.2 M |

**Table 3-4: TE buffer**

| Reagents | Concentration/amount |
|---|---|
| Tris-HCl | 10 mM |
| EDTA | 1 mM |

### 3.4.2 How to deal with RNA: lab equipment and solutions preparation

RNA is more susceptible to degradation than DNA. It can be easily degraded in smaller components by the action of a type of nuclease know as Ribonuclease (commonly abbreviated RNase). RNase A is a single-strand specific endoribonuclease that is resistant to metal chelating agents and can survive to prolonged boiling or autoclaving but can be inactivated by the histidine-specific alkylating agent, diethyl pyrocarbonate (DEPC).

In order to reduce the presence of RNases during the course of experimentation it is important to follow some laboratory procedures:

1. Wear always gloves because ungloved fingers can introduce bacteria into solutions resulting in RNase contamination.
2. All plastic material (such as eppendorf tubes, Sarstedt tubes) is considered RNAse -free, according to the label before opening the original bag; so, always with gloves, it has been handled and then sterilized by autoclaving. The original bag must be closed to avoid dust deposition and dedicated only to RNA.
3. Glassware and everything that can be in contact with RNA must be soaked in 0.1 M NaOH for 1 hour and then rinsed properly with distilled water and autoclaved at 120°C for 20 min. Autoclaving a solution, without this treatment, kill contaminating bacteria, but RNases, liberated from them and already present, are still active.
4. All the working solutions must be treated with DEPC (Diethyl Pyrocarbonate). DEPC is effective as a nuclease inhibitor; each solution is treated with 0.1% DEPC and shaken. The DEPC hydrolyzes over time to form ethanol and carbon dioxide. After an overnight incubation, solutions can be autoclaved to destroy the DEPC and later containers opened to allow the $CO_2$ release. All solutions, except buffers containing Tris, were subjected to this procedure. Also distilled water was treated with DEPC and then used for pellet solubilisation and also for rinsing steps of all RNA lab ware.

### 3.4.3 RNA extraction from roots and leaves of quinces and pears

Frozen samples used to extract RNA have been pooled from hydroponic culture material as described above. RNA extraction was carried out on pooled leaves as on pooled roots; each extraction was done in two repetitions in order to obtain enough material to accomplish expression analyses. For each organ, one repetition included 32 samples (4 genotypes X 2 treatments X 4 timings), so that the total extractions performed have been 128 (32 samples X 2 repetitions X 2 organs).

The chosen RNA extraction protocol is a basic method that utilizes SDS/phenol for the isolation of RNA from plant tissues; it has been taken from Wilkins and Smart (1996) and modified on the basis of need and plant material features.

Before taking out sample from -80°C refrigerator, one 13 ml tube was prepared with extraction buffer (Table 3-5 reagent first step) and 1% (w/v) PVP and 1% (w/v) PVPP. Extraction buffer tube and a phenol:chloroform (5:1) aliquot at 4.5 pH (Ambion) were both put in a pre-warmed 65°C bath to keep them at constant temperature.

**Table 3-5: RNA extraction buffer**

| Reagents first step | Final concentration/amount in extraction buffer |
|---|---|
| Tris HCl pH 8.5 | 100 mM |
| NaCl | 100 mM |
| EDTA pH 8 | 5 mM |
| SDS | 1% w/v |
| DEPC $H_2O$ | to volume |
| PVP | 1% w/v |
| PVPP | 1% w/v |
| Reagent second step | |
| ascorbic acid | 5 mM |
| Proteinase K | 100 µg |
| β-mercaptoethanol | 2.5% v/v |

Plant material was weighted (1 g for 10 ml extraction buffer) and immediately put in the pre-chilled porcelain mortar and ground with liquid nitrogen and pestle until obtaining a fine powder. Immediately before transferring extraction buffer into powdered samples, hot buffer was refined with 2.5% (v/v) o β-mercaptoethanol, 5 mM ascorbic acid and (0.1 mg/ml) Proteinase K (reagents second step) and then immediately added to powdered samples and strongly vortexed to well homogenize powder and buffer. After that, samples were incubated at 65°C for 10 min.

One volume of pre-warmed phenol:chloroform (5:1) at 4.5 pH (Ambion) was poured in samples which were replaced in 65°C bath for other 10 min vortexing periodically. The pH of phenol is important since chromosomal DNA will end up in the phenol phase if the pH is acid (around pH 5). Phenol is very corrosive and will severely burn the skin. Safety precautions such as gloves, protective eyewear, a lab coat, and working in a fume hood are critical. Phenol extraction is a commonly used method for deproteinization of nucleic acids (Ausubel et al., 1987, Wallace 1987, Sambrook et al., 1989). Most proteins are more soluble in phenol than in the aqueous phase. Conversely, nucleic acids are more soluble in the aqueous phase. Centrifugation of the mixture will yield two phases; the lower phase is the organic phase and will contain proteins, usually as a white flocculent ring at the interface. The upper aqueous phase will contain nucleic acids (Ambion Inc., QC Form 0376). During the incubation time, ultra-centrifuge Optima (Beckman) was set at 13000 rpm and 4°C; then samples were centrifuged at these conditions for 10 min. Supernatants were put into new tubes paying attention not to touch the white interface and everything was placed on ice to avoid RNA degradation during extraction. One volume of phenol:chloroform:isoamylic alcohol (25:24:1-Fluka) from the fridge was add to supernatants, tubes vigorously vortexed and placed in centrifuge at 13000 rpm and 4°C for 10 min. Upper phases were collected again and the addition of phenol:chloroform:isoamylic alcohol (25:24:1) with subsequent centrifugation was repeated as previous step. To new supernatants, one volume of chloroform:isoamylic alcohol (24:1-Fluka) was added and then another step of vortex and centrifugation as above. After this last centrifugation the upper phase was collected measuring the correct volume obtained and placing it in a 13 ml Sarstedt tube. Depending on different volumes, 0.1 volume of 3 M sodium acetate

(pH 4.8) and 1 volume of isopropanol (stored at -20°C) were incorporated and tubes shacked by inversion. In this step, solution appeared viscous and nucleic acids started to aggregate. To better promote acid nucleic precipitation, all samples were put 1 hour at -80°C. After that freezing incubation, tubes were put on ice to defrost and later loaded into the centrifuge for 30 min at 13000 rpm and 4°C. Supernatant of each tube was discarded carefully to save nucleic acid pellets which were straightway washed with 70% cold ethanol. After a 5 min centrifugation, the ethanol was removed and pellets kept on ice under sterile hood to make them dry.

When no more drops were present inside tubes, 2X TBE was added on pellets to dissolve them, after that also 4 M LiCl was supplied in order to separate DNA from RNA (respective final concentrations: 1X TBE and 2 M LiCl). This step was extended overnight on ice. The use of lithium chloride in RNA precipitation is a fast, convenient method of isolating transcripts from "*in-vitro*" transcription reactions with very low carryover of unincorporated nucleotides. A major advantage of lithium chloride is that it does not efficiently precipitate either protein or DNA (*Applied Biosystem*, Technical Bulletin #160).

The day after, tubes were centrifuged for 30 min at 13000 rpm and 4°C and supernatant containing DNA was discarded, instead RNA pellet rinsed with 70% cold ethanol. After a brief centrifugation pellet was re-suspended in an appropriate volume of DEPC water. If pellet dissolves quickly it means that RNA is clean, really important feature for further steps.

Then RNA was precipitated with 1 volume of cold isopropanol and 0.1 volume of 3 M sodium acetate (pH 4.8) for 1 hour at -80°C; later samples were defrosted slowly on ice and centrifuged 5 min at 13000 rpm and 4 °C. Pellets were washed again with 70% ethanol as previously described and when they looked dry, 20 µL DEPC $H_20$ was incorporated to solubilise RNA. Failure to remove traces of ethanol will inhibit RNA resuspension.

RNA dissolved in water was incubated on ice for minimum 30 min in refrigerator to be sure that all the acid nucleic of interest was completely dissolved before measuring its concentration.

This method described above is the general protocol for RNA extraction from plant tissue and, generally, it considers one gram of plant material for 10 ml of extraction

buffer, but firstly this protocol was adopted in small volumes, making calculation to have 1 ml of buffer and 100 mg of plant material. This modification was done only for the first repetition of extraction from leaves; the second one was performed with about 0.5 g of plant material and 5 ml of buffer to get more RNA than in the previous one.

RNA extraction from roots was more complicated than for leaves because of difficulties in grinding step and in polysaccharides content of this particular material. To cope with difficulty in manually grinding frozen roots, where resilience of cell wall to rupture is more pronounced, 50-70 mesh particle size white quartz ($SiO_2$-Sigma) was used to facilitate cell disruption (Fig. 3-12). Sand in virtue of its micro-roughness rips off cell walls and helps in homogenization of plant material (http://matcmadison.edu/biotech/resources/proteins/labManual/chapter_4.htm; Eggermont et al., 1996).

**Methods of Cell Disruption**

| Technique | Example | Principle |
|---|---|---|
| **Gentle** | | |
| Enzyme Digestion | Lysozyme treatment of bacteria | Cell wall digested, leading to osmotic disruption |
| Hand Homogenizer | Liver tissue | Cells forced through narrow gap |
| Mincing, (grinding) | Muscle | Shear force |
| **Moderate** | | |
| Blade Homogenizer | Muscle tissue, most animal tissues, plant tissues | Chopping action breaks up large cells, shears apart smaller ones |
| Grinding with abrasive (Alumina, sand) | Plant tissues Bacteria | Microroughness rips off cell walls |
| **Vigorous** | | |
| French Press | Bacteria, plant cells | Cells forced through small orifice at very high pressure; shear force disrupts cells |
| Ultrasonication | Cell suspensions | Microscale high-pressure sound waves cause disruption by shear forces and cavitation |
| Bead mill | Cell suspensions | Rapid vibration with glass beads rips cell walls off |
| Manton-Gaulin homogenizer | Cell suspensions | Same as French press but larger scale |

**Fig. 3-12: Method of cell disruption, with particular interest to sand (circled in red).**

About polysaccharides content in roots, several attempts have been done, like adding a step with 0.5 calcium hydroxide ($Ca(OH)_2$) or with 2-butossiethanol, but they did not give any improvement. So, for RNA extraction from roots, protocol was maintained equal to leaves' one apart from sand addition and the amount of buffer

utilized was 5 ml for both repetition, because RNA yield from roots is a bit lower than in leaves.

### 3.4.4 RNA quantification

To measure RNA concentration by optical density a Nanodrop ND-1000 UV-Vis spectrophotometer (http://www.nanodrop.com) has been used. It is a really precise instrument that provides reliable readings and only 1 µL for each sample is necessary to get the RNA quantification directly expressed in ng/µL.


**Fig. 3-13: Nanodrop ND-1000**

The concentration of RNA should be determined by measuring the absorbance at 260 nm ($A_{260}$). An absorbance of 1 unit at this wavelength corresponds to 40 µg of RNA per ml ($A_{260}$ = 1 => 40 µg/ml).

This relation is valid only for measurements in water. Therefore, if it is necessary to dilute the RNA sample, this should be done in water.

RNA purity was estimated by the ratio between the absorbance values at 260 and 280 nm ($A_{260}/A_{280}$) and between 260 and 230 nm ($A_{260}/A_{230}$); respectively indicate contaminations by proteins and phenols and polysaccharides. The $A_{260}/A_{280}$ ratio should fall in the range of 1.7-2.1 and $A_{260}/A_{230}$ of 1.8 or more to say that RNA is of good quality without limiting contaminations (Fleige and Pfaffl, 2006).

### 3.4.5 RNA integrity

The most frequently used procedure for estimating RNA integrity is fractionating 1 µg of a total RNA sample on a 1% denaturing agarose gel in 1X TAE, but also a conventional DNA non-denaturating gel was effective previous denaturation of RNA sample at 95°C for 5 min.

Ethidium bromide staining of the RNA should reveal the 18S and 28S ribosomal RNA (rRNA) bands. Theoretically if these bands are discrete (i.e. there is no significant smearing below each band) and the 28S rRNA band is approximately twice as intense as the 18S rRNA band, then the mRNA in the sample is of good quality

(Palmer and Prediger, 2004). Otherwise if a smear appears in the lane it means that RNA is degraded and it can't be used for next analyses.

### 3.4.6 RNA precipitation

For each sample, RNA was precipitated using a salt/ethanol method, the same used for DNA (Crouse and Amorese 1987; Sambrook et al., 1989). In particular, 0.1 volumes of 3 M sodium acetate pH 4.8 and 2.5 volumes of 100% cold ethanol were added to each RNA samples dissolved in water. Samples treated in this way need to be stored at cold temperature, preferably at -80°C, so good RNA quality can be maintained.

These samples were shipped in dry ice to Purdue University, West Lafayette, IN where it was planned to carry out molecular analyses at Center for Plant Environmental Stress Physiology Horticulture and Landscape Architecture Department, in collaboration with Prof. Ray Bressan.

### 3.4.7 DNase I treatment

Although on agarose gels, RNA samples did not show any DNA contamination, that is clearly recognizable as a heavy band that does not run fast in electrophoresis; RNA needs to be cleaned with DNAse I treatment before performing retrotranscription step.

Total extracted RNA has been digested by enzyme DNAse I (Amersham Pharmacia, Biotech) following protocol described in Paris, 2006 (phD dissertation). This enzyme is supplied in 10 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 10 mM $MgCl_2$, 50 % glycerol.

RNA used was previously precipitated in ethanol and sodium acetate for shipment and then stored at -80°C until further steps. Immediately before DNAse I digestion, RNA samples were centrifuged at 14000 rpm for 30 min at 4 °C and supernatants containing ethanol discarded. Pellets were rinsed with cold 70 % ethanol and then let them dry on ice. At the end each pellet was resuspended in a proper volume of DEPC water, usually 20-40 µL were enough for almost all samples. After solubilization step, 1 µL of RNA was quantified again and 20 µg transferred in a new RNAse-free tube. DNAse enzyme needs a buffer to work properly; it was composed

by 40 mM Tris-HCl pH 7.5 and 6 mM $MgCl_2$. The reaction mix was constituted as follow (Table 3-6):

**Table 3-6: DNaseI treaction mix**

| Component | Amount |
|---|---|
| Total RNA | 20 µg |
| DNAse I | 1 µL |
| 10X Buffer | 3 µL |
| DEPC water | to reach 30 µL |

The reaction was executed at 37°C inside an incubator for 20 min. After that step, a new phenol extraction was performed. In each tube 20 µL of DEPC water was added and then also 50 µL of phenol:chloroform:isoamylic alcohol (25:24:1-Fluka) were incorporated. Tubes were vigorously vortexed and placed in centrifuge at 15000 rpm and 4°C for 5 min. Upper phases were collected again in new RNAse-free tubes. The lower phases in the original tube were used for a second extraction step; inside these tubes 25 µL of DEPC water was added. A subsequent centrifugation was repeated as before and new supernatants were collected and incorporated with the previous ones. The precipitation was accomplished with 0.1 volume 5 M sodium acetate and 2.5 volumes of 100% ethanol (stored at -20°C) in each tube followed by an incubation period of 1 hour at -20°C. After that chill incubation, tubes were put on ice to defrost and later loaded into the centrifuge for 20 min at 15000 rpm and 4°C. Supernatant of each tube was discarded carefully and pellets washed with cold 80% ethanol. After a 5 min centrifugation, the ethanol was removed and pellets kept on ice under sterile hood to make them dry. To solubilise RNA, 20 µL DEPC $H_20$ was incorporated and after that RNA was quantified as previously.

### 3.4.8 Retrotranscription

Retrotranscription (RT reaction) is a process in which single-stranded RNA is reverse transcribed into complementary cDNA by using total cellular RNA. RT reaction is also called first strand cDNA synthesis. This synthesis took place in a RNAse-free tube in a 20 µL of reaction with different steps following ThermoScript™ RT-PCR System's protocol (Invitrogen).

First step involved components listed in the next table 3-7; this mixture was incubated at 65°C for 5 min and then placed on ice.

**Table 3-7: First step Retrotranscription mix**

| Components | Amount |
|---|---|
| Total RNA (10 pg-5 µL) | X µL |
| Oligo $(dT)_{20}$ (50 µM) | 1 µL |
| 10 mM dNTP mix | 2 µL |
| DEPC water | to 12 µL |

After that, the 5X cDNA Synthesis Buffer (250 mM Tris acetate pH 8.4, 375 mM potassium acetate, 40 mM magnesium acetate, and stabilizer) was vortexed properly for 5 sec and used to prepare a reaction mix on ice as follow (Table 3-8):

**Table 3-8: Second step Retrotranscription mix**

| Component | Amount for 1 reaction |
|---|---|
| 5X cDNA Synthesis Buffer | 4 µL |
| DTT 0.1 M | 1 µL |
| RNaseOUT™ (40 U/ µL) | 1 µL |
| DEPC water | 1 µL |
| ThermoScript ™ RT (15 units/µL) | 1 µL |
| Total volume per reaction | 8 µL |

In each tube placed on ice, 8 µL of this mix reaction were pipetted and transferred immediately to a thermal cycler preheated to the appropriate cDNA synthesis temperature (55°C) and incubated for 1 hour. Last step of retrotranscription reaction was incubation at 85°C for 5 min. From synthetized cDNAs an aliquot of 2 µL was used to check if they worked properly by PCR amplification for one housekeeping gene. Leftovers of cDNAs were stored at -20°C for later expression analyses.

PCR was performed with 2 µL of cDNA at 58°C of annealing for 40 cycles using actin primers (Table 3-9) designed on apple for qRT-PCR (by Dr. Roberta Paris, PhD

dissertation thesis). PCR products should be around 80 bp and they were checked on a 2% agarose gel with DNA 100 bp ladder on a side.

**Table 3-9: Actin primers pair on apple**

| Primers pair name | gene | Target genotype | Forward 5' to 3' | Reverse 5' to 3' |
|---|---|---|---|---|
| **ACT** | actin | apple | TATGAAGGGTATGCCCTCCC | CTGTAAGATCACGACCCGCC |

## 3.5. Cloning and sequencing

### 3.5.1 Heterologous primers design for cloning

To proceed with the isolation of fragments of SOS1, HKT1 and NHX1 genes involved in SOS pathway in pears and quinces genotypes; primers design was necessary. Having specific primers for a candidate gene gives the possibility to adopt a PCR approach with DNA/cDNA as template for reaction. First of all it has been important looking for all candidate gene sequences belonged to different species present in database (http://www.ncbi.nlm.nih.gov/) in order to mach them together and highlight conserved regions (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

If the gene of interest hasn't been studied yet in a particular plant species, it is better to take in considerations related sequences obtained by database research that showed higher homology, discarding species genetically distant. Anyway sometimes it is difficult to discover some strategic conserved regions to design primers because, although sequences have been selected, homology does not result 100%. For amplification of related sequences from different organisms, or for "evolutionary PCR", it is possible to design "degenerate" primers, in order to increase the chances of getting PCR product. A PCR primer sequence is called *degenerate* if some of its positions have several possible bases. The degeneracy of the primer is the number of unique sequence combinations it contains (Linhart and Shamir, 2005).

Starting from NHX gene, a literature research was performed to find out main studies about this $Na^+/H^+$ antiporter located on tonoplast.

Between all papers about this gene, a work carried out by Hanana et al., 2007 was really useful because it showed the phylogenetic tree (Fig. 3-14) of NHX-like vacuolar cation/$H^+$ antiporters.

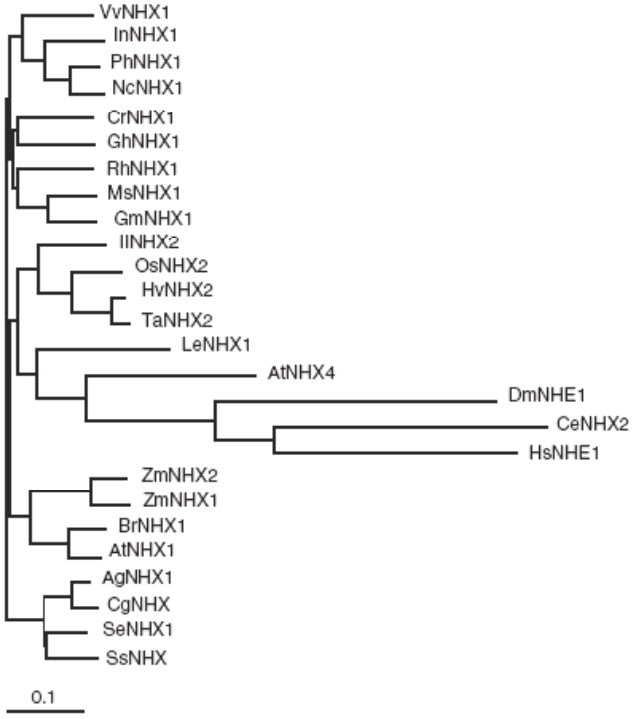



**Fig. 1** Phylogenetic tree of NHX-like vacuolar cation/$H^+$ antiporters. VvNHX1, *Vitis vinifera* (AAV36562); PhNHX1, *Petunia hybrida* (BAB56105); CrNHX1, *Citrus reticulata* (AAT36679); NcNHX, *Nierembergia caerulea* (BAB56106); MsNHX, *Medicago sativa* (AAR19085); InNHX1, *Ipomoea nil* (BAD91201); GhNHX1, *Gossypium hirsutum* (AAM54141); GmNHX, *Glycine max* (AAR27596); IlNHX2, *Iris lactea* (AAU81619); AgNHX1, *Atriplex gmelini* (BAB11940); RhNHX, *Rosa hybrida* (BAD93487); SeNHX1, *Salicornia europaea* (AAN08157); CgNHX, *Chenopodium glaucum* (AAQ72785); SsNHX, *Suaeda maritima* subsp. salsa (AAK53432); HvNHX1, *Hordeum vulgare* (BAC56698); OsNHX1, *Oryza sativa* (AAQ74383); TaNHX2, *Triticum aestivum* (AAK76738); ZmNHX2, *Zea mays* (AAP20429); BrNHX1, *Brassica napus* (AAO38856); ZmNHX1, *Zea mays* (AAP20428); LeNHX1, *Lycopersicon esculentum* (CAC84522); AtNHX4, *Arabidopsis thaliana* (AAM08405); DmNHE1, *Drosophila melanogaster* (AAD32689); CeNHX2, *Caenorhabditis elegans* (AAA62524); AtNHX1, *Arabidopsis thaliana* (AAD16946); HsNHE1, *Homo sapiens* (AAF25592).

**Fig.3-14: Phylogenetic tree of NHX-like vacuolar cation/H+ antiporters by Hanana et al., 2007**

Each accession number related to NHX and reported in that paper was checked in NCBI data bank and between 25 sequences only 18 were chosen to be matched together. Discarded sequences were or not referred to NHX1 or not plant sequences or not mRNA.

From this NHX1, in mRNA sequences alignment, four conserved regions were found out and on these four degenerate primers were designed (two forward and two reverse Table 3-10) paying attention on bases divergence frequencies and assigning the correct letter in the “degenerate code” (i.e. if base A and C are both present in the alignment put M, fig.3-15).

If degeneracy is high (>5 degenerated bases on 20 bp length primers) annealing specificity of primer decreases during PCR reaction, so that it can be possible not to obtain any PCR products. This happens more frequently if degenerated bases are mainly located at 3’ of primer where polymerase is used to stick to star replication and so correctly amplifying the DNA fragment included between two primers. For this reason it is better to avoid putting degenerated bases at 3’ because it needs to be a stable attachment for polymerase.

| |
|---|
| W=A or T |
| S=C or G |
| R=A or G |
| Y=C or T |
| K=G or T |
| M=A or C |
| B=C, G or T |
| D=A, G or T |
| H=A, C or T |
| V=A, C or G |
| N=A, C, G, or T |

**Fig. 3-15: Letters for degeneracy of primers.**

**Table 3-10: Degenerated primers designed to clone NHX1.**

| gene | primer name | 5'-3' | degeneracy | Tm °C | bp |
|---|---|---|---|---|---|
| NHX1 | **Deg1FOR** | GC**R**AC**N**GATTCTGT**N**TGCAC | 3 | 61 | 20 |
| NHX1 | **Deg2REV** | CAT**D**AG**M**CCAGCCCACCA**W**A | 3 | 61.6 | 20 |
| NHX1 | **Deg3FOR** | G**N**TGGATGAA**Y**GA**R**TCCATC | 3 | 59 | 20 |
| NHX1 | **Deg4REV** | A**RD**GCATCCAT**V**CCAACATA | 3 | 56.2 | 20 |

Stock primers (100 µM) were diluted in sterile water to a ready-to-use concentration of 10 µM. Degenerated NHX primers were tested by PCR reaction at different annealing temperature, adding 19 µL of PCR reaction mix, as described in the table 3-11 to 1 µL of template (DNA or cDNA from leaves of genotypes in trial). Template utilized for PCR was genomic DNA extracted from MC and Farold®40 (chosen to compare one pear and one quince).

**Table 3-11: PCR reaction mix**

| Reagents | µL per sample |
|---|---|
| sterile water | 8.85 |
| GoTaq® 5X Green Flexi buffer (Promega) | 4 |
| $MgCl_2$ 25 mM (Promega) | 2 |
| dNTP 10 mM (Sigma) | 2 |
| primer for 10 µM (Operon) | 1 |
| primer rev 10 µM (Operon) | 1 |
| GoTaq® polymerase 5U/µL (Promega) | 0.15 |
| DNA/cDNA | 1 |
| Total volume | 20 |

**Table 3-12: PCR amplification program for NHX1**

| amplification step | temperature °C | time | cycles |
|---|---|---|---|
| denaturation | 94 | 3 min | 1 |
| annealing | **55** | 45 sec | 38 |
| extension | 72 | 40 sec | |
| denaturation | 94 | 40 sec | |
| annealing | **55** | 45 sec | 1 |
| extension | 72 | 10 min | |

PCR reaction was carried out in a thermocycler MJ Research, Inc, PTC 100 adopting a program as described in the table 3-12. After several attempts on different annealing temperatures, 55°C was chosen as correct annealing for primers. Actually only Deg1FOR and Deg2REV worked at that temperature, but the other pair of primer (Deg3FOR and Deg4REV) did not amplified any PCR product also at different temperatures. So Deg1FOR and Deg2REV were chosen to be good primers for amplification of a putative fragment of NHX1 in both species.

After PCR reaction, samples were ready to be loaded in a gel, without loading blue dye thanks to the Green Flexi buffer (Promega) that contains blue and yellow dye. The blue dye migrates at the same rate as a 3-5 kb DNA fragment in a 1% agarose gel, instead yellow dye runs at a rate faster than primers (<50bp); like this it is possible to better check time running. Sample were loaded on a 1% agarose gel with 0.1% v/v ethidium bromide solution and the presence of PCR product was double-checked by a transilluminator.

It was clear on gel that Deg1FOR and Deg2REV primers amplified a fragment similar in size to the theoretical one for cDNA and one band >1000 bf on genomic DNA.

Also for the other two candidate genes, several degenerated primer pairs have been designed from alignments of related sequences, already submitted in NCBI nucleotide databases.

Primers tested by PCR did not get any result neither for SOS1 nor for HKT1.

Prof. Bressan's lab owns a large primers collection for genes involves in salt stress response used in previous experiments on *Arabidopsis thaliana* and *Thellungiella halophila*; so SOS1 and HKT1 primers have been tested also on pear and quince DNA to find out possible PCR product.

In the next table are reported the two primers pairs that worked efficiently for these genes on pear and quince. The number associated to the name indicates the position of that primer in *Arabidopsis*'s sequence of that gene.

**Table 3.13: Prof. Bressan's lab primers used for SOS1 and HKT1 cloning**

| Primer | Sequence (5'-3') | bp |
|---|---|---|
| SOS1-992F | GTGTTGTCATTGCTGAAGGCATT | 23 |
| SOS1-1480R | GCCAGTCAGCAGGTCCTA | 18 |
| HKT1-670F | CCCACGAATGAGAACATGATCATCTT | 26 |
| HKT1-1015R | CAACGATCCAACCAACTTCTCGTA | 24 |

For SOS1 and HKT1, PCR program was changed with different annealing temperatures, respectively at 56°C (Table 3-14) and 60°C (Table 3-15). In addition, considering the genetic distance between *Arabidopsis* or *Thellungiella* and pear and quince, PCR mix reaction was modified putting 4-fold primers to increase the possibility to have an amplified product.

These primers amplified a fragment for each gene similar in size to the theoretical one for cDNA.

**Table 3-14: PCR amplification program for SOS1**

| amplification step | temperature °C | time | cycles |
|---|---|---|---|
| denaturation | 94 | 3 min | 1 |
| annealing<br>extension<br>denaturation | **56**<br>72<br>94 | 45 sec<br>40 sec<br>40 sec | 38 |
| annealing<br>extension | **56**<br>72 | 45 sec<br>10 min | 1 |

**Table 3-15: PCR amplification program for HKT1**

| amplification step | temperature °C | time | cycles |
|---|---|---|---|
| denaturation | 94 | 3 min | 1 |
| annealing<br>extension<br>denaturation | **60**<br>72<br>94 | 45 sec<br>40 sec<br>40 sec | 38 |
| annealing<br>extension | **60**<br>72 | 45 sec<br>10 min | 1 |

### 3.5.2 Extraction and purification of DNA from gel

When putative bands, correct in theoretical length, were visualized on 1% agarose gels, another PCR amplification was performed using the same condition to the original one, but changing the green buffer with the 5X colorless GoTaq® Flexi Buffer (provided with the other components).

Amplifications were carried out starting from genomic DNA but also from cDNA as templates. New PCR products were loaded in a gel and visualized bands were excised from it, using a sterile sharp blade on the transilluminator (properly cleaned with pure ethanol before starting). Each fragment appeared pink in an agarose gel with EtBr looked at transilluminator, so the excision was made carefully paying attention to cut only band and minimizing the size of the gel slice by removing extra agarose.

DNA extraction from each fragment was carried out with QIAquick Gel Extraction Kit Protocol (Qiagen) and following the spin protocol.

Each band sample was weighted in a colorless 2 ml eppendorf tube and later 3 volumes of Buffer QG were added to tubes (100 mg = 100 µL) that were put to incubate at 50°C for 10 min (until the gel slice was completely dissolved). During incubation it was useful vortexing tubes every 2-3 min. After that, bands were perfectly melted and the color of the mixture appeared yellow. Isopropanol (1 volume) was pipetted in each sample and tubes mixed; this step increases the yield of DNA fragments <500 bp and >4 kb. Kit is composed by QIAquick spin columns and relative collection tubes, so each sample was applied into a QIAquick spin column (maximum 800 µL) and centrifuged at 10,000 g for 1 minute at RT. The flow-through was discarded and column replaced in its collection tube and, in order to remove all traces of agarose, other 0.5 ml of Buffer QG were poured in each column and centrifuged for 1 min. Using this kit, DNA is absorbed by a membrane placed inside QIAquick spin column. DNA was washed with 0.75 ml of Buffer PE, the column was let stand for 2-4 min for a better result and then centrifuged for 1 min. One other centrifugation was necessary after discarding the flow-through to remove all the ethanol contained in the Buffer PE. Each QIAquick column was put in a new tube and DNA eluted adding 50 µL of sterile water directly on the center of membrane; with a 1 min centrifugation, DNA was collected in tubes.

DNA was checked on a gel adding 1 volume of loading Dye to 5 volume of purified DNA. If each DNA appeared on agarose gel it was considered suitable for further steps of cloning procedure.

### 3.5.3 Preparation of Bacteria medium

The most used medium for bacteria growth is LB, whose composition is reported in Table 3-16:

**Table 3-16: LB medium**

| Components | amount |
|---|---|
| Bacto Triptone | 1% w/v |
| Bacto Yeast | 0.5% w/v |
| NaCl | 1% w/v |
| distilled $H_20$ | to volume |

LB was used in two different ways: liquid and solid, so that for the first state the recipe was done like in the table 3-16; instead to become solid it was necessary to add to the other components 1.5% w/v Bacto™Agar (Becton Dickinson, USA). Both LB needed to be sterilized before use by autoclaving 20 min at 121°C. Sterilization was fundamental to guarantee the growth of bacteria on media without any contamination; and after autoclaving, these media can be opened only under a sterile hood.

When the temperature of LB with agar was just lower 50°C, 2 ml of ampicillin (50 mg/ml) were added; if the media is still too hot the antibiotic risks to be degraded. After ampicillin, 500 µL IPTG 1M stock solution (Table 3-17) and 1.6 ml X-gal (50 mg/ml) (Table 3-18) were incorporated in 1 L of LB.

The bottle was shaken and medium poured into sterile Petri plates (Ø=9 cm). Plates took some time to solidify under the hood and, when ready, they were stored at 4°C covered with aluminum foil because X-gal needs dark conditions.

**Table 3-17: IPTG stock solution (1 M)**

| IPTG stock solution (1 M) | amount |
|---|---|
| IPTG | 1.42 g |
| distilled $H_2O$ | To 5 ml final volume |
| Solution was filter-sterilized and stored at 4°C | |

**Table 3-18: X-gal stock solution (50 mg/ml)**

| X-gal stock solution (50 mg/ml) | amount |
|---|---|
| 5-bromo-4-chloro-3-indolyl-β-D-galactoside | 100 mg |
| N,N'-dimethylformamide | 2 ml |
| Covered with Al foil and stored at -20°C | |

### 3.5.4 Preparation of competent cells

Competent cells used in Prof. Bressan's lab for transformation by electroporation were Escherichia coli strain XL-1 Blue. From a competent cells tube stored in -80°C an aliquot of cells was scooped out and spread on a LB plate with ampicillin. In this way, after a overnight incubation at 37°C, bacteria were grown and colonies were distinguished in the way that a single colony was caught and placed in a 5 ml LB liquid medium (with 5 µL of tetracycline 5 mg/ml sterilized by filtration) and let it grow for one night at 37°C shaked at 200 rpm. This is very important as it eliminates all the steps needed to remove salts. Salts are known to reduce the efficiency of electrotransformation and cause arcing in the electroporation cuvette.

XL-1 cells are tetracycline resistant and during the preparation of competent cells, it is important to add this antibiotic to maintain the proper osmotic pressure and avoid the loss of episome. XL1-Blue cells are endonuclease (endA) deficient, which greatly improves the quality of miniprep DNA, and are recombination (recA) deficient, improving insert stability. The hsdR mutation prevents the cleavage of cloned DNA by the EcoK endonuclease system. The lacI$^{q}$ZΔM15 gene on the F´ episome allows blue-white color screening (Stratagene, XL1-Blue Competent Cells 200249-11 Revision #074003, 2004).

Fresh over-night culture of bacteria was inoculated in 1L of fresh YEP media (Table 3-19) and cells grow at 37°C with shaking (about 200 rpm).

**Table 3.19: YEP medium**

| Component | amount |
|---|---|
| Bacto Peptone | 2% w/v |
| Yeast Extract | 1% w/v |
| distilled $H_20$ | to volume |

Cells should be harvested between an OD (at 600 nm) ranging from 0.5 to 0.9. So after 3 hours of shaking, a small aliquot of bacteria was measured at spectrophotometer, taking as blank the same volume of fresh YEP media. When the OD reading was 0.84, cells were stopped growing and transferred in two 500 ml plastic bottles sterilized and chilled on ice for 5 min. After that, bottles were centrifuged for 10-15 min at 4°C at 4,500 g; higher g-value can affect cells and therefore reduces electrotransformation efficiency. The supernatant medium was discarded and since culture media did not contain added salts, it was not so important to remove traces of medium as in original protocol. Pellets were resuspended and washed twice in cold glycerol 10% (sterile) and later they were centrifuged again and liquid discarded. Pellets were dissolved in 3 ml each of cold 10% glycerol and later joined together. These competent cells could be used fresh or be frozen. Several 60 µL aliquots of XL-1 Blue cells were pipetted in 500 µL sterile tubes and immediately frozen in liquid nitrogen. About 100 tubes of competent cells were stored in -80°C and ready to be used for transformation.

### 3.5.5 Preparation of recombinant plasmid

To proceed with cloning steps pGEM-T easy vector kit (Promega- Fig. 3-16) was used. The main character of this step is pGEM T-easy vector (3015 bp), it has peculiar features that make it suitable for that procedure:

1. it has a region for ampicillin resistance, important for further selection for transformed cells;
2. it owns a lacZ operon sequence codifying for β-galattosidase, where it is placed the insertion site for gene fragment. If DNA fragment integrates in that point, enzyme is inactivated and colony appears white.
3. pGEM-T easy Vector has two polymerase promoters: T7 and SP6 adjacent to cloning site, this helps later on in sequencing process, that exploits presence of these generic primers able to attach to these promoters.

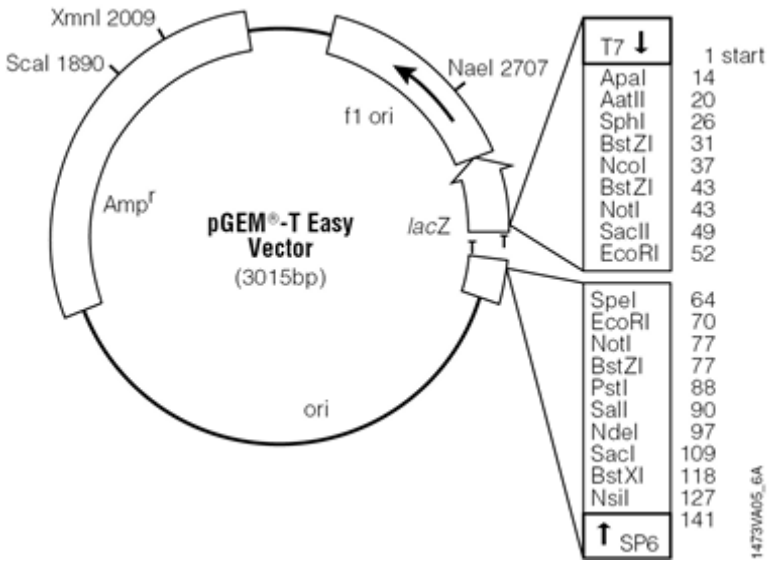



**Fig. 3-16: pGEM-T easy vector**

The reaction to obtain recombinant plasmid is made by several components (Table 3.20). First of all, this reaction needed cDNA or DNA previously eluted from agarose gel and visually quantified on a new gel. Once determined how much DNA is required (in µL), in each tube all the other components were added. T4 DNA Ligase is an enzyme able to join blunt end and cohesive end termini as well as repair single stranded nicks in duplex DNA, RNA, DNA/RNA hybrids (BioLabs, New England). This enzyme works properly in presence of its 10X buffer that contains: 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 µg/ml BSA (pH 7.5 at 25°C).

**Table 3.20: Ligation reaction mix**

| Components | Amount/ reaction |
|---|---|
| 10X T4 DNA ligase reaction buffer | 1 µL |
| pGEM T-easy vector | 0.33 µL |
| T4 DNA Ligase (400000 U/ml-BioLabs) | 0.5 µL |
| (c)/DNA eluited from gel | X µL |
| Sterile distilled $H_20$ | (8-X) µL |
| Final volume | 10 µL |

In pGEM-T easy vector kit, plasmid is already opened and ready to host a DNA fragment.

For each DNA fragment eluted from gels, a new tube was prepared with the correct amount of DNA (different in volume depending on each sample) and all the other reagents as reported in the following table. Mixtures were slightly shaked and incubated at 4°C overnight.

### 3.5.6 Transformation of E.Coli by electroporation

The day after, ligation reaction was ready to be used; therefore competent cells tubes were defrosted on ice. Each tube containing 60 µL of cells was labeled with gene and genotype abbreviation. Defrosted cells were gently flicked and 3 µL of each ligation reaction (that is pGEM-T easy vector with gene fragment of interest integrated in its ring) were pipetted in the tube. After a gentle flicking to mix cells with vector, tubes were placed on ice for 20 min.

Also E.Coli Pulser® Cuvettes (Bio-Rad) were positioned on ice; one for each transformation event. Cuvettes used had a gap of 0.2 cm (Fig. 3-17).

During this incubation time, one sterile tube named as each transformation event was prepared with 1 ml SOC medium (Table 3-21 and 22).

Beyond this, Gene Pulser II System (BioRad- Fig. 3-18) was switched on and Pulse Controller was set at 2.5 kV for electroporation protocol commonly used (see bulletin 311235 BioRad).

**Fig. 3-17: E.Coli Pulser® Cuvettes (Bio-Rad).**

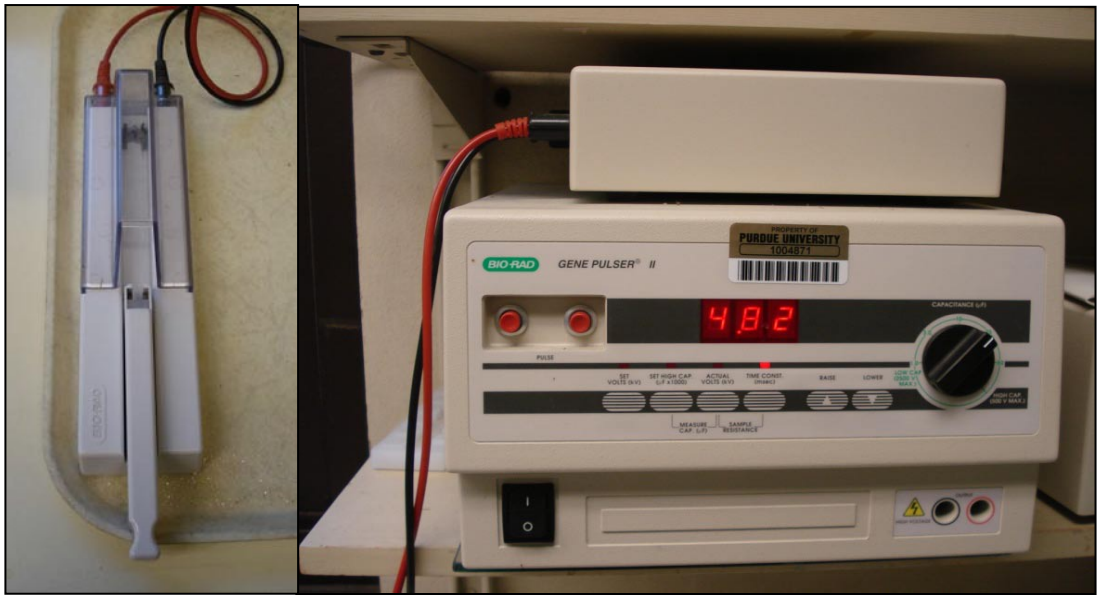



**Fig. 3-18: Gene Pulser II System (BioRad); on the left the cuvette chamber and on the right the display.**

**Table 3.21: SOC medium**

| Components | | Amount |
|---|---|---|
| Bacto Triptone | A | 2% w/v |
| Bacto Yeast Extract | B | 0.5% w/v |
| NaCl (1 M) | C | 1% (v/v) |
| KCl (1 M) | D | 0.25% (v/v) |
| $Mg^{2+}$ (2 M) filter-sterilized | E | 1% (v/v) |
| Glucose (2 M) filter-sterilized | F | 1% (v/v) |
| distilled $H_20$ | G | To volume |
| A, B, C, D mixed and autoclaved. E, F, G added at RT. The final pH should be 7.0 | | |

**Table 3-22: 2 M $Mg^{2+}$ stock**

| Components | Amount in 100 ml |
|---|---|
| $MgCl_2$ . 6 $H_2O$ | 20.33 g |
| $MgSO_4$ . 7 $H_2O$ | 24.65 g |
| distilled $H_20$ | To volume |

Electroporation is a powerful, highly efficient technique for introducing nucleic acids, proteins, and other molecules into a wide variety of cells. A high-intensity electric field transiently permeabilizes the membrane, inducing gaps in the phospholipid bilayer, enabling uptake of exogenous molecules from the surrounding medium. In particular, extracellular genetic material passes through these transient gaps and is assimilated by the target cells' DNA. This technique has been used to introduce nucleotides, RNA, proteins, carbohydrates, dyes, and virus particles into prokaryotic and eukaryotic cells. Electroporation is considered the most popular, most versatile, and most efficient transformation method for several cell types (Bulletin 5542 Bio-Rad).

After incubation time on ice, total amount of each tube was pipetted in one corresponding labeled E.Coli Pulser® Cuvettes, paying attention not to create bubbles that can interfere with electric field. Each cuvette was immediately re-placed on ice and closed with proper lid.

Gene Pulser II System is supplied with a cuvette chamber with lid; cuvette should be positioned properly in its holder, with aluminum electrode plates adherent to cuvette electrodes, in order to get firm contact and to deliver pulses with the correct electric field. With cuvette in correct position, two "pulse" buttons were pushed together until an indicator tone was produced indicating a successful pulse delivery. In the Gene Pulser II System a display showed the time constant of each measurement.

Cells-vector solutions with high salt concentrations might cause an electrical discharge (known as arcing), which often reduces the viability of the bacteria. In this situation a noisy blast is clear and E. Coli cells can't be considered efficient anymore, so a new competent cells tube must be prepared and ligation reaction incorporated as before.

When electroporation had good result, 1 ml of SOC medium was poured inside the cuvette to rinse it properly and then, all the content was pipetted back into the tube. This tube enclosed XL-1 Blue cells transformed with pGEM-T easy vector containing gene fragment.

At the end of electroporation step, all the E.Coli Pulser® Cuvettes were washed with 1 M NaOH, shaking for 15 min, in order to clean all residues and reuse them for the

next transformation. After this time they were rinsed and sprayed with ethanol and laid under sterile hood to dry out.

### 3.5.7 Plating of bacteria

Tubes, containing cells transformed with ligation reactions, were placed in a shaker at 150 rpm for 1 hour at 37°C (pre-heated before electroporation). After 1 hour of growing, tubes were centrifuged at 5000 g for 5-10 min to pellet down cells; so as it was possible to discard 800 µL of SOC media supernatant and to resuspend all cells grown in a minimum volume of 200 µL.

LB/ampicillin/X-gal/IPTG plates prepared before were acclimatized at RT under sterile hood conditions. Glass Pasteur pipettes were sprayed with denatured alcohol and flamed on Bunsen and then folded in two points (as figure 3-19 shows) to obtain a spreader. Transformation cultures were plated in duplicate in LB/ampicillin/X-gal/IPTG plates, pipetting in the middle of each plate 100 µL of cells and spreading circularly with the spreader in order to cover the entire plate surface. Plates with bacteria were kept open for 5 minutes under the hood to allow them to absorb all the bacteria and, after that, Petri dishes were closed and transferred up-side-down in a bacteria incubator constantly maintained at 37°C for overnight growth (16-24 hours).

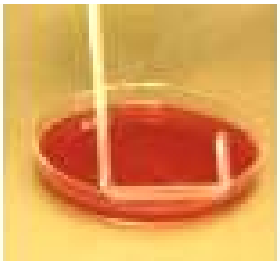


**Fig. 3-19: Plating with spreader**

### 3.5.8 Selection of transformed colonies

Ampicillin presence in LB medium is essential because only transformed cells with plasmid inside can grow, thanks to the ampicillin resistance fragment incorporated into the vector; therefore un-transformed ones can't survive. X-gal and IPTG are useful to distinguish between transformed cells, containing gene fragment of interest, against those with plasmid closed without any insert integration.

Sequence codifying for β-galattosidase inside vector needs X-gal as chromogenic substrate and IPTG as promoter for a blue precipitate. So cells with closed plasmid with no insert are able to perform this reaction and colonies appear blue. Otherwise in cells transformed with recombinant vector, β-galattosidase sequence is interrupted by insert and this event prevents the blue coloration of cells, which appear white. This color difference in plates allows a quick screening of transformed white colonies.

The day after overnight incubation, plates appeared well covered by colonies mostly blue, but also several white ones were present and named on plate (Fig. 3-20).

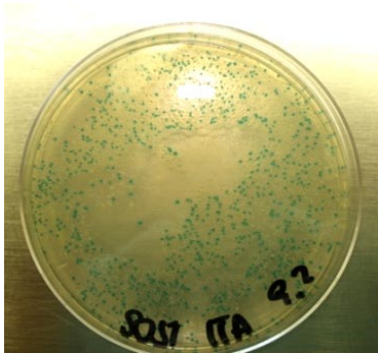


**Fig. 3-20: Plate with transformed (white) and untransformed (blue) colonies.**

### 3.5.9 Singularization of white colonies

Each white selected colony was picked up using sterile toothpick and uniformly spread in a sector of a new LB/ampicillin/X-gal/IPTG plate previously divided with a marker in 8 sectors (Fig. 3-21). As many white colonies as possible were chosen and singularized in new plates (about 24 colonies for each transformation with a recombined plasmid); after that they were incubated at 37°C overnight. Singularization step is important in order to separate transformants belonged to the same colony; sometimes happen that one white colony, after spreading, appears bluish instead of white again. In this way it is possible to eliminate false positive and at the end, reduce the number of positive transformed colonies. The day after among all white sectors, the most spread colonies were chosen and single white outdistance colonies were picked up with sterile toothpicks. Immediately after, each toothpick touched an empty sterilized PCR tube and afterwards it was placed inside a glass tube containing 5 ml of LB medium and 5 µL of ampicillin (50 mg/ml) for later liquid culture.

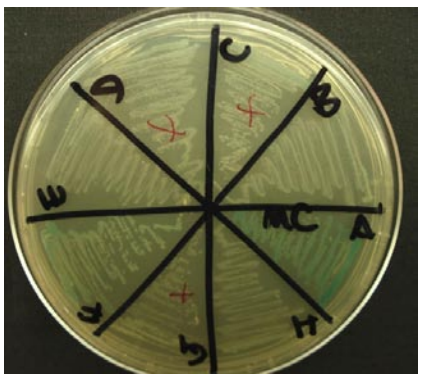


**Fig. 3-21: Plate used for singularization of transformed cells.**

### 3.5.10 "Colony" PCR reaction

For each kind of insert (i.e. NHX insert from cDNA or NHX insert from gDNA) for all genes of interest, 10 good white colonies were selected (=10 tubes touched by toothpicks) and used for a PCR in order to check if chosen transformants effectively contained the insert inside the vector.

So PCR was carried out using different master mixes, one for each gene (SOS1, NHX1, HKT1) using specific primers already adopted in the first part, for obtaining good gene fragments (see tables 3-10 and 3-13 for primers). PCR programs were maintained unchanged as the amplification before transformation; the only difference involved volumes in master mix, in fact in "Colony" PCR, 1 µL of DNA used in normal amplification was substituted by 1 µL more of sterile water to reach anyway the total volume of 20 µL.

In this case three different "colony" PCR reactions were performed because of different annealing temperatures of 3 primer pairs for candidate genes.

PCR products were loaded on a 2% agarose gel to check the size of each insert that should correspond to the theoretical length between primers paired. To better visualize bands size, 5 µL of a Gene Ruler 100 bp DNA ladder was loaded in the first lane of gel.

### 3.5.11 Liquid cultures preparation

As already mentioned in paragraph "Singularization of white colonies", LB/ampicillin tubes were prepared and a toothpick with a transformed colony was inserted in each of them. Tubes were incubated at 37°C in a shaker at 150 rpm. This further growth step allowed to double-checked if, actually, all the selected colonies were transformed, therefore ampicillin resistant (Fig. 3-22).

If in the team of 10 colonies one did not grow in this step, it was abandoned.

**Fig. 3-22: Liquid culture of transformed cells.**

### 3.5.12 Glycerol stocks

To prepare glycerol stocks, an aliquot of fresh liquid culture (387.5 µL) was transferred in a new sterile tube with the addition of (112.5 µL) sterile 80% glycerol. Proportion between bacteria culture and glycerol was: 77.5% and 22.5%. In this way, transformed bacteria cultures can be stored for long period at -80°C.

### 3.5.13 Plasmid extraction

Small-scale purifications of plasmid DNA, better known as minipreps, are commonly used in molecular biology procedures. In literature a lot of minipreps protocols are used and several can be both laborious and time-consuming, particularly when large numbers of minipreps are performed in parallel (Technical bulletin Part#TB117 Promega). In this experiment, it has been chosen to adopt the Wizard® Plus Minipreps DNA purification System (Fig. 3-23) that eliminates many problems and provides a simple and reliable method for rapidly isolate plasmid DNA.

From each liquid culture, 3 ml were centrifuged in three times in eppendorf tubes for 1-2 min at 10000 g at RT to collect pellet of cells. Once obtained the pellets, all the supernatants were discarded and tubes turned upside-down on a paper towel to remove excess media. Pellets were resuspended in 200 µL of Cell Resuspension Solution (Table 3-23) and immediately later the same volume of Cell Lysis Solution (Table 3-24) was added and tubes inverted four times; at this point cell suspensions should clear instantly.

**Fig. 3-23: Wizard® Plus Minipreps DNA purification System.**

The same volume of Neutralization solution was added (Table. 3-25) and tubes inverted four times.

Lysated cells were centrifuged at 10,000 g for 5 or 15 min until new pellets were evident.

For each minipreps, a Wizard® Minicolumn was prepared and attached to a 3 ml syringe, removing the plunger. Wizard Minipreps DNA purification Resin needed to be mixed vigorously and if aggregates were present, it should be warmed up to dissolve crystals. In each syringe 1 ml of resuspended resin was poured and carefully all of the cleared lysate was transferred in the syringe barrel with resin.

Gently pushing the plunger, mixture was introduced into the Minicolumn; syringe was detached from Minicolumn and plunger removed then barrel inserted again and inside it 2 ml of Column Wash Solution were pipetted (table 3-26). This washing step consisted in the complete passage of solution with ethanol through Minicolumn.

After that each Minicolumn (named with abbreviation of gene of interest and genotype) was put in a new eppendorf tube and centrifuged at 10,000 g for 2 min to dry the resin. Minicolumn was put in a new eppendorf tube again and 30 µL of nuclease free water were poured inside it; after 1 minute of incubation all samples were centrifuged at 10,000 g for 20 sec to elute the plasmid DNA.

This DNA was loaded in a 1% agarose gel to check the efficiency of Wizard Plus Minipreps DNA purification System.

**Table 3-23: Cell Resuspension Solution**

| Components | Final concentration |
|---|---|
| Tris-HCl pH7.5 | 50 mM |
| EDTA | 10 mM |
| RNase A | 100 µg/ml |
| distilled $H_2O$ | to volume |
| After addition of RNase A store at 4°C | |

**Table 3-24: Cell Lysis Solution**

| Components | Final concentration |
|---|---|
| NaOH | 0.2 M |
| SDS | 1 % |
| distilled $H_2O$ | to volume |

**Table 3-25: Neutralization Solution**

| Components | Final concentration |
|---|---|
| Potassium acetate pH 4.8 | 1.32 M |
| distilled $H_2O$ | to volume |

**Table 3-26: Column Wash Solution**

| Components | Final concentration |
|---|---|
| Tris-HCl pH 7.5 | 8.3 mM |
| EDTA | 40 µM |
| Potassium acetate | 80 mM |
| distilled $H_2O$ | to volume |
| 95% ethanol was added before with final concentration of 55% | |

### 3.5.14 Digestion of plasmid DNA

Before sending all plasmids to be sequenced, it has been decided to digest them to double-check the presence of a correct insert. Depending on the concentration of plasmid DNA visualized on agarose gel, an aliquot was put in a new tube and water was added to reach 15 µL of volume. After that a digestion mix was assembled as reported in the table 3-27 and 35 µL of that was pipette in each tube with plasmid DNA.

**Table 3-27: Digestion mix**

| Components | µL for 1 reaction |
|---|---|
| NEBuffer EcoRI (10X) | 5 |
| EcoRI | 0.8 |
| distilled $H_2O$ | 29.2 |

Restriction endonucleases such as EcoRI recognize specific palindrome sequences and cleave a phosphodiester bond on each strand at that sequence (Fig. 3-24). Enzyme buffers are specifically formulated to provide the salt concentration for optimal enzyme activity (http://www.neb.com; Sambrook at al., 1989).

The digestion was carried out overnight at 37°C and the day after, EcoRI needed to be inactivated at 65°C for 20 min. In each tube 150 µL of sterile water was added and then 20 µL of 3 M sodium acetate and 200 µL of isopropanol. Precipitation was performed at -20°C for almost 15 min. Tubes were then placed in centrifuge for 10 min to maximum speed and supernatants discarded. Digested DNA pellets were dried out in speed vacuum at 60°C and later resuspended in 10 µL of water.

After digestion with a restriction endonuclease the resulting DNA fragments were separated by agarose gel electrophoresis and their size could be estimated.

In each lane more than one band could be visible: a band > 3000 bp represented cut plasmid (pGEM-T easy vector) and it was present in all samples; the other bands were the cloned inset that, in its turn, could be cut too by restriction enzyme and the sum of the size of the 2 bands should be equal to the length of each insert.

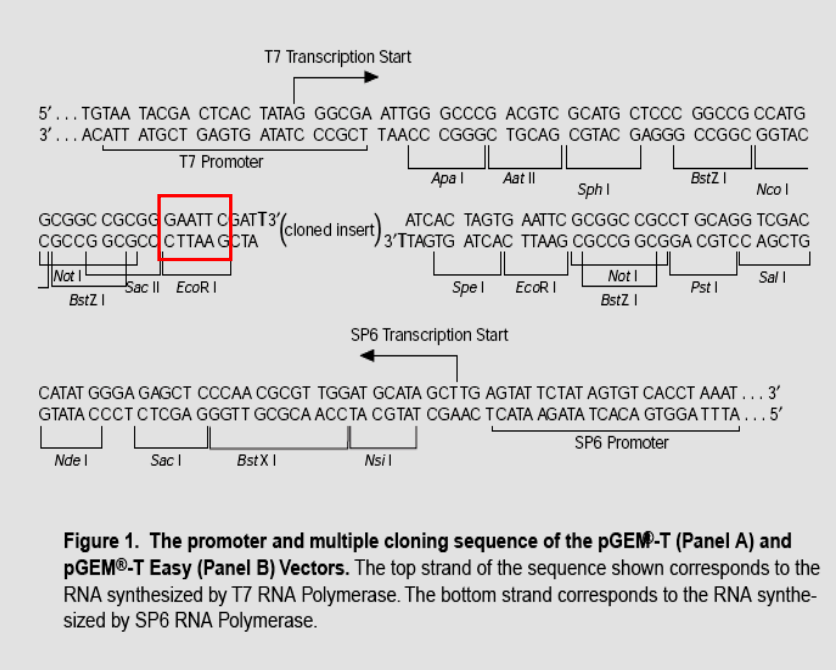



**Figure 1. The promoter and multiple cloning sequence of the pGEM®-T (Panel A) and pGEM®-T Easy (Panel B) Vectors.** The top strand of the sequence shown corresponds to the RNA synthesized by T7 RNA Polymerase. The bottom strand corresponds to the RNA synthesized by SP6 RNA Polymerase.

**Fig. 3-24: EcoRI site of cleavage in the pGEM-T Easy Vector, palindrome sequence in red box.**

### 3.5.15 Quantification of plasmids and sequencing

Each plasmid was quantified and, in a final volume of 10 µL, they have been prepared with the concentration equal to 0.3 µg/µL. All samples were brought to Purdue Genomics Core Facility with a form filled with all information about vector, template size and primer for sequencing (T7 or SP6 or both).

### 3.5.16 Sequences analyses

Once sequences came back, it was necessary to "clean" them from fragments not belonging to the insert. So pGEM-T easy vector complete sequence (www.promega.com) was used to eliminate some possible plasmid bases matching it with sequence results by means of ClustalW software (http://www.ebi.ac.uk/clustalw/). At this point, original and specific primers (for SOS1, NHX1 and HKT1) were found in each sequence and only this fragment,

delimited by them, was aligned to all gene sequences submitted in databases using one of the most widely used bioinformatics programs: BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). In BLAST, the query was submitted asking to compare it in "nucleotide blast" and selecting as choose search set "others" (excluding in this way human and mouse databases).

In conclusion, for each candidate gene, 4 sequences have been cloned: one for each genotype. Totally 12 partial sequences were obtained from cDNA.

## 3.6 Quantitative gene expression

### 3.6.1 qRT-PCR primers design

For gene expression study, the primers design step is very important; normally DNA primers should be between 15 and 25 bases long to maximize specificity, with a G/C content of around 50% (Nolan et al., 2006). In particular for qRT-PCR, primes must be designed specifically on cloned target sequences (cDNA) and they must be able to amplify a fragment between 60 and 120 bp long, at the same annealing temperature.

Primers for qRT-PCR were designed with the help of *Primer Express 2.0* software (related to ABI PRISM) that is specialized in designing primers for quantitative reall time PCR, proposing better pairs on each template according to the next amplification conditions. In this way 9 pairs of primers have been planned with the annealing temperature commonly set at 60 °C (Table 3-28).

Obtained cDNA sequences for each gene were aligned together and it was clear that for both pears as quinces, sequences matched not 100% but really closed; so primers were designed on the 100% homologue fragment of the 2 cDNA sequences.

So for NHX1 as for SOS1, two pairs of primers were used, one for pears and one for quinces.

For HKT, some differences were present comparing cDNA sequence of BA29 and the one belonged to MC; since it was difficult to find good common place to design primers; it has been considered convenient planning 2 different pairs of primers: one for cMC SOS1 sequence (CoHKT f+r) and one for cBA29 SOS1 sequence (BAHKT f+r).

All qRT-PCR primers were tested on respective cDNA target with PCR reactions. Amplifications were performed with 2 µL of cDNA (25 ng) at 60°C of annealing and 40 cycles to verify that these primers worked properly and that PCR products were specific and short as planned on sequences. For this reason PCR products were loaded on 2% agarose gel with a gene ruler on a side; if they confirmed expectations they could be considered suitable for qRT-PCR.

**Table 3-28: qRT-PCR primers designed with Primer Express 2.0**

| Primers pair name | gene | Target genotype | Forward<br>5' to 3' | Reverse<br>5' to 3' |
|---|---|---|---|---|
| **QUI** | NHX1 | Quinces | GGGAGGGCGTTGTTAACGAT | TGGGTGAGATCAAAGCTCTGAA |
| **PEAR** | NHX1 | Pears | TGTGGTTCTTTTCAATGCTATTCAG | CCCAGAAAGTGCAAGGCAAT |
| **PeSOS1** | SOS1 | Pears | TGGATCCACTACCCAATTTGCT | AATATTCGTATCTTTGAGGCTGGTAAA |
| **CoSOS1** | SOS1 | Quinces | CAAGTTTCCCGGTTCATAGTTGTT | TTCCAGTCCAAACCATATCCAAA |
| **BAHKT** | HKT1 | BA29 | GGCTCTTAATCCCTCAAGTACTAATGG | TTGTGATCTTGTAAAGTCCCCAAAT |
| **PeHKT** | HKT1 | Pears | ACGAACTCATTCCTTCAAGTGACTT | TGTTAGGGTTTCTGATGATACAGCTT |
| **CoHKT** | HKT1 | (MC) | CTCGGTTCGTCTTTGTTTTCTTC | CACTCAAAGGCGCAAAAGAGA |
| **pELF1** | elF1α | Pears/Quinces | GCATGCTTTGCTTGCTTTCAC | TGGTGGCATCCATCTTGTTG |
| **ACT** | actin | Pears/Quinces | TATGAAGGGTATGCCCTCCC | CTGTAAGATCACGACCCGCC |

In this table are reported two pairs of qRT-PCR primers for NHX1, two for SOS1 and three for HKT1 (as explained in the text).
Minimum length of a primer is 20 bp and maximum is 27 bp.
Last two lines are relative to two possible housekeeping genes: elongation factor 1 (pear) and actin (apple);
sequences used to design these primers were respectively: AY338249.1 and DT002474 (NCBI databases).

### 3.6.2 Choice of housekeeping gene

In literature, there are several papers that face the topic of the choice of the correct housekeeping gene in plant expression studies. In general, the most used housekeeping genes are: 18S and actin. So in this experiment it has been tried to use 18S gene, but it was not successful. The reason was that 18S primers, designed on *P. communis* 18S ribosomial RNA gene (AF195619.1-gDNA), worked properly on pears cDNA but did not work on quinces. Therefore attention moved from 18S gene to actin one. Actin is frequently used as housekeeping gene in fruit plant expression studies and a lot of works give evidence of this (Fonseca et al., 2005, Paris R. PhD dissertation thesis, Pierantoni L. PhD dissertation thesis). But Nicot and co-workers (2005) claimed that in plant stress studies is important to choose one or several internal control genes which should not fluctuate during treatments. This study on potato suggests at the end the use of elongation factor1α as the most stable among seven housekeeping genes tested. This result suggested that could be a right decision integrating efl1α in the candidate housekeeping genes.

These two candidate housekeeping genes were tested on cDNAs of different salt stress timings (different level of necrosis) by qRT-PCR in order to understand which one was the most stable in this salt stress experiment. Specific results of this test will be discussed later in results section.

### 3.6.3 qRT-PCR

Quantitative Real Time PCR was carried out with the ABI PRISM 7000 instrument (Applied Biosystem) and with the QuantiTect® SYBR Green PCR system (Qiagen).

For each gene the qRT-PCR reaction mix was composed as reported in table 3-28:

**Table 3-28: qRT-PCR reaction mix**

| Reagents | µL per sample |
|---|---|
| Rnase free water water | 6 |
| 2X QuantiTect® SYBR Green PCR Master MIX (Qiagen) | 10 |
| primer for | 1 |
| primer rev | 1 |
| cDNA (25 ng) | 2 |
| Total volume | 20 |

2X QuantiTect® SYBR Green PCR Master Mix was composed by:

- HotStarTaq DNA Polymerase: is a modified form of a recombinant 94 kDa DNA polymerase (EC 2.7.7.7). It is provided in the kit in inactive state (no enzymatic effect at RT). It is activated by 10 min at 95°C;
- QuantiTect® SYBR Green PCR Buffer;
- dNTP mix (ultrapure quality);
- Fluorescent dyes: SYBR Green I and ROX.

ABI PRISM 7000 can run one 96-wells-plate a time; in each plate one target gene was analyzed with actin gene in parallel.

Each genotype was studied in 4 stages: T0 (= no stress) and T2, T3 and T4 (3 stages during salt stress) and in two organs: leaves and fine roots. So 8 cDNA were synthesized for each genotype (leaves and roots) and then diluted to have 25 ng of template in each well. Each plate was repeated 2 times and each cDNA was repeated in 3 wells.

qRT-PCR program was 40 cycles long as reported in table 3-29:

**Table 3-29: qRT-PCR program**

| stage | T°C | Time | cycles |
|---|---|---|---|
| 1 | 50 | 2 min | |
| 2 | 95 | 10 min | |
| 3 | 95 | 15 sec | 40 |
| 4 | 60 | 1 min | |
| 5 | 72 | 30 sec | |
| 6 | 65 → 95 | 20 min | |

Step 2 is fundamental for HotStarTaq DNA Polymerase activation.

Step 5 is usual extension step that in this case includes also fluorescence data collection. At the end step 6 is dedicated to the visualization of melting curves to verify absence of nonspecific products.

Each PCR took about 2 hours and 20 min; after that data were immediately available to be checked for absence of unspecific products and to be downloaded.

### 3.6.4 qRT-PCR data analysis

In qRT-PCR two different methods of analyzing data exist: absolute quantification and relative quantification. The first method should be performed in situations where it is important to determine the input copy number of the transcript of interest; usually it is related to a standard curve. In other situation, the exact number of transcripts can be unnecessary; instead reporting the relative change in gene expression could be more meaningful (Livak and Schmittgen, 2001).

Relative, or comparative quantification, measures the relative variation in mRNA expression levels. It determines the changes in steady state mRNA levels of a gene across multiple samples and expresses it relative to the levels of another RNA. This method of analyzing data, that has been adopted in this experiment, does not need of any calibration curves or standards, but only a "reference" gene with known sequence (Pfaffl, 2006). To investigate the physiological changes in gene expression this method is adequate for the most purposes (Pfaffl, 2001), as in this experiment. In particular relative quantification is so called because the gene of interest is expressed in relation to another "reference" gene and the level of expression depends on differences (Δ) between threshold cycles $C_t$ of these two genes (Pfaffl, 2006). $C_t$ value can be modified, but in this experiment the ABI PRISM 7000 default value was maintained.

The choice of housekeeping gene is a critical step because this "reference" gene should not vary by experimental conditions. Genes like 18SrRNA, tubulin, actin, ubiquitin, elongation factor 1 were chosen in pre-genomic era because of their notorious housekeeping role in basic cellular processes (primary metabolism and cell structure maintenance) and in this context are simply known as "reference" genes. One problem associated with them is that the so important stability is not always constant (Czechowski et al., 2005)

The comparative $C_t$ method ($\Delta\Delta C_t$) assumes constant PCR efficiency of the gene of interest, "reference gene" and same value in all studied samples. This constant efficiency is considered equal to 2 (Kim et al., 2003; Ramakers et al., 2003). So the amount of target is equal to $2^{-\Delta\Delta Ct}$ and this value represents the mean fold change in expression of the target gene at each time point of the experiment normalized with the reference gene (i.e. actin).

$\Delta\Delta C_t$ is calculated using the following equation 1 (modified from Pfaffl, 2001):

$$\Delta\Delta C_t = [\Delta C_{t\ target} - \Delta C_{t\ reference}] \qquad \textbf{eq. 1}$$

The mean fold change in expression of the gene target at each time point was calculated with eq. 2 derived from equation 1 (Livak and Schmittgen, 2001):

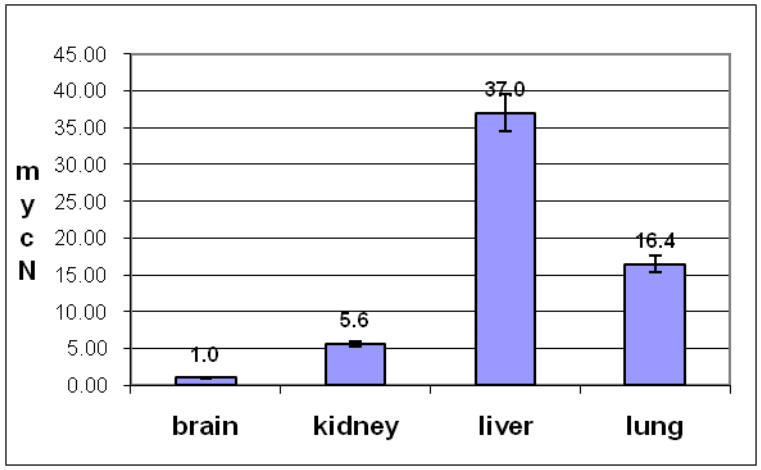
$$\Delta\Delta C_t = (C_{t,target} - C_{t,actin})_{time\ x} - (C_{t,target} - C_{t,actin})_{time\ 0} \qquad \textbf{eq. 2}$$

For the untreated control samples, $\Delta\Delta C_t$ equals zero and $2^0$ equals 1; so the variation in gene expressed relative to untreated control equals one by definition.

All calculations were done according to detailed procedure illustrated in User Bulletin # 2 (ABI PRISM 7700 Sequence Detection System) and reported as example in next table 3-30.

**Table 3-30: Example of calculation of ΔΔCt method using and experiment of qRT-PCR reported in User Bulletin # 2 (ABI PRISM 7700 Sequence Detection System). Brain was considered the calibrator. Error bars derived from ΔΔCt+ s and ΔΔCt- s (standard deviation).**

| | gene target | housekeeping | gene devst | housekeeping devst | ΔCT c-myc-gapdh | ΔΔCt | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ct av c-myc | ct av gapdh | | | | $\Delta CT-\Delta CT_{brain}$ | c-myc N | dev | ΔΔCt + | ΔΔCt - | 2^- | 2^- | negative | positive |
| **brain** | 30.49 | 23.63 | 0.15 | 0.09 | 6.86 | 0 | 1.00 | 0.17 | 0.17 | -0.17 | 0.89 | 1.13 | 0.11 | 0.13 |
| **kidney** | 27.03 | 22.66 | 0.06 | 0.08 | 4.37 | -2.49 | 5.62 | 0.10 | -2.39 | -2.59 | 5.24 | 6.02 | 0.38 | 0.40 |
| **liver** | 26.25 | 24.6 | 0.07 | 0.07 | 1.65 | -5.21 | 37.01 | 0.10 | -5.11 | -5.31 | 34.56 | 39.64 | 2.45 | 2.63 |
| **lung** | 25.83 | 23.01 | 0.07 | 0.07 | 2.82 | -4.04 | 16.45 | 0.10 | -3.94 | -4.14 | 15.36 | 17.62 | 1.09 | 1.17 |



# Chapter 4: MATERIALS AND METHODS

## A LONG-TERM EXPERIMENT

### 4.0 The aim of the experiment

Since soil salinity and saline water are two important aspects of modern agriculture, it has been decided to focus on these topics related to Abbé Fétel, which represents the main pear cultivar produced in Emilia-Romagna region. This experiment is an open field trial that involves potted trees of this variety, or grafted in three rootstocks or own-rooted, all subjected to a saline irrigation of 5 dS/m electrical conductivity. This study will last four years, so that it is planned to destructively harvest every year three plants per genotype and to evaluate elemental distribution in the different organs by mineral analyses, comparing plant treated with NaCl against control plants irrigated with fresh water. In this way it will be possible to understand how pear and its rootstocks behave in a long-term salt condition and to confirm or reject hypotheses made in previous studies (Musacchi et al., 2006a; Boland et al., 1997; Meyers et al., 1995).

Other purposes of this study are:

- Evaluate effects of irrigation by saline water (5 dS/m) on physiological and yield parameters of cultivar Abbé Fétel differently grafted and its rootstocks separately planted.
- Verify differences between genotypes in their ability to uptake, transfer and storage $Na^+$ and $Cl^-$ ions.
- Discriminate among pears rootstocks which one is the more suitable for Abbé Fétel in salt stress conditions for long period and identify the threshold in salinity that pear is able to tolerate.

### 4.0.1 Plant material

For this experiment on salinity, one-year-old plants from the nursery were used. Seven different types of plants have been chosen:

1. pear cv. Abbé Fétel own-rooted;
2. pear cv. Abbé Fétel grafted on BA29;
3. pear cv. Abbé Fétel grafted on MC;

4. pear cv. Abbé Fétel grafted on Farold®40;
5. clonal pear rootstock Farold®40;
6. quince BA29;
7. quince EMC.

Abbé Fétel is the main Italian pear cultivar and spread mainly in Emilia Romagna and for this reason it has been chosen for this trial. These selected rootstocks are widely diffused in Italy; in particular quinces are adopted in high density plantings and Farold®40 instead for traditional orchard (low density).

Until the planting moment, one-year-old trees were conserved in a cold room (4°C) to keep them dormant. Plants were briefly watered once a week in order to avoid their drying up. The field project counted six rows, each divided in six blocks which included one plant for combination. Therefore each line was constituted by 42 trees and the total of plants used for the trial was 252. In addition other 21 plants, three for each combination, were immediately destroied and divided in parts.

## 4.1 Field project and its realization

In February 2007, the plot of land available for the trial (77 m x 12 m) located in the experimental station of the University of Bologna (Cadriano, BO) was worked (prepared) and later squared using surveyor's cross, surveyor's stakes, string and sticks to correctly divide space to plant trees. According to the project, the field was shared in six rows each far from the others 1.8 m and the distance between trees was 1.5 m. In each row, 42 holes excavated from soil were made, using a drill and on the bottom of holes a layer of gravel was put to guarantee a correct drainage (Fig. 4-1).

**Fig. 4-1: One hole excavated by drill with gravel on the bottom.**

The plant system was provided with concrete piles placed along each rows and driven into the ground and also steel cables bound on them to ensure a support for all trees. The plan predicted to fill holes in the field with pots; in fact, in order to manage to

destroy each year a group of plants for mineral analyses, trees should be planted in pots. Before filling pots, the soil mixture was prepared combining in a concrete mixer about 60% of sand with 40 % of clay soil.

So this soil was used to fill 40 L plastic pots (36 cm Ø and 41 cm h).

**Fig. 4-2: Group of trees of same genotype/ combination.**

At this point, plants were taken out from the cold room, tagged with their genotype name and divided in groups (Fig. 4-2). All pots were half-filled with the prepared mixture soil (after some gravel in the bottom) and each plant was transplanted in a pot. One-year-old quinces were small and fitted properly in pots; instead almost all the grafted pears were bigger so as to require a root pruning to reduce the volume before transplanting. Later all pots were fulfilled until 4/5 of the capacity with more soil to cover plant roots.

The field project was planned as a randomized block design with six blocks for row and six adjacent rows: three assigned to control plants alternated with other three assigned to plants later irrigated with saline water. In order to accomplish the randomized block design, the plant placement was organized along row, following a random extraction procedure, in way of having always seven plants for block, one for each combination/genotype. This randomly extraction allowed to decide the order of plants in each row and to draw a map of the field as reported (Map. 1).

**Fig. 4-3: How pots were placed in the holes in the field.**

According to the map, pots were arranged and placed in the holes (Fig.4-3); in this way the tree root apparatus could grow under temperature conditions similar to those of a normal tree in an orchard. Since holes in the field were bigger than

the pot diameter, so after having placed all trees in their position, soil left over was tucked in around pots. This operation was carried out giving special attention to align all trees on the row as much as possible to facilitate the next connection of them with cables.

The irrigation system was formed of three 1,000 L tanks, three independent pipe lines with two drippers (2 L/h) in each pot to provide irrigation. Each line was connected with one 1,000 L tank by polyethylene black pipes and it was provided with an electric pump, an electric valve to control the leak of liquid that can outflow because of the depression inside the system. A bigger black pipe came from the tap of the tank and smaller black distribution pipes were inserted in the bigger one to provide water in three rows by drippers (at 2 bars). All the irrigation system was managed by two power stations that were properly set in order to distribute daily the correct volume of water/nutrition at a precise moment in the day.

The first line was connected to the 1,000 L tank assigned to fresh water or fertigation that provide it to all the 252 plant in this field. This first line had an own power and control station set to distribute solutions daily. The other two lines, instead, were regulated by the same power supply, because they should distribute the same amount of solution contemporarily. The second line was assigned to dispense saline water to rows 2, 4, 6. The third line was involved in the water supply at rows 1, 3, 5.

**Fig. 4-4: Irrigation system formed of three 1,000 L tanks and power systems.**

**Map. 1: Map of the experiment orchard for the long term experiment on salt stress applied to pears and quinces.**
**Row 1, 3 , 5= control irrigation; Row 2, 4, 6 =NaCl treatment via irrigation**
**Legend: A-BA= Abbé Fétel/BA29, A-MC= Abbé Fétel/MC, A-OHF= Abbé Fétel/Farold®40, OHF=Farold®40, AUTO= Abbé Fétel own rooted, BA=BA29.**

| block | tree | ROW 1 | tree | ROW 2 | tree | ROW 3 | tree | ROW 4 | tree | ROW 5 | tree | ROW 6 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BL 1 | 42 | OHF | 42 | AUTO | 42 | A-BA | 42 | A-BA | 42 | MC | 42 | MC |
| | 41 | A-BA | 41 | A-BA | 41 | OHF | 41 | A-MC | 41 | OHF | 41 | BA |
| | 40 | A-OHF | 40 | OHF | 40 | AUTO | 40 | AUTO | 40 | A-BA | 40 | A-OHF |
| | 39 | BA | 39 | MC | 39 | BA | 39 | A-OHF | 39 | A-MC | 39 | AUTO |
| | 38 | A-MC | 38 | A-OHF | 38 | A-OHF | 38 | MC | 38 | A-OHF | 38 | A-BA |
| | 37 | MC | 37 | A-MC | 37 | MC | 37 | OHF | 37 | AUTO | 37 | A-MC |
| | 36 | AUTO | 36 | BA | 36 | A-MC | 36 | BA | 36 | BA | 36 | OHF |
| BL 2 | 35 | A-BA | 35 | BA | 35 | A-BA | 35 | A-BA | 35 | A-BA | 35 | AUTO |
| | 34 | A-MC | 34 | OHF | 34 | MC | 34 | MC | 34 | BA | 34 | A-OHF |
| | 33 | MC | 33 | MC | 33 | BA | 33 | AUTO | 33 | MC | 33 | A-MC |
| | 32 | AUTO | 32 | A-MC | 32 | A-MC | 32 | A-OHF | 32 | A-OHF | 32 | OHF |
| | 31 | OHF | 31 | A-BA | 31 | A-OHF | 31 | OHF | 31 | OHF | 31 | MC |
| | 30 | BA | 30 | AUTO | 30 | AUTO | 30 | BA | 30 | A-MC | 30 | BA |
| | 29 | A-OHF | 29 | A-OHF | 29 | OHF | 29 | A-MC | 29 | AUTO | 29 | A-BA |
| **BL 3** | 28 | A-MC | 28 | AUTO | 28 | A-BA | 28 | MC | 28 | A-MC | 28 | MC |
| | 27 | AUTO | 27 | A-BA | 27 | AUTO | 27 | A-MC | 27 | BA | 27 | A-OHF |
| | 26 | MC | 26 | A-MC | 26 | A-MC | 26 | AUTO | 26 | MC | 26 | OHF |
| | 25 | A-BA | 25 | MC | 25 | MC | 25 | OHF | 25 | OHF | 25 | A-BA |
| | 24 | BA | 24 | OHF | 24 | BA | 24 | BA | 24 | A-BA | 24 | AUTO |
| | 23 | A-OHF | 23 | BA | 23 | OHF | 23 | A-OHF | 23 | A-OHF | 23 | BA |
| | 22 | OHF | 22 | A-OHF | 22 | A-OHF | 22 | A-BA | 22 | AUTO | 22 | A-MC |
| BL 4 | 21 | A-MC | 21 | A-MC | 21 | OHF | 21 | BA | 21 | A-OHF | 21 | A-MC |
| | 20 | OHF | 20 | MC | 20 | BA | 20 | AUTO | 20 | BA | 20 | A-BA |
| | 19 | AUTO | 19 | BA | 19 | A-OHF | 19 | A-MC | 19 | AUTO | 19 | BA |
| | 18 | A-OHF | 18 | A-OHF | 18 | AUTO | 18 | A-BA | 18 | A-BA | 18 | A-OHF |
| | 17 | A-BA | 17 | OHF | 17 | MC | 17 | A-OHF | 17 | A-MC | 17 | AUTO |
| | 16 | MC | 16 | A-BA | 16 | A-BA | 16 | MC | 16 | MC | 16 | OHF |
| | 15 | BA | 15 | AUTO | 15 | A-MC | 15 | OHF | 15 | OHF | 15 | MC |
| BL 5 | 14 | A-MC | 14 | A-BA | 14 | OHF | 14 | AUTO | 14 | A-BA | 14 | BA |
| | 13 | BA | 13 | A-OHF | 13 | A-OHF | 13 | BA | 13 | A-OHF | 13 | A-MC |
| | 12 | A-OHF | 12 | AUTO | 12 | MC | 12 | A-MC | 12 | BA | 12 | MC |
| | 11 | A-BA | 11 | BA | 11 | AUTO | 11 | OHF | 11 | A-MC | 11 | OHF |
| | 10 | OHF | 10 | A-MC | 10 | A-BA | 10 | MC | 10 | MC | 10 | A-OHF |
| | 9 | AUTO | 9 | MC | 9 | BA | 9 | A-OHF | 9 | AUTO | 9 | A-BA |
| | 8 | MC | 8 | OHF | 8 | A-MC | 8 | A-BA | 8 | OHF | 8 | AUTO |
| BL 6 | 7 | A-MC | 7 | A-OHF | 7 | MC | 7 | A-MC | 7 | A-OHF | 7 | A-OHF |
| | 6 | OHF | 6 | AUTO | 6 | OHF | 6 | A-BA | 6 | OHF | 6 | OHF |
| | 5 | MC | 5 | OHF | 5 | A-OHF | 5 | A-OHF | 5 | A-BA | 5 | AUTO |
| | 4 | BA | 4 | A-BA | 4 | A-MC | 4 | AUTO | 4 | BA | 4 | A-MC |
| | 3 | A-BA | 3 | BA | 3 | AUTO | 3 | BA | 3 | MC | 3 | BA |
| | 2 | A-OHF | 2 | MC | 2 | BA | 2 | OHF | 2 | AUTO | 2 | MC |
| | 1 | AUTO | 1 | A-MC | 1 | A-BA | 1 | MC | 1 | A-MC | 1 | A-BA |

### 4.1.1 Irrigation, fertigation and salt application

The controlled irrigation and fertigation started on June, 20$^{th}$ in 2007 and again on May, 19$^{th}$ in 2008.

The amount of solutions applied was determined calculating the maximum water capacity in the pot and therefore avoid to waste of water or nutrient solution; based on this, the time needed to irrigate was set in the automatic power station.

The three lines were independent because the fertigation line did not always follow the same setting, in fact application of nutrient solution respected needs of trees that change depending on the development stage and environmental conditions.

Fertigation is an efficient way, extensively used in commercial agriculture, to provide nutrient solutions or soil amendments to plant through the irrigation system.

Fertilizers adopted in this experiment were:

1. Bolikel (Aventis) is a product containing: 6.5% iron soluble in water and 6.5% iron chelate that is a form of iron readily absorbed by plants, especially in alkaline soils. It contains also 2-hydroxy-4-methylphenylacetic acid (EDDHMA) that acts as a chelating agent.
2. Idrofloral 20-9-10 (Cifo) is a NPK fertilizer containing: 20% Nitrogen (8.5% N nitric and 11.5% ammonia nitrogen), 9% Phosphor pentoxide ($P_2O_5$) soluble in water, 10% Potassium Oxide ($K_2O$) soluble in water, 2% Magnesium oxide (MgO) soluble in water, 16% sulphur trioxide ($SO_3$).

At the beginning the application of nutrients was stronger because plants showed a clear nutrient deficiency situation; all trees received daily a nutrient solution containing 2 g of iron chelate and 1.43 g of 20-9-10. After a couple of week the quantity of iron chelate per plant was reduced at 0.5 g/day because the beginning iron deficiency seemed ameliorated.

Daily the fertigation line dispensed solution for 30 min (9:00 AM to 9:30 AM) corresponding to approximately 1,000 ml/pot. Instead the other two lines distributed fresh/saline water for 20 min (9:30 AM-9:50 AM) equivalent to about 666 ml/pot. The fertigation tank should be refilled every 4 days with 1,100 g iron chelate and 1,080 g of 20-9-10 dissolved in water.

The fertigation was stopped on August 1st and replaced with fresh water, that was applied regularly in parallel with the other two lines and, for some days during summer, the volume was increased in order to cope the drought and high temperature. The other two tanks dispensed about 85 L/day each, according to the setting of their power station so the tank replenishment should be done more or less every 11 days.

The fresh water tank, assigned to irrigate control plants, was filled with normal water coming from the waterworks. Using a portable EC-meter it was possible to control also the electrical conductivity of fresh water inside the tank; it was on the average 0.65 dS/m. Saline water was obtained adding a proper amount of NaCl to the same fresh water. The amount of salt to be added was calculated based on the electrical conductivity of 5 dS/m reported in a previous work (Musacchi et al., 2006a) which represented the short-term experiment on salinity applied to pears and quinces a lot alike to the current long-term one. It is well known that a solution with an electrical conductivity of 10 dS/m corresponds to 100 mM NaCl; so that to have saline water at 5 dS/m inside the tank, it was established to make a 50 mM NaCl solution. Practically to fill this tank, 2920 g of cooking rock NaCl was weighted, put in a container and dissolved in a smaller volume of fresh water, 8 L were enough because NaCl solubility in water at 20°C is 365 g/L. The complete dissolution of salt in water was facilitated by using a manual drill with a whisk; only when all the rock salt was solubilized this “mother solution” was poured into the big tank assigned to salt treatment. The container was rinsed twice to be sure to transfer all salt needed into the tank; after that the tank was filled to the volume of 1,000 L with fresh water. To avoid the sedimentation of salt inside the tank, initially the solution was stirred by a stick and later an aquarium pump was dipped into it (and the power cable plugged into the power station nearby) to keep the solution in movement. The electrical conductivity was later measured inside the tank and it was on the average 5.7 dS/m.

In 2007, salt treatment started on 19th July and ended on 3rd October, so the treatment last 75 days and the total amount of NaCl applied corresponded at 1.67 g NaCl per day to every plant of rows 2, 4, 6.

In 2008, salt treatment started on 10th June and ended on 12th October, so the treatment last 125 days and the total amount of NaCl applied corresponded at 1.70 g NaCl per day to every plant of rows 2, 4, 6.

### 4.1.2 Water content in the soil and electrical conductivity

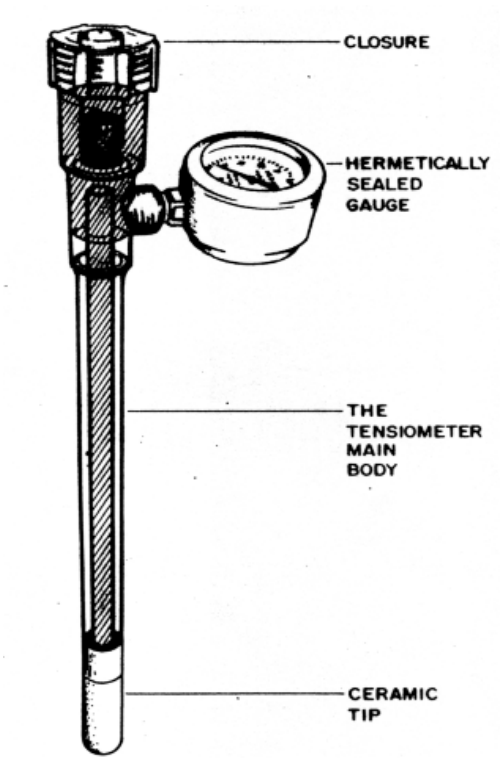



**Fig. 4-5: Scheme of a tensiometer**

In a trial like this, it was important to monitor the water content inside pots. To absolve this need six tensiometers (EcoSearch, Italy - Fig. 4-5) were installed in each row on the fourth pots that were chosen as representative for its row.

A tensiometer is an instrument used to determine soil moisture tension, moreover evaluating rain effect and estimating the water penetration into soil.

Tensiometer consists of a plastic tube ending with a porous ceramic cup, and inside should be filled with distilled water. On the top of the tube, the tensiometer is provided with an inserted vacuum gauge that can measure the pressure inside it expressed in centibar. Its principle of working consists in water inside the device that tries to reach equilibrium with the water in the soil, generating a tension. As the soil dries up, it draws back water from the tensiometer through the porous cup; this event creates a depression that is measured by the vacuum gauge. So the instrument was buried quite deeply into soil (10 cm from the bottom of the pot) and than attached by mud; this step is necessary because ceramic cup must be in close contact with soil. After having poured water in the inner tube and ensured that no bubbles were present, the instrument was hermetically closed by its lid. In particular, the model “Jet Fill” is provided with a top reservoir with a pushing button that permits easily the exit of bubbles from the inner tube; instead in the older models a straw should be used to remove air. This instrument needs some days to

build a close contact with soil, before giving true measurements of moisture conditions.

As water is supplied to the soil, the vacuum inside the tube pulls moisture from the soil and pressure inside decreases; usually when soil is saturated of water the scale of the vacuum gauge indicates 0÷10 centibar. When instead it indicates about 30 centibars it means that it is time to start drop irrigation.

In addition two suction lysimeters (EcoSearch, Italy) for each row were placed randomly in order to measure the electrical conductivity of the soil solution, comparing control plants and "salted" plants.

A suction lysimeter is a non-permanent hydrological device that collects liquid from the pores spaces of soil and allows obtaining sample for electrical conductivity measurement at different depths and determining the soluble constituents in the drainage. Lysimeter experiments under outdoor conditions have been used since the late 18$^{th}$ century to study the movement of water, soluble salts and heavy metals, to evaluate the environmental behaviour of pesticides and their potential risk on water contamination and to monitor the impact of saline irrigation water (Grundmann et al., 2008; Bowman et al., 2006; Robbins and Willardson 1980).

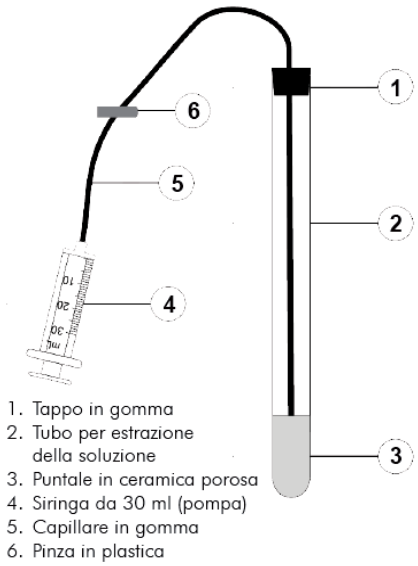



**Fig. 4-6: Different parts componing a lysimeter.**

A suction lysimeter looks like a tensiometer with some differences (Fig. 4-6), it consists of a porous ceramic cup, a PCV tube (60 cm) that represents also the collector chamber for sample liquid, a rubber stopper to hermetically seal the chamber where is inserted, in the middle, a transparent, flexible plastic tube that extends down enough to reach the ceramic cup. So the instrument, similarly to tensiometer, needs to be buried for half of its length into soil (in the middle of the pot) surrounded by mud to guarantee a continuum between cup and soil (Hubbell and Sisson, 2003 Patent application).

The lysimeter accomplishes his function by applying a vacuum (around 70-80 centibar) with a proper vacuum pump inserted in the flexible plastic tube. Before disconnecting the pump from the device, it is fundamental to close the tube with a clamp in order to maintain the depression inside the chamber that will permit the collection of liquid sample when soil will be saturated. After a couple of days the depression inside the tube accumulates sample that can be drawn from the suction lysimeter using a 60 ml syringe connected to the flexible plastic tube. In this experiment, the sampling of liquid inside the chamber was carried out after a week. It happened, several times during these two years that the collection chamber was empty and so no sample could be recovered; this can be due to the excess of evapotranspiration or to the insufficient irrigation applied to the orchard.

Each drawn sample was poured in a 100 ml plastic bottle in order to measure the electrical conductivity of the solution and in this way keep under control the level of salinity applied through watering. If all 12 lysimeters provided samples, six solutions were relative to control plants and the other six to salted trees.

Conductivity is the capacity of a solution to conduct an electrical current. Therefore electrical conductivity (EC) is a measurement of the total concentration of ions in a solution or total dissolved salts (TDS); but it does not distinguish which salts are present at specific concentration.

**Fig. 4-7: Portable EC-meter.**

For example, an EC meter can be used in modern agriculture for measuring how many nutrients to put in the water without causing an excess in fertilization; or in the food industry for measuring the salinity of the samples as part of the quality control.

The use of an EC-meter is an easy and quick way to obtain the ionic strength of a solution; it is portable and measures can be carried out directly in the field (Fig. 4-7).

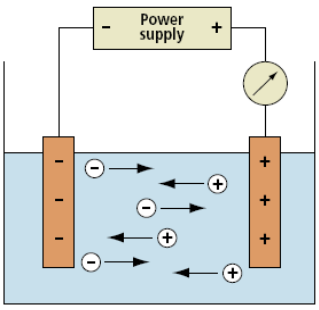



**Fig. 4-8: Electric field applied in the EC-meter probe.**

The passage of an electric current through a solution is measured via a probe with two metal prongs one cm apart. An electrical field between two electrodes (Fig. 4-8) is applied and the

electrical resistance of the solution is measured. Therefore the higher the EC, the easier it is for electrical current to move through the solution, thus a higher EC value.

It is well known that the conductivity of a solution is very dependent on the temperature; in fact EC value increases with temperature. To face with this aspect, some EC-meters, such as portable EC-meter Crison used in the study, are provided with an internal temperature sensor integrated in the probe, able to calculate the temperature compensation and give back an EC value referred to 25°C.

Electrical conductivity is measured in millisiemens per centimeter (mS/cm) equivalent to decisiemens per meter (dS/m).

## 4.2 Estimation of vegetative activity

To evaluate the vegetative activity, the growth rate was measured on two shoots for each genotype or combination (where possible) for each row.

During the vegetative rest, in February 2007, the diameter of the plant trunk (collar) was measured using calipers. In the grafted plants, this measure was carried out 10 cm above the grafting point; instead in the other genotypes (rootstocks and Abbé Fétel own-rooted) the edge of pots was kept as reference for the evaluation.

To estimate the vegetative growth of trees, the growth activity was measured on two brunches per genotype in each row.

Additionally in March 2008 all the shoots of the year, all flower buds and number and length in different combinations were measured. In March 2009, estimation of shoots number and average shoot length was performed contemporarily with the first tree destruction (T1).

## 4.3 Gas exchange measurements

Gas exchange measures were carried out once per month during the four-months period of salt stress imposition; always in the morning using a portable gas exchange system LI-COR 6400 (Bioscience-Nebraska USA). How to use LI-COR 6400 has already been explained in the previous section “Methodological deepening- Gas exchange measurements and LI-COR 6400”.

This measure in the field was made with the GaAsP PAR sensor integrated in the Li-COR 6400 head/IRGA, the one that allows setting a PAR that can be different from the real one, but in this way data can be compared without worrying about variations in meteorological conditions (i.e. shining sun and later shading clouds) during the 2-3 hours of measurement. In particular, every time the gas exchange measurements were performed (2007 and 2008), PAR was set at 1300 µmol $m^{-2}s^{-1}$ that correspond to a full field light condition in a sunny day.

Since the field was too big for carrying out the measures in all its trees, the block number 3 was chosen as the most representative of the field and only on those plants, physiological parameters were evaluated.

For each plant three fully expanded leaves exposed to light were chosen to measure photosynthesis, transpiration and stomatal conductance.

In 2007 it was possible to perform measurements each month starting from June, when the salt stress was not imposed yet until September; instead in 2008 the first measure could be done only in July when the salinity irrigation had already started.

## 4.4 Plant destruction at T0 and T1

At the beginning of experiment, 21 plants, three for each combination/genotype, were rinsed with distilled water to eliminate the residue of soil in roots and destroyed dividing in parts, such as: branches, trunk (Fig. 4-9), coarse roots and fine roots (≤ 2 mm Ø). The fresh weight of the different plant parts was valued, samples were dried in a ventilated oven at 60 °C and dry weights were determined after one week because wooden parts take longer to dry up.


**Fig. 4-9: Example of trunk divided in small pieces for future mineral analyses.**

A representative subsample of each dried organ was ground into powder using a Mill (Fritsch P14, Fritsch Germany) to pass a 0.2 mm mesh; these fine products were conserved in plastic sealed bags named with genotype, organ, replicate number (1, 2 3) and date (T0= beginning of the experiment). These powdered samples will be

used for determining content in macronutrients and micronutrients by atomic absorption spectrophotometry as previously described.

In March 2009, before the vegetative resumption, again three trees per combination/genotype were destructively harvested from row 5 (control) and row 6 (NaCl) and divided in organs to get a second collection of samples that will represent the T1. So in the next future, the idea is to compare the content in macronutrients and micronutrients between T0 samples (at the beginning of the experiment 2007) and T1 samples after two cycles of salt treatment at 5 dS/m of CE. This proposal has the aim to investigate particularly on sodium and chloride in order to clarify how they have been drawn in, where they have been mainly transported and stored in the different plant combinations and genotypes under study.

## 4.5 Unexpected heat stress

In the middle of July 2008 after a really hot weather week end, some trees in the field appeared seriously damaged; they showed leaves turning brown and sometimes becoming shriveled (Fig. 4-10). This event was probably caused by a temporary malfunctioning of the power station that distributed irrigation combined with high temperature.

**Fig. 4-10: Clear effect of heat stress.**

Temperature above normal is sensed by all organisms as heat stress. This stress combined with drought alters cellular homeostasis and can lead to irreversible damage to plant function and development (Kotak et al., 2007). A previous study carried out by Hall (2001) asserts that high temperatures can have direct harmful effects linked to hot tissue temperatures or indirect effects related to the plant-water-deficits that can arise because of high evaporative demands (Hall 2001). Trying to avoid trees death, a conspicuous quantity of fresh water was distributed to the entire field without any distinction. The damage concerned four trees with a severe damage and other sixteen with symptoms less serious.

**Fig. 4-11: Effect of the heat stress relief: new shoots and new flowering.**

Considering that the physiology of those plants was definitely compromised because of this additional stress, they were excluded from the experiment that is no gas exchange measurements or leaf samplings were done anymore from July 15$^{th}$, in trees affected by heat/ drought stress located in block number 3.

About one month later, new shoots (Fig. 4-11) came out from damaged plants and some of these resumed flowering; this behavior can mean that the heat/drought stress ended enough soon to allow plants to recover. It has been reported that photosynthetic pigments are involved in flowering regulation; for instance an increase in photosynthetic activity in the new shoots can accelerate the passage to a reproductive phase (Buchanan et al., 2003).

## 4.6 Leaf sampling

During 2008 salt stress imposition, three leaf samplings, one each month (July, August, September) were done from the block number 3 (from plant number 22 to 28) as decided for gas exchange measurements. 13 ml plastic tubes (Sarstedt) were named indicating number of row, number of plant, block number 3, genotype/combination and date. The sampling was performed harvesting 15 leaves from each tree, possibly full expanded leaves and avoiding damaged ones. Leaves were roughly cleaned, rolled all together and inserted inside proper tube then immediately frozen in liquid nitrogen and stored at -80°C.

This collected material will be useful for future biochemical analyses (that will not be part of this thesis) such as antioxidant enzymes activities (i.e. SOD, CAT), evaluation of proline and malon-dialdehyde (MDA) contents.

## 4.7 Fruit production (2008)

In summer 2008, the first production of Abbé Fétel pears was observed only in the three grafted combinations (Abbé Fétel/Farold®40, Abbé Fétel/MC, Abbé Fétel/BA29); Abbé Fétel own-rooted usually presents a longer juvenility phase, in this case only two pears were produced but not considered.

As first fruit production it was characterized by few fruits per plant and not present in all the trees; but fruits were harvested from the entire field anyway in September, 9$^{th}$. In addition, pears did not look like commercial standard ones because of some rottenness and cracks; in fact any treatments haven't been applied during the summer to preserve fruits from pathogens or diseases. So for each tree, fruits were counted and weighted keeping them separated for rows; all these data were recorded. At the end of harvest, it was clear that keeping fruits separated in 6 rows did not have any meaning for the reduced number of fruits. Therefore all fruits coming from the same combination in every control rows (1, 3, and 5) were gathered in one crate (Fig. 4-12); the same was done for fruits originated by "salted" plants (row 2, 4 and 6). At the end six crates were collected and size of all fruits was measured using a tool that thanks to several holes large from 55 mm to 90 mm allow to quickly determine the diameter of fruit. In this way each combination had fruits divided in size classes and it was possible to analyze the effect of NaCl two-year impositions on fruit size.


**Fig. 4-12: Crate of pears of Abbé Fétel/BA29 from control rows.**

## 4.8 Qualitative analyses

Although the production was so reduced, qualitative analyses were carried out anyway in order to have some preliminary ideas of the effect of saline irrigation of fruits quality in this particular experiment and compare them with what already is known in literature.

Qualitative analyses started dividing fruits for combination and treatment, later only the best 20 fruits were selected for following steps.

The non-destructive steps:

1. fruit weight:

   each fruit was weighted in a precision scale;

2. evaluation of percentage of rustiness:

   this value was assigned by an operator looking each pear and considering the percentage of fruit affected by these histological and superficial damages (that are later “fixed” by suberification process) on the total area of fruit.

After the first two steps, the next ones were destructive:

3. evaluation of firmness of the flesh:

   each pear was peeled on the two opposite sides and firmness was determined with a manual penetrometer equipped with an 8-mm-diameter probe. This instrument measures the resistance that flesh exerts to the probe pressure ($Kg/cm^2$). This parameter is linked to the ripening stage of the fruit; it decreases with the flesh softening.

4. Soluble sugars in the flesh:

   skin was removed from all fruits that later were cut in smaller pieces and put into a juice extractor (Mulinex) gathering 4 fruits together to obtain, in this way, 5 juices from 20 initial fruits. Soluble solids were determined by a digital handheld refractometer (Fig. 4-13) that measures fluid concentration as the sugar content using only one drop of juice. During ripening, in fact starch is converted in disaccharides


**Fig. 4-13: Digit portable refractometer.**

and monosaccharides which give the sugary taste to ripened fruits. Sugar content is expressed by Brix degrees (°Brix) also known as refractometric dried substance (RDS).

5. pH and acidity of juice:

   an aliquot of 10 ml was diluted with an equivalent amount of water. The pH and the content of acids, in particular malic acid (the most present in pears) were determined by a titration system with automatic sample changer. The instrument works based on a complete chemical reaction between the analyte (juice) and a reagent (titrant) of known concentration, in this case NaOH which is added to the sample.

## 4.9 Natural leaf falling and analyses

The three trees per combination/genotype, from row 5 (control) and row 6 (NaCl), that will be destructively harvested in February-March 2009 as mentioned before, were already selected in October 2008. Selected plants did not suffer for any problems during the season and appeared in good condition. They were completely wrapped by anti-hail net that was tied up to the trunk; in this way it was possible to collect all the foliage without any loss waiting for a natural defoliation (Fig. 4-14). Genotypes and combinations showed differences in time of defoliation; in fact it took about three months to have all 42 trees bare. When trees let all leaves fall off, they were harvested from the net and put in a bag. All leaves, taking part to the T1 sampling, were counted and weighted (fresh weight) and put in a ventilated oven to dry up at 60°C for four days. After this period leaves were weighed again to obtain the dry weight (see paragraph 6.4). This dry material will be used for elemental determination by atomic absorption spectrophotometry.

It is important to say that these leaves were not washed before placing in the oven because this step, usually done, could break leaves that were not completely fresh anymore.

**Fig. 4-14: Selected trees wrapped in net for collection of leaves.**

# Chapter 5: RESULTS AND DISCUSSION

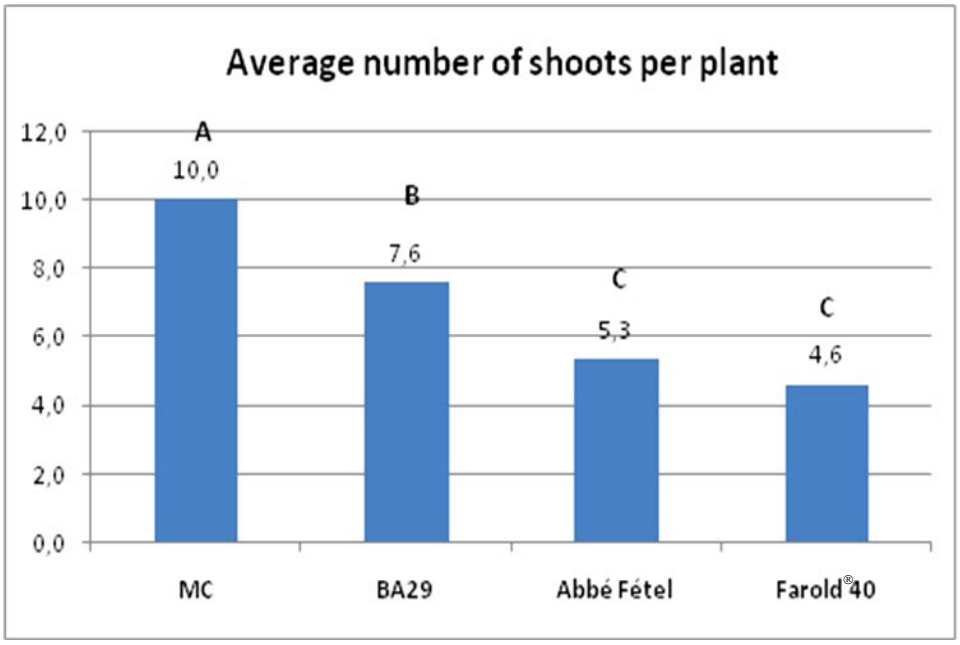
A SHORT-TERM EXPERIMENT

## 5.0 Physiological measurements

### 5.0.1 Shoot length

The measure of shoot number and length at T0 was carried out after five weeks of hydroponic culture. From statistic analyses (SAS), it appeared that at T0, plants grown in the two benches (to keep separate for later stress imposition control and treated plants) did not show any significant difference in shoot number per plant and shoot average length. The difference in shoots number was evident among genotypes; in fact quince MC presented the highest average with 10 as shoots number per plant, followed by BA29 with 7.6, while both pear genotypes showed a lower number of shoots, around 5 per plant (Graph 5-1).

These plants grew differently according to genotypes, in fact pears developed less shoots, but longer than those of quinces that were shorter, but more in number per plant (Fig. 5-1).




**Graph 5-1: Differences in number of shoots among genotypes after five weeks of hydroponic culture with no salt application yet (T0). Capitol letters indicate difference among genotypes according to SNK test p<0.05.**

**Fig. 5-1: Control plants of Farold®40 (on the left) and MC (on the right) after five weeks of growth in hydroponic culture; different genotypes developed different number of shoots.**

Differences among genotypes appeared also comparing the average length of shoots; Farold®40 in fact presented the longest shoot average length with 16.7 cm, significantly different ($p<0.01$) from the other three genotypes that had comparable length around 11 cm. From this comparison for shoot length in date 1 (T0), the interaction treatment*genotype resulted significant, in fact SNK test classified differently "control" pears plants and "control" quinces (respectively A and B) and plants located in the bench labeled "NaCl" were differently classified: Farold®40 as the genotype with highest average shoot length (A for SNK) and Abbé Fétel with the minimum length reported (B for SNK) (Tab. 5-1 date1).

As mentioned in Chapter 3, shoot length was checked again after seven weeks from the beginning of the experiment (date2 in Tab. 5-1) and at the end of the experiment (date3 in Tab. 5-1) corresponding respectively to T2 and T4 sample timings (see appendix 1). In these two dates of measurements, plants labeled as "NaCl" were effectively exposed to salt stress (80 mM turned in T2 at 90 mM); in this way it could be possible to evaluate the effect of this stress on shoot length.

In date 2 (T2) it has been observed a situation similar to T0, in fact treatment did not affect significantly shoot length of "NaCl" plants compared to the corresponding "control" plants; but the difference in shoots length among genotypes, regardless of the treatment, was maintained as appeared in T0 ($p<0.01$). Also in T2 the interaction between treatment and genotype appeared significant, so that it was possible to observe that in quinces, salt treatment imposed for 13 days did not influence shoot length, while for Abbé Fétel the osmotic effect of salinity underlined

a reduction in shoot length that was confirmed by plant aspect. Abbé Fétel plants in T2 appeared in fact a bit wilty in comparison to Farold®40 or quinces.

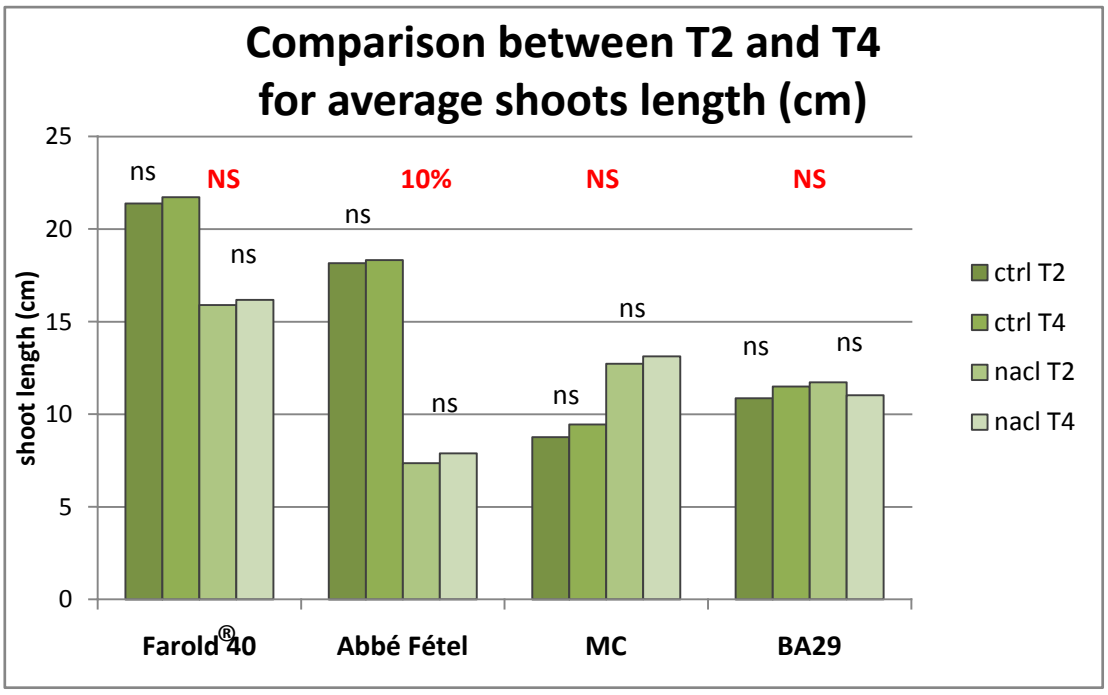
However the difference in shoot length for Abbé Fétel "control" against "NaCl" plants did not result statistically meaningful for $p<0.05$, but significant at 10% (Tab. 5-1 date2).

In date 3, that corresponded to T4 (end of experiment), the difference in shoot length among "control" plants and "NaCl" ones was significant with $p<0.05$, the former registered a major shoot length than the latter; while among genotypes, regardless of the treatment, the situation pointed out again the significant higher shoot length for Farold®40 respect to the other three considered genotypes (Tab. 5-1 date3). Also in T4 the interaction between treatment and genotype appeared significant, so that it could be observed that in quinces, salt treatment imposed for 21 days did not affect shoot length as found in T2, therefore we can say that the stress progressing did not highly decrease shoot average length.

Since the square means of errors of these three dates of measurement were comparable it was allowed to evaluate differences among dates using contrasts (SAS). From this analysis, it emerged that between T0 and T2, differences in shoot length were significant with $p<0.01$. It means that "control" plants continued to grow from date 1 to date 2, while NaCl plants stopped and remained stable with no statistically meaningful differences. The comparison between date 2 and date 3 pointed out that the situation at T4 did not change from T2 in term of shoot length; the increase in average length for "control" shoots from 13.8 cm to 14.3 cm per plant seemed significant, but only at 10% (Tab. 5-1). The comparison between T2 and T4 dates underlined that the behavior of the four genotypes in terms of shoot length did not change with the advance of the stress (Graph 5-2). It is well known that the first effect of salinity on plants is the inhibition of growth (Tester and Davenport, 2003), data indicated a general slowdown of growth for all our four genotypes cultured in hydroponic system and exposed up to 90 mM NaCl. This limitation of the growth could be due to a different allocation of energies due to salinity conditions. In fact plant needs to face the osmotic effect of the stress providing an osmotic adjustment that requires a high level of energy. This can be a

possible explanation for growth reduction (Sotiropoulos and Dimassi, 2004; Munns and Tester, 2008).

Comparing "control" with "NaCl" plants, it can be noticed that the former continued growing while the latter stopped lengthening shoots. Probably the growth activity of "control" plants from T0 to T4 was not so evident because of environmental conditions in the chamber; in fact respect to an open field experiment this hydroponic culture trial had a limitation in light (as mentioned in the methodological part) made on purpose to try to avoid a supplemental stress as photo-oxidative stress that could influence plant responses to salt stress in case it occurred. The main disadvantage of this choice is the consequent low growing rate of "control" plants probably caused by limiting light rather than nutrients deficiency.




**Graph 5-2: Comparison between average shoot length at T2 and T4. ns indicate according to SNK test ($p<0.05$) not significant in the comparison between control or NaCl plants between T2 and T4 and NS indicate not significant differences for treatment, while for Abbé Fétel the significance is 10% (red letters).**

**Table 5-1: Measure of average shoot length at T0 (no stress) and T2 and T4 for all plants in trial. Significance: *=p<0.05, **=p<0.01, ***=p<0.001; NS= not significant. Capital letters distinguish according to SNK in horizontal way, while small letters discriminate in vertical way. 1Va 2 and 2Vs indicate the contrast between dates.**

| shoot length | | Date1 (T0) | 1Vs2 | Date2 (T2) | 2Vs3 | Date3 (T4) |
|---|---|---|---|---|---|---|
| **Treatment (trt)** | **control** | 12.7 | | 13.8 | | 14.3 |
| | **NaCl** | 12.4 | | 12.2 | | 12.3 |
| | *signif* | *ns* | ** | *ns* | *ns* | * |
| **Genotype (gen)** | **Farold®40** | 16.7 a | | 18.3 a | | 18.6 a |
| | **Abbé Fétel** | 11.8 b | | 11.7 b | | 12.0 b |
| | **MC** | 10.6 b | | 11.3 b | | 11.3 b |
| | **BA29** | 10.9 b | | 10.7 b | | 11.3 b |
| | *Significance* | ** | *ns* | ** | *ns* | ** |
| ***Interaction gen*trt*** | | * | ** | * | * | * |
| ***Interaction gen*trt*data*** | | ** | | | | |

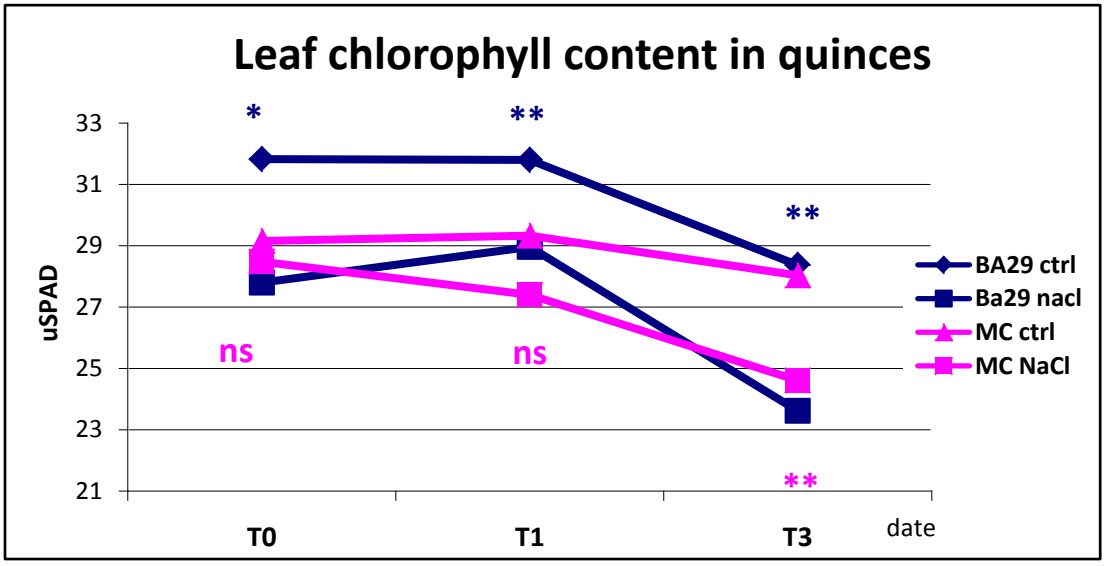
| **Date1 (T0)** | **Farold®40** | | **Abbé Fétel** | | **MC** | | **BA29** | | *Significance* |
|---|---|---|---|---|---|---|---|---|---|
| **control** | 19.1 | A | 18.0 | A | 8.9 | B | 10.3 | B | * |
| **NaCl** | 16.4 | A | 8.3 | B | 12.3 | AB | 11.4 | AB | * |
| *Significance* | ns | | ns | | ns | | ns | | |
| **Date2 (T2)** | **Farold®40** | | **Abbé Fétel** | | **MC** | | **BA29** | | *Significance* |
| **control** | 21.4 | A | 18.2 | A | 8.8 | B | 10.9 | B | * |
| **NaCl** | 15.9 | A | 7.4 | B | 12.7 | AB | 11.7 | AB | * |
| *Significance* | ns | | ns (10%) | | ns | | ns | | |
| **Date3 (T4)** | **Farold®40** | | **Abbé Fétel** | | **MC** | | **BA29** | | *Significance* |
| **control** | 21.7 | A | 18.3 | A | 9.5 | B | 11.5 | B | * |
| **NaCl** | 16.2 | A | 7.9 | B | 13.1 | AB | 11.0 | AB | * |
| *Significance* | ns | | ns (10%) | | ns | | ns | | |

### 5.0.2 Leaf chlorophyll content

The leaf chlorophyll concentration was estimated at T0 (date 1 and measurements were repeated after one week of salt stress (T1) and at the end of May, after 16 days of exposure to NaCl (T3) (see paragraph 3.1 and appendix 1).

At T0 (date 1), before stress imposition, Chl measure did not show any significant differences in leaf chlorophyll content (uSPAD) among "control" and "NaCl" plants as expected. Among genotypes instead, the chlorophyll content in both pears resulted higher than that in quinces, this difference was statistically significant with $p<0.01$; in fact pear leaves appeared greener and more shining than quince ones. At this date, unlikely, the interaction treatment*genotype resulted significant (Tab. 5-2), in fact Farold®40 and BA29 plants located in "NaCl" bench (without salt imposition yet) reported a lower chlorophyll content than the corresponding "control" plants.

At T1 (date 2), data reported that salt treatment did not affect the leaf chlorophyll content comparing all "control" plants (ctrl) against "NaCl" plants and that the different amounts of SPAD units were maintained as before the stress imposition, that is greater values for pears leaves than for quince ones ($p<0.01$). In this measurement pear leaves exposed to NaCl were starting to become necrotic, but we tried to choose leaves still green without considering light green young leaves, despite they were not affected by necrosis. At T3 (date 3), pear leaves subjected to salt stress were almost totally necrotic and shriveled so it was not possible to perform the SPAD measurement on these plants, so that for this date pears were excluded from evaluation and in table 5-2 data reported in T3 column are referred only to quinces. At T3 both quinces registered a significant decrease in leaf chlorophyll content ($p<0.01$) in comparison with their corresponding "control" plants. BA29 plants were characterized also in T0 by a lower value in uSPAD in "NaCl" plants, so we can say that MC plants probably showed this decrease in chlorophyll later than the other genotypes. In fact, MC plants at T1 reported not significant differences in uSPAD comparing with their "control", only in T3 the decrease became meaningful ($p<0.01$) (Graph 5-3).




**Graph 5-3: Trend of leaf chlorophyll content (uSPAD) in both quinces at T0 (unstressed) and T1 and T3. Pink lines are relative to MC (ctrl and NaCl) and blue lines to BA29 (ctrl and NaCl). The significance is reported with the two colours and must be associated to the two means at each time for each genotype (i.e. BA29 ctrl T0 against BA29 NaCl T0, p<0.05=*).**

Evidences reported that chlorophyll decrease results in reduced stomatal aperture (Shimshi, 1967), so during salt stress, a specific signaling to close stomata exists in order to try to avoid dehydration for excessive transpiration (Bartels and Sunkar, 2005). Also recent studies proved that salt stress condition decreases chlorophyll content and in particular that NaCl has effect on chlorophyll, inhibiting a precursor of chlorophyll synthesis (Santos 2004; Eryılmaz 2006; Jaleel et al., 2008).

In rice seedlings, grown in hydroponics and exposed to 130 mM NaCl, it has been reported that chlorophyll fluorescence significantly decreased after 4 days of stress and this may be attributed to salt-stress-induced inhibition of photosynthetic electron transport (Kim et al., 2005). Cinelli and colleagues (2003) carried out a hydroponic experiment on peach rootstocks and revealed that a decrement of chlorophyll content occurred after the addition to the nutrient solution of sodium bicarbonate in sensitive genotypes and the highest value of Chl belonged to the genotype more tolerant to iron-induced chlorosis (Cinelli et al., 2003). Iron-induced chlorosis can be a problem also for quince rootstocks as BA29, and also in this case the ability to maintain a high value of uSPAD can mean a greater tolerance to lime soil and high culture pH (Marino et al., 2000).

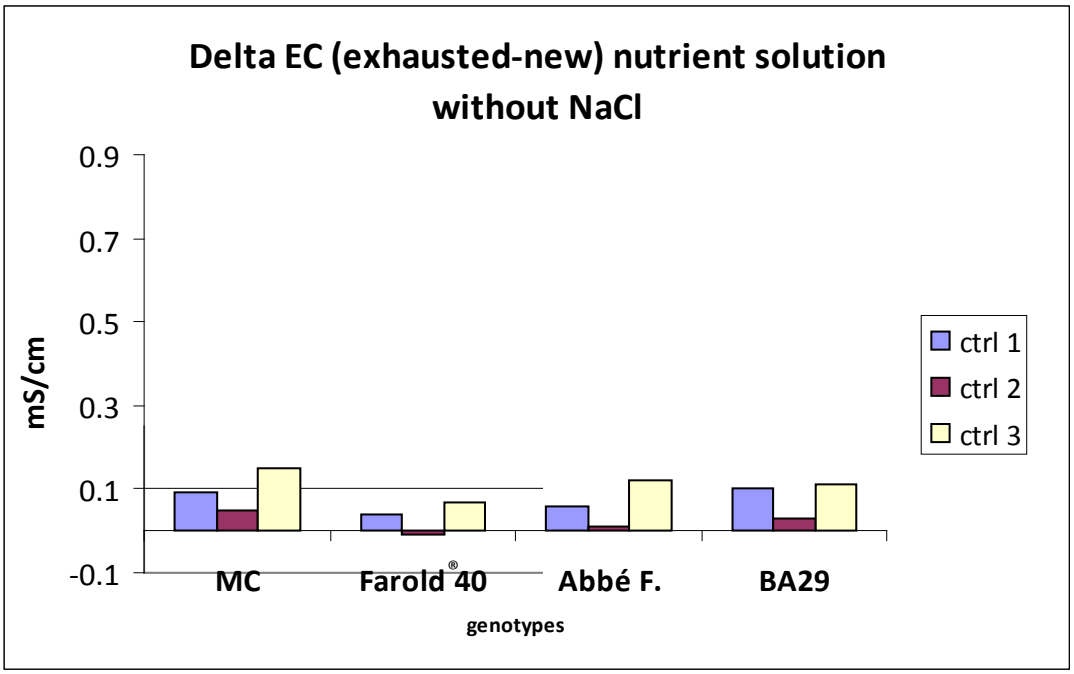
**Table 5-2: Measure of leaf chlorophyll content in three dates: at T0 (no stress) and T1 and T3 for all plants in trial. Significance: *=p<0.05, **=p<0.01, ***=p<0.001; NS= not significant.Capital letters distinguish according to SNK in horizontal way, while small letters discriminate in vertical way. Slash indicates absence of material.**

| uSPAD | | **Date 1 (T0)** | **Date 2 (T1)** | **Date 3 (T3)** |
|---|---|---|---|---|
| Treatment | **ctrl** | 34.92 | 34.72 | 28.2 a |
| | **NaCl** | 34.31 | 34.31 | 24.1 b |
| | *Significance* | ns | ns | ** |
| Genotype | **Farold®40** | 41.49 a | 42.18 a | / |
| | **Abbé Fétel** | 39.61 a | 41.62 a | / |
| | **MC** | 28.82 b | 28.36 b | 26.32 |
| | **BA29** | 29.81 b | 30.38 b | 25.99 |
| | *Significance* | ** | ** | ns |
| ***Interaction gen*trt*** | | ** | ns | ns |

| **uSPAD** | | | | | |
|---|---|---|---|---|---|
| **Date 1 (T0)** | **Farold®40** | **Abbé Fétel** | **MC** | **BA29** | *Significance* |
| **ctrl** | 42.57 A | 39.43 B | 29.155 D | 31.825 C | * |
| **nacl** | 40.4 A | 39.79 A | 28.475 B | 27.295 B | * |
| *Significance* | * | ns | ns | * | |

### 5.0.3 EC of Hoagland solution

Every times that the new nutrient solutions (1/2 Hoagland) replaced the exhausted ones, placed 3 days before, the electrical conductivity of the exhausted one was measured using a EC-meter (mS/cm) and the value compared with the EC in the previous new solution in order to understand what happened to salts in hydroponic solution. For this kind of measure, carried out in order to check solutions and to have an idea of the correspondence between salt concentration and relative electrical conductivity, the reading was only one for tank, so that it was not possible to perform a statistical analysis on these data. But we can analyze the variation only qualitatively. In "control" tanks the initial EC, when the nutrient solution was new, corresponded to about 0.8 mS/cm and after 3 days of hydroponic culture the same reading reported an EC increase of about 0.07 mS/cm as average among four "control" tanks. So in general, in "control" tanks the electrical conductivities of exhausted solutions were almost always increased respect to those registered for "NaCl" exhausted solutions as Graph 5-4 shows.

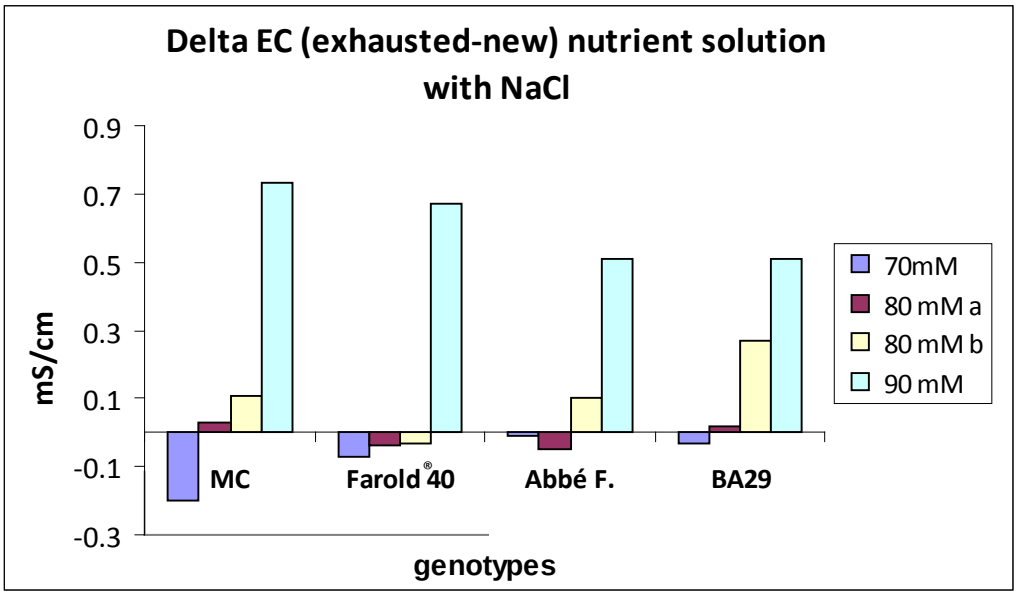



**Graph 5-4: Trend of the delta EC between exhausted solution and new solution in "control" tanks. Blue histograms are referred to 17th May (ctrl 1), Bordeaux to 21st May (ctrl 2), and yellow to 24th May (ctrl 3).**

On the other hand the same investigation was carried out in "salted" tanks. Starting from May 15th, when salt was added to Hoagland solution for the first time, the average of electrical conductivities of the four new nutrient solutions added with 70 mM of NaCl was around 6.7 mS/cm. Initially it has been noted that the first two

times, in general, the exhausted solutions registered a decrease in EC. This fact can be evident looking at the Graph 5-5, where the first histogram for each genotype lies in the negative part of the graph meaning that the difference in EC between exhausted and new solutions gave a negative value.

If the electrical conductivity is a measurement of the total concentration of ions in a solution or total dissolved salts, we can suppose that the variation of EC value can be correlated with the uptake of NaCl by plants in solution. Despite the fact that EC does not distinguish which salts are present at specific concentration in solution (Haughton, 2004), it can be possible that, being NaCl at so higher concentration respect to the other salts present in Hoagland solution, its mobility from solution to root system or the opposite can likely give the answer to this difference between exhausted and new solution. It is well known that EC measurement depends also on temperature, in fact higher EC values can be due to higher temperature, but this idea must be excluded in our particular case, because the growth chamber had a set optimal temperature and the EC-meter recorded the value with a thermal compensation (see paragraph 4.1.2). Following this argument we can assume that for example both pears absorbed NaCl until the end of the first salt stress week (May 21$^{st}$ included) because the delta EC was negative until that date.




**Graph 5-5: Trend of the delta EC among exhausted solutions and new solutions in "NaCl" tanks. Blue histograms are referred to May 17$^{th}$ when stress corresponded to 70 mM NaCl, Bordeaux to May 21$^{st}$ when stress increased to 80 mM NaCl, yellow to May 24$^{th}$ still at 80 mM NaCl and cyan to May 31$^{st}$ when salt reached 90 mM.**

Later it seems that Abbé Fétel started to expel NaCl before Farold®40 that registered a positive EC delta only at 90 mM NaCl and graphically was negative for all the other measurements. On the contrary, BA29 seems to have quickly stopped to take up salts at the first increase in NaCl concentration (first exhausted solution at May 21$^{th}$) as well as MC, which appeared to release more than the other genotype at 90 mM NaCl (cyan histograms in Graph 5-5). It is important to remember that in saline condition in addition to the probable NaCl uptake it has been proved that also $K^+$ leaks occur and also this ion could alter the EC of the solutions (Shabala and Cuin, 2008).

The supposed early extrusion of salts from some plants could be linked to their delay in necrosis onset; in fact for instance, quinces appeared to release more salts into the solution from 80 mM and the necrosis on their leaves appeared about one week later than in pears. Following this hypothsis, Farold®40 was the first genotype in trial to show necrosis and this can be associated with the supposed prolonged uptake of salts as negative histograms of delta EC show in Graph 5-5 (necrosis development will be discussed later in paragraph 5.0.7).

This supposition can be supported by some observations, such as that the electrical conductivity estimates the nutrient content of the substrate solution (Burnett et al., 2005), that EC of aqueous extracts of growing media is used as a non-specific indicator of nutrient status (Timmer and Parton, 1984) and that increasing EC diminished growth and photosynthesis rate in tomato (Schwarz et al., 2001). In addition if EC reaches about 4 mS/cm, plants can start to show nutrients deficiency even though they are in excess; this phenomena is linked to osmosis and to the competition among ions to be adsorbed (Haughton, 2004).

For example evidence reported that, in a highly saline environment, $Na^+$ competes with $K^+$ altering the normal potassium absorption resulting in toxicity risks for cells (Blumwald et al., 2000; Carvajal et al., 2000) and that NaCl establishes a large electrochemical gradient favouring the passive entry of salt ions through different kinds of transporters in plasma membrane (Sun et al., 2009).

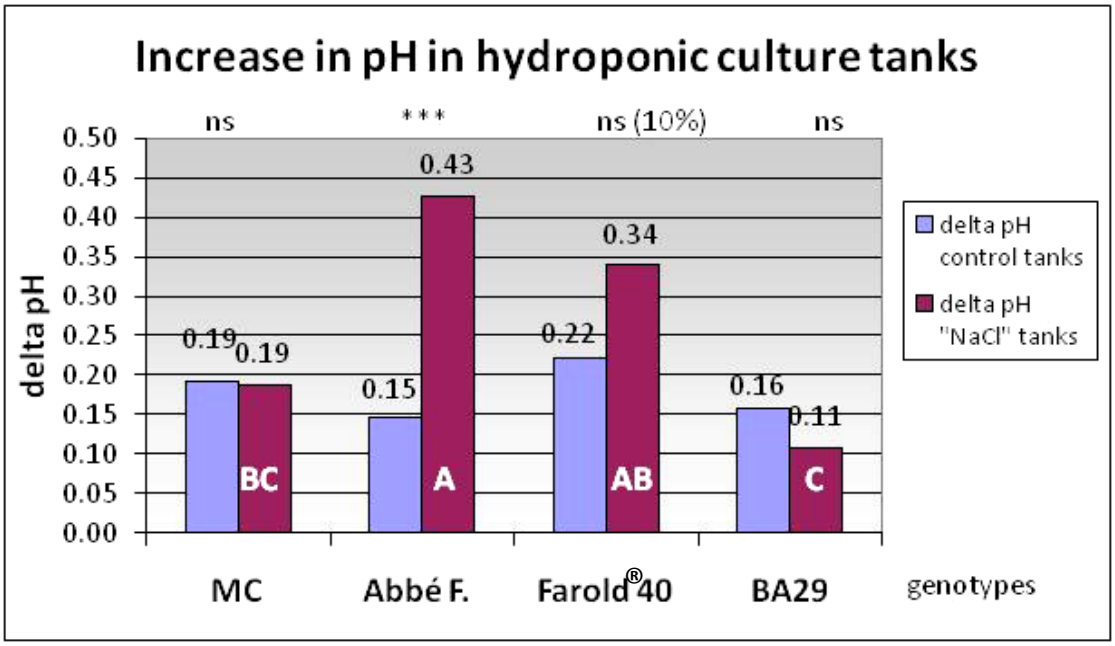
### 5.0.4 pH of Hoagland solution

Every time that the new 1/2 Hoagland solutions replaced the exhausted ones, placed 3 days before, the pH of the exhausted one was measured using a portable pH-meter and the value compared with the initial pH adjusted to 6 with KOH in all new solutions (Marino et al., 2000). For this kind of measure, we considered all pH variation data available from the beginning of salt imposition until the end, without distinguish among different gradual increases in NaCl concentration; while unlike for EC values distinction had been done because electrical conductivity is more strictly linked to salt concentration. In general, for all tanks in trial an increase in pH has been reported regardless of the treatment. For this evaluation we had enough data to carry out a statistical analysis (SAS) that showed a significant difference ($p<0.05$) among increases of pH in "control" tanks against "NaCl" ones; "NaCl" exhausted solutions registered a higher increase in delta pH respect to "control" exhausted solutions. Additionally, genotypes appeared different with $p<0.05$ but SNK did not discriminated among them. Also the interaction treatment*genotype resulted significant with $p<0.05$, so that each genotype was analyzed to understand the meaning of this interaction. From the comparison among "control" and "NaCl" solutions in tanks for Farold®40 and both quinces, the increase in pH did not appear significant (Tab. 5-3), whereas the increase in pH recorded for Abbé Fétel solutions resulted highly significant ($p<0.001$). These differences were better visualized in Graph 5-6. It has been reported that probably the most important factor that can modify the plant growth in relation to nutrient uptake is pH (Haughton, 2004) and that changes induced by roots in rhizosphere pH are linked to the nutritional status of plants (Marschner, 1997). Plants will change the composition of the nutrient solutions, upon contact, by depleting specific nutrients more rapidly than others, removing water from the solution, and altering the pH by excretion of either acidity or alkalinity. Care is required not to allow salt concentrations to become too high, but nutrients to become too depleted, or pH to go far from the desired value (Hoagland and Arnon, 1950).

**Table 5-3: Measurements of increase in pH among exhausted and new solutions in each tank in trial (Control and NaCl). Significance according to SAS proc GLM: *=p<0.05, **=p<0.01, ***=p<0.001; ns= not significant. Capital letters distinguish according to SNK in horizontal way, while small letters discriminate in vertical way.**

| Increase in pH | | |
|---|---|---|
| Treatment | **Ctrl** | 0.179 b |
| | **NaCl** | 0.266 a |
| | *signif* | * |
| Genotype | **Farold®40** | 0.281 |
| | **Abbé Fétel** | 0.287 |
| | **MC** | 0.190 |
| | **BA29** | 0.132 |
| | *Significance* | * |
| ***Interaction gen*trt*** | | * |

| increase in pH | Farold®40 | Abbé Fétel | MC | BA29 | *Significance* |
|---|---|---|---|---|---|
| **Ctrl** | 0.222 | 0.147 | 0.192 | 0.157 | ns |
| **NaCl** | 0.340 AB | 0.427 A | 0.188 BC | 0.108 C | * |
| signif | ns (10%) | *** | ns | ns | |




**Graph 5-6: Differences in increase in pH for each tank in trial. On the top in black is reported for each genotype the significance of the comparison among "control" and "NaCl" tanks. White capitol letter on Bordeaux histograms indicate the SNK distinction among "NaCl" tanks (ns for "control" tanks not reported).**

For this latter reason nutrient solution for hydroponic culture must be changed about every three days in order to re-establish and control the nutrient uptake and guarantee continued growth. The concentration of nutrient solution changes with the growth of the plant and this can lead to a variation in acid-base balance (pH) which may be responsible for precipitation of certain essential elements so they become unavailable for plants (Hoagland and Arnon, 1950). It has been reported that increase in net release of proton (acidification) is related to increase in cation/anion uptake ratio (Marschner, 1997) and similarly it can be argued that the alkalinization can be related to decrease in cation/anion ratio and to release of $OH^-$. It is possible to transport the concept well known for rhizosphere that a nutrient solution can become more alkaline when plant adsorbs nitrate and more acid when the nitrogen form adsorbed is ammonium (Nye, 1981), to our case of hydroponic nutrient solutions. Nitrate is almost the unique N form taken up by plant roots growing in calcareous soils, where crops frequently suffer from Fe deficiency (Kosegarten et al., 2004).

We may suppose that Abbé Fétel, which registered this significant increased in delta pH (pH exhausted solution- pH new solution) in saline condition against its corresponding control, had a different adsorption of elements and probably for what concerns nitrogen in this stress condition respect to a normal situation. This idea should be proved in the future by further analyses of nitrate and ammonium concentrations on the exhausted solutions. It has been hypothesized also that salt could affect the pH gradient across the plasmalemma and in this way alter also potassium uptake (Martinez and Lauchli, 1994).

### 5.0.5 Gas exchange measurements

Gas exchange measures were carried out twice during the entire experiment: the first one at T1 and the last one at T3, always in the morning using a portable gas exchange system LICOR 6400 as described in the methodological part (par.3.1.2). During T1, gas exchange measure was done on all four genotypes (“control” and “NaCl”), instead in T3, foliage necrosis was so expanded in both pear genotypes that it was not feasible to conduct the measures on them.

In the first measure, carried out after one week of salt imposition (80 mM), "NaCl" plants showed a significant higher photosynthetic activity than "control" plants with $p<0.01$, while no meaningful differences were reported among the genotypes. But for what concern the interaction treatment*genotype, it resulted significant, so that it was necessary to go deep in it to find out which genotype presented a peculiar performance. This analysis revealed that MC plants subjected to salt stress presented a higher photosynthetic activity in comparison to corresponding unstressed plants ($p<0.001$); the other genotypes did not present differences in photosynthetic activity among stressed and "control" plants (Tab. 5-4). This behavior was already noticed in a previous study carried out by Musacchi and colleagues (2002) where leaf photosynthesis was unaffected by a salt stress applied with 5 dS/m irrigation water (Musacchi et al., 2002). The increase in photosynthetic activity reported in MC leaves exposed to salinity can be explained as a consequence of the salt imposition that, at the beginning, can have a stimulating effect on plant efficiency. According to the literature, this effect can be correlated with the positive influence that chloride ion has on the good efficiency of the water-splitting system at the oxidizing site of PSII (Xu et al., 2000). Moreover it has been recently underlined by Munns and Tester (2008) that a mechanism of salinity tolerance, classified as part of the so called "tissue tolerance", implies at a photosynthetic level, a delay in ion toxicity in chloroplasts (Munns and Tester, 2008); this clue, in addition to MC's delay in leaf necrosis onset, let us think that this genotype showed in this way a good adaptability to medium-high saline condition.

For the other two parameters we noticed a significant ($p<0.01$) decrease in both stomatal conductance and transpiration for all plants exposed to NaCl if compared with their corresponding controls, whereas it has not been recorded differences in genotypes (Tab. 5-4).

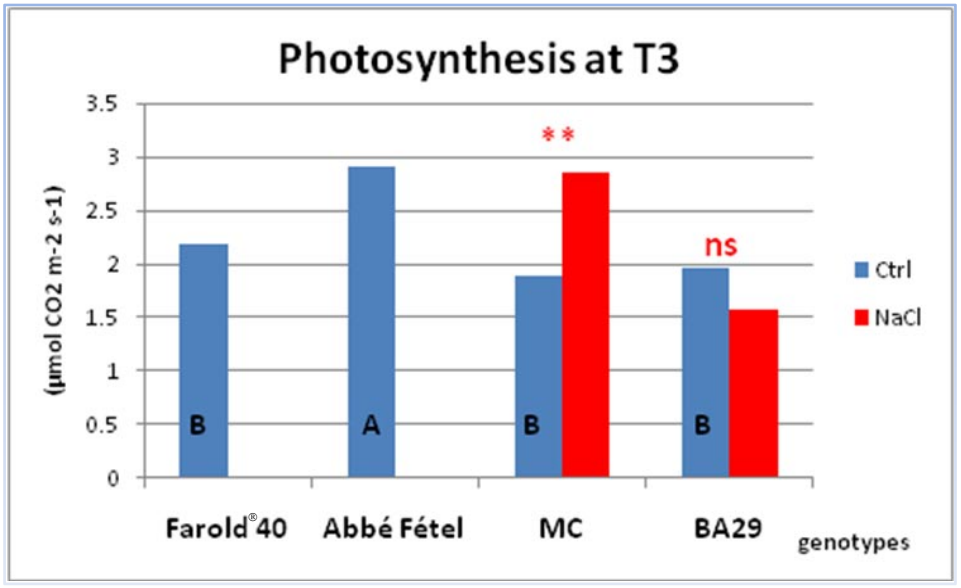
As mentioned before, at T3, gas exchange measurements were unbalanced because of the expanded necrosis on pears leaves in both genotypes, so that the evaluation of these physiological parameters were performed on the four genotypes in "control" bench and in the two quinces on the "NaCl" benches. So in "control" bench, genotypes showed a different photosynthetic activity, underling Abbé Fétel with the higher value and the other three genotypes did not differ significantly each

other (Graph 5-7, blue histograms). For what concerns "NaCl" bench, the comparison was made among quinces because they presented leaves still alive and quite green to be utilized. The analysis revealed a significant increase ($p<0.01$) in photosynthetic activity of MC exposed to salt stress respect to the corresponding MC "control" plants; while BA29 did not showed significant changes among control and "NaCl" plants (Graph 5-7, red histograms). Also stomatal conductance and transpiration were analyzed only in relation to the two quinces under salt stress; these data confirmed the same trend found in T1 that is a meaningful decrease in these parameters in stressed plants as reported in Table 5-5.

**Table 5-4: Measurements of gas exchange parameters (photosynthesis, stomatal conductance, transpiration). Significance according to SAS proc GLM: *=p<0.05, **=p<0.01, ***=p<0.001; ns= not significant. Capital letters distinguish according to SNK in horizontal way, while small letters discriminate in vertical way.**

| **T1** | | **Photosynthesis (µmol $CO_2$ $m^{-2}$ $s^{-1}$)** | **Stomatal conductance (mol $H_2O$ $m^{-2}$ $s^{-1}$)** | **Transpiration (mmol $H_2O$ $m^{-2}$ $s^{-1}$)** |
|---|---|---|---|---|
| Treatment | **Ctrl** | 1.146 b | 0.015 a | 0.381 a |
| | **NaCl** | 1.673 a | 0.009 b | 0.212 b |
| | *Significance* | ** | ** | ** |
| Genotype | **Farold®40** | 1.113 | 0.012 | 0.297 |
| | **Abbé Fétel** | 1.605 | 0.009 | 0.230 |
| | **MC** | 1.661 | 0.010 | 0.244 |
| | **BA29** | 1.335 | 0.016 | 0.381 |
| | *Significance* | *ns* | *ns* | *ns* |
| ***Interaction gen*trt*** | | * | *ns* | *ns* |

| **Photosynthesis (µmol $CO_2$ $m^{-2}$ $s^{-1}$)** | | | | | | |
|---|---|---|---|---|---|---|
| **T1** | | **Farold®40** | **Abbé Fétel** | **MC** | **BA29** | *Significance* |
| **trt** | **Ctrl** | 1.169 | 1.567 | 0.89 | 1.091 | ns |
| | **NaCl** | 1.062 B | 1.663 B | 2.497 A | 1.547 B | * |
| *Significance* | | ns | ns | *** | ns | |




**Graph 5-7: Photosynthetic activity at T3. For both pears data for NaCl condition are missed because of necrosis. Blue histograms are relative to "control" plants and black capitol letter on them point out SNK discrimination with p<0.05 only for those four means. Red histograms, ns and asterisks are referred only to quinces (**=p<0.01).**

**Table 5-5: Measurements of gas exchange parameters (stomatal conductance, transpiration) at T3 only for quinces. Significance according to SAS proc GLM: *=p<0.05, **=p<0.01, ***=p<0.001; ns= not significant. Small letters discriminate in vertical way.**

| T3 | | Stomatal conductance (mol $H_2O$ $m^{-2}$ $s^{-1}$) | Transpiration (mmol $H_2O$ $m^{-2}$ $s^{-1}$) |
|---|---|---|---|
| Treatment | **Ctrl** | 0.021 a | 0.554 a |
| | **NaCl** | 0.004 b | 0.124 b |
| | *signif* | *** | *** |
| Genotype | **MC** | 0.013 | 0.371 |
| | **BA29** | 0.012 | 0.321 |
| | *signif* | *ns* | *ns* |
| ***Interaction gen*trt*** | | * | ** |

| Stomatal conductance | | | | |
|---|---|---|---|---|
| **T3** | | **MC** | **BA29** | *Significance* |
| **trt** | **crtl** | 0.024 | 0.017 | * |
| | **NaCl** | 0.002 | 0.007 | ns |
| | *Significance* | *** | ** | |

| Transpiration | | | | |
|---|---|---|---|---|
| **T3** | | **MC** | **BA29** | *Significance* |
| **trt** | **crtl** | 0.662 | 0.453 | * |
| | **NaCl** | 0.063 | 0.181 | ns |
| | *Significance* | *** | ** | |

The explanation of this physiological situation of NaCl-exposed plants in trial is difficult, in fact literature has been reported that photosynthetic response to drought and salinity stress is highly complex. Several variables are involved in the comprehension of the possibility to reach acclimation or not such as: stress intensity, duration and rate of progression (Chaves et al., 2009).

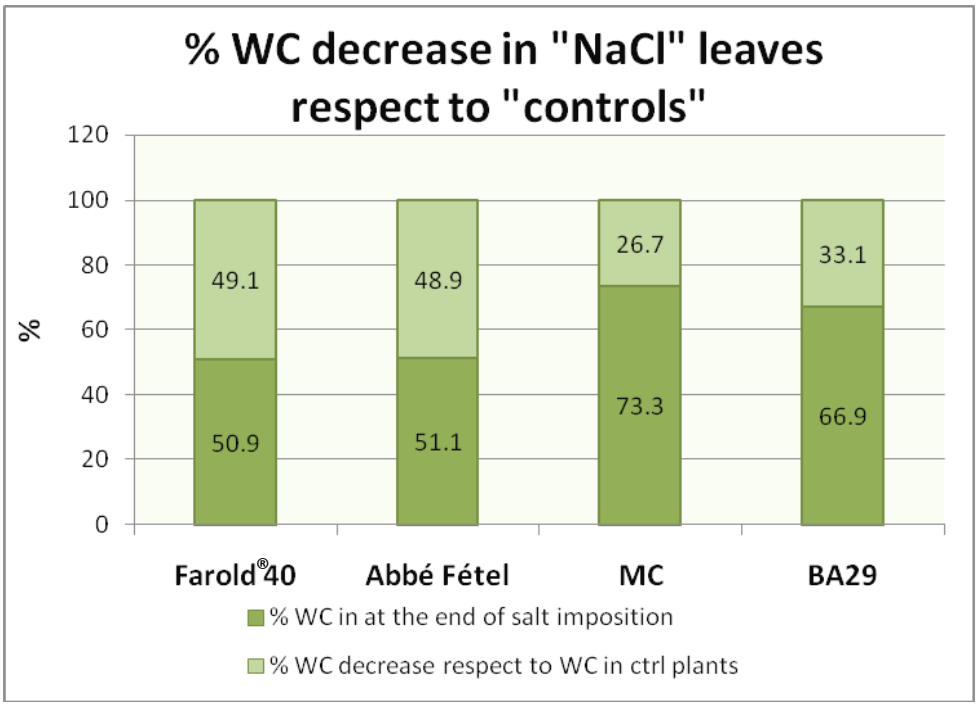
Evidences reported that under a mild stress, a small decline in stomatal conductance can have a protective effect against salt and water stress, by favoring plant water saving and improving water-use efficiency by the plant (Chaves et al., 2009). Transpiration rate is really important in saline condition because it has been clarified that any factor that is able to reduce the amount of transpiration would also diminish salt concentration in the leaves and so that prolong their life (Munns and Termaat, 1986).

It is well known that a slowly imposed stress triggers an osmotic adjustment in plants as a mechanism to face the loss of water for dehydration and to try to decrease water potential and maintain cell turgor. In addition it has been reported that salt-acclimated plants show an increase in primary metabolism that involve amino acids, nitrogen and carbohydrates, this can be part of the osmotic adjustment; moreover it has been pointed out that N metabolism exploits a positive effect on photosynthesis under salinity stress (Garg et al., 2006; Chaves et al., 2009). Taking in consideration all this aspects related to plant response to salt stress, we can suppose that MC genotype under saline condition could have decreased its transpiration to reduce root-to-shoot salts transport and have triggered an osmotic signalling in order to obtain an osmotic adjustment (OA) and for doing this, photosynthesis was maintained higher in order to provide carbon source to contribute to compatible osmolites synthesis (i.e. proline or others).

### 5.0.6 Water content

At the end of the experiment each plant was divided in organs: leaves, shoots, trunk, coarse roots and fine roots. The fresh weight (FW) of the different parts was valued, then samples were dried for several days in oven and then dry weights (DW) were determined. The water content (WC) was calculated as percentage of FW of different organs using this formula: WC (%) = (FW-DW)/FW *100 (Kim et al., 2005); four means for each organs (and for each treatments) were used to perform statistical analysis.

Comparing the effect of salinity on water content in "control" plants respect to "stressed" plants (Ctrl against NaCl), it was possible to observe that in fine roots, coarse roots, trunk and shoots differences among WC values in "control" and "stressed" plants were not significant (Table 5-6). Only for leaves, a highly significant decrease in water content was registered among "control" plants and "NaCl" plants ($p<0.001$) as effect of salinity, but not among genotypes. It could be interesting to point out that WC differences among "control" and "NaCl" plants for each genotype underlined a greater decrease in percentage around 49% for both pears while for quinces was respectively around 27% for MC and 33% for BA29



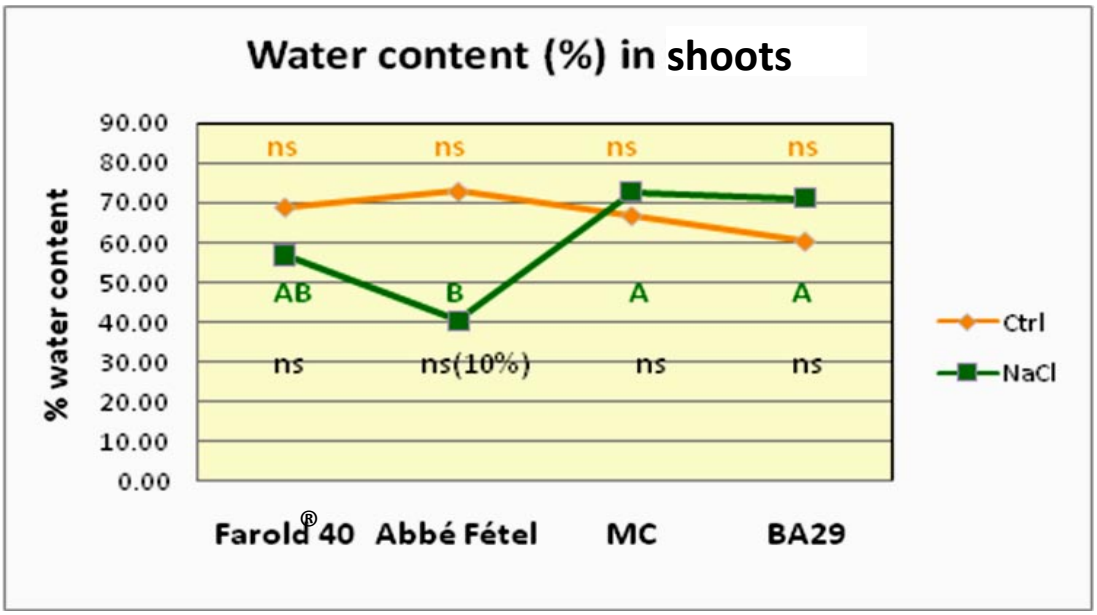

**Graph 5-8: Decrease in leaf water content (WC %) of "NaCl" plants respect to "control" plants (considering 100% the WC of control leaves at the end of the trial and calculating the relative decrease). Pear leaves showed a greater decrease in WC respect to quinces.**

(Graph 5-8).

In fine roots, genotypes differed for water content regardless to the treatment and Farold®40 emerged as its higher WC respect to the other genotypes and MC as the lower WC (Tab. 5-6). Also for coarse roots, Farold®40 appeared to have the higher WC value and BA29 the lower WC value for that organ.

Water content differences referred to trunk were not relevant for the statistical analysis, so no significant observations can be done on this organ. For what concerns shoots, not significant differences were reported among genotypes, but the interaction treatment*genotype resulted meaningful with $p<0.05$ (Tab. 5-6 part in yellow).

From Graph 5-9, it appears clear the meaning of the interaction, comparing only "control" plants together no differences were reported (ns), while evaluating only "NaCl" plants together we noticed that Abbé Fétel registered the lowest WC value respect to the other genotypes (quinces showed highest WC values both classified as A by SNK test). Unlikely comparing each "control" and "NaCl" plants for each genotype no significant differences were registered, but only because the reduced number of plants for tanks. For Abbé Fétel, for instance, the decrease from WC content in "control" plants and the corresponding value in stressed ones is meaningful at 10%, so probably if the number of plants producing the means were higher, the discrepancy would be significant for this genotype (Graph 5-9).




**Graph 5-9: Differences in water content (WC %) among "control" plant shoots (ns in orange) and among "NaCl" shoots (capitol letter in dark green come from SNK test) and among treatments (Ctrl against NaCl, significance in black).**

**Table 5-6: Percentage of water content in every organs of plant for "control" and "NaCl" plants at the end of the trial (T4). Significance according to SAS proc GLM: *=p<0.05, **=p<0.01, ***=p<0.001; ns= not significant. Small letters discriminate according to SNK in vertical way. Abbé Fétel WC value for fine roots was absent because that genotype missed this part, so statistical analysis for this organ was carried out only on three genotypes. In yellow is reported the analysis of interaction treatment*genotype.**

| % WATER CONTENT | | | | | | |
|---|---|---|---|---|---|---|
| **T4** | | **Fine Roots** | **Coarse Roots** | **Trunk** | **Shoots** | **Leaves** |
| Treatment | **Ctrl** | 55.88 | 55.10 | 50.91 | 66.15 | 67.85 |
| | **NaCl** | 59.40 | 55.76 | 49.26 | 61.50 | 41.57 |
| | *Significance* | *ns* | *ns* | *ns* | *ns* | *** |
| Genotype | **Farold®40** | 63.15 a | 62.16 a | 50.33 | 61.96 | 48.41 |
| | **Abbé Fétel** | / | 57.22 ab | 50.97 | 53.45 | 51.01 |
| | **MC** | 54.68 b | 54.51 ab | 51.57 | 69.63 | 59.06 |
| | **BA29** | 56.01 ab | 49.42 b | 47.61 | 65.57 | 54.88 |
| | *Significance* | ** | ** | *ns* | *ns* | *ns* |
| ***Interaction gen*trt*** | | *ns* | *ns* | *ns* | * | *ns* |

| shoots % water content | | | | | |
|---|---|---|---|---|---|
| **T4** | **Farold®40** | **Abbé Fétel** | **MC** | **BA29** | *Significance* |
| **Ctrl** | 68.84 | 72.82 | 66.74 | 60.21 | ns |
| **NaCl** | 56.80 AB | 40.54 B | 72.51 A | 70.92 A | * |
| *Significance* | ns | ns (10%) | ns | ns | |

Salt stress is known to be linked to a dehydration process inside the plant; this event can be related to loss in transpiration or reduction in water uptake or both. Dehydration in plant appears as a decrease in water content that leads to wilting and obviously is also related to a decrease or inhibition of growth rate (de Lacerda et al., 2003). It has been reported that, as a consequence of a decrease in water content, the increase in proline content can be noticed as part of the osmotic adjustment; in fact the increase in solutes concentration allows maintaining or restoring the original water content/uptake (Mahajan and Tuteja, 2005). This mechanism acts in the so called "dehydration avoidance" (Ingram and Bartels, 1996; de Lacerda et al., 2003; Verslues et al., 2006). Both pears showed a similar performance in leaf water content decrease, their WC reduced around 50% means that plants in that saline condition suffered for water deficiency as reported for drought stress; this idea can match with the reduction in shoot length reported for Abbé Fétel stressed plants that was significant only at 10%. On the other hand we can assume that quinces exploited the early decrease in water content to trigger the production of solutes (i.e. proline or other osmolites), as reported in literature. This could have helped them to limit further water loss and in this way the reduced decreases in their leaves respect to pears ones and can be explained, taking in consideration that transpiration was already significantly reduced as reported in previous paragraph. Another aspect of the dehydration avoidance is that a decrease in water content can also trigger the ABA accumulation, which is known, leads to stomatal closure to prevent further loss of water content (Verslues et al., 2006). To better understand which was the main mechanism adopted by pears and which by quinces to face this salt stress in relation to water loss, it would be necessary to deeper investigate on osmotic adjustment in terms of proline or sugar accumulation, either on ABA content or in dehydration proteins such as LEA and in particular dehydrins. LEA proteins in fact are more abundant in salt-tolerant plants and are involved in the desiccation survival (Moons et al., 1995; Ingram and Bartels, 1996). Data reported on shoots suggested that Abbé Fétel was the genotype that worse faced salt-induced dehydration because it seemed that after the significant leaf wilting, the desiccation process proceeded to shoots where the WC decrease

resulted significant only at 10% respect to the other genotypes which did not register water loss in shoots.

### 5.0.7 Leaf necrosis

Since a hydroponic culture of quinces and pears together exposed to NaCl stress has never been done before, we had no idea about the duration of plants in this condition so we gradually increased salt concentration until 90 mM (around 10 dS/m) in order to see clear symptoms of the stress and to understand the behavior of our four genotypes in this stressful situation. The salt stress (70 mM NaCl) was imposed on May 15$^{th}$ and at the sixth day (T1) some symptoms of salt stress were evident on the external leaf lamina of older leaves in Farold®40 and Abbé Fétel (Fig. 5-2, see appendix 1). At the same time, both quinces did not show any necrotic symptoms, but only a beginning of chlorosis, so leaves were becoming a bit yellowish. At T2, corresponding to 13 days of stress, the situation showed advanced necrosis for pears and chlorotic appearances for quinces as illustrated in figure 5-3. After 16 days of NaCl stress (T3) the situation in pears was worse and worse, because necrosis almost covered the entire leaf area leading to the death of these leaves for desiccation, in fact their consistence was similar to leaves put in an oven to dry up. In quinces instead the necrosis proceeded from the leaf tip and at this time has not reached the half of the leaf area yet, but its progress was really clear on older leaves (Fig. 5-3). At T4, that was the last day of experiment, all the treated pear leaves were totally dead, but at each apex new leaves were emerging and appeared completely green. Quinces instead showed advanced necrosis and in particular more BA29 than MC that can be considered the genotype that delayed more than the others the advance of salt-induced necrosis (Fig. 5-3).

**Fig. 5-2: Beginning of necrosis in Farold®40 leaf exposed to salt stress.**

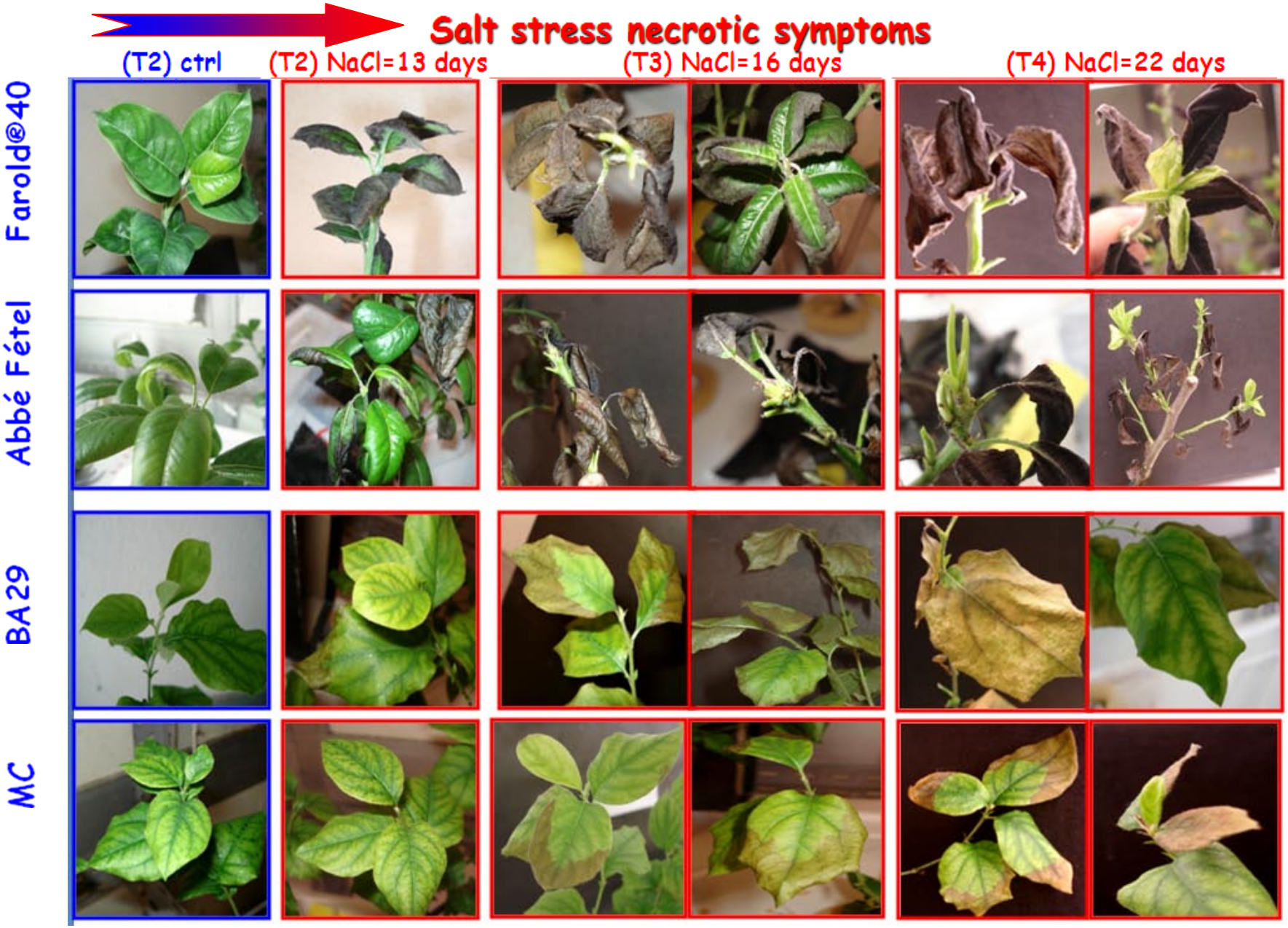



**Fig.5-3: Advance of necrotic symptoms onset in the four genotypes in trial cultured in hydroponics and exposed up to 90 mM NaCl stress for 22 days.**

It is well known that both sodium and chloride can cause toxic effects on plant, for some species one can be more dangerous than the other. From previous experiments carried out in our Department with pear species and salinity irrigation we did not visualized any necrotic symptoms at 5 dS/m (Musacchi et al., 2006a), while in BA29 somaclones selected "*in vitro*", after two years of 7 dS/m water irrigation necrotic symptoms appeared (data not shown) and this was useful to perceive the time of onset of symptoms.

Fruit species subjected to excessive accumulation of toxic ions caused by salt stress can show necrosis of leaf tips that becomes quite soon marginal necrosis. In citrus, chlorosis and bronzing leaves occur without a well-defined necrosis. With the continued accumulation of chloride, the effects become more severe leading to progressively premature fall of leaves, defoliation, desiccation of shoots and, in extreme cases, to plant death (Flagella et al., 1999).

Plants, as other eukaryotic organisms, have developed the possibility to induce death in particular groups of cells in order to deal with abiotic or biotic stresses. This kind of mechanisms is part of the so called programmed cell death (PCD) which includes apoptosis and necrosis, the latter is a consequence of traumatic events (Buchanan et al., 2003). Necrosis is also related to senescence, in fact leaves, before dying, percept a signal of early senescence and consequently that part of plants, still active, changes its metabolism in order to transfer and re-allocate essential elements and important compounds to another plant part that will survive to the stress (Buchanan et al., 2003).

Considering that for a plant the sacrifice of a part of its structure constitutes an adaptive strategy in nature to survive to a stress episode (Zhu, 2002), we can suppose that for pear genotypes the complete desiccation of expanded leaves, already formed before salt imposition, could be a mechanism adopted to survive at this saline stress. This hypothesis can be supported by the evidence that both genotypes recovered because they managed to generate new leaves in the while salt stress persisted at 90 mM. This behavior can posit the possibility of a glycophyte species such as *Pyrus* to find the way to tolerate a salt stress. The most dramatic examples of dehydration tolerance in nature are "desiccation-tolerant" plants that can recover from a fully air-dried state during which these plants are in a

metabolically dormant state that is in many ways similar to seed dormancy (Verslues et al., 2006), but despite the "recovery" of pears to the salt stress we cannot consider them as "desiccation-tolerant" plants.

In conclusion, it has been pointed out that the increased ability to continue production of new leaves means increased osmotic tolerance, whereas tissue tolerance is evident primarily by the increased survival of older leaves (Munns and Tester, 2008), from this definition it can be possible to hypothesize that pears belong to the first salt-tolerance mechanism, while quinces seem to behave more likely to the tissue tolerance system.

### 5.0.8 Roots development

In literature several hydroponic culture experiment are reported and some of them suggested ways to follow the roots growth under some stresses. For example, root elongation rates were quantified by marking the positions of the root apices on the side of the Plexiglas box, containing plants, at various times (Verslues et al., 1998) or distal 10 mm region of each root was marked with indelible ink to track the further inhibition or growth (Watt, 2003). In the present experiment, all these suggestions were considered, but finally it has been decided not to measure roots development because for our experimental system it was not feasible and roots were really heterogeneous and in some case fine roots really limited or absent.

So the root development was followed taking pictures of them.

After some days of culture in nutrient solution, all plants gradually developed new white fine roots as in figure 5-4. At the moment of stress imposition, generally, all root systems in "NaCl" bench appeared in a good condition; obviously they appeared different according to the genotype, in fact quinces presented mostly fine white and brown roots, while pears had essentially coarse roots and in particular Farold®40 presented the most developed radical apparatus with also fine roots, instead Abbé Fétel almost only coarse, as already mentioned. During the stress progress, root systems were checked and rinsed every time the solution was replaced, it has been noticed that in quinces some white roots turned brown, but still new roots were forming (figure 5-5), instead in both pears roots, the development was not so clear, in fact they looked like as in a steady state during the

salt stress period. Between T3 and T4 (see appendix 1), when the foliage in both pears was almost totally covered by necrosis we questioned about the possibility of a sudden root death, so that with some cuttings we realized that cortex and stele were still white and consequently root still alive. The next regeneration of leaves in fact can be taken as a confirmation of the correct functioning of roots also in pears.

**Fig. 5-4: BA29 roots development after 5 weeks of normal hydroponic culture.**

**Fig. 5-5: BA29 roots after three weeks of NaCl stress, white roots were still growing.**

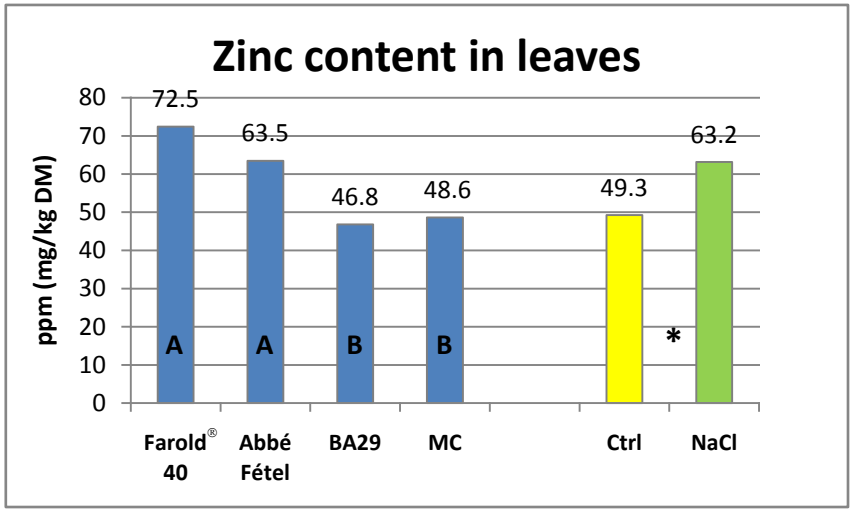
## 5.1 Mineral analyses

### 5.1.1 Micro-elements

Micro-elements, which have been analyzed by an atomic absorption spectrophotometer (SpectrAA200, Varian, Australia) using a calibration curve for each one, were zinc (Zn), copper (Cu), manganese (Mn), iron (Fe). As definition micro-elements or micronutrients should be present in plant tissues at low concentration in comparison with macro-elements, but this classification has been criticized (as already mentioned in chapter 2) because sometimes differences in micro and macro-elements are not so evident. Since it is well known that salt stress provokes an unbalance in ion homoeostasis, effects on membrane selectivity has a great influence on nutrients uptake (Kozlowski, 1997), we will try to figure out changes in nutrients content induced by salinity during this hydroponic culture. Being in low concentration, micronutrients do not play a direct role in osmoregulation or in maintaining electrochemical equilibrium, but being metals act important roles as cofactor for enzymes (Marschner, 1997). In general elements will be reported in tables as ppm that are equivalent to mg/kg of dry matter.

**Table 5-7: Zinc content in all five organs of plants expressed as ppm (mg/kg dry matter). "Control" plants correspond to "-NaCl". Significance according to SAS proc GLM: *=p<0.05, **=p<0.01, ***=p<0.001; NS= not significant. Small letters discriminate according to SNK in vertical way. Slash indicates absence of material.**

| Zn | trunk | | | leaves | | | | | | shoots | | | | fine roots | | | | coarse roots | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ctrl | + NaCl | signif | Ctrl | | + NaCl | | signif | | Ctrl | + NaCl | | signif | Ctrl | | + NaCl | signif | Ctrl | + NaCl | signif |
| **genotype** | ppm | | | ppm | | | | | | ppm | | | | ppm | | | | ppm | | |
| Farold®40 | 11.73 | 15.46 | NS | 68.23 | a | 75.63 | a | NS | | 24.23 | 38.56 | a | * | 60.2 | a | 32.4 | ** | 12.79 | 11.68 | NS |
| Abbé Fétel | 11.14 | 18.43 | NS | 46.38 | b | 74.87 | a | NS | | 26.14 | 43.16 | a | * | 47.4 | ab | / | | 11.78 | 16.32 | NS |
| BA29 | 8.65 | 11.95 | NS | 41.27 | b | 52.31 | b | NS | | 23.06 | 24.96 | b | NS | 32.1 | b | 37.7 | NS | 7.87 | 10.78 | NS |
| MC | 13.9 | 14.40 | NS | 44.49 | b | 52.79 | b | NS | | 29.73 | 37.15 | a | * | 55.0 | a | 39.1 | * | 12.45 | 14.81 | NS |
| signif | NS | NS | | * | | * | | | | NS | * | | | * | | NS | | NS | NS | |
| **treatment** | | | | | | | | | | | | | | | | | | | | |
| Ctrl | 11.36 b | | | 49.27 b | | | | | | 25.86 b | | | | 49.10 a | | | | 11.02 | | |
| + NaCl | 14.83 a | | | 63.17 a | | | | | | 35.48 a | | | | 36.40 b | | | | 13.2 | | |
| signif | * | | | * | | | | | | * | | | | ** | | | | NS | | |
| ***Interaction* gen * trt** | NS | | | NS | | | | | | NS | | | | * | | | | NS | | |




**Graph 5-10: Zinc content in ppm in leaves. Discrimination among genotypes according to SNK with p<0.05 and on the right comparison between control and "NaCl"**

**Zn**

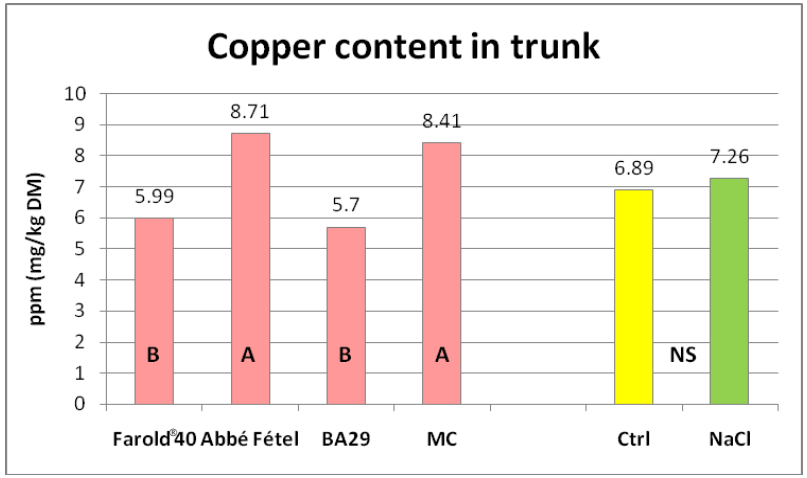
Mineral analyses for this element show in aerial part of "NaCl" plants (leaves, trunk, shoots) significant increases in Zn ($p<0.05$) were reported as consequence of the treatment. While in fine roots, a decrease in Zn content was registered and in coarse roots no meaningful changes were seen comparing "NaCl" and "control" plants (Tab. 5-7). This let us think about a possible re-allocation of Zn directed to leaves respect what happens in "control" plants. In trunk and coarse roots no significant changes were reported. In leaves, instead, Zn content was higher in both pears and lower in both quinces, regardless to the treatment (Graph 5-10). Zinc participates in chlorophyll formation (Salisbury and Ross, 1992), so the lower content of it regardless of the treatment, in quinces can be explained with quinces' tendency to chlorosis ad reported in paragraph 5.0.2. In addition it seems that many enzymes contain tightly bound zinc that is essential for their function (Marschner, 1997; Salisbury and Ross, 1992), so maybe some of these enzyme were required to face the salt stress for example acting in osmotic adjustment in leaves, where "NaCl" treatment resulted in an increased Zn content (Graph 5-10). Other possibility to explain this increase for this element in salt condition, regardless to the genotype, can be its key role in CuZnSOD, which is an important enzyme taking part in detoxification signalling in plant against ROS. CuZnSOD in fact is the most spread isoform of SODs in all cell compartments and acts protecting nucleic acids against oxidation and contributing in lignifications process (Alscher et al., 2002).

**Cu**

The analyses of this element revealed that the salinity contributed to a significant increase in copper content in shoots, but in all the other organs the Cu amount did not seem affected by NaCl (Tab. 5-8). Probably this can be due to the fact that copper is rarely deficient in plants, because they need so little of it (Salisbury and Ross, 1992). Looking deeper into differences among "control" plants and "NaCl" plants, it appeared that only Farold®40 registered a significant increment of Cu content in "salted" shoots. It has been reported that copper plays an important role for two enzymes acting in lignin biosynthesis: polyphenol oxidase and diamine oxidase and, in Cu-deficient tissues, it is reported an accumulation of polyphenols.

**Table 5-8: Copper content in all five organs of plants expressed as ppm (mg/kg dry matter). "Control" plants correspond to "-NaCl". Significance according to SAS proc GLM: *=p<0.05, **=p<0.01, ***=p<0.001; NS= not significant. Small letters discriminate according to SNK in vertical way. Slash indicates absence of material.**

| Cu | trunk | | | | | leaves | | | shoots | | | fine roots | | | coarse roots | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ctrl | | + NaCl | | signif | Ctrl | + NaCl | signif | Ctrl | + NaCl | signif | Ctrl | + NaCl | signif | Ctrl | + NaCl | | signif |
| **genotype** | | | | | | | | | | | | | | | | | | |
| Farold®40 | 4.85 | c | 6.85 | bc | NS | 28.76 | 36.46 | NS | 11.81 | 21.41 | * | 50.9 | 36.1 | NS | 6.74 | 7.71 | ab | NS |
| Abbé Fétel | 7.68 | ab | 9.4 | a | NS | 20.21 | 21.24 | NS | 15.65 | 17.44 | NS | 26.0 | / | | 6.11 | 6.89 | ab | NS |
| BA29 | 6.06 | bc | 5.34 | c | NS | 24.84 | 26.68 | NS | 11.17 | 12.32 | NS | 21.6 | 25.5 | NS | 2.35 | 2.75 | b | NS |
| MC | 8.85 | a | 7.97 | ab | NS | 20.77 | 18.2 | NS | 14.28 | 18.44 | NS | 51.8 | 26.2 | * | 4.30 | 9.23 | a | * |
| signif | * | | * | | | NS | NS | | NS | NS | | NS | NS | | NS | * | | |
| **treatment** | | | | | | | | | | | | | | | | | | |
| Ctrl | 6.89 | | | | | 23.78 | | | 12.96 b | | | 41.43 | | | 4.54 | | | |
| + NaCl | 7.26 | | | | | 25.94 | | | 17.40 a | | | 29.26 | | | 6.63 | | | |
| signif | NS | | | | | NS | | | * | | | NS | | | NS | | | |
| *Interaction* | NS | | | | | NS | | | NS | | | NS | | | NS | | | |




**Graph 5-11: Copper content in ppm in trunks. Discrimination among genotypes according to SNK with p<0.05 and on the right comparison between "control" and "NaCl".**

An external supply of copper demonstrated to rapidly trigger the lignifications process (Marschner, 1997).

Since it is known that a mechanism taking part of the dehydration avoidance in plant is the cell wall hardening (Verslues et al., 2006), we could suppose that this doubling in copper content in Farold®40 shoots, as a consequence of salinity, can be explained as a way to face the possible dehydration, which had already hit leaves, in order to thicken cell wall and survive to the desiccation. This copper effect can also be responsible for the not significant decrease in water content (WC) reported for Farold®40 shoots in Graph 5-9, while on the contrary, for Abbé Fétel where the reduction in WC was significant only 10%, no increase in copper was found.

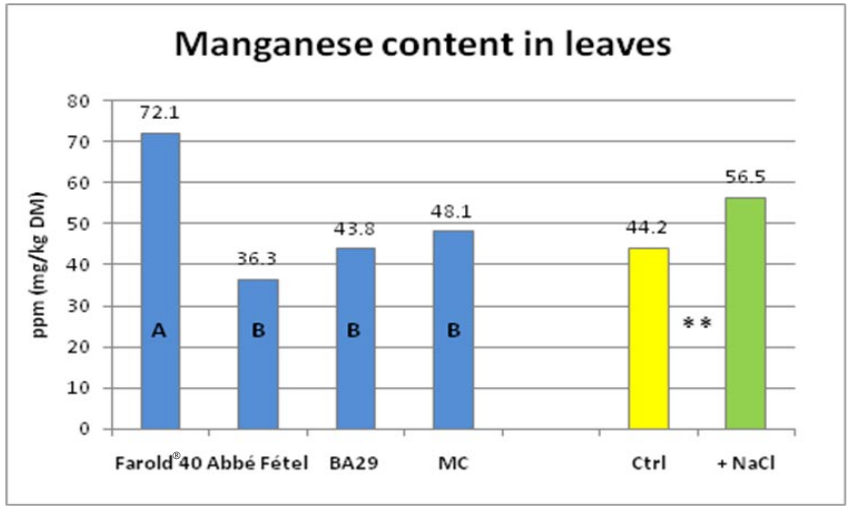
For what concerns trunks, a discrimination based on copper content was done regardless of the treatment (not significant) and MC and Abbé Fétel showed higher levels than the other two genotypes (Graph 5-11). At fine root level only MC emerged for its Cu content decrease in "NaCl" plants respect to "control" maybe related to a lower level of lignifications. In MC coarse roots, on the contrary, it has been reported an increase in Cu amount due to salinity and possibly related to enhance of lignification (Tab. 5-8). It is important to remember that Cu acts also in several enzymes/proteins activities such as cythochrome oxidase (mithocondria), ascorbate oxidase, CuZnSOD and plastocyanin (chloroplast) (Salisbury and Ross, 1992; Marschner, 1997).

**Mn**

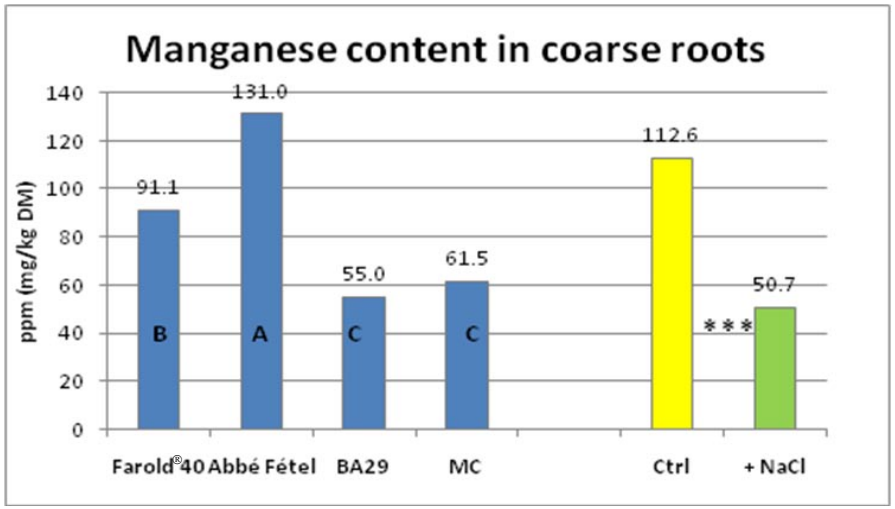
In salt stressed leaves, we found a significant increase in manganese content (Graph 5-12) and looking inside different genotypes, it appeared that the significant increase in Mn amount in "NaCl" leaves regards to Farold®40. The clearest function of Mn in plant cell is the participation in PSII to the water splitting system during photosynthesis (Taiz and Zeiger, 1996; Marschner, 1997). Since in the harvesting of leaves at the end of the experiment for mineral analyses, also the new generates ones were picked up and grouped with the older, probably can be the presence of young leaves to contribute to this meaningful increase of Mn. This perhaps because being Mn so strictly related to photosynthesis, the production of new leaves can enhance this accumulation.

**Table 5-9: Manganese content in all five organs of plants expressed as ppm (mg/kg dry matter). "Control" plants correspond to "-NaCl". Significance according to SAS proc GLM: *=p<0.05, **=p<0.01, ***=p<0.001; NS= not significant. Small letters discriminate according to SNK in vertical way.**

| Mn | trunk | | | leaves | | | | | shoots | | | | fine roots | | | coarse roots | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ctrl | + NaCl | signif | Ctrl | | + NaCl | | signif | Ctrl | | + NaCl | signif | Ctrl | + NaCl | signif | Ctrl | | + NaCl | | signif |
| **genotype** | | | | | | | | | | | | | | | | | | | | |
| Farold®40 | 20.56 | 10.51 | NS | 56.45 | a | 83.84 | a | * | 11.42 | b | 19.17 | NS | 2282.1 | 1340.9 | ** | 126.28 | b | 64.67 | ab | * |
| Abbé Fétel | 18.33 | 24.88 | NS | 29.42 | b | 40.94 | b | NS | 12.18 | b | 13.2 | NS | 2106.7 | / | | 182.19 | a | 96.92 | a | * |
| BA29 | 9.93 | 11.14 | NS | 42.85 | ab | 44.82 | b | NS | 16.16 | ab | 15.31 | NS | 2234.6 | 898.5 | ** | 81.29 | c | 28.78 | b | ** |
| MC | 13.61 | 10.13 | NS | 43.71 | ab | 52.54 | b | NS | 21.72 | a | 17.77 | NS | 2166.9 | 771.9 | ** | 98.85 | bc | 24.1 | b | *** |
| signif | NS | NS | | * | | * | | | * | | NS | | NS | NS | | * | | * | | |
| **treatment** | | | | | | | | | | | | | | | | | | | | |
| Ctrl | 14.81 | | | 44.19 b | | | | | 16.16 | | | | 2227.9 a | | | 112.6 a | | | | |
| + NaCl | 13.45 | | | 56.51 a | | | | | 16.57 | | | | 1003.8 b | | | 50.7 b | | | | |
| signif | NS | | | ** | | | | | NS | | | | *** | | | *** | | | | |
| *Interaction* gen * trt | NS | | | NS | | | | | NS | | | | NS | | | NS | | | | |




**Graph 5-12: Mn content in ppm in leaves. Discrimination among genotypes according to SNK with p<0.05 and on the right the comparison "Control" against "NaCl".**




**Graph 5-13: Mn content in ppm in coarse roots. Discrimination among genotypes according to SNK with p<0.05 and on the right the comparison "Control" against "NaCl".**

In salt-stressed barley plants it has been noticed that manganese concentrations in shoots were highly correlated with RGR (relative growth rate) (Cramer et al., 1990).
At leaf level it is important to know that manganese has also a role as cofactor of MnSOD that is less spread than CuZnSOD, but is active in mitochondria and peroxisomes in order to detoxify this compartment from ROS inevitably produced both with respiration and as a consequence of salt stress. To support this has been described that transcript levels of mitochondria MnSOD were strongly induced in leaves of NaCl-tolerant varieties of *Pisum sativum* L. exposed to a long-term NaCl treatment (70 mM) (Hermandez et al., 2000).
Several studies have found that manganese exerts antioxidant effects in plant tissues (Aktas et al., 2005). Kawano et al. (2002) showed that $Mn^{2+}$ and $Zn^{2+}$ protect against ROS production induced in tobacco cell suspension by incubation with NaCl.
Moreover, this element participates to several other metabolic processes such as tricarboxilic acid cycle, shikimic acid pathway and activates several peroxidases (Marschner, 1997). So this Mn efficiency lets think that the so significant decrease ($p<0.001$) reported in fine and coarse roots (Tab. 5-9), as a consequence of salinity, implied a reduction of activity at root levels (Graph 5-13). A manganese deficiency in plant implies a severe reduction in carbohydrates content particularly evident in the roots end probably responsible for the depression in root growth (Marschner, 1997). A significant decline in Mn was also observed in roots in salt-stressed cucumber plants and in that experiment Mn rose again in roots when putrescine was applied (Shi et al., 2008). Therefore this decisive decline in “NaCl” roots can be due to a demand for this element from leaves in order to act in the photosynthetic complex and as part of a detoxification and repair system. Inevitably, this kind of hypothetical “Mn re-allocation” led to a deficiency of this element at root levels, and this possibility can be related to an inhibition or reduction of root growth and formation of new lateral roots. At the end of the experiment when plants were harvested, root appeared alive but steady in growing. The statistical analysis of Mn content in coarse roots in “NaCl” plants showed that both pears reported a comparable value for this element (for SNK: “a” and “ab”) distinguished from that of quinces (both classified by SNK as b).

**Fe**

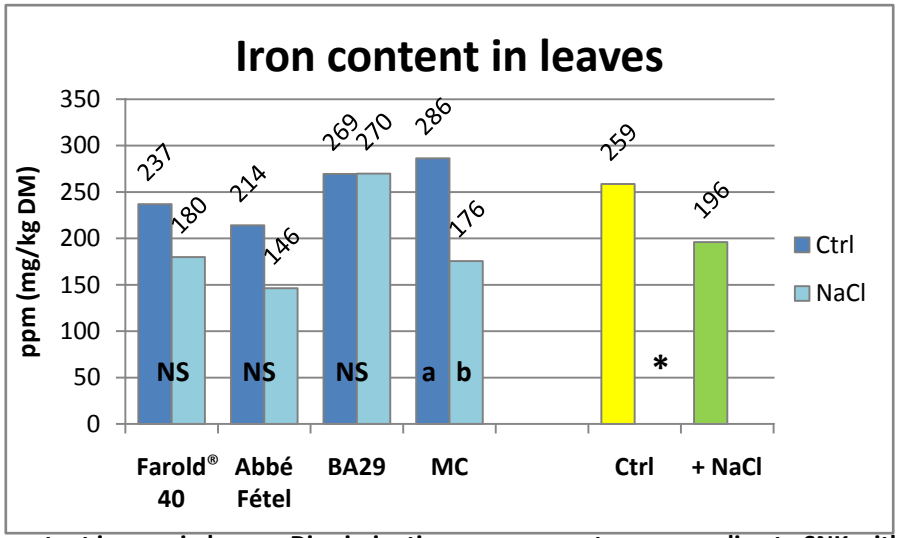
The iron contents did not undergo any alterations at root levels as consequence of salinity, while in salt-stressed shoots only MC exhibited a significant increase in Fe content (Tab. 5-10). In trunks, the only significant difference reported for Fe content concerned only genotypes of "control" plants, showing the higher amounts in MC and Abbé Fétel and lower in Farold®40 and BA29.

Comparing "NaCl" leaves and "control" ones, it appear that the former decreased their Fe content respect to the latter (with $p<0.05$) and this seems mainly due to the decrease reported in MC leaves (Graph 5-14), because the other three genotypes showed not significant changes. This behavior of salt-stressed leaves can be associated with the significant chlorophyll content reduction encountered just in MC at the end of the experiment (par. 5.0.2). This decrease was already reported in cucumber leaves under salt stress (Shi et al., 2008). In fact in plant, iron plays a really important role as a component of enzymes active in electron transfer (redox reactions). When iron deficiency occurs an internerval chlorosis appears on leaves, this because $Fe^{2+}$ is required by some enzymes to catalyze certain reaction of chlorophyll synthesis (Taiz and Zeiger, 1996). In addition iron accumulated in the older leaves results relatively immobile in the phloem because it tends to precipitate in leaf cells as oxides or organic and inorganic compounds. In leaves the most steady stable form of iron is in chloroplasts where is located the iron-protein complex known as phytoferritin (Salisbury and Ross, 1992). Moreover Fe represents the cofactor for the enzyme FeSOD, mainly located in chloroplasts and important as ROS scavenger in coordination with CAT (Marschner, 1997). Probably in the case of MC, a potential increase in Fe for this purpose was not necessary also because this genotype, as mentioned before, was the last to be seriously affected by salt; so maybe the detoxification process could not be the first tolerance mechanism adopted by it.

During salt stress, increased oxidative stress could result in the formation of hydroxyl radicals from a reaction between ferrous iron and $H_2O_2$ (Fenton reaction).

**Table 5-10: Iron content in all five organs of plants expressed as ppm (mg/kg dry matter). "Control" plants correspond to "-NaCl". Significance according to SAS proc GLM: *=p<0.05, **=p<0.01, ***=p<0.001; NS= not significant. Small letters discriminate according to SNK in vertical way.**

| Fe | trunk | | | | | leaves | | | | shoots | | | | fine roots | | | | coarse roots | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ctrl | | + NaCl | | signif | Ctrl | | + NaCl | signif | Ctrl | | + NaCl | | signif | Ctrl | | + NaCl | signif | Ctrl | | + NaCl | signif |
| **genotype** | | | | | | | | | | | | | | | | | | | | | | |
| Farold®40 | 82.08 | ab | 78.34 | | NS | 236.87 | | 179.81 | NS | 119.96 | | 131.04 | b | NS | 2086.6 | | 1885 | NS | 186.39 | | 162.77 | NS |
| Abbé Fétel | 104.24 | a | 93.56 | | NS | 214.08 | | 146.28 | NS | 96.71 | | 80.24 | b | NS | 1730.8 | | / | | 189.36 | | 223.57 | NS |
| BA29 | 58.59 | b | 72.33 | | NS | 269.49 | | 269.86 | NS | 140.1 | | 132.26 | b | NS | 1982.8 | | 2030.8 | NS | 112.05 | | 160.72 | NS |
| MC | 115.03 | a | 78.8 | | NS | 286.41 | | 175.6 | * | 159.54 | | 219.7 | a | * | 2070.90 | | 2181.5 | NS | 173.39 | | 238.44 | NS |
| signif | * | | NS | | | NS | | NS | | NS | | * | | | NS | | NS | | NS | | NS | |
| **treatment** | | | | | | | | | | | | | | | | | | | | | | |
| Ctrl | 88.4 | | | | | 258.64 a | | | | 134.76 | | | | | 2046.8 | | | | 160 | | | |
| + NaCl | 79.9 | | | | | 196.00 b | | | | 144.85 | | | | | 2032.4 | | | | 194.6 | | | |
| signif | NS | | | | | * | | | | NS | | | | | NS | | | | NS | | | |
| *Interaction* genotype * trt | NS | | | | | NS | | | | NS | | | | | NS | | | | NS | | | |




**Graph 5-14: Fe content in ppm in leaves. Discrimination among genotypes according to SNK with p<0.05 on the right difference between Ctrl and NaCl.**

The decrease of iron in our salt-stressed MC leaves can mean a reduction in hydroxyl radicals, the most dangerous reactive oxygen species produced in living cells, during salt stress (Parker et al., 2006).

Another attractive aspect is the involvement of iron in the lipoxygenase (LOX) enzyme that catalyzes the peroxidation of polyunsatured fatty acids. This lipid peroxidation is typical of senescence processes in cells and tissues and LOX activity resulted positively correlate with chlorophyll content in leaves of iron-deficient plants (Marschner, 1997).

### 5.1.2 Macro-elements

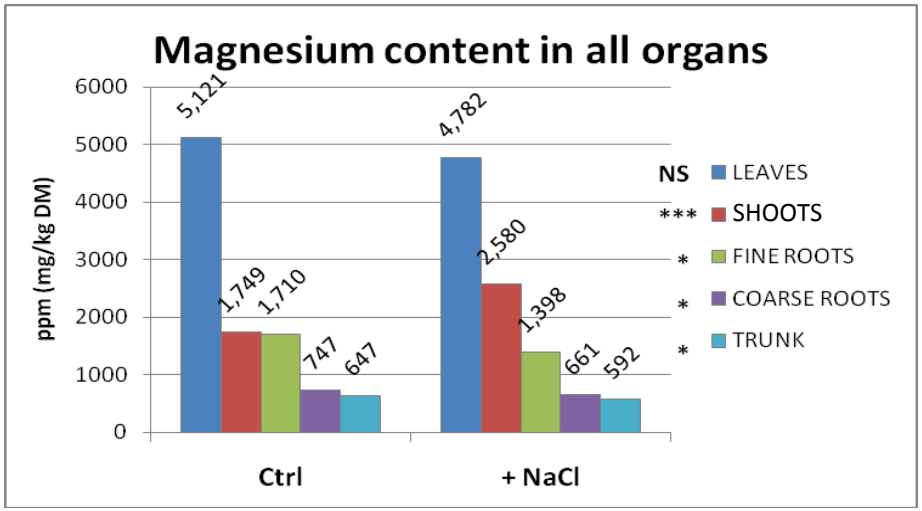
Macro-elements have been analyzed by an atomic absorption spectrophotometer using a calibration curve for each one and diluting samples according to estimated amount present in different organs. In general also these elements will be reported in tables as ppms that are equivalent to mg/kg dry matter, obtained via calculations with dilution factor. Macro-elements analyses for this experiment have been: Magnesium (Mg), Potassium (K) and Calcium (Ca). Nitrogen (N), sulphur (S) and phosphorus (P) were not determined in this trial. Breafly macronutrients are considered essential elements for plants for several reasons, such as: their deficiencies cannot allow plant to complete its life cycle, they take part of any crucial molecules or components and they should act directly inside the plant (Salisbury and Ross, 1992).

**Mg**

Mineral analyses for this element showed in radical part of “NaCl” plants significant decreases in magnesium ($p<0.05$) compared with “control” plants; these were reported as a consequence of the treatment. Among genotypes Farold®40 was the main responsible for the statistical significance of these data, because it registered in both types of roots an important decrease in Mg ppms (Tab. 5-11). Magnesium in plant is an essential element and it acts in several metabolic processes such as photosynthesis, respiration, DNA and RNA synthesis (Salisbury and Ross, 1992; Taiz and Zeiger, 1996).

**Table 5-11: Iron content in all five organs of plants expressed as ppm (mg/kg dry matter). "Control" plants correspond to "-NaCl". Significance according to SAS proc GLM: *=p<0.05, **=p<0.01, ***=p<0.001; NS= not significant. Small letters discriminate according to SNK in vertical way.**

| Mg | trunk | | | | | leaves | | | | | shoots | | | | | fine roots | | | | | coarse roots | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ctrl | | + NaCl | | signif | Ctrl | | + NaCl | | signif | Ctrl | | + NaCl | | signif | Ctrl | | + NaCl | | signif | Ctrl | | + NaCl | | signif |
| **genotype** | | | | | | | | | | | | | | | | | | | | | | | | | |
| Farold®40 | 754.4 | a | 755.4 | a | NS | 4434.7 | b | 3924.8 | b | NS | 1434.5 | | 3081.2 | a | ** | 2424.5 | a | 1350.4 | | ** | 1235.0 | a | 806.2 | a | ** |
| Abbé Fétel | 581.2 | b | 721.4 | a | * | 4570.8 | b | 4384.7 | ab | NS | 2254.7 | | 3096.1 | a | NS | 1277.3 | bc | / | | | 630.3 | b | 828.4 | a | NS |
| BA29 | 597.2 | b | 442.6 | b | *** | 6030.3 | a | 5564.4 | a | NS | 1656.5 | | 2198.3 | b | NS | 1102.5 | c | 1351.2 | | NS | 586.0 | b | 504.95 | b | NS |
| MC | 649.6 | b | 481.6 | b | *** | 4999.8 | ab | 5154.4 | ab | NS | 1824.1 | | 2073.8 | b | NS | 1603.6 | b | 1491.4 | | NS | 598.7 | b | 546.8 | b | NS |
| signif | * | | * | | | * | | * | | | NS | | * | | | * | | NS | | | * | | * | | |
| **treatment** | | | | | | | | | | | | | | | | | | | | | | | | | |
| Ctrl | 647.1 a | | | | | 5120.5 | | | | | 1748.8 b | | | | | 1710.2 a | | | | | 746.5 a | | | | |
| + NaCl | 592.2 b | | | | | 4781.9 | | | | | 2580.1 a | | | | | 1397.7 b | | | | | 661.1 b | | | | |
| signif | * | | | | | NS | | | | | *** | | | | | * | | | | | * | | | | |
| *Interaction* gen * trt | *** | | | | | NS | | | | | NS | | | | | *** | | | | | *** | | | | |




**Graph: 5-15: Mg content in different organs of plants in trial regardless of the genotype, in the comparison between "Ctrl" and "NaCl" plants. Significance reported near legend.**

Also in salt-stressed trunks, a decrease in Mg content was registered, but the behavior of the four genotypes differed, in fact the interaction genotype* treatment results significant ($p<0.001$). Quinces in fact reported a significant and similar Mg decrease, while Abbé Fétel an increase and Farold®40 no meaningful variations.

Instead in shoots the salt treatment seemed to significantly increase the amount of Mg; also for this organ the genotype more responsible for this increment is Farold®40. It has been reported that magnesium possesses a good mobility from older leaves when roots are insufficiently supplied (Marschner, 1997).

So it can be possible that we found a higher amount of Mg in Farold®40 salt-stressed shoots because magnesium was moving from leaves to roots. A low concentration of magnesium in roots can be explained with a competition in the uptake with other cations like $K^+$, $NH_4^+$, $Ca^{2+}$ and $Mn^{2+}$ and this assertion can concern also $Na^+$ because it is worth noting that sodium competes with potassium for its uptake and therefore in this salinity condition its concentration was particularly high respect to other elements in the nutrient solution. The lower concentration of magnesium on salt-stressed roots (Farold®40) can also be due to a limitation in carbohydrates supply that leads to a depression of growth as mentioned for manganese (Marschner, 1997). The magnesium accumulation was inhibited in roots after salt stress also in cucumber plants in hydroponic system exposed to 100 mM NaCl for seven days (Shi et al., 2008).

In Graph 5-15 are illustrated all the Mg contents allocated in all the five organs considered in the mineral analyses with the relative statistical results (on the left); it appears clear that regardless of the treatment, leaves are the main Mg storage organ and roots the less important in terms of amount of magnesium (ppm). Magnesium content in leaves was not affected by treatment as it appeared in table 5-11; in this main organ, in general, Mg is involved in chlorophyll structure and is combined with ATP and therefore acts in several reaction that need energy.

**K**

Potassium content analyses showed that in this experiment the two organs mainly involved in K accumulation were shoots and leaves, both in "control" and "NaCl" plants. In fact looking at the comparison between treatments no significant differences emerged (Tab. 5-12). Almost all potassium found in plant tissues is taken up by roots, the majority of K in a plant is found in stems and leaves. It is not so surprising that $K^+$ transport systems in roots are deeply studied, but in contrast, despite the majority of potassium is found in the upper part of plant, the study of $K^+$ transport and regulation in leaves is much more limited (Shabala, 2003).

Salinity influenced neither K amount in salt-stressed trunks. Root exhibited a significant decrease in K content in both kinds of roots, for fine roots $p>0.05$, while for coarse roots $p<0.001$ as appears in Graph 5-16 (with different colours). The decrease of $K^+$ content in roots under salinity was found also in the salt-sensitive *Medicago intertexta* (Zahran et al., 2007).

In fine roots, Farold®40 was distinguished from the other genotypes for its meaningful decrement in potassium content, while both quinces did not show any changes towards this element, but among the genotypes under stress condition they presented the highest K values also in comparison with Farold®40 that apparently had a stronger root system. The same observation can be translated also in coarse roots, where again quinces emerged for their elevated K content (not significantly changed from "control" plants) despite the more scarce coarse roots than those of pears (Graph 5-16). Both pears instead registered a decline in potassium content as a consequence of salinity, but differently: for Farold®40 the decrease interested fine roots (that were absent for the other pear genotype), while for Abbé Fétel was for coarse roots. Salt stress significantly reduced the K content in roots also in cucumber plant subjected to similar experiment (Shi et al., 2008).

The lack of decrease in K content in quinces roots could be perhaps explained with a compartmentalization of K into vacuoles to compensate the prominent uptake of sodium. Since it is worth noting that $Na^+$ and $K^+$ are in competition for influx into the plants for their physicochemical similarities and for using common transporters (1.2.2.3 a), but contrary to $Na^+$, $K^+$ has some more important functions in plant, such as: 1) it is required for osmotic balance, 2) it plays a role in opening/closing stomata

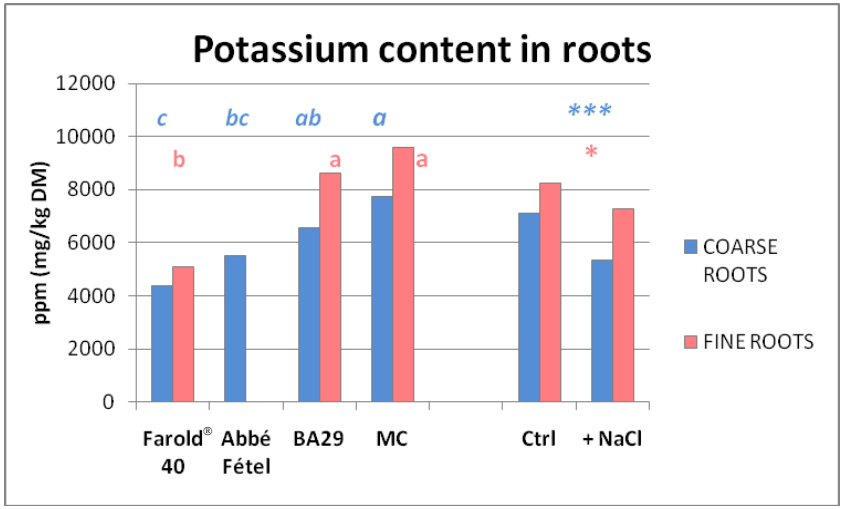
and 3) it is also an essential co-factor for many enzymes like piruvate kinase (Maathius and Amtmann, 1999; Mahajan and Tuteja, 2005).

When charged $Na^+$ crosses the plasma membrane a significant depolarization is reported, which consequently implies a impossibility from $K^+$ to passively enter and, at the same time, a dramatical increase of $K^+$ leakage through depolarization-activated outward-rectifying (KOR) $K^+$ channels (Shabala and Cuin, 2008). This explanation can represent what happened for pears; that is, an important sodium uptake caused a potassium leakage that decreased significantly K content in pears roots. All this hypotheses should be reviewed in the light of sodium distribution in salt-stressed plant, thinking also to $K^+/Na^+$ ratio, whose high value maintained in cytosol can be a critical aspect of plant salt tolerance despite not much direct experimental evidences have been presented supporting this hypothesis (Shabala and Cuin, 2008).

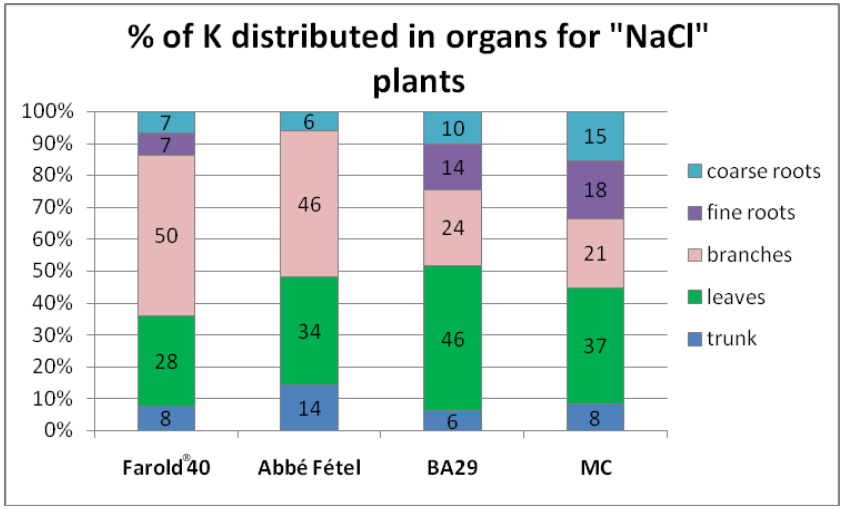
It seemed important to underline the doubling in K content for Farold®40 salt-stressed shoots respect to the corresponding "control" shoots. In the case of shoots instead, both pears showed a higher K amount than both quinces (Tab. 5-12). The increase of K in shoots can be involved in the osmotic adjustment, being $K^+$ a good candidate to be accumulated in the cytosol; in fact, it is the potassium cytosolic homeostasis rather than vacuolar content that is essential for plant metabolic processes (Shabala and Cuin, 2008). In addition it could be that this increase in salt-stressed-Farold®40 shoots means increased rates of $K^+$ circulation through the vascular tissue, pointing towards a long distance redistribution of $K^+$ between roots and shoots (Shabala and Cuin, 2008). With regard to leaves, comparing "NaCl" leaves together, it emerged that BA29 had the highest content of potassium respect to the other three genotypes and that MC reported a significant ($p<0.01$) decrease due to salinity. In Graph 5-17 the K distribution in percentage among different NaCl-stressed-organs in all four genotypes was represented and this gave an idea of how quinces stored the main amount of K in leaves and secondly in shoots, while pears firstly in shoots and then in leaves. Moreover in quinces around 25-30% of total K found in plant was located in roots while for pears only 6-14%.

**Table 5-12: Potassium content in all five organs of plants expressed as ppm (mg/kg dry matter). "Control" plants correspond to "-NaCl". Significance according to SAS proc GLM: *=p<0.05, **=p<0.01, ***=p<0.001; NS= not significant. Small letters discriminate according to SNK in vertical way.**

| K | trunk | | | | | leaves | | | | | shoots | | | | fine roots | | | | | coarse roots | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ctrl | | + NaCl | | signif | Ctrl | | + NaCl | | signif | Ctrl | + NaCl | | signif | Ctrl | | + NaCl | | signif | Ctrl | | + NaCl | | signif |
| **genotype** | | | | | | | | | | | | | | | | | | | | | | | | |
| Farold®40 | 3951 | b | 4469 | b | NS | 15497 | b | 16268 | b | NS | 14241 | 28763 | a | * | 6118 | b | 4086 | b | * | 5172 | b | 3806 | c | NS |
| Abbé Fétel | 6128 | a | 7459 | a | NS | 21486 | a | 17897 | b | NS | 20241 | 23973 | a | NS | 9324 | a | / | | | 9036 | a | 3140 | c | * |
| BA29 | 4373 | b | 3590 | b | NS | 25717 | a | 26587 | a | NS | 15161 | 13929 | b | NS | 8980 | a | 8296 | a | NS | 7155 | a | 5983 | b | NS |
| MC | 4645 | b | 4288 | b | NS | 24569 | a | 18868 | b | ** | 14939 | 11004 | b | NS | 9690 | a | 9519 | a | NS | 7629 | a | 7871 | a | NS |
| signif | * | | * | | | * | | * | | | NS | * | | | * | | * | | | * | | * | | |
| **treatment** | | | | | | | | | | | | | | | | | | | | | | | | |
| Ctrl | 4629 | | | | | 21431 | | | | | 15662 | | | | 8263 a | | | | | 7133 a | | | | |
| + NaCl | 4784 | | | | | 20039 | | | | | 19113 | | | | 7300 b | | | | | 5338 b | | | | |
| signif | NS | | | | | NS | | | | | NS | | | | * | | | | | *** | | | | |
| *Interaction* gen * trt | NS | | | | | NS | | | | | NS (5.2%) | | | | NS | | | | | ** | | | | |




**Graph 5-16: K content in roots in four genotypes regardless to treatments. Quinces showed higher content. On the right, significance of the treatment (Ctrl against NaCl); pink letters and asterisk are referred to fine roots, while blue to coarse roots.**




**Graph 5-17: K distribution in percentage among different NaCl-stressed-organs in all four genotypes. At the total K amount was assigned 100% and % of K divided in each organ was calculated.**

**Ca**

Ca, K, Mg can influence each other, but also for their characteristics result in some way similar; in this experiment we noticed that at root level in salt-stressed plants, Ca, K, Mg decreased, instead only Ca and Mg contents diminished in “NaCl” trunks. Starting from the bottom of the plants, it has been reported for this trial, that calcium content regardless of the genotype was higher in fine roots and in leaves (Tab. 5-13 and Graph 5-18). For both kinds of roots (in coarse root only MC), salinity caused a decrease in Ca content, more evident in fine roots for MC as well as Farold®40 ($p<0.05$), but also in MC coarse roots.

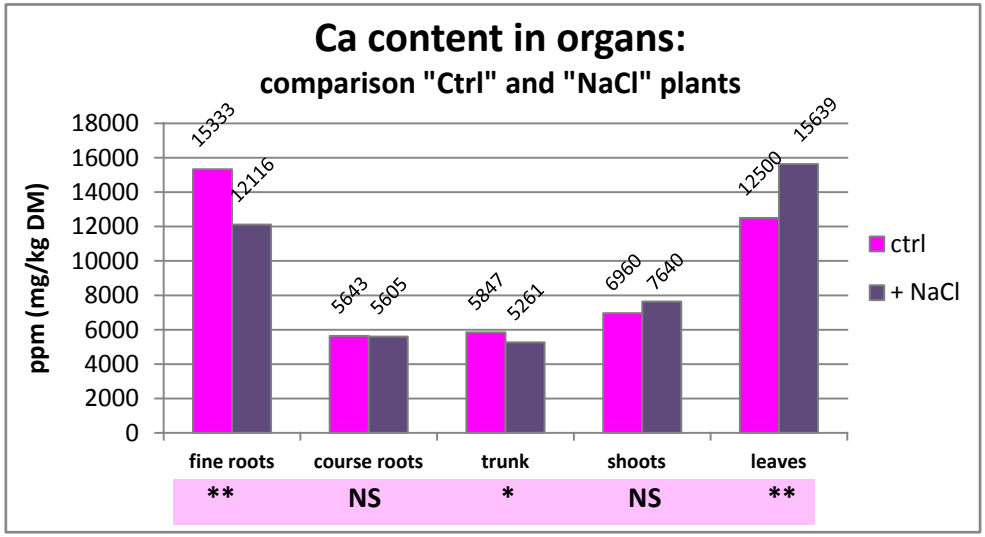
The statistical analysis of the effect of treatment pointed out that also in trunk it was observed a decline in calcium content under salt stress, but looking better at each genotype, it appeared that the main significance of this is attributed to MC that registered a decrease with $p<0.01$ between “control” plants and the corresponding “NaCl” plants. In shoots, no meaningful changes were observed as a consequence of salinity in calcium amount, only the difference in the content of this element among genotypes, both pears in fact presented more calcium in this organ than quinces. After all, the only organ that reported an increase of this nutrient was the foliage. Salt-stressed leaves in fact showed a rise in calcium contend due significantly to the contribution of quinces; which increased their content of Ca respect to pears leaves, which did not report meaningful changes respect to their corresponding “control” plants (Tab. 5-13).

Calcium is one of the most complicate elements to be interpreted in its distribution at the whole plant level, because, as reported in chapter 1 par 1.4, it covers several roles. Since this nutrient is taken up by roots and transported to shoot via xylem (White and Broadly, 2003), the Ca distribution into salt-stressed plants favouring leaves as final organ of accumulation, let us think immediately about a translocation of calcium from the bottom to the top. This can be supported by the fact that calcium acts with structural role in plasma membrane, as a second messenger into the cytoplasm, but also into organelles as storage or as in the case or vacuoles to regulate levels of toxic anions (Salisbury and Ross, 1992; Mills and Benton Jones 1996). Indeed, calcium is known as a regulatory molecule, the most-ubiquitous cellular second messengers involved in almost all phases of plant life such as growth and

development (Shao et al., 2008b). It can also be possible that the increase of calcium in quinces stressed leaves is a way to face the stress, triggering some other $Ca^{2+}$ dependent signallings, such as SOS pathway and be bound to SOS3 that activate SOS2 and then SOS1, but also phospholipid signaling. Another quality of calcium is that it ameliorates salt toxicity symptoms in several plants (Shabala et al., 2006). Application of Ca to senescing leaves reduced the rate of senescence and the effect was additive to the cytokinin deferral of senescence (Poovaiah and Leopold, 1973; Ferguson et al., 1983; Buchanan et al., 2003) and this evidence could correlate the increase amount of calcium in quinces stressed leaves with the fact that quinces respect to pears withstood longer to the progress of necrosis. Maybe in this case calcium could have played an important role in salt-stressed leaves, in order to contain their senescence progress. Moreover, it has been reported that the rise in $[Ca^{2+}]_{cyt}$ in plant seems to be caused by non selective cation channels (NSCCs) which play also a crucial role in salinity stress (Dimidchik and Maathius, 2007). In fact it has been experimented that high extracellular $[Ca^{2+}]$ inhibits plasma membrane NSCCs that intercede $Na^+$ influx, and proper this down regulation in $Na^+$ entrance can elucidate the ameliorative effect of $Ca^{2+}$ in salt conditions, because other $Na^+$-inward-transporters are not affected by calcium (Shabala et al., 2006)**.** Also this aspect could have had relevance for quinces leaves under salt stress; maybe it could explain the difference between pears leaves' behaviour and quinces' one; in fact blocking NSCC for the entrance of sodium thanks to the higher amount of calcium available (in quinces leaves), could imply a delay in onset of necrosis because $Na^+$ had less possibilities to penetrate cells. In addition, external $Ca^{2+}$ blocks also DA-NSCC (depolarization activated NSCC) to avoid $K^+$ efflux and maintain ion homeostasis (Dimidchik and Maathius, 2007). This aspect was reported both for leaves and for roots; calcium always supports $K^+$ transport and prevents $K^+$ loss (Shabala et al., 2006). In fact in fine roots, where the decrease of calcium was quite important, data reported also a decline in potassium as a consequence of salinity, this confirmed the fact that Ca and K are strictly linked in particular in salt conditions. At the end, we can say that calcium triggers several signalling pathways which can proceed contemporarily and cross-talk as always happens during salt stress responses. So it is possible that some of these ideas can co-exist and working together determining plant responses to this salt stress.

**Table 5-13: Calcium content in all five organs of plants expressed as ppm (mg/kg dry matter). "Control" plants correspond to "-NaCl". Significance according to SAS proc GLM: *=p<0.05, **=p<0.01, ***=p<0.001; NS= not significant. Small letters discriminate according to SNK in vertical way. # this mean doesn't consider Abbé Fétel to balance the absence in "NaCl".**

| Ca | trunk | | | | | leaves | | | | | shoots | | | | | fine roots | | | | | coarse roots | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | - NaCl | | + NaCl | | signif | - NaCl | | + NaCl | | signif | - NaCl | | + NaCl | | signif | - NaCl | | + NaCl | | signif | - NaCl | | + NaCl | | signif |
| **genotype** | | | | | | | | | | | | | | | | | | | | | | | | | |
| Farold®40 | 5343 | c | 4864 | b | NS | 12896 | | 16224 | | NS | 6803 | b | 8468 | b | NS | 17836 | a | 12288 | | * | 6174 | a | 6289 | ab | NS |
| Abbé Fétel | 6465 | b | 6418 | a | NS | 11325 | | 14191 | | NS | 9121 | a | 10055 | a | NS | 16950 | ab | / | | | 5379 | ab | 7097 | a | NS |
| BA29 | 4249 | d | 3842 | c | NS | 13185 | | 16092 | | * | 6355 | b | 6157 | c | NS | 13043 | b | 12069 | | NS | 4281 | c | 4082 | c | NS |
| MC | 7515 | a | 6209 | a | ** | 12105 | | 15688 | | * | 6603 | b | 6485 | c | NS | 15119 | ab | 11992 | | * | 6739 | a | 5323 | bc | * |
| signif | * | | * | | | NS | | NS | | | * | | * | | | * | | NS | | | * | | * | | |
| **treatment** | | | | | | | | | | | | | | | | | | | | | | | | | |
| Ctrl | 5847 a | | | | | 12500 a | | | | | 6960 | | | | | 15332 a# | | | | | 5643 | | | | |
| + NaCl | 5261 b | | | | | 15639 b | | | | | 7640 | | | | | 12116 b | | | | | 5605 | | | | |
| signif | * | | | | | ** | | | | | NS | | | | | ** | | | | | NS | | | | |
| *Interaction* gen* trt | NS | | | | | NS | | | | | NS | | | | | NS | | | | | NS | | | | |

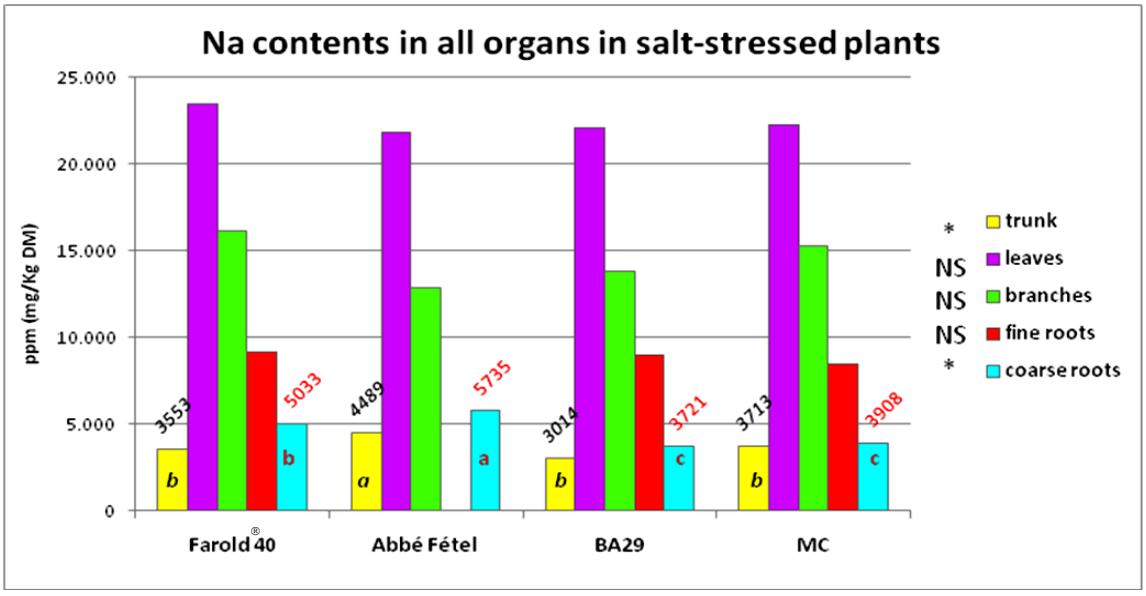



**Graph 5-18: Ca content in all organs for the four genotypes underlining the effect of treatment. On the bottom in the pink box, significance of the treatment (Ctrl against NaCl).**

### 5.1.3 Na

NaCl stress lasted for about three weeks at a final concentration of 90 mM. For fruit trees this concentration was considered to be lethal for plants, we planned to apply this kind of stress in order to observe onset and progress of salinity symptoms and to understand the behaviour of these genotypes in this extreme condition. In Graph 5-19, it appears clear that the distribution of Na in salt-stressed plants of different genotypes did not differ too much; in fact in general, sodium resulted mostly to be accumulated in leaves, then in shoots and in fine roots. The two organs with less amount of sodium resulted coarse roots and trunk. In all the comparisons between "control" and "NaCl" plants it resulted that salinity caused a highly significant ($p<0.001$) increase in Na content in all the different organs, because, respect to normal sodium content in unstressed plants, the rise was really elevated (Tab. 5-14).

In leaves, shoots and in fine roots, where increments of sodium were so high, we did not get any significant differences between genotypes (NS), the only variation observed was in percentage of significance, but the increases always resulted meaningful (see yellow boxes in Tab. 5-14). Probably this result was the consequence of a too high salt stress imposition for too long, in this way is less easy and immediate to understand the preferential allocation of sodium for each genotype under salinity condition.




**Graph 5-19: Na distribution in five organs for all genotypes exposed to NaCl stress (regardless of "control" plants). In Abbé Fétel, fine roots were absent. On the left of the legend are reported the statistical results and on the yellow histograms (trunk) small letter discriminated means based on SNK test ($p<0.05$), while red small letter on cyan histograms are referred to coarse roots. The other organs did not show significant differences.**

**Table 5-14: Sodium content in all five organs of plants expressed as ppm (mg/kg dry matter). "Control" plants correspond to "-NaCl". Significance according to SAS proc GLM: *=p<0.05, **=p<0.01, ***=p<0.001; NS= not significant. Small letters discriminate according to SNK in vertical way.**

| Na | trunk | | | | leaves | | | shoots | | | fine roots | | | coarse roots | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ctrl | + NaCl | | signif | Ctrl | + NaCl | signif | Ctrl | + NaCl | signif | Ctrl | + NaCl | signif | Ctrl | + NaCl | | signif |
| **genotype** | ppm | | | | ppm | | | ppm | | | ppm | | | ppm | | | |
| Farold®40 | 170 | 3553 | b | *** | 112 | 23473 | ** | 314 | 16161 | ** | 989 | 9095 | *** | 409 | 5033 | b | *** |
| Abbé Fétel | 104 | 4489 | a | ** | 158 | 21812 | * | 130 | 12817 | * | 749 | / | | 462 | 5735 | a | *** |
| BA29 | 214 | 3014 | b | *** | 142 | 22086 | *** | 275 | 13806 | *** | 842 | 8989 | *** | 373 | 3721 | c | *** |
| MC | 160 | 3713 | b | *** | 241 | 22241 | *** | 361 | 15290 | *** | 834 | 8406 | *** | 299 | 3908 | c | *** |
| *significance* | NS | * | | | NS | NS | | NS | NS | | NS | NS | | NS | * | | |
| **treatment** | | | | | | | | | | | | | | | | | |
| Ctrl | 171 | | | | 168 | | | 288 | | | 863 | | | 372 | | | |
| + NaCl | 3640 | | | | 22442 | | | 14632 | | | 8830 | | | 4524 | | | |
| *significance* | *** | | | | *** | | | *** | | | *** | | | *** | | | |
| *Interaction gen* trt* | * | | | | NS | | | NS | | | NS | | | *** | | | |

In coarse roots, data showed that Abbé Fétel accumulated more sodium than the other genotypes; followed by Farold®40 (respectively "a" and "b") and both quinces presented a comparable sodium amount. This led to think that pears were able to accumulate more sodium in coarse roots than quinces did, this idea is only based on mineral content analyses, later it will be discussed at the light of $Na^+$-transporters expression in leaves and roots. Similarly, Abbé Fétel emerged for its higher content of sodium in salt-stressed trunk respect to the other genotypes, whose Na amount in trunk was similar (for SNK: "a" for Abbé Fétel and "b" for the others).

Generally, as reported by Boland at al., (1997) referring to a 30 year-old pear orchard exposed to salinity, sodium tends to be stored in roots, trunk and shoots for several years, in order to save leaves from high concentration of this ion. So wood represents a sink for salt, but after some years the "wood capacity of $Na^+$ storage" ends and this ion starts to accumulate in leaves (Boland et al., 1997).

So in our case of hydroponic culture experiment, the time to see effects as consequence of imposed stress was shortened for several reasons, such as: application of 90 mM NaCl at nutrient solution without the "buffer effect" of soil implies a massive uptake of sodium of all the four genotypes, and three-weeks-long-salt treatment imposed to one-year old plants was suitable to see gradualism in salinity effects, but caused also a noticeable accumulations of Na in the whole plant, regardless of the genotype. Probably if we hadn't been interested to see the advance of symptoms in all plants, we could have interrupted the experiment earlier or have applied lower NaCl concentration, in order to avoid this kind of "sodium saturation" of the whole plant. In that hypothetical case probably we could have seen differences among genotypes in the allocation of sodium.

In quinces, the lower amount of sodium accumulated in coarse roots and trunk respect to pears gave the impression that the "wood capacity of $Na^+$ storage" in quinces was saturated earlier than in pears or that the "sodium threshold" in quinces was lower than in pears, in fact it has been reported that it can be different among species and depending on the tree age (Boland et al., 1997). Another possibility could be that quinces altered expression of genes determinant for the extrusion of sodium at the cell root levels (i.e. SOS1).

So in both cases the sodium threshold was exceeded and for quinces probably the delay in onset of toxicity symptoms in leaves, despite the "earlier saturation of Na storage capacity" than that of pears, could partially be due to ameliorative effect of calcium. But the better thing to do could be, taking in consideration all these aspects with the addition of $K^+/Na^+$ ratio.

Several papers reported the importance of this ratio, in fact high $K^+/Na^+$ ratio can be more essential than simply maintaining low $Na^+$ (Chinnusamy, 2005; Parida and Das, 2005), this relevance is also pointed out by *sos1*, *sos2* and *sos3 Arabidopsis* mutants that showed to be defective also in potassium nutrition, as well as in sodium transporters (Tester and Davenport, 2003).

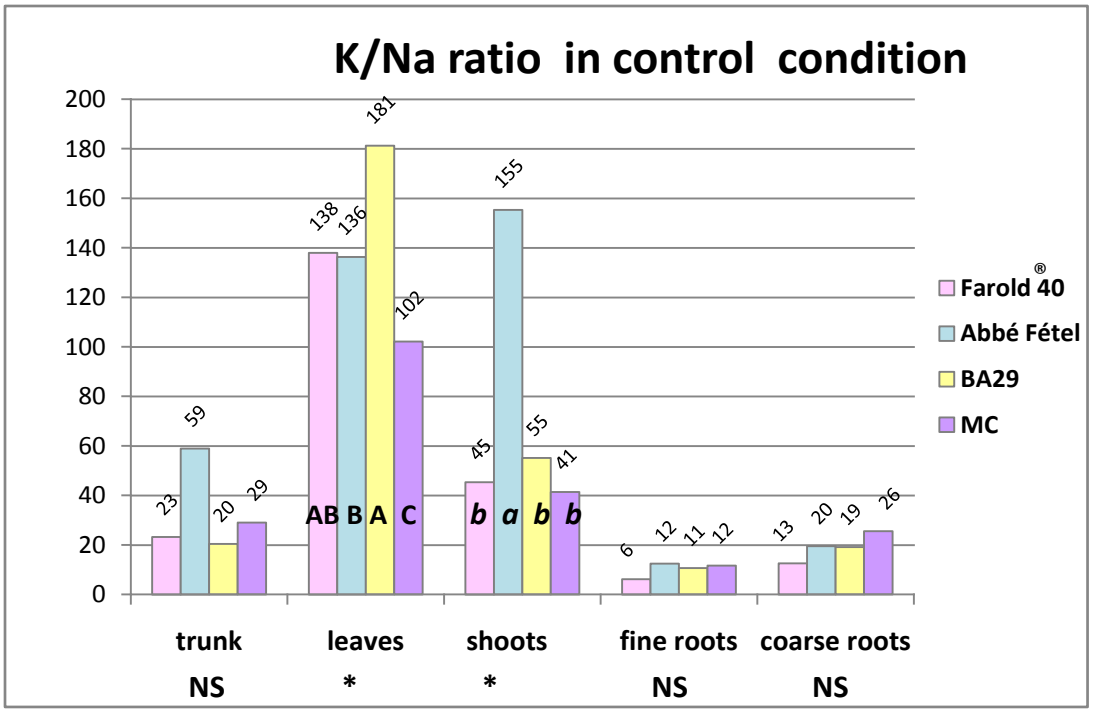
In normally unstressed condition, the $K^+/Na^+$ ratio in the cytosol is high (around 100) while under salinity it falls dramatically due to excessive accumulation of $Na^+$ in the cytosol and to the leak of potassium from the cell (Blumwald, 2000; Chen et al., 2005). So it is the cytosolic $K^+/Na^+$ ratio that determines cell metabolic competence and, at the end, the ability of a plant to survive in saline environments (Shabala and Cuin, 2008). So in this experiment we tried also to explain the behaviour of different genotypes thinking to the $K^+/Na^+$ ratio in different organs, keeping separated "control" plants from "NaCl" ones.

In unstressed plants it appeared clear how $K^+/Na^+$ ratio resulted higher mainly in leaves and shoots (respectively 144 and 64 as mean values, separated by SNK as A and B) and particularly lower in both kinds of roots (differences not significant) (Graph 5-20). The same was done for NaCl-stressed plants and it was immediately evident how the ratio severely decreased; for "control" plant ratio values were between 6 and 181, while for stressed plants the maximum $K^+/Na^+$ ratio value was 2.78; this only to demonstrate the drastically decreased that we registered under salinity.

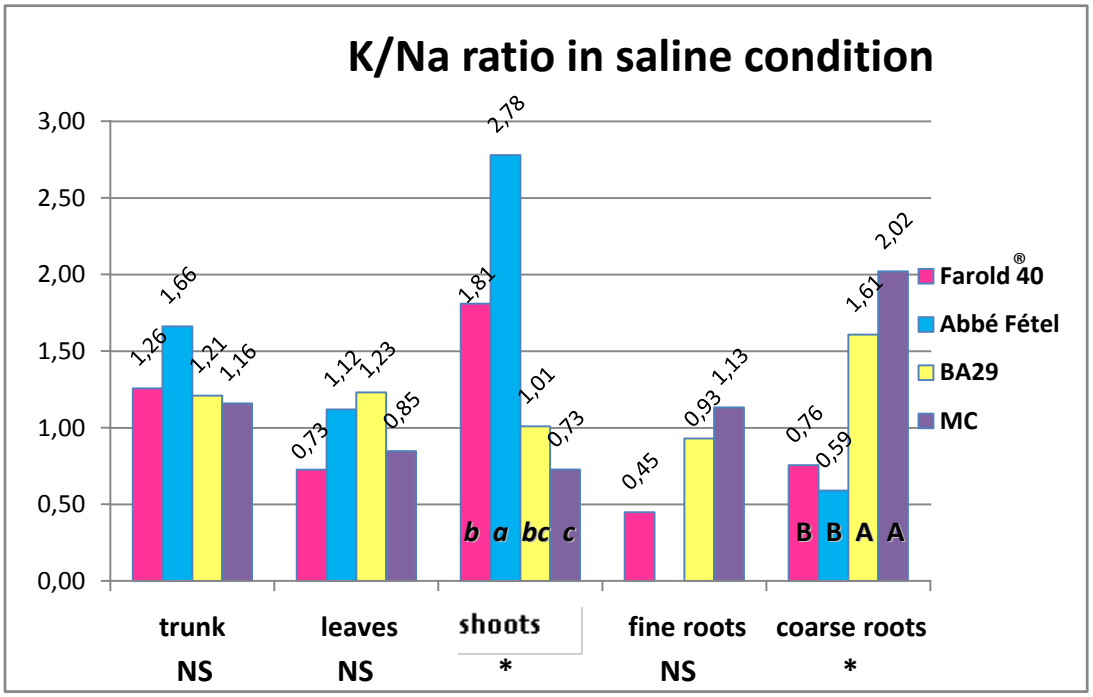
In this stress condition the classification of organs with higher ratio changed, in fact the new situation showed shoots as organ with higher value (A for SNK test) and fine roots as part of the plant with lower $K^+/Na^+$ ratio (B for SNK test).

Comparing trends of genotypes for different organs between "control" and "stress" situations, it emerged that in coarse roots quinces registered a less drastic reduction in $K^+/Na^+$ ratio than that reported in pears, differences among genotypes

were significant with p<0.05 and pointed out how quinces reported a similar behaviour (both reported as A, see Graph 5-21) different from pears' one (B in Graph 5-21).




**Graph 5-20: K/Na ratio in all five organs and differences among genotypes. In "control" plants this ratio is higher in leaves and shoots and lower in both kinds of roots. On the bottom of each organ is reported the significance according to SAS and on the histograms letters are referred to SNK test (p<0.05) discrimination.**




**Graph 5-21: K/Na ratio in all five organs and differences among genotypes. In "stressed" plants this ratio seems higher in coarse roots and shoots and lower in leaves. On the bottom of each organ is reported the significance according to SAS and on the histograms letters are referred to SNK test (p<0.05) discrimination.**

This led to re-examine the consideration about the lower amount of sodium in quinces roots subjected to salt-stress at the light of $K^+/Na^+$ ratio; in fact it could be possible that the higher ratio in quinces means a particular ability of BA29 and MC to extrude back sodium from roots, in order to favour the positive effect of potassium and contain in this way the detrimental effect of sodium.

This calculation of the $K^+/Na^+$ ratio in different organs as exactly the ratio between K content and Na content in the same organ obtained by AAS analyses was recently argued, because actually the important parameter seems to be the cysosolic $K^+/Na^+$ ratio. The argument is based on the assertion that the "surrogate" $K^+/Na^+$ ratio (obtained by AAS analyses) doesn't take into consideration the intracellular compartmentalization of each of the two ions (Shabala and Cuin, 2008). And this diminishes the predictive value of the $K^+/Na^+$ ratio often used to screen plants for salt tolerance (Chen et al., 2005). The problem relies on the difficulties to measure the content of both ions into compartments, in fact nowadays some methods like NMR or energy-dispersive X-ray microanalyses are employed for this purpose (Shabala and Cuin, 2008).

It has been demonstrated that bread wheat has a superior salinity tolerance than durum wheat probably due to the presence in chromosome 4D of the locus Kna1 responsible for the classic $K^+/Na^+$ discrimination, which was subject also to QTL analyses suggesting that $K^+/Na^+$ ratio could be a hereditable trait, but not in all the species (Shabala and Cuin, 2008; Munns and Tester, 2008).

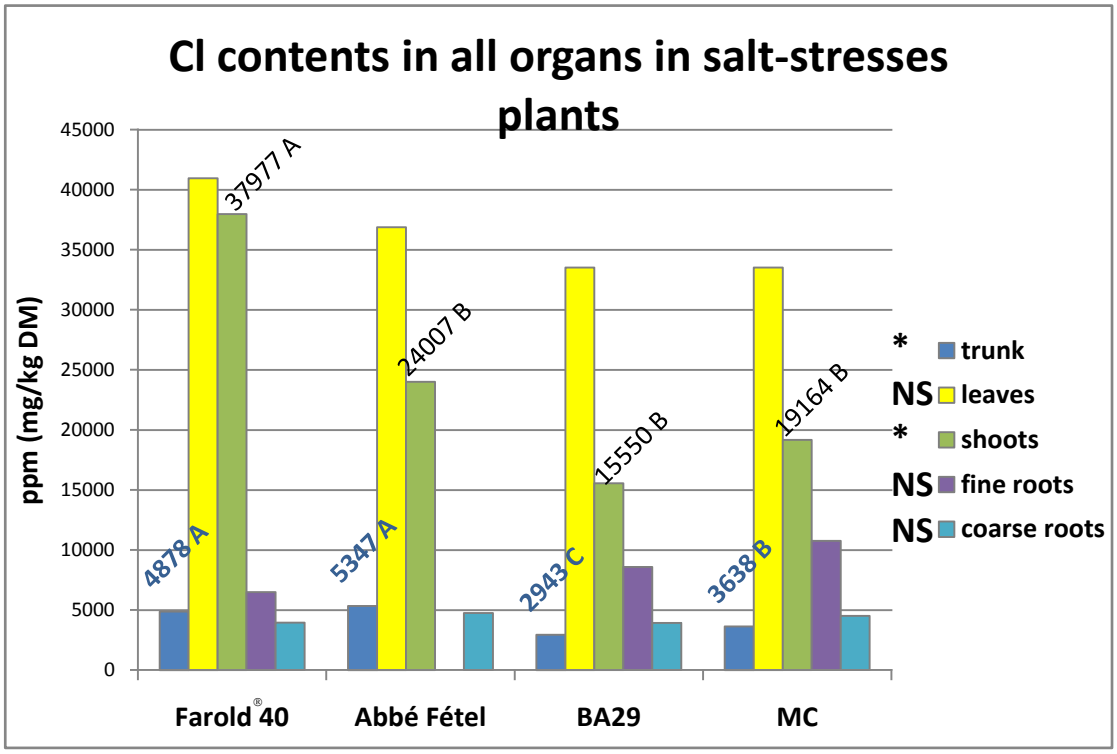
In conclusion, a comparison between *Thelungiella alophila* and *Arabidopsis thaliana* about the potassium content under saline conditions, pointed out that the former is able to increase the K content in mesophyll, while the latter register a classical decline reported also in other glycophytes (Shabala and Cuin, 2008).

### 5.1.4 Cl

During salt stress, both ions $Na^+$ and $Cl^-$ are absorbed by roots and are transported to shoots, with chloride more rapidly than $Na^+$ and in higher concentration, $Cl^-$ seems to be the main responsible of the onset of first symptoms of toxicity (Boland et al., 1997). But additionally, it should be considered also that having the plant cells negative electrical potential, sodium can passively enter the cell, while chloride cannot unless the cytosolic concentration is very low or the membrane potential is depolarized (Munns, 2005; Munns and Tester, 2008).

Since, generally, it results difficult to discriminate which of the two key ions in salinity stress is the main responsible for toxicity; we decide to evaluate also the content of chloride in all the organs, in order to clarify uptake, transport and accumulation of this ion during salinity.

In Graph 5-22, it appears evident that the distribution of chloride in salt-stressed plants of different genotypes did not seem to vary a lot; in fact, in general, Cl has been accumulated mostly in leaves, then in shoots and lastly in fine roots. On the other hand, Cl resulted present in lower amounts in trunk and coarse roots.




**Graph 5-22: Cl distribution in five organs for all genotypes exposed to NaCl stress (regardless of "control" plants). In Abbé Fétel, fine roots were absent. On the left of the legend are reported the statistical results and on the blue histograms (trunk) letter discriminated means based on SNK test ($p<0.05$), while black letters near numbers on green histograms are referred to shoots. The other organs did not show significant differences.**

**Table 5-15: Chloride content in all five organs of plants expressed as ppm (mg/kg dry matter). Significance according to SAS proc GLM: *=p<0.05, **=p<0.01, ***=p<0.001; NS= not significant. Small letters discriminate according to SNK in vertical way.**

| Cl | trunk | | | | leaves | | | shoots | | | | fine roots | | | coarse roots | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ctrl | + NaCl | | signif | Ctrl | + NaCl | signif | Ctrl | + NaCl | | signif | Ctrl | + NaCl | signif | Ctrl | + NaCl | signif |
| **genotype** | | ppm | | | | ppm | | | ppm | | | | ppm | | | ppm | |
| **Farold®40** | 322 | 4878 | **a** | *** | 526 | 40961 | ** | 471 | 37977 | **a** | *** | 1379 | 6488 | * | 676 | 3948 | *** |
| **Abbé Fétel** | 376 | 5347 | **a** | *** | 565 | 36883 | *** | 614 | 24007 | **b** | *** | / | | | 916 | 4740 | *** |
| **BA29** | 294 | 2943 | **c** | *** | 560 | 33522 | ** | 620 | 15550 | **b** | * | 689 | 8587 | * | 328 | 3918 | *** |
| **MC** | 294 | 3638 | **b** | *** | 710 | 33522 | *** | 790 | 19164 | **b** | ** | 4848 | 10762 | NS (10%) | 392 | 4506 | *** |
| *significance* | NS | * | | | NS | NS | | NS | * | | | NS | NS | | NS | NS | |
| **treatment** | | | | | | | | | | | | | | | | | |
| Ctrl | 322 | | | | 595 | | | 634 | | | | 2305 | | | 572 | | |
| + NaCl | 4125 | | | | 35921 | | | 25149 | | | | 8806 | | | 4247 | | |
| *significance* | *** | | | | *** | | | *** | | | | *** | | | *** | | |
| *Interaction* gen* trt | *** | | | | NS | | | ** | | | | NS | | | NS | | |

A comparison between "control" and "NaCl" plants showed that salt stress determined a highly significant increase in Cl content ($p<0.001$) in all considered organs (Tab. 5-15), the same was reported for sodium in paragraph 5.1.3. This can be due to the concentration of salt stress imposed to plants in trial.

From table 5-15, it is also clear that the comparisons for each genotype for the increase in Cl content between "control" and "salt-stressed" condition, resulted always significant with just variation in percentage of significance, only the raise noticed in MC fine roots did not appear meaningful (only at 10%).

In salt stressed coarse roots, the increase in chloride content was equally significant (with $p<0.001$) for all four genotypes and in general (regardless of the genotype) the increase was around 7-fold respect to Cl content in control. Instead, for fine roots under salinity, the accumulation of chloride was 4-fold higher than the normal content in control fine roots. Also in that organ, no discrimination among genotypes was noticed (a part from the non-significance of increase registered in MC). However, MC reported numerically the highest value of chloride amount in fine roots, but it did not turn out significant (Tab. 5-15).

In leaves, again the saline stress did not allow to distinguis different behaviour among genotypes, but in general the raise of chloride under salinity was really impressive, corresponding to around 60-fold respect to the normal chloride content in unstressed leaves in our experimental condition. Despite statistical analyses did not show meaningful differences among leaf Cl contents in different genotypes (Tab. 5-15), numerically the highest values for this element were found in both pears leaves (around 3.6-4% of DM).

The two organs that reported differences, among genotypes, in chloride amount have been trunk and shoots. In trunk, in the comparison between "control" and "NaCl" the increase of Cl content determined by salinity was around 13-fold. Trunk in unstressed condition reported a quantity of chloride around 0.03% with no distinction among genotypes, while under salinity the increase was in all cases highly meaningful ($p<0.001$) and genotypes showed different values. In fact Farold®40 showed together with Abbé Fétel own-rooted the highest values of Cl amount in trunks (0.5% both "a" for SNK), whereas a lower value was registered by

MC (0.36%, “b” for SNK) and at the end BA29 presented the minimum Cl amount for trunk, equal to 0.29%.

The increase of chloride in “NaCl” treated shoots, regardless of the genotypes, was really high and 40-fold respect to the corresponding content in unstressed shoots. While in “control” shoots the Cl contents were similar in all four genotypes and around 0.06%, under salinity genotypes showed different amounts of Cl in shoots. Farold®40, also in this organ, registered the highest Cl content (“a” for SNK), whereas the other three genotypes shows comparable values (Tab. 5-15) according to SNK discrimination (“b” for MC, BA29 and Abbé F.).

So, in salt stressed trunk and shoots, where was possible to observe differences among genotypes, we can say that in both organs quinces showed lower chloride contents than pears, but the absence of significant differences at root level cannot allow to decide if quinces are better chloride excluders than pears. Farold®40, in fact with the highest Cl contents in trunk and shoots, let think to be a better chloride accumulator than Cl excluder. In olive plants it has been observed that the accumulation of chloride into the shoots is different (like in our case), while the uptake and transport of $Cl^-$ are usually lower than for $Na^+$ (Gucci and Tattini, 1997). Moreover, evidences reported for olive revealed that the leaf $Cl^-$ content after five weeks of salt stress was 3-fold greater than that in roots (Tattini et al., 1995). The same was found in our case, where the amount of Cl in total roots was around 1.3% while in leaves 3.6%.

As well as for sodium, the tolerance to high concentration of external chloride is due to the capacity of the plant to limit the uptake of this anion by the roots and subsequently the transport to shoots (Xu et al., 2000). According to this we can say that probably Farold®40 was quite sensitive to chloride in this experiment of hydroponic culture with salt stress imposition. In addition, it is important to underline that chloride, despite its essential role in photosynthesis under normal condition, when in excess can cause toxicity as well as sodium. But not only Cl accumulation and translocation are important, rather mostly the rate of its uptake (Xu et a., 2000; Levy and Syvertsen, 2004). If chloride is effectively more rapid in its kinetics uptake than sodium (Boland et al., 1997) we can suppose that in Farold®40 (and perhaps also for Abbé F.), Cl entrance and translocation have been more rapid

than in the others genotypes. This rapid adsorption of $Cl^-$ could have determined the earlier onset of necrosis in both pears; in fact evidence reported that chloride toxicity coincides with the apparent bronzing and injury of leaves and consequent defoliation (Xu et al., 2000; Levy and Syvertsen, 2004).

It has been pointed out in citrus that rootstocks with low growth vigor have good Cl exclusion ability, while some of vigorous citrus rootstocks exhibit poor Cl tolerance (Levy and Syvertsen, 2004). This idea can be transferred to our pear rootstock; in fact Farold®40 is classified as a vigorous rootstock (as shown in figure 1.5) respect to quince MC. Farold®40, being a fast-growing tree, probably used more water than quinces and its leaves (or in general upper part of plant) could have been exposed to relatively more chloride in transpiration stream from saline water than low-vigour quinces.

In this experiment we cannot go deeper in the toxicity effect details of chloride and sodium, because we can refer only to mineral analysis and not more specific investigations have been planned.

In conclusion, we can assert that our salt stress caused an increase of both $Na^+$ and $Cl^-$ in all organs of the plants in trial and that both pears showed higher accumulation of both ions respect to quinces (compare graph. 5-19 and 5-22).

In general when a salt stress is imposed usually the tendency is to judge $Na^+$ as the main responsible for all the physiological answers of plant, but it should be considered also chloride effects, because for some species (citrus, grapevine) it is considered the more toxic ion (Munns and Tester, 2008). In this experiment we decided to study sodium transporters as determinants in salt stress response mechanism because less is known about chloride transport processes at whole plant level (Munns and Tester, 2008).

## 5.2 Cloning and identification of fragments of $Na^+$-transporter-genes

### 5.2.1 NHX1

As reported in details in paragraph 3.5.0, "Heterologous primers design for cloning" after the step of degenerated primers design, based on the similarity of 18 sequences aligned, both primers pairs were tested and only the couple Deg1FOR and Deg2REV gave as result some interesting PCR products (Fig. 5-6). The theoretical product size according to the position of these two primers on *Arabidopsis* NHX1 (#at5g27150.1 TAIR see Appendix 2) was 670 bp. The obtained bands from both cDNAs visualized on agarose gel fell within 600 and 700 bp and this gave a good indication of the truth of the fragment. As appears in figure 5-6, at the beginning only two of four genotypes were used to test primers and for further cloning steps; indeed MC was chosen as representative for quince genotypes, while Farold®40 (OHF in figure) for pears.

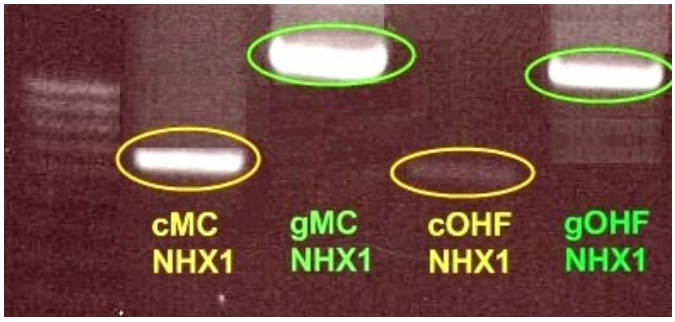



**Fig. 5-6: PCR products using the degenerated primers Deg1FOR and Deg2REV designed for NHX1. cMC and cOHF (=Farold®40) mean products obtained from cDNA and gMC and gFarold from genomic DNA.**

Bands obtained from genomic DNA resulted longer (>1000 bp) than the previous ones from cDNA, this is probably due to the presence of one or more introns. All bands described were excised from agarose gel and DNA extracted as reported in paragraph 3.5.2 "Extraction and purification of DNA from gel".

After the transformation for each inserts (4) a "colony PCR" was performed and from the agarose gel it was possible to check the correct size of the insert that was again between 600-700 bp before the sequencing step (Figure 5-7). In this way we chose the top colonies to carry on that additionally resulted transformed in the liquid culture (Fig. 3-22) and minimum two cultures for each kind of inserts were used to extract plasmids as described in the methodological part (par 3.5.13).

Verified the presence of a satisfying plasmid, all samples were sent to be

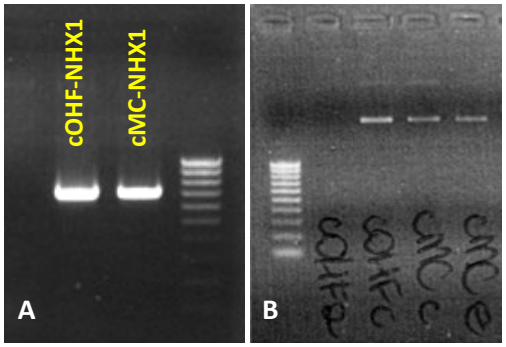



**Fig. 5-7: A) cFarold®40 (OHF) and cMC NHX1 putative bands obtained from the "colony PCR". On the right the 100 bp-gene ruler indicates that both bands are around 700 bp. B) Part of the plasmids containing the NHX1 inserts obtained from cloning and sent to sequence.**

sequenced. A "sequence cleaning job" was done on sequences results as described in paragraph 3.5.16, in order to obtain a correct fragment to compare with all sequences submitted in databases. In all fragments, specific primers used from the beginning have been found. The position of primers determines the length of the cloned fragment. Farold®40 fragment length obtained starting from cDNA was 706 bp as reported in figure 5-8 (top). MC fragment length obtained from cDNA was 708 bp as reported in figure 5-8 (bottom).

**cOHF-NHX1 (706 bp)**

GCGACGGATTCTGTATGCACNTTGCAGGTGCTCAATCAAGATGAGACACCTTTACTCTACAGCTC
TTGTGTTCGGGGAGGGTGTCGTTAACGATGCGACATCTGTGGTTCTTTTCAATGCTATTCAGAGC
TTTGATCTCACCCACCTTGATTCCGGCATTGCCTTGCACTTTCTGGGAAACTTCTTCTATTTGTT
TTTCGCAAGCACCATGCTAGGAGTGTTTGCAGGGCTGCTTAGTGCTTACATTATCAAAAAGCTTT
ATTTTGGAAGGCACTCTACGGATCGTGAGGTTGCTCTTATGATGCTCATGGCATACCTGTCATAT
ATACTGGCTGAATTATTCTATTTGAGTGGCATTCTCACCGTGTTCTTTTGTGGGATCGTGATGTC
GCATTACACTTGGCACAATGTGACTGAGAGTTCAAGAGTTACGACCAAGCATGCTTTCGCAACAT
TGTCATTTGTTGCCGAAATATTTATCTTCCTTTATGTTGGTATGGATGCCTTGGACATTGAAAAG
TGGAGATTTGTAAGTGACAGTCCTGGAACATCTGTGGCGGTGAGTTCAATACTGCTAGGTCTTCT
TATGCTAGGAAGAGCAGCTTTTGTTTTCCCCCTACCATTTTTGTCCAACTTAGCAAAGAAAAACC
AACATGAGAAAATCAGCATCCAGCAGCAAGTGATAATATGGTGGGCTGGTCTAATG

**cMC-NHX1 (708 bp)**

GCAACTGA(A)TTCTGTGTGCACATTGCAGGTGCTAAATCAGGATGAGACACCATTACTCTACAG
TCTTGTACTTGGGGAGGGCGTTGTTAACGATGCGACATCTGTGGTCCTTTTCAATGCTATTCAGA
GCTTTGATCTCACCCACATTGATCCCAGTATTGCTTTGCATTTTATAGGCAACTTCTCATATTTG
TTTTTCGCAAGCACTATGCTAGGAGTGTTTGCAGGGCTGCTTAGTGCTTACATCATCAAAAAGCT
TTATTTCGGAAGGCACTCTACGGATCGTGAGGTTGCTCTTATGATGCTCATGGCATACTTGTCAT
ATATACTGGCTGAATTATTCTATTTGAGTGGCATTCTCACTGTGTTCTTTTGTGGGATCGTGATG
TCGCATTACACTTGGCACAATGTGACTGAGAGTTCAAGAGTTACGACCAAGCATGCTTTCGCGAC
CTTGTCATTTGTTGCCGAAACATTTATCTTCCTTTATGTTGGTATGGATGCTTTGGACATTGAAA
AGTGGAGATTTGTAAGTGACAGTCCTGGAACATCAGTGGCAGTGAGTTCAATACTGCTAGGTCTT
GTTATGCTTGGAAGAGCAGCTTTCGTTTTCCCCCTATCATTCTTGTCGAACTTAACAAAGAAAAA
CCAACATGATAAAATTAGCCTTCGGCAGCAAGTTATAATATGGTGGGCTGGGCTCATG

**Fig. 5-8: cOHF NHX1 on the top and on the bottom cMC NHX1 putative fragments. The green and pink sequences correspond respectively to Deg1FOR and Deg2REV where in bold and underlined are the letter of degeneracy. In blankets one additional base found in the sequence.**

The putative NHX1 fragments belonged to both genotypes were aligned with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and they effectively resulted highly similar to others NHX, $Na^+/H^+$ vacuolar antiporters present in databases (Tab. 5-16).

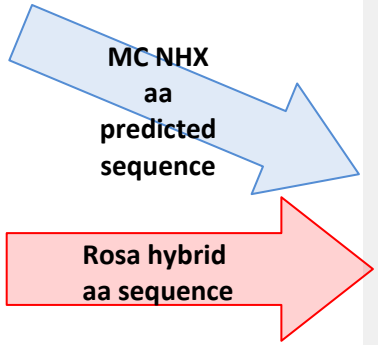
At the beginning also sequences obtained from genomic DNA were taken in consideration, but sending to sequence from only one primer (T7) we did not obtain the entire length of fragment as it appeared in agarose gel (Figure 5-6 lane 2 and 4). After the elimination of some plasmid bases, the alignment of these genomic fragments (gFarold®40 and gMC) resulted both similar at around 90% to NHX1 of several species such as *Suaeda japonica, Glycine max, Trifolium.* We decided to not proceed with the cloning of the full length fragment (>1000 bp) because it was not essential for the genes expression study.

Both our putative fragments (from cDNA) resulted to be homologous at 88-89% to vacuolar NHX of *Rosa hybrid* cultivar Watarase (accession number AB199912.1) that as full length gene is 2000 bp, so, obtained pear and quince fragments covered about one third of the theoretical length of RhNHX. Among all the other NHX genes similar to cOHF-NHX and cMC-NHX, we found some tree species such as *Populus euphratica, Populus tomentosa* and *Eucalyptus camaldulensis x Eucalyptus globules* with homologies above 80% and really low E values. This confirmed the successful cloning of about 700 bp fragment of NHX in pear Farold®40 and in MC quince.

In literature and databases the only NHX gene from fruit trees has been found in *Vitis vinifera* cv. Cabernet Sauvignon (VvNHX1 reported in Hanana et al., 2007), so, since the majority of genes reported in Tab 5-16 as the most similar to our queries are classified as NHX1, we will refer to the new cloned fragments as NHX1 (mRNA) partial sequence of Farold®40 and MC. Using ClustalW, the two cloned fragments relative to NHX1 were aligned to visualize the level of similarity and as reported in appendix 3, the two sequences resulted highly similar with few different bases along the entire fragment. We utilized ORF Finder (section of NCBI) to predict the better frame of the amino acid sequence starting from our cloned cDNA sequence (we utilized as example cMC-NHX1). With the correct frame we did a BLAST and as first hit resulted the amino acid sequence of *Rosa hybrida* (Fig. 5-9).

**Table: 5-16: Results of BLASTn alignment of the two putative fragments of NHX1 (cOHF and cMC) with databases (NCBI). In the first column are reported the species that presented more similarity with both our queries. In the second column is reported the name of the gene. In orange the homology (%) between cOHF NHX1 and each NHX homologous gene; in yellow the homology (%) between cMC NHX1 and each NHX1 .homologous gene. E value= Expectation value. The number of different alignents with scores equivalent to or better than S that are expected to occur in a database search by chance. The lower the E value, the more significant the score of alignment. On the right are reported references linked to each genesimilar to our queries.**

| Species accession number in NCBI | gene (mRNA) | % homology with cOHF putative NHX1 | E value | % homology with cMC putative NHX1 | E value | Reference |
|---|---|---|---|---|---|---|
| *Rosa hybrida* (cultivar watarase) #AB199912.1 | NHX vacuolar $Na^+/H^+$ | **89** | 0 | **88** | 0 | Kagami and Suzuki, 2005 |
| *Populus euphratica* #EU382999 | $Na^+/H^+$ antiporter (NHX2) putative salt tolerance protein | **83** | 0 | **83** | $1e^{-179}$ | Wu et al., Unpublished |
| *Populus tomentosa* #AY660749.1 | PtNHX1 | **83** | 0 | **83** | $5e^{-179}$ | Zhang Unpublished |
| *Glycine Max* #AY972078.1 | GmNHX1 tonoplast $Na^+/H^+$ antiporter | **83** | 0 | **82** | $1e^{-174}$ | Li et al., 2006 |
| *Citrus x paradisi* #AY028416.2 | cNHX1 | **83** | $2e^{-178}$ | **82** | $1e^{-170}$ | Porat et al., 2002 |
| *Trifolium repens* #EU109427.1 | NHX1 $Na^+/H^+$ antiporter | **82** | $2e^{-173}$ | **82** | $1e^{-165}$ | Unpublished |
| *Salicornia europaea* (Halophyta) #AY131235.1 | $Na^+/H^+$ antiporter Nhx1 | **81** | $8e^{-152}$ | **80** | $1e^{-144}$ | Unpublished |
| *Zoysia japonica* #EU 333827.2 | $Na^+/H^+$ antiporter (NHX1) | **77** | $2e^{-108}$ | **77** | $1e^{-109}$ | Du and Xia Unpublished |
| *Eucalyptus camaldulensis x Eucalyptus globules* #EU033971.1 | NHX $Na^+/H^+$ antiporter | **82** | $8e^{-167}$ | **/** | / | Faridah et al., Unpublished |




>gi|BAD93487.1| vacuolar Na+/H+ antiporter [Rosa hybrid cultivar]
Length=543

Score = 431 bits (1109), Expect = 2e-119, Method: Compositional matrix adjust.
Identities = 212/233 (90%), Positives = 220/233 (94%), Gaps = 0/233 (0%)

```
Query  2    NSVCTLQVLNQDETPLLYSLVLGEGVVNDATSVVLFNAIQSFDLTHIDPSIALHFIGNFS  61
            +SVCTLQVL+QDETPLLYSLV GEGVVNDATSVVLFNAIQSFDLTH+D  IAL F+GNF
Sbjct  163  DSVCTLQVLHQDETPLLYSLVFGEGVVNDATSVVLFNAIQSFDLTHLDSRIALKFMGNFL  222

Query  62   YLFFASTMLGVFAGLLSAYIIKKLYFGRHSTDREVALMMLMAYLSYILAELFYLSGILTV  121
            YLFFASTMLGV  GLLSA+IIKKLYF RHSTDREVALMMLMAYLSYILAELFYLSGILTV
Sbjct  223  YLFFASTMLGVITGLLSAFIIKKLYFARHSTDREVALMMLMAYLSYILAELFYLSGILTV  282

Query  122  FFCGIVMSHYTWHNVTESSRVTTKHAFATLSFVAETFIFLYVGMDALDIEKWRFVSDSPG  181
            FFCGIVMSHYTWHNVTESSRVTTKHAFATLSFV ETFIFLYVGMDALDIEKWRFVSDSPG
Sbjct  283  FFCGIVMSHYTWHNVTESSRVTTKHAFATLSFVCETFIFLYVGMDALDIEKWRFVSDSPG  342

Query  182  TSVAVSSILLGLVMLGRAAFVFPLSFLSNLTKKNQHDKISLRQQVIIWWAGLM  234
            TSVAVSSILL LVMLGRAAFVFPLSF SNL KKNQ +KISL+QQV+IWWAGLM
Sbjct  343  TSVAVSSILLSLVMLGRAAFVFPLSFFSNLFKKNQSEKISLQQQVVIWWAGLM  395
```

**Fig. 5-9: Alignment between Rosa hybrid amino acid sequence and the predicted MC-NHX1 amino acid sequence. The two sequences showed a 90% of identity.**

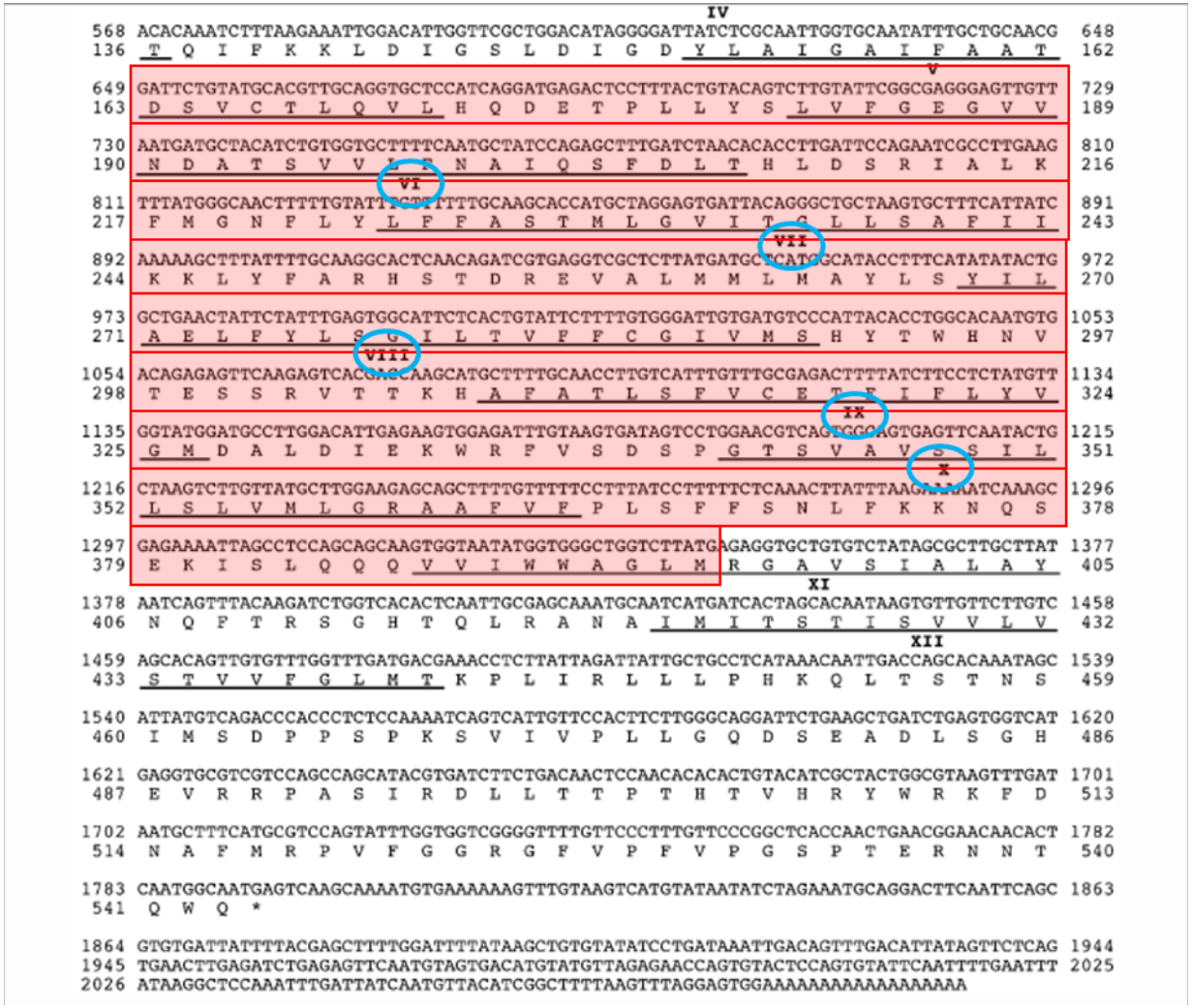



**Fig. 5-10: Nucleotide and amino acid sequence of RhNHX1 cDNA of Rosa hybrid (Kagami and Suzuchi, 2005). Putative transmembrana regions are underlined and indicated by Roman numerals. Red rectangular region identifies part of the amino acid sequence homologous with predicted MC-NHX1 aa sequence and in blue cycles the transmembrane regions included.**

From figure 5-10, we can see that our sequence (called query) ranges from aa 163 to 395 of the *Rosa hybrida* sequence. In the reference related the Rosa hybrida NHX1 (Kagami and Suzuchi, 2005), authors provided a representation of the predicted protein structure and we modified that picture to better understand which part of the vacuolar $Na^+/H^+$ antiporter can be coded by our NHX1 fragment.

It is easy to understand from that figure that there are 5 trans-membrane regions that are common to both "aa" sequences. These regions are hydrophobic regions that move through the membrane and take part to the antiporter structure that contains 12 putative hydrophobic regions, so our "aa" sequence covered less than half of the entire predicted protein in *Rosa hybrida* (Kagami and Suzuchi, 2005). According to the study carried out by Kagami and Suzuki (2005), the fifth and the sixth transmembrane regions are fundamental for the antiporter activity, in fact differences in these parts of the protein can determine the direction of the ion movement (sodium efflux or influx). In figure 5-10, it has been underlined a red area corresponding to the similarity between RhNHX1 and our cloned NHX1 fragments, the amino acid number 163 (the first in the red area) is known as "Asp residue" and it resulted to be involved in the exchange activity of the antiporter and conserved among several NHX1 sequences (GhNHX1, AtNHX1, OsNHX1, PeNHX1 etc…)

After having obtained these two NHX1 fragment from Farold®40 and MC, we enlarged the cloning section also to Abbé Fétel and BA29 to have a clear idea about the similarities between them. From BA29's cDNA we cloned a successful 705 bp fragment that resulted highly homologous to several NHX vacuolar $Na^+/H^+$ antiporters the same reported in Tab. 5-16.

Using ClustalW, the two quinces cloned fragments relative to NHX1 were matched together to visualize the similarity; this output was reported in appendix 4, the two sequences resulted highly similar (score=98) with only few different bases.

The same procedure was done starting from cDNA of Abbé Fétel and using always the same pair of primers Deg1FOR and Deg2 REV. The cloned sequence resulted 705 bp long, highly similar to cFarol40-NHX (score=94, see appendix 4) and was confirmed by BLAST search as a NHX1 fragment.

In conclusion, NHX1, sodium/hydrogen antiporter located in the tonoplast was successfully partially cloned in all four genotypes starting from corresponding cDNAs.

### 5.2.2 SOS1

As mentioned before in the methodological part, also for SOS1 were designed degenerated primers from alignment of related sequences, already submitted in NCBI nucleotide databases, but after several tempts, we realized that they did not work well either on genomic DNA either on cDNA. In the first case the problem probably was due to the presence of introns that probably could have avoided the correct annealing of primers. Arabidopsis SOS1 as genomic full-length gene is 6076 bp long with 22 introns inside. Then Prof. Bressan's lab primers for SOS1 used for *Arabidopsis thaliana* and *Thelungiella halophila* (Tab. 3-13) were tested on cDNA of MC and Farold (chosen again as representative of quinces and pears for preliminary cloning) and provided interesting PCR products. From agarose gel, it was possible to visualize two apparently identical bands, one for genotype, with a size of around 500 bp according to the gene ruler on a side (Figure 5-11 A).

All the other steps were performed as for NHX1 until the achievement of plasmids containing the two different inserts.

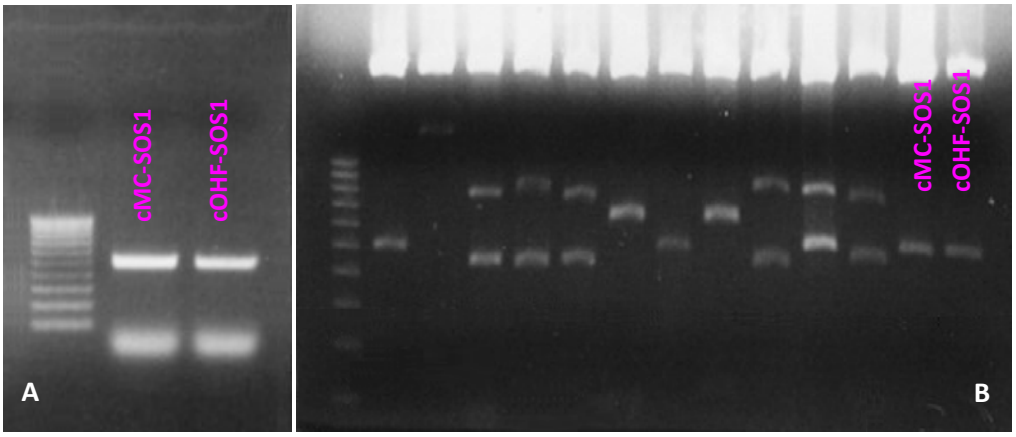



**Fig. 5-11: A) cMC SOS1 and cFarold®40 (OHF) SOS1 putative bands obtained from PCR with the primer in table 3-13. B) The digested plasmids the gel confirmed the presence of an insert of correct length (around 500 bp) plus the cut plasmid >3000 bp on the top.**

All plasmids were digested as described in 3.5.14 and the gel confirmed the presence of an insert of correct length (around 500 bp) plus the cut plasmid >3000

bp on the top, as visualized in figure 5-11 B. These plasmids were sent to be sequenced and the resulting sequences were cleaned from pGEM vector's bases in order to obtain a correct fragment to compare with all sequences submitted in databases. In both fragments, original SOS1 primers have been found; their positions determine the length of the cloned fragment.

For Farold®40, the length of the fragment obtained starting from cDNA was 488 bp while MC was 490 bp for as reported in figure 5-12.

The putative SOS1 fragments belonged to both genotypes were aligned with BLAST and they effectively were highly similar to others SOS1, $Na^+/H^+$ plasma membrane antiporters present in databases (Tab. 5-17 for cOHF-SOS1 and see Fig. 5-13).

The ClustalW alignment of our two fragments gave as result a score equal to 70 and we noticed for cMC-SOS1 that translating nucleotide (490 bp) sequence into amino acid sequence (142 aa) and "blasting" the new one, several more results were obtained and reported in table 5-18. As for NHX1, also the SOS1 cloning was extended to the other two genotypes in trial: Abbé Fétel and BA29.

**cOHF SOS1 (488bp)**

( ) TGTTGTCATTGCTGAAGGCATTCTCGACAGCGATAAGATTGCGTACCAAGGGAGTTCATG
GGGATATCTTTTTCTACTATACTTATACATCCAACTATCGCGTTGTGTTGTTGTTGGAGTTC
TGTATTCATTTTTATGTCGCGTTGGCTATGGCTTGGATTGGAAAGAAGCCATTATACTCGTA
TGGTCTGGTTTGAGGGGTGCAGTGGCGCTCTCACTTTCTTTATCCGTGAAGCAATCAAGCGG
AAATTCATTTCTCAGCACCGAGACAGGAACAATGTTTATTTTCTTCACTGGTGGAATCGTGT
TCCTGACTCTGATAGTTAATGGATCCACTACCCAATTTGCTCTGCGCCTTCTTCGCATGGAC
GGTTTACCAGCCTCAAAGATACGAATATTGGATTATACAAAGTATGAAATGCTGAATAAGGC
CTTACAAGCGTTCGAAGATCTAGGAGACGATGAAGAGTTAGGACCTGCTGACTGGC

**cMC-SOS1 490bp**

( ) TGTTGTCATTGCTGAAGGCATTCTTAGTGGTGAAAATATTTTAATCACGGAAAATCTTGG
TTGTACCTCATTCTTCTCTATGTTTATGTACAAGTTTCCCGGTTCATAGTTGTTGCGGTTTC
ATTTCCCCTTCTCCGGTGTTTTGGATATGGTTTGGACTGGAAAGAGGCTATAATCCTTATAT
GGTCAGGTCTGCGAGGGGCTGTAGCATTGTCACTTTCACTGTCAGTGAAGCAAACTAGCGAC
AGCTCTTCACTTATCAGTTCTGACACAGGAGTCCTGTTTGTTTTCTTCACTGGTGGAATTGT
CTTCTTGACACTAATTGTGAATGGTTCCACCACACAATTCATTTTACGCCTTCTAGATCTGG
ACAAGCTATCAGCAGCTAAGAGGCGTGTGCTGGAGTACACAAAATATGAAATGTTGAACAAA
GCATTAGAGGCTTTTGGTGATCTTGGAGATGACGAGGAGTTAGGACCTGCTGACTGGC

**Fig. 5-12: Putative SOS1 cloned sequences from cDNA of Farold®40 (on the top) and from cDNA of MC (on the bottom). In both sequences forward (green) and reverse (pink) primers were found. In the forward primers the two blankets mean that in comparison with the designed primers a "G" base is missed at 5'. The reverse SOS1 primer instead was found as the original one.**

```
>gb|EF207775.1|  Thellungiella halophila salt-overly-sensitive 1 (SOS1) mRNA,
complete cds
Length=3441

 Score =  896 bits (485),  Expect = 0.0
 Identities = 487/488 (99%), Gaps = 0/488 (0%)
 Strand=Plus/Plus

Query  1     TGTTGTCATTGCTGAAGGCATTCTCGACAGCGATAAGATTGCGTACCAAGGGAGTTCATG  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  975   TGTTGTCATTGCTGAAGGCATTCTCGACAGCGATAAGATTGCGTACCAAGGGAGTTCATG  1034

Query  61    GGGATATCTTTTTCTACTATACTTATACATCCAACTATCGCGTTGTGTTGTTGTTGGAGT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1035  GGGATATCTTTTTCTACTATACTTATACATCCAACTATCGCGTTGTGTTGTTGTTGGAGT  1094

Query  121   TCTGTATTCATTTTTATGTCGCGTTGGCTATGGCTTGGATTGGAAAGAAGCCATTATACT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1095  TCTGTATTCATTTTTATGTCGCGTTGGCTATGGCTTGGATTGGAAAGAAGCCATTATACT  1154

Query  181   CGTATGGTCTGGTTTGAGGGGTGCAGTGGCGCTCTCACTTTCTTTATCCGTGAAGCAATC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1155  CGTATGGTCTGGTTTGAGGGGTGCAGTGGCGCTCTCACTTTCTTTATCCGTGAAGCAATC  1214

Query  241   AAGCGGAAATTCATTTCTCAGCACCGAGACAGGAACAATGTTTATTTTCTTCACTGGTGG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1215  AAGCGGAAATTCATTTCTCAGCACCGAGACAGGAACAATGTTTATTTTCTTCACTGGTGG  1274

Query  301   AATCGTGTTCCTGACTCTGATAGTTAATGGATCCACTACCCAATTTGCTCTGCGCCTTCT  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1275  AATCGTGTTCCTGACTCTGATAGTTAATGGATCCACTACCCAATTTGCTCTGCGCCTTCT  1334

Query  361   TCGCATGGACGGTTTACCAGCCTCAAAGATACGAATATTGGATTATACAAAGTATGAAAT  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1335  TCGCATGGACGGTTTACCAGCCTCAAAGATACGAATATTGGATTATACAAAGTATGAAAT  1394

Query  421   GCTGAATAAGGCCTTACAAGCGTTCGAAGATCTAGGAGACGATGAAGAGTTAGGACCTGC  480
             |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  1395  GCTGAATAAGGCCTTACAAGCGTTTGAAGATCTAGGAGACGATGAAGAGTTAGGACCTGC  1454

Query  481   TGACTGGC  488
             ||||||||
Sbjct  1455  TGACTGGC  1462
```

**Fig. 5-13: cSOS1 cloned sequences from cDNA of Farold®40 (query) aligned with Thellungiella halophila salt-overly-sensitive 1 (SOS1) mRNA (sbjct). They had 99% identities.**

The putative SOS1 fragment obtained from Abbé Fétel cDNA was long 489 bp and compared by BLAST, resulted to be highly homologous to the same SOS1 genes reported in table 5-17. cAbbé Fétel-SOS1 was really a fragment of this plasma membrane antiporter for the extrusion of sodium, because it resulted similar to *Pisum sativum* SOS1, to *Arabidopsis* SOS1 (87% homology), to *Thellungiella halophila* SOS1 (91%) and to *Brassica napus* SOS1 (92%).

With regard to BA29, we found some difficulties in cloning from cDNA of these genotype using the same SOS1 primers of table 3-13 and, at the end, we cloned from BA29 cDNA utilizing the pair of primers designed for qRT-PCR (CoSOS1f+r) a small fragment of 72 bp totally identical to partial cMC-SOS1 previously discussed. Despite the smaller length of this sequence in comparison to those of the other three genotypes for SOS1, we could be quite confident of its identity being 100% homologous to cMC-SOS1 partial sequence.

**Table: 5-17: Results of BLASTn alignment of cOHF putative SOS1 with databases (NCBI). In the first column are reported the species that presented more similarity with both our queries. In the second column is reported the name of the gene. In pink the homology (%) between cOHF SOS1 and each SOS1 homologous gene. E value= Expectation value. The number of different alignents with scores equivalent to or better than S that are expected to occur in a database search by chance. The lower the E value, the more significant the score of alignment. On the right are reported references linked to each gene similar to our queries.**

| Species accession number in NCBI | gene (mRNA) | % homology with cOHF putative SOS1 | E value | Reference |
|---|---|---|---|---|
| *Thellungiella halophila* #EF207775.1 | salt-overly-sensitive 1 (SOS1) | **99** | 0 | Oh et al., 2007 |
| *Brassica napus* #EU487184 | sodium:hydrogen antiporters (SOS1) | **92** | 0 | Unpublished |
| *Arabidopsis thaliana* # NM_126259.3 | SALT OVERLY SENSITIVE 1); sodium:hydrogen antiporter (SOS1) | **90** | $3e^{-179}$ | Unpublished |
| *Pisum sativum* EF219135.1 | SOS1 protein-like mRNA | **90** | $1e^{-177}$ | Unpublished |

**Table: 5-18: Results of BLASTp alignment of cMC putative SOS1 with databases (NCBI). In the first column are reported the species that presented more similarity with both our queries. In the second column is reported the name of the protein. In pink the homology (%) between cMC-SOS1 and each SOS1 homologous SOS1 proteins. E value= Expectation value. The number of different alignents with scores equivalent to or better than S that are expected to occur in a database search by chance. The lower the E value, the more significant the score of alignment. On the right are reported references linked to each protein similar to our queries.**

| Species accession number in NCBI | Protein | % homology with cMC putative SOS1 | E value | Reference |
|---|---|---|---|---|
| *Ricinus communis* #EEF40533 | sodium/hydrogen exchanger plant | **81** | $6e^{-62}$ | Unpublished |
| *Populus euphratica* #ABF60872 | $Na^+/H^+$ antiporters putative role in salt tolerance | **81** | $3e^{-61}$ | Unpublished |
| *Solanum lycopersicum* #CAG30524 | putative plasmalemma $Na^+/H^+$ antiporter | **78** | $8e^{-54}$ | Unpublished |
| *Arabidopsis thaliana* #AAF76139 | SALT OVERLY SENSITIVE 1; sodium:hydrogen antiporter (SOS1) | **76** | $6e^{-57}$ | Shi et al., 2000 |
| *Chenopodium quinoa* #ABS72166 | salt overly sensitive 1 (SOS1) | **73** | $1e^{-55}$ | Unpublished |
| *Thellungiella halophila* #ABN04857 | salt overly sensitive 1 (SOS1) | **73** | $6e^{-54,}$ | Oh et al., 2007 |

### 5.2.3 HKT1

For HKT1, high-affinity $K^+$ transporter and low-affinity $Na^+$ transporter (in normal condition), the same steps as for SOS1 were carried out. Also for this gene we utilized primers that have been used successfully to clone HKT1 in *Arabidopsis* and *Thellungiella*, they were HKT1-670F and HKT1-1015R (as reported in table 3-13). PCR products obtained using these primers and as templates cDNAs of the two representatives for species (MC and Farold®40 as for the previous cloned genes) were loaded on an agarose gel. The visualized bands were good and less that 400 bp long as nearly what we expected from primers positions on AtHKT1 sequence (Figure 5-14).

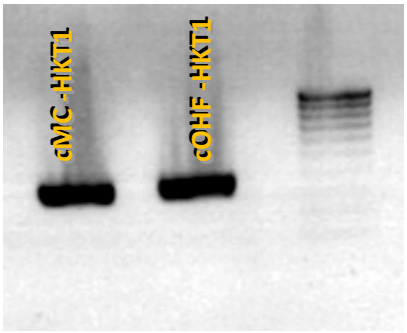



**Fig. 5-14**: **cMC HKT1 and cFarold®40 (OHF) HKT1 putative bands obtained from PCR with the primer in table 3-13.**

All the further transformation steps proceeded regularly and once obtained the plasmids; correct length of each inserts was checked by digestion of the vector.

After the confirmation of the correct length of inserts, plasmids were quantified and sent to be sequenced.

Cloned sequences were “cleaned” from not necessary parts and location of the two original primers was found without any modifications in the sequence. The exact length of the two putative HKT1 fragments was the same for both and corresponded to 336 bp (Fig. 5-15).

The two 336 bp-long fragments were aligned together by ClustalW and they got a score of 91, presenting some mismatches in the middle of the sequence (see appendix 6). The putative HKT1 fragments were immediately confirmed as effectively partial HKT1 sequence of quince MC and pear Farold®40.

**cOHF-HKT (336 bp)**

CCCACGAATGAGAACATGATCATCTTTCGCAAGAACTCTGGTCTCCTCTGGCTCTTAATCCC
TCAAGTACTAATGGGAAACACATTGTTCCCTTGTTTCTCGGTGTTGTCCATTTGGGGACTTT
ACAAGATCACAAAGCGAGACGAGTTTAGTTACATTCTCAAGAACCACAAAAAGATGGGATAC
TCTCATCTATTCTCCGTTCGTCTATGTGTTCTTCTTGGATTGACAGTGTTAGGGTTTCTGAT
GATACAGCTTCTTCTCTTTTCCGCCTCTGAGTGGAGCTCCAAGTCACTTGAAGGAATGAGTT
CGTACGAGAAGTTGGTTGGATCGTTG

**cMC-HKT (336 bp)**

CCCACGAATGAGAACATGATCATCTTTCGCAAGAACTCTGGTCTCATCTGGCTCTTGATCCC
TCAAGTACTAATGGGAAACACTTTGTTCCCTTGCTTCTTGGTTATGCTCATATGGGGACTTT
ACAAGATCACAAAGCGTGAAGAGTTTAGTTACATTCTCAAGAACCACAAGAAGATGGGATAC
TCTCATCTACTCTCGGTTCGTCTTTGTTTTCTTCTTGGATTAACAGTGTTAGGGTTTCTAAC
GATACAGCTTCTTCTCTTTTGCGCCTTTGAGTGGAGCTCTGAGTCTCTCCAAGGTATGAGCT
CATACGAGAAGTTGGTTGGATCGTTG

**Fig. 5-15: Putative HKT1 cloned sequences from cDNA of Farold®40 (on the top) and from cDNA of MC (on the bottom). In both sequences forward (green) and reverse (pink) primers were found. The two sequences are identically long (336 bp).**

BLASTn search found as the most similar sequence to our both queries (inserting a fragment one at a time) *Arabidopsis thaliana* HKT1 with a homology of 91% and *Thellungiella halophila* HKT1 with 87%. In table 5-19 are reported more detailed information about this BLASTn search.

As before, the cloning of this gene was extended to the other two genotypes, utilizing the same primers and same PCR conditions. So that BA29 and Abbé Fétel cloned fragments from corresponding cDNA were 336 bp long as the other two fragments cloned from MC and Farold®40 cDNAs and they were confirmed to belong to HKT1. In particular cAbbé-HKT1 and cFarold®40-HKT1 resulted after a ClustalW alignment, exactly identical as figure 5-16 shows.

**Table: 5-19: Results of BLASTn alignment of cOHF and cMC putative HKT1 fragments with databases (NCBI). In the first column are reported the species that presented more similarity with both our queries. In the second column is reported the name of the gene. In pink the homology (%) between cOHF HKT1 and cMC with each HKT1 homologous gene. E value= Expectation value. The number of different alignents with scores equivalent to or better than S that are expected to occur in a database search by chance. The lower the E value, the more significant the score of alignment. On the right are reported references linked to each gene similar to our queries.**

| Species accession number in NCBI | Gene (mRNA) | Details | % homology with cMC and cOHF putative HKT1 | E value | Reference |
|---|---|---|---|---|---|
| *Arabidopsis thaliana* #NM_117099.5 | HKT1 High-affinity $K^+$ transporter; Sodium ion transmembrane transporter | encodes a sodium transporter (HKT1) expressed in the phloem. Mutants over-accumulate sodium in shoot tissue and have reduced sodium in phloem sap and roots. | **91** | $7e^{-131}$ | unpublished |
| *Thellungiella halophyla* #EF025500 | High-affinity $K^+$ transporter | **/** | **87** | $5e^{-112}$ | unpublished |
| *Suaeda salsa* # AY530754.2 | HKT1 | **/** | **75** | $9e^{-08}$ | |

```
CLUSTAL 2.0.10 multiple sequence alignment

auto            GAGCTCTCCATATGGTCGACCTGCAGGCGGCCGCGAATTCACTAGTGATTCCCACGAATG 60
ohf             ----------------------------------------------------CCCACGAATG 10
                                                                     **********

auto            AGAACATGATCATCTTTCGCAAGAACTCTGGTCTCCTCTGGCTCTTAATCCCTCAAGTAC 120
ohf             AGAACATGATCATCTTTCGCAAGAACTCTGGTCTCCTCTGGCTCTTAATCCCTCAAGTAC 70
                ************************************************************

auto            TAATGGGAAACACATTGTTCCCTTGTTTCTCGGTGTTGTCCATTTGGGGACTTTACAAGA 180
ohf             TAATGGGAAACACATTGTTCCCTTGTTTCTCGGTGTTGTCCATTTGGGGACTTTACAAGA 130
                ************************************************************

auto            TCACAAAGCGAGACGAGTTTAGTTACATTCTCAAGAACCACAAAAAGATGGGATACTCTC 240
ohf             TCACAAAGCGAGACGAGTTTAGTTACATTCTCAAGAACCACAAAAAGATGGGATACTCTC 190
                ************************************************************

auto            ATCTATTCTCCGTTCGTCTATGTGTTCTTCTTGGATTGACAGTGTTAGGGTTTCTGATGA 300
ohf             ATCTATTCTCCGTTCGTCTATGTGTTCTTCTTGGATTGACAGTGTTAGGGTTTCTGATGA 250
                ************************************************************

auto            TACAGCTTCTTCTCTTTTCCGCCTCTGAGTGGAGCTCCAAGTCACTTGAAGGAATGAGTT 360
ohf             TACAGCTTCTTCTCTTTTCCGCCTCTGAGTGGAGCTCCAAGTCACTTGAAGGAATGAGTT 310
                ************************************************************

auto            CGTACGAGAAGTTGGTTGGATCGTTGAATCGAATTCCCGCGGCCGCCATGGCGGCCGGGA 420
ohf             CGTACGAGAAGTTGGTTGGATCGTTG-------------------------------- 336
                *************************

auto            GCATGCGACGTCGGGCCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTC 480
ohf             ------------------------------------------------------------
```

**Fig. 5-16: HKT1 cloned sequences from cDNA of Farold®40 (ohf) and from cDNA of Abbé Fétel (auto). They were aligned by ClustalW and they got a 100 as score.**

### 5.2.4 Actin

For all the experiment, actin primers from apple (see table 3-28) were used to check the condition of new cDNA, aware that they worked properly in apple as in pear.

At the end of cloning phase, it has been decided to confirm that PCR products, with these actin primers on pears and quinces cDNA, were effectively pear and quince actin small fragments. So that these bands were cloned and sequencing results validated previous suppositions. Actin fragments cloned from cDNA of Abbé Fetel and from cDNA of MC matched with high score *Pyrus communis* actin mRNA sequence (AF386514) submitted in NCBI database, respectively 98 and 96 (Fig. 5-17). The confirmed actin fragment was 64 bp long. So these actin primers were also used for qRT-PCR analyses after an evaluation of the stability of transcripts, as described in the next paragraph.

```
actAbbeF.       ------------------------------------------------------------
AF386514        CTATATGCTAGTGGACGTACAACTGGTATTGTTCTCGACTCAGGAGATGGTGTGAGCTAC 360
actMC           ------------------------------------------------------------


actAbbeF.       ------------TATGAAGGGTATGCCCTCCCACATGCCATCCTTCGTTTGGACCTGGCG 48
AF386514        ACAGTTCCAATTTATGAAGGGTATGCCCTCCCACATGCCATCCTTCGTTTGGACCTGGCA 420
actMC           ------------TATGAAGGGTATGCCCTCCCGCATGCCATCCTTCGTTTGGACCTGGCG 48
                            ******************** *************************


actAbbeF.       GGTCGTGATCTTACAG---------------------------------------------- 64
AF386514        GGTCGTGATCTTACAGATGCTCTCATGAAAATTTTGACTGAACGTGGTTACTCTTTCACC 480
actMC           GGTCGTGATCTTACAG---------------------------------------------- 64
                ****************


actAbbeF.       ------------------------------------------------------------
AF386514        ACAACTGCTGAGCGGGAAATTGTGAGGGACATGAAGGAGAAGTTAGCCTATATTGCTCTT 540
actMC           ------------------------------------------------------------
```

**Fig. 5-17: ClustalW alignment among the two cloned fragments of actin from MC and Abbé Fétel and the submitted *Pyrus communis* actin mRNA (AF386514).**

### 5.3.0 Housekeeping gene choice for qRT-PCR during salt stress

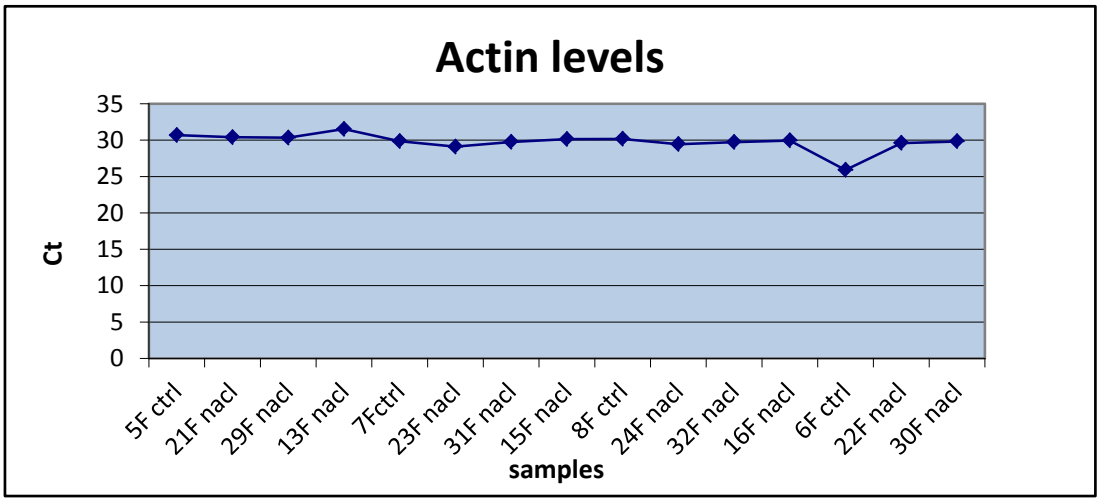
As mentioned before in paragraph 3.6.2, two candidate housekeeping genes were tested on cDNAs from different salt stress timings (different level of necrosis, see appendix 1) by qRT-PCR, in order to understand which one could be the most stable in this salt stress experiment. The candidate genes were actin and elF1α.

The first gene is well known and widely used, while the second one was suggested by Nicot and colleagues (2005) as the most stable in potato subjected to salt stress.

To evaluate the stability of housekeeping genes, RNA transcription levels were measured for several “control” and “stressed” samples. The RNA transcriptional profiles referred to actin and elongation factor were reported on Graphs (Fig. 5-18).

As definition the housekeeping gene should be as much stable as possible, so comparing actin and elf1α it appeared evident how the first one was steadier than the other that showed some evident fluctuations between genotypes, organs and treatment.

In conclusion, actin, which has been considered always expressed in all stages of development and in all plant organs as relatively constant, was chosen in this experiment as housekeeping gene for qRT-PCR analyses (Heid et al., 1996).



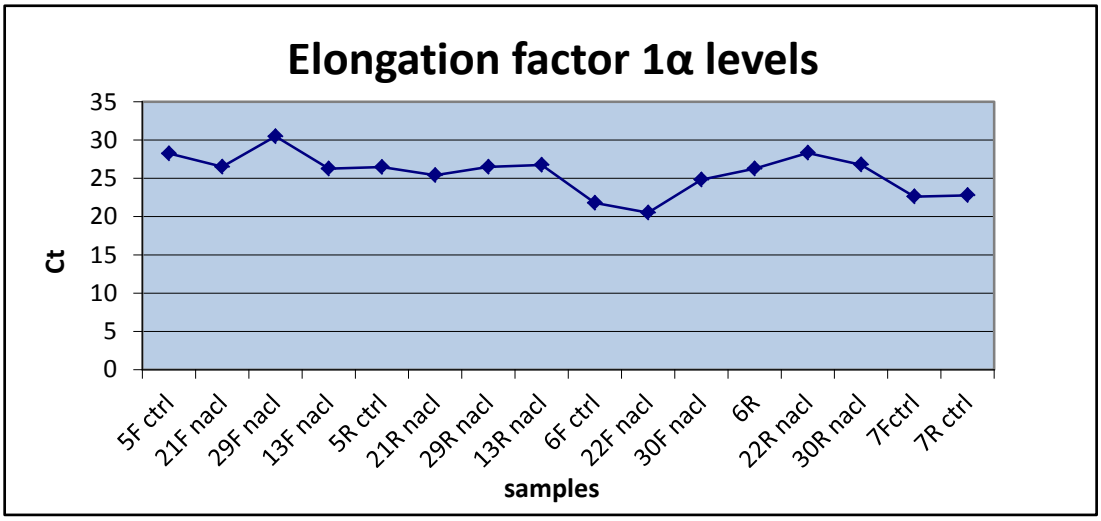




**Fig. 5-18: RNA transcriptional levels of housekeeping gene tested; on the top actin and on the bottom elongation factor 1 α, both presented as Ct mean value in different samples (i.e. 5F= MC leaves at T0, 21F= MC leaves at T2 and so on) in the same qRT-PCR plate.**

### 5.4.0 qRT-PCR

In paragraph 3.6.1 were reported all the primers pairs designed for qRT-PCR, they were tested on respective cDNA target with PCR reactions. Amplifications were performed with cDNA (25 ng) at same conditions established for next real-time step, to verify that these primers worked properly and that PCR products were specific and short as planned on sequences. So that PCR products were loaded on 2% agarose gel with a gene ruler on a side; they confirmed expectations because they were below the 100 bp gene ruler band (Figure 5-19 and 5-20). The qRT-PCR primers designed were reputed suitable for real time gene expression.

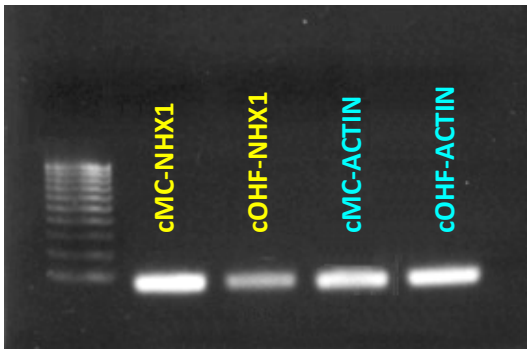



**Fig. 5-19: Bands obtained by PCR in order to test NHX1 primers for qRT-PCR (see paragraph 3.6). From the left: cMC (cDNA) and cFarold®40 (OHF) as templates and the same cDNA used with actin**

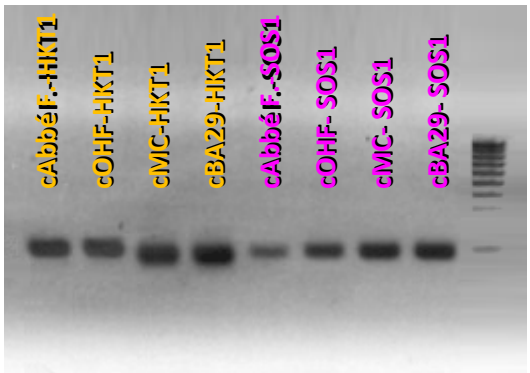



**Fig. 5-20: Bands obtained by PCR in order to test HKT1 and SOS1 primers for qRT-PCR (see paragraph 3.6). From the left: four cDNA (2 for pears and 2 for quinces) in orange for HKT1 and the same but in pink for SOS1. All the bands were below or similar to the 100 bp gene ruler band as expected.**

### 5.4.1 NHX1 expression

The analyses of NHX1 expression were carried out by real-time PCR, as mentioned before in paragraph 3.6.3 using cDNA obtained from RNA of leaves and roots of all four genotypes. The aim of this study was to investigate on different expression of our three genes (NHX1, SOS1, HKT1) in the main plant organs such as: roots, where firstly salinity is sensed, and leaves where the stress signal arrives in order to alter shoot function (Munns and Tester, 2008).

Starting from NHX1, the first gene cloned in this experiment, the expression of this gene at T0 (that was the day before the salt stress imposition = "-NaCl") was compared with the other timings (T2, T3 and T4) when all four genotypes were exposed to salinity (respectively 80 mM at T2 and 90 mM at T3 and T4).

This comparison aimed to understand the behavior of NHX1 in response to salinity in our four genotypes in both the selected organs.

The level of expression of each gene was calculated according to the ΔΔCt method (reported in User Bulletin # 2 ABI PRISM 7700 Sequence Detection System) as described in 3.6.4, where also an example of calculation was reported.

So the amount of target is equal to $2^{-\Delta\Delta Ct}$ and this value represents the mean fold change in expression of the target gene at each time point of the experiment normalized with the reference gene (actin). The normalized amount of target (NHX1N) is a unit-less number that can be used to compare the relative amount of target in different samples. One way to make this comparison is to designate one of the samples as a calibrator (in table 3-30 the brain was the calibrator), in this case the each sample at T0 was selected as calibrator for each genotype.

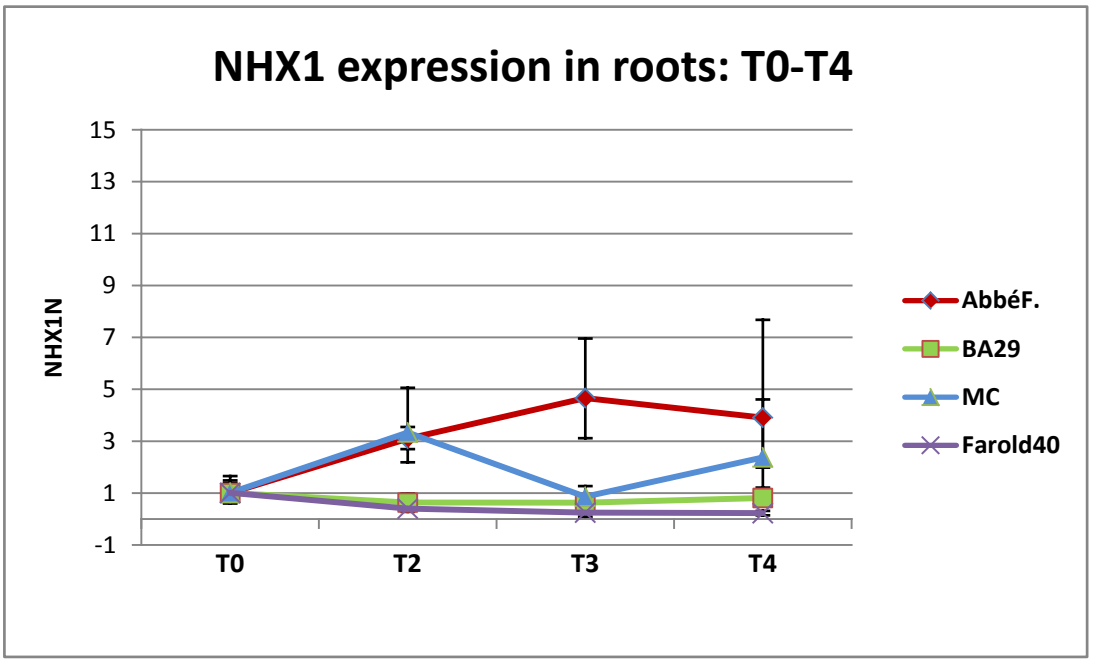
So the expression level of each calibrator is always 1 and comparing it to other samples, it is possible to value if the target gene is up or down regulated as a consequence of the treatment. As appeared in the methodological part, all the values were affected by a standard deviation calculated according to "Standard Deviation Calculation Using the Comparative Method" (User Bulletin # 2 ABI PRISM 7700) that will provide the height of error bars in all next presented Graphs.

In the roots of four genotypes in trial, NHX1 expression differed depending on the genotype, in fact, as Graph 5-23 shows, it seems that in BA29 and in Farold®40, NHX1 had a similar down regulation behavior, while in MC and Abbé Fétel, the

expression of this gene appeared to be up-regulated after the salt imposition. In particular at T2, MC and Abbé Fétel seemed to react similarly in terms of increase of NHX1 expression in roots, but at T3 they moved away each other, indeed the former reported a decrease in NHX1 expression probably as a consequence of the further increase in salt concentration (between T2 and T3 the concentration changed from 80 mM to 90 mM) while the latter remained quite stable according to error bars.

Since the discrimination of each genotype's behavior at each salt treatment timings resulted quite difficult to explain, it has been decided to represent the change in expression in a easier way, that was to compare T0 (named as "-NaCl") against the average of fold change in expression of the target gene in T2, T3 and T4, in order to obtain a unique histogram that gathered the expressions of the gene (in three times) in a single value during the salt stress imposition, regardless of the real concentration. Our interest was to understand if these selected genes were involved in the salt-stress responses of the four genotypes in trial and not exactly their precise behavior following the proceeding of salt stress.

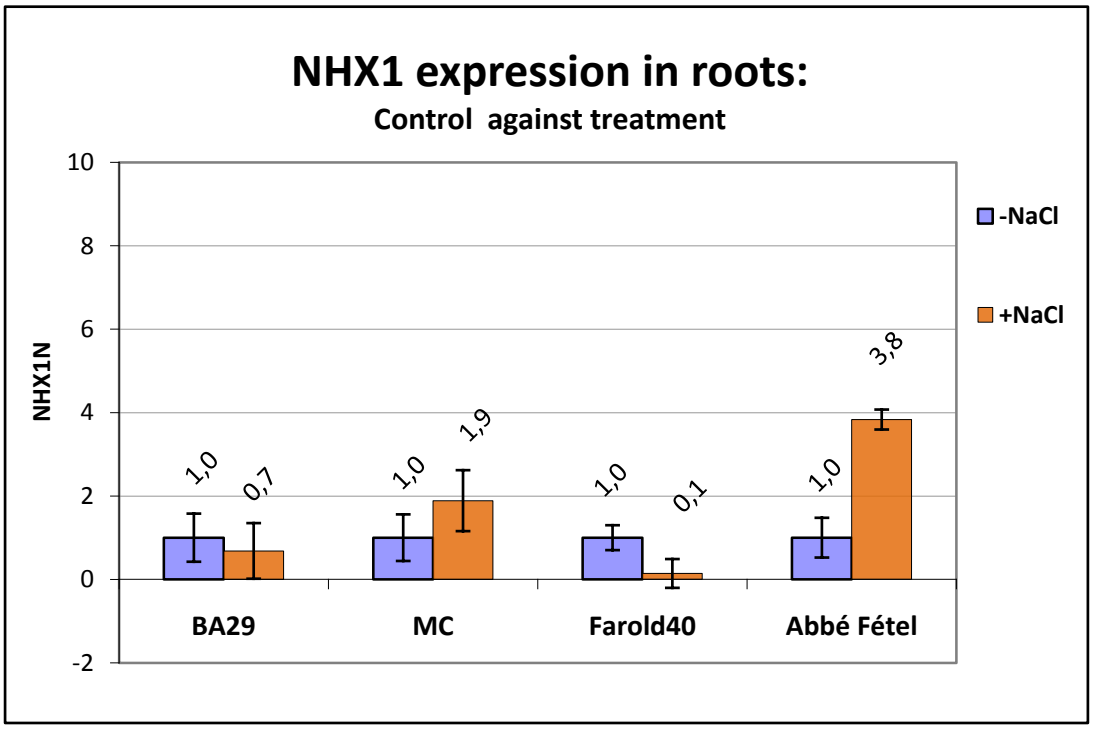
So this "pooling calculation" of gene expression followed the original method with only some variations such as the application of standard errors as error bars.




**Graph 5-23: NHX1 expression in roots of each genotype in all the dates of sample harvesting: T0= before the stress, T2=80 mM NaCl, T3=90 mM and T4=90 mM and last day of experiment. In each point, error bars are drawn corresponding to ΔΔCt+s (positive) and ΔΔCt-s (negative) as calculated in User Bulletin # 2 ABI PRISM 7700.**

In this way the NHX1 expression in roots appears more clear (Graph 5-24). In fact in BA29 roots, effectively NHX1 did not change significantly its expression and at the end this was valid also for MC, considering the error bars.

In pears instead NHX1 expression appeared clearer in fact, in Farold®40 the down-regulation of the target gene was convincing and as meaningful as the up-regulation of the same NHX1 in Abbé Fétel roots.




**Graph 5-24: NHX1 expression (normalized with actin using ΔΔCt method) in roots of four genotypes. Histograms in blue represent expression of the gene at T0 (-NaCl) and orange histograms describe the mean value of expression in 3 dates of treatment (T2, T3, T4) for the same gene (+NaCl).**

It is well known that NHX1 is responsible for the compartmentalization of sodium inside the vacuole and this is a really important mechanism that takes part to salt stress responses in order to prevent the accumulation of this ion inside the symplast. As mentioned before evidence showed that this gene in *Arabidopsis* is responsive to hyperosmotic stress and not only to salinity and that it is also constitutively expressed in normal conditions and also involved in potassium accumulation into vacuoles (Bartel and Sunkar, 2005; Zahran et al., 2007; Hanana et al., 2007). In *Gossipium hirsutum,* it has been demonstrated that GhNHX1 responded with an up-regulation to treatments with 400 mM KCl and 100 µM ABA confirming a non-specific salt stress response (Wu et al., 2004).

Deletion mutants have shown that the function of AtNHX1 is not restricted to salt tolerance (Apse et al., 2003).

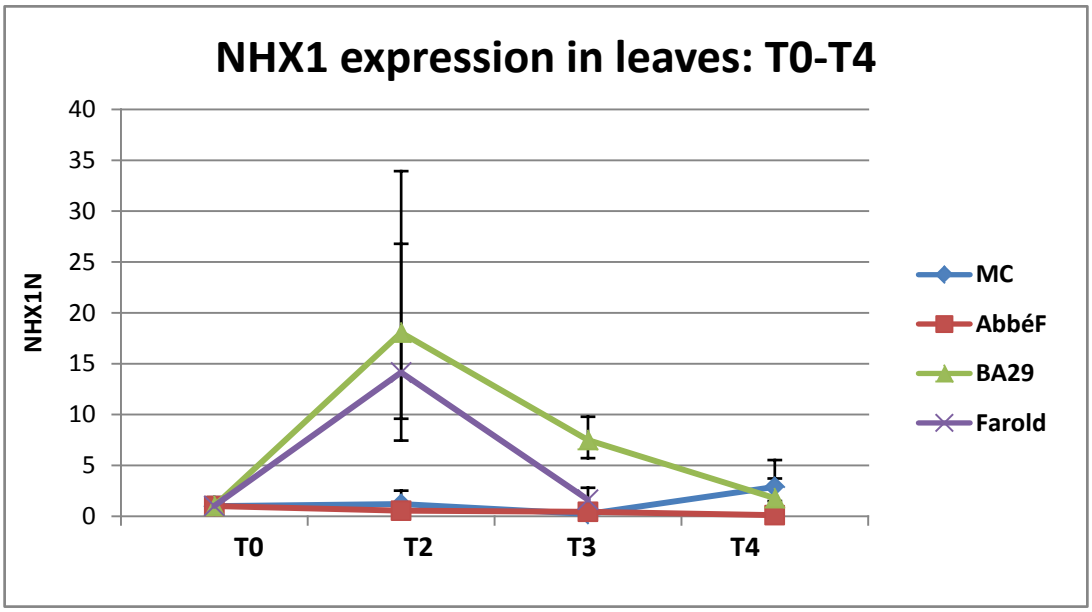
Maybe the different trends of NHX1 expression in roots among genotypes in this experiment reflected these observations, for example BA29 can be one of that species where NHX1 works constitutively and Abbé Fétel can represent a plant that adopted the vacuolar sequestration of sodium as "salt-defensive strategy" to try to maintain osmotic balance. For what concerns NHX1 expression in MC roots, its trend in Graph 5-23 suggested that this gene was up-regulated at T2 as consequence of one week of salt stress, but at T3 it decreased maybe as result of the increment of salinity concentration. At T4 in fact NHX1 expression seemed to start rising again, but according to error bars this probably was not meaningful. This trend advised that in MC roots, NHX1 expression can be dependent on the level of adaptation of this genotype to salinity, indeed after the first week of salt stress this gene appeared to be involved in the response to salt stress. In literature is often reported that experiments overexpressing AtNHX1 can provide more salt tolerant plants thanks to the increase in activity of this tonoplast sodium transporters (Apse et al., 1999; Wang et al., 2003). We should take in consideration that in the present experiment we are dealing with wild type plants and we are considering only the normal expression of this gene under salinity in generally classified sensitive plants. It has been reported also that this gene could be constitutively active, but also up regulated by high salinity. The down regulation in NHX1 expression in Farold®40 roots under salt-stress can remind the behavior of *Plantago media,* a sensitive species that did not show any NHX1 activity (Blumwald et al., 2000).

It is important to point out that AtNHX1 transcripts are widely distributed in plants excepts for roots tips (Shi and Zhu, 2002; Martinoia et al., 2007), if this observation, made in the model plant *Arabidopsis*, can be transferred to other plant such as quinces and pears, we should considered that for RNA extraction have been utilized fine roots pooled together without any discrimination between tip or inner part of the roots. In some cases where so precise details of a gene are available, it might be interesting to investigate expression not only in organs (like in our case) but also in smaller sub parts of organs, because gene expression can widely differ among dissimilar differentiated cells.

NHX1 expression could be read at the light of the $K^+/Na^+$ ratio; in a specific organ (i.e. roots) in fact an up regulation of NHX1 can imply a higher $K^+/Na^+$ ratio in the cytosol, because the increase in activity of this antiporter can ameliorate the cytosolic condition reducing the concentration of the toxic ion $Na^+$ and favoring potassium content. *Vice versa* a NHX1 down regulation can mean that sodium sequestration was not necessary anymore because a $K^+/Na^+$ ratio was already high or, one step back, that the considered genotype possesses a "sodium excluder" capacity. In our case the NHX1 expression at root level is referred only to fine roots and the $K^+/Na^+$ ratio shown in Graph 5-21 for the five organs evidenced that in fine roots not significant variations are present and additionally this ratio is the so called "surrogate" ratio, because it isn't able to discriminate between compartments.

So it could be a good idea to investigate NHX1 expression correlating it with the $K^+/Na^+$ ratio measured with the new method capable to discriminate each of the two ions into organelles and cytosol (Shabala and Cuin, 2008).

Also at leaf level NHX1 expression differed among four genotypes in trial, in fact, as Graph 5-25 shows, Abbé Fétel did not registered important variations in NHX1 expression, neither in T4 where RNA was extracted from new leaves grown under




**Graph 5-25: NHX1 expression in leaves of each genotype in all the dates of sample harvesting: T0= before the stress, T2=80 mM NaCl, T3=90 mM and T4=90 mM and last day of experiment. In each point, error bars are drawn corresponding to ΔΔCt+s (positive) and ΔΔCt-s (negative) as calculated in User Bulletin # 2 ABI PRISM 7700. For Farold®40, T4 value was not available sampled because several small leaves were growing but not enough to extract RNA.**

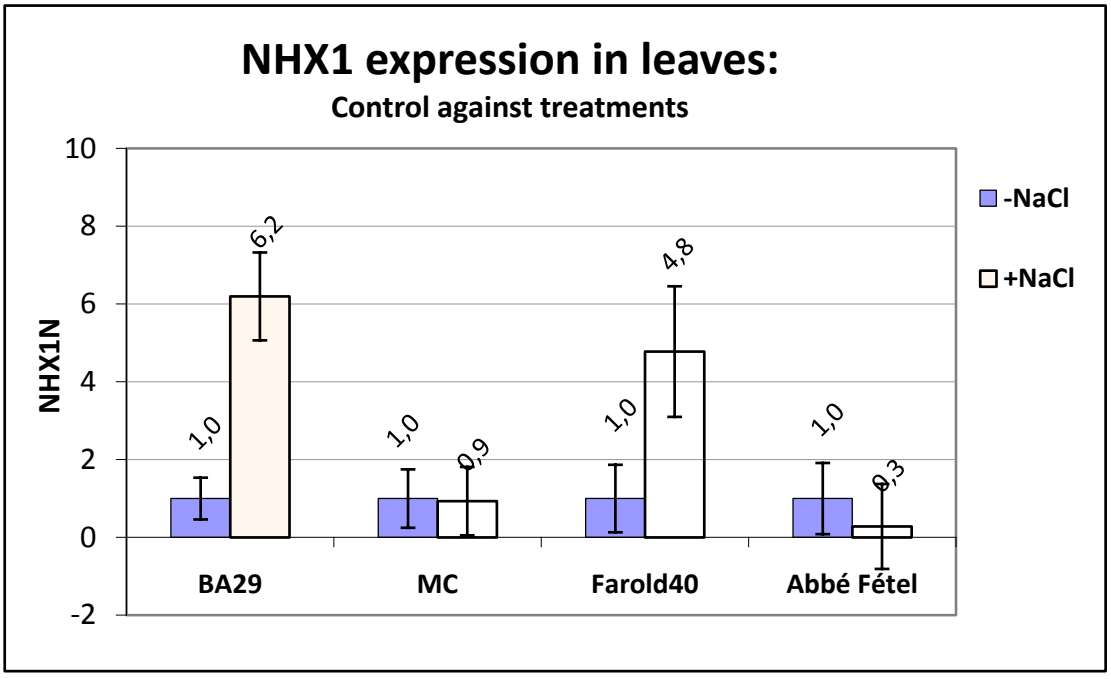
salinity. In MC leaves, NHX1 did not change significantly in comparison with T0, only at T4 it seemed to be up-regulated, but probably not in a significant way.

Interesting trends were recorded for BA29 and Farold®40 because both presented an increase in NHX1 expression in leaves as a consequence of salinity (at T2) followed by a decrease at T3 (90 Mm NaCl) and a further decrease in T4 only for BA29 (T4 sample for Farold®40 was not harvested).

NHX1 expression "pooling" together T2, T3 and T4, clarified the general behavior of each genotype during the three-weeks-salt stress. Graph 5-26 shows that BA29 and Farold®40 registered at leaf level a general and significant up-regulation of NHX1, while the other two genotypes did not show any meaningful variations in the comparison between samples at T0 (-NaCl) and samples under salinity.

To explain the absence of induction of NHX1 expression in MC and Abbé Fétel it can be hypothesized that in that tissue they did not need to up-regulate NHX1 maybe because, like some halophytic species, no more sodium accumulates in the cytosol or that they adopted an alternative way to get rid of excess of $Na^+$.

It has been shown that SOS pathway is also involved in the regulation of the tonoplast $Na^+/H^+$ antiporter activity and in particular SOS2 seemed to be




**Graph 5-26: NHX1 expression (normalized with actin using ΔΔCt method) in leaves of four genotypes. Histograms in blue represent expression of the gene at T0 (-NaCl) and orange striped histograms describe the mean value of expression in 3 dates of treatment (T2, T3, T4) for the same gene (+NaCl).**

responsible for NHX1 increase in activity if added "*in vitro*" to tonoplast vesicles (Qiu et al., 2004). According to this finding, we can also think that if SOS2 is able to regulate NHX1, a connection between SOS1 and this tonoplast antiporter can exist too, since SOS2, activated by the interaction with SOS3, leads to the activation of SOS1. So if SOS1 is up regulated probably also NHX1 is more expressed in the same tissue. This observation was true in leaves of BA29 and Farold®40, where it has been reported an increase in NHX1 activity; in the same organ in fact also an up-regulation of SOS1 gene was noticed (SOS1 expression will be discussed in next paragraphs).

Thinking to the sequestration of sodium under salinity in these four genotypes we can just underline that at roots level a NHX1 up-regulation that lasted for all the three-weeks-salt stress was found only in Abbé Fétel, while MC had an increase only in T2. At leaf level, instead the other two genotypes, BA29 and Farold®40, emerged showing an increase in compartmentalization activity into vacuoles.

Despite the fact that at leaves levels the salt treatment did not significantly alter the potassium content in Farold®40, Abbé Fétel and BA29, while it decreased with $p<0.01$ in MC, it could be hypothesized that the up-regulation of NHX1 at leaf level in BA29 and Farold®40 is linked to the slight increase (not resulted statistically significant in table 5-12) of $K^+$ content. This potential explanation came from observations done by Hanana and colleagues (2007), they in fact proved that NHX1 in *Vitis vinifera* showed higher affinity for $K^+$ than for $Na^+$ through measurement of rates of cation-dependent $H^+$ efflux using fluorescence quenching (Hanana et al., 2007). This result confirmed the duality of NHX1 role in plant, it acts to maintain potassium homeostasis, but also to sequester sodium into vacuoles. In several studies moreover, a greater $K^+$ leaf concentration was found linked to the tolerant phenotype (Pardo et al., 2006).

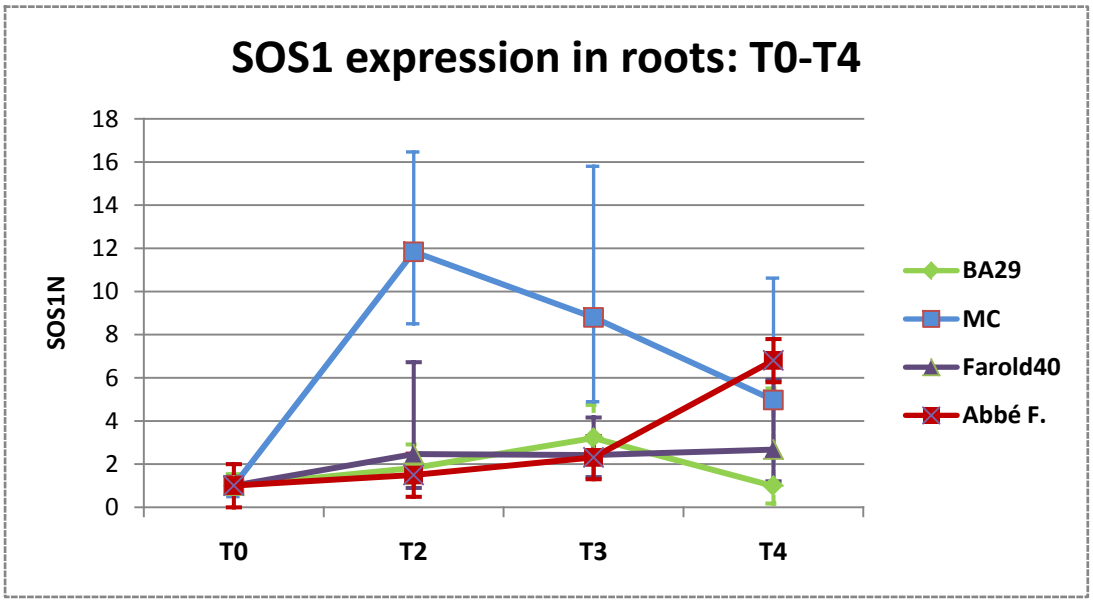
Contrary to expectations, the two pear genotypes did not show a common behavior in NHX1 expression under salinity in both tissues considered and the same was noticed for quinces.

In general, NHX1 expression was in some cases induced by salt stress in roots and in leaves, but it showed highest levels in leaves, this result was confirmed by a similar study performed by Wu and colleagues (2004) using salt-tolerant cotton variety

ZM3. They found out by Northern blot that at 250 mM, NHX1 expression in roots was lower than that reported in stems and leaves. Moreover they understood that GhNHX1 mRNA accumulation was more abundant in seedlings treated with higher concentration of NaCl (250 and 400 mM), in fact up to 150 mM NaCl the probe signal of Northern was quite weak and also that GhNHX1 was rapidly induced by salt stress in fact its expression did not change from 3 to 24 hours (Wu et al., 2004). The maximum level of expression of CgNHX1 (*Chenopodium glaucum*) was reached similarly to GhNHX1 at 400 mM NaCl concentration (Li et al., 2008). At the light of these observations it cannot be excluded that also the other genotypes, where no variation in expression was detected in salt-stressed samples, could present an up-regulation of NHX1 under higher NaCl concentration.

### 5.4.2 SOS1 expression

The analyses of SOS1 expression at root level pointed out newsworthy trends during the three-weeks-salt stress impositions for genotypes in trial. From Graph 5-27 it appears evident how SOS1 expression in MC roots diverged from the behavior of the same gene in the other genotypes at the same date of sampling. At T2 in fact, when the salt-stress had been imposed for 13 days, SOS1 in MC roots was clearly up-regulated in such a way that resulted significantly different from the slight increase in expression reported for the other three genotypes (see error bars at T2).




**Graph 5-27: SOS1 expression in roots of each genotype in all the dates of sample harvesting: T0= before the stress, T2=80 mM NaCl, T3=90 mM and T4=90 mM and last day of experiment. In each point, error bars are drawn corresponding to ΔΔCt+s (positive) and ΔΔCt-s (negative) as calculated in User Bulletin # 2 ABI PRISM 7700.**

At T3 the situation of SOS1's up regulation exclusively higher in MC roots is maintained with not meaningful differences among data obtained at T2 for the same genotype and organ. In this date the NaCl imposition reached 90 mM, but this last increase in salt concentration seems not to have affected SOS1 expression for MC roots. Also the other three genotypes manifested various increases in SOS1 expression that however, according to the error fluctuations were lower than that estimated for MC.

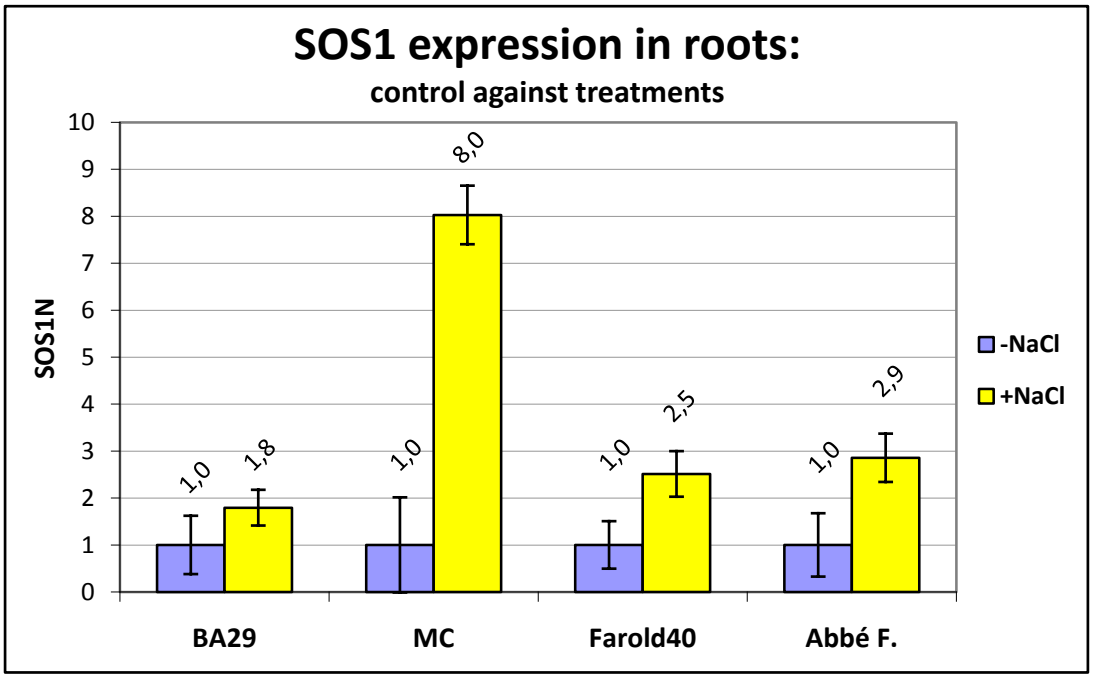
The situation at T4 showed that SOS1 expression in MC apparently seems to decrease, but error bars did not allow distinguishing between the state at T3 and at T4. In general, in Farold®40, Abbé Fétel and BA29, at T4, the expression of this gene,

fundamental for the salt stress tolerance, resulted not significantly different from SOS1 transcripts level reported in MC at the same date. A peculiar behavior emerged in Abbé Fétel at T4; in fact SOS1 was up-regulated respect to the level of expression reported at T3 for the same species. SOS1 expression in Abbé Fétel roots at T4 resulted higher than that occurred in BA29. The rising SOS1 expression in roots of Abbé Fétel seems to work against the final trends presented in the other genotypes for the same gene. A potential explanation of this behavior can be linked to the renewal of new healthy leaves from Abbé Fétel during the last part of the experiment as it appears in figure 5-3. In effect at T3 none plant of this species presented any new leaves, rather all the older foliage was dead, and after 6 days of persisting salt stress- at T4- several new leaves appeared as a presumable adaptive response to salinity. These two observations put together led us to suppose that up-regulation of SOS1 in the roots of this genotype permitted a new growth in this quite high salt concentration.

On the other hand, also Farold®40 reported a similar behavior regarding the formation of new leaves at T4 (see again figure 5-3) and in this case the increase of SOS1 expression did not appear so evident as for the other pear genotype (Graph 5-26), but indeed the difference between the two genotypes for SOS1 up-regulation in the comparison within control (-NaCl) and treatments pooled together (as described for NHX1) did not differ very much, highlighting however a higher increase in SOS1 level in Abbé Fétel roots than that in Farold®40 radical system (Graph 5-28).

The decrease of inhibition of cell expansion and lateral bud development are reported as plant mechanisms taking part of the so defined “osmotic tolerance”, but candidate genes responsible for this aspect are still unknown (Munns and Tester, 2008). Even though SOS1 was classified as a candidate gene involved in the accumulation of sodium in shoots (jointly to HKT) and leading to an increase in osmotic adjustment as osmotic tolerance mechanism (Munns and Tester, 2008), it may be possible that this function might contribute also to the vegetative renewal driven by still unknown determinants.

To support this hypothesis, Shi and co-workers (2002b) reported that transgenic *Arabidopsis* plants overexpressing SOS1 showed a better root growth, PSII activity and survival under salt stress conditions as parameters that led to improve the salt tolerance (Bartels and Sunkar, 2005; Shi et al., 2002b).




**Graph 5-28: SOS1 expression (normalized with actin using ΔΔCt method) in roots of four genotypes. Histograms in blue represent expression of the gene at T0 (-NaCl) and yellow histograms describe the mean value of expression in 3 dates of treatment (T2, T3, T4) for the same gene (+NaCl).**

Even though MC plants exposed to salt stress did not behave like pears, these considerations about the possible role of SOS1 can be extended to MC's situation. In fact the so evident up regulation of the antiporter responsible for removing of sodium from the cell (Shi et al., 2002a) in MC roots from the beginning of the stress exposure let us think that this gene can be closely involved in the manifest delay of the onset of salt stress symptoms in MC foliage. The higher level of SOS1 expression in MC roots respect to those registered for the other genotypes, led to suppose that SOS1 increased $Na^+$ efflux and thereby reduced the sodium accumulation in MC cells. This was only partially confirmed by the lower amount of sodium in salt stressed MC coarse roots respect to the $Na^+$ content in the same organ in other pear genotypes (BA29 resulted similar to MC, see Tab. 5-13).

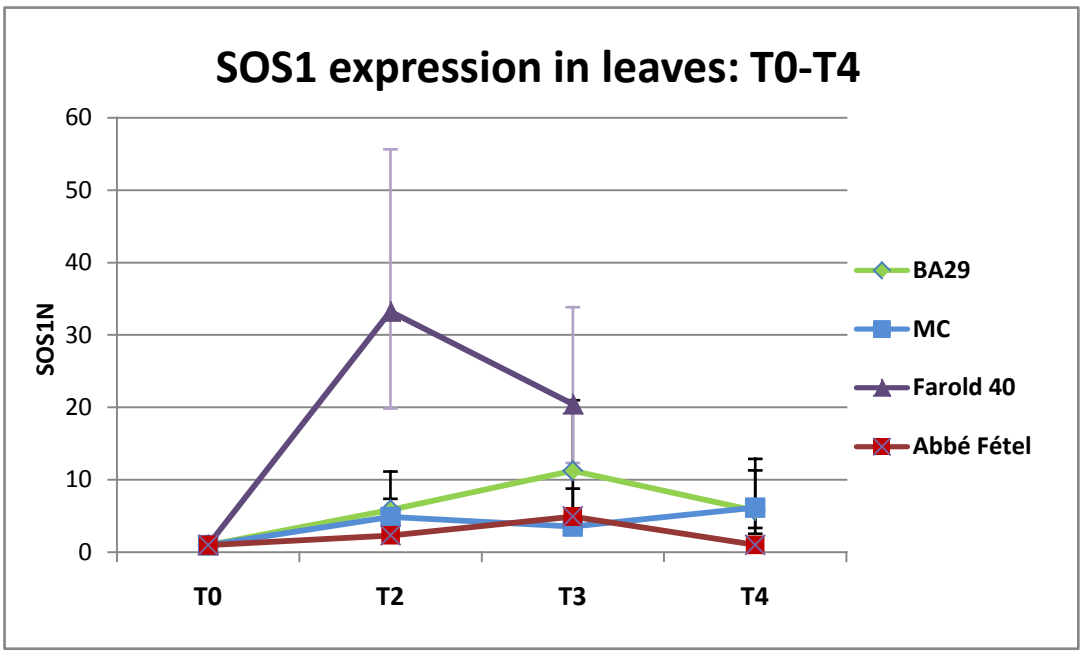
In general, looking at Graph 5-28 it appears clear that in all the genotypes with exception for BA29, at root level an up-regulation of SOS1 (variable according to genotypes) was registered in the period of stress from T2 to T4. This suggested that also in our species in trial the SOS1 emerged as a key gene involved in salt stress responses.

However the behavior and the defined role of SOS1 are still largely discussed also for model plants as mentioned in paragraphs 1.2.2.3.c and 1.2.2.4. Contrary to NHX1, AtSOS1 resulted to be mainly expressed in epidermal cells at the root tips and in the parenchyma cells at the xylem/symplast boundary of roots for the xylem loading, but also in stems and leaves.

In previous paragraph, on results of mineral content analysis, it has been hypothesized that pears were able to accumulate more sodium in coarse roots than quinces; this idea should be reviewed looking also at SOS1 expression in roots. But as described in methodological part RNA extraction was carried out from fine roots, so the results of SOS1 expression were referred to fine roots and probably, considering the specificity of behaviour according to the different part of the root for this sodium transporter (see inner and outer part of the root and knock-out experiment on SOS1 par. 1.2.2.4) it might be risky and fake thinking of roots in general without maintaining distinctions even if so easy as coarse and fine roots.

At leaf level for all genotypes, an increase in SOS1 expression was registered during the three-weeks of salt exposure (Graph 5-29). Immediately at T2, in Farold®40 it has been registered a significant increase in SOS1 expression respect both to T0 SOS1 level in its leaves and to the SOS1 levels of the other genotypes at the same date.

At T3 instead, the error fluctuations cancelled the meaning of differences among the four genotypes and the same was noticed at T4; where the value referred to Farold®40 missed because, as already hinted, the sampling of live leaves was not possible as the growing new leaves were still few and small to be enough for RNA extraction.




**Graph 5-29: SOS1 expression in leaves of each genotype in all the dates of sample harvesting: T0= before the stress, T2=80 mM NaCl, T3=90 mM and T4=90 mM and last day of experiment. In each point, error bars are drawn corresponding to ΔΔCt+s (positive) and ΔΔCt-s (negative) as calculated in User Bulletin # 2 ABI PRISM 7700. For Farold®40, T4 value was not available.**

In graph 5-30 instead the SOS1 expression trends were represented comparing the SOS1 expression level without salt stress against the expression of this sodium antiporter in the samples exposed to NaCl up to 90 mM, from T2 to T4, pooled together as done for the other genes.

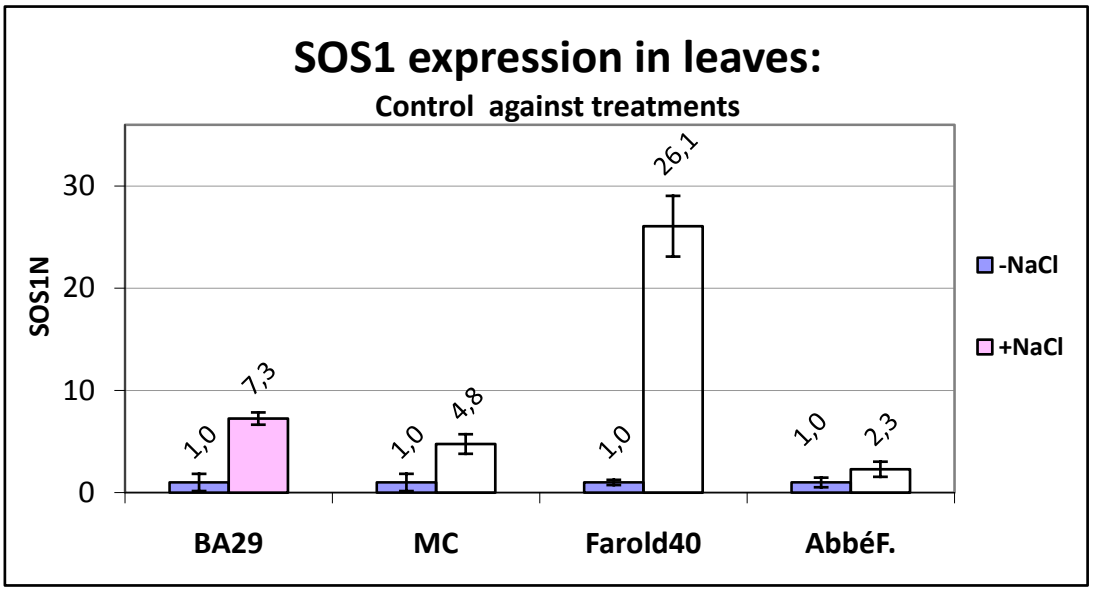
Looking at these data it appeared evident the highest SOS1 expression in leaves was found in Farold®40, followed by the SOS1 increase in transcripts in both quinces.

In Abbé Fétel, at leaf level, it seems that SOS1 did not covered any role in the response of the genotype to salt stress, while its expression increased at roots level.

It is really difficult to explain SOS1 expression at leaf level, because it is not completely clear the destiny of sodium expelled from cells in leaves.

Shi and colleagues (2002a) claimed with the SOS1 promoter-GUS gene fusion experiment that SOS1 has an important role in regulating the sodium transport in long distance, acting in loading/unloading xylem. Moreover, when salinity is moderate it seems that SOS1 works loading sodium into xylem to storage $Na^+$ ions in mesophyll cells with the help of NHX1, but when the salt concentration increased the capacity of storage of mesophyll cells is rapidly saturated. So in this case, according to the model proposed by Shi and co-workers (2002a) SOS1 can function

both to restrict the $Na^+$ uptake from root tips and to retrieve $Na^+$ from the xylem in the mature roots (Shi et al., 2002a).




**Graph 5-30: SOS1 expression (normalized with actin using ΔΔCt method) in leaves of four genotypes. Histograms in blue represent expression of the gene at T0 (-NaCl) and pink striped histograms describe the mean value of expression in 3 dates of treatment (T2, T3, T4) for the same gene (+NaCl).**

Increase in SOS1 expression in Farold®40 leaves, respect to values in the other genotypes, can provide an explanation for the regeneration of new leaves after the complete death of the older ones. In fact SOS1 can potentially have expelled sodium back to mature roots, as proposed by Shi et al., (2002a) in order to try to preserve the upper part of the plant, to guarantee the water uptake and consequently to allow the growth recovery, even if salt stress was still applied. Following this reasoning, if this retrieval of sodium occurred in salt stressed-Farold®40 plants, we should find a higher amount of Na content in coarse roots of this genotype in comparison with that present in the other plants in trial. Looking again at the sodium content analyzed in stressed plants at the end of the experiment we can pointed out that effectively Farold®40 presented in coarse roots the second highest amount of sodium after that found in Abbé Fétel.

SOS1 in fact over-expressed in *Arabidopsis* plants improved salt tolerance with reduced $Na^+$ accumulation in shoots. SOS1 probably retrieves $Na^+$ from the xylem (Shi *et al.*, 2002b). In *Thellungiella*, the halophytic *Arabidopsis* relative, TheSOS1

seems to act both excluding $Na^+$ from roots tips, and also protecting cells in elongation zone during first salt stress stages (Bressan, personal communication). It has been reported that the upward flux of sodium ions to shoots appeared to be more constrained and regulated in *Thellungiella* than in its glycophyte relative *Arabidopsis* (Wang et al., 2006). A possible explanation can be the higher level of SOS1 mRNA in *Thellungiella* than in *Arabidopsis* under normal unstressed condition that is able to increase in both roots and shoots if plants are exposed to salt stress (Bressan, personal communication).

A study carried out in *Populus euphratica*, a poplar species known for its high salt tolerance, demonstrated through a Western blot analysis that the PeSOS1 protein was significantly up-regulated (5 to 10 times more than control) in leaves of plants treated with 200 mM NaCl for 24 and 48 hours. Salt also was able to determine the up-regulation of $H^+$-ATPase in the plasma membrane that works to provide the necessary proton force to allow the antiporters SOS1 activity (Wu et al., 2007).

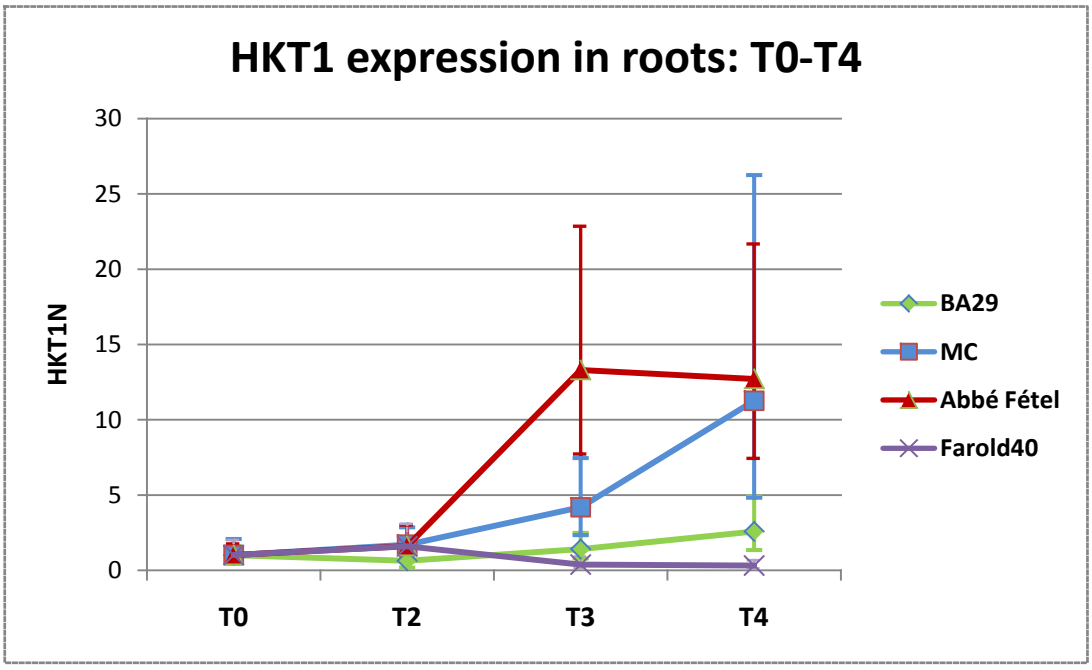
However, the role of SOS1 in plant salinity tolerance remains uncertain, and also its involvement in halophytes salt tolerance are still completely unknown. Measurement of the effects of SOS1 knock-out on long distance transport of $Na^+$ is confused because most experiments were done in non-transpiring conditions (Munns and Tester, 2008).

### 5.4.3 HKT1 expression

The last gene to study in this experiment participates to the entrance of sodium inside the cell, together with NSCC and LC1 transporters, other possible ways for $Na^+$ to influx (Botella et al., 2005).

qRT-PCR analyses carries out on roots samples at different times for this gene showed that at T2 in all the four genotypes, HKT1 did not increase respect to control levels. At T3, the situation changed completely, in fact Abbé Fétel reported a significant up- regulation of HKT1 that lasted more or less unaltered at T4 (Graph. 5-31). When a HKT1 increase was reported in Abbé Fétel (T3), at the same time in BA29 and Farold®40 HKT1 mRNA levels did not vary considerably.

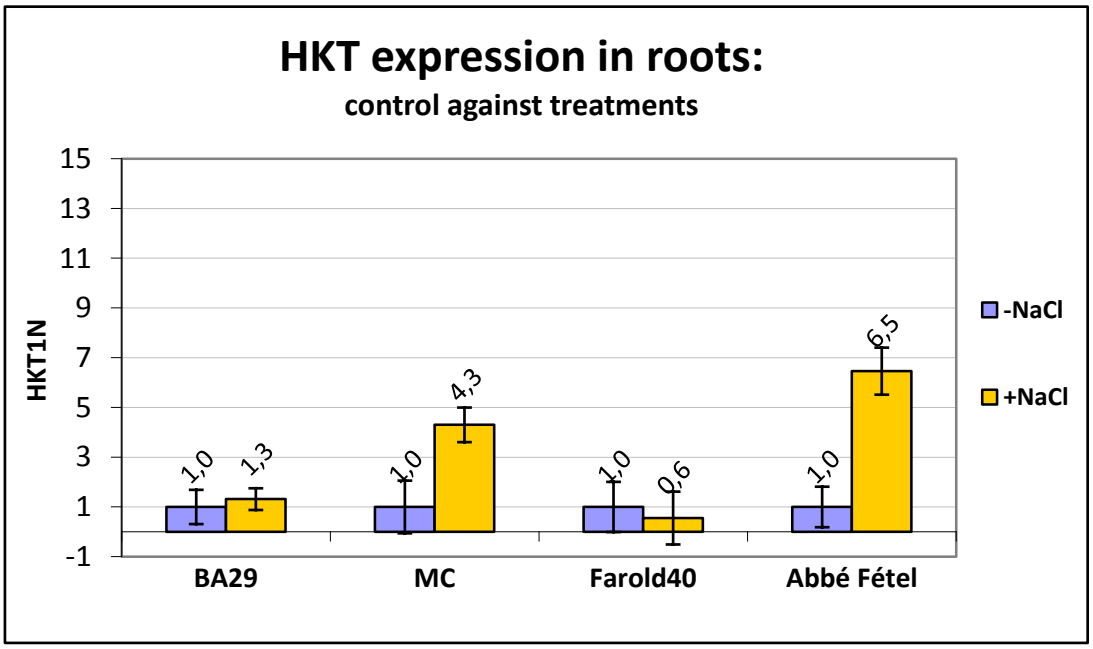
In MC roots instead a further trend emerged; in fact HKT1 expression seemed to gradually increase with the progress of the salt stress. In fact, comparing HKT1 mRNA level at T0 and the same at T4, the significant increase in expression appeared evident, probably as consequence of salinity (Graph 5-31).




**Graph 5-31: HKT1 expression in roots of each genotype in all the dates of sample harvesting: T0= before the stress, T2=80 mM NaCl, T3=90 mM and T4=90 mM and last day of experiment. In each point, error bars are drawn corresponding to ΔΔCt+s (positive) and ΔΔCt-s (negative) as calculated in User Bulletin # 2 ABI PRISM 7700.**

If we visualize data according to the pooling of treatments (Graph 5-32), the situation point out easily that BA29 and Farold®40 did not register a particular behavior linked to HKT1 during the three weeks of salinity. So we can suppose that for these genotypes either this gene works as in normal condition despite salinity or that for them it doesn't represent a further problem since HKT1 did not increase with its activity the influx of sodium in their roots.

Following this suppositions we decided to not discuss further on about HKT1's behavior in roots of these two genotypes. Returning to Graph 5-32, it has been noticed that HKT1 expression was higher in Abbé Fétel salt stressed roots than in MC ones.




**Graph 5-32: HKT1 expression (normalized with actin using ΔΔCt method) in roots of four genotypes. Histograms in blue represent expression of the gene at T0 (-NaCl) and orange histograms describe the mean value of expression in 3 dates of treatment (T2, T3, T4) for the same gene (+NaCl).**

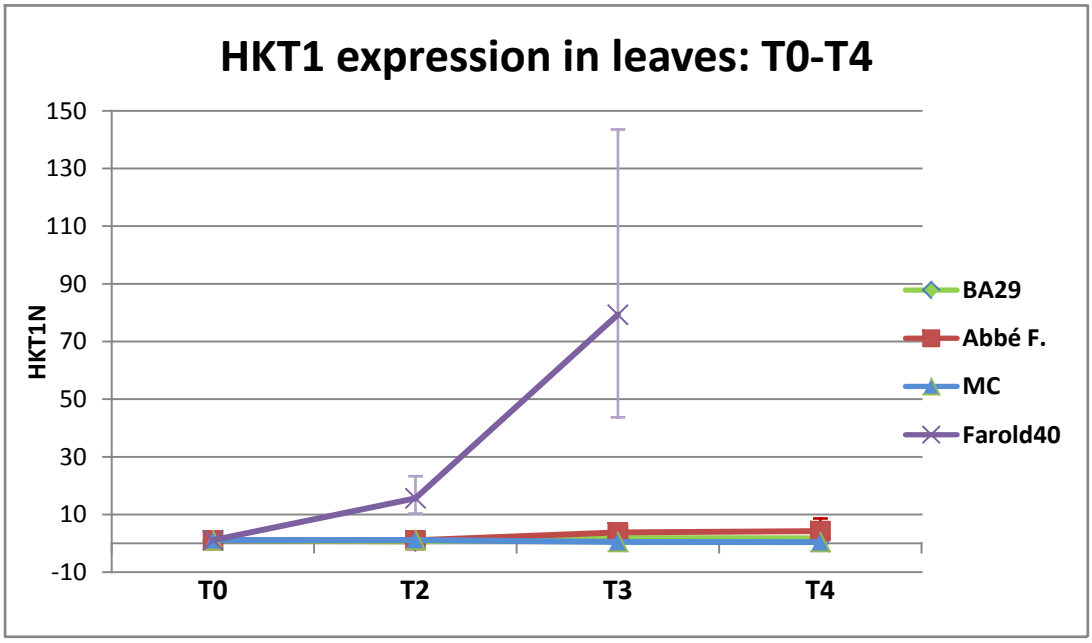
Firstly this situation let think that Abbé Fétel had a higher HKT1 mRNA levels under salt stress that implied an elevated amount of sodium in roots. This seemed to be confirmed by our mineral analyses that reported for Abbé Fétel coarse roots the highest content of sodium registered among the four genotypes subjected to salt stress. Moreover in Graph 5-21 it appeared that $K^+/Na^+$ ratios in coarse roots of Abbé Fétel and Farold®40 were the lowest among the four evaluated; this

established that, despite the original function of HKT1 as high-affinity-potassium transport, in this case it did not work to promote potassium entrance.

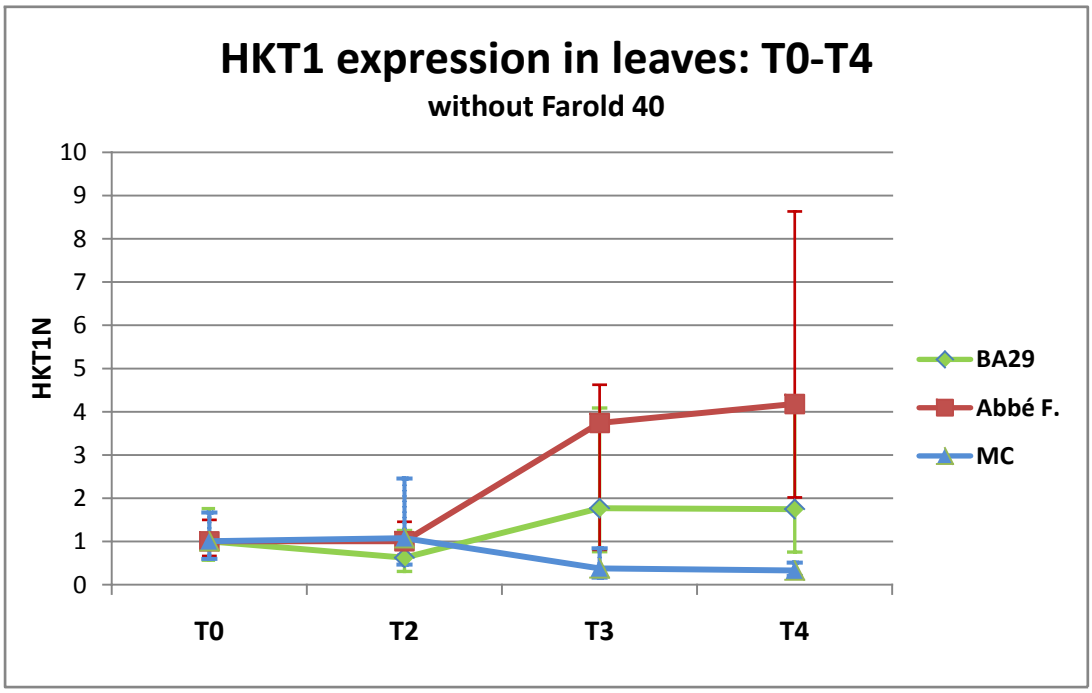
But, considering that recent studies on *Arabidopsis* claimed that, despite HKT1 drives sodium inside the cell, it seems that at whole plant level, its main role is reducing the net $Na^+$ influx into shoots by retrieving it from xylem (Davenport et al., 2007; Munns and Tester, 2008). Reviewing the previous hypothesis at the light of this recent finding, it is possible to assume that HKT1 up-regulation in Abbé Fétel roots under salinity can signify a reduction of sodium accumulation into the shoots. This idea can be positively supported by the evidence of the regeneration of new leaves occurred at some days from the end of the experiment.

With regard to MC roots, the HKT1 expression trend showed a gradual increase in the HKT1 mRNA level, this led to think that probably for this genotype the action of HKT1 in retrieving Na from xylem is not the first mechanism to adopt in case of salinity stress. In fact looking at Graph 5-27 about the SOS1 expression in roots it was evident that in MC, SOS1 activity covered a more important role under salinity than in the other genotypes.

Some authors reported that the complex SOS3-SOS2, acting in SOS pathway as activator of SOS1, negatively regulates the AtHKT1 activity (Zhu, 2003; Gong et al., 2004). This observation can be used to motivate the slow increase of HKT1 in MC roots respect to that of Abbé Fétel roots. In fact at T2, SOS1 expression in MC roots reached its maximum for that organ and our genotypes, while HKT1 expression in MC roots was still almost at a steady state similar to T0. It is possible to postulate a probable HKT1 inhibition by the complex that triggers SOS1, this because if SOS1 is so expressed, it means that upstream the complex SOS3-SOS2 is activated. The SOS1 predominance in MC roots as mechanism to respond to salinity can be confirmed by the fact that in coarse roots of MC salt-stressed plants, the highest $K^+/Na^+$ ratio was registered among all four genotypes. This fact can support the idea that if SOS1 plays in removing sodium from cell consequently the ionic balance will move to favor potassium and the ratio will increase respect to both pears (Graph 5-21).




**Graph 5-33: HKT1 expression in leaves of each genotype in all the dates of sample harvesting: T0= before the stress, T2=80 mM NaCl, T3=90 mM and T4=90 mM and last day of experiment. In each point, error bars are drawn corresponding to ΔΔCt+s (positive) and ΔΔCt-s (negative) as calculated in User Bulletin # 2 ABI PRISM 7700.**




**Graph 5-34: Graph 5-32: HKT1 expression in leaves of 3 genoytpes excluding Farold®40 in all the dates of sample harvesting: T0= before the stress, T2=80 mM NaCl, T3=90 mM and T4=90 mM and last day of experiment. In each point, error bars are drawn corresponding to ΔΔCt+s (positive) and ΔΔCt-s (negative) as calculated in User Bulletin # 2 ABI PRISM 7700.**

At leaf level, HKT1 expression is more difficult to understand. As it appears in graph 5-33, HKT1 expression followed two different trends, apparently in Abbé Fétel, MC and BA29 no evident variations under salinity were reported, while in Farold®40 this $K^+$- $Na^+$ transporter demonstrated to be highly up-regulated, more than 10-fold respect to mRNA levels in the other genotypes.

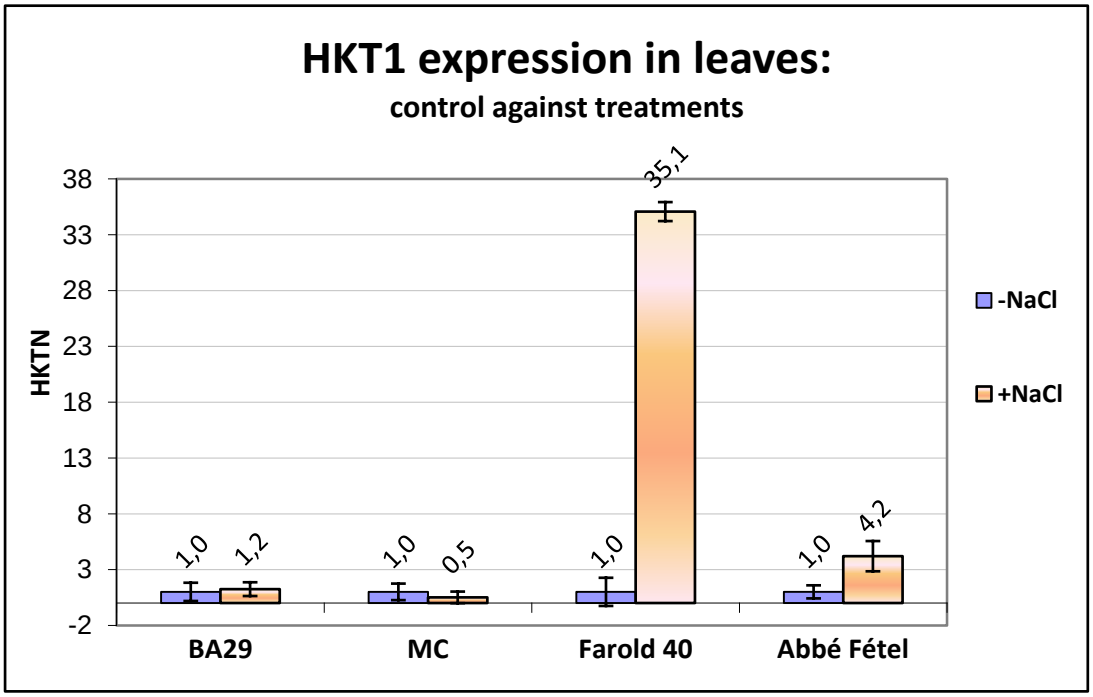
For this genotype the sample at T4 is absent as explained before, so the last result of HKT1 expression is referred to T3. Since this difference in values among HKT1 in Farold®40 leaves and in the other genotypes did not allow to analyze deeper the other trends, we decided to exclude HKT1 expression in Farold®40 leaves and represent data again in Graph 5-34 with the remaining three genotypes.

Here it is clear that at T2 HKT1 expression did not change among genotypes and the same can be said for T3 data, because of errors bars applied to mean values. In the last timing of sampling (T4) it can be seen that HKT1 mRNA levels were different: in MC in fact this gene seemed down- regulated and however its expression resulted lower than that found in Abbé Fétel.

In general, at leaf level it seemed that in both pears, HKT1 was up-regulated by salinity (values were however completely different each other), while in both quinces, HKT1 expression appeared not to be induced by treatment applied because variations were not meaningful respect to control at T0 (Graph 5-35).

Even though HKT1 functions in roots were well studied in *Arabidopsis*, its function in shoots is still obscure and the discussion about the two hypotheses of retrieval from xylem and recirculation from phloem is still open (Munns and Tester, 2008).

Evidence from other studies showed that *hkt1* mutants *Arabidopsis* plants presented a higher accumulation of sodium into shoots than wild type plants and that at root level these *hkt1* mutants accumulated less $Na^+$ than wild types. In the same experiment also *sos1* mutants and *sos1-hkt1* double mutants were evaluated and it has been noticed that the former showed a three-fold accumulation of $Na^+$ in roots, but the same $Na^+$ amount found in *hkt1* mutants in shoots; and the latter accumulated two-fold $Na^+$ content in roots, which demonstrates that the salt sensitivity of *sos1* mutants can be due to excessive root $Na^+$ accumulation, partially decreased by knocking out AtHKT1 (Apse and Blumwald, 2007; Davenport et al., 2007).




**Graph 5-35: HKT1 expression (normalized with actin using ΔΔCt method) in leaves of four genotype. Histograms in blue represent expression of the gene at T0 (-NaCl) and orange histograms describe the mean value of expression in 3 dates of treatment (T2, T3, T4) for the same gene (+NaCl).**

These experiments clarified that these genes are connected together and their relative activities may determine the situation inside the plant with regard to Na uptake, translocation and accumulation. But the comprehension is still confused about HKT1, in fact null *hkt1* mutant also confer sensitivity to high NaCl levels; this confirmed again that HKT1 functions in regulating $Na^+$ in leaves by unloading sodium from the xylem in both shoots and roots (Rus et al., 2006).

In our hydroponic cultural experiment, the high HKT1 expression in Farold®40 leaves was not easily to be explained, because it could be that HKT1 was involved in the $Na^+$ entrance in that tissue coming from vascular tissues or that this gene located in the plasma membrane of xylem parenchyma cells in the shoots worked to retrieve sodium from xylem and drive it back to the roots, where it can be expelled or stored in vacuoles of mature roots.

To try to figure out which one of the two possibilities is more close to the real mechanism; we can re-evaluate the data about $K^+/Na^+$ ratio. Unlikely at leaf level these data were not able to discriminate between genotypes, but shoots (immediately below leaves) revealed in salt stressed plants that Farold®40

registered the second highest value of this ratio. This could mean that in the upper part of the treated Farold®40 plants, the balance moved to favor potassium and this implies that a so high expression of HKT1 in leaves did not cause a excessive $Na^+$ accumulation. Moreover, salt stressed pear coarse roots presented the lowest values of $K^+/Na^+$ ratio (<1) and this can indicate that sodium fluxes were driven downward. This hypothesis could survive thinking that in this case HKT1 located in the shoots might act in reducing $Na^+$ entrance from shoots.

It is not still unknown how to explain the exact function of HKT1 at leaf level, since recently it has been excluded the possibility of its involvement in phloem recirculation, despite its certain role in Na retrieval from xylem.

Further investigations, such as measures of pH and $Na^+$ concentrations in different compartments, would be useful in our trial to try to better explain the behavior of these four genotypes exposed to salt stress in terms of Na distribution at whole plant level.

Probably in general, this complicate topic still needs experiments and studies on other important determinants that probably with their activity can fill and clarify these large gaps remained in our knowledge about $Na^+$ transport.

## 5.5 Conclusion

With this hydroponic culture experiment that involved four different genotypes, two pears and two quinces, it has been possible to observe their behaviors when a salt stress up to 90 mM NaCl was imposed for about three weeks. In fact in a previous experiment carried out by Dondini and colleagues (2002) on pears (Abbé Fétel and William) grown in sand and exposed to 7 dS/m of NaCl for six months, plants showed decreases in growth, photosynthesis and dry weight, but the onset of symptoms has not been mentioned (Dondini et al., 2002). So since pears and quinces rootstocks, have never been studied before in salinity condition up to 90 mM NaCl in a hydroponic system, we wanted to experiment the onset and the development of salinity symptoms on different genotypes, applying a salt concentration medium-high for pear, considering that it is classified as salt sensitive plant, as most of fruit trees species.

The salt imposition was applied to half of the plants in trial after five weeks of normal growth in Hoagland nutrient solution. From the day before the stress started (T0), some physiological measurements and evaluations were carried out on plants until the end of the experiment that was established according to the degree of leaf necrosis.

The first plant parameter affected by salinity is the growth rate; in fact plants under salinity are subjected to a reduction of new shoots growth and a decrease in leaf area, as a first effect of the osmotic component of salt stress. After this first osmotic phase, a second phase starts when $Na^+$ (and $Cl^-$) ions are accumulated in concentration that rapidly become toxic for the plant and in particular for older leaves.

Farold®40 and Abbé Fétel plants exposed to salt stress exhibited the first symptoms of leaf necrosis after just a week of salt stress, while, on the contrary, quinces showed a delay of one week in developing necrosis respect to both pears.

It can be hypothesized that in our experiment the osmotic phase lasted longer for quinces than for pears, where rapidly arose the ionic phase that determined the complete death of the foliage. In literature it has been reported that the ionic effect can overpower the osmotic one when the salt concentration is quite high or in

species that are not able to cope and control high sodium levels. The osmotic phase, in fact, may last from few hours to days, before toxic ion levels reach leaves.

In addition, both salt-stresses pears, at the end of the trial, registered a greater decrease in water contents respect to quinces exposed to the same stress; and indeed the former appeared wilting because of dehydration process, while the latter showed only a sort of growth slowdown. An idea about this is linked to the concept of "dehydration avoidance", in fact it may be that quinces exploited their decrease in water content as a signaling to trigger production of solutes, taking part of the bigger process known as osmotic adjustment; while pears could not be able to adopt this strategy at the same time (but probably later) and so their water content decrease was more severe than in quinces.

This experiment has been focused on monitoring the morphologic, physiological and symptomatic processes under salinity, trying to explain different genotypes responses to NaCl stress by both mineral analyses data and by gene expression of three of the main determinants in salt tolerance mechanism: NHX1, SOS1, and HKT1.

By mineral analysis data, it emerged that some of the plant essential elements changed in their content in different organs under salinity; this sometimes can be a negative consequence of a large sodium uptake, for instance the $K^+$ decrease at root levels, or, on the other hand, a positive alteration arranged by plant in order to achieve a salt stress tolerance. It has been seen for example in Farold®40 that some increases in macro and/or micro nutrients may in some ways contribute to overcome the apparent leaf death, such as copper involved in cell wall hardening, manganese linked to carbohydrates content and potassium required for osmotic balance in plant. In addition some other elements resulted involved in the multiple processes triggered in plant by the salt stress perception, among them zinc that represents a cofactor for CuZnSOD acting in detoxification signaling, iron whose decrease in leaf content avoids the further formation of ROS and eventually calcium that showed its well-known ameliorative effect on the detrimental result of salt stress, in quinces leaves.

In this experiment, however the aspect related to osmotic adjustment was not faced directly as several studies have instead done; for future experiments it could

be interesting to evaluate the content of same crucial osmolytes such as proline, glycine betaine or mannitol, during the proceed of salt stress, in order to try to support the present hypotheses with experimental results.

Apparently both quinces could be retained less sensitive to salt stress than pears, but nevertheless they have been affected by it, showing a loss in chlorophyll content and a decrease in growth, stomatal conductance and transpiration after two weeks of NaCl imposition. But almost at the end of the experiment both pear genotypes showed a surprising renewal of new leaves from apexes which seemed already dead, this unexpected response muddled ideas that pears could be more sensitive than quinces to salt stress. These two *Pyrus* genotypes in trial, in fact, demonstrated, through this clear physiological response, the ability to find the way to tolerate a salt stress.

According to a recent classification, the mechanisms of salinity tolerance fall into three categories: osmotic tolerance, $Na^+$ exclusion and tissue tolerance (Munns and Tester, 2008). From this classification, it seems quite easy to distinguish a plant based on its response to salt stress, but real experiments have proved that the distinction is not so explicit, in fact different lines of cereals use diverse combinations of the three tolerance mechanisms to enhance their tolerance to salt stress at a whole plant level (Rajendran et al., 2009). Therefore these strategies do not exclude each other, rather they can operate together to reach the salt tolerance. This explanation supports our idea that the four genotypes exposed to salt stress differently responded to the stress and that probably each species found a peculiar way to cope with salinity, without selecting one clear and unique of these mechanisms, but taking advantages by combining them, differently.

The ability of Farold®40 and Abbé Fétel to produce new leaves, from apparently "dead" foliage, let us hypothesize that these genotypes increased their osmotic tolerance during the salt stress, since, according to definition, this means to be able to tolerate the drought component of salinity and to maintain a leaf expansion or in this case to restart growing. Leaf shedding may be a way in which some plants like avocado adapts to high chloride conditions (Xu et al., 2000).

On the other hand, both quinces - MC in a more pronounced way than BA29 - showed the ability to increase survival of older leaves and the delay in the onset of

symptoms despite the high leaf Na and Cl concentrations, this mechanism was defined as tissue tolerance. MC, in addition during the stress exposure, presented also an increase in photosynthetic activity, response less common for salt sensitive plants than the decrease in photosynthesis, but Ashraf, 2004 (*Beta vulgaris*) for mild saline condition. Previously in pears it has been reported that photosynthesis was unaffected by medium salinity level (5 dS/m) (Musacchi et al., 2002). This in fact may be explained perhaps with a delay both in ion toxicity in chloroplasts and in premature senescence of older leaves (carbon source) as plant processes acting in the tissue tolerance (Munns and Tester, 2008).

On this aspect, the differences between pears and quinces behaviors under salinity appeared clearly, because Abbé Fétel and Farold®40 presented at the end of the experiment high leaf $Na^+$ contents and high leaf damage, while MC and BA29 lower leaf damage with the same level of $Na^+$ content in leaves, for this reason pears before acquiring osmotic tolerance and regenerate new leaves were deemed sensitive to salinity.

Behind the proposal to classify quinces salt-stress responses as tissue tolerance, several aspects were evaluated and it resulted that MC and BA29, despite they belong to the same mechanism, responded quite differently to salinity. This clearly emerged studying the expression of NHX1, SOS1 and HKT1 acting in salt stress tolerance. Between the two quinces, it has been noticed that while BA29 was increasing its sequestration of sodium into leaf vacuoles (NHX1) under NaCl stress, MC enhanced temporarily its ability to compartmentalize Na in root vacuole. Another difference in quinces behaviors under salinity was that MC invested energy mainly at roots levels, both in extruding sodium from root cells (SOS1) and in retrieving the same ion from xylem (HKT1), while BA29 reacted to the salt stress mostly at leaf level where up regulations of SOS1 and NHX1 were verified.

Also both pears, which have been associated in osmotic tolerance mechanism for their capacity to regenerate new leaves under running salt stress, were characterized by different responses to NaCl. In fact, Farold®40 under salinity exhibited its responses mainly at leaf level with important increases in expression of SOS1 and HKT1, but also showed a raised sequestration of $Na^+$ into leaf vacuoles. On the side of this evident role that these sodium transporters covered for this pear

exposed to salinity, probably other plant adaptive processes such as osmotic adjustment and modification of long-distance signaling took place, but to validate this hypothesis, further specific analyses should be carried out in the future.

Abbé Fétel subjected to 90 mM NaCl stress reacted increasing the compartmentalization of sodium into roots vacuoles more than the other genotypes, but also activating SOS1 and HKT1 mainly at root levels. This underlines that probably this pear invested principally in avoiding the transport of sodium (and chloride) up to shoots, this may explain the enhancement of activity of these genes involved both in retrieving Na from xylem and avoiding its loading into the transpiration stream.

In conclusion each genotype in trial showed a proper response to salt stress that was the sum of its ability in $Na^+$ exclusion, osmotic tolerance and tissue tolerance.

In general we can say that both pears showed a predominant osmotic tolerance with the contribution of sodium exclusion, while quinces exhibited a tissue tolerance in addition to some plant processes belonged to both osmotic tolerance and sodium exclusion mechanisms. Further studies will be necessary to better understand these salt tolerance mechanisms, shedding light also on chloride responsibility.

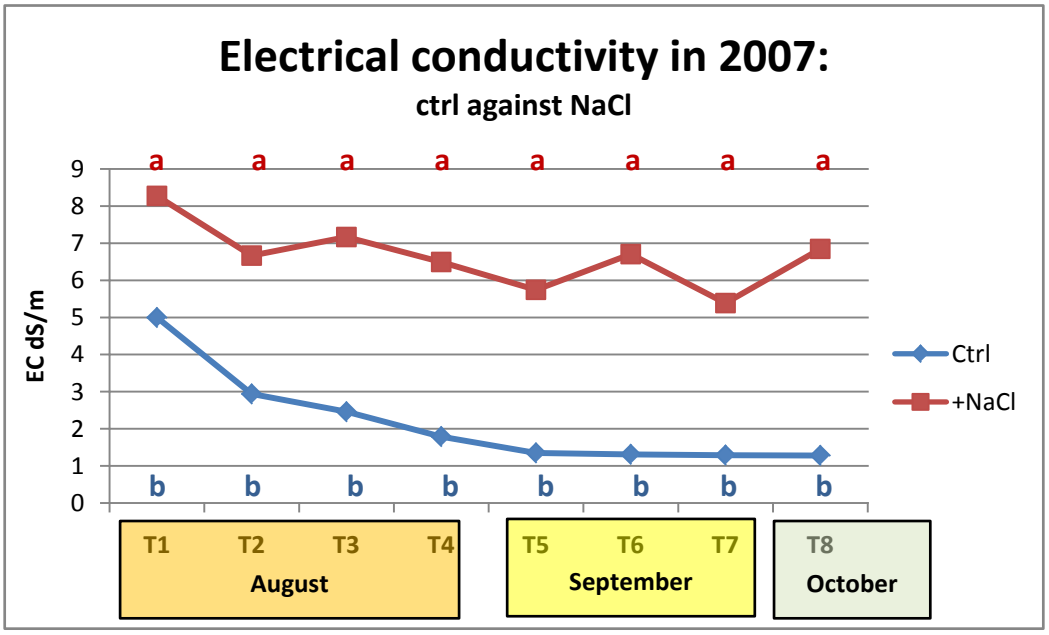
# Chapter 6: RESULTS AND DISCUSSION

A LONG-TERM EXPERIMENT

## 6.0 Field measurements

### 6.0.1 Electrical conductivity measurement in soil solutions

Data collected from suction lysimeters, placed as described in paragraph 4.1.2, are reported in table 6-1 for the year 2007. They showed that differences in electrical conductivity between solutions picked up from unstressed rows and those from "salt-stressed" rows, resulted always significant (according to SAS) for all the dates (T1÷T8) took in consideration. This confirmed the correct distribution of both fertigation and saline water irrigation according to the schedule. In fact the electrical conductivity should not go down 5 dS/m in the rows where salt treatment was applied and higher values were due to the additional effect that fertilizer had on electrical conductivity. In particular at T1, EC values appeared quite high; this was caused by the initial elevated application of fertigation to guarantee a proper plant growing and development. After the T1 measurement, EC values decreased both for "control" plants and for "stressed" plants, which however continued to show clear fluctuations in these EC values (graph 6-1).



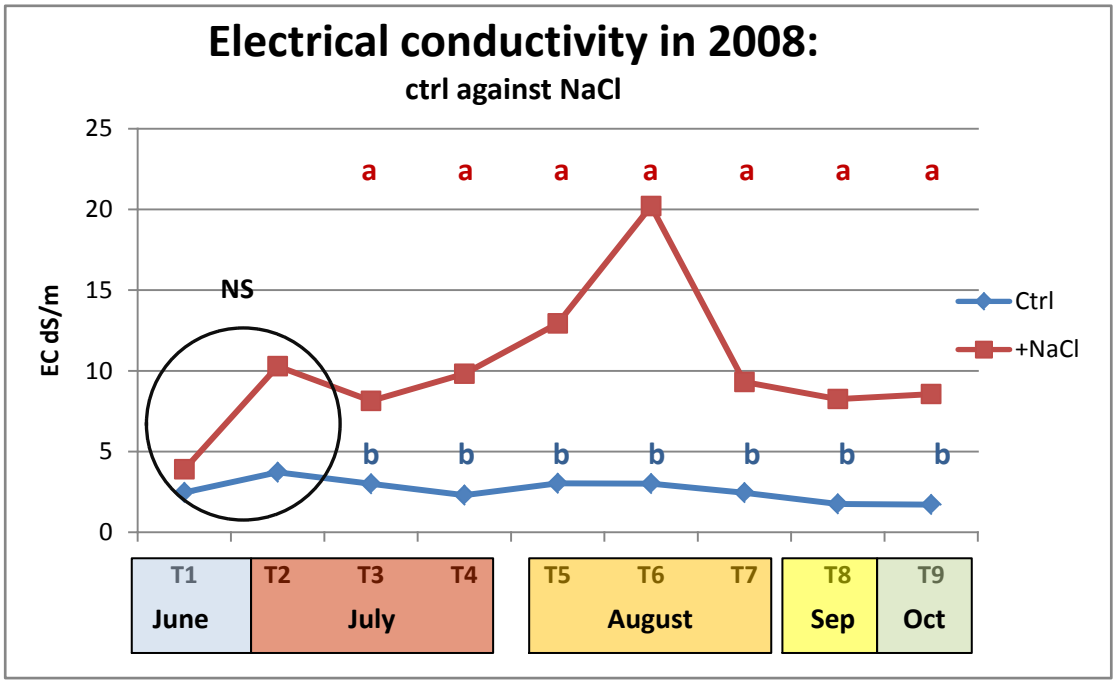

**Graph 6-1: Trend of electrical conductivity (dS/m) of soil solutions collected with suction lysimeters and measured by EC-meter, in "control" and "NaCl" rows in 2007 from August to October.**

**Tab. 6-1: - Electrical conductivity (dS/m) measured in soil solutions of potted trees by suction lysimeters in 2007.**
**"Ctrl" corresponds to measures made in rows without treatment (1, 3, 5) and "NaCl" to measures in rows where salt was applied (2, 4, 6).**

| Rows | T1 | | T2 | | T3 | | T4 | | T5 | | T6 | | T7 | | T8 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **2007** | **5-Aug** | | **16-Aug** | | **22-Aug** | | **28-Aug** | | **7-Sep** | | **17-Sep** | | **24-Sep** | | **3-Oct** | |
| **Ctrl** | 5.00 | b | 2.94 | b | 2.46 | b | 1.79 | b | 1.35 | b | 1.31 | b | 1.29 | b | 1.28 | b |
| **NaCl** | 8.27 | a | 6.66 | a | 7.16 | a | 6.49 | a | 5.74 | a | 6.70 | a | 5.38 | a | 6.84 | a |
| Significance | * | | *** | | *** | | ** | | * | | ** | | ** | | ** | |

**Tab. 6-2: - Electrical conductivity (dS/m) measured in soil solutions of potted trees by suction lysimeters in 2008.**
**"Ctrl" corresponds to measures made in rows without treatment (1, 3, 5) and "NaCl" to measures in rows where salt was applied (2, 4, 6).**

| Rows | T1 | T2 | T3 | | T4 | | T5 | | T6 | | T7 | | T8 | | T9 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **2008** | **23-Jun** | **17-Jul** | **22-Jul** | | **29-Jul** | | **4-Aug** | | **14-Aug** | | **18-Aug** | | **22-Sep** | | **2 Oct** | |
| **Ctrl** | 2.47 | 3.72 | 3.00 | b | 2.30 | b | 3.03 | b | 3.01 | b | 2.45 | b | 1.75 | b | 1.71 | b |
| **NaCl** | 3.89 | 10.28 | 8.13 | a | 9.81 | a | 12.93 | a | 20.20 | a | 9.30 | a | 8.24 | a | 8.55 | a |
| Significance | NS | NS | ** | | * | | ** | | ** | | * | | * | | * | |




**Graph 6-2: Trend of electrical conductivity (dS/m) of soil solutions collected with suction lysimeters and measured by EC-meter, in "control" and "NaCl" rows in 2008 from June to October.**

In 2008, the EC measurements of solutions in the potted plants started before respect to the previous year. In graph 6-2 and table 6-2, it appears clear that EC values referred to rows irrigated with normal water were quite stable; they oscillated from 1.71 to 3.72 dS/m. The trend relative to mean values of electrical conductivity in NaCl-treated-rows was fairly inconstant during the season from June to October.

At T1 and T2 the differences in EC between "control" and "NaCl" plants did not result significant, because the saline water concentration was not stabilized. In all the other dates, EC differences resulted significant ($p<0.05$ or $p<0.01$).

At T5 it has been noticed that the conductivity of the "salt-stressed" plants was rising, providing values doubled respect to the predicted EC of the saline water inside the 1000L tank. After the maximum EC level registered in T6, the electrical conductivity measured in next dates lowered at suitable values.

## 6.1 Vegetative growth

### 6.1.1 Shoot length and elongation

From July to September 2007, the shoot length was measured five times on two shoots for each genotype/combination (where possible) for each row. In date T1 and T2 (table 6-3), salt treatment has not applied yet, but only fertigation was dispensed to promote a suitable growth of the plants. At T1 the means of shoots length between control plants and (future) salt stressed plants were similar, respectively 31.3 and 36.6 cm (difference not significant). At T2, the means of shoot length in “control” and in “NaCl” plants were similar with the latter numerically higher but not statistically significant; the elongation between the first two measures did not result meaningful neither.

Salt stress application at the concentration equal to 5 dS/m caused a slowdown of growth in “NaCl” plant regardless of the genotype, in fact shoots elongation T3-T2 and T4-T3 resulted lower for “NaCl” shoots. At T5 the final shoots length resulted lower for “NaCl” shoots in comparison with “Ctrl” ones with values of respectively 63.16 cm and 65.64 cm. This result was similar to the lateral shoots growth reduction reported by Myers and co-workers (1995) on pear trees cv. William during the seventh season of saline irrigation (around 2 dS/m) (Myers et al., 1995).

A different shoot growth pattern was observed among genotypes and grafted trees; both quinces rootstocks (MC and BA29) showed the highest shoots elongation among all plants in trial regardless the treatment, while Abbé Fétel own-rooted, Abbé F./Farold®40 reported a lower growing rate and at the beginning of August (T3) the first genotype stopped growing.

BA29 and Farold®40 registered the highest shoots elongation at the end of measures (T5-T4) and the former also reported the greater total shoots length at T5 (94 cm) while Farold®40 registered the lowest length among genotypes with 28.7 cm. Abbé Fétel own-rooted instead started growing with quinces, but in less than 1 month stopped the growth, maybe because of the particular hot temperature (see appendix 7). As reported in table 6-3, combination Abbé Fétel/MC and Abbé Fétel/BA29 were not considered for this measurement because none of these plants presented any shoots in active growth. Quinces demonstrated to have an earlier vegetative resumption than that of Farold®40.

**Table 6-3: Shoots length and elongation (cm) of some genotypes/combinations in trial in 2007, those not present in the table were not active in growth (Abbé Fétel/MC and Abbé Fétel/BA29 were not considered for this measurement). Dates underlined mean that salt stress was applied, while normal dates mean that the measurement was performed before NaCl application. Significance according to SAS proc GLM: *=p<0.05, **=p<0.01, ***=p<0.001; NS= not significant. Small letters discriminate according to SNK in vertical way**

| Treatment | **5-July T1 length** | | **13-July T2 length** | | **elongation T2-T1** | | **1-August T3 length** | | **elongation T3-T2** | | **16-August T4 lengh** | | **elongation T4-T3** | | **7-Sept T5 lengh** | | **elongation T5-T4** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Ctrl** | 31.3 | | 41.22 | | 9.92 | | 47.56 | | 6.33 | | 54.82 | | 7.25 | | 65.64 | | 6.6 | |
| **NaCl** | 36.64 | | 46.68 | | 10.04 | | 51.32 | | 4.64 | | 57.08 | | 5.76 | | 63.16 | | 6.08 | |
| *Significance* | ns | | ns | | ns | | ns | | * | | ns | | * | | * | | ns | |
| Genotype/ combination | | | | | | | | | | | | | | | | | | |
| **Abbé F. own r.** | 44.79 | a | 58.08 | a | 13.29 | | 59.08 | ab | 1.00 | b | 59.08 | ab | 0 | c | 59.08 | b | 0 | b |
| **Abbé Fétel/Farold®40** | 26.89 | a | 34.89 | b | 8.00 | | 40 | b | 5.11 | ab | 43.78 | b | 3.78 | bc | 52 | b | 3.13 | b |
| **Farold®40** | 3.5 | b | 5.86 | c | 2.36 | | 10 | c | 4.14 | ab | 18 | c | 8.00 | b | 28.71 | c | 10.71 | a |
| **BA29** | 48.08 | a | 60.33 | a | 12.25 | | 69.17 | a | 8.83 | a | 82.33 | a | 13.17 | a | 94.33 | a | 12 | a |
| **MC** | 31.66 | a | 42 | ab | 10.33 | | 49.83 | ab | 7.83 | a | 57.5 | ab | 7.67 | b | 69.27 | b | 6.64 | ab |
| *Significance* | ** | | *** | | ns | | *** | | *** | | *** | | *** | | *** | | *** | |
| *Interaction gen*trt* | ns | | ns | | ns | | ns | | ns | | ns | | ns | | ns | | ns | |

### 6.1.2 Trunk section area and growth rate

During plant winter dormancy in 2007 and 2008, the diameter of plant trunk was measured (as described in paragraph 4.2) in order to estimate the area of the trunk section ($cm^2$) and to evaluate the effect of salinity on this growth parameter.

In both years we did not noticed any significant differences between area of "control" plants and "NaCl" plants regardless of genotypes, despite the growth rate ($cm^2$) was slight lower in salt-stressed plants, but not statistically significant (Tab. 6-4).

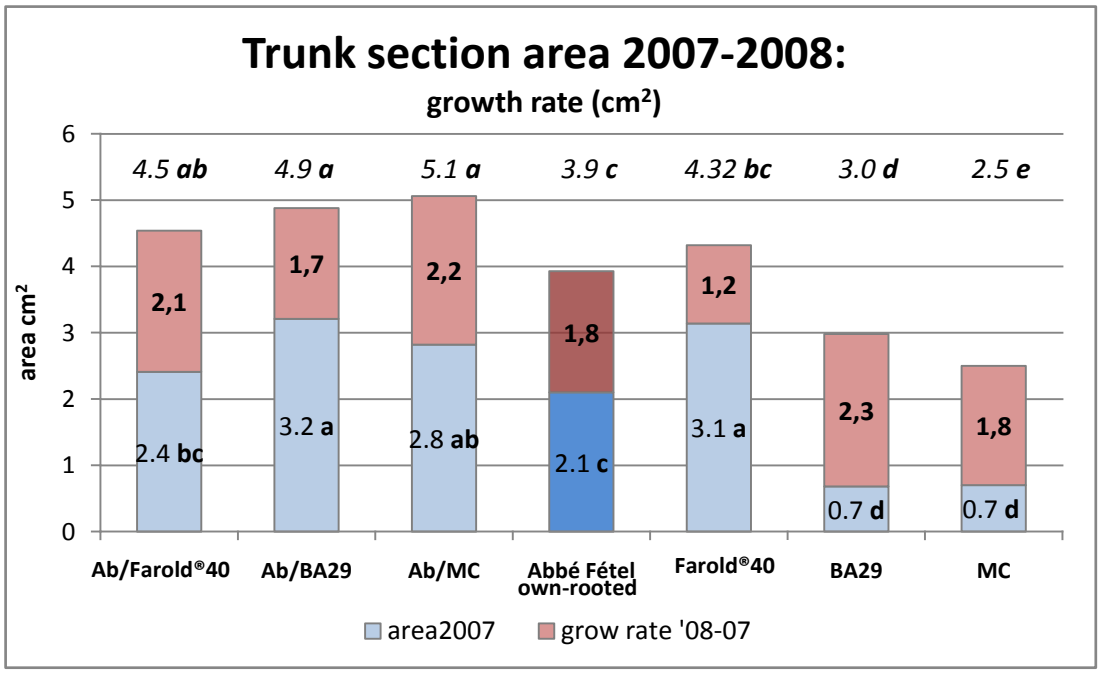
**Table 6-4: Trunk area in $cm^2$ calculated on the diameter of collar measured for each plant in 2007 and 2008. In the third column the growth rate between the two years is reported.**

| Treatment | **AREA 2007 ($cm^2$)** | | **AREA 2008 ($cm^2$)** | | **growth rate($cm^2$)** |
|---|---|---|---|---|---|
| **Ctrl** | 2.00 | | 4.06 | | 2.06 |
| **NaCl** | 2.01 | | 3.89 | | 1.88 |
| *Significance* | ns | | ns | | ns |
| Genotype/combination | | | | | |
| **Abbé Fétel** | 2.10 | c | 3.93 | c | 1.83 |
| **Abbé Fétel/Farold®40** | 2.42 | bc | 4.54 | ab | 2.13 |
| **Abbé Fétel/BA29** | 3.21 | a | 4.88 | a | 1.68 |
| **Abbé Fétel/MC** | 2.82 | ab | 5.06 | a | 2.25 |
| **Farold®40** | 3.14 | a | 4.32 | bc | 1.18 |
| **BA29** | 0.69 | d | 2.99 | d | 2.30 |
| **MC** | 0.70 | d | 2.50 | e | 1.80 |
| *Significance* | *** | | *** | | *** |
| *Interaction* trt*gen | ns | | ns | | ns |

On the other hand, variations in trunk section area resulted significant among the seven different genotypes/combinations both in 2007 and in 2008 and different was also the growth rate. The highest area values in both years were relative to Abbé Fétel/BA29 (in 2007 the value was similar to Farold®40 and in 2008 this value was similar also for Abbé Fétel/MC).

On the contrary, MC and BA29 emerged for their lowest trunk areas among all genotypes in both years, but also for high growth rate with increases respectively of

1.80 and 2.30 $cm^2$. Abbé Fétel own-rooted and Abbé Fétel/BA29 reported similar increments respectively of 1.80 and 1.70 $cm^2$. Abbé Fétel/Farold®40 and Abbé Fétel/MC exhibited a comparable growth rate (graph 6-3).




**Graph 6-3: Trunk section areas measured at collar level in 2007 and in 2008 (winter). Light blue histograms are relative to 2007 areas and the red part represents the growth rate between the two years. On the top of histograms are reported the mean values of 2008 areas. All the letters near values discriminate means according to SNK. Ab=abbreviation of Abbé Fétel.**

A statistical analysis has been carried out only for Abbé Fétel combinations and Abbé Fétel own-rooted. From data about Abbé Fétel and its grafting combinations, it emerged that the decrease in growth rate between "control" plants and "NaCl" plants was not significant at 5%, but at 8%, this let us hypothesize that next year this difference can be magnified. The interaction between genotypes*treatment resulted significant in table 6-5, this was due to Abbé Fétel own-rooted that in salt-stressed plants reported a diameter greater than reported for "control" plant of the same genotype.

Instead, looking at single genotype/rootstock, the decrease in growth rate (relative to area of trunks) resulted significant with $p<0.01$ between "control" and "NaCl" plants as a consequence of saline treatment. Also among genotypes the growth rate resulted different, in fact in areas of 2008 means were higher for both pears (Abbé Fétel own-rooted and Farold®40) and lower for both quinces (table 6-6).

**Table 6-5: Trunk areas measured in 2007 and 2008 for the Abbé Fétel combinations and Abbé Fétel own-rooted comparison between "Control" and "NaCl" plants.**

| Treatment | **AREA 2007 ($cm^2$)** | | **AREA 2008 ($cm^2$)** | | **Growth increase ($cm^2$)** |
|---|---|---|---|---|---|
| **Control** | 2.48 | | 4.61 | | 2.13 |
| **NaCl** | 2.75 | | 4.57 | | 1.82 |
| *Significance* | * | | ns | | ns (8%) |
| Genotypes/combinations | | | | | |
| **Abbé F. Own-rooted** | 2.10 | b | 3.92 | b | 1.83 |
| **Abbé F./Farold®40** | 2.41 | b | 4.54 | a | 2.13 |
| **Abbé F./BA29** | 3.21 | a | 4.88 | a | 1.67 |
| **Abbé F./MC** | 2.82 | a | 5.06 | a | 2.24 |
| *Significance* | *** | | *** | | ** |
| *Interaction gen*trt* | ** | | ** | | ns |

**Table 6-6: Trunk areas measured in 2007 and 2008 for the Abbé Fétel own-rooted and its rootstocks, comparison between "Control" and "NaCl" plants.**

| Treatment | **AREA 2007 ($cm^2$)** | | **AREA 2008 ($cm^2$)** | | **Growth increase ($cm^2$)** |
|---|---|---|---|---|---|
| **Control** | 1.65 | a | 3.61 | a | 1.96 |
| **NaCl** | 1.35 | b | 3.19 | b | 1.84 |
| *Significance* | * | | ** | | ** |
| Genotypes/Rootstocks | | | | | |
| **Abbé F. Own-rooted** | 2.10 | b | 3.93 | a | 1.83 |
| **Farold®40** | 3.14 | a | 4.32 | a | 1.18 |
| **BA29** | 0.68 | c | 2.99 | b | 2.30 |
| **MC** | 0.70 | c | 2.50 | c | 1.80 |
| *Significance* | *** | | *** | | *** |
| *Interaction gen*trt* | ns (5.3%) | | ns | | ns |

### 6.1.3 Winter measurements of vegetative activity and flower bud numbers (2008)

In March 2008, all the one-year-shoots were measured for their length and in number, in addition the number of flower buds were determined in the entire trial. In table 6-7 all these data were reported and it appeared evident that comparing "control" and "NaCl" plants on these parameters, regardless of the genotype, none apparent differences resulted significant, despite numerically the mean of total length of salt-stressed one-year-old shoots was higher than that belonged to "control" one-year-old shoots.

Among genotypes/combinations instead all differences reported in table 6-7 were significant (with $p<0.001$). It is interesting to underline that the highest total length of one-year-old shoots was registered for MC followed immediately by BA29, while the lowest belonged to the combination Abbé F./Farold®40. The combination with maximum number of shoots registered was Abbé F./MC, while Abbé Fétel own-rooted, Abbé F./Farold®40 and BA29 presented similar number of shoots corresponding to the lowest among genotypes in trial.

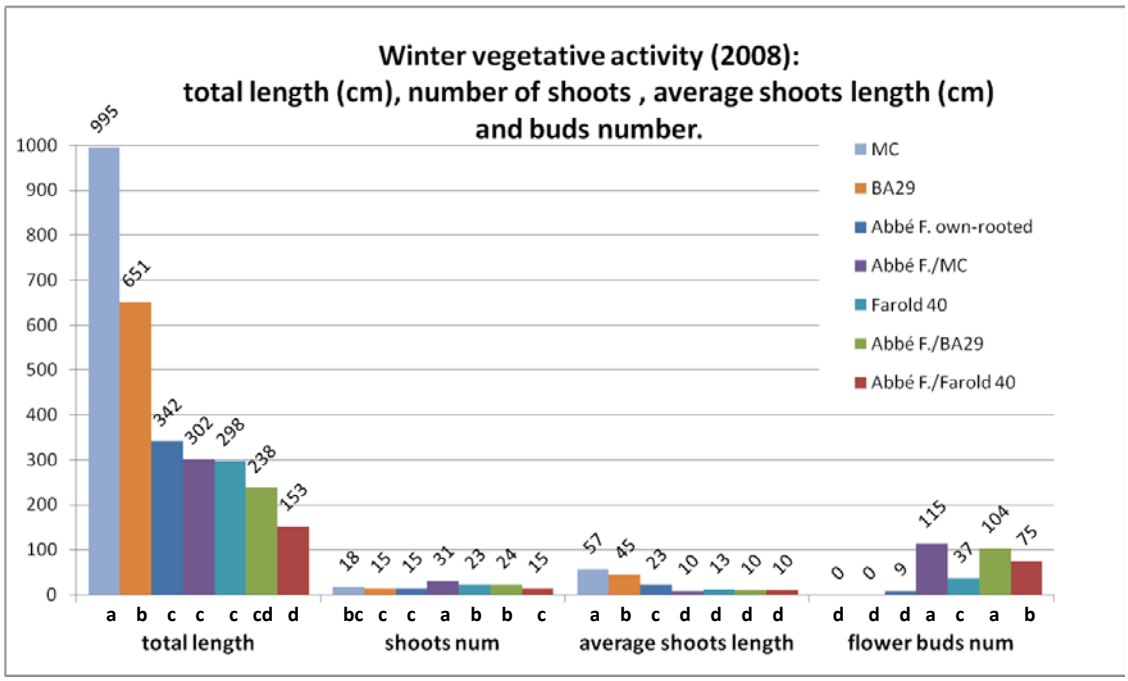
**Tab 6-7: Winter measurements of vegetative activity and bud numbers (March 2008).**

| Treatment | Total shoot length (cm) | | Shoots number | | Average length (cm) | | Flower buds number | |
|---|---|---|---|---|---|---|---|---|
| **Control** | 402.14 | | 19.85 | | 23.27 | | 48.61 | |
| **NaCl** | 448.50 | | 20.47 | | 24.66 | | 48.59 | |
| *Significance* | ns | | ns | | ns | | ns | |
| Genotype/combination | | | | | | | | |
| **AbbéF. Own-rooted** | 341.58 | c | 15.08 | c | 22.98 | c | 9.08 | d |
| **Abbé F./Farold®40** | 153.00 | d | 15.08 | c | 10.13 | d | 74.83 | b |
| **Abbé F./BA29** | 237.83 | cd | 23.66 | b | 10.15 | d | 103.75 | a |
| **Abbé F./MC** | 301.92 | c | 30.75 | a | 9.74 | d | 115.41 | a |
| **Farold®40** | 297.75 | c | 23.33 | b | 12.77 | d | 37.16 | c |
| **BA29** | 650.50 | b | 15.16 | c | 44.91 | b | 0.00 | d |
| **MC** | 994.67 | a | 17.91 | bc | 57.08 | a | 0.00 | d |
| *Significance* | *** | | *** | | *** | | *** | |
| *Interaction gen*trt* | ns | | ns | | ns | | ns | |

At the same time, Abbé F./MC showed the lowest mean length of shoots and the maximum number of flower buds, probably due to the dwarfing effects of quince C. Both the combination of Abbé Fétel on quinces showed a comparable behavior, the only statistically significant difference regarded the number of shoots. In fact Abbé F./BA29 counted 24 shoots, while Abbé F./MC 31.

The lowest number of flower buds was counted on Abbé F. own-rooted which, on the other hand, reported a mean length of branches superior respect to corresponding graft combinations Abbé F./MC, Abbé F./BA29, Abbé F./Farold®40.

All the differences in these vegetative parameters can be visualized in graph 6-4.




**Graph 6-4: In horizontal axis the four parameters evaluated in 2008 are present and for each of them differences between genotypes are shown. For total and mean length, values on top of histograms are in cm, while for shoots and flower buds are number. On the bottom of histograms are reported letters coming from SNK discrimination present also in table 6-7.**

We analyzed separately the Abbé Fétel combinations to reduce the variability induced by the use of non grafted rootstocks.

Abbé Fétel own-rooted exhibited the highest total length of shoots (this average was similar to that of Abbé F./MC) and also the maximum average length of shoots among the other three combinations in trial. On the contrary, the highest numbers both of shoots and flower buds were counted in the combination Abbé F./BA29. With regard to number of flower buds, it is important to underline that Abbé

F./BA29 showed a quite similar number (around 104) compare to Abbé F./MC (115) (Tab. 6-8). In table 6-9, it appeared evident that among the un-grafted plants/rootstocks, Farold®40 seems to be the one with higher number of flower buds, but the shortest one-year-shoots as average length. Both in table 6-8 and 6-9 the salt treatment did not influence in a significant way any of these vegetative parameters evaluated, despite numerically differences can be seen.

**Table 6-8: Winter vegetative parameters evaluated on March 2008 on Abbé Fétel own-rooted and its grafted combinations.**

| Treatment | Total shoot length (cm) | | Shoots number | | Average length (cm) | | Flower buds number | |
|---|---|---|---|---|---|---|---|---|
| Control | 241.58 | | 20.54 | | 12.37 | | 76.38 | |
| NaCl | 275.58 | | 21.83 | | 14.14 | | 75.17 | |
| Significance | ns | | Ns | | ns | | ns | |
| **Genotypes** | | | | | | | | |
| **AbbéF. Own-rooted** | 341.58 | a | 15.25 | c | 22.99 | a | 9.08 | c |
| **Abbé F./Farold®40** | 153.00 | b | 15.08 | b | 10.13 | b | 74.83 | b |
| **Abbé F./BA29** | 237.83 | ab | 23.67 | a | 9.75 | b | 103.75 | a |
| **Abbé F./MC** | 301.92 | a | 30.75 | c | 10.15 | b | 115.42 | a |
| Significance | ** | | *** | | *** | | *** | |
| *Interaction gen*trt* | ns | | ns | | ns | | ns | |

**Table 6-9: Winter vegetative parameters evaluated on March 2008 on single rootstocks and Abbé Fétel own-rooted.**

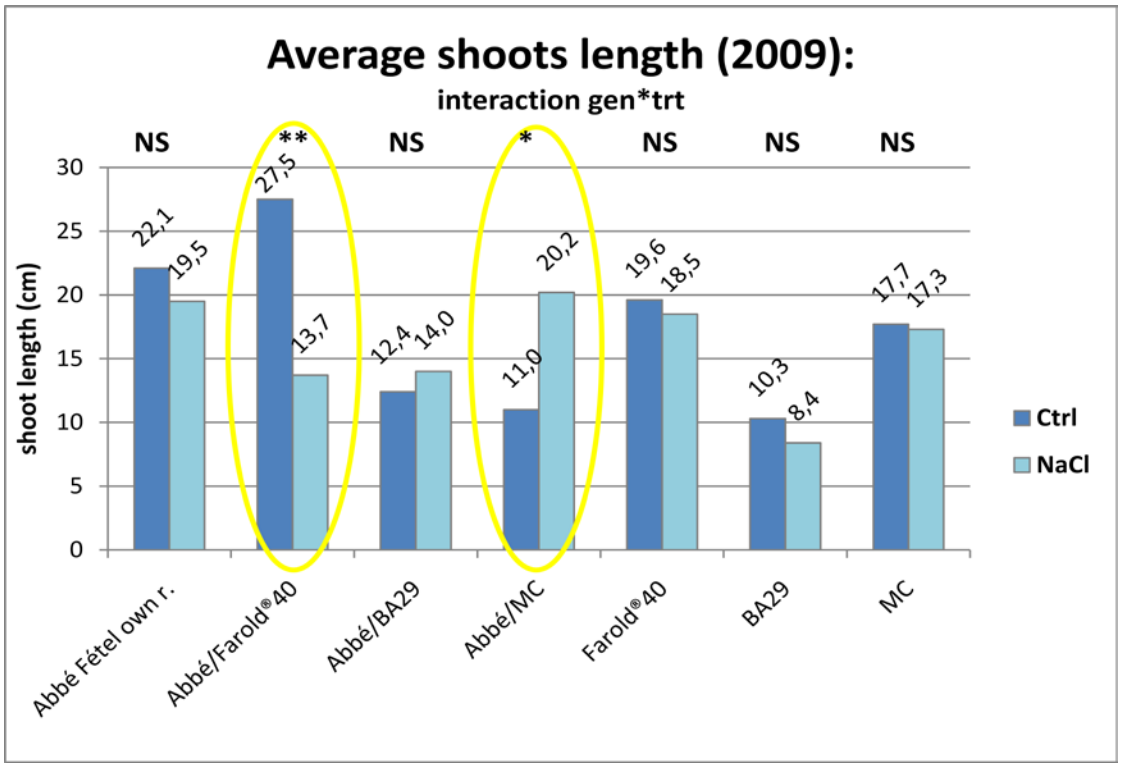
| Treatment | Total shoot length (cm) | | Shoots number | | Average length (cm) | | Flower buds number | |
|---|---|---|---|---|---|---|---|---|
| Control | 534.12 | | 18.21 | | 32.92 | | 11.125 | |
| NaCl | 608.12 | | 17.62 | | 35.96 | | 12 | |
| Significance | ns | | ns | | ns8.8 | | ns | |
| **Genotypes** | | | | | | | | |
| **Abbé Fétel** | 341.58 | c | 15.25 | b | 22.98 | c | 9.083 | b |
| **Farold®40** | 297.75 | c | 23.33 | a | 12.78 | d | 37.167 | a |
| **BA29** | 650.50 | b | 15.17 | b | 44.92 | b | 0 | b |
| **MC** | 994.67 | a | 17.92 | b | 57.08 | a | 0 | b |
| Significance | ** | | *** | | *** | | *** | |
| *Interaction* Tesi*pi | ns | | ns | | ns | | ns | |

### 6.1.4 Winter measurements of vegetative activity at T1 (2009)

In March 2009, during the first tree destruction (T1), the length and number of one-year-shoots were measured. In table 6-10, shoots number and average shoots length (cm) were reported and differences between "control" and "NaCl" plants did not resulted significant. Instead comparing all genotypes/combinations together significant differences appeared. MC and Abbé Fétel/BA29 exhibited respectively the highest and the lowest number of new shoots. The longest shoots (in average) belonged instead to Abbé Fétel own-rooted and Abbé Fétel/Farold®40, while the shortest to BA29. The interaction genotype*treatment resulted significant (with $p<0.05$) for the average shoots length, so probably a different behavior among genotypes was hidden in this measurement. Indeed the analysis of interaction showed that Abbé Fétel/MC and Abbé Fétel/ Farold®40 had an opposite behavior in response to salt treatment. In fact the former reported a significant increase ($p<0.05$) in shoots length, while the latter a meaningful decrease as effect of salinity (Graph 6-5).

**Table 6-10: Winter vegetative parameters evaluated on March 2009 on all genotypes in trial. Small letters discriminate in a vertical way.**

| Treatment | **Shoots number** | | **Average shoots length (cm)** | |
|---|---|---|---|---|
| **Ctrl** | 88.9 | | 17.0 | |
| **NaCl** | 85.1 | | 16.1 | |
| *Significance* | NS | | NS | |
| Genotype/combination | | | | |
| **Abbé Fétel own-rooted** | 55.7 | c | 20.8 | a |
| **Abbé Fétel/Farold®40** | 53.2 | c | 20.6 | a |
| **Abbé Fétel/BA29** | 22.5 | c | 13.2 | ab |
| **Abbé Fétel/MC** | 39.0 | c | 15.6 | ab |
| **Farold®40** | 36.3 | c | 19.1 | a |
| **BA29** | 143.0 | b | 9.3 | b |
| **MC** | 275.0 | a | 17.5 | a |
| *Significance* | *** | | ** | |
| *Interaction* trt*gen | NS | | * | |




**Graph 6-5: Analysis of the interaction gen*trt for average shoots length (2009) that appeared in table 6-A. On the top of histograms, significance is reported. Important differences are pointed out by yellow circles.**

**Table 6-11: Winter vegetative parameters evaluated on March 2009 on Abbé Fétel own-rooted and its combinations in trial. Small letters discriminate in a vertical way.**

| Treatment | **Shoot number** | | **Average shoots length (cm)** | |
|---|---|---|---|---|
| **Ctrl** | 45.9 | | 17.7 | |
| **NaCl** | 38.7 | | 16.0 | |
| *Significance* | NS | | NS | |
| Genotype/combination | | | | |
| **Abbé Fétel own r.** | 55.7 | a | 20.8 | a |
| **Abbé Fétel/Farold®40** | 53.2 | a | 20.6 | a |
| **Abbé Fétel/BA29** | 22.5 | b | 13.2 | b |
| **Abbé Fétel/MC** | 39.0 | ab | 15.6 | b |
| *Significance* | * | | ** | |
| *Interaction trt*gen* | NS | | ** | |

**Table 6-12: Winter vegetative parameters evaluated on March 2009 on Abbé Fétel own-rooted and its rootstocks in trial. Small letters discriminate in a vertical way.**

| Treatment | Shoot number | | Average length (cm) | |
|---|---|---|---|---|
| **Ctrl** | 127.1 | | 17.4 | |
| **NaCl** | 128.1 | | 15.9 | |
| *Significance* | NS | | NS | |
| Genotype/combination | | | | |
| **Abbé Fétel own r.** | 55.7 | c | 20.8 | a |
| **Farold®40** | 36.3 | c | 19.1 | a |
| **BA29** | 143.0 | b | 9.3 | b |
| **MC** | 275.0 | a | 17.5 | a |
| *Significance* | *** | | ** | |
| *Interaction trt*gen* | NS | | NS | |

Analyzing separately Abbé Fétel own-rooted with its grafting combinations the variability was reduced and SNK showed significant differences among them ($p<0.001$). In fact, if previously all pears values resulted similar (Tab. 6-10, “c” for SNK), in tab. 6-11 it was possible to notice that the highest number of shoots was reported by Abbé Fétel own-rooted while the lowest by Abbé Fétel/BA29 (table 6-11).

From the comparison among Abbé Fétel and its combinations, an evident difference appeared between Abbé Fétel own-rooted or grafted on Farold®40 and both combination on quinces. The former reported higher average shoots lengths than those of the latter (SNK discrimination was respectively “a” and “b”.

On the other hand, comparing rootstocks together, it was clear that MC was the most vegetative active with in average 275 shoots, whereas Farold®40 the less productive one in terms of new shoots (table 6-12).

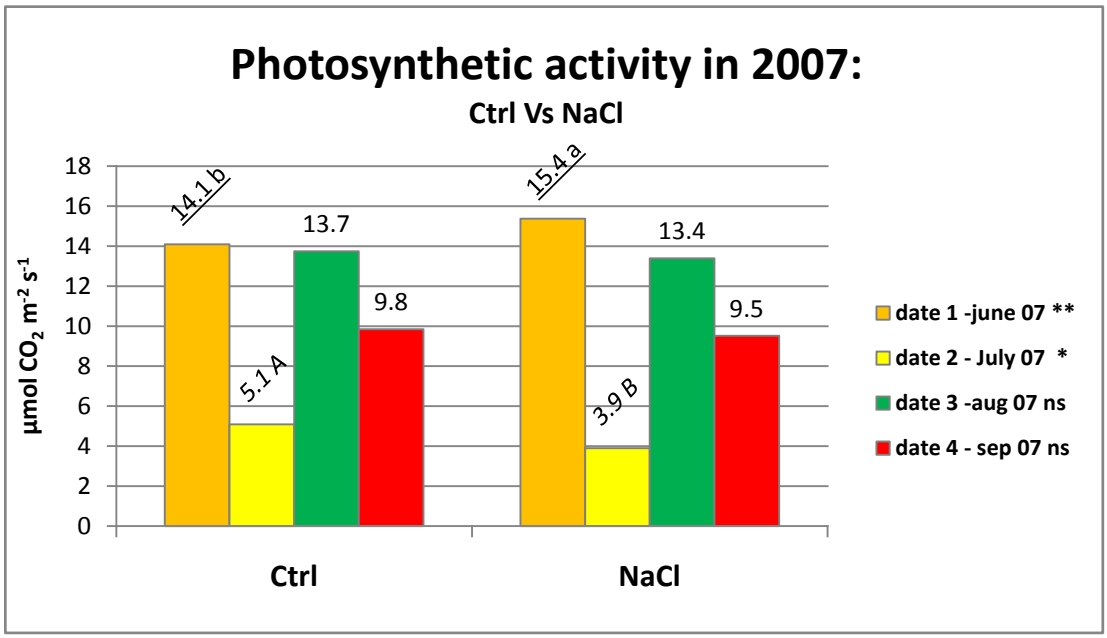
## 6.2 Gas exchange measurements

### 6.2.1 Gas exchange measurements in 2007

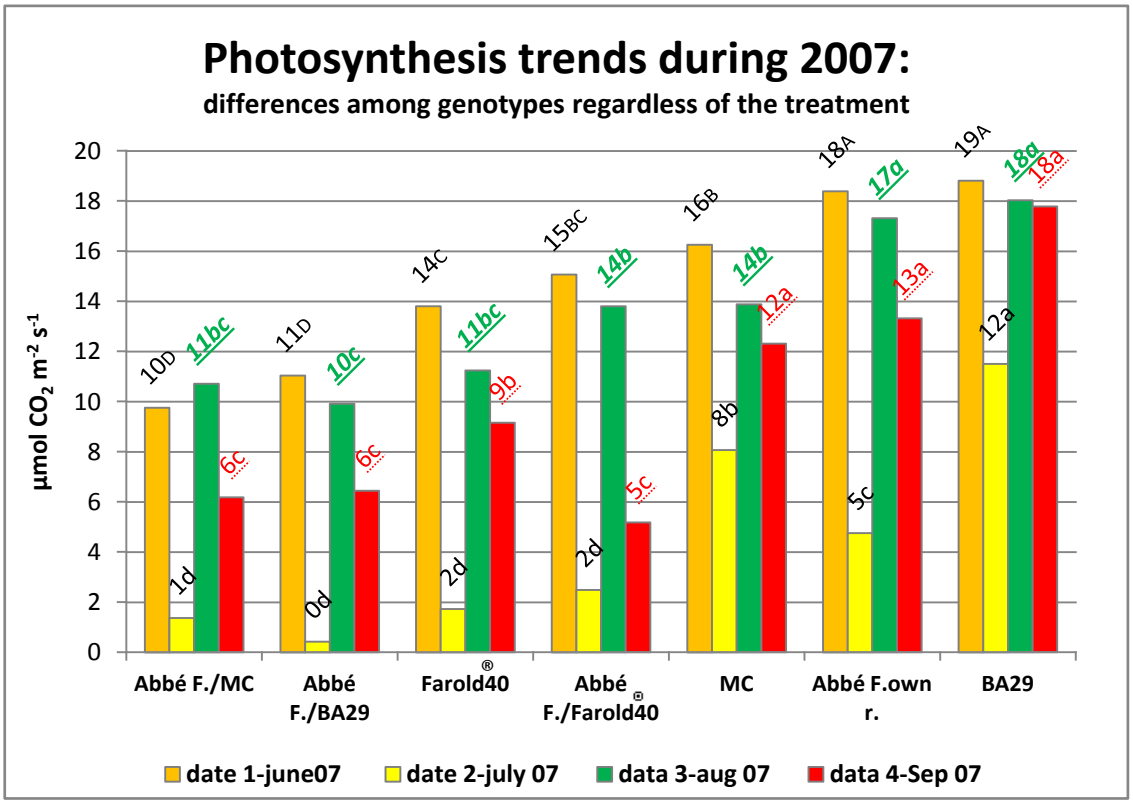
The gas exchange measurements were carried out four times during the season, two times before salt treatment (date 1 and 2) and two during the stress imposition (date 3 and 4). So, starting from date 3, it was possible to compare "control" against "NaCl" plants to evaluate the effect of salt treatment on photosynthesis.

From graph 6-6, it appeared evident that the only significant differences between "control" and "NaCl" plants were relative to date 1 (June 07) and date 2 (July 07) where treatment was not applied. These differences in photosynthesis were due probably only to variability among plants. In date 2, the average photosynthetic values resulted really lower than in the other dates because of the high temperature registered in July 2007 (appendix 7).

Analyzing data with regard to genotypes/combinations, without taking in consideration the effect of treatment, it emerged that the seven combinations behaved differently in terms of photosynthesis during the four months of measurements. From graph 6-7, it showed that BA29 and MC were the rootstocks that maintained their photosynthetic activities always higher than that of all the other kinds of plants in the four dates.




**Graph 6-6: Photosynthetic activity trends of "control" and "NaCl" plants during 2007 from date 1 to date 4. Near the legend the significance is reported for the comparison "Ctrl"-"NaCl" in each date.**




**Graph 6-7: Trends of photosynthesis in the seven different types of plants in 4 measurements, one for month. Data are means for each genotype regardless the treatment applied to rows. Date 1 and 2 are referred to the period before the salt stress imposition. Letters present near each value are referred to the SNK discrimination made separately for each date.**

In fact to point out the BA29's behavior we can underline that, despite the high temperature of July 07, BA29 registered a high photosynthetic activity comparable with that of Farold®40 in August 07 where temperature were less severe (graph 6-7).

Among Abbé F. own-rooted and its grafted combinations it emerged that the former was the genotype that showed the highest photosynthetic activity in comparison with the other genotypes belonging to this sub-group, and in a decrescent scale followed Abbé F./Farold®40 and then Abbé F./BA29 and Abbé F./MC that presented a similar trend during the four dates of measurements. This observation confirmed the previous one reported for Abbé Fétel own-rooted in the study carried out by Musacchi and colleagues (2002).

In date 2, all the photosynthetic values were lower than the month before; this was caused by the temperature that in that moment was over 40°C.

Also the other parameters measured at the same time with the photosynthetic activity were considered during the four month of experiment.

The stomatal conductivity did not show any significant differences between "control" and "NaCl" plants during the dates, except for date 2, where the lower value reported for "stressed" plants resulted significant respect the control one. It could be possible that the effect of temperature had influenced also this measure.

Considering the different genotypes at the light of these two physiological parameters, it appeared evident that they showed different behaviors both for stomatal conductivity such as for transpiration. In date 1, where the situation in the orchard was similar for all plants (because NaCl treatment was not started yet), differences between treatments were not significant as appeared in table 6-13 (date 1). Variations among genotypes were significant with $p<0.001$ for all the parameters evaluated through gas exchange measurements.

Genotypes that showed higher values for both stomatal conductance and transpiration in date 1 (unstressed situation) were: BA29, MC and Abbé Fétel own-rooted (the last only for the second parameter mentioned). The situation in date 2 and 3 did not differ in a meaningful way so we decided to report only the situation in September 2007 corresponding to date 4.

**Table 6-13: Gas exchange measurements in date 1 (22 June 2007).**

| Treatment | **Photosynthesis ($\mu$mol $CO_2$ $m^{-2}$ $s^{-1}$)** | | **Stomatal conductance (mol $H_2O$ $m^{-2}$ $s^{-1}$)** | | **Transpiration (mmol $H_2O$ $m^{-2}$ $s^{-1}$)** | |
|---|---|---|---|---|---|---|
| **Control** | 14.098 | b | 0.225 | | 5.355 | |
| **NaCl** | 15.373 | a | 0.231 | | 5.321 | |
| Significance | ** | | ns | | ns | |
| Genotypes/comb. | | | | | | |
| **Farold®40** | 13.809 | c | 0.200 | c | 4.779 | b |
| **Abbé F. /Farold®40** | 15.073 | bc | 0.217 | c | 5.094 | b |
| **BA29** | 18.811 | a | 0.307 | a | 6.811 | a |
| **Abbé F./BA29** | 11.045 | d | 0.171 | d | 4.253 | c |
| **MC** | 16.261 | b | 0.299 | a | 6.695 | a |
| **Abbé F. /MC** | 9.759 | d | 0.127 | e | 3.356 | d |
| **Abbé F. Own r.** | 18.389 | a | 0.275 | b | 6.378 | a |
| Significance | *** | | *** | | *** | |
| *Interaction gen*trt* | ns | | ns | | ns | |

At that time (date 2) the effect of the NaCl treatment, effectively imposed on half of plants in trial, did not appear affecting the stomatal conductance and transpiration respect to "control" plants.

**Table 6-14: Gas exchange measurements in date 4 (12 September 2007).**

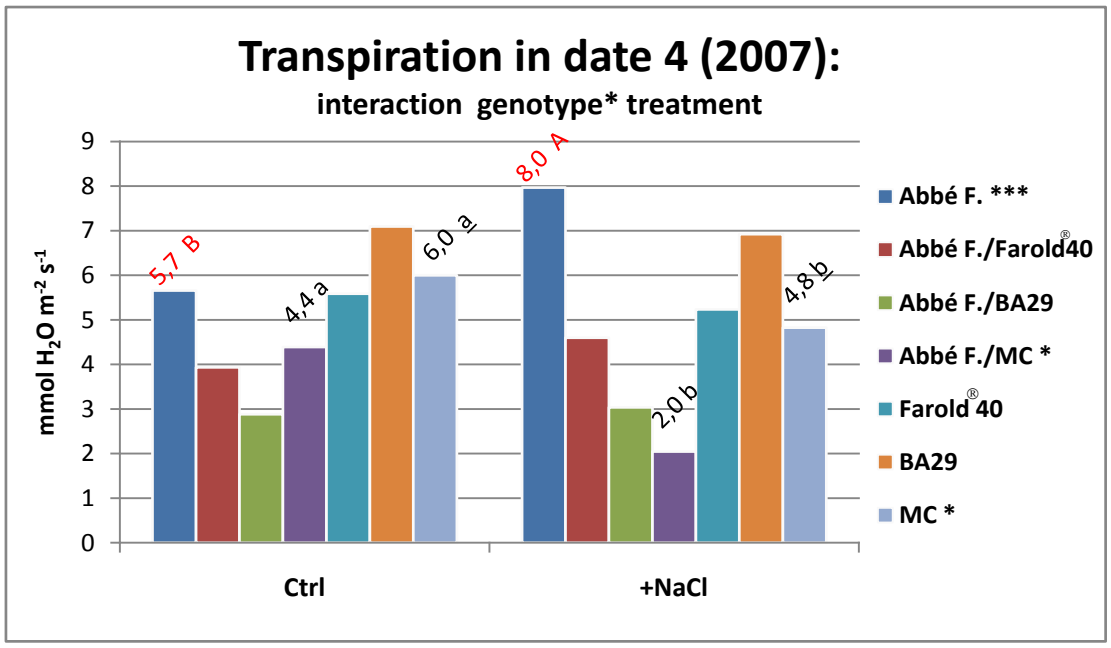
| Treatment | **Photosynthesis ($\mu$mol $CO_2$ $m^{-2}$ $s^{-1}$)** | | **Stomatal conductance (mol $H_2O$ $m^{-2}$ $s^{-1}$)** | | **Transpiration (mmol $H_2O$ $m^{-2}$ $s^{-1}$)** | |
|---|---|---|---|---|---|---|
| **Control** | 9.841 | | 0.331 | | 5.037 | |
| **NaCl** | 9.518 | | 0.312 | | 4.973 | |
| Significance | ns | | ns | | ns | |
| Genotypes/comb. | | | | | | |
| **Farold®40** | 9.155 | b | 0.351 | b | 5.412 | b |
| **Abbé F. /Farold®40** | 5.180 | c | 0.240 | c | 4.251 | c |
| **BA29** | 17.779 | a | 0.450 | a | 7.011 | a |
| **Abbé F./BA29** | 6.438 | c | 0.168 | c | 2.960 | d |
| **MC** | 12.307 | a | 0.367 | b | 5.412 | b |
| **Abbé F. /MC** | 6.177 | c | 0.205 | c | 3.220 | d |
| **Abbé F. Own r.** | 13.317 | a | 0.474 | a | 6.813 | a |
| Significance | *** | | *** | | *** | |
| *Interaction gen*trt* | ns | | * | | ** | |

Differences reported among genotypes in date 4 were not so far from those noticed in date 1 without stress. In September, the statistical analyses of gas exchange measurements pointed out the significance (with $p<0.01$) of interaction between genotype and treatment (table 6-14). As appears in graph 6-8, transpiration did not change in a meaningful way for BA29, Farold®40, Abbé F./BA29 and Abbé F./Farold®40 in the comparison between "control" plant and "NaCl" ones; while this parameter decreased significantly in Abbé F./MC and MC plants exposed to salinity. The relevance of this interaction is due to the behavior of Abbé Fétel own–rooted, this genotype in fact showed an increase of transpiration under salt stress condition and this difference resulted highly significant ($p<0.001$). It could be interesting to underline that regardless of the treatment, the three genotypes with the most low values of transpiration were referred to Abbé F. grafted on its three rootstocks in trial (graph 6-8).

In conclusion, with regard to the photosynthetic activity of plants in trial in 2007, measurements revealed that significant differences were present among genotypes in terms of different photosynthetic activity regardless of treatment.

During three months of saline treatment, the genotype among the rootstocks/un-grafted plants that showed the lowest value of photosynthesis was Farold®40, while

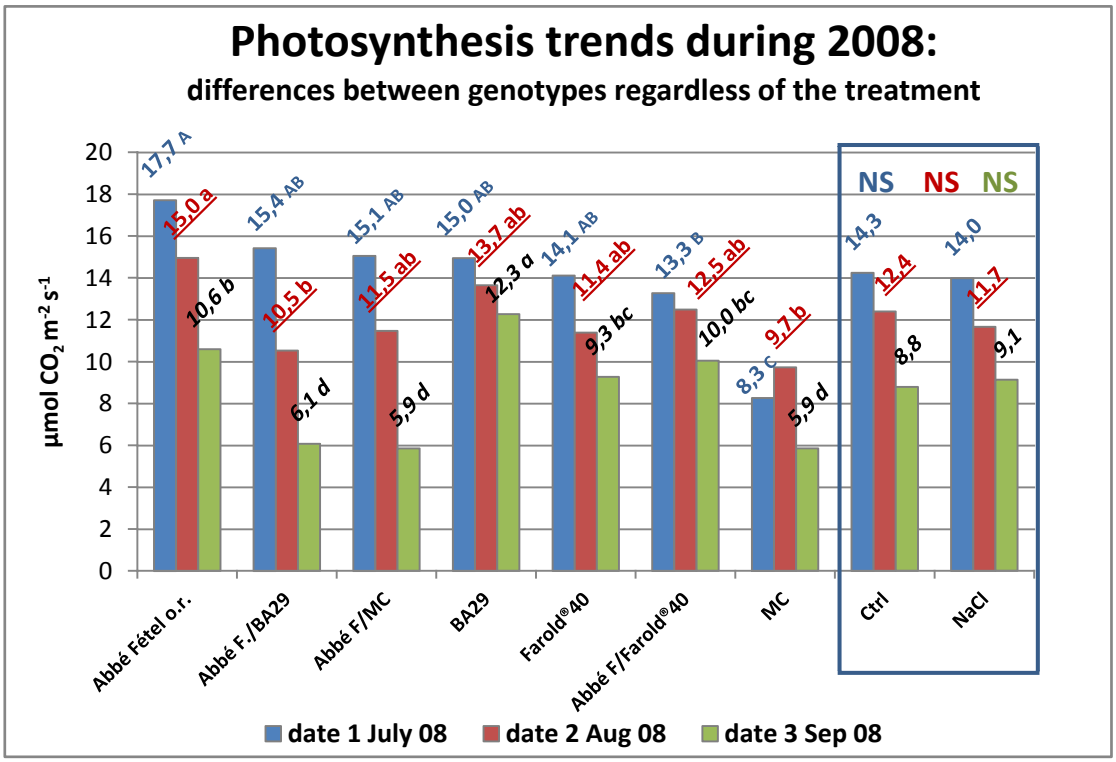
among pears and grafted combinations Abbé Fétel own-rooted appeared to be the most active in terms of photosynthesis.




**Graph 6-8: Analysis of interaction genotype*treatment resulted significant in date 4 for transpiration. On the right of the legend the significance is reported and on the top of histograms only meaningful differences are pointed out. Clear controversial behavior of Abbé F. own-rooted is underlined with red circles.**

### 6.2.2 Gas exchange measurements in 2008

In 2008, the salt treatment started before respect to the previous year and in addition the first gas exchange measurement carried out in date 1 corresponded to July 21$^{th}$ when NaCl treatment was already started. Analyzing all the data together in date 1, we realized that the difference between "control" and "NaCl" plants in terms of photosynthetic activity was not significant and that among all the kinds of plants the one that reported the highest value of photosynthesis was Abbé Fétel own-rooted followed by Abbé F./BA29, Abbé F./MC, BA29 and Farold®40 that all four reported comparable values (classified by SNK with "ab", see graph 6-9). Instead, keeping separate the two usual groups in order to maximize differences in particular between combinations (regardless of treatment), it appeared that among Abbé F. own-rooted and its grafting combinations, the highest value of photosynthesis was referred to Abbé F. own-rooted, while the lowest to Abbé F./Farold®40 and the two combinations on quinces reported a similar activity (Table 6-15).




**Graph 6-9: Trends of photosynthetic activity during 2008 from July to September. Differences among genotypes/combinations and between "Ctrl" and "NaCl" are visualized and on the top of histograms values and significance are reported. Letters are referred to SNK discrimination made for each date separately.**

Also separating plants in these two groups, the numeric variations between "control" and "NaCl" plants were always not significant in both sub-groups (table 6-16). In general, the stomatal conductivity in both groups followed the trends of photosynthesis and with slight fluctuations also transpiration (see table 6-15 and 16). In both cases of analyses the interaction genotype* treatment did not result significant.

Examining all together the data relative to date 2 (August 2008), it has been confirmed that the difference between "control" and "NaCl" plants in terms of photosynthetic activity was not significant and that among all the combinations, differences were significant with $p<0.05$. The genotype that registered the highest photosynthesis was again Abbé Fétel own-rooted (like in date 1) followed by Abbé F./MC, Abbé F./Farold®40, BA29 and Farold®40. These genotypes reported four comparable values (classified by SNK with "ab", see graph 6-9), while Abbé F./BA29 reported a decrease respect to the previous four genotypes and respect to date 1.

**Table 6-15: Gas exchange measurements on Abbé Fétel own-rooted and its grafted combinations in date 1 (July 21th, 2008).**

| Treatment | **Photosynthesis ($\mu$mol $CO_2$ $m^{-2}$ $s^{-1}$)** | | **Stomatal conductance (mol $H_2O$ $m^{-2}$ $s^{-1}$)** | | **Transpiration (mmol $H_2O$ $m^{-2}$ $s^{-1}$)** | |
|---|---|---|---|---|---|---|
| **Control** | 15.03 | | 0.167 | | 3.73 | |
| **NaCl** | 15.70 | | 0.147 | | 3.65 | |
| *Significance* | NS | | NS | | NS | |
| Combination | | | | | | |
| **Abbé F. own root.** | 17.72 | a | 0.194 | a | 4.56 | a |
| **Abbé F./BA29** | 15.43 | ab | 0.149 | ab | 3.57 | bc |
| **Abbé F./MC** | 15.06 | ab | 0.154 | ab | 3.80 | ab |
| **Abbé F./Farold®40** | 13.28 | b | 0.132 | b | 2.84 | c |
| *Significance* | * | | ** | | *** | |
| *Interaction gen*trt* | NS | | NS | | NS | |

**Table 6-16: Gas exchange measurements on Abbé Fétel own-rooted and its rootstocks in date 1 (July 21th, 2008).**

| Treatment | **Photosynthesis ($\mu$mol $CO_2$ $m^{-2}$ $s^{-1}$)** | | **Stomatal conductance (mol $H_2O$ $m^{-2}$ $s^{-1}$)** | | **Transpiration (mmol $H_2O$ $m^{-2}$ $s^{-1}$)** | |
|---|---|---|---|---|---|---|
| **Control** | 14.04 | | 0.157 | | 3.49 | |
| **NaCl** | 13.49 | | 0.134 | | 3.32 | |
| *Significance* | NS | | NS (5.1%) | | NS | |
| Combination | | | | | | |
| **Abbé F. own root.** | 17.72 | a | 0.194 | a | 4.56 | a |
| **BA29** | 14.95 | ab | 0.147 | b | 3.56 | b |
| **Farold®40** | 14.12 | b | 0.152 | b | 3.32 | b |
| **MC** | 8.27 | c | 0.089 | c | 2.17 | c |
| *Significance* | *** | | *** | | *** | |
| *Interaction gen*trt* | NS | | NS | | NS | |

From table 6-17, it resulted that among Abbé F. own-rooted and its grafting combinations, the highest value was again reported by Abbé F. own-rooted, while the lowest photosynthetic activity was similar for Abbé F. grafted on both quinces. The combination Abbé F./Farold®40 had a photosynthesis immediately lower to Abbé F. own-rooted (ab for SNK) contrary respect to the previous month, where its value was the lowest among the combinations (b for SNK; Table 6-15 and 6-17).

**Table 6-17: Gas exchange measurements on Abbé Fétel own-rooted and its grafted combinations in date 2 (August 22th, 2008).**

| Treatment | **Photosynthesis (µmol $CO_2$ $m^{-2}$ $s^{-1}$)** | | **Stomatal conductance (mol $H_2O$ $m^{-2}$ $s^{-1}$)** | **Transpiration (mmol $H_2O$ $m^{-2}$ $s^{-1}$)** |
|---|---|---|---|---|
| **Control** | 12.97 | | 0.157 | 4.37 |
| **NaCl** | 11.76 | | 0.146 | 4.41 |
| *Significance* | NS | | NS | NS |
| Genotype/combination | | | | |
| **Abbé F. own-rooted** | 14.96 | a | 0.172 | 4.81 |
| **Abbé F. /BA29** | 10.52 | b | 0.14 | 4.44 |
| **Abbé F. /MC** | 11.47 | b | 0.154 | 4.09 |
| **Abbé F. /Farold®40** | 12.49 | ab | 0.139 | 4.2 |
| *Significance* | * | | NS | NS |
| *Interaction gen*trt* | NS | | NS | NS |

**Table 6-18: Gas exchange measurements on Abbé Fétel own-rooted and its rootstocks in date 2 (August 22th, 2008).**

| Treatment | **Photosynthesis (µmol $CO_2$ $m^{-2}$ $s^{-1}$)** | | **Stomatal conductance (mol $H_2O$ $m^{-2}$ $s^{-1}$)** | | **Transpiration (mmol $H_2O$ $m^{-2}$ $s^{-1}$)** | |
|---|---|---|---|---|---|---|
| **Control** | 12.85 | | 0.130 | | 3.57 | |
| **NaCl** | 12.02 | | 0.132 | | 3.93 | |
| *Significance* | NS | | NS | | NS | |
| Genotypes | | | | | | |
| **Abbé F. own-rooted** | 14.96 | a | 0.172 | a | 4.81 | a |
| **BA29** | 13.65 | ab | 0.138 | b | 4.07 | b |
| **Farold®40** | 11.39 | bc | 0.140 | b | 3.97 | b |
| **MC** | 9.73 | c | 0.074 | c | 2.14 | c |
| *Significance* | *** | | *** | | *** | |
| *Interaction gen*trt* | NS | | NS | | NS | |

In table 6-18 the behavior in terms of photosynthesis of the other group (gathering three rootstocks and Abbé Fétel own-rooted) was reported and this comparison showed again that the highest value was referred to Abbé F. own-rooted, while the lowest to MC and, despite the difference was small, Farold®40 presented an activity lower than quince BA29.

Also analyzing plants as separated in these two groups, the numeric differences between "control" and "NaCl" plants were always small and not significant in both sub-groups.

In general, the stomatal conductivity and transpiration resulted not significant in the comparison between Abbé F. and its grafted combinations regardless of treatment (table 6-17), while in table 6-18 these two parameters varied according to genotypes as for photosynthesis. In both cases of analyses the interaction genotype* treatment did not result significant.

In the last gas exchange measurement relative to September 2008 (date 3), analyzing the data of all seven genotypes/combinations together, we noticed that differences among genotypes were significant with $p<0.001$ with a SNK discrimination in 4 classes and that the highest value was reported by BA29, while Abbé F. own-rooted was not at its maximum anymore after three months of experiment. The effect of salinity did not influence the photosynthetic capacity neither in date 3 (Ctrl against NaCl= NS).

Dividing genotypes in the usual two groups, interesting differences emerged.

As reported in table 6-19, the highest levels of photosynthetic activity were measured in Abbé F. own-rooted as well as in Abbé F./Farold®40 which in date 1 was the combination that reported the lowest value in its group. So, apparently, its performance improved during these months or, better, the performances of the other genotypes belonged to this group statistically decreased ($p<0.05$) while that of Abbé F./Farold®40 was maintained constant during the three dates of measurements (data not shown). Also stomatal conductivity and transpiration resulted statistically meaningful, comparing genotypes together regardless of treatment as reported in table 6-19.

Differently from previous observations done in date 1 and 2, the interaction between genotype and treatment was meaningful with $p<0.01$, this can signify that

one or more genotypes/combinations, belonging to this group, were in some way affected by salinity. To better understand the meaning of this interaction, data were reported on graph 6-10. From this graph, the interaction appeared evident, in fact two combinations on four analyzed, registered a significant difference in photosynthetic activity between "control" value and the corresponding "NaCl" one. Abbé F./Farold®40 registered a significant ($p<0.05$) decrease of photosynthesis under salinity, while, at the same conditions Abbé F./MC showed a more meaningful ($p<0.01$) increase in this parameter moving from 4.2 to 7.5 µmol $CO_2$ $m^{-2}$ $s^{-1}$.

A similar increase in photosynthetic activity under salinity has been already seen in the previous short-term experiment with regard to MC rootstock exposed to a salt stress equal to 90 mM NaCl. In some way this behavior could be linked to an improved performance of the plant subjected to this kind of stress.

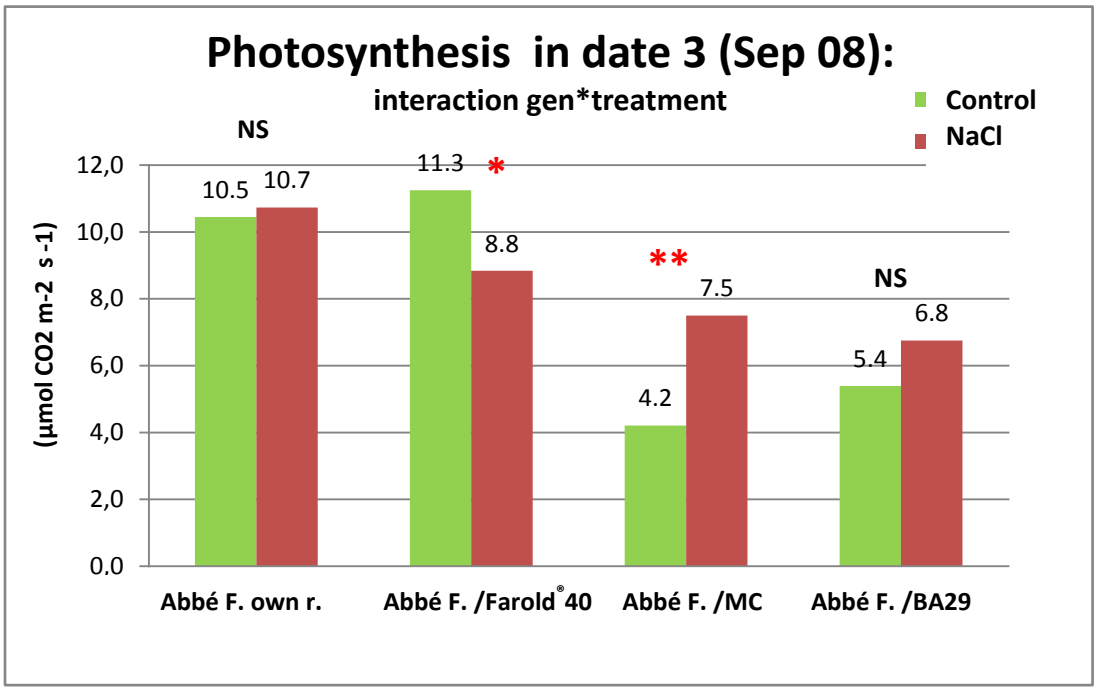
In the other group that aimed to compare essentially rootstocks performances in terms of gas exchange parameters, it appeared clear how BA29 resulted the rootstock with the highest value of photosynthesis as well as stomatal conductivity and transpiration.

**Table 6-19: Gas exchange measurements on Abbé Fétel own-rooted and its grafted combinations in date 3 (September22th, 2008).**

| Treatment | **Photosynthesis (µmol $CO_2$ $m^{-2}$ $s^{-1}$)** | | **Stomatal conductance (mol $H_2O$ $m^{-2}$ $s^{-1}$)** | | **Transpiration (mmol $H_2O$ $m^{-2}$ $s^{-1}$)** | |
|---|---|---|---|---|---|---|
| **Control** | 7.82 | | 0.113 | | 1.49 | |
| **NaCl** | 8.45 | | 0.123 | | 1.69 | |
| *Significance* | NS | | NS | | NS | |
| Genotypes/combinations | | | | | | |
| **Abbé F. own-rooted** | 10.59 | a | 0.154 | a | 2.00 | a |
| **Abbé F. /BA29** | 6.07 | b | 0.092 | b | 1.41 | ab |
| **Abbé F. /MC** | 5.85 | b | 0.148 | a | 1.17 | b |
| **Abbé F. /Farold®40** | 10.04 | a | 0.083 | b | 1.88 | a |
| *Significance* | *** | | *** | | ** | |
| *Interaction gen*trt* | ** | | NS | | NS | |

On the contrary, MC appeared to be the rootstock with the lowest values for all these three parameters among this group (table 6-20).

In this case interaction genotype*treatment did not appeared significant.




**Graph 6-10: Analysis of interaction gen*trt in date 3 (2008) for photosynthesis. On the top of histograms significance is reported.**

**Table 6-20: Gas exchange measurements on Abbé Fétel own-rooted and its rootstocks in date 3 (September 22th, 2008).**

| Treatment | Photosynthesis (µmol $CO_2$ $m^{-2}$ $s^{-1}$) | | Stomatal conductance (mol $H_2O$ $m^{-2}$ $s^{-1}$) | | Transpiration (mmol $H_2O$ $m^{-2}$ $s^{-1}$) | |
|---|---|---|---|---|---|---|
| **Control** | 10.21 | | 0.164 | | 2.08 | |
| **NaCl** | 10.21 | | 0.146 | | 1.86 | |
| *Significance* | NS | | NS | | NS | |
| Genotypes | | | | | | |
| **Abbé F. own-rooted** | 10.59 | b | 0.154 | b | 2.00 | ab |
| **BA29** | 12.27 | a | 0.190 | a | 2.35 | a |
| **Farold®40** | 9.27 | bc | 0.148 | bc | 1.88 | ab |
| **MC** | 8.63 | c | 0.120 | c | 1.54 | b |
| *Significance* | *** | | *** | | * | |
| *Interaction gen*trt* | NS | | NS | | NS | |

So apparently Abbé F. own-rooted performance lowered during these months respect to the other genotypes belonged to this group (table 6-20), this decrease in its performance was significant (with $p<0.05$) but it cannot be charged with salinity. Similar decrease through dates was reported for Farold®40, while both quinces maintained a level of performance quite constant in terms of photosynthesis (table 6-21) from July to September 2008 (regardless of the treatment).

**Tab 6-21: Photosynthetic trend among dates for Abbé F. own-rooted and its rootstocks. Small letters distinguish according to SNK in vertical way, while capital letters in horizontal way.**

| Genotypes | **Photosynthesis ($\mu$mol $CO_2$ $m^{-2}$ $s^{-1}$) date 1** | | **Photosynthesis ($\mu$mol $CO_2$ $m^{-2}$ $s^{-1}$) date 2** | | **Photosynthesis ($\mu$mol $CO_2$ $m^{-2}$ $s^{-1}$) date 3** | | *Signifi.* |
|---|---|---|---|---|---|---|---|
| **Abbé F. own rooted** | 17.72 | Aa | 14.96 | B | 10.59 | Cb | * |
| **BA29** | 14.95 | ab | 13.65 | | 12.27 | a | NS |
| **Farold®40** | 14.12 | Ab | 11.39 | B | 9.27 | Bb c | * |
| **MC** | 8.27 | c | 9.73 | | 8.63 | c | NS |
| *Significance* | *** | | *** | | *** | | |

Looking at variations among different dates of measurements, it appeared evident that both “control” and “NaCl” showed a decrease in photosynthetic activity and that values at date 3 were lower than the other in date 1 and 2.

## 6.3 Plant destruction at T0

In 2007, 21 plants were destroyed and fresh and dry weights were determined.

In Table 6-22, it has been reported the water content (% WC) present in every organs, derived from plant destruction, of each genotype/combination in trial at T0.

At T0, in fine and coarse roots the differences of WC (%) did not result significant, while highly meaningful ($p<0.001$) were differences among genotypes in trunk and branches. In the three grafting combinations, the differences in WC in rootstock part resulted significant with $p<0.01$ and SNK discriminated two classes, where Abbé Fétel/Farold®40 emerged for the higher water content in this "organ" respect to the other combinations in trial. Abbé Fétel/Farold®40 also showed the higher water content (%) both in trunk and in branches, while BA29 the lowest WC in trunk.

It is well known in fact that salt stress determines a decrease in water content for its osmotic component and the capacity to go on in uptake water and maintain WC in these conditions represent a tolerance mechanism (Verslues et al., 2006).

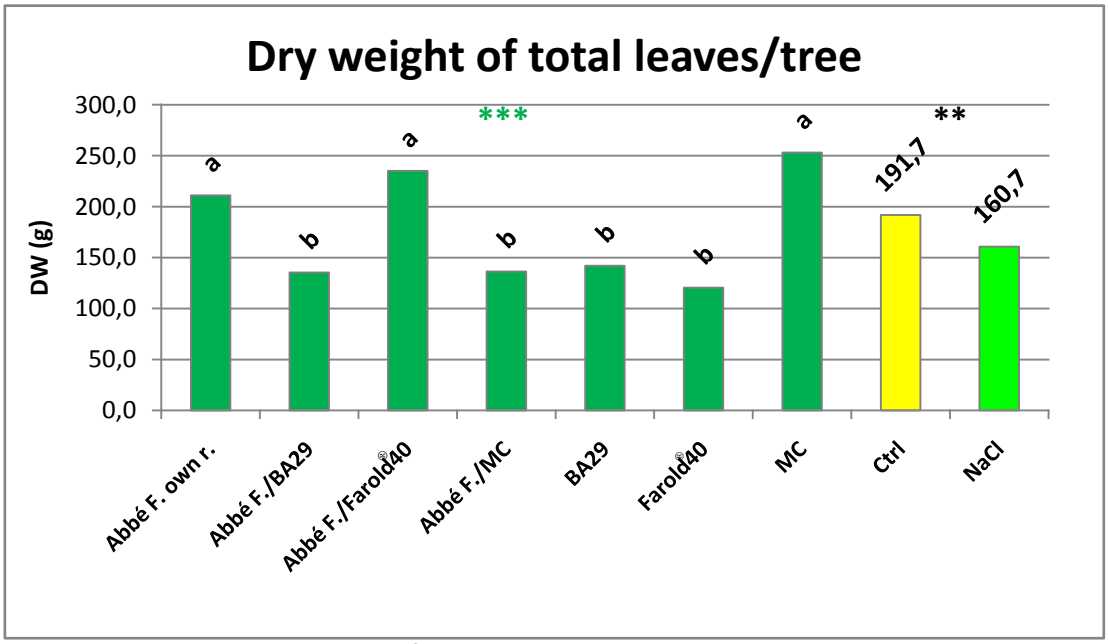
**Table 6-22: Water content (%) in organs separated from plants at T0 before stress imposition. Slashes indicate the absence of that organ.**

| Water content % | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **genotype** | **Fine Roots** | **Coarse Roots** | **Rootstock** | | **Trunk** | | **Branches** | |
| **Abbé F./BA29** | 42.7 | 51.5 | 43.4 | b | 49.2 | bc | 48.5 | b |
| **Abbé F./Farold®40** | 45.0 | 51.4 | 52.7 | a | 57.4 | a | 56.0 | a |
| **Abbé F./MC** | 48.5 | 49.5 | 44.5 | b | 49.1 | bc | 47.6 | b |
| **Abbé F. own r.** | 51.1 | 51.0 | / | | 52.9 | b | / | |
| **BA29** | 31.1 | / | / | | 39.0 | d | / | |
| **Farold®40** | 34.7 | 48.7 | / | | 47.0 | c | 44.7 | b |
| **MC** | 48.5 | / | / | | 44.6 | c | / | |
| *Significance* | NS | NS | ** | | *** | | *** | |

## 6.4 Leaf analyses at T1

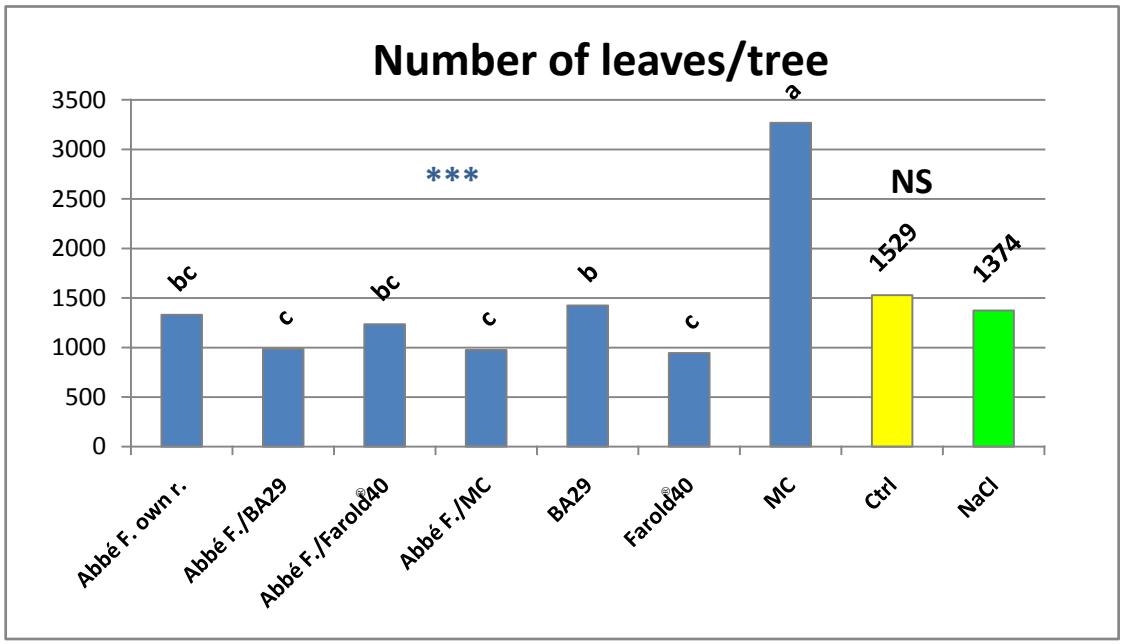
Genotypes and combinations showed variations in defoliation time (winter 2008-2009). The combinations with earlier defoliation were Abbé Fétel/MC and Abbé Fétel/BA29 followed by Farold®40 and the last genotypes, which let leaves naturally fall down, were both quinces. All the leaves were counted and weighted (fresh weight) and then let them dry in an oven.

Analyzing all trees together, it emerged that differences between genotypes/combinations resulted always highly significant ($p<0.001$) whereas, only the dry weight of total foliage per tree resulted affected by salinity. This pointed out that leaves exposed to salt stress reported a lower dry weight respect to those present in trees normally watered (significance: $p<0.01$). In graph 6-11 variations among the seven kinds of plants in terms of dry weight of total leaves per tree were represented. Abbé F. own-rooted, Abbé F./MC and MC showed the highest values of dry weight, while the other genotypes presented similar behaviors. The decrease in dry weight between "control" and "NaCl" leaves was quite similar to that reported in the experiment carried out by Musacchi and colleagues (2006a); but in that case this decrease in DW (g tree-1) caused by saline treatment did not appear significant. In general, the application of saline water in that case implied on the average a reduction of dry weight of the whole tree.




**Graph 6-11: Effect of the genotype/combination and NaCl treatment on dry weight (g per tree) of leaves. Dark green asterisks report significance of differences among genotypes, while black asterisks between "control" and "NaCl" leaves.**

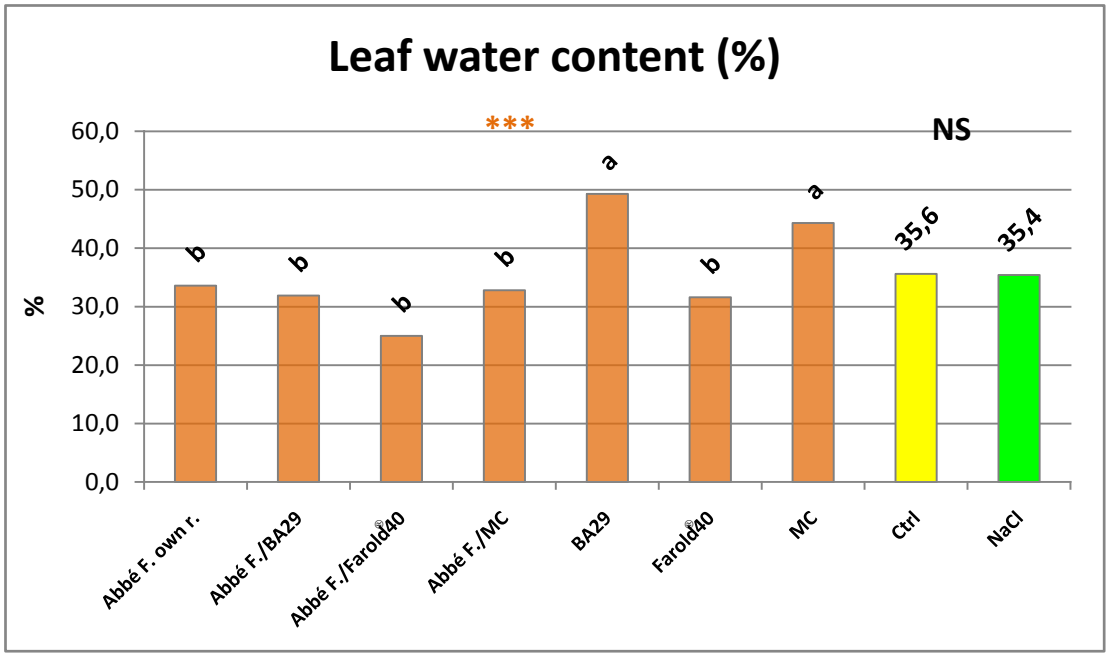
On the other hand, also that time Abbé Fétel grafted on quinces showed similar dry weights (total leaves) (Musacchi et al., 2006a) and with values comparable with the present ones of Abbé F./MC and Abbé F./BA29.




**Graph 6-12: Effect of the genotype/combination and NaCl treatment on number of leaves per tree. Dark blue asterisks report significance of differences among genotypes, while NS between "control" and "NaCl" leaves indicates not significant.**

Instead in the graph 6-12 were represented the distributions and differences in number of leaves in each genotype/combinations and it is clear that MC presented a superior number of leaves (3267) while Abbé F./BA29, Abbé F./MC and Farold®40 showed the lowest numbers of leaves per tree (around 970). Since genotypes differed largely for their number of leaves, the saline treatment did not affect in a meaningful way the number of leaves produced per tree (Ctrl vs NaCl: NS in graph 6-12). The percentage of leaf water content appeared greater in both quinces (graph 6-13) while the other genotype/combination seemed similar, perhaps this can be due to the different leaf typology, the quince one in fact appeared thicker and smaller respect to pear leaf.

A statistical analysis of Abbé Fétel combinations and Abbé Fétel own-rooted confirmed that the only aspect that seems to be affected by salinity was the leaf total dry weight per tree, whereas the other comparisons between "control" and "NaCl" leaves resulted not significant (Tab. 6-23).




**Graph 6-13: Effect of the genotype/combination and NaCl treatment on leaf water content (%). Orange asterisks report significance of differences among genotypes, while NS between "control" and "NaCl" leaves indicates not significant.**

In terms of leaf water content (%), different combinations did not present differences, while the difference found for dry weight of total leaves per tree confirmed the distribution previously reported in graph 6-11. Instead, dividing trees in these two subgroups allowed to better point out differences in number of leaves. In fact with regard of pears, the highest number of leaves was found in Abbé Fétel own-rooted trees followed by Abbé Fétel/Farold®40, while both combinations on quinces resulted comparable (Tab. 6-23) and the general significance among genotypes/combinations was for $p<0.05$.

In all the discussed cases the interaction genotype*treatment did not result significant.

In the second subgroup, where all rootstocks and Abbé Fétel were compared together, a new significant difference came out comparing "control" and "NaCl" leaves in term of number per trees. It seems that salinity caused the reduction in number of leaves per plant; this result has already been noticed in other plants exposed to salinity, where number of leaves decreased with increasing levels of salt and drought stress (Dahal et al., 2003; Suárez and Medina, 2005; El-Sharabasy et al., 2008).

**Table 6-23: Parameters evaluated after natural defoliation in 2008 in Abbé F. combinations, such as number leaves, dry weight (DW) total leaves per tree and WC (%).**

| Genotypes/ combinations | **Number leaves/tree** | | **D.W. Total leaves/tree (g)** | | **Leaf water content (%)** |
|---|---|---|---|---|---|
| **Abbé F. own r.** | 1331 | a | 211.0 | a | 33.6 |
| **Abbé F./BA29** | 985 | b | 135.4 | b | 31.9 |
| **Abbé F./Farold®40** | 1235 | ab | 235.1 | a | 25.0 |
| **Abbé F./MC** | 975 | b | 136.3 | b | 32.8 |
| *Significance* | * | | *** | | NS |
| Treatment | | | | | |
| **Ctrl** | 1190 | | 198.0 | a | 31.5 |
| **NaCl** | 1073 | | 160.8 | b | 30.2 |
| *Significance* | NS | | * | | NS |
| *Interaction gen*trt* | NS | | NS | | NS |

**Table 6-24: Parameters evaluated in Abbé Fétel own-rooted and its rootstocks after natural defoliation in 2008, such as number leaves/tree, dry weight (DW) total leaves per tree and leaf WC (%).**

| Genotypes/ combinations | **Number leaves/tree** | | **D.W. Total leaves/tree (g)** | | **Leaf water content (%)** | |
|---|---|---|---|---|---|---|
| **Abbé F. own r.** | 1331 | b | 211.0 | b | 33.6 | b |
| **BA29** | 1425 | b | 141.9 | c | 49.3 | a |
| **Farold®40** | 945 | c | 120.4 | c | 31.6 | b |
| **MC** | 3267 | a | 253.1 | a | 44.3 | a |
| *Significance* | *** | | *** | | *** | |
| Treatment | | | | | | |
| **Ctrl** | 1866 | a | 198.5 | a | 39.8 | |
| **NaCl** | 1618 | b | 164.8 | b | 39.6 | |
| *Significance* | * | | * | | NS | |
| *Interaction gen*trt* | NS | | NS | | NS | |

Looking into different genotypes, MC showed the highest number of leaves while Farold®40 the lowest for this group. The other two parameters did not show any additional and significant differences respect to the previously analyzed data (Tab.

6-24). In fact also in table 6-24, variations among genotypes/combinations for the three parameters appeared highly significant.

It was confirmed again that salinity affected the leaf total dry weight per tree, underlining a decrease in DW in the “salt-stressed” total leaves.

## 6.5 Plant destruction at T1

In table 6-25, the dry weights have been reported relativly to each organs at T1: shoots (1 year), two and three-year-old-branches, trunk, flower buds on spur and on shoots, coarse and fine roots. It resulted that the DW decreased in “salt stressed” organs, such as: shoots, two-year-old branches and coarse roots, while this parameter was not affected in the other organs (Table 6-25). An opposite and peculiar trend was reported for three-year-old branches that exhibited an increase in dry weight in “NaCl” plants. This behaviour could be due to the accumulation effect of sodium and chloride, after two year of saline treatment. In fact, sodium tends to be stored in roots, trunk and branches for several years, because tree wood represent, under salinity, a sort of sink for this ion that however has a limited capacity of storage (Myers et al., 1995; Boland et al., 1997). Among genotypes, the highest dry weight in three-year-branches was found in Abbé Fétel/BA29, followed by Abbé Fétel/MC.

The dry weight of the whole tree was affected by salinity (graph 6-14), exhibiting a decrease equal to 10% respect to “control” plants. In addition, in graph 6-14 it appeared that, regardless of the treatment, the three rootstocks accumulated less dry matter (g) than all the Abbé Fétel’s combinations.

Significant differences ($p<0.001$) among genotypes/combinations emerged in all organs except for flower buds on one-year- shoots.

In shoots, regardless of the treatment, Abbé Fétel own-rooted and grafted on Farold®40 showed highest values of DW (g), while in trunk, all weights related to pears were higher than those of quinces and clearly distinguished by SNK. The interaction treatment*genotype resulted significant only in shoots DW (with $p<0.05$), from a deeper analysis, it was noticed that really the salinity did not indiscriminately affect the dry weight in all the genotypes/combinations. In fact only Abbé Fétel/Farold®40 and Farold®40 presented shoots with a significant reduction

in dry weigh as a consequence of the treatment (graph 6-15). Abbé Fétel/Farold®40 at T0 was the combination with the highest percentage in water content in branches and trunk and, at T1, registered an unexpected decrease ($p<0.01$) in shoots DW equal to 68% respect to "control". Farold®40 instead decreased for 50% the shoots DW ($p<0.05$), this decrease was less severe than in Abbé Fétel/Farold®40 (graph 6-15).

At root level, Abbé Fétel own-rooted and grafted on Farold®40 showed the highest values of DW in coarse roots and, on the contrary, the lowest in fine roots. This situation in fine roots confirmed results obtained by Musacchi and co-workers (2006a) in the same conditions. Oppositely, Abbé Fétel/BA29 presented the highest DW of fine roots among all genotypes/combinations and Farold®40 the lowest one. At whole plant level, the greatest accumulation of dry matter, regardless of the treatment, was found in Abbé Fétel/BA29 followed by Abbé Fétel/MC that reported a value similar (according to SNK) to Abbé Fétel own-rooted and grafted on Farold®40 (Tab. 6-25 and graph 6-14).

With regard to DW of flower buds on spur, we noticed that Abbé Fétel /BA29 presented the highest weight respect to the other genotypes/combinations probably due to a rootstock effect.

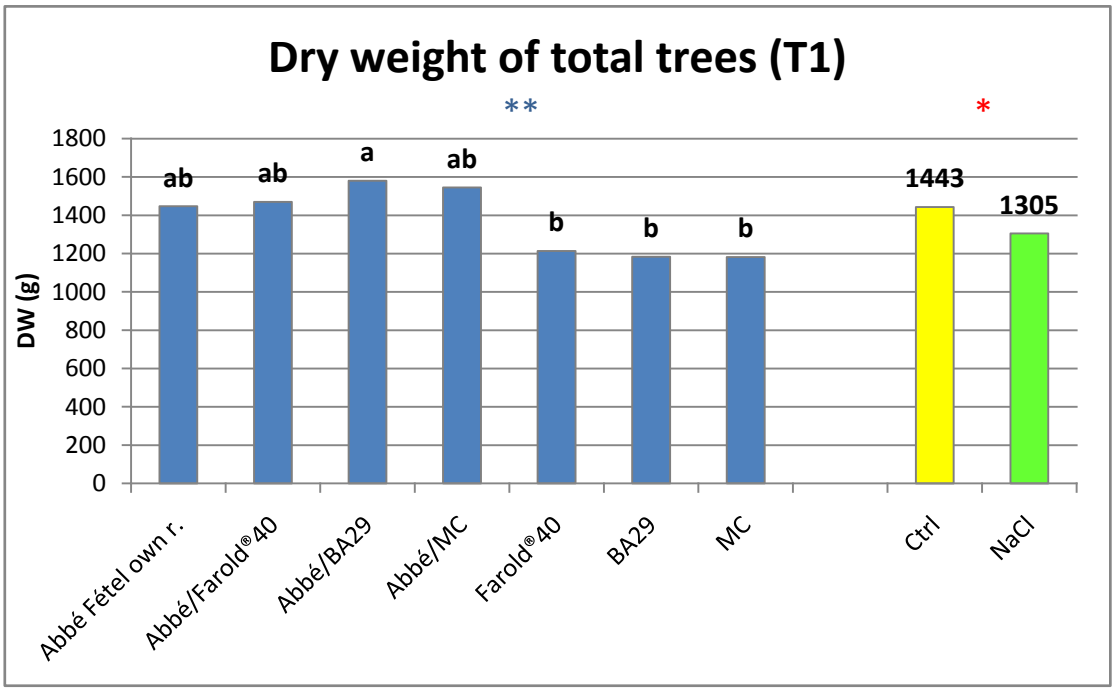
In table 6-26, water content percentages at T1 were reported for the different organs. It was clear that salinity did not lead to a significant decrease in any of the considered organs. But in fine roots, it has been registered a significant increase ($p<0.01$) in WC %: "control" fine roots showed a WC equal to 70%, while "salt-stressed" ones 73%. This fact may be explained with an increase in water flow through fine roots probably due to some changes in hydraulic resistance by roots structure modification (Rodriguez et al., 1997) induced under prolonged mild salinity. It has been reported that pericycle cells generate lateral root primordia, but it is unknown how salinity modulates this cellular process linked to development of lateral roots (Storey et al., 2003).

**Tab. 6-25: Dry weight (g) of all organs separated from T1 trees (March 2009). Significance: *= p<0.05, **= p<0.01, ***= p<0.001, NS= not significant. Values characterized by same small letters are not significantly different with p<0.05 (SNK test). # for trunk we summed DW of original trunk (3-year-old) and 2-year old trunk where present. Slashes indicate the absence of organ.**

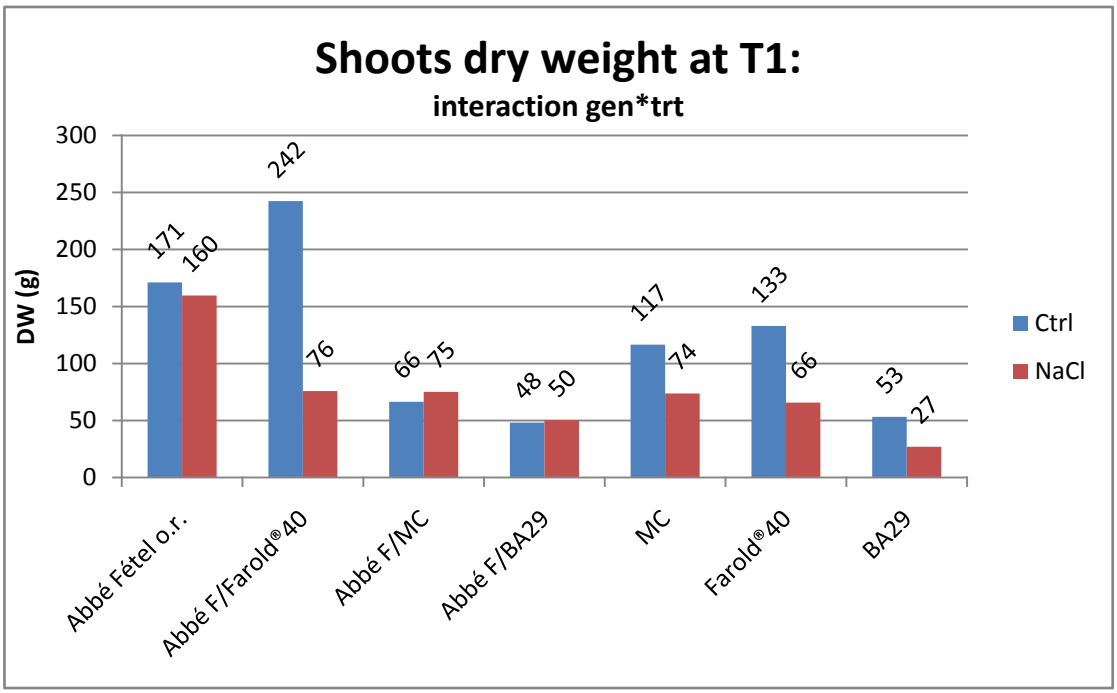
| DW (g) organs at T1 | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treatment | **Shoots** | | **Branches 2 years** | | **Branches 3 years** | | **Trunk#** | | **Flower buds on spur** | | **Flower buds on shoots** | **Fine Roots** | | **Coarse Roots** | | **Total tree** | |
| **Ctrl** | 116.3 | a | 157.0 | a | 132.44 | | 382.47 | a | 5.76 | | 7.67 | 174.18 | | 518.79 | a | 1443 | a |
| **NaCl** | 75.3 | b | 102.0 | b | 182.75 | | 320.28 | b | 5.67 | | 6.70 | 156.72 | | 460.75 | b | 1305 | b |
| *Significance* | ** | | *** | | * | | NS (7%) | | NS | | NS | NS | | * | | * | |
| Genotype/combination | | | | | | | | | | | | | | | | | |
| **Abbé Fétel own r.** | 165.4 | a | 106.6 | c | 57.3 | c | 472.35 | a | 2.23 | b | 8.63 | 58.57 | c | 589.07 | a | 1447 | ab |
| **Abbé/Farold®40** | 159.1 | a | 97.2 | c | 124.2 | bc | 400.76 | a | 4.30 | b | 10.01 | 91.40 | c | 603.75 | a | 1470 | ab |
| **Abbé/BA29** | 49.2 | b | 71.9 | c | 241.1 | a | 467.83 | a | 12.04 | a | 3.33 | 275.53 | a | 538.06 | ab | 1580 | a |
| **Abbé/MC** | 70.2 | b | 70.4 | c | 190.4 | ab | 394.12 | a | 7.03 | b | 8.36 | 180.65 | b | 371.05 | c | 1545 | ab |
| **Farold®40** | 99.3 | b | 103.5 | c | 115 | bc | 381.61 | a | 2.74 | b | 5.13 | 85.96 | c | 420.53 | bc | 1213 | b |
| **BA29** | 40.1 | b | 217.0 | b | / | | 242.46 | b | / | | / | 253.38 | ab | 429.93 | bc | 1183 | b |
| **MC** | 95.2 | b | 273.7 | a | / | | 170.25 | b | / | | / | 207.59 | ab | 506.55 | ab | 1182 | b |
| *Significance* | *** | | *** | | ** | | *** | | *** | | NS (7%) | *** | | *** | | ** | |
| *Interaction* trt*gen | * | | NS | | NS | | NS | | NS | | NS | NS | | NS | | NS | |

**Tab. 6-26: Percentage in water content (%) calculated as WC (%) = (FW-DW)/FW *100 for all organs separated from T1 trees (March 2009). Significance: *= p<0.05, **= p<0.01, ***= p<0.001, NS= not significant. Values characterized by same small letters are not significantly different with p<0.05 (SNK test). # for trunk we summed DW of original trunk (3-year-old) and 2-year old trunk where present.**

| Water content (%) organs at T1 | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Treatment** | **Shoots** | | **Branches 2 years** | | **Branches 3 years** | **Trunk#** | | **Flower buds on spur** | **Flower buds on shoots** | **Fine Roots** | | **Coarse Roots** | |
| **Ctrl** | 50.0 | | 48.8 | | 47.9 | 48.3 | | 52.2 | 55.5 | 70.3 | b | 50.8 | |
| **NaCl** | 50.7 | | 49.3 | | 48.9 | 48.3 | | 56.2 | 57.5 | 73.0 | a | 51.5 | |
| *Significance* | NS | | NS | | NS | NS | | NS | NS | ** | | NS | |
| Genotype/combination | | | | | | | | | | | | | |
| **Abbé Fétel own r.** | 51.4 | a | 50.5 | a | 49.5 | 50.3 | a | 54.4 | / | 75.2 | a | 49.6 | b |
| **Abbé/Farold®40** | 51.6 | a | 50.9 | a | 48.1 | 50.3 | a | 54.6 | 55.0 | 78.0 | a | 53.2 | a |
| **Abbé/BA29** | 53.3 | a | 50.3 | a | 48.0 | 50.5 | a | 54.0 | 58.4 | 67.1 | b | 53.9 | a |
| **Abbé/MC** | 52.5 | a | 51.4 | a | 48.4 | 49.2 | a | 54.6 | 56.9 | 68.7 | b | 55.4 | a |
| **Farold®40** | 51.4 | a | 48.6 | a | 47.9 | 48.3 | a | 52.2 | 53.4 | 76.4 | a | 52.3 | a |
| **BA29** | 46.0 | b | 45.9 | b | / | 44.7 | b | / | / | 68.0 | b | 45.2 | c |
| **MC** | 45.7 | b | 44.5 | b | / | 43.9 | b | / | / | 69.2 | b | 47.2 | bc |
| *Significance* | *** | | *** | | NS | *** | | NS | NS | *** | | *** | |
| *Interaction trt*gen* | NS | | NS | | NS | NS | | NS | NS | NS | | NS | |




**Graph 6-14: Effect of the genotype/combination and NaCl treatment on DW (g) of total trees. Dark blue asterisks report significance among genotypes, while red asterisk between "control" and "NaCl" trees.**




**Graph 6-15: Analysis of the interaction genotype*treatment resulted significant in table 6-25 for Shoots DW (g). With yellow circles want underline the genotype with peculiar behaviors. On the top of histograms is reported the significance between "Ctrl" and "NaCl" for each genotype/combination.**

Among genotypes/combinations, differences in WC resulted significant ($p<0.001$) in shoots, two-year-old branches, trunk and both kinds of roots. From these data emerged that pears were statistically distinguished from quinces (respectively “a” and “b”), the former presented higher values in WC % than the latter ones, mainly in the upper part of the plant.

In fine roots, the highest percentages of water content were found in Abbé Fétel/Farold®40, Farold®40, and Abbé Fétel own-rooted. Abbé Fétel/Farold®40, Farold®40 showed also the highest value of WC also in coarse roots.

This let us think to a greater water flow through these roots in the mentioned genotypes, regardless of the treatment. These two genotypes reported the longest shoots in average among all combinations in 2009 (tab. 6-10) and this shoot growth can support the hypothesis of a greater water uptake by roots.

Analyzing Abbé Fétel own-rooted and its combinations, reducing the variability due to rootstock, we noticed that among pear combinations no significant differences were found respect to those in table 6-26 (data not shown).

The dry material collected at T1 will be used in the next future to carry out mineral analyses in order to clarify the physiological mechanisms that regulate the different uptake, transport and accumulation of important nutrients after two years of water saline irrigation.

## 6.6 Fruit production analysis

During summer 2008, this experimental orchard produced for the first time some fruits. The only combinations involved in bearing have been: Abbé Fétel/Farold®40 Abbé Fétel/MC and Abbé Fétel/BA29, while Abbé Fétel own-rooted plants have not yet produced fruit because of their longer juvenile phase. From table 6-27, it appeared clear that the yield level was low with an average of 4.6 fruits for tree corresponding to slight more than 1 kg in weight. All fruits coming from the same combination in every "control" rows (1, 3, and 5) were gathered (paragraph 4.7), and the same was done for fruits originated by "salted" plants (row 2, 4 and 6).

In addition, it appeared from table 6-20 that, for this first year of scarse production, none significant differences in number of fruits and yield per tree (kg) emerged between "control" and "NaCl" fruits, but neither among different combinations. Also interaction between combinations and treatments was not meaningful.

At the end of harvesting, six crates were collected keeping separate treatments and combinations and size of all fruits was measured.

In this way each combination had fruits divided in size classes and it was possible to analyze the effect of two-year-NaCl-stress on fruit size.

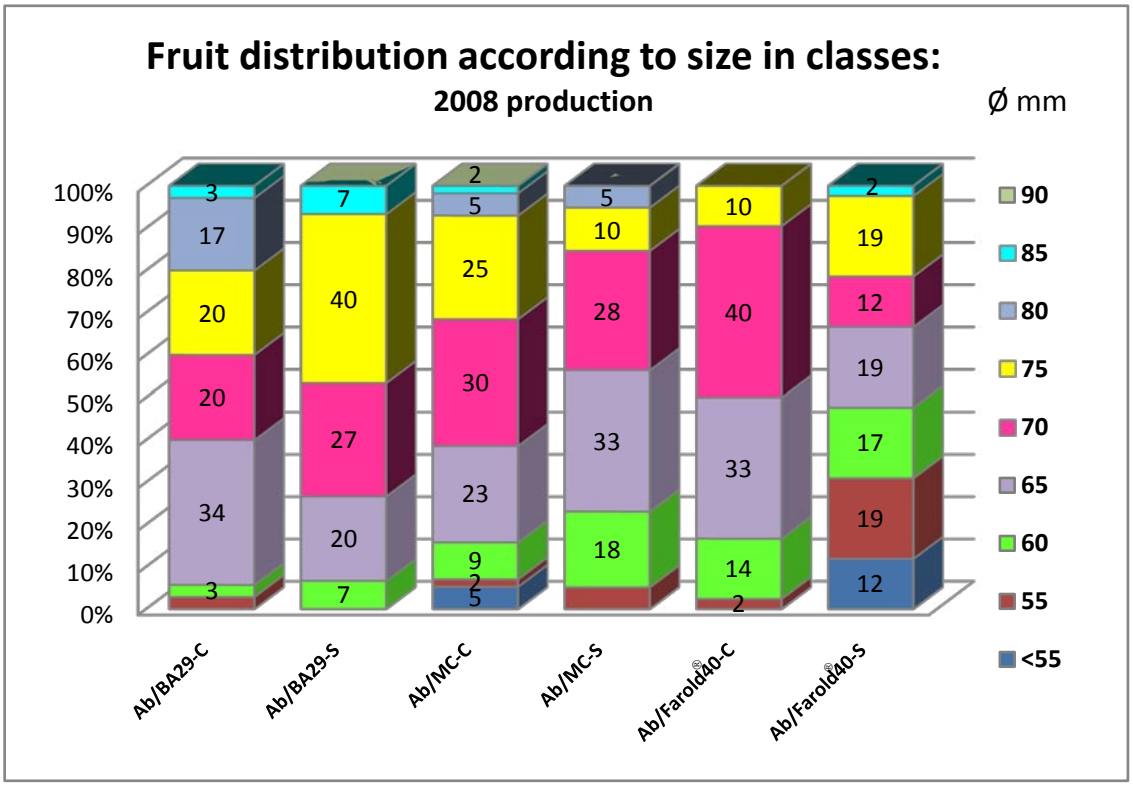
**Table 6-27: Fruits production in the second growing season (2008): number of fruits/trees and yield/trees in Kg.**

| Combinations | **Num Fruits /tree** | **Yield/tree (kg)** |
|---|---|---|
| **Abbé Fétel/Farold®40** | 4.83 | 0.9 |
| **Abbé Fétel/MC** | 4.85 | 1.17 |
| **Abbé Fétel/BA29** | 4.25 | 1.06 |
| *significance* | NS | NS |
| Treatment | | |
| **Ctrl** | 4.82 | 1.12 |
| **NaCl** | 4.55 | 0.95 |
| *significance* | NS | NS |
| *Interaction trt*comb.* | NS | NS |

From Graph 6-16, it appeared evident the different distribution of fruits into the size classes and the effect that the salt treatment had on fruit size. Comparing together "control" fruits of each combination, we can notice that apart from Abbé Fétel/Farold®40, whose distribution of fruit was 50% in the class below 70 mm and 50% over 70 mm, the other two combinations showed to have more fruits in the class over 70 mm with a percentage of 60% of the total fruits.

The same consideration was done for "NaCl" fruits and their size division appeared different; in fact for Abbé Fétel/Farold®40 and Abbé Fétel/MC the percentage of fruits with a size equal or over to 70 mm decreased respect to "control" fruits.

Instead, the increase in percentage of fruits with the superior size for Abbé Fétel/BA29, exposed to saline irrigation, is manifest (increase around 13%). This tendency has already been noticed in a study carried out by Musacchi and colleagues (2000a) where fruits of Abbé Fétel from Mezzano (location characterized by water for irrigation with electrical conductivity around 5 dS/m) showed an increase in fruit size, in particular for 65 mm and 70 mm classes, respect to same fruits coming from trees not exposed to salt stress in Cadriano (Fig. 1-12) (Caliandro et al., 2000; Musacchi et al., 2000a). In general, evidences reported that Abbé Fétel grafted on quinces has the advantage of a better size of fruit and shape (Colombo and Bolognesi, 2008).




**Graph 6-16: Abbé Fétel (Ab) fruits distribution in size classes (production 2008).Each genotype is identified with a "c" for "control" fruits and "s" for salt stressed ones. Later shadows characterize the size class ">70 mm".**

## 6.7 Qualitative analyses

In literature it has been reported that, in some cases, the irrigation with moderate saline water can improve the quality and the nutritional value of fruits, this was found for example in some cultivars of tomato (Cuartero et al., 1999; Sgherri et al., 2008) and in strawberry (Keutgen and Pawelzik, 2008). So, despite the production was so reduced, we decided to analyze some qualitative aspects of this first pear production in order to underline the effect of two seasons of saline water irrigation on fruit quality. As described previously in paragraph 4.8, the first non-destructive steps of analyses have been the evaluation of percentage of russeting and the weight of each fruits. In table 6-28 all the qualitative parameters were reported with their statistical significance in the comparison between "control" versus "salt-stressed" fruits (NaCl) and among combinations, regardless of the treatment.

With regard to russeting (%), it appeared evident that fruits obtained from "NaCl-stressed" plants presented a higher percentage of russeting (18.6%) on the total area of fruit in comparison with that shown by "control" fruits (13.5%). In particular among combinations, Abbé Fétel/BA29 emerged for its superior percentage of russeting on fruit skin (Figure 6-1), up to 19.6%, while the other two combinations presented a similar percentage (between 13 and 14%).

The same observation has been done by Musacchi and colleagues (2000a) in the experiment that compared fruits obtained by an high density planting of Abbé Fétel grafted on MC quince and irrigated with saline water (Mezzano orchard) with those obtained by the same combination grown in a normal condition (Cadriano orchard).

**Fig. 6-1: Pears obtained from "ctrl" Abbé F./BA29 tree (on the left) and from "NaCl" tress (on the right).**

**Table 6-28: Qualitative analyses of Abbé Fétel fruits obtained from different grafting combinations and exposed to saline water irrigation (5 dS/m) for two seasons (2007-2008). Significance: *= p<0.05, **= p<0.01, ***= p<0.001, NS= not significant. Values characterized by same small letters are not significantly different with p<0.05 (SNK test).**

| Combination | Fruit mean weight (g) | | Firmness of flesh ($kg/cm^2$) | | °Brix | | pH | Acidity (g/L malic ac.) | | % russeting on fruit skin | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Abbé Fétel/Farold® 40** | 214.7 | **b** | 4.43 | **b** | 16.64 | **b** | 4.16 | 1.69 | **b** | 13.3 | **b** |
| **Abbé Fétel/MC** | 256.7 | **a** | 5.17 | **a** | 18.24 | **a** | 4.16 | 1.89 | **ab** | 19.6 | **a** |
| **Abbé Fétel/BA29** | 236.3 | **ab** | 4.25 | **b** | 17.27 | **ab** | 4.17 | 1.98 | **a** | 14.2 | **b** |
| *significance* | ** | | * | | ** | | NS | ** | | * | |
| Treatment | | | | | | | | | | | |
| **Ctrl** | 247.4 | **a** | 4.31 | **b** | 17.48 | | 4.15 | 1.82 | | 13.5 | **b** |
| **NaCl** | 223 | **b** | 5.04 | **a** | 17.34 | | 4.18 | 1.90 | | 18.6 | **a** |
| *significance* | * | | ** | | NS | | NS | NS | | ** | |
| *Interaction combin*trt* | NS | | NS | | NS | | NS | NS | | ** | |

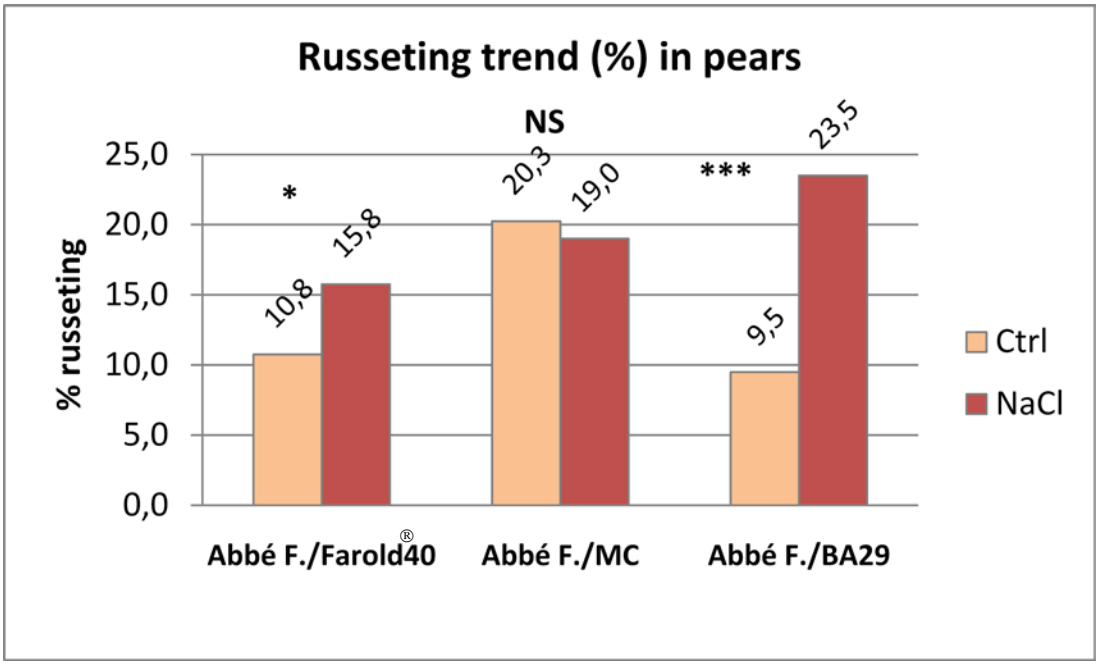
The former “salt-stressed” situation provided pears with around 37% of russeting on the skin, while in the latter condition the percentage lowered around 20 to 30% depending on the different training system (Musacchi et al., 2000a).

It is well known that russeting is the formation of cork cells (periderm) in the fruit skin as a reaction to the death of epidermal and hypodermal tissue. The causes of russeting formation are several and vary from the weather to the type of cultivar and the nature of cuticle (Gildemacher et al., 2006; de Jong and van der Maas, 2007). Other conditions able to increase russet development can be nitrogen excess, boron deficiency, growth stress, deficit in gibberellins, moisture, some fertilizer and fungicides, but also water stress (Gildemacher et al., 2006; Gil et al., 1994). Evidences reported that high fruit concentration of K and Mg appeared to influence russeting, respectively showing negative and positive correlation (Sanchez et al., 2001). In addition, it has been suggested that a way to control russeting development is to avoid large changes in electrical conductivity of the nutrient solution (http://www.ces.ncsu.edu/depts/hort/greenhouse_veg/topics/waterfert_pages/russet.html).

From all these considerations, we may hypothesize that in our case the increase in percentage of russeting in “NaCl” fruits can be due to a water stress that is tightly connected to saline treatment (as described in the paragraph 1.1.2).

In addition, the application of NaCl to rows 2, 4 and 6 caused a raise of EC (paragraph 6.0.1), which could be implied in the more significant percentage of russeting in “salt stressed” fruits.

From table 6-28, the interaction combination*treatment resulted significant so probably a different behavior in response to salinity in terms of russeting can be hidden among combinations. The statistical analysis of the interaction clarified the situation, pointing out that in Abbé F./MC the difference between “control” and “NaCl” fruits in terms of russeting (%) was not really significant, while the other two grafting combinations showed significant increases, respectively with $p<0.05$ for Abbé F./Farold®40 and with $p<0.001$ for Abbé F./BA29 (graph 6-17).




**Graph 6-17: Analysis of interaction combination*treatment: comparison between "control" and "NaCl" fruits in terms of percentage of russeting. Abbé Fétel/BA29 reported the most significan increase in % of russeting.**

From the other results obtained, it appeared clear that salinity caused a significant ($p<0.05$) reduction in average weight per fruit equal to 10%, in fact "control" fruit registered a value of 247 g compared to 223 g in salt treated pears. For this parameter also differences among combinations resulted meaningful, underlining Abbé F./MC fruits with the highest weight, and Abbé F./Farold®40 the lowest (with $p<0.01$). Abbé F./MC fruits showed an average weight comparable with the value reported for fruits of the same combination obtained from a high density planting at its 5$^{th}$ years (Musacchi, 2006c). In addition, it has been reported that Abbé Fétel grafted on Farold®40 usually produces a fruit not so elongated as Abbé Fétel standard fruit, with the tendency to russeting and with a size lower than average (Bolognesi and Colombo, 2008).

In addiction also the flesh firmness resulted affected by salt stress, in fact it seemed that the treatment led to an increase of firmness respect to control fruits. Evaluating combinations' behaviors, it has been noticed the same trend reported for fruit weight, with the most hard flesh assigned to Abbé F./MC fruits.

In general, we can say that the saline treatment on trees in trial led to obtain fruits with a higher percentage of russeting (%), a high firmness of the flesh (kg/cm$^2$), but with a lower weight per fruit (g). Also pears harvested from Mezzano orchard and

reported in Musacchi et al., 2000a showed an increase in firmness if compared with normally grown pears of the same cultivar. Moreover, this trend has been found also in tomato exposed to salinity, in fact also for this plant, NaCl caused a reduction in fruit weigh and an increase of firmness in comparison with corresponding control plants (Mizraha et al., 1986). The fruit weight decrease was reported also for strawberry exposed to long-term salt stress and this reduction may be caused by the inhibition or slowdown of water uptake and consequently reduced water transport to fruit (Keutgen and Pawelzik, 2008).

The other three parameters evaluated by destructive steps of qualitative analyses (paragraph 4.8) did not underline any significant differences between "control" fruits and "salt-stressed" ones. In particular the measurement of pear juice pH did not show differences neither between diverse combinations in trial and the mean value was 4.16. With regard to the soluble sugars and acidity of the flesh, Abbé F./MC and Abbé F./BA29 presented the highest values for both parameters (respectively for °Brix and acidity) in comparison with Abbé F./Farold®40 which showed 1.64 °Brix for soluble sugars level and 1.69 g/L malic acid (Tab. 6-28).

In general, this first year of production provided few fruits and not exactly well-shaped, so all the aspects above evaluated have to be reviewed next year, when the yield will hopefully be better and maybe the effect of salinity will be much more evident on fruits respect to what emerged in 2008.

## 6.8 Conclusion

This long-term experiment was planned as an open field trial that aimed to study in four years the behavior of seven different combinations and genotypes such as Abbé Fétel own-rooted, but also the same cultivar grafted on its most used rootstocks (BA29, MC and Farold®40) plus the same rootstocks separately planted.

Salt stress was applied through saline (NaCl) water irrigation with an electrical conductivity in average never less than 5 dS/m. In this context, we discussed results obtained from the first two years of trial, that is 2007 and 2008, but the experiment will continue for two more years, in order to observe the effect of salinity in the long-term. In fact, evidences reported that effect of salinity on perennial, deciduous woody plants increase in succeeding years, for this reason it resulted important to carry out studies on this kind of stress in the long-term (Myers et al., 1995).

Salts in soil water may cause an inhibition of plant growth firstly because the ability of the plant to take up water is reduced and later because of the specific ion-excess effect that affect transpiring leaves (Greenway and Munns, 1980). So the first aspects, evaluated in this experiment, concerned with vegetative growth and physiological trees responses to salinity. It appeared that at the end of the season the shoots length resulted significantly diminished by salinity ($p<0.05$) and that, during the months of treatment, the shoots elongation resulted in some dates lower in “NaCl” plants than in “control” ones. Single rootstocks showed to continue growing until the end of the season (September) while Abbé Fétel own-rooted arrested shoot elongation in the middle of August. The growth has been evaluated also in terms of increase of trunk section area. This parameter was not modified by salinity if compared Abbé Fétel own-rooted with its graft combinations, whereas for rootstocks apart, the increase in trunk area was limited by salt stress. In general in 2008, all the three grafted combinations of Abbé Fétel showed the highest trunk section areas in comparison with the other plants and regardless of the treatment.

Winter measurements, such as number and length of shoots and number of flower buds, did not underline any significant differences between plants watered with normal water and those exposed to saline irrigation. Another important aspect to consider was the behavior of trees in trial in terms of photosynthetic activity, stomatal conductance and transpiration. In both the years it has been registered

that, regardless of the genotypes or combinations, the three gas exchange parameters were not influenced by salinity. Among plants in trial, Abbé Fétel own-rooted emerged for its highest values of photosynthetic activity both in 2007 and 2008; stomatal conductance and transpiration followed the same trend.

On the contrary, the combinations that reported the lowest leaf photosynthetic activity were those grafted on quinces. These results on gas exchange responses confirmed previous data obtained by Musacchi and colleagues (Musacchi et al., 2002) on the same genotypes exposed to identical saline water irrigation, but for only one vegetative season. This let us think that after two years of saline treatment on *Pyrus* species (and *Cydonia oblonga* rootstocks), no relevant chances in trees physiological responses have been induced by salt stress.

In a long-term experiment on pear trees, investigating the effect of salinity on yield and fruit quality results also important in order to understand if a saline irrigation can be applied to a pear orchard without altering the commercial fruit value of pear cv. Abbé Fétel. From the first fruit production, obtained in 2008, we achieved an average of 1 Kg of fruit per tree and really few fruits per plant, and not significant differences in number of fruits and yield per tree were noticed, comparing fruits from normally-grown-plants and fruits coming from “salt-stressed”-trees.

Measuring the size of fruits, it emerged that salinity tended to reduce the percentage of fruits “over 70 mm”-size-class. This observation was partially and already done by Myers and colleagues that reported, after seven years of salt stress, an increase in the proportion of fruit in the smallest size class as a consequence of salinity (Myers et al., 1995). However, among combinations in trial their behavior was not the same in terms of decrease in pears size under salinity, in fact Abbé Fétel/BA29 showed an increase of 13% of fruits “over 70 mm”-size-class respect to the corresponding fruits from “control” plants.

Regardless of the genotype, “salt-stressed” plants provided fruits with a significant reduction in average weight; an increase in flesh firmness and a rising of russeting percentage respect to “control” fruits, these results reflected some observations previously performed by Musacchi and colleagues (Musacchi et al., 2000a).

On the other hand, soluble sugar content, pH and acidity did not result altered by salinity. Among different combinations, Abbé Fétel/MC fruit appeared to have fruits

with the highest weight average, flesh firmness and soluble sugar content (°Brix), regardless of the saline treatment.

From natural defoliation, we noticed that salinity caused a decrease in dry weight of total leaves per tree if compared with "control" trees, whereas number of leaves and leaf water content (%) did not seem affected by the treatment. While, among genotypes and combinations, Abbé Fétel own-rooted and grafted on Farold®40 showed the highest values in DW of leaves per trees, regardless of the treatment.

In conclusion, among all plants in trial, it is still untimely to decide which one is the best grafting combination for pear cultivar Abbé Fétel under salinity conditions. However, it is worth noting the behavior of Abbé Fétel/MC, in fact it resulted unaffected by salinity in terms of vegetative growth parameters, showing the highest trunk area and number of bud, but also reporting a significant increase in photosynthetic activity respect to its "control" in the last gas exchange measurement in September 2008. This result went in the opposite direction respect to the other genotypes that did not change or rather decreased their net leaf photosynthesis under salinity. With regard to fruit quality, again pears produced by Abbé Fétel/MC emerged for their significant characteristics, but further years of experiment on all these trees exposed to saline water irrigation will be useful to better appreciate their responses to this stress in the long-term. Additionally, the prolonged salinity imposition for more years will lead us to understand the salt stress "threshold" that pear is able to tolerate and it will allow to discriminate genotypes for their ability to exclude, accumulate or transfer Na and Cl ions inside the different tissues and organs of the plant.

# Chapter 7: GENERAL CONCLUSION

Salinity is one of the most severe environmental factors limiting the productivity of agricultural crops. In Italy, the most salt-affected soils are retrieved in semi-arid regions, particularly in the southern part of the country, and amounted to 450,000 ha. In addition, the quantity of water available for agriculture is decreasing in the world, so poor quality water such as saline one will be inevitably used for this purpose. This aspect, together with the actual spread of fertigation, can cause problems in the growing of fruit trees.

A deeper study on the characteristics of different pear rootstocks and their ability to face a salt stress can provide useful information for the future orchard management under this kind of condition. This work has been planned in order to investigate pear and quinces response to salt stress with a double approach: a short-term experiment carried out in a hydroponic culture system and a long-term trial in open field. The genotypes chosen for these experiments corresponded to the most used rootstocks nowadays adopted for pear culture. In particular, among quinces, MC and BA29 were chosen and Farold®40 as a pear clonal rootstock in order to be compared with Abbé Fétel own-rooted.

In the hydroponic culture experiment, these four genotypes have been exposed to salinity up to 90 mM NaCl through the nutrient solution, in order to evaluate the threshold of stress that plants were able to tolerate and moreover to observe the onset of salt toxicity symptoms. Since the concentration of saline treatment applied was considered medium-high for pear, according to previous studies, we could see the gradual development of necrosis. In fact, Farold®40 and Abbé Fétel own-rooted, exposed to salt stress, exhibited the first symptoms of leaf necrosis after just a week of salt stress, while, on the contrary, quinces showed a delay of one week in developing necrosis respect to both pears. In addition, salt-stressed pears appeared wilting, and this reflected a higher decrease in water contents in these plants respect to quinces, which showed only a sort of growth slowdown. It can be supposed that in our study the osmotic phase - the first step of the salt stress onset - lasted longer for quinces than for pears, where rapidly arose the ionic phase (the second step) that determined the complete death of the foliage.

Although both quinces could be retained apparently less sensitive to salt stress than pears, they anyway have been affected by it, showing a loss in chlorophyll content and reduction in stomatal conductance and transpiration under salinity. On the other side, pears in the same condition demonstrated the ability to find the way to tolerate a salt stress regenerating new leaves. This experiment had the aim to monitor, in addition to physiological and symptomatic responses, differences in mineral contents and in gene expression of three of the main determinants in salt tolerance mechanism such as NHX1, SOS1 and HKT1, as a consequence of the treatment. Between the two quinces, it has been noticed that, while BA29 was increasing its sequestration of sodium into leaf vacuoles (NHX1) under NaCl stress, MC temporarily enhanced its ability to compartmentalize Na in root vacuoles.

On the other hand, Farold®40 under salinity exhibited responses mainly at leaf level with important increases in expression of SOS1 and HKT1, but also showed a raised sequestration of $Na^+$ into leaf vacuoles. Finally, Abbé Fétel own-rooted reacted to salt stress raising the compartmentalization of sodium into roots vacuoles more than the other genotypes, but also activating SOS1 and HKT1 principally at root levels. Mineral content results showed also that some of the essential elements changed in their amount in different organs of the plant under salinity; this sometimes can be a negative consequence of a large sodium uptake (i.e. potassium), but in other cases, increases in macro and/or micro nutrients may in some ways contribute, in pears, to overcome the apparent leaf death or in quinces, ameliorate the detrimental effects of salt stress (i.e. calcium).

In conclusion, we can say that each genotype showed a peculiar response to salt stress that was the sum of its ability in $Na^+$ exclusion, osmotic tolerance and tissue tolerance. In particular, pears seemed to show a likely osmotic tolerance with the contribution of sodium exclusion, while quinces exhibited a tissue tolerance in addition to some processes, belonging to both osmotic tolerance and sodium exclusion mechanisms.

In the long-term experiment, we confirmed the hypothesis that pear, despite its classification as a salt-sensitive fruit tree, can be cultivated for two years with a saline water irrigation equal to 5 dS/m, without showing any salt toxicity symptoms or serious limitations on plant development and yield.

In fact, it has been reported that, for instance, gas exchange measurements, numbers of buds and leaves, as well as pear yield were not affected by a two-years-salt stress. On the contrary, it appeared that at the end of the season the shoots length decreased significantly in "salt–stressed" plants, but also that the three grafted combinations of Abbé Fétel reported the highest trunk section areas in comparison with the other plants in trial. Among them, Abbé Fétel own-rooted emerged for its highest photosynthetic activity both in 2007 and in 2008, where at the end also Abbé Fétel/MC reported a meaningful increase in photosynthesis respect its "control". These behaviors can be explained reconnecting results emerged by hydroponic system with physiological responses from the long-term experiment. In fact the short-term experiment pointed out that both Abbé Fétel and MC faced the salt stress trying to limit the sodium transport up to the shoots. They invested energies differently, but both on the activity of the main sodium transporters, increasing sodium exclusion and compartmentalization at root level. This kind of salt stress responses in these genotypes could have acted in order to support the photosynthetic activity under salinity, as reported from *"in field"* measurement.

In conclusion, among all plants in trial, it is still early to decide which one is the best grafted combination for pear cultivar Abbé Fétel under salinity conditions. However, Abbé Fétel grafted on MC, but also Abbé Fétel own-rooted resulted interesting for their peculiar behaviors under salt stress conditions.

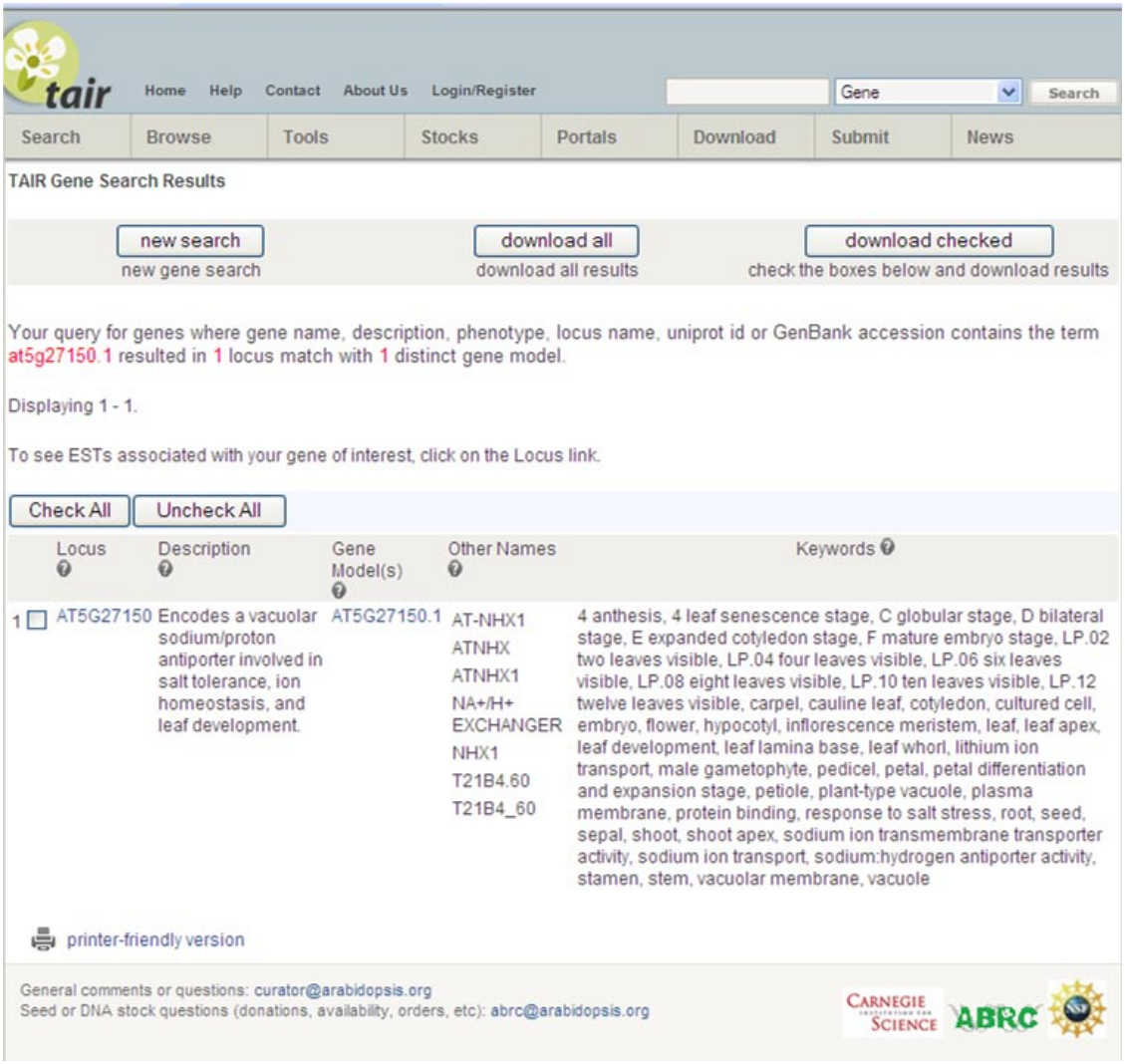
Nevertheless further investigations on physiological and molecular aspects will be necessary to deepen the knowledge of salt stress responses in pear.

# Appendix 1

## Journal of pear and quince hydroponic culture

| | |
|---|---|
| 4/10/2007 | start with hydroponic culture of 4 genotypes (2 pears: Farold®40 and Abbé Fétel own-rooted and 2 quinces: MC and BA29). |
| 5/14/2007 = **T0** | sampling of roots and leaves from all the genotypes for RNA extraction (without NaCl). |
| 5/15/2007 | 70 mM NaCl imposition to Hoagland solution<br>No sampling. |
| 5/17/2007 | 80 mM NaCl imposition to Hoagland solution (gradual increase in concentration). |
| 5/22/2007=**T1** | sampling ONLY leaves from all the genotypes (80mM NaCl treatment),<br>I didn't extract RNA from these samples because I couldn't take root samples (not abundant) in order to not compromise the experiment.<br>Start of necrosis in Farold®40 and Abbé Fétel leaves. |
| 5/28/2007=**T2** | sampling of roots and leaves from all the genotypes for RNA extraction (80mM NaCl) after that:<br>90 mM NaCl imposition to Hoagland solution (final NaCl concentration for my experiment).<br>Necrosis in Farold®40 and Abbé Fétel leaves and chlorosis in quinces. |
| 5/29/2007 | Start of necrosis in BA29 |
| 5/31/2007=**T3** | sampling of roots and leaves from all the genotypes for RNA extraction (90mM NaCl).<br>All quinces show incipient necrosis (also MC) |
| 6/6/2007=**T4** | last sampling of roots and leaves from all the genotypes for RNA extraction (90mM NaCl).<br>All pears have old leaves dead but they show new leaves in particular in Abbé Fétel (enough to sampling=15F) and some starting ones in Farold®40. |
| 6/7/2007 | end of the experiment and plant destruction for mineral analysis. |

# Appendix 2




**TAIR web page with detailed description of At-NHX1 and its function.**

# Appendix 3

```
CLUSTAL W (1.83) multiple sequence alignment

cMC             GCAACTGAATTCTGTGTGCACATTGCAGGTGCTAAATCAGGATGAGACACCATTACTCTA 60
cOHFcNHX        GCGAC-GGATTCTGTATGCACNTTGCAGGTGCTCAATCAAGATGAGACACCTTTACTCTA 59
                ** ** * ******** ***** ************ ***** ************ *********

cMC             CAG-TCTTGTACTTGGGGAGGGCGTTGTTAACGATGCGACATCTGTGGTCCTTTTCAATG 119
cOHFcNHX        CAGCTCTTGTGTTCGGGGAGGGTGTCGTTAACGATGCGACATCTGTGGTTCTTTTCAATG 119
                *** *******  * ********* ** ************************ ***********

cMC             CTATTCAGAGCTTTGATCTCACCCACATTGATCCCAGTATTGCTTTGCATTTTATAGGCA 179
cOHFcNHX        CTATTCAGAGCTTTGATCTCACCCACCTTGATTCCGGCATTGCCTTGCACTTTCTGGGAA 179
                ***************************** ***** ** * ***** ***** *** * ** *

cMC             ACTTCTCATATTTGTTTTTCGCAAGCACTATGCTAGGAGTGTTTGCAGGGCTGCTTAGTG 239
cOHFcNHX        ACTTCTTCTATTTGTTTTTCGCAAGCACCATGCTAGGAGTGTTTGCAGGGCTGCTTAGTG 239
                ******  ********************** ************************************

cMC             CTTACATCATCAAAAAGCTTTATTTCGGAAGGCACTCTACGGATCGTGAGGTTGCTCTTA 299
cOHFcNHX        CTTACATTATCAAAAAGCTTTATTTTGGAAGGCACTCTACGGATCGTGAGGTTGCTCTTA 299
                ******* ******************* ************************************

|
cMC             TGATGCTCATGGCATACTTGTCATATATACTGGCTGAATTATTCTATTTGAGTGGCATTC 359
cOHFcNHX        TGATGCTCATGGCATACCTGTCATATATACTGGCTGAATTATTCTATTTGAGTGGCATTC 359
                ******************* ************************************************

cMC             TCACTGTGTTCTTTTGTGGGATCGTGATGTCGCATTACACTTGGCACAATGTGACTGAGA 419
cOHFcNHX        TCACCGTGTTCTTTTGTGGGATCGTGATGTCGCATTACACTTGGCACAATGTGACTGAGA 419
                **** ************************************************************

cMC             GTTCAAGAGTTACGACCAAGCATGCTTTCGCGACCTTGTCATTTGTTGCCGAAACATTTA 479
cOHFcNHX        GTTCAAGAGTTACGACCAAGCATGCTTTCGCAACATTGTCATTTGTTGCCGAAATATTTA 479
                ********************************** ** ********************* ******

cMC             TCTTCCTTTATGTTGGTATGGATGCTTTGGACATTGAAAAGTGGAGATTTGTAAGTGACA 539
cOHFcNHX        TCTTCCTTTATGTTGGTATGGATGCCTTGGACATTGAAAAGTGGAGATTTGTAAGTGACA 539
                **************************** ****************************************

cMC             GTCCTGGAACATCAGTGGCAGTGAGTTCAATACTGCTAGGTCTTGTTATGCTTGGAAGAG 599
cOHFcNHX        GTCCTGGAACATCTGTGGCGGTGAGTTCAATACTGCTAGGTCTTCTTATGCTAGGAAGAG 599
                *************** ****** ************************** ******** ********

cMC             CAGCTTTCGTTTTCCCCCTATCATTCTTGTCGAACTTAACAAAGAAAAACCAACATGATA 659
cOHFcNHX        CAGCTTTTGTTTTCCCCCTACCATTTTTGTCCAACTTAGCAAAGAAAAACCAACATGAGA 659
                ******** ************** **** ****** ******* ********************* *

cMC             AAATTAGCCTTCGGCAGCAAGTTATAATATGGTGGGCTGGGCTCATGA 707
cOHFcNHX        AAATCAGCATCCAGCAGCAAGTGATAATATGGTGGGCTGGTCTAATG- 706
                **** *** * * ********** ****************** ** ***
```

**Alignment by Clustal W between cloned fragments of cMC NHX1 and cOHF NHX1.**

# Appendix 4

```
CLUSTAL 2.0.10 multiple sequence alignment

ba29            GCAAC-GGATTCTGTATGCACATTGCAGGTGCTAAATCAGGATGAGACACCATTACTCTA 59
MC              GCAACTGAATTCTGTGTGCACATTGCAGGTGCTAAATCAGGATGAGACACCATTACTCTA 60
                ***** * ******** ************************************************

ba29            CAGTCTTGTATTTGGGGAGGGCGTTGTTAACGATGCGACATCTGTGGTCCTTTTCAATGC 119
MC              CAGTCTTGTACTTGGGGAGGGCGTTGTTAACGATGCGACATCTGTGGTCCTTTTCAATGC 120
                ********** ************************************************************

ba29            TATTCAGAGCTTTGATCTCACCCACATTGATCCCAGTATTGCTTTGCATTTTATAGGCAA 179
MC              TATTCAGAGCTTTGATCTCACCCACATTGATCCCAGTATTGCTTTGCATTTTATAGGCAA 180
                ************************************************************

ba29            CTTCTCATATTTGTTTTTCGCAAGCACTATGCTAGGAGTGTTTGCAGGGCTGCTTAGTGC 239
MC              CTTCTCATATTTGTTTTTCGCAAGCACTATGCTAGGAGTGTTTGCAGGGCTGCTTAGTGC 240
                ************************************************************

ba29            TTACATTATCAAAAAGCTTTATTTCGGAAGGCACTCTACGGATCGTGAGGTTGCTCTTAT 299
MC              TTACATCATCAAAAAGCTTTATTTCGGAAGGCACTCTACGGATCGTGAGGTTGCTCTTAT 300
                ****** ************************************************************

ba29            GATGCTCATGGCATACTTGTCATATATACTGGCTGAATTATTCTACTTGAGTGGCATTCT 359
MC              GATGCTCATGGCATACTTGTCATATATACTGGCTGAATTATTCTATTTGAGTGGCATTCT 360
                ************************************************** ***************

ba29            CACTGTGTTCTTTTGTGGGATCGTGATGTCGCATTACACTTGGCACAATGTGACTGAGAG 419
MC              CACTGTGTTCTTTTGTGGGATCGTGATGTCGCATTACACTTGGCACAATGTGACTGAGAG 420
                ************************************************************

ba29            TTCAAGAGTTACGACCAAGCATGCTTTCGCGACCTTGTCATTTGTTGCCGAAACATTTAT 479
MC              TTCAAGAGTTACGACCAAGCATGCTTTCGCGACCTTGTCATTTGTTGCCGAAACATTTAT 480
                ************************************************************

ba29            CTTCCTTTATGTTGGTATGGATGCTTTGGACATTGAAAAGTGGTGATTTGTAAGTGACAG 539
MC              CTTCCTTTATGTTGGTATGGATGCTTTGGACATTGAAAAGTGGAGATTTGTAAGTGACAG 540
                ************************************************ ***************

ba29            TCCTGGAACATCAGTGGCAGTGAGTTCAATACTGCTAGGTCTTGTTATGCTTGGAAGAGC 599
MC              TCCTGGAACATCAGTGGCAGTGAGTTCAATACTGCTAGGTCTTGTTATGCTTGGAAGAGC 600
                ************************************************************

ba29            AGCTTTCGTTTTCCCCCTATCATTCTTGTCGAACTTAACAAAGAAAAACCAACATGATAA 659
MC              AGCTTTCGTTTTCCCCCTATCATTCTTGTCGAACTTAACAAAGAAAAACCAACATGATAA 660
                ************************************************************

ba29            AATTAGCCTTCGGCAGCAAGTTATAATATGGTGGGCTGGTCTAATG 705
MC              AATTAGCCTTCGGCAGCAAGTTATAATATGGTGGGCTGGGCTCATG 706
                ******************************************* ** ***
```

**Alignment by ClustalW between cBA29-NHX1 and cMC-NHX1. They got a score of 98, only some bases are different.**

# Appendix 5

**>7F S2 (SOS1 cDNA auto) 489bp**

GCCAGTCAGCAGGTCCTAACTCTTCATCGTCTCCAAGATCTTCAAGCGCTTGTAAGGCCTTA
TTCAGCATTTCAAACTTTGTATAGTCCAATATTCGTATCTTTGTGGCTGGTAATCCGTCCAT
GCCAAGAAGCCGTAGAGCAAATTGGGTAGTGGATCCATTAATTATCAGAGTTAGAAACACAA
TTCCACCTGTGAAGAAAATACACAATGTTCCTGTCTCCGTGCTGAGAAATGAATTTCCGCTA
GATTGCTTCACAGATAAAGAAAGCGAGAGCGCCACCGCACCCCTCAAACCAGACCATACGAG
TATAATGGCTTCTTTCCAGTCCAAGCCATAACCAACACATCGTAGCAGTGGAAATAAAGCTC
CAACAACAATACAACGAGATAAATGGATGTAAAAGTATAGTAGAAAAAGAAATCCCCATGAA
GTCCCTTGGTAGGCAATCTTATCGCTGTCGAGAATGCCTTCAGCAATGACAACAC

**SOS1 partial cloned sequence from cDNA of Abbé Fétel, length 489 bp.**

# Appendix 6

```
SeqA Name       Len(nt)   SeqB Name       Len(nt)   Score
=========================================================
1    cOHF-HKT   336       2    cMC-HKT    336       91
=========================================================
```

*PLEASE NOTE: Some scores may be missing from the above table if the alignment was done using multiple CPU mode. Pl*

View Output File

**Alignment**

Show Colors | View Alignment File

```
CLUSTAL 2.0.10 multiple sequence alignment


cOHF-HKT        CCCACGAATGAGAACATGATCATCTTTCGCAAGAACTCTGGTCTCCTCTGGCTCTTAATC 60
cMC-HKT         CCCACGAATGAGAACATGATCATCTTTCGCAAGAACTCTGGTCTCATCTGGCTCTTGATC 60
                ********************************************** ********** ***

cOHF-HKT        CCTCAAGTACTAATGGGAAACACATTGTTCCCTTGTTTCTCGGTGTTGTCCATTTGGGGA 120
cMC-HKT         CCTCAAGTACTAATGGGAAACACTTTGTTCCCTTGCTTCTTGGTTATGCTCATATGGGGA 120
                ********************** *********** **** ***  **  *** ******

cOHF-HKT        CTTTACAAGATCACAAAGCGAGACGAGTTTAGTTACATTCTCAAGAACCACAAAAAGATG 180
cMC-HKT         CTTTACAAGATCACAAAGCGTGAAGAGTTTAGTTACATTCTCAAGAACCACAAGAAGATG 180
                ******************* ** ****************************** ******

cOHF-HKT        GGATACTCTCATCTATTCTCCGTTCGTCTATGTGTTCTTCTTGGATTGACAGTGTTAGGG 240
cMC-HKT         GGATACTCTCATCTACTCTCGGTTCGTCTTTGTTTTCTTCTTGGATTAACAGTGTTAGGG 240
                *************** **** ******** *** ************* ************

cOHF-HKT        TTTCTGATGATACAGCTTCTTCTCTTTTCCGCCTCTGAGTGGAGCTCCAAGTCACTTGAA 300
cMC-HKT         TTTCTAACGATACAGCTTCTTCTCTTTTGCGCCTTTGAGTGGAGCTCTGAGTCTCTCCAA 300
                ***** * ******************** ***** ************  **** **  **

cOHF-HKT        GGAATGAGTTCGTACGAGAAGTTGGTTGGATCGTTG 336
cMC-HKT         GGTATGAGCTCATACGAGAAGTTGGTTGGATCGTTG 336
                ** ***** ** ************************
```

**ClustalW alignment between the first two putative HKT1 cloned fragments starting from cOHF (=Farold®40) and cMC. The two sequences presented some difference in between but the recorded score is 91.**

# Appendix 7

**Temperature and rain (January 2007-August 2008)**
**(http://www.comune.bologna.it/iperbole/piancont/noterapide/meteo/2008/)**

**Temperature e precipitazioni. Stazione di Bologna - Area Urbana**

**da Gennaio 2007 a Agosto 2008**

| Anni | Temperatura | | Temperatura media mensile | | | Precipitazione totale (mm.) | Numero di giorni con pioggia [1] (gg.) | Umidità relativa media mensile (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Massima assoluta (°C) | Minima assoluta (°C) | Media giornal. (°C) | Massima giornal. (°C) | Minima giornal. (°C) | | | valore massimo giornaliero | valore minimo giornaliero | valore medio giornalierio |
| 2007 | | | | | | | | | | |
| Gennaio | 17,0 | 0,8 | 6,7 | 10,4 | 3,4 | 11 | 5 | 90 | 66 | 78 |
| Febbraio | 17,8 | -0,6 | 8,2 | 11,8 | 5,0 | 35 | 5 | 87 | 60 | 73 |
| Marzo | 22,8 | 2,6 | 10,9 | 15,1 | 6,9 | 100 | 8 | 81 | 48 | 64 |
| Aprile | 28,0 | 7,2 | 16,8 | 22,2 | 11,2 | 20 | 1 | 74 | 35 | 54 |
| Maggio | 32,7 | 10,5 | 20,5 | 25,8 | 15,6 | 42 | 7 | 69 | 34 | 52 |
| Giugno | 33,7 | 12,9 | 23,2 | 28,2 | 18,8 | 132 | 7 | 72 | 37 | 55 |
| Luglio | 38,0 | 14,3 | 27,0 | 32,9 | 20,9 | 1 | 1 | 53 | 22 | 37 |
| Agosto | 35,2 | 15,4 | 24,2 | 29,6 | 19,1 | 19 | 5 | 68 | 33 | 51 |
| Settembre | 29,6 | 10,3 | 19,6 | 25,1 | 14,2 | 25 | 3 | 71 | 30 | 51 |
| Ottobre | 26,6 | 5,1 | 14,5 | 18,5 | 11,0 | 134 | 7 | 81 | 50 | 66 |
| Novembre | 17,6 | 1,4 | 8,8 | 12,2 | 5,8 | 17 | 2 | 81 | 57 | 70 |
| Dicembre | 14,1 | -4,3 | 4,8 | 8,1 | 2,1 | 41 | 4 | 81 | 55 | 68 |
| **Anno** | **38,0** | **-4,3** | **15,4** | **20,0** | **11,2** | **576** | **55** | **76** | **44** | **60** |
| 2008 | | | | | | | | | | |
| Gennaio | 17,5 | -0,4 | 6,2 | 9,1 | 3,9 | 49 | 9 | 87 | 64 | 75 |
| Febbraio | 15,6 | -2,0 | 6,9 | 10,9 | 3,5 | 16 | 3 | 80 | 53 | 66 |
| Marzo | 26,7 | 1,3 | 10,5 | 14,8 | 6,5 | 54 | 8 | 77 | 42 | 60 |
| Aprile | 23,5 | 6,1 | 13,9 | 18,6 | 9,4 | 31 | 9 | 78 | 38 | 58 |
| Maggio | 29,1 | 8,9 | 18,4 | 23,2 | 13,6 | 133 | 7 | 76 | 41 | 59 |
| Giugno | 35,4 | 11,0 | 22,6 | 27,9 | 17,8 | 88 | 8 | 77 | 40 | 59 |
| Luglio | 34,4 | 15,6 | 25,7 | 31,1 | 20,3 | 39 | 4 | 66 | 29 | 48 |
| Agosto | 35,7 | 15,9 | 26,1 | 31,8 | 20,1 | 10 | 2 | 63 | 28 | 45 |
| Settembre | | | | | | | | | | |
| Ottobre | | | | | | | | | | |
| Novembre | | | | | | | | | | |
| Dicembre | | | | | | | | | | |
| **Anno** | | | | | | | | | | |

NOTA BENE: Stazione meteo collocata in sede ARPA-SIM, Viale Silvani 6 - Bologna. Altezza 30m sul piano stradale.

(1) Si considerano giorni di pioggia solo quelli in cui la quantità di precipitazioni è uguale o superiore ad 1mm.

Fonte: ARPA-SIM - Agenzia Regionale Prevenzione e Ambiente dell'Emilia-Romagna - Servizio IdroMeteorologico

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