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PHYSIOLOGICAL AND BIOCHEMICAL ASPECTS OF IRON NUTRITION IN GRAPEVINE

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Esame finale anno 2012

To my beloved wife Carolina and my little son Nicolás

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CHAPTER I

INTRODUCTION

1.1. Iron nutrition in humans and animals

Iron (Fe) is one of the most important micronutrients in the human diet and a large segment of the world's population does not ingest enough iron to meet daily dietary requirements. Therefore, Fe deficiency and Fe deficiency anemia (IDA) are estimated to affect 30-50% of the world's population (Vasconcelos and Grusak, 2006), being especially prevalent in developing countries where food intakes can be severely low. In some populations, Fe deficiency is estimated to reach 85% (Kapur et al., 2002). The iron present in the human body is mostly in a stored form, and losses are usually minimal. However, dietary intake of Fe is needed to replace the Fe lost by passage of stool and urine, shedding of skin, and sweating. After exercising, a person can loose up to 1 mg of iron (Vellar, 1968), and on average the looses represent around 0,9 mg of iron per day for an adult male and 0,8 mg per day for and adult female (DeMaeyer et al., 1989).

Iron has an important function as a component of a number of proteins, including enzymes and hemoglobin. It is crucial for optimal physical performance and cognitive development. At least four major classes of Fe-containing proteins exist in the mammalian system: iron containing enzymes (hemoglobin, myoglobin, cytochromes), Fe-sulfur enzymes (flavoproteins, heme-flavoproteins), proteins for Fe storage and transport (transferring, lactoferrin, ferritin, hemosiderin), and other Fe-containing or activated enzymes (sulfur, non-heme enzymes) (Institute of Medicine, 2001). Nutritional Fe is usually divided into heme Fe, which is absorbed unaffected by other food components, and non-heme Fe, which is envisioned as "free" or as weak complexes (Theil, 2004). Heme Fe contributes only 10-15% of the total Fe intake (1 to 3 mg day^{-1}), in diets of developed countries but may provide a substantial amount of the total absorbed iron. Where meat is consumed extensively (e.g. Argentina and New Zealand) this contribution can rise to almost 50%

(Vasconcelos and Grusak, 2006). Heme Fe intake is negligible for the majority of people in many developing countries, due to cultural constraints and the high cost of animal products. For this reason, non heme Fe is the main source of dietary Fe for most people in the world.
The total content of a given nutrient in any food is not always a good indicator of its nutritional quality, because not all of the nutrients in food are absorbed (Grusak and Dallapena, 1999). Plant foods can contribute significantly to human nutrition and health because they contain almost all essential human nutrients (Grusak and Dallapena, 1999). However, not all plant sources provide the same amount of Fe, and the amount of Fe ingested is directly proportional to the portion size that is consumed. Guthrie and Picciano (1995) reported that the contributions of various food groups to the iron content in North American diet were as follows: cereals (43%); meat, fish, and poultry (22%); vegetables and beans (20%); eggs (3%); fruit (3%); and others sources combined (9%). In China grain products are the major food sources of Fe (38%), with vegetables and legumes contributing 14 and 7% respectively. Al least of 66% of the Fe consumed in North America and 59% for Chinese comes from plant sources (Vasconcelos and Grusak, 2006).

1.2. Iron in agricultural soils

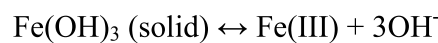
1.2.1. Iron in the soil

Iron is the fourth element more abundant in the terrestrial crust, after oxygen, silicon and aluminum (Jackson, 1958). It is present in the soil principally as primary minerals of Fe(II) (ferromagnesian silicates). These minerals are degraded by oxidation and hydrolysis, releasing Fe^{2+} and this can be oxidized to Fe^{3+}. The Fe^{3+} precipitates principally as oxides, oxyhydroxides and secondary hydroxides of Fe -denominated oxides of Fe- and are associated to clay minerals. Only a few fraction may be passed to other secondary minerals of Fe(III) or complexed by organic matter in the soil (Chen and Barak, 1982).
Iron oxides are very stable due to their low solubility, and the most common species forms are the goethite (α-FeOOH), hematite (α-Fe_2O_3), ferrihydrite ($Fe_5O_8H \cdot 4H_2O$), lepidocrocite (γ-FeOOH), maghemite (γ-Fe_2O_3) and magnetite (Fe_3O_4). Iron form stable complexes also with solid organic compounds in solution, and organic matter play an

important role for avoiding the crystallization of ferrihydrite to hematite and goethite (Schwertmann, 1964). Iron associated to organic matter is probably in a strongly crystalline form (Loeppert and Hallmark, 1985). The Fe(III) oxides can be dissolved by microbial reduction to Fe(II) or can be complexed by organic ligands. Environmental factors such as temperature, pH, and water can modulate these reactions (Schwertmann, 1985).

The solubility of Fe is controlled by the more soluble Fe oxide and the soil pH. According to Lindsay and Schwab (1982), the solubility of oxides decreases in the following order: ferrihydrite > maghemite > lepidocrocite > hematite > goethite. In highly oxygenated soils, Fe is present as Fe(III), and it may pass to Fe(II) in reducing conditions.

The concentration of Fe(III) in the soil solution is controlled by the balance with Fe hydroxides present in the solid phase, through the following reaction:



$$Fe(OH)_3 \text{ (solid)} \leftrightarrow Fe(III) + 3OH^-$$

The balance of the reaction is moved to hydroxide formation, and is highly influenced by the pH of the soil solution. If pH increases one unit in the soil, the concentration of Fe(III) ion increases thousand times (Lindsay and Schwab, 1982). As a consequence, if the pH of the soil is relatively low, Fe is more availability for plants.

Calcium carbonate may influence Fe reactions in the soil, due to modifications induced by $CaCO_3$ in the pH of soil solution. In a range of pH in the soil of 7-9, the tree forms more abundant on Fe are Fe $(OH)_2^+$, Fe $(OH)_3$ and Fe $(OH)_4^-$, where the minimum level of solubility, in equilibrium conditions with the Fe oxide more soluble, are in the range of pH 7,4-8,5 (Lindsay and Schwab, 1982). If Fe^{3+} is in solution, plants can take up it, and the solubility and speed of dissolution of Fe oxides determinate the concentration of Fe in the soil solution. In calcareous soils, the secondary Fe oxides more common are goethite and hematite. In lower proportion are ferrihydrite and, rarely, lepidocrocite and maghemite (Schwertmann, 1991).

The concentration, mineralogy and crystallization of Fe oxides affect the availability of Fe for the plant (Loeppert and Hallmark, 1985). It has been demonstrated the importance of low crystal forms of Fe oxides on the Fe chlorosis prevention, and ferrihydrite is the form

of inorganic Fe more readily available for plant uptake, because it has a large specific surface and solubility respect to crystal oxides (Vempati and Loeppert, 1986).
The Fe oxides can be quantified by an extraction with ammonium oxalate at pH 3 (Schwertmann, 1964). The properties of Fe_{ox} as chlorophyll content predictor have been demonstrated in sorghum (Morris et al., 1990), chickpea (del Campillo y Torrent, 1992), olive (Benítez et al., 2002) and grapevine (Reyes et al., 2006) cultivated in calcareous soils. $Iron_{ox}$ becomes from low crystal Fe oxides of Fe complexed by the organic fraction and the partial solution of Fe oxides less soluble, as lepidocrocite, maghemite and magnetite (Borggaard, 1982). Others methods of low crystal Fe oxides extraction are with citrate-ascorbate at pH 6, assessed in olive (Benítez et al., 2002) and grapevine (Reyes et al., 2006), and with hydroxylamine unbuffered (de Santiago and Delgado, 2006).

1.2.2. Influence of calcareous soils on iron chlorosis

A calcareous soil is defined as a soil which the pH is controlled by carbonates present in the solid phase. Calcareous soils generally present pH values of 7,5-8,5, and higher levels (until 9) can be found in soils with a high sodium bicarbonate ($NaHCO_3$) concentrations. Generally, calcite ($CaCO_3$) is the dominant carbonate form present in the soil, and in several cases the dolomite [$CaMg(CO_3)_2$] is also present. The carbonate concentration in the soil can reach values of 80% (Loeppert et al., 1994). The chemical reactivity of carbonates changes in function of the mineralogy and dimension of the particles. As a consequence, the reactivity parameters as the distribution of the particles dimension, "active calcareous", or the reactivity of acids, represent an appropriated index for estimating the chlorosing power of the soils (del Campillo et al., 1992).
Generally, Fe chlorosis in plants is highly related to the soil content of carbonates, and the percent of "active lime" has been largely considered the main indicator parameter of chlorosis risk on plants. Yaalon (1957) observed that the chlorosis level has been related with active lime concentration, and established that 10% of active lime in the soil was the critic level for the more susceptible species. Some criticism has been raised about the clay-sized fraction of calcium carbonates (Patruno and Cavazza, 1989). Recent studies demonstrated that active lime is not necessarily a soil property strongly related with

chlorosis severity in plants (del Campillo and Torrent, 1992; Benitez et al., 2002; Reyes et al., 2006).

Juste and Pouget (1972) proposed the “chlorosing power index” (CPI) defined as the ratio of the active calcareous (%) to the square of the amount of neutral ammonium oxalate extractable Fe (mg kg^{-1}) [CaCO3 / (easily extractable Fe)] x 10^4. Later, Courbé et al. (1982) proposed the Fe extractable with EDTA to calculate CPI. Other method for predicting the incidence of Fe chlorosis and the chlorophyll concentration in plants was utilized in grapevine by del Campillo and Torrent (1992) and Reyes et al. (2006) based on the product of Fe_{ox} (g kg^{-1}) x $CaCO_3$ (g kg^{-1}) x 10^4.

The pH and bicarbonate concentration in the soil is controlled by calcite ($CaCO_3$) and the partial pressure of CO_2 in the soil atmosphere (Loeppert, 1986). The bicarbonate concentration in equilibrium with calcite and atmospheric CO_2 (P_{CO2}=0,00035) is around 1 mM. Increases of CO_2 partial pressure (P_{CO2}) induce a decrease in the pH and an increase of HCO_3^- ion concentration. Bicarbonate ion decreases the solubility of Fe in the soil, because increases the pH and favors the oxidation of Fe^{2+} to low solubility compounds (Lindsay, 1984), increasing the risks of Fe chlorosis incidence in plants. In calcareous soil solution, $CaCO_3$ is in equilibrium as the follow reaction:

$$CaCO_3 + CO_2 + H_2O \leftrightarrow Ca^{2+} + 2\ HCO_3^-$$

The soil solution could has higher calcite concentrations (until 40 times) respect to the theoretically expected (Inskeep and Bloom, 1986), due to kinetic inhibitions that avoid the $CaCO_3$ precipitation. As a consequence, in the field, the effective concentration of bicarbonate in the soil can reach higher levels than those expected by the chemical equilibrium (Loeppert et al., 1994).

In soils saturated by water excess, Fe(III) ion is reduced to Fe(II) by bacteria’s that utilize Fe oxides as electron acceptors in respiration process. The excess of Fe(II) accumulation, associated to a scarce O_2 availability, may be phytotoxic for plants. A high soil compaction impairs the gaseous interchange between the soil atmosphere and the exterior, increases CO_2, and consequently HCO_3^- concentration (Mengel et al., 1984). In an initial step, organic matter can reduce Fe chlorosis incidence in plants, due to the stabilization of Fe in

amorphous oxides, that are highly available for plants (Loeppert and Hallmark, 1985), and because it is a good chelating agent (Bloom, 1981). High organic matter content may increase microbial activity and consequently the CO_2 produced by microorganisms, increasing the risks of chlorosis incidence in plants.

Clay texture is an additional factor that influences Fe availability in the soil. A fraction of Fe oxides particles (of nanometric dimension) may be adsorb in the clay surface or as Fe interstitial hydroxide into clay particles (Loeppert and Hallmark, 1985; del Campillo and Torrent, 1992). In several species cultivated in calcareous soil, it has been recorded a good correlation between clay content in the soil and leaf chlorophyll concentration in plants (del Campillo and Torrent, 1992; Benítez et al., 2002). In soils with high pH values, clay has a low retention capacity to Fe^{3+} and Fe^{2+} ions.

Root growth also influences Fe nutrition in plants, principally due to the exploration capacity into the soil, the higher rate to release of root exudates (H^+, phytosiderophores, phenols, etc.) and enzyme activity in roots. In soybean culture, temperatures <12 °C and >26 °C favor Fe chlorosis incidence, possibly due to a lower root growth and enzyme activity (Inskeep and Bloom, 1986).

1.3. **Iron nutrition in plants**

1.3.1. Metabolic functions of iron

The photosynthetic apparatus of plants represents one of the most Fe-enriched cellular systems (Spiller and Terry, 1980). In chloroplasts, an important role of Fe is related to its association with metalloproteins of the thylakoid electron transfer chain, through two prosthetic groups: heme and Fe-sulfur clusters. The terminal enzyme of heme biosynthesis, ferrochelatase, catalyses the insertion of ferrous Fe into protoporphyrin IX, the last common intermediate between heme and chlorophyll synthesis (Cornah et al., 2002). Most ferrochelatase activity is associated with plastids, which therefore represent the principal site of heme biosynthesis in higher plant cells (Cornah et al., 2002). Two ferrochelatase genes have been isolated in Arabidopsis: *At*FCII encodes a chloroplastic protein in shoots, whereas the *At*FCI product is dually targeted to plastids and mitochondria in all tissues

(Singh et al., 2002). In roots, the pool of heme is mostly found outside the plastids, and so there must be a heme export machinery on the plastid envelope (Briat et al., 2007).

Electron flow in the thylakoids also requires Fe-S clusters. In vitro studies of the import of the ferredoxin precursor have been demonstrated that chloroplasts are able to synthesize Fe-S clusters autonomously (Li et al., 1990). Characterization of the molecular factors involved in this biogenesis is recent. In vivo biogenesis, assembly and repair of Fe-S clusters have been well documented in bacteria. Three bacterial systems coexist, termed NIF (nitrogen fixation), ISC (iron-sulfur cluster), and SUF (mobilization of sulfur). Plants have the ability to synthesize Fe-S clusters both in mitochondria and plastids. In Arabidopsis, a complete SUF system and a NifS-like cysteine desulfurase are present in plastids (Balk and Lobréaux, 2005), whereas mitochondria contain homologs of the ISC system (three IscU-like desulfurases and the IscS-like desulfurase). The chloroplast Fe-S biogenesis machinery works as complexes. Production of S from cysteine by the chloroplastic desulfurase NifS protein (Léon et al., 2002) is activated by SufE (Briat et al., 2007). Protein complexes have also been identified in the plastidic SUF system. *At*NAP7 is a functional plastidic ABC/ATPase (Xu and Moller, 2004) that is involved in the maintenance and repair of oxidatively damaged Fe-S clusters. *At*NAP7 can interact with the SufD homolog *At*NAP6 (Xu and Moller, 2004). An *Arabidopsis thaliana* homolog of SufB, *At*NAP1, has been shown to interact with *At*NAP7, suggesting the presence of aNAP1–NAP7–NAP6 complex in chloroplasts (Xu et al., 2005) *At*NAP1, whose expression is down regulated by Fe-deficiency, is an Fe-stimulated ATPase that exhibits an optimum activity at a physiological Fe concentration. This suggests that, in conjunction with *At*NAP7, *At*NAP1 could act as an iron sensor in plastids, fueling Fe-S assembly and repair in response to changes in Fe levels. Furthermore, laf6, an *At*NAP1 knockout mutant, exhibits a photomorphogenic phenotype and has reduced chlorophyll content (Briat et al., 2007).

Several steps in photosynthetic pigment metabolism and chloroplast ultrastructure are dependent on Fe (Briat et al., 1995). Indeed, the characteristic symptom of plant iron deficiency is chlorosis, an interveinal leaf yellowing that is caused by a decrease in chlorophyll content. Furthermore, chloroplast proteome composition is modified in response to iron deficiency: the amount of proteins from electron transfer complexes decrease, whereas proteins that are involved in carbon fixation increase (Andaluz et al.,

2006). Chlorosis results from the proteolytic loss of photosynthetic components, including both photosystems and the cyt b6/f complex. The antenna protein complexes are differentially affected by iron deficiency, with Light Harvesting Complexi (LHCI) being drastically reduced whereas LHCII abundance remains fairly constant. The loss of Photosystem I (PSI) in response to Fe-shortage modifies the polypeptide composition of LHCI (Naumann et al., 2005), highlighting the existence of an antenna remodeling program that could be required to bypass the light sensitivity that results from PSI loss. This adaptation is a sequential process. It starts with uncoupling the antenna from the PSI core, followed by specific degradation of LHCs and induction of new LHCs, ending with the assembly of new antenna complexes in Fe-deficient cells (Briat et al., 2007). The initial uncoupling of the LHCI antenna from PSI could be regulated by the PSI-K subunit of PSI. PSI-K accumulation is influenced by the activity of Crd1, a candidate Fe-containing enzyme in chlorophyll biosynthesis, which has been proposed as a key target of plastid Fe-deficiency (Moseley et al., 2002). Recent genetic approaches have protein Nitrogen Fixation Unit2 (NFU2), which also required along with the P-loop ATPase High Chlorophyll Fluorescence101 (HCF101) for assembly of PSI clusters (Lezhneva et al., 2004). The putative function of the chloroplast-localized IscA protein (a SufA homolog) as a Fe-S cluster scaffold is less clear, because an iscA null mutant carries no defect, neither in plant growth nor in the biogenesis of major iron-sulfur proteins (Briat et al., 2007).

1.3.2. Iron absorption by roots

Depending or their response to Fe-deficiency, plants are classified into Strategy I and Strategy II to take up Fe from the soil. Non-grasses (dicotyledonous and non graminaceous monocotyledonous plants) activate a reduction-based Strategy I under Fe-starvation conditions, whereas grasses (monocotyledonous graminaceous plants) activate a chelation-based Strategy II.

Strategy I plants increase the reducing capacity and enhanced net excretion of protons and organic compounds into the rhizosphere, lowering the pH of the soil solution and increasing the solubility of Fe^{3+} (Marschner, 1995; Schmidt, 2003; Kim and Guerinot, 2007; Cesco et al., 2010). For every one unit drop in pH, Fe^{3+} becomes a 1000 fold more

soluble. One of the physiological mechanisms of Strategy I plants for downloading the pH in the rhizophere is the increase of ATPase enzyme in the cell plasma membrane of roots. The responsible proton-ATPases are not yet identified at a molecular level, and several proton-ATPases of the AHA (Arabidopsis H^+-ATPase) family are suggested to be involved in this process (Kim and Guerinot, 2007). AHA7 is up-regulated in response to Fe-deficiency and its expression is dependent on FIT1 (Fe-deficiency induced transcription factor 1), implicating AHA7 as part of the Fe-deficiency response (Colangelo and Guerinot, 2004). Cucumber species also has at least one Fe-regulated proton ATPase, whose expression is induced in Fe-deficient roots enhancing the accumulation of *Cs*HA1 gene transcripts, which were hardly detectable in leaves (Santi et al., 2005).

Phenolic compounds are frequently reported as one of the main components of root exudates in response to Fe-deficiency in strategy I plants (Cesco et al., 2010; Piagnani et al., 2003). Considering the mechanisms by which phenolic compounds regulate the mobility of Fe in the rhizosphere, it is widely accepted that the reducing and complexing properties play an important role. Ksouri et al. (2006), showed an increase in phenols index (an estimation of plant ability to release phenol compounds in the medium) in two Fe-chlorosis tolerant rootstoks (140R and Khamri) grown under Fe-shortage medium (1 µM Fe), whereas this increase was not observed in plants grown in presence of bicarbonate (20 µM Fe + 10 mM HCO_3^-). In vitro experiments, Cesco et al. (2010) observed that also flavonoids (genistein, quercetin and kaempferol) may reduce Fe(III). The magnitude of this capacity was higher when Fe was supplied as Fe(III)-EDTA rather than as Fe(III)-hydroxide. Phenolic compounds can also affect the availability of Fe in the rhizosphere through the formation of Fe complexes/chelates (Cesco et al., 2010). The chelating capacities of phenolic compounds could allow them to dissolve Fe-oxides and/or poorly soluble Fe minerals, such as goethite, by enhancing the rate of ligand-promoted dissolution of these minerals, as proposed for phytosiderophores released by graminaceus (Kraemer et al. 2006).

An important mechanism of Strategy I plants for reduce Fe^{3+} in the rizosphere is by increasing the activity of ferrichelate reductase (FCR) enzyme in the cell plasma membrane of roots. The corresponding Arabidopsis gene of ferrichelate reductase, FRO2, was identified and mapped. The FRO2 gene is expressed in the epidermal cells of Fe-deficient

roots and is thought to be the main Fe(III) chelate reductase in roots. Like FRO2, FRO3 and FRO5 are expressed in roots. However FRO3 is predominantly expressed in the vascular cylinder of roots, suggesting a role in Fe re-absorption from the root apoplast (Kim and Guerinot, 2007). The shoot-specific FRO genes are FRO6, FRO7, and FRO8. The promoter of FRO6 contains multiple light responsive elements and a FRO6 promoter driven reporter gene is activated upon exposure to light (Feng et al., 2006). The *Ps*FRO1 mRNA accumulates in Fe deficient roots, however, the mRNA is also seen throughout the root, suggesting that *Ps*FRO1 may also play a role in Fe transport within the plant. The *Le*FRO1 mRNA is detected both in roots and shoots, indicating a role in Fe mobilization in the shoots. The *Le*FRO1 localizes to the plasma membrane in onion epidermal cells and confers Fe(III) reductase activity when expressed in yeast (Li et al., 2004).

Plants of Strategy I transport Fe^{2+} inside the roots by IRT1, a member of the ZIP (ZRT, IRT-like proteins) metal transporter family. The IRT1 gene was identified in Arabidopsis, and is expressed in the epidermal cells of Fe-deficient roots and localizes to the plasma membrane. IRT1 transport multiple divalent metals (Fe, Zn, Mn and Cd) (Kim and Guerinot, 2007; Schmidt, 2003). IRT1 orthologs have been found in other Strategy I plants as well as in rice, which is a Strategy II plant. In tomato, *Le*IRT1 and *Le*IRT2 are specifically expressed in roots (Kim and Guerinot, 2007).

Strategy II plants, such as grasses, are characterized by release to the rhizosphere small molecular weight compounds known as the mugineic acid (MA) family of phytosiderophores (PS) in response to Fe-deficiency (Kim and Guerinot, 2007). PS have high affinity for Fe^{3+} and efficiently bind Fe^{3+} in the rhizosphere. Fe^{3+}-PS complexes are then transported into the plant roots via a specific transport system (Kim and Guerinot, 2007). The chelation strategy of grasses is more efficient than the reduction strategy of dicotyledonous and thus allows grasses to survive under more drastic Fe-deficiency conditions (Kim and Guerinot, 2007).

The family of mugineic acids includes 2′-deoxymugineic acid (DMA), mugineic acid (MA), 3-epihydroxymugineic acid (epiHMA), 3-epihydroxy-2’deoxymugineic acid (epiHDMA), distichonic acid (DA), 3-hydroxymugineic acid (HMA), 3-hydroxy-2’deoxymugineic acid (HDMA), and recently have been reported the two novel PS avenic acid A (AVA) and 2’-hydroxyavenic acid (HAVA) (Ueno et al., 2007). Each grass

produces its own sets of MAs and increases the production and secretion of MAs in response to Fe-deficiency. Thus, tolerance to Fe-deficiency is correlated with the amounts and the types of PS secreted (Marschner, 1995). Barley secretes large amounts of many types of PS, including MA, HMA, and epi-HMA and is therefore more tolerant of low Fe-availability (Bashir et al., 2006). It has been reported that phytosiderophores secretion in *Festuca rubra* -an efficient species for phytosiderophores secretion- is characterized by a daily rhythm and is influenced by temperature at root level (Ma et al. 2003). In the generation of MA, nicotianamine (NA) is the key intermediate that is produced from the condensation of three molecules of S-adenosyl methionine by nicotianamine synthase (NAS) (Kim and Guerinot, 2007). Nicotianamine is present not only in grasses but in non-grasses as well. NA can bind various metals including Fe^{2+} and Fe^{3+}, but is not secreted, suggesting a role for NA in intra- and intercellular metal transport in both for Strategy I and Strategy II plants (Kim and Guerinot, 2007). In Strategy II plants, grass-specific nicotianamine aminotransferase (NAAT) converts NA to the intermediate 3'-keto DMA (Kanazawa et al., 1994). Subsequent reduction of the 3-keto intermediate produces DMA, the common precursor of all other MAs (Fig. 1). Deoxymugineic acid then undergoes hydroxylation and produces other types of MAs. Two barley genes, Ids2 and Ids3, encode dioxygenases and are thought to catalyze the formation of epi-HMA and epi-HDMA in Fe-deficient roots (Nakanishi et al., 2000).

A high-affinity uptake system for specific complexes transports Fe-PS into the epidermal cells of Fe-deficient roots. The yellow-stripe 1 (ys1) maize mutant showed a defect in uptake of Fe-PS, resulted in Fe-deficiency, and plants developed interveinal chlorosis (yellow-stripe) (Curie et al., 2001). A transposon tagging of the YS1 gene enabled its molecular cloning. YS1 encodes an Fe-PS transporter, an integral membrane protein with 12 putative transmembrane domains that belongs to the oligopeptide transporter (OPT) superfamily. YS1 mRNA accumulates in response to Fe-deficiency, further suggesting that YS1 function in Fe uptake from the soil (Kim and Guerinot, 2007). In addition, YS1 is also expressed in the shoots, suggesting a role for YS1 in the intercellular transport of Fe in the plant shoots. A barley ortholog, HvYS1, is only expressed in the roots and seems to be specific for uptake of Fe(III)–PS (Murata et al., 2006).

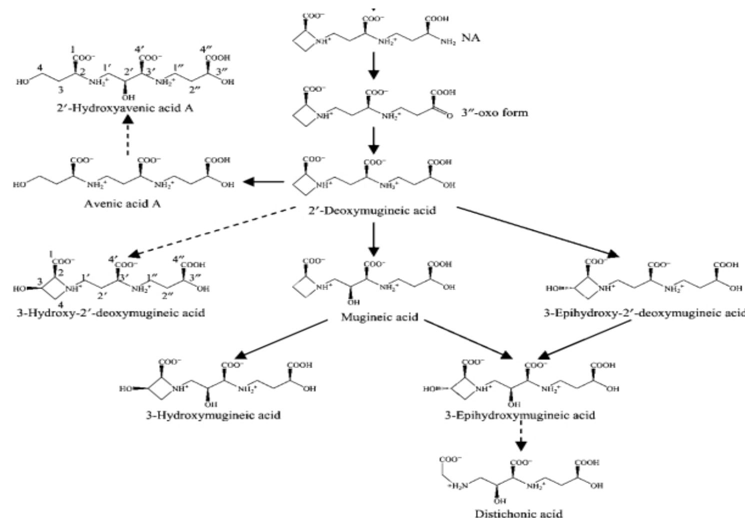


Figure 1. Mugineic family phytosiderophores (MAs). DMA, 2′-deoxymugineic acid; MA, Mugineic acid; epiHMA, 3-epihydroxymugineic acid; epiHDMA, 3-epihydroxy-2'deoxymugineic acid; DA, distichonic acid; HMA, 3-hydroxymugineic acid; HDMA, 3-hydroxy-2'deoxymugineic acid; AVA, avenic acid A; HAVA 2'-hydroxyavenic acid A (Ueno et al., 2007).

Strategy II plants can also take up Fe^{2+} like Strategy I plants. Rice, for example, in addition to having the ability to transport Fe-PS complexes, is able to transport Fe^{2+} via *Os*IRT1 (Kim and Guerinot, 2007). Like IRT1 in Arabidopsis, the *Os*IRT1 and the *Os*IRT2 genes are expressed predominantly in roots and are induced in response to Fe deficiency. However no increases of FRO-like gene expression or Fe(III) chelate reductase activity have been detected in Fe-deficient rice roots. It is plausible that rice can compensate for the lack of effective Fe(III) chelate reductases because of its wetland culture (Kim and Guerinot, 2007). In paddy fields, the equilibrium of Fe^{3+}/Fe^{2+} is shifted in the direction of Fe^{2+} due to the deficiency of oxygen in the soil. The adoption of a Fe^{2+} acquisition strategy can be especially advantageous for rice, since rice plants are not very efficient at Fe^{3+} uptake via Strategy II (Kim and Guerinot, 2007).

Under iron deficiency, plants may increase the activity of phosphoenolpyruvate carboxylase enzyme (PEPC) for fixing bicarbonate, the main causal factor of Fe chlorosis (Mengel, 1994) into phosphoenolpyruvate, generating oxaloacetate, which can be easily converted to malate. In cytoplasm the equilibrium between carboxylation (CO_2 fixation)

and decarboxylation is regulated mainly by the pH sensitivity of PEP carboxylase and the malic enzyme (Marschner, 1995). An increase in pH activates PEP carboxylase (synthesis of oxaloacetate increased). If anions are taken up in excess and thus proton-anion cotransport predominates, the pH of cytoplasm decreases and the malic enzyme is activated. De Nisi and Zocchi (2000) have reported that extractable PEPCase activity was increased by 4-fold in the absence of Fe in *Cucumis sativus* L. about 5 days after Fe starvation. In kiwifruit, this kind of responses have been reported (Rombolà, 1998; Rombolà et al., 1998; Rombolà et al., 2002), and higher PEPC activity associated with higher citric acid concentration in roots and lower xylem sap pH have been observed in the more tolerant genotype to Fe chlorosis D1 as compared with the sensitive genotype Hayward (Rombolà et al., 2002). The increase in PEPCase specific activity and concentration seem to indicate that under Fe deficiency the enzyme regulation might be, in part, exerted at the transcriptional level.

The rate of oxygen consumption can increase under Fe deficiency. López-Millán et al. (2000) showed that O_2 can be used by the FCR itself when plants are grown in complete absence of Fe. This reaction could generate H_2O_2, or other reactive oxygen species (ROS), in fact, some of the enzymes involved in the detoxification of these compounds were found to be overexpressed in some species submitted to Fe-limited conditions (Vigani et al., 2009). Microarray analysis of *A. thaliana* grown in such conditions (Thimm et al., 2001) has revealed the induction of some components of the mitochondrial electron transport chain (cytochrome c reductase and oxidase), suggesting that the observed increase in respiration activity in response to Fe deficiency involves transcriptional regulation of genes encoding for respiratory chain enzymes. However, it is not clear how the mitochondrial respiration activity participates in the increase in O_2 consumption observed in roots in these growth conditions. Vigani et al. (2009) observed that mitochondria undergo structural changes under Fe deficiency. In this work, enzyme assays revealed that lack of Fe induced a decrease in the activities of respiratory complexes that was proportional to the number of Fe atoms in each complex. Also, they proposed that mitochondria and the electron transport chain are an important target of Fe limitation and mitochondria modify their function to meet higher demands for organic acids while restricting the activity of enzymes with Fe cofactors.

Moreover, other biochemical criteria such as catalase activity were found to be strongly correlated with the content of Fe "active" fraction in, for instance, chickpea (*Cicer grietinum*) suggesting the role of these enzymes in the response of plants is dealing with this abiotic constraint (Iturbe-Ormaetxe et al., 1995). In grapevine, Ksouri et al. (2006) have reported that the activity of catalase has been affected differently by the Fe-deficiency, according to the genotype. Malonydialdehyde (MDA) is a good indicator of the possible induction of oxidative damage caused by Fe-deficiency. Malonydialdehyde contents was found to be significantly higher in the young leaves of the sensitive variety (Balta4).

1.3.3. Long-distance Fe transport and sensors

Once Fe enters the root symplast, it is required to be bound by chelating compounds and then, Fe-chelator complexes move through intercellular connections into the stele through the diffusion gradient. The mechanism of Fe efflux into the xylem vessels of plants is not yet clearly understood. Recently, a tri-Fe(III), tri-citrate complex (Fe_3Cit_3) was found by Rellán-Álvarez et al. (2010) in the xylem sap of Fe-deficient tomato (*Solanum lycopersicum* Mill.) resupplied with Fe, by using an integrated mass spectrometry approach based on exact molecular mass, isotopic signature and retention time. This complex has been modeled as having an oxo-bridged tri-Fe core. A second complex, a di-Fe(III), di-citrate complex was also detected in Fe-citrate standards along with Fe_3Cit_3, with the allocation of Fe between the two complexes depending on the Fe to citrate ratio. Citrate transported Fe(III) to veins in the young leaves but not into the interveinal area. Thus, NA enhanced Fe transport to the veins and interveinal areas in young leaves. For Fe uptake by leaf cells, proton pumping into the cell wall establishes a favorable pH surrounding for the reductase and prevents repulsion of negatively charged $FeCit_2^{3-}$ ions at the plasma membrane, which represent the major iron species in the xylem (Abadía et al., 2002). The FRD3 gene encodes a transmembrane protein that is suggested to transport citrate, and is expressed in the root pericycle and vascular cylinder, indicating a role for FRD3 in citrate efflux into xylem vessels (Kim and Guerinot et al., 2007). YS1 transports Fe(III)-PS and Fe(II)–NA complexes. *At*YSL1 mRNA is expressed in the vasculature of roots and shoots; more specifically in the xylem parenchyma surrounding xylem tubes (Kim and Guerinot,

2007). In the phloem, it is presumed that Fe is transported as a Fe(III) or Fe(III)-ITP complex. In addition to ITP, NA has been demonstrated to function in Fe transport in the phloem, based on its ubiquitous presence in plant tissues and its ability to form stable complexes with Fe^{2+} at neutral and weakly alkaline pH (von Wiren et al., 1999).
The signaling pathways underlying systemic responses are, in principle, more complex because one sensor for sensing the nutrient status of the shoot and another in the root that perceives the long-distance signal coming from the shoot are required. FRD3 is expressed in roots under iron-replete and iron-deficient conditions and is up regulated by iron deficiency (Schmidt, 2003). A sensor of the iron status in leaves has not yet been identified. Reciprocal grafting studies showed that FER is required in the roots and not in the shoots (Brown et al., 1971). The target genes of FER have not yet been identified, but recent evidence indicates that, in addition to transcriptional control, post-translational regulation of proteins involved in iron acquisition occurs when iron becomes rapidly available (Schmidt, 2003). The free-radical gas nitric oxide (NO) is an attractive candidate for the translation of the Fe-deficiency signal. Nitric oxide mediated ferritin regulation has been shown in Arabidopsis (Murgia et al., 2002). Nitric oxide was shown to act downstream of iron through the Fe-dependent regulatory sequence (IDRS) (Petit et al., 2002) of the AtFER1 promoter, suggesting that NO plays an important role in the regulation of iron homeostasis in plants (Murgia et al., 2002).

1.3.4. Intracellular Fe transport

Studies conducted with mutants illustrate that Fe homeostasis at cellular level is highly regulated and plants may develop either Fe-deficiency or Fe toxicity in normal soils depending on where they lose the control. The knowledge concerning how plants regulate cellular Fe homeostasis and intracellular Fe transport is limited, however several observations have suggested that vacuoles play a role in accumulating excess Fe and releasing Fe into the cytosol when external Fe supply is sub-optimal. Upon Fe overload, the concentrations of NA are increased in the vacuoles, while NA is detected in the cytosol under normal or Fe-deficient conditions (Kim and Guerinot, 2007). It is not yet known whether Fe translocates into the vacuole as Fe-NA complexes or whether specific

transporters for NA are present and the Fe-NA complexes then form in the vacuole. Several results obtained by experiment with mutants suggesting NA is required to maintain vacuolar Fe in a soluble form (Becker et al., 1995). The Arabidospis VIT1 (Vacuolar Iron Transporter 1) was recently identified as an Fe^{2+} transporter that functions in vacuolar Fe storage (Kim et al., 2006). VIT1 localizes to the vacuolar membrane, and it is expressed in the vasculature with increased expression seen during embryo and seed development. VIT1-mediated vacuolar Fe transport plays an important role in Fe localization in seeds. Members of the *Nramp* (natural resistance associated macrophage proteins) gene family mediate the uptake of a variety of divalent cations.

Chloroplasts requires a higher amount of Fe in order to maintain the structural and functional integrity of the thylakoid membranes, and thus the chloroplast is highly sensitive to Fe-deficiency. Iron can be stored in plant cells in the stroma of plastids as ferritin. Ferritin is a Fe storage protein with the ability to store up to 4500 atoms of Fe. Arabidopsis contains four genes that encode ferritin (AtFer1-4). All four proteins are predicted to contain transit peptides for delivery to the plastid. mRNA of *At*Fer1, *At*Fer3 and *At*Fer4 were increased upon excess Fe treatment in both roots and leaves (Petit et al., 2001). In grapevine, Oláh et al. (2004) applied molecular breeding methods to improve the quality, stress and disease resistance of grapevines by using the gene encoding the iron binding protein, ferritin, cloned from *Medicago sativa*. Somatic embryos (with size of 1-2 mm) of Richter 110 were treated with *Agrobacterium tumefaciens* EHA101 (pRok2:Ferr). Therefore, by sequestering the intracellular Fe involved in generation of the very reactive hydroxyl radicals through Fenton-reaction, the increased overall ferritin concentration results in increased protection of plant cells from oxidative damage induced by a wide range of stresses (Oláh et al., 2004). Later, the effect of *Medicago sativa* ferritin gene (MsFer) on abiotic stress tolerance was tested by Zok et al. (2010) using transgenic *Vitis berlandieri* x *Vitis rupestris* cv Richter 110 grapevine rootstock. In this work, leaf discs from transgenic plants maintained higher photosynthetic activity after NaCl, tert-butyl-hydroperoxide (t-BHP) or paraquat treatment than control ones. These results indicate that the increased production of ferritin significantly improved abiotic stress tolerance in transgenic grapevine plants.

1.4. **Iron deficiency in plants**

1.4.1. Iron-deficiency and chlorosis in grapevine

Iron chlorosis is on of the main nutritional deficiency of grapevine cultivated on alkaline and calcareous soils (Tagliavini and Rombolà, 2001). In Italy more than 50% of the viticultural soils (~350.000 ha) have more than 10% total carbonates (Bavaresco et al., 2010), and some cultural practices how as irrigation and high doses applications of macroelements, favor Fe chlorosis incidence.

The principal symptoms of iron chlorosis in grapevine could be the loose of green color of intervenial areas on young leaves due to the chlorophyll synthesis inhibition. Under severe Fe chlorosis, leaves can loose completely the green color and necrosis could appear on leaf blade. Kosegarten et al. (1999) shown that under Fe-deficiency, poor leaf growth and retardation of leaf formation occurred, even when leaves are green, and could be overcome by increased Fe supply to the plant. Hence, they evidenced that under alkaline nutritional conditions, the suppression of leaf formation and reduced leaf growth are Fe deficiency symptoms. Cultivation on calcareous soils may induce a reduction of cluster growth, shortening of internodes and reduction of shoots growth of 50% in grapevines without chlorosis symptoms (Gruber and Kosegarten, 2002).

Iron-deficiency may induce a dramatic reduction of orchard economical life cycle as well (Rombolà and Tagliavini, 2006). Lime stress conditions may decrease leaf and whole canopy photosynthesis and total dry matter production (Bavaresco and Poni, 2003). Depending on the severity of Fe chlorosis, fines and superficial roots can die, reducing the vigor of the vines. Regarding yield and grape quality parameters, Fe-deficiency induces a reduction of yield production and fruit set, decreases the berry diameter and the cluster compactness and induce a loss of red color in grapes (Bavaresco et al., 2010). In berries, Fe-scarcity causes peroxidase activity depression, increase trans-resveratrol concentration (stilbenic compound) and induces an accumulation of anthocyanins and polyphenols (Bavaresco et al., 2010; Sijmons et al., 1985).

1.4.2. Main causal factors of iron deficiency

The concentration of bicarbonate in the soil is considered the mayor factor for induce Fe chlorosis in plants cultivated in calcareous soils (Mengel et al., 1984; Romera et al., 1992). In calcareous soils, bicarbonate concentration can reach values up to 9–15 mM (Boxma, 1972). In several model plants (*Arabidopsis thaliana* L., *Cucumis sativus* L., *Pisum sativum* L. *and Lycopersycon esculentum* Mill.) it has been demonstrated that bicarbonate, at extremely high concentrations (20-30 mM), could induce Fe chlorosis by inhibiting the expression of ferric reductase, iron transporter and the H^+-ATPase genes (Lucena et al., 2007). In grapevine, it has been demonstrated that 140 Ruggeri rootstock is able to withstand high concentrations of bicarbonate in the soil (10 mM), without showing strong decreases in leaf chlorophyll and plant biomass (Ksouri et al., 2005). Ollat et al. (2003) reported that Fe-limitation or addition of 5 mM bicarbonate had a larger effect on citrate than on malate concentrations in grapevine roots, so it has been suggested a procedure using bicarbonate effect on root tip citrate concentration for screening limestone-tolerant *Vitis* rootstocks. Mengel et al. (1994) hypothesized that bicarbonate-induced chlorosis was caused by transport of bicarbonate into the stele leading to an alkalinization of the xylem sap and, in turn, of the leaf apoplast. Several authors have been tested Mengel hypothesis by measuring the effects of bicarbonate on the xylem sap pH but not final conclusions were reached (Wegner and Zimmermann, 2004). Using a novel xylem pH probe enabling *in situ* measurements, Wegner and Zimmermann (2004) clearly demonstrated that bicarbonate induced alkalinisation of the xylem sap in intact maize seedlings, supporting Mengel hypothesis.

In nutrient solution culture experiments, a supply of bicarbonate results in inhibited Fe acquisition and a subsequent decreased concentration of Fe in the leaf dry matter. This is indicated by a close positive relationship between chlorophyll and total Fe concentration in the upper leaves. In contrast to nutrient solution experiments, in some pot experiments particularly with calcareous soil, and in field experiments under certain conditions, no such close correlation can be observed and a higher Fe concentration can even be found in young chlorotic leaves than in green leaves. This phenomenon is called "the chlorosis paradox" (Römheld, 2000). For this phenomenon, two possible explanations have been hypothesized by some authors. The first hypothesis is related to the Fe inactivation in the leaves caused

by the alkalinization of the apoplast induced by bicarbonate. In a chlorotic leaf, the existence of Fe pools which are somehow inactivated has been proposed by Mengel (1994) and demonstrated by some authors (Kosegarten and Englisch, 1994; Kosegarten et al., 1999; Kosegarten et al., 2001). In plants grown under alkaline nutritional conditions, interveinal microsites of increased apoplastic pH have been found and in these microsites Fe^{3+} reduction is depressed inducing leaf Fe chlorosis. According to these authors, this inactivation may be the primary factor in Fe deficiency. In contrast, Römheld (2000) suggested that Fe inactivation is a secondary effect occurring in a leaf after the occurrence of Fe chlorosis, principally because high HCO_3^- concentration in the soil would lead to a decrease in the uptake and availability of iron for canopy growth, so the higher Fe concentration in chlorotic leaves would be the final consequence of the leaf growth inhibiting by bicarbonate. So they suggested that the scarce dimension of chlorotic leaves, as a consequence of limited Fe availability, would concentrate iron in the chlorotic leaf tissue in a higher amount than green leaves.

Most plants can take up of both ammonium (NH_4^+) and/or nitrate (NO_3^-) ions for metabolic functions. The uptake of these two nitrogen (N) forms is controlled by genotype, plant development, physiological status, and by soil properties such as texture, structure, water content and pH (Loulakakis and Roubelakis-Angelakis 2001). In calcareous soils, NO_3^- is the prevalent form of inorganic nitrogen (N) in the soil solution, and NH_4^+ is rapidly nitrified. Nitrate uptake is normally mediated by two-proton co-transport, which can increase the pH in the rhizosphere or neutralize the protons released by roots to increase Fe solubility in the soil or in the apoplast (Kosegarten et al., 2004; Nikolic and Römheld, 2002). In this sense, NO_3^- behaves similarly to bicarbonate or some biological buffers, preventing the presence of acid microsites in the rizosphere (Lucena, 2000). Consequently, the presence of NO_3^- in the soil contributes to induce Fe-deficiency chlorosis in plants cultivated in lime soils (Mengel et al., 1994). It has been hypothesized by Kosegarten et al. (1999) that under NO_3^- nutrition a high apoplastic pH in leaves depresses Fe^{3+} reductase activity and thus the subsequent Fe^{2+} transport across the plasmalemma, inducing Fe chlorosis. It was demonstrated that NO_3^- nutrition significantly increased apoplastic pH at distinct interveinal sites (pH >6,3) and was confined to about 10% of the whole interveinal

leaf apoplast (Kosegarten et al., 2001). These apoplastic pH increases are supposed to be related to growing cells of a young leaf.
In contrast, NH_4^+ uptake induce an acidification of the rhizosphere, due to the excretion of protons via the H^+-ATPase, favoring the reduction of Fe(III). In grapevine, positive effects of NH_4^+ supply in the nutrient solution for Fe chlorosis prevention have been reported (Jimenez et al., 2007). In order to improve the effectiveness of NH_4^+ to prevent/cure Fe chlorosis in plants, it is important to develop effective strategies for maintaining medium-low concentration of NH_4^+ in the soil, slowing down the oxidation of NH_4^+ to NO_3^- (nitrification process). In this context, the employment of nitrification inhibitors applied to the soil could be an interesting approach for optimizing N and Fe nutrition simultaneously. Some authors have been reported the effectiveness of 3,4-Dimethylpyrazole phosphate (DMPP) as nitrification inhibitor (Zerulla et al., 2001; Irigoyen et al., 2003). The utilize of DMPP at rates of 0,5-1,5 kg ha^{-1} are sufficient to achieve optimal nitrification inhibition in the substrate, reducing significantly NO_3^- leaching and N_2O emission, and improving crops yield (Zerulla et al., 2001). It has been observed that the effectiveness of DMPP may be drastically reduced in soils with extremely high temperatures (30 °C). This factor should be properly taken into account in warm climates (Irigoyen et al., 2003).

1.5. **Management techniques for controlling iron chlorosis**

This section is referred to the management techniques for controlling Fe chlorosis adopted in the experiments performed within the Doctoral Thesis.

1.5.1. Grapevine rootstocks

The use of tolerant rootstocks may represent an economical and efficient method for preventing Fe chlorosis. It is well known that the susceptibility degree to Fe chlorosis in grapevine is highly variable for different genotypes (Table 2). *Vitis vinifera* and *Vitis berlandieri* species have been classified as highly tolerant and tolerant to Fe chlorosis respectively, whereas *Vitis riparia* is characterized by a high susceptibility to Fe chlorosis (Tagliavini and Rombolà, 2001; Ollat et a., 2003; Jimenez et al., 2007; Brancadoro et al.,

1995). *Vitis rupestris* genotype has been classified as slightly susceptible to Fe chlorosis (Tagliavini and Rombolà, 2001). Some studies focalized to determining mechanisms responses to Fe-deficiency in grapevine rootstocks reported that genotypes from *Vitis vinifera* and *Vitis berlandieri* display an enhanced ability to reducing Fe at the root level by the FCR enzyme, and releasing protons into the rhizosphere under low external availability (Rombolà and Tagliavini, 2006; Jimenez et al., 2007; Dell'Orto et al., 2000; Brancadoro et al., 1995). In addition, it has been demonstrated that tolerant genotypes may increasing the activity of PEPC enzyme and the concentration of organic acids in roots (particularly citric acid) (Ollat et al., 2003; Rombolà et al., 2002; Rombolà and Tagliavini, 2006; Jimenez et al., 2007). In spite of this, in grapevine the use of tolerant rootstocks do not always provide satisfactory results as a consequence of different responses to Fe chlorosis of grafting combinations and negative traits associated to excessive plant vigor (Rombolà and Tagliavini, 2006).

Table 2. Iron chlorosis susceptibility for main genotypes (rootstocks) for grapevines (Tagliavini and Rombolà, 2001).

Rootstock species	Genotype	Degree of susceptibility
Vitis berlandieri		Tolerant
V. berlandieri x V. riparia	420 A	Tolerant
	Kober 5BB; 225 Ruggeri; SO4	Moderately tolerant
V. berlandieri x V. rupestris	140 Ruggeri	Highly tolerant
	775 Paulsen; 1103 Paulsen; 110 Richter	Moderately tolerant
	779 Paulsen	Tolerant
V. riparia		Highly susceptible
V. rupestris		Slightly susceptible
V. vinifera		Highly tolerant
V. vinifera (cv. Chasselas) x V. berlandieri	41 B	Highly tolerant
V. berlandieri x (V. vinifera x V. berlandieri)	Fercal	Highly tolerant

It has been reported that ATPase activity, may be utilized as an efficient method for selecting tolerant rootstocks for iron chlorosis (Dell'Orto et al., 2000). However, Piagnani et al. (2003) conducted an experiment in callus culture (on internodes, leaves, and roots tissues), in which Fe-deficiency did not affect the H^+-ATPase activity in the microsomal

fraction of any considered culture sample. These results are in contrast with other studies (Piagnani and Zocchi, 1997), and leads to assume that its role as marker of Fe-efficiency at tissue culture level is still ambiguous in the case of grapevine. In addition, the Fe chlorosis tolerant genotypes Cabernet Sauvignon and Pinot Blanc submitted to Fe-deficiency showed to be more efficient to decrease the pH of the nutrient solution and accumulate organic acids in roots (malate, succinate and particularly citrate) than the Fe chlorosis susceptible genotype *Vitis riparia* (Jimenez et al., 2007; Brancadoro et al., 1995).

In grapevines submitted to Fe-depletion, Jimenez et al. (2007) measured a higher FCR and PEPC enzymes activity in roots of cv Cabernet Sauvignon (*V. vinifera*, a tolerant genotype) than in Gloire de Montpellier (*V. riparia*, a susceptible genotype). In contrast to previous results, Piagnani et al. (2003) showed that culture responses were clearly linked to the Fe-efficiency status of the genotype. Calli of Cabernet Sauvignon leaf explants from green tissues grown on Fe-deficient medium showed up to a five-fold increase in the FCR enzyme activity as compared to material grown in Fe-sufficiency conditions. However, Fe-deficient from *Vitis riparia*, either did not change in case of root derived cultures, or depressed the FCR activities in the case of leaf and internode derived cultures.

1.5.2. Iron chelates

The prevention/cure of Fe chlorosis with Fe-chelates is a very widespread and effective agronomical practice in vineyards and orchards. Iron-chelates can be supplied *via* foliar applications or diluted in the irrigation water to the soil. The number, amount and frequency of Fe-chelates application depends on the crop species and the severity of Fe chlorosis. For foliar applications, low Fe concentrations should be used (eg. 2 mM) in order to avoid Fe toxicity in leaves. Foliar sprays have been largely assessed (Lucena, 2006), and in some cases good results have been obtained. Álvarez-Fernández et al. (2004) studied the effectiveness of foliar fertilization with Fe-DTPA to re-green chlorotic pear trees, and they concluded that foliar applications of low-stability synthetic Fe-chelates (eg. HEDTA, EDTA, DTPA and others) cannot offer a good alternative for the full control of Fe chlorosis, but could be useful in conditions where chlorosis is not severe (Lucena, 2006). In contrast, the application of most stable chelates (o,o-EDDHA/Fe(III) and analogous) to the

soil are able to maintain Fe in the soil solution and transport it to the roots, also in highly calcareous soils. In Fe-deficiency chlorotic vineyards, generally Fe-chaltes are applied to the soil at doses of 20-40 g of chelate per plant during the season (Rombolà and Tagliavini, 2006). In Concord grapevine genotype (*Vitis labrusca* Bailey), the application of Fe-EDDHA increased the active Fe concentration in leaves linearly with the application rate (Smith and Cheng, 2007). However, Fe chelates are highly stable and soluble in the soil, and consequently may increase the risk of leaching of metals and chelating agents in the deep soil layers and in the water table. In addition, synthetic Fe(III)-chelates are expensive, require repeated applications, and therefore widely used only in high-value crops such as fruit trees. (Rombolà e Tagliavini, 2006).

Synthetic Fe(III)-chelate fertilizers are derived from polyaminocarboxylic acids which have high affinity for Fe(III), such as ethylenediamine tetraacetic acid (EDTA) (Lucena, 2006). These chelates are obtained by carrying out first the synthesis of the chelating agents and then incorporating Fe(III) from inorganic salts. Synthetic Fe(III)-chelates are remarkably effective as soil fertilizers, even in calcareous soils, because Fe is bound to the chelating agent over a wide range of pH values and therefore remains soluble. In the particular case of calcareous soils, synthetic Fe(III)-chelates from chelating agents with phenolic groups (e.g., the ethylenediamine-N-N0bis(o-hydroxyphenylacetic) acid; o,oEDDHA) are very effective Fe fertilizers (Abadía et al., 2011).

Also, the Fe(III)-chelates of two new biodegradable, synthetic chelating agents structurally similar to EDTA, N-(1,2-dicarboxyethyl)-D,L-aspartic acid (IDHA) and ethylenediaminedisuccinic acid (EDDS) have been assessed as plant Fe-sources. Both chelates were successfully used as Fe fertilizers in several plant species both when applied to the foliage (Ylivainio et al., 2004) and in hydroponics (Lucena et al., 2008). In general, these two chelates had an efficacy similar to that of Fe(III)-EDTA. However, Fe(III)-IDHA was not as effective as Fe(III)-EDTA in foliar sprays to peach trees, with the efficacy being dependent on the surfactant used (Fernandez, et al., 2008). The performance of Fe(III)-EDDS was markedly dependent of the soil pH, being more efficient in acid soils. Recently, the Fe(III)-chelates of three chelating agents structurally similar to o,oEDDHA, N,N0-bis(2-hydroxy-5-methylphenyl) ethylendiamine-N,N0-diacetic acid (HJB), N,N0-bis(2-hydroxybenzyl) ethylenediamine-N,N0-diacetic acid (HBED) and 2-(2-((2-

hydroxybenzyl)amino)ethylamino)-2-(2-hydroxyphenyl)acetic acid (DCHA), have also been proposed as Fe fertilizers (Abadía et al., 2011). Iron(III)-HJB and Fe(III)-HBED were introduced because they have a much higher purity than fertilizers based on Fe(III)-EDDHA, with no optical isomers or other by-products being present, while Fe(III)-DCHA has an intermediate stability between those of Fe(III)-o,oEDDHA and Fe(III)-o,pEDDHA. Although Fe(III)-DCHA is capable of maintaining Fe in soluble forms in soil solutions, its effectiveness with plants is still to be confirmed. Several studies have assessed the effectiveness of Fe-compounds such as regio-isomers (e.g., Fe(III)-o,p-EDDHA), poly-condensation products and other by-products derived of the industrial synthesis procedures of the phenolic Fe(III)-chelate fertilizers. For instance, Fe(III)-o,pEDDHA has been found to be as effective as Fe(III)-o,oEDDHA in nutrient solutions (Rojas et al., 2008), but not in calcareous soils, and a mixture of Fe(III)-EDDHA poly-condensate byproducts was not effective in chlorosis correction (Schenkeveld et al., 2007).

1.5.3 Intercropping with graminaceus species

Graminaceus species are able to release into the rhizosphere high affinity Fe (III) chelating compounds (phytosiderophores), solubilizing Fe and taking it up by roots as intact Fe-phytosiderophore complexes (Ma and Nomoto, 1996). It has been shown that intercropping of some species with graminaceous species prevents Fe chlorosis in co-cultivated plant as effectively as synthetic Fe chelates (Rombolà et al., 2003; Ammari and Rombolà 2010), due to their abilities of solubilizing soil Fe through phytosiderophore secretion (Ma et al., 2003; Ueno et al., 2007; Cesco and Rombolà, 2007). Iron chlorosis prevention on intercropped plants depends on the effectiveness of graminaceous species used to release phytosiderophores (Ammari and Rombolà 2010). It has been reported that phytosiderophores secretion in *Festuca rubra* -an efficient species for phytosiderophores secretion- is characterized by a daily rhythm and is influenced by temperature at root level (Ma et al. 2003). In grapevine, recently it has been shown that intercropping of cv. Merlot grafted on the hybrid 3309 C (*V.riparia* x *V. rupestris*) with graminaceous species prevents iron chlorosis as effectively as synthetic Fe chelates (Bavaresco et al., 2010). Intercropped

systems can enhance root growth, chlorophyll content, active Fe content, and in reductase activity (Zuo and Zhang, 2008).

In citrus species, Cesco et al. (2006) have reported that the highly susceptible to Fe cholorosis citrumelo ''Swingle'' (*Poncirus trifoliate x Citrus paradise)*, and the moderately tolerant *Citrus aurantium* L. could take up ^{59}Fe from ^{59}Fe-hydroxide supplied only in presence of Fe-deficient graminaceous species (*Hordeum vulgare* L., P*oa pratensis* L. and *Festuca rubra* L. in the nutrient solution. This effect was particularly evident for the susceptible citrus rootstock. In the same study, no effect was found when Fe-sufficient grasses has been employed, indicating that there must be a Fe-deficiency in the medium to render effective the management control with grass species. In addition, the uptake of ^{59}Fe by the susceptible citrus rootstock increased in proportion to the amount of 2'-deoxymugineic acid (DMA), the major PS released by Fe-deficient *F. rubra*, present in the uptake solution, demonstrating the importance of the compound for improvement of chlorosis.

Although significant progress have been evolved in recent years regarding Fe nutrition in intercropping systems, there is still a lack of knowledge about Fe behave in plants at molecular level. Xiong et al. (2008) investigated the effects of intercropping on iron uptake genes expression in roots of peanut, and indentified the influence of intercropping on genes expression related to phytosiderophores synthesis and their transcription factor 2 (*Zm*IRO2) in roots of maiz. Results suggest that intercropping may play an important role in up-regulated AhNramp Fe(II) transporter intracellular vesicles in peanut plants. In maiz roots, microarray analysis showed that 117 genes are up-regulated by monocropping and 312 by intercropping, and these genes play an important role in improve iron nutrition of peanut intercropped in calcareous soil. Recent studies have shown that the presence of graminaceous species may stimulate, in co-cultivated species, the expression of *AhFRO1* and *AhIRT1* genes encoding proteins responsible for Fe absorption and such changes have been mainly attributes to soil Fe level, cultivation conditions and phenological phase (Ding et al., 2009; Ding et al., 2010).

The above mentioned evidences indicated that vineyards adopting intercropping with grasses properly selected may represent a sustainable tool for controlling Fe chlorosis avoiding or reducing the need of applying synthetic Fe-chelates.

Literature cited

Abadía, J., Lopez-Millan, A.F., Rombolà, A. and Abadía, A. 2002. Organic acids and Fe deficiency: a review. Plant and Soil, 241: 75-86.

Abadía, J., Vázquez, S., Rellán-Álvarez, R., Jendoubi, H.E., Abadía, A., Álvarez-Fernández, A. and López-Millán, A.F. 2011. Towards a knowledge-based correction of iron chlorosis. Plant Physiology and Biochemistry, doi:10.1016/j.plaphy.2011.01.026.

Ammari, T., Rombolà, A.D. 2010. Overcoming iron deficiency chlorosis in citrus through intercropping with perennial grass species. *In*: Acta Horticulturae. May 2008, Faro, Portugal, 19-23, vol. 868, p. 327-330.

Andaluz, S., Lopez-Millan, A.F., De Las Rivas, J., Aro, E.M., Abadia, J. and Abadia, A. 2006. Proteomic profiles of thylakoid membranes and changes in response to iron deficiency. Photosynthesis Research 89: 141-155.

Balk, J. and Lobréaux, S. 2005. Biogenesis of iron–sulfur proteins in plants. Trends in Plant Science, 10: 324-331.

Bashir, K., Inoue, H., Nagasaka, S., Takahashi, M., Nakanishi, H., Mori, S. and Nishizawa, N.K. 2006. Cloning and characterization of deoxymugineic acid synthase genes from graminaceous plants. Journal of Biology and Chemistry, 281: 32395–32402.

Bavaresco, L. and Poni, S. 2003. Effect of calcareous soil on photosynthesis rate, mineral nutrition, and source-sink ratio of table grape. Journal of plant nutrition, 26 (10&11): 2123-2135.

Bavaresco, L., van Zeller, M.I., Civardi, S., Gatti, M. and Ferrari, F. 2010. Effects of traditional and new methods on overcoming lime-induced chlorosis of grapevine. American Journal of Enology and Viticulture, 61(2): 186-190.

Becker, R., Fritz, E. and Manteuffel, R. 1995. Subcellular localization and characterization of excessive iron in the nicotianamine-less tomato mutant chloronerva. Plant Physiology, 108: 269–275.

Benítez, M.L., Pedrajas, V.M., del Campillo, M.C. and Torrent, J. 2002. Iron chlorosis in olive in relation to soil properties. Nutrient Cycling in Agroecosystems, 62: 47–52.

Bloom, P.R. 1981. Metal-organic matter interactions in soil. In: Chemistry in the soil environment. American Society of Agronomy Madison, Special Publication, 44: 129-150.

Borggaard, O.K. 1982. Selective extraction of the amorphous iron oxides by EDTA from selected silicates and mixtures of amorphous and crystalline oxides. Clay Miner, 17: 365-368.

Boxma, R. 1972. Bicarbonate as the most important soil factor in lime induced chlorosis in the Netherlands. Plant and Soil, 37: 233–243.

Brancadoro, L., Rabotti1, G., Scienza, A. and Zocchi, G. 1995. Mechanisms of Fe-efficiency in roots of *Vitis spp.* in response to iron deficiency stress. Plant and Soil, 171: 229-234.

Briat, J.F., Curie, C. and Gaymard, F. 2007. Iron utilization and metabolism in plants. Plant Biology, 10:276–282.

Briat, J.F., Fobis-Loisy, I., Grignon, N., Lobréaux, S., Pascal, N., Savino, G., Thoiron, S., von Wiren, N., Van Wuytswinkel, O. 1995. Cellular and molecular aspects of iron metabolism in plants. Biology fo the Cell, 84: 69-81.

Brown, J.C., Chaney, R.L. and Ambler, J.E. 1971. A new tomato mutant inefficient in the transport of iron. Physiologia Plantarum, 25: 48–53.

Cesco, S. and Rombolà, A. 2007. Nutrient management at the rhizosphere through intercropping. In: Robert M. Goodmann. Encyclopedia of Plant and Crop Science. London: Taylor & Francis, p. 1 – 3, ISBN/ISSN: 978-0-8247-0944-0.

Cesco, S., Rombolà, A.D., Tagliavini, M., Varanini, Z., Pinton, R. 2006. Phytosiderophores released by graminaceous species promote ^{59}Fe-uptake in citrus. Plant and Soil, 287: 223–233.

Cesco, S., Neumann, G., Tomasi, N., Pinton, R. and Weisskopf, L. 2010. Release of plant-borne flavonoids into the rhizosphere and their role in plant nutrition. Plant and Soil, 329: 1–25.

Chen, Y. and Barak, P. 1982. Iron nutrition in calcareous soils. Advances in Agronomy, 35:217-240.

Colangelo, E.P. and Guerinot, M.L. 2004. The essential basic helix-loop-helix protein FIT1 is required for the iron deficiency response. Plant Cell, 16: 3400–3412.

Cornah, J.E., Singh, D.P., Roper, J.M., Smith, A.G. 2002. Measurement of ferrochelatase activity using a novel assay suggests that plastids are the major site of haem biosynthesis in both photosynthetic and nonphotosynthetic cells of pea (*Pisum sativum* L.). Biochemical Journal, 362: 423-432.

Courbé, C., Morlat, R., Robichet, J. and Venin, G. 1982. L'altération des formations glauconieuses dans le vignoble du Val de Loire: quelques aspects de la libération du fer et application agronomique. Agronomie 2(8): 745-754.

Curie, C., Panaviene, Z., Loulergue, C., Dellaporta, S.L., Briat, J.-F. and Walker, E.L. 2001. Maize yellow stripe1 encodes a membrane protein directly involved in Fe(III) uptake. Nature, 409: 346–349.

De Carolis, E. and De Luca, V. 1994. 2-Oxoglutarate-dependent dioxygenase and related enzymes: Biochemical characterization. Phytochemistry, 36:1093 –1107.

De Nisi, P. and Zocchi, G. 2000. Phosphoenolpyruvate carboxylase in cucumber (*Cucumis sativus* L.) roots under iron deficiency: activity and kinetic characterization. Journal of Experimental Botany, 51: 1903–1909.

de Santiago, A. and Delgado, A. 2006. Predicting iron chlorosis of lupin in calcareous spanish soils from iron extracts. Soil Science Society of American Journal, 70: 1945-1950.

del Campillo, M.C. and Torrent, J. 1992. Predicting the influence of iron chlorosis in calcareous soils of southern Spain. Communications in Soil Science and Plant Analysis, 23: 399-416.

Dell'Orto, M., Brancadoro, L., Scienza, A. and Zocchi, G. 2000. Use of biochemical parameters to select grapevine genotypes resistant to iron-chlorosis. Journal of Plant Nutrition, 23(11&12): 1767-1775.

DeMaeyer, E.M., Dallman, P., Gurney, J.M., Hallberg, L., Sood, S.K. and Srikantia, S.G. 1989. Preventing and controlling iron deficiency anemia through primary health care: a guide for health administrators and program managers. World Health Organization, Geneva, Switzerland.

Ding, H., Duan, L., Wu, H., Yang, R., Ling, H., Li, W-X. and Zhang, F. 2009. Regulation of *Ah*FRO1, an Fe(III)-chelate reductase of peanut, during iron deficiency stress and intercropping with maize. Physiologia Plantarum, 136: 274–283.

Ding, H., Duan, L., Li, J., Yan, H., Zhao, M., Zhang, F and Li, W-X. 2010. Cloning and functional analysis of the peanut iron transporter *Ah*IRT1 during iron deficiency stress and intercropping with maize. Journal of Plant Physiology, 167: 996–1002.

Feng, H., An, F., Zhang, S., Ji, Z., Ling, H.-Q. and Zuo, J. 2006. Light-regulated, tissue- and cell differentiation-specific expression of the *Arabidopsis* Fe(III)-chelate reductase gene AtFRO6. Plant Physiology, 140: 1345–1354.

Fernández, V., Del Río, V., Pumariño, L., Igartua, E., Abadía, J. and Abadía, A. 2008. Foliar fertilization of peach (*Prunus persica* (L.) Batsch) with different iron formulations: effects on re-greening, iron concentration and mineral composition in treated and untreated leaf surfaces. Scientia Horticulturae, 117: 241-248.

Gruber, B. and Kosegarten, H. 2002. Depressed growth of non-chlorotic vine grown in calcareous soil is an iron deficiency symptom prior lo leaf chlorosis. Journal of Plant Nutrition and Soil Science, 165: 111-117.

Grusak, M.A. and Dellapena, D. 1999. Improving the nutrient composition of plants to enhance human nutrition and health. Annual Review of Plant Physiology and Plant Molecular Biology, 50, 133-161.

Guthrie, B. and Picciano, M.F. 1995. Macronutrient minerals, In Human Nutrition, Mosby Year Book, St. Louis, USA, pp. 334-351.

Inskeep, W.P. and Bloom, P.R. 1986. Effects of Soil Moisture on Soil pCO_2, Soil Solution Bicarbonate, and Iron Chlorosis in Soybeans. Soil Science Society of American Journal, 50: 946–952.

Institute of Medicine. 2001. Dietary reference intakes for vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium and zinc. National Academy Press, Washington, D.C., USA.

Irigoyen, I., Muro, J., Azpilikueta, M., Aparicio-Tejo P. and Lamsfus, C. 2003. Ammonium oxidation kinetics in the presence of nitrification inhibitors DCD and DMPP at various temperatures. Australian Journal of Soil Research, 41(6): 1177-1183.

Iturbe-Ormaetxe, I., Moran, J.F. Arrese-Igor, C., Gogorcena, Y. Klucas, R.V. and Becana, M. 1995. Activated oxygen and antioxydant defenses in iron deficient pea plants. Plant Cell and Environment, 18: 421–429.

Jackson, M.L. 1958. Soil chemical analysis. Prentice-Hall, New York.

Jimenez, S., Gogorcena, Y., Hévin, C., Rombolà, A.D., and Ollat, N. 2007. Nitrogen nutrition influences some biochemical responses to iron deficiency in tolerant and sensitive genotypes of *Vitis*. Plant and Soil, 290: 343-355.

Juste, C. and Pouget, R. 1972. Appréciation du pouvoir chlorosant des sols par un nouvel indice faisant in-tervenir le calcaire actif et le fer facilement extrac-tible: Application au choix des porte-greffes de la vigne. Comptes Rendus de L Academie des Agricole, 58: 352-357.

Kanazawa, K., Higuchi, K., Nishizawa, N., Fushiya, S., Chino, M. and Mori, S. 1994. Nicotianamine aminotransferase activities are correlated to the phytosiderophore secretion under Fe deficient conditions in Gramineae. Journal of Experimental Biology, 46: 1903–1906.

Kapur, D., Agarwal, K. N. and Agarwal, D. K. 2002. Nutritional anemia and its control, Indian Journal of Pediatry, 69: 607-616.

Kim, S.A., Punshon, T., Lanzirotti, A., Li, L., Alonzo, J.M., Ecker, J.R., Kaplan, J. and Guerinot, M.L. 2006. Localization of iron in *Arabidopsis* seed requires the vacuolar membrane transporter VIT1. Science, 314: 1295–1298.

Kim, S.A. and Guerinot, M.L. 2007. Mining iron: Iron uptake and transport in plants. FEBS Letters, 581: 2273–2280.

Kosegarten, H., Englisch, G. 1994. Effects of various nitrogen forms on the pH in leaf apoplast and on iron chlorosis of *Glycine max* L. Z. Planzenernähr. Bodenk, 157: 401–405.

Kosegarten, H., Hoffmann, B., Mengel, K. 1999. Apoplastic pH and Fe^{3+} Reduction in intact sunflower leaves. Plant Physiology, 121: 1069–1079.

Kosegarten, H., Hoffmann, B., Mengel, K. 2001. The paramount influence of nitrate in increasing apopastic pH of young sunflower leaves to induce Fe deficiency chlorosis, and the re-greening effect brought about by acidic foliar sprays. Journal of Plant Nutrition and Soil Science, 164: 155–163.

Kosegarten, H., Hoffmann, B., Roco, E., Grolig, F., Glüsenkamp, K.-H. and Mengel, K. 2004. Apoplastic pH and FeIII reduction in young sunflower (*Helianthus annuus*) roots. Physiologia Plantarum, 122: 95–106.

Ksouri, R., M'rah, S., Gharsalli, M. and Lachaâl, M. 2006. Biochemical responses to true and bicarbonate-induced iron deficiency in grapevine genotypes. Journal of Plant Nutrition, 29: 305-315.

Ksouri, R., Gharsalli, M. and Lachaal. M. 2005. Physiological responses of Tunisian grapevine varieties to bicarbonate-induced iron deficiency. Journal of Plant Physiology, 162: 335-341.

Kraemer, S.M., Crowley, D.E. and Kretzschmar, R. 2006. Geochemical aspects of phytosiderophores-promoted iron acquisition by plants. Advances Agronomy, 91: 1-46.

Léon, S., Touraine, B., Briat, J.F., Lobrèaux, S. 2002. The AtNFS2 gene from *Arabidopsis thaliana* encodes a NifS-like plastidial cysteine desulphurase. Biochemical Journal, 366: 557-564.

Lezhneva, L., Amann, K., Meurer, J. 2004. The universally conserved HCF101 protein is involved in assembly of [4Fe–4S]-clustercontaining complexes in *Arabidopsis thaliana* chloroplasts. Plant Journal, 7: 174-185.

Li, H.M., Theg, S.M., Bauerle, C.M., Keegstra, K. 1990. Metal-ion-center assembly of ferredoxin and plastocyanin in isolated chloroplasts. Proceedings of the National Academy of Science, 87: 6748-6752.

Li, L., Cheng, X. and Ling, H.-Q. 2004. Isolation and characterization of Fe(III)-chelate reductase gene LeFRO1 in tomato. Plant Molecular Biology, 54: 125–136.

Lindsay, W.L. 1984. Soil and plant relationships associated with iron deficiency with emphasis on nutrient interactions. Journal of Plant Nutrition, 7: 489-500.

Lindsay, W.L. and Schwab, A.P. 1982. The chemistry of iron in soils and its availability to plants. Journal of Plant Nutrition, 5: 821-840.

Loeppert, R.H. 1986. Reactions of iron and carbonates in calcareous soils. Journal of Plant Nutrition, 9: 195-215.

Loeppert, R.H. and Hallmark, C. T. 1985. Indigenous soil properties influencing the availability of iron in calcareous soils. Soil Science Society American Journal, 49: 597-603.

Loeppert, R.H., Wei, L.C. and Ocumpaugh, W.R. 1994. Soil factors influencing the mobilization of iron in calcareous soils, *In:* Biochemistry of metal micronutrients in the rhizosphere. J.A. Manthey, D.E. Crowley and D.G. Luster (Eds.), CRC Press, Florida, pp. 343-360.

López-Millán, A.F., Morales, F., Andaluz, S., Gogorcena, Y., Abadía, A., De Las Rivas, J. and Abadía, J. 2000. Responses of sugar beet roots to iron deficiency. Changes in carbon assimilation and oxygen use. Plant Physiology, 124: 885–897.

Loulakakis, K.A. and Roubelakis-Angelakis, K.A. 2001. Nitrogen assimilation in grapevine. In: Roubelakis-Angelakis KA (ed) Molecular biology and biotechnology of the grapevine. Kluwer Academic Publishers, Dordrecht/Boston/London, pp 59–85.

Lucena, J.J. 2000. Effects of bicarbonate, nitrate and other environmental factors on iron deficiency chlorosis. A review. Journal of Plant Nutrition, 23(11&12): 1591-1606.

Lucena, J.J. 2006. Synthetic iron chelates to correct iron deficiency in plants. *In* Iron Nutrition in Plants and Rhizospheric Microorganisms (Eds, J, Abadía and L, Barton), Springer, Berlin, Germany, pp. 103-128.

Lucena, C., Romera, F.J., Rojas, C.L., García, M.J., Alcántara, E. and Pérez-Vicente, R. 2007. Bicarbonate blocks the expression of several genes involved in the physiological responses to Fe deficiency of Strategy I plants. Functional Plant Biology, 34: 1002–1009.

Lucena, J.J., Sentis, J., Villén, M., Lao, T. and Pérez-Sáez, M. 2008. IDHA chelates as a micronutrient source for green bean and tomato in fertigation and hydroponics, Agronomy Journal, 100: 813-818.

Ma, J.F. and Nomoto, K. 1996. Effective regulation of iron acquisition in graminaceous plants. The role of mugineic acids as phytosiderophores. Physiologia Plantarum, 97: 609-617.

Marschner, H. 1995. Mineral nutrition of higher plants. 2nd ed. Academic Press, London.

Mengel, K. 1994. Iron availability in plant tissues-iron chlorosis on calcareous soils. Plant and Soil, 165: 275–283.

Mengel, K., Breininger, M.T. and Bübl, W. 1984. Bicarbonate, the most important factor inducing iron chlorosis in vine grapes on calcareous soil. Plant and Soil, 81: 333-344.

Morris, D.R., Loeppert, R.H. and Moore, T.J. 1990. Indigenous soil factors influencing iron chlorosis of soybean in calcareous soil. Soil Science Society of American Journal, 54: 1329-1336.

Moseley, J.L., Page, M.D., Pergam, N., Eriksson, M., Quinn, J., Soto, J., Theg, S., Hippler, M., Merchant, S. 2002. Reciprocal expression of two diiron enzymes affecting photosystem I and light-harvesting complex accumulation. Plant Cell, 14: 673-688.

Murata, Y., Ma, J.F., Yamaji, N., Ueno, D., Nomoto, K. and Iwashita, T. 2006. A specific transporter for iron(III)-phytosiderophore in barley roots. Plant Journal, 46: 563–572.

Murgia, I., Delledonne, M. and Soave, C. 2002. Nitric oxide mediates iron-induced ferritin accumulation in *Arabidopsis*. Plant Journal, 30(5): 521–528.

Nakanishi, H., Yamaguchi, H., Sasakuma, T., Nishizawa, N.K. and Mori, S. 2000. Two dioxygenase genes Ids3 and Ids2 from *Hordeum vulgare* are involved in the biosynthesis of mugineic acid family phytosiderophores. Plant Molecular Biology, 44: 199–207.

Naumann, B., Stauber, E.J., Busch, A., Sommer, F., Hippler, M. 2005. N-terminal processing of Lhca3 is a key step in remodeling of the photosystem I-light-harvesting complex under iron deficiency in *Chlamydomonas reinhardtii*. Journal of Biology and Chemistry, 280: 20431-20441.

Nikolic, M. and Römheld, V. 2002. Does high bicarbonate supply to roots change availability of iron in the leaf apoplast? Plant and Soil, 241: 67–74.

Oláh, R., Tóth, A., Ruthner, S., Korbuly, J. and Szegedi, E. 2004. Genetic transformation of rootstock cultivar Richter 110 with the gene encoding the ironbinding protein, ferritin. Acta Horticulturare (ISHS), 652:471-473.

Ollat, N., Laborde, B., Neveux, M., Diakou-Verdin, P., Renaud, C. and Moing, A. 2003. Organic acid metabolism in roots of various grapevine (*Vitis*) rootstocks submitted to iron deficiency and bicarbonate nutrition. Journal of plant nutrition, 26 (10 & 11): 2165–2176.

Patruno, A. and Cavazza, L. 1989. Osservazioni sul calcare attivo. Proceedings "Fertilità del suolo e nutrizione delle piante". May 6-8, S. Agnello, Sorrento, Italy.

Petit, J.M., Briat, J.F. and Lobréaux, S. 2001. Structure and differential expression of the four members of the *Arabidopsis thaliana* ferritin gene family. Biochemistry Journal, 359: 575-582.

Petit, J.M., van Wuytswinkel, O., Briat, J.F. and Lobréaux, S. 2002. Characterization of an iron-dependent regulatory sequence involved in the transcriptional control of *At*Fer1 and *Zm*Fer1 plant ferritin genes by iron. Journal of Biology and Chemistry, 276: 5584–5590.

Piagnani, C., De Nisi, P., Espen, L. and Zocchi, G. 2003. Adaptive responses to iron deficiency in callus cultures of two cultivars of *Vitis* spp. Journal of Plant Physiology, 160: 865–870.

Piagnani, C. and Zocchi, G. 1997. Physiological responses of grapevine (*Vitis spp*,) callus cultures to iron-deficiency. Journal of Plant Nutrition, 20: 1539–1549.

Rellán-Álvarez, R., Giner-Martínez-Sierra, J., Orduna, J., Orera, I., Rodríguez-Castrillón, J.A., García-Alonso, J.I., Abadía, J. and Álvarez-Fernández, A. 2010. Identification of a tri-iron(III), tri-citrate complex in the xylem sap of iron-deficient tomato resupplied with iron: new insights into plant iron long-distance transport. Plant Cell Physiology, 51(1): 91–102.

Reyes, J.M., del Campillo, M.C. and Torrent, J. 2002. Soil properties influencing iron chlorosis in grapevines grown in the Montilla-Moriles area, southern Spain. Community of Soil Science and Plant Analysis, 37: 1723-1729.

Rojas, C., Romera, R., Alcántara, E., Pérez-Vicente, R., Sariego, C., García-Alonso, J.I., Boned, J. and Martí, G. 2008. Efficacy of Fe (o,o-EDDHA) and Fe (o,p-EDDHA) isomers in supplying Fe to strategy I plants differs in nutrient solution and calcareous soil. Journal of Agriculture and Food Chemistry, 56: 10774-10778.

Rombolà, A.D. 1998. Aspetti fisiologici e biochimici della nutrizione ferrica in actinidia (*A. deliciosa*). Ph.D. Thesis. Dipartimento di Colture Arboree. Università degli Studi di Bologna, Italy.

Rombolà, A., Baldi, E., Franceschi, A., Ammari, T., Minguez, J. and Tagliavini, M. 2003. Prevenzione della clorosi ferrica dell'actinidia (*Actinidia deliciosa*) mediante consociazione temporanea con specie Graminacee. In: Atti del convegno nazionale "Actinidia, la novità frutticola del XX secolo". November 21, Verona, Italy.

Rombolà, A.D., Brüggemann, W., López-Millán, A.F., Tagliavini, M., Abadía, J., Marangoni, B. and Moog, P.R. 2002. Biochemical responses to iron deficiency in kiwifruit (*Actinidia deliciosa*). Tree Physiology, 22: 869–875.

Rombolà, A.D., Brüggemann, W., Tagliavini, M., Moog, P.R. 1998. Meccanismi biochimici di tolleranza alla clorosi ferrica in Actinidia (*Actinidia deliciosa*). Proceedings IV° Giorntae Scientifiche Società Orticola Italiana. April 1-4, Sanremo, Italy, pp. 395-396.

Rombolà, A.D. and Tagliavini, M. 2006. Iron nutrition of fruit tree crops. *In* Iron Nutrition in Plants and Rhizospheric Microorganisms (Eds, J, Abadía and L, Barton), Springer, Berlin, Germany, pp. 61-83.

Romera, F.J., Alcántara, E. and de la Guardia, M.D. 1992. Effects of bicarbonate, phosphate and high pH on the reducing capacity of Fe-deficient sunflower and cucumber plants. Journal of Plant Nutrition, 15: 1519-1530.

Römheld, V. 2000. The chlorosis paradox: Fe inactivation as a secondary event in chlorotic leaves of grapevine. Journal of Plant Nutrition, 23(11&12), 1629-1643.

Santi, S., Cesco, S., Varanini, Z. and Pinton, R. 2005. Two plasma membrane H^+-ATPase genes are differentially expressed in iron-deficient cucumber plants. Plant Physiology and Biochemistry, 43: 287–292.

Schenkeveld, W.D.C., Reichwein, A.M., Temminghoff, E.J.M. and van Riemsdijk, W.H. 2007. The behavior of EDDHA isomers in soils as influenced by soil properties. Plant and Soil, 290: 85-102.

Schmidt, W. 2003. Iron solutions: acquisition strategies and signaling pathways in plants. TRENDS in Plant Science, 8: 188-193.

Schwertmann, U. 1964. Differenzierung der eisenoxide des bodens durch extraktion mit ammoniumoxalat-lösung.. Z. Pflanzenernähr, Bodenkd, 105:194-202.

Schwertmann, U. 1985. The effect of pedogenic environments on iron oxide minerals. Advances in Soil Science, 1: 171-200.

Schwertmann, U. 1991. Solubility and dissolution of iron oxides. *In*: Iron nutrition and interactions in plants. Y. Chen and Y. Hadar (Eds.). Kluwer Academic Publishers, Netherlands, pp. 3-27.

Sijmons, P.C., Kolattukudy, P.E. and Bienfait, H.F. 1985. Iron deficiency decreases suberization in bean roots through a decrease in suberin-specific peroxidase activity. Plant Physiology, 78: 115-120.

Singh, D.P., Cornah, J.E., Hadingham, S., Smith, A.G. 2002. Expression analysis of the two ferrochelatase genes in *Arabidopsis* in different tissues and under stress conditions reveals their different roles in haem biosynthesis. Plant Molecular Biology, 50: 773-788.

Smith, B. and Cheng, L. 2006. Fe-EDDHA alleviates chlorosis in 'Concord' grapevines grown at high pH. HortScience, 41(6): 1498-1501.

Spiller, S. and Terry, N. 1980. Limiting factors in photosynthesis. II. Iron stress diminishes photochemical capacity by reducing the number of photosynthetic units. Plant Physiology, 65: 121-125.

Tagliavini, M. and Rombolà, A.D. 2001. Iron deficiency and chlorosis in orchard and vineyard ecosystems. European Journal of Agronomy, 15: 71-92.

Theil, E.C. 2004. Iron, ferritin and nutrition. Annual Review of Nutrition, 24: 327-343.

Thimm, O., Essigmann, B., Kloska, S., Altmann, T. and Buckhout, T.J. 2001. Response of *Arabidopsis* to iron deficiency stress as revealed by microarray analysis. Plant Physiology, 127: 1030–1043.

Ueno, D., Rombolà, A.D., Iwashita, T., Nomoto, K. and Ma, J.F. 2007. Identification of two novel phytosiderophores secreted by perennial grasses. New Phytologist, 174: 304–310.

Vasconcelos, M. and Grusak, M.A. 2006. Status and future developments involving plant iron animal and human nutrition. *In* Iron Nutrition in Plants and Rhizospheric Microorganisms (Eds, J, Abadía and L, Barton), Springer, Berlin, Germany, pp. 01-22.

Vellar, O.D. 1968. Studies on sweat losses of nutrients. Scandinavian Journal of Clinical & Laboratory I, 21: 157-167.

Vempati, R.K. and Loeppert, R.H. 1986. Synthetic ferrihydrite as a potential iron amendment in calcareous soils. Journal of Plant Nutrition, 9: 1039-1052.

Vigani, G., Maffi, D. and Zocchi, G. 2009. Iron availability affects the function of mitochondria in cucumber roots. New Phytologist, 182: 127–136.

von Wiren, N., Klair, S., Bansal, S., Briat, J.-F., Khodr, H., Shioiri, T., Leigh, R.A. and Hider, R.C. 1999. Nicotianamine chelates both Fe(III) and Fe(II). Implications for metal transport in plants. Plant Physiology, 119: 1107–1114.

Wegner, L.H. and Zimmermann, U. 2004. Bicarbonate-induced alkalinization of the xylem sap in intact maize seedlings as measured in situ with a novel xylem ph probe. Plant physiology, 136: 3469–3477.

Xiong, H., Duan, P., Wu, H., Ling, H., Zhang, F. and Zuo, Y. 2008. The effects of peanut and maize intercropping on iron uptake genes expression in plants. XIV International Symposium on Iron Nutrition and Interactions in Plants. XIV ISINIP. October 11-15, Beijing, PR China.

Xu, X.M., Adams, S., Chua, N.H., Moller, S.G. 2005. AtNAP1 represents an atypical SufB protein in *Arabidopsis* plastids. Journal of Biology and Chemistry, 280: 6648-6654.

Xu, X.M., Moller, S.G. 2004. AtNAP7 is a plastidic SufC-like ATP-binding cassette/ATPase essential for *Arabidopsis* embryogenesis. Proceedings of Natural Academy of Science, 101: 9143-9148.

Yaalon, D.H. 1957. Problems of soil testing on calcareous soils. Plant and Soil, 8: 275-288.

Ylivainio, K., Jaakkola, A. and Aksela, R. 2004. Effects of Fe compounds on nutrient uptake by plants grown in sand media with different pH. Journal of Plant Nutrition, 167: 602-608.

Zerulla, W., Barth, T., Dressel, J., Erhardt, K., von Locquenghien, K.H., Pasda, G., Rädle, M. and Wissemeier, A.H. 2001. 3,4-Dimethylpyrazole phosphate (DMPP) – a new nitrification inhibitor for agriculture and horticulture. Annual Introduction of Biology and Fertility of Soils, 34: 79–84.

Zok, A., Oláh, R., Hideg, É., Horváth, V.G., Kós, P.B., Majer, P., Váradi, G. and Szegedi, E. 2010. Effect of *Medicago sativa* ferritin gene on stress tolerance in transgenic grapevine. Plant Cell Tissues and Organ Culture, 100: 339–344.

Zuo, Y. and Zhang, F. 2008. Effect of peanut mixed cropping with graminaceous species on micronutrient concentrations and iron chlorosis of peanut plants grown in a calcareous soil. Plant and Soil, 306: 23–36.

CHAPTER II

OBJECTIVES OF THE WORK

As it has been discussed in the state of the art (Chapter I), the investigations previously conducted on plant Fe nutrition are highly interdisciplinary. However, the knowledge on Fe nutrition physiology in woody plants is still scant and scientific advances are highly required and welcome. Grapevine, a crop displaying special agronomic, economical and scientific relevance, represents a model plant for investigating the physiology of woody species. By the Doctoral dissertation, various physiological and biochemical aspects associated to Fe nutrition have been studied. The research has been focused on the following specifics aims:

1. Physiological and biochemical response mechanisms of grapevine to Fe-deficiency and bicarbonate nutrition.
2. Response mechanisms to Fe-deficiency in *Vitis* genotypes displaying different Fe-chlorosis tolerance under field conditions.
3. Effectiveness and physiological implications of ecologically and economically sustainable management techniques for preventing iron chlorosis in grapevine

These objectives have been pursued through three specific experiments performed under controlled conditions.

PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES OF THE IRON CHLOROSIS TOLERANT 140 RUGGERI GRAPEVINE ROOTSTOCK TO FE-DEFICIENCY AND BICARBONATE

Introduction

In many important viticultural areas of the Mediterranean basin, characterized by soils with high concentration of active lime and alkaline pH, iron (Fe) chlorosis is one of the main nutritional deficiency of grapevine (Tagliavini and Rombolà, 2001). This nutritional disorder can cause a dramatic reduction of vineyard economical life cycle as well as grape yield, quality losses and shoot and root growth reduction (Bavaresco et al., 2003).

Grapevine belongs to Strategy I plants, therefore under Fe-deficiency it is able to increase Fe reductase activity and enhance net excretion of protons and root organic compounds (eg. organic acids, phenols), lowering the pH and increasing the solubility of Fe(III) (Brancadoro et al., 1995; Ksouri et al., 2006; Jimenez et al., 2007). The prevention/cure of Fe chlorosis with Fe chelates is a very widespread agronomical practice in vineyards, nevertheless it implies high costs and potential environmental and health risks. Such consequences strongly suggest the need of adopting alternative strategies for managing Fe nutrition according to soil and plant parameters. In this context, the use of tolerant rootstocks may represent an economical and efficient method for preventing Fe chlorosis. It is well known that the susceptibility level to Fe chlorosis in grapevine is highly variable in function of the genotype, and 140 Ruggeri has been classified as highly tolerant rootstock to Fe chlorosis (Ksouri et al., 2006; Tagliavini and Rombolà, 2001). Rootstock 140 Ruggeri is an hybrid from *Vitis berlandieri x Vitis rupestris*, that inherited the high tolerance lime soil from *Vitis berlandieri* and to drought stress from *Vitis rupestris* (Eynard and Dalmasso, 1990). Ksouri et al. (2006) found that the high tolerance of 140 Ruggeri to Fe chlorosis is in part due to a high roots Fe(III)-reductase activity and ability to release phenol compounds in the medium. Currently this rootstock is largely employed in south Mediterranean and North Africa viticulture areas, characterized by lime soils and drought environmental conditions.

Bicarbonate is one the main factor causing Fe-chlorosis in Strategy I plants (Mengel et al., 1984). In calcareous soils bicarbonate concentration can reach values up to 9–15 mM (Boxma, 1972). Nevertheless, the mode of action of bicarbonate in plants and the mechanisms involved in Fe-chlorosis induction are not well understood. By using morphological and physiological criteria, it has been demonstrated that 140 Ruggeri rootstock is able to withstand high concentrations of bicarbonate in the soil (10 mM), showing only a slight/moderate decreases in leaf chlorophyll and plant biomass (Ksouri et al., 2005). In several studies bicarbonate has been routinely included in the nutrient solution for exacerbating expected Fe-deficiency symptoms and stimulating response mechanisms (Lopez-Millán et al., 2000; Lopez-Millán et al., 2009; Rombolà et al., 2005). In a research performed on grapevine rootstocks, Nikolic et al., (2000) concluded that bicarbonate-induced Fe chlorosis was caused by an inhibition of Fe uptake and translocation due to an inhibition of Fe(III) reduction by root cells, but these processes were less inhibited in chlorosis-resistant rootstocks. Mengel et al., (1994) hypothesized that bicarbonate-induced chlorosis was caused by a transport of bicarbonate into the stele leading to an alkalinization of the xylem sap and, in turn, of the leaf apoplast. Several authors have been tested Mengel hypothesis by measuring the effects of bicarbonate on the xylem sap pH but not final conclusions were reached (Wegner and Zimmermann, 2004). Using a novel xylem pH probe enabling *in situ* measurements, Wegner and Zimmermann (2004) clearly demonstrated that bicarbonate induced alkalinization of the xylem sap in intact maize seedlings, supporting Mengel hypothesis. In several model plants (*Arabidopsis thaliana* L., *Cucumis sativus* L., *Pisum sativum* L. *and Lycopersycon esculentum* Mill.) it has been demonstrated that bicarbonate, at extremely high concentrations (20-30 mM), could induce Fe chlorosis by inhibiting the expression of ferric reductase, iron transporter and the H^+-ATPase genes (Lucena et al., 2007). Others authors (Donnini et al., 2009; Jelali et al., 2011; Rombolà, 1998) utilized the addition of bicarbonate and Fe to the nutrient solution in order to mimic the field conditions, where Fe deficiency in plants is caused by the low availability of Fe in the soil induced by the presence of bicarbonate. Results obtained in experiments that have adopted this methodological approach, generally indicate that plants grown in the presence of bicarbonate combined with Fe (+Fe+BIC) are less affected than plants submitted to absolute Fe-deficiency (-Fe). In the field, it has been observed that

several woody crops can tolerate lime soils, growing and producing normally without the need of adopting any specific agronomic techniques to control Fe chlorosis. Moreover, some plants –as calcicoles- are able to overcome high bicarbonate concentrations in the substrate without displaying chlorotic symptoms (Donnini et al., 2012), and some of them have shown positive effects of bicarbonate on root growth (Lee and Woolhouse, 1969).
In view of the above exposed background, this work studied the physiological and biochemical response mechanisms of grapevines to Fe and bicarbonate in the substrate as independent and combined factors. For this purpose, a nutrient solution experiment with two level of Fe and two levels of bicarbonate has been conducted on the Fe chlorosis tolerant rootstock 140 Ruggeri. This experimental approach has allowed to identifying the effects of each factor and their interaction, establishing distinctly the response of plants to the factors.

Materials and Methods

Vegetal material, growth conditions and treatments

The experiment was conducted at the Experimental Station of the Agriculture Faculty (Cadriano, Bologna). Micropropagated plants of the Fe chlorosis tolerant genotype cv 140 Ruggeri (*Vitis berlandieri x Vitis rupestris)* were acclimated in peat for 3 weeks and pruned maintaining one main shoot on each plant. The plants (36) were transferred to 35 l plastic containers covered with aluminum and filled with 30 l of a half Hoagland nutrient solution continuously aerated, with 9 plants for each container. The growth chamber was programmed for 16 hours photoperiod (150-200 µmol photons m^{-2} s^{-1} at leaf level) at 25-30°C and 8 hours of darkness at 25°C, with 70-75% relative humidity. Plants were treated with two levels of Fe and two level of bicarbonate in the solution. The treatments established were: 1) 50 µM of Fe(III)-EDTA + 5 mM of $KHCO_3$ (+Fe +BIC); 2) 50 µM of Fe(III)-EDTA + 0 mM of $KHCO_3$ (+Fe -BIC); 3) 0 µM of Fe(III)-EDTA + 5 mM of $KHCO_3$ (-Fe +BIC); 4) 0 µM of Fe(III)-EDTA + 0 mM of $KHCO_3$ (-Fe -BIC). The potassium supplied in the treatments with $KHCO_3$ was balanced with K_2SO_4 in the

treatments without bicarbonate. The composition of the nutrient solution half Hoagland was: 2,5 mM KNO_3; 2 mM $MgSO_4$; 1 mM KH_2PO_4; 2,5 mM $Ca(NO_3)_2$; 4,6 µM $MnCl_2$; 23,2 µM H_3BO_3; 0,06 µM Na_2MoO_4; 0,4 µM $ZnSO_4$; 0,19 µM $CuSO_4$. The nutrient solution was renewed twice a week, the pH was monitored daily at 9:00 am and was adjusted to 7,4 and 6,0 in the containers with and without bicarbonate respectively after every renewal with HCl 0,1 M. The plants were grown on these conditions for 20 days.

Leaf chlorophyll content and plant growth

Leaf chlorophyll content was periodically monitored during the experiment on five points of the first completely expanded leaf with the portable chlorophyll meter SPAD MINOLTA 502 (Osaka, Japan). When apical leaves of -Fe plants showed severe Fe-chlorosis symptoms (SPAD index value of around 5), the experiment was concluded and the plants were divided into roots, main shoot, lateral shoots and leaves for dry weight determinations, total leaf area and following analysis.

Enzyme assays and protein concentration in roots

At the end of the experiment, root tip (20–30 mm long) samples were collected from each plant, rinsed in deionized water, weighed, deep-frozen in liquid nitrogen, and kept at –80°C for enzyme activity analysis. The activity of phosphoenolpyruvate carboxylase (PEPC), malate dehydrogenase (MDH), citrate synthase (CS), and isocitrate dehydrogenase ($NADP^+$-IDH) were determined. The extraction of enzymes was performed as described by Jimenez et al., (2007).
Phosphoenolpyruvate carboxylase was determined by coupling its activity to malate dehydrogenase- catalyzed NADH oxidation (Vance et al., 1983). MDH activity was determined by monitoring the increase in absorbance at 340 nm, due to the enzymatic reduction of NAD^+ (Smith, 1974). CS activity was assayed by monitoring the reduction of acetyl CoA to CoA with DTNB at 412 nm (Srere, 1967). $NADP^+$-IDH was assayed as described by Goldberg and Ellis (1974), by monitoring the reduction of $NADP^+$ at 340 nm. Protein concentration was determined by the Bradford method, using BSA as standard

(Bradford, 1976). Data obtained in enzyme assays were referred to protein concentration of roots.

Determination of kinetic properties of PEPCase

Kinetic analysis was performed in the standard buffer by varying each time the HCO_3^- concentration. The substrate dependence of PEPC to HCO_3^- concentration was characterized determining the PEPC activity with different concentrations of HCO_3^- in 9 points in a range from 0-10 mM. Decarbonated water was used for the determination of HCO_3^- kinetic. V_{max} and Km values were calculated using the Eadie-Hofstee plot.

Organic acids concentration in roots

The organic acids concentration was determined according to Neumann (2006). Frozen samples of root tips collected at the end of the experiment were submerged in a pre-cooled (4 °C) mortar with liquid nitrogen. After evaporation, the tissue was grind and homogenized with a pestle. For extraction and deproteinization, 5% H_3PO_4 was utilized. Organic acids were quantified by HPLC with 250 x 4 mm LiChrospher 5 um RP-18 column (SUPELCO INC., PA 16823-0048 USA). HPLC elution buffer was 18 mM KH_2PO_4, pH 2,1 adjusted with H_3PO_4. Samples were read at 210 nm for 40 minutes (Neumann, 2006).

Xylem sap collection and analysis

Xylem sap was collected from each plant at the end of the experiment applying a pressure of 5 bar to the root system by a Schölander pressure chamber, as described by Rombolà et al., (2002). Approximately 500–1000 µl of xylem sap per plant was collected with microcapillary tubes in Eppendorf capsule of 2 ml and then were frozen at -20°C for organic acids and pH determination. The pH of the xylem samples was determined with a microelectrode (Hamilton AG, CH-7402 Bonaduz, Switzerland). Organic acids were quantified by HPLC with 250 x 4 mm LiChrospher 5 um RP-18 column (SUPELCO INC., PA 16823-0048 USA) as described by Neumann et al., (2006).

Statistics

Data were analyzed by a two-way (Fe x BIC) analysis of variance with SAS software (SAS Institute, Cary, NC). If significant interactions between factors were obtained by the F-test, it was calculate the standard error of the interaction means (SEM), and in this case, treatments are significantly different when the difference between data is higher than 2 x SEM. Significant differences between factor levels were detected by the F-test ($p \leq 0.05$), and in this case, statistical comparisons are between levels of each factor.

Results

Chlorophyll content and plants growth

At the end of the experiment, plants grown under Fe-deficiency showed a decrease (-1.9-fold) of chlorophyll content. SPAD index values of 9.5 and 4.6 were recorded in the first fully expanded leaf of +Fe and –Fe treatment respectively. The presence of bicarbonate in the nutrient solution increased leaf chlorophyll content of 1.5-fold, with SPAD index values of 8.5 and 5.6 in +BIC and –BIC, respectively. Iron deficiency decreased roots biomass of 0.3 g plant^{-1} DW (-13%) whereas and bicarbonate nutrition induce an increase of 0.9 g plant^{-1} DW (+56%) (Tab. 1). Total leaf area and biomass yield of main shoot, lateral shoots, leaves, and total biomass did not present significant differences due to experimental factors (Tab. 1). However, Fe-deficiency induced a trend to decrease total biomass yield, and the presence of bicarbonate tendentially increased it.

pH of the nutrient solution

After seven days of treatment, Fe deficient plants decreased the pH of nutrient solution (Fig. 1). In presence of bicarbonate the pH reached values above 7.4, while when bicarbonate was not added in the nutrient solution pH values were below 6.3. The

differences in the pH of nutrient solution between levels of Fe were slightly higher in presence of bicarbonate (Fig. 1).

Enzyme activities and protein concentration in root extracts

At the end of the experiment, the activity of PEPC and some key enzymes of Krebs cycle were determined in the root tip extracts. Iron deficiency induced an increase of 56% in PEPC activity (Tab. 2), whereas bicarbonate decreased the activity of this enzyme by 43% (Tab. 2). MDH activity did not change in response to Fe level (Tab. 2), whereas bicarbonate supply reduced the activity of MDH by 27% (Tab. 2). CS and $NADP^+$-IDH data showed an interaction between factors. Iron deficiency increased the activity of these enzymes in bicarbonate fed-plants, opposite effects were recorded in –BIC plants (Tab. 2).

Kinetic properties of PEPCase

The saturation kinetics curves of PEPCase were established by addition of different concentrations of bicarbonate -in a range of 0 to 10 mM- to the buffer assay. The assays showed that bicarbonate concentration in the buffer assay increased the activity of PEPCase in all treatments, until a concentration of around 5 mM (data not shown). With concentrations of bicarbonate between 5 mM and 10 mM, the activity of the enzyme did not change significantly (data not shown). Iron deficiency increased the rate of PEPCase activity, reaching a higher V_{max} than Fe-sufficient plants (Tab. 3). In contrast, bicarbonate did not modify the V_{max} (Tab. 3), however, a tendentially lower V_{max} (23%) was recorded in bicarbonate-fed plants (Tab. 3). *Km* was not altered by treatments (Tab. 3).

Organic acids concentration in roots and xylem sap

At the end of the experiment, the major organic acids present in root extracts were tartaric, followed by malic, citric and ascorbic acids (Tab. 4). Significant interactions between iron and bicarbonate were recorded. Regardless to bicarbonate treatment, Fe-deficiency increased citric acid concentration in roots (Tab. 4). However, the effect of Fe was less

pronounced in bicarbonate-fed plants. Iron shortage enhanced the concentration of tartaric acid only in –BIC plants (73%), whereas no differences were detected in plants submitted to bicarbonate nutrition (Tab. 4). Limiting Fe did not alter malic acid level, while bicarbonate enhanced by 50% malic acid concentration in the roots (Tab. 4). In –BIC plants, root ascorbic acid concentration was lowered by Fe-deficiency, whereas Fe status did not affect ascorbic acid levels in +BIC roots (Tab. 4). Iron deficiency enhanced by 2-fold total organic acids concentration in the roots in –BIC plants (Tab. 4), whereas, under bicarbonate nutrition, similar levels were recorded in +Fe and –Fe plants (Tab. 4).

In xylem sap, the predominant organic acid was malic acid, followed by citric and ascorbic acids (Tab. 5). Iron deficiency increased the concentration of citric acid without affecting the pH of the xylem sap (Tab. 5). Bicarbonate decreased citric acid concentration and increased xylem sap pH (Tab. 5). Malic, ascorbic and total organic acids concentration did not change in response to Fe and bicarbonate levels (Tab. 5).

Discussion

Under Fe-deficiency conditions (-Fe), the Fe chlorosis tolerant rootstock 140 Ruggeri showed a marked decrease of leaf chlorophyll content (SPAD index values of 9.5 and 4.6 in +Fe and –Fe respectively) associated to a reduction of root biomass (Tab. 1). Such symptoms as well as a reduction of shoot growth, have been previously described in grapevine and other woody species (Bavaresco et al., 2003; Jimenez et al., 2007; Rombolà and Tagliavini, 2006).

The presence of bicarbonate in the nutrient solution (5 mM) stimulated chlorophyll content of apical leaves (SPAD index values of 8.5 and 5.6 in +BIC and -BIC respectively) and root biomass (Tab. 1). Opposite results have been reported for chlorophyll content in *Pisum sativum* (Barhoumi et al., 2007; Jelali et al., 2011), *Pyrus communis* (Donnini et al., 2009), *Cydonia oblonga* (Donnini et al., 2009), *Medicago ciliaris* (M'sehli et al., 2009). A stimulation of root biomass induced by bicarbonate has been reported in *Medicago ciliaris* (M'sehli et al., 2009). In this species, the presence of bicarbonate increased root dry weight in the line more tolerant to Fe chlorosis, and did not affected root growth in the line less

tolerant to Fe chlorosis. It is well known that species originating in calcareous soils (calcicoles) are more tolerant to bicarbonate ion than species from acid soils (calcifuges) (Woolhouse, 1966). Interestingly, the root growth of the calcifuge species *Deschampsia fiexuosa* was inhibited by 1 mM HCO_3 whereas that of the calcicole species *Arrhenatherum elatius* was stimulated by bicarbonate at concentrations up to 4 mM (Lee and Woolhouse, 1969). In a pot experiment, similar results were observed in pear and quince genotypes grown on soils with different $CaCO_3$ content (Tagliavini et al., 1993). In *Parietaria diffusa* species, the bicarbonate supply induced a shorter root system, with the appearance of structures similar to "proteoid roots", that provide an enhanced surface of contact between plant and soil (Donnini et al., 2012). The presence of such structures in Fe-sufficient plants grown with bicarbonate but not in Fe-deficient plants suggests that this should not be a specific response to Fe deficiency but to a more general condition of low nutrient availability (Donnini et al., 2012). The increase of root biomass in bicarbonate fed-plants may be interpreted as a plant reaction for enhancing the contact surface between roots and rizosphere, releasing of nutrient-solubilizing compounds and taking up of mineral elements scarcely available due to the high soil pH. These results indicate that rootstock 140 Ruggeri behaves as a calcicole species.

In the present work, starting from seven days after treatments imposition, Fe-deficient plants decreased the pH of nutrient solution regardless to bicarbonate level (Fig. 1). These results are in line with Ksouri et al., (2005) and demonstrate the capacity of this Fe-chlorosis tolerant genotype to activate mechanisms for decreasing the pH of medium under Fe-deficiency conditions. In grapevine, the chlorosis-tolerant genotype Cabernet Sauvignon (*Vitis vinifera*) strongly decreased the pH by around 3 units after 7 days of Fe depletion, whereas the chlorosis-susceptible genotype Riparia Gloire de Montpellier slightly decreases the pH of nutrient solution (by around 1 unit) (Jimenez et al., 2007).

The rootstock 140 Ruggeri reacted to Fe-deficiency increasing the carbon fixation in roots by a higher activity PEPCase. In grapevine, Fe-deficiency increased the PEPCase activity in roots of the chlorosis-tolerant genotype Cabernet Sauvignon by around 3-fold, whereas the chlorosis-susceptible genotype Gloire de Montpellier increased PEPC activity by around 2.5-fold (Jimenez et al., 2007). Moreover, similar results have been observed in other species submitted to Fe-deficiency (eg. *Capsicum annuum* L., *Cucumis sativus* L.,

Pisum sativum, *Beta vulgaris* L., *Actinida deliciosa*, *Pyrus comunis*). The increase in PEPC activity in Fe-deficiency roots was associated with a higher concentration of some organic acids (citrate and tartrate, discussed later). These results support the significance of carbon fixation by this enzyme for organic acid accumulation, a typical response of Strategy I plants to Fe-deficiency (Abadía et al., 2002; López-Millán et al., 2000; Rombolà et al., 2002).

The stimulus observed by Fe-deficiency in the V_{max} of PEPC without modifying the substrate-affinity of the enzyme (*Km*) (Tab. 3) indicates that Fe-deficiency probably increases the concentration of the enzyme in roots of 140 Ruggeri, at as been commented for *Cucumis sativus* L. by De Nisi and Zocchi et al., (2000). Microarray data presented by Thimm et al., (2001) reported an induction of PEPCase expression genes in roots of *Arabidopsis thaliana* cv Landsberg submitted to Fe-deficiency. In the grapevine genotype 140 Ruggeri, information regarding the concentration of root PEPC is necessary for establishing if this protein, in Fe-deficient plants, is more or less under transcriptional regulation.

The presence of bicarbonate decreased the activity of PEPC and MDH, whereas the effect on the activity of CS and $NADP^+$-IDH depended on the Fe status of plants (Tab. 2). A decrease in PEPCase activity induced by bicarbonate was also observed in *Pisum sativum* (Jelali et al., 2010) and in *Parietaria diffusa* (Donnini et al., 2012). Several hypothesis have been proposed for explaining this effect. This enzyme is negatively regulated by L-malate (Chollet et al., 1996). López-Millán et al., (2000) proposed that concentrations of 0.5 mM of malic acid could be an inhibitory effect on PEPC activity, and in this experiment bicarbonate increased malic acid concentration in roots reaching 0.64 mM, 52% higher than in roots grown without bicarbonate. Recently, Donnini et al., (2012) suggested that in the calcicole species *Parietaria diffusa*, the presence of bicarbonate in the substrate changes significantly the responses of plant to Fe-deficiency. The PEP produced by glycolysis is in part channeled into the shikimate pathway and converted into phenolics rather than assimilated by PEPC (Donnini et al., 2012). Data showed a clear reduction on PEPC regardless of Fe level indicating a *per se* effect of bicarbonate on the enzyme activity. An adverse effect of bicarbonate on PEPC activity has been observed in various species including those tolerant to calcareous soils. Therefore, in such conditions the lower

bicarbonate fixation inside roots cells could lead to an increased of ion accumulation into cytoplasm compartments. In this context, which are the fate and the possible role of bicarbonate? Mengel et al., (1994) hypothesized that bicarbonate may be transported into the stele leading an alkalinization of the xylem sap and, in turn, of the leaf apoplast. Several authors have tested Mengel hypothesis by measuring the effects of bicarbonate on the xylem sap pH but not final conclusions were reached (Wegner and Zimmermann, 2004). However, using a novel xylem pH probe enabling *in situ* measurements, Wegner and Zimmermann (2004) clearly demonstrated that bicarbonate induced alkalinization of the xylem sap in intact maize seedlings, supporting Mengel hypothesis. It has been reported that increases in the pH of xylem sap can increasing the concentration of CO_2 dissolved products (Levy et al., 1999). Results of this experiment showed that bicarbonate increased xylem sap pH (Tab. 5), suggesting that this ion was presumably loaded into the xylem and transported to the shoots and leaves. The increase of leaf chlorophyll content induced by bicarbonate observed in this experiment suggest that this ion may slow down leaf chlorophyll degradation as occurs in green vegetables submitted to high CO_2 atmosphere in postharvest treatments (Eason et al., 2007). The possibility that xylem sap bicarbonate represents a source of carbon for leaf Rubisco (Stringer and Kimmerer, 1993) is an intriguing subject for further researches.

The activities of TCA cycle enzymes in roots indicate that response mechanisms to Fe-deficiency and bicarbonate are complex and the accumulation of organic acids may change according to these factors or in interaction between them. The presence of bicarbonate in the nutrient solution slowed down the activity of MDH regardless to Fe level. In contrast, bicarbonate decreased the activity of CS and $NADP^+$-IDH only under Fe-sufficiency conditions. The slowdown of CS and $NADP^+$-IDH in bicarbonate fed-plants resulted in an accumulation of malate in roots, in spite of the PEPCase reduction induced by bicarbonate. This behavior represents a typical response of calcicole plants to bicarbonate (discussed later). Under Fe-deficiency conditions the presence of bicarbonate resulted into a higher activity of CS in roots. This result indicates that under concomitant occurrence of Fe-deficiency and bicarbonate stress conditions, this genotype primarily reacts to Fe-deficiency increasing the synthesis of citric acid by CS. In this case, considering that bicarbonate decreases MDH activity, and consequently oxaloacetate concentration inside

mitochondrion also decrease it, is possible that oxaloacetate synthesized in the cytoplasm enter directly to the mitochondrion and it is utilized by CS for accumulating citric acid in roots. Iron nutrition did not modify MDH activity in roots (Tab. 2). In addition, recently it has been demonstrated that under Fe-deficiency, a GroEl-like chaperone facilitate folding the mitochondrial MDH (Rodríguez-Celma et al., 2011), causing an accumulation of the MDH precursors.

Iron deficient roots of 140 Ruggeri grown in presence of bicarbonate showed a higher CS and $NADP^+$-IDH activity (Tab. 2). Both TCA cycle enzymes (CS and $NADP^+$-IDH) have been reported as enzymes that respond to Fe-deficiency in root tissues of some species (eg. *Beta vulgaris* L., *Pisum sativum, Lycopersicon esculentus* L.). In addition, *Arabidopsis thaliana* showed an induction of $NADP^+$-IDH genes expression in roots of Fe-deficient plants (Thimm et al., 2001), confirming the role of these proteins in Fe-deficiency responses. Moreover, several authors have suggested that CS activity is a typical mitochondrial marker (Hood et al., 1989; Williams et al., 1986) and also it is generally proportional to the number of mitochondria (Vigani et al., 2009).

In plants grown without bicarbonate in the nutrient solution, Fe-deficiency decreased the activity of CS and $NADP^+$-IDH in roots (Tab. 2). A possible cause for explaining this effect on CS may be that citric acid concentration in Fe-deficient roots grown without bicarbonate was already quite high compared with the others treatments (Tab. 4), possibly due to a strong decrease of aconitase activity, a Fe containing enzyme (Tab. 2). We may speculate that the decrease in aconitase activity reduced the production of isocitrate (the substrate of $NADP^+$-IDH) and consequently the activity of $NADP^+$-IDH.

The addition of bicarbonate in the nutrient solution induced and increases of malate concentration in roots (Tab. 4) without increasing its concentration in xylem sap (Tab. 5). In Fe-sufficient plants of *Pisum sativum*, bicarbonate increased malate concentration at root level. Thus effect was more pronounced in the Fe-deficiency tolerant cv Kelvedon than Fe-deficiency sensitive Lincoln (Jelali et al., 2010). The supplying of bicarbonate increased the exudation of malate only in cv Kelvedon. In *Parietaria diffusa* species, Donnini et al., (2012) showed that bicarbonate increased the concentration and release of malic acid by roots. It has been demonstrated that enhanced organic acid concentrations and release from

the roots are typical responses of calcicole plants to the presence of bicarbonate in the soil, and could play a strong role in plant tolerance to bicarbonate excess (Ström, 1997).

The interactions found between Fe and BIC factors indicate that bicarbonate level modulates the concentrations of tartrate, citrate and ascorbate in the roots of 140R rootstock as a response to Fe-deficiency (Tab. 4). The major organic acid present in root extracts were tartrate, followed by malate and citrate and in lower concentration ascorbate (Tab. 4). Iron deficiency increase citrate concentration in roots (Tab. 4) for both levels of bicarbonate in the nutrient solution, and in xylem sap (Tab. 5). This is a common result in many plant species and genotypes with a medium-high level of tolerance to iron chlorosis, and several authors suggested that citrate concentrations in roots could be used as a biochemical marker of Fe-chlorosis tolerance level (Ollat et al., 2003; Rombolà et al., 2002). However, the differences in root citrate concentration between Fe levels recorded in plants grown in +BIC are lower than in –BIC conditions (Tab. 4). This effect may be attributed to a higher citrate exudation by roots in -Fe+BIC than in -Fe-BIC, due to a double response to Fe-deficiency and bicarbonate stress, reducing the accumulation of citrate inside root cells in -Fe+BIC plants (Donnini et al., 2012; Ström, 1997).

Tartaric acid was the mayor organic acid founded in roots of 140 Ruggeri (Tab. 4) This organic acid is very specific to *Vitis* species (Ruffner, 1982), and is one of the main acids in berries. In the present experiment, Fe-deficiency increased the concentration of tartaric acid in plants grown in absence of bicarbonate (Tab. 4), according to that found by Ollat et al. (2003) for Fe-chlorosis tolerant genotype Cabernet Sauvignon genotype. Reverse effects were found for Fe-chlorosis sensitive genotypes 101-14 and Gloire de Montpellier, suggesting that the increase of tartrate concentration in roots is a specific response of tolerant genotypes. No differences were induced by Fe-deficiency in +BIC plants, presumably due to the high tartaric acid concentration in roots of Fe-sufficient plants. In addition, tartaric acid was not found in the xylem sap, probably due to a high root exudation rate of this acid as calcicole plants that release more organic acids than calcifuge plants (Rengel, 2002; Ström, 1997) or due to the transport of this acid as caftaric acid, amino acids or sugars. Results obtained showed that Fe-deficiency decreased ascorbic acid concentration in roots grown in absence of bicarbonate (Tab. 4). It is well known that that ascorbate is a synthesis precursor of tartaric acid (DeBolt et al., 2006; Keates et al., 2000;

Kostman et al., 2001), therefore it is possible that the higher concentration of tartrate recorded in roots of -Fe-BIC than in +Fe-BIC, produced a decrease in ascorbate concentration.

Conclusions

The present work shows that the Fe-chlorosis tolerant 140 Ruggeri rootstock strongly reacts to Fe-deficiency activating several response mechanisms at different physiological and biochemical levels. The methodological approach adopted in this experiment has allowed to demonstrating that the presence of bicarbonate in the nutrient solution can modulate several response mechanisms to Fe-deficiency, shifting the activity of PEPC and TCA cycle enzymes and the accumulation/translocation of organic acids in roots. Moreover, this genotype increased root biomass and root malic acid concentration as a response to high bicarbonate levels in the substrate, displaying a behavior typical of calcicole plants. In addition, bicarbonate enhanced the leaf chlorophyll content of the plants, suggesting a possible role of bicarbonate in chlorophyll degradation processes. Further investigations are needed to clarify the metabolic fate of bicarbonate in the xylem and the possible effects on graft combinations.

3.6. Literature cited

Abadía, J., López Millán, A.F., Rombolà, A. and Abadía, A. 2002. Organic acids and Fe deficiency: a review. Plant and Soil, 241: 75–86.

Barhoumi, Z., Rabhi, M. and Gharsalli, M. 2007. Effect of two nitrogen forms on the growth and iron nutrition of pea cultivated in presence of bicarbonate. Journal of Plant Nutrition, 30: 1953–1965.

Bavaresco, L., E. Giachino, and S. Pezzutto. 2003. Grapevine rootstock effects on lime-induced chlorosis, nutrient uptake, and source-sink relationships. Journal of Plant Nutrition, 26: 1451-1465.

Boxma, R. 1972. Bicarbonate as the most important soil factor in lime induced chlorosis in the Netherlands. Plant and Soil, 37: 233–243.

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72: 248-254.

Brancadoro, L., Rabotti, G., Scienza, A. and Zocchi, G. 1995. Mechanisms of Fe-efficiency in roots of *Vitis* spp. in response to iron deficiency stress. Plant and Soil, 171: 229-234.

Chollet, R., Vidal, J. and O'Leary, M.H. 1996. Phosphoenolpyruvate carboxylase: a ubiquitous, highly regulated enzyme in plants. Annual Review of Plant Physiology and Plant Molecular Biology, 47: 273–298.

De Nisi, P. and Zocchi, G. 2000. Phosphoenolpyruvate carboxylase in cucumber (*Cucumis sativus* L.) roots under iron deficiency: activity and kinetic characterization. Journal of Experimental Botany, 51(352): 1903-1909.

DeBolt, S., Cook, D.R. and Ford, C.M. 2006. L-Tartaric acid synthesis from vitamin C in higher plants. Biological Sciences and Plant Biology, 103(14): 5608-5613.

Donnini, S., Castagna, A., Ranieri, A. and Zocchi, G. 2009. Differential responses in pear and quince genotypes induced by Fe deficiency and bicarbonate. Journal of Plant Physiology, 166: 1181-1193.

Donnini, S., De Nisi, P., Gabotti, D., Tato, L. and Zocchi, G. 2012. Adaptive strategies of *Parietaria diffusa* (M.&K.) to calcareous habitat with limited iron availability. Plant, Cell & Environment, doi: 10.1111/j.1365-3040.2012.02481.x

Eason, J.R., Ryan, D., Page, B., Watson, L. and Coupe, S.A. 2007. Harvested broccoli (*Brassica oleracea*) responds to high carbon dioxide and low oxygen atmosphere by inducing stress-response genes. Postharvest Biology and Technology, 43: 358–365.

Eynard, I. and Dalmasso, G. 1990. La scelta del portinnesto. *In:* Viticoltura moderna, manuale pratico. IX Ed. Pp. 261-289.

Goldberg, D.M. and Ellis, G. 1974. Isocitrate dehydrogenase. *In*: Bergmeyer HU (ed) Methods of enzymatic analysis. Verlag Chemie/Academic Press, New York, EEUU, pp 183–189.

Hood, D., Zak, R. and Pette, D. 1989. Chronic stimulation of rat skeletal muscle induces coordinate increases in mitochondrial and nuclear mRNAs of cytochrome c oxidase subunits. European Journal of Biochemistry, 179: 275–280.

Jelali, N., Salah, I.B., M'sehli, W., Donnini, S., Zocchi, G. and Gharsalli, M. 2011. Comparison of three pea cultivars (*Pisum sativum*) regarding their responses to direct and bicarbonate-induced iron deficiency. Scientia Horticulturae, 129: 548–553.

Jimenez, S., Gogorcena, Y., Hévin, C., Rombolà, A.D., and Ollat, N. 2007. Nitrogen nutrition influences some biochemical responses to iron deficiency in tolerant and sensitive genotypes of Vitis. Plant and Soil, 290: 343-355.

Keates, S.E., Tarlyn, N.M., Loewus, F.A. and Franceschi, V.R. 2000. L-Ascorbic acid and L-galactose are sources for oxalic acid and calcium oxalate in *Pistia stratiotes*. Phytochemistry, 53: 433-40.

Kostman, T.A., Tarlyn, N.M., Loewus, F.A. and Franceschi, V.R. 2001. Biosynthesis of L-ascorbic acid and conversion of carbons 1 and 2 of L-ascorbic acid to oxalic acid occurs within individual calcium oxalate crystal idioblasts. Plant Physiology, 125: 634-40.

Ksouri, R., Gharsalli, M. and Lachaal. M. 2005. Physiological responses of Tunisian grapevine varieties to bicarbonate-induced iron deficiency. Journal of Plant Physiology, 162: 335-341.

Ksouri, R., M'rah, S., Gharsalli, M. and Lachaâl, M. 2006. Biochemical responses to true and bicarbonate-induced iron deficiency in grapevine genotypes. Journal of Plant Nutrition, 29: 305–315.

Lee, J.A. and Woolhouse, H.W. 1969. Root growth and dark fixation of carbon dioxide in calcicoles and calcifuges. New Phytology, 68: 247-255.

Levy, P.E., Meir, P., Allen, S.J. and Jarvis, P.G. 1999. The effect of aqueous transport of CO_2 in xylem sap on gas exchange in woody plants. Tree Physiology, 19: 53-58.

López-Millán, A.F., Morales, F., Andaluz, S., Gogorcena, Y., Abadía, A., De Las Rivas, J. and Abadía, J. 2000. Responses of sugar beet roots to iron deficiency. Changes in carbon assimilation and oxygen use. Plant Physiology, 124: 885–897.

López-Millán, A.F., Morales, F., Gogorcena, Y., Abadía, A. and Abadía, J. 2009. Metabolic responses in iron deficient tomato plants. Journal of Plant Physiology, 166: 375-384.

Lucena, C., Romera, F.J., Rojas, C.L., García, M.J., Alcántara, E. and Pérez-Vicente, R. 2007. Bicarbonate blocks the expression of several genes involved in the physiological responses to Fe deficiency of Strategy I plants. Functional Plant Biology, 34: 1002–1009.

Neumann, G. 2006. Root exudates and organic composition of plant roots. In: Luster J, Finlay R (eds) Handbook of methods used in rhizosphere research. Swiss Federal Research Institute WSL, Birmensdorf, 536 pp.

Nikolic, M., Römheld, V. and Merkt, N. 2000. Effect of bicarbonate on uptake and translocation of ^{59}Fe in two grapevine rootstocks differing in their resistance to Fe deficiency Chlorosis. Vitis, 39(4): 145-149.

Mengel, K., Breininger, M.T. and Bübl, W. 1984. Bicarbonate, the most important factor inducing iron chlorosis in vine grapes on calcareous soil. Plant and Soil, 81: 333–344.

Mengel, K., Planker, R. and Hoffmann. 1994. Relationship between leaf apoplast pH and iron chlorosis of sunflower (*Helianthus annuus* L.). Journal of Plant Nutrition, 17: 1053–1065.

M'sehli, W., Dell'Orto, M., De Nisi, P., Donnini, S., Abdelly, C., Zocchi, G. and Gharsalli, M. 2009. Responses of two ecotypes of Medicago ciliaris to direct and bicarbonate-induced iron deficiency conditions. Acta Physiologiae Plantarum, 31: 667–673.

Ollat, N., Laborde, B., Neveux, M., Diakou-Verdin, P., Renaud, C. and Moing, A. 2003. Organic acid metabolism in roots of various grapevine (*Vitis*) rootstocks submitted to iron deficiency and bicarbonate nutrition. Journal of plant nutrition, 26(10&11): 2165–2176.

Rengel, Z. 2002. Genetic control of root exudation. Plant and Soil, 245: 59–70.

Rodríguez-Celma, J., Lattanzio, G., Grusak, M.A., Abadía, A., Abadía, J. and López-Millán, A.F. 2011. Root responses of *Medicago truncatula* plants grown in two different iron deficiency conditions: changes in root protein profile and riboflavin biosynthesis. Journal of Proteome Research, 10: 2590–2601.

Rombolà, A.D. 1998. Aspetti fisiologici e biochimici della nutrizione ferrica in actinidia (*A. deliciosa*). PhD thesis. Dipartimento di Colture Arboree, Università degli Studi di Bologna, Bologna, Italy.

Rombolà, A.D., Brüggemann, W., López-Millán, A.F., Tagliavini, M., Abadía, J., Marangoni, B. and Moog, P.R. 2002. Biochemical responses to iron deficiency in kiwifruit (*Actinidia deliciosa*). Tree Physiology, 22: 869–875.

Rombolà, A.D., Gogorcena, Y., Larbi, A., Morales, F., Baldi, E., Marangoni, B., Tagliavini, M and Abadía, J. 2005. Iron deficiency-induced changes in carbon fixation and leaf elemental composition of sugar beet (*Beta vulgaris*) plants. Plant and Soil, 271: 39-45.

Rombolà, A.D. and Tagliavini, M. 2006. Iron nutrition of fruit tree crops. *In* Iron Nutrition in Plants and Rhizospheric Microorganisms (Eds, J, Abadía and L, Barton), Springer, Berlin, Germany, pp. 61-83.

Ruffner, H.P. 1982. Metabolism of tartaric and malic acids in *Vitis*: a review. Vitis, 2: 247–259.

Smith, F. 1974. Malate dehydrogenase. In: Bergmeyer HU (ed) Methods of enzymatic analysis. Verlag Chemie/Academic Press, New York, EEUU, pp. 163–175.

Srere, P.A. 1967. Citrate synthase. In: Colowick SP, Kaplan NO (eds) Methods in enzymology. Academic Press, New York, pp 3–11.

Stringer, J.W. and Kimmerer, T. W. 1993. Refixation of xylem sap CO_2 in *Populus deltoides*. Physiologia Plantarum, 89: 243–251.

Ström, L. 1997. Root exudation of organic acids: importance to nutrient availability and the calcifuge and calcicole behaviour of plants. Oikos, 80: 459–466.

Tagliavini, M., Bassi, D. and Marangoni, B. 1993. Growth and mineral nutrition of pear rootstocks in lime soils. Scientia Horticulturae, 54:13-22.

Tagliavini, M. and Rombolà, A.D. 2001. Iron deficiency and chlorosis in orchard and vineyard ecosystems. European Journal of Agronomy, 15: 71-92.

Thimm, O., Essigmann, B., Kloska, S., Altmann, T. and Buckhout, T.J. 2001. Response of arabidopsis to iron deficiency stress as revealed by microarray analysis. Plant Physiology, 127: 1030–1043.

Vance, C.P., Stade, S., Maxwell, C.A. 1983. Alfalfa root nodule carbon dioxide fixation. I: association with nitrogen fixation and incorporation into amino acids. Plant Physiology, 72: 469–473.

Vigani, G., Maffi, D. and Zocchi, G. 2009. Iron availability affects the function of mitochondria in cucumber roots. New Phytologist, 182: 127–136.

Wegner, L.H. and Zimmermann, U. 2004. Bicarbonate-induced alkalinization of the xylem sap in intact maize seedlings as measured in situ with a novel xylem ph probe. Plant physiology, 136: 3469–3477.

Williams, R.S., Salmons, S., Newsholme, E., Kaufman, R.E. and Mellor, J. 1986. Regulation of nuclear and mitochondrial gene expression by contractile activity in skeletal muscle. Journal of Biological Chemistry, 261: 376–380.

Woolhouse, H.W. 1966. Comparative physiological studies on *Deschampsia fiexuosa*, *Holcus mollis*, *Arrhenatherum elatius* and *Koeleria gracilis* in relation to growth on calcareous soils. I. Growth and root respiration. New Phytology, 65 (1): 22-31.

3.7. Figures and tables

Table 1. Total leaf area (cm^2 $plant^{-1}$) and biomass yield of organs (g $plant^{-1}$ DW) determined at the end of experiment at two levels of bicarbonate (+BIC and -BIC) grown under Fe-sufficient (+Fe) and Fe-deficient (–Fe) conditions. Data are means of nine replicates.

	Leaf area	Roots	Main Shoot	Lateral shoots	Leaves	Total
Bicarbonate (BIC)						
+ BIC	183,1	2,5	0,7	0,2	1,0	4,4
- BIC	188,8	1,6	0,7	0,3	1,1	3,7
Significance	n.s.	***	n.s.	n.s.	n.s.	n.s.
Iron (Fe)						
+ Fe	193,1	2,2	0,7	0,3	1,1	4,3
- Fe	178,7	1,9	0,7	0,2	1,1	3,9
Significance	n.s.	*	n.s.	n.s.	n.s.	n.s.
B x Fe interaction	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Abbreviation and symbols: n.s., *, *** = not significant and significant at $p \le 0.05$ and $p \le 0.001$ levels, respectively.

Figure 1. Changes in the pH of the nutrient solution of 35-l containers with plants grown at two levels of bicarbonate (+BIC and -BIC) under Fe-sufficient (+Fe) and Fe-deficient (–Fe) conditions. The nutrient solution was renewed twice a week during the experiment (↓).

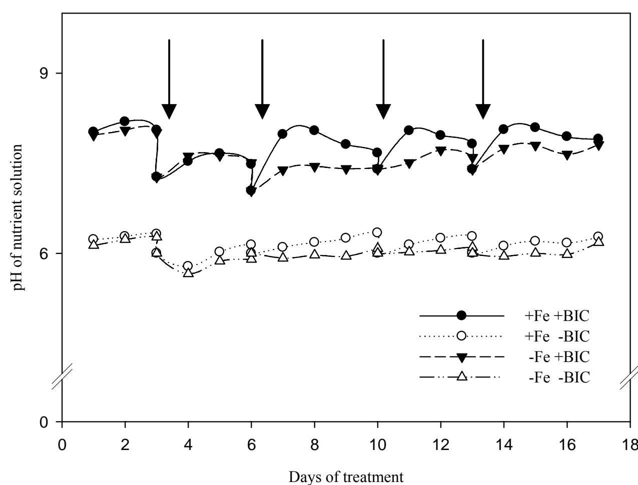


Table 2. Activities (nmol mg^{-1} protein min^{-1}) of PEPC, MDH, CS, $NADP^+$-IDH and protein concentration (mg g^{-1} FW) measured in root tip extracts of 140 Ruggeri grapevines grown

at two levels of bicarbonate (+BIC and - BIC) under Fe-sufficient (+Fe) and Fe-deficient (–Fe) conditions. Data are means of nine replicates.

	PEPC	MDH	CS		$NADP^+$-IDH		Protein
Bicarbonate (BIC)			+Fe	-Fe	+Fe	-Fe	
+ BIC	20,2	188,2	5,5	9,4	13,2	18,3	30,5
- BIC	35,5	257,8	10,8	6,0	28,8	19,2	30,0
Significance	**	*					n.s.
Iron (Fe)							
+ Fe	21,0	237,4					33,9
- Fe	32,8	208,6					26,7
Significance	**	n.s.					*
B x Fe interaction	n.s.	n.s.	*		*		n.s.
SEM[2]			0,37		0,72		

[1] Abbreviation and symbols: n.s., *, ** = not significant and significant at p≤0.05 and p≤0.01 levels, respectively.
[2] SEM = standard error of the interaction means.

Table 3. Kinetic parameters *Km* (mM of $NaHCO_3^-$) and V_{max} (nmol mg^{-1} protein min^{-1}) of phosphoenolpyruvate carboxylase activity (PEPC) in extracts of grapevine 140 Ruggeri roots grown at two levels of bicarbonate (+BIC and - BIC) under Fe-sufficient (+Fe) and Fe-deficient (–Fe) conditions. Data are means of three replicates.

	Km	V_{max}
Bicarbonate (BIC)		
+ BIC	0,10	25,8
- BIC	0,08	33,7
Significance	n.s.	n.s.
Iron (Fe)		
+ Fe	0,11	18,9
- Fe	0,08	40,6
Significance	n.s.	**
B x Fe interaction	n.s.	n.s.

Abbreviation and symbols: n.s., ** = not significant and significant at p≤0.01 level.

Table 4. Effects of bicarbonate (+BIC and – BIC) and Fe supply (+Fe and –Fe) on organic acids concentration (mg g^{-1} FW) in roots samples. Data are means of nine replicates.

	Citrate		Tartrate		Malate	Ascorbate		Total	
Bicarbonate (BIC)	+ Fe	- Fe	+ Fe	- Fe		+Fe	-Fe	+Fe	-Fe
+ BIC	0,37	0,73	1,37	1,25	O,64	0,031	0,031	2,3	2,7
- BIC	0,22	0,91	0,93	1,61	0,42	0,038	0,018	1,6	3,0
Significance					*				
Iron (Fe)									
+ Fe					0,47				
- Fe					0,59				
Significance					n.s.				
B x Fe interaction	*		***		n.s.	*		*	
SEM[2]	0,065		0,072			0,004		0,21	

[1] Abbreviation and symbols: n.s., *, *** = not significant and significant at p≤0.05 and p≤0.001 levels, respectively.
[2] SEM = standard error of the interaction means.

Table 5. Effects of bicarbonate (+BIC and – BIC) and Fe supply (+Fe and –Fe) on the pH and organic acids concentration (μM) in xylem sap. Data are means of nine replicates.

	pH	Citrate	Malate	Ascorbate	Total
Bicarbonate (BIC)					
+ BIC	6,78	78,2	314,3	18,1	410,7
- BIC	6,26	108,5	294,7	18,4	421,6
Significance	*	*	n.s.	n.s.	n.s.
Iron (Fe)					
+ Fe	6,61	76,9	308,8	18,8	404,4
- Fe	6,42	106,2	302,2	17,9	426,2
Significance	n.s.	**	n.s.	n.s.	n.s.
B x Fe interaction	n.s.	n.s.	n.s.	n.s.	n.s.

Abbreviation and symbols: n.s., *, ** = not significant and significant at p≤0.05 and p≤0.01 levels, respectively.

CHAPTER IV

RESPONSE MECHANISMS OF GRAPEVINE ROOTSTOCKS TO SEVERE IRON DEFICIENCY

4.1. Introduction

Iron (Fe) is the fourth most abundant element in the Earth's crust and requirements of this essential element by woody crops are relatively low (eg. 50-100 mg Fe kg^{-1} SS) (Tagliavini and Rombolà, 2001). However, in many important viticultural areas of the Mediterranean basin, characterized by soils with high concentration of active lime and alkaline pH, the Fe availability for plants is extremely low due to its scarce solubility in the soil (Miller et al., 1984; Mengel, 1994). The typical Fe deficiency symptom is the intervenial leaf yellowing starting from apical leaves (Fe chlorosis), and the reduction of shoots and roots growth (Rombolà and Tagliavini, 2006). Moreover, this nutritional disorder can cause a dramatic reduction of vineyard economical life cycle as well as grape yield (Bavaresco et al., 2003). Increased soluble solids concentration in berries of Fe chlorotic vines have been reported (Bavaresco and Poni, 2003). Iron deficiency may also reduce the synthesis of some components of grape red color as anthocyanins, due the decreases of anthocyanidin synthase activity that requires Fe (De Carolis and De Luca, 1994).
Grapevine belongs to Strategy I plants, therefore under Fe-deficiency it is able to increase Fe reductase activity and enhance net excretion of protons and root organic compounds (eg. organic acids, phenols), lowering the pH and increasing the solubility of Fe(III) (Brancadoro et al., 1995; Ksouri et al., 2006; Jimenez et al., 2007). Iron chelates are usually effective but do not represent a sustainable approach to prevent or cure Fe-deficiency (Tagliavini and Rombolà, 2001). Soil applied Fe-chelates are expensive, require repeated applications and due to their high stability and solubility, increase the risk of leaching of metals and chelating agents in the deep soil layers and in the water table (Rombolà et al., 2002a; Rombolà e Tagliavini, 2006). Such consequences strongly suggest the need of

adopting alternative strategies for managing Fe nutrition according to soil and plant parameters. The use of tolerant rootstocks may represent an economical and efficient method for preventing Fe chlorosis. It is well known that the susceptibility degree to Fe chlorosis in grapevine is highly variable for different genotypes. *Vitis vinifera* and *Vitis berlandieri* species have been classified as highly tolerant and tolerant to Fe chlorosis respectively, whereas *Vitis riparia* is characterized by a high susceptibility to Fe chlorosis (Tagliavini and Rombolà, 2001; Ollat et a., 2003; Jimenez et al., 2007; Brancadoro et al., 1995). *Vitis rupestris* genotype has been classified as slightly susceptible to Fe chlorosis (Tagliavini and Rombolà, 2001). Studies focalized on response mechanisms to Fe-deficiency in grapevine rootstocks reported that genotypes from *Vitis vinifera* and *Vitis berlandieri* display an enhanced ability to reducing Fe at the root level by the FCR enzyme, and releasing protons into the rhizosphere under low external availability (Rombolà and Tagliavini, 2006; Jimenez et al., 2007; Dell'Orto et al., 2000; Brancadoro et al., 1995). In addition, in various species it has been demonstrated that tolerant genotypes may increased the activity of PEPC enzyme and the concentration of organic acids in roots (particularly citric acid) (Ollat et al., 2003; Rombolà et al., 2002b; Rombolà and Tagliavini, 2006; Jimenez et al., 2007). Therefore, these parameters have been used as biochemical markers for screening Fe chlorosis tolerant genotypes (Rombolà and Tagliavini, 2006). Under field conditions, plants often face prolonged periods of scarce Fe availability, which are successfully overcome without adverse effects on leaf chlorophyll content and yield. Nevertheless, most of the experiments on grapevine have studied biochemical response mechanisms to Fe-shortage during short periods, therefore, information concerning the behavior of grapevine genotypes under prolonged Fe-deficiency is scarce.

The main objective of the present work was to compare physiological and biochemical response mechanisms to a severe Fe-deficiency in *Vitis* genotypes with different tolerance degree to Fe-chlorosis. For this purpose, a nutrient solution experiment was conducted, in which three rootstocks characterized by a different susceptibility degree to Fe chlorosis have been submitted to two levels of Fe in the nutrient solution.

4.2. Materials and Methods

4.2.1. Vegetal material, growth conditions and treatments

The experiment was conducted in a greenhouse at the Agriculture Faculty of Bologna University. Micropropagated plants of rootstocks 101-14 (*Vitis riparia* x *Vitis rupestris*), 110R (*Vitis berlandieri* x *Vitis rupestris*) and SO4 (*Vitis berlandieri* x *Vitis riparia*) were acclimated in peat for 1 month and pruned maintaining one main shoot on each plant. The plants (36) were transferred to 10 l plastic containers covered with aluminum and filled with 8 l of a half Hoagland nutrient solution continuously aerated, with 6 plants for each container. The temperature of the greenhouse was 25-30°C with 70-75% relative humidity, and it was applied the external photoperiod (16 hours of light and 8 hours of darkness).
The three grapevine genotypes were treated with two levels of Fe in the nutrient solution. The treatments established were: 1) 101-14 rootstock grown with 10 µM of Fe-EDDHA (101-14 +Fe); 2) 110 Richter rootstock grown with 10 µM of Fe-EDDHA (110 Richter +Fe); 3) SO4 rootstock grown with 10 µM of Fe-EDDHA (SO4 +Fe); 4) 101-14 rootstock grown without Fe (101-14 -Fe); 5) 110 Richter grown without Fe (110 R –Fe); 6) SO4 rootstock grown without Fe (SO4 –Fe). The composition of the nutrient solution half Hoagland was: 2,5 mM KNO_3; 1 mM $MgSO_4$; 1 mM KH_2PO_4; 2,5 mM $Ca(NO_3)_2$; 4,6 µM $MnCl_2$; 23,2 µM H_3BO_3; 0,06 µM Na_2MoO_4; 0,4 µM $ZnSO_4$; 0,19 µM $CuSO_4$. The nutrient solution was renewal twice a week, the pH was monitored daily at 9:00 am and was adjusted to 6,0 after every renewal with HCl 0,1 M. Plants were grown in these conditions for 32 days.

4.2.2. Leaf chlorophyll content and plants growth

Leaf chlorophyll content was periodically monitored during the experiment on five points of the first completely expanded leaf with the portable chlorophyll meter SPAD MINOLTA 502 (Osaka, Japan). When apical leaves of -Fe plants showed extremely severe Fe-chlorosis symptoms (SPAD value < 6), the experiment was concluded and the plants were divided into roots, main shoot and leaves for dry weight determinations and following analysis.

4.2.3. Enzyme assays and protein concentration in roots

At the end of the experiment, root tip (20–30 mm long) samples were collected from each plant, rinsed in deionized water, weighed, deep-frozen in liquid nitrogen, and kept at –80°C for enzyme activity analysis. The activity of phosphoenolpyruvate carboxylase (PEPC), malate dehydrogenase (MDH), citrate synthase (CS), and isocitrate dehydrogenase ($NADP^+$-IDH) were determined. The extraction of enzymes was performed as described by Jimenez et al. (2007).
Phosphoenolpyruvate carboxylase was determined by coupling its activity to malate dehydrogenase- catalyzed NADH oxidation (Vance et al., 1983). MDH activity was determined by monitoring the increase in absorbance at 340 nm, due to the enzymatic reduction of NAD^+ (Smith, 1974). CS activity was assayed by monitoring the reduction of acetyl CoA to CoA with DTNB at 412 nm (Srere, 1967). $NADP^+$-IDH was assayed as described by Goldberg and Ellis (1974), by monitoring the reduction of $NADP^+$ at 340 nm. Protein concentration was determinate by the Bradford method, using BSA as standard (Bradford, 1976). Data obtained in enzyme assays were referred to protein concentration of roots.

4.2.4. Determination of kinetic properties of PEPCase

Kinetic analysis was performed in the standard buffer by varying each time the HCO_3^- concentration. The substrate dependence of PEPC to HCO_3^- concentration was characterized determining the PEPC activity with different concentrations of HCO_3^- in 9 points in a range from 0-10 mM. Decarbonated water was used for the determination of HCO_3^- kinetic. V_{max} and Km values were calculated using the Eadie-Hofstee plot.

4.2.5. Organic acids concentration in roots

The organic acids concentration in roots was determined according to Neumann (2006). Frozen samples of root tips collected at the end of the experiment were submerge in a pre-cooled (4 °C) mortar with liquid nitrogen. After evaporation, the tissue was grind and

homogenized with a pestle. For extraction and deproteinization, 5% H_3PO_4 was utilized. Organic acids were quantified by HPLC with 250 x 4 mm LiChrospher 5 um RP-18 column (SUPELCO INC., PA 16823-0048 USA). HPLC elution buffer was 18 mM KH_2PO_4, pH 2,1 adjusted with H_3PO_4. Samples were read at 210 nm for 40 minutes (Neumann, 2006).

4.2.6. Statistics

Data were analyzed by a two-way (Fe x Genotype) analysis of variance with SAS software (SAS Institute, Cary, NC). If significant interactions between factors were obtained by the F-test, it was calculate the standard error of the interaction means (SEM), and in this case, treatments are significantly different when the difference between data is higher than 2 x SEM. Significant differences between factor levels were detected by the F-test ($p \leq 0,05$), and in this case, statistical comparisons are between levels of each factor.

4.3. Results

4.3.1. Chlorophyll content and plants growth

During the first period of the experiment (until 14 days from treatments imposition), Fe-deficiency decreased the leaf chlorophyll content regardless to the genotype (Tab. 1). In the following period, an interaction between factors was detected. Iron-deficiency strongly decreased the chlorophyll content in 101-14 rootstock. The rootstock 110 Richter was less affected by Fe-deficiency (Tab. 1). Iron deficiency reduced the chlorophyll content in SO4 genotype with an intermediate intensity (Tab. 1).
Shoot length of vines was influenced by Fe and genotype during the experiment (Tab. 2). During the first period of the experiment (until 21 days from treatments imposition), genotypes displayed differences in the length of the main shoot independently of Fe level. Plants of 101-14 showed a higher shoot length as compared with the others rootstocks (Tab. 2). Starting from 14 days after treatments imposition, Fe-deficiency significantly decreased the length of shoots (Tab. 2). At the end of the experiment data showed an interaction

between factors. Iron-deficiency decreased shoot length by -80% in 101-14 rootstock, by -71% in SO4 and by -51% in 110 Richter.

Data of organs biomass recorded at the end of the experiment showed interactions between genotype and Fe level (Tab. 3). Iron deficiency decreased the biomass of roots, shoots, leaves and total weight of plants. The highest decrease has been recorded in 101-14 genotype, and the lowest decreases have been recorded in 110 Richter (Tab. 3). For SO4 rootstock, the effect of Fe-deficiency on dry biomass was intermediate (Tab. 3).

4.3.2. pH in the nutrient solution

After 3 days from the renewal of the nutrient solution, roots of –Fe plants decreased the pH of the nutrient solution (Fig. 1). The highest pH difference was measured for 101-14 rootstock (Fig. 1). In case of 110 Richter and SO4, smaller differences between +Fe and –Fe plants were recorded.

4.3.3. Enzyme activities and protein concentration in root extracts

At the end of the experiment, the activity of PEPC and some key enzymes of Krebs cycle have been determined in the root tip extracts. In roots of 101-14 and SO4 genotypes, Fe-deficiency decreased the PEPC activity by -68% and -81% respectively (Tab. 4), whereas Fe-deficiency did not change PEPC activity in plants of 110 Richter (Tab. 4). Iron deficiency induced a decrease in the root activity of MDH in 101-14 (-36%) and SO4 (-46%) genotypes (Tab. 4). The root activity of $NADP^+$-IDH has been different for rootstocks, regardless to Fe level (Tab. 4). CS activity and protein concentration in roots were not influenced by treatments (Tab. 4).

4.3.4. Kinetic properties of PEPCase

The saturation kinetics curves of PEPCase were established by addition of different concentrations of bicarbonate -in a range of 0 to 10 mM- to the buffer assay. In roots of 101-14 and SO4 genotypes, Fe-deficiency decreased the V_{max} of PEPCase activity by -46%

and -62% respectively than Fe-sufficient plants (Tab 5). In contrast, Fe-deficiency did not modify the V_{max} in roots of 110 Richter (Tab. 5). *Km* was not altered by treatments (Tab. 5).

4.3.5. Organic acids concentration in roots

At the end of the experiment, the major organic acids present in root extracts were malic, followed by citric and ascorbic acids (Tab. 6). Significant interactions between Fe level and genotype were recorded. Iron deficiency increased citric acid concentration in roots of the three genotypes. The highest increase has been recorded in 101-14 genotype (27-fold) (Tab. 6). In roots of 101-14 genotype, Fe-deficiency induced an increase of malic acid concentration by 54%. In contrast, in 110 Richter and SO4 genotypes Fe-deficiency decreased the concentration of malic acid by -35% and -27% respectively (Tab. 6). Iron status and genotype did not influence ascorbic acid levels in roots (Tab. 6). Iron deficiency enhanced by 2,7-fold total organic acids concentration in roots of 101-14 genotype, whereas, similar values were recorded in 110 Richter and SO4 rootstocks (Tab. 6).

4.4. Discussion

Until 14 days from treatments imposition, a negative effect of Fe-deficiency on leaf chlorophyll content was recorded in the three genotypes (Tab. 1). During the following period, an interaction between Fe level and genotype has been found (Tab. 1). At the end of the experiment, Fe-deficiency decreased the chlorophyll content of genotypes by -99,6% in 101-14, -92% in SO4 and -72% in 110 Richter. During the period between 14 and 21 days of treatment, Fe-shortage reduced the shoot length of plants regardless to the genotype (Tab. 2). After this period, the adverse effect of the prolonged Fe-deficiency on main shoot length changed according to the genotype. At the end of the experiment, Fe-deficiency decreased the shoot length in 101-14 by -80%, in SO4 by -71% and in 110 Richter by -51% (Tab. 2). The total biomass of organs has been reduced by Fe deprivation by -78% in 101-14, -62% in SO4 and -48% in 110 Richter genotypes (Tab. 3).

Data on leaf chlorophyll content and plant growth clearly indicate that short period of Fe-depletion (14-21 days) did not discriminate among genotypes. However, when plants were submitted to a prolonged Fe-deficiency (32 days), 110 Richter rootstock exhibited the lowest reduction of chlorophyll content and biomass as compared to control, suggesting the highest tolerance to severe Fe-deficiency. In contrast, a dramatic reduction of chlorophyll content and biomass has been observed in 101-14 genotype. The rootstock SO4 exhibited an intermediate behavior.

The degree of Fe chlorosis severity showed genotypes is in line with tolerance levels reported in literature (Tagliavini and Rombolà et al., 2001). 110 Richter rootstock is an hybrid between the Fe chlorosis tolerant species *Vitis berlandieri* and the Fe chlorosis slightly susceptible species *Vitis rupestris*. In contrast, 101-14 rootstock was originated from *Vitis rupestris* and the Fe-chlorosis highly susceptible species *Vitis riparia* (Tagliavini and Rombolà, 2001; Ollat et a., 2003; Jimenez et al., 2007; Brancadoro et al., 1995). The tolerance level of the originating species may also explain the intermediate Fe chlorosis symptoms exhibited by SO4 genotype, an hybrid from *Vitis berlandieri x Vitis riparia.*

The biomass yield data indicate a significant higher growth rate in Fe-sufficient 101-14 as compared to the other genotypes, and this effect exacerbated the differences between +Fe and -Fe for 101-14 genotype. This result may be due to a relatively higher ability of 101-14 Fe-sufficient plants to take advantage from the nitrate availability in the nutrient solution. Nitrate reductase -a key enzyme for nitrogen assimilation- contains an Fe ion on each subunit (Campbell, 1999; Rouze and Caboche, 1992). In addition, the nitrate-reducing capacity of a plant system depends in part from Fe availability (Campbell, 1999). In this experiment, the presence of Fe in the nutrient solution possibly promoted the assimilation of nitrogen, stimulating the growth rate in 101-14 rootstock. This effect may induce modifications in the rate of nitrate uptake of 101-14 rootstock, and consequently in the pH of the nutrient solution. Noteworthy, 101-14 showed higher differences in pH the nutrient solution between +Fe and -Fe levels (pH 7,0 and 6,3 in +Fe and –Fe respectively) (Fig. 1), as a result of the higher plant biomass that promoted the uptake of some anions increasing the pH in the nutrient solution. Nitrate uptake is associated with the proton consumptions via $2H^+/NO_3^-$ symport, leading to an increase in the pH of the outer solution (Mengel and Kirkby, 2001). In view of the above exposed effect, the higher differences detected in the

pH of the nutrient solution of 101-14 genotype are due to an interaction of Fe and nitrate uptake rather than a intrinsic capacity of this Fe chlorosis sensitive rootstock to enhancing net excretion of protons and root organic compounds for lowering the pH under Fe-deficiency conditions. In fact, this hypothesis is supported by the high degree of Fe-deficiency chlorosis symptoms generally exhibited in the field by 101-14 genotype.
The interactions obtained in PEPC and MDH enzymes activity in roots indicate differences in the response to a prolonged Fe-deficiency between genotypes. The severe Fe-deficiency did not modify the activity of PEPC and MDH in 110 Richter rootstock, whereas in 101-14 and SO4 genotypes Fe-deficiency decreased these enzymes activities in roots (Tab. 4). In addition, Fe-deficiency did not modified the V_{max} of PEPC in 110 Richter roots, whereas 101-14 and SO4 rootstocks submitted to Fe-depletion showed a decreases in by about -46% and -62% respectively than Fe-sufficient plants (Tab. 5). $NADP^+$-IDH and CS enzyme activities in roots did not change according to Fe-deficiency, whereas $NADP^+$-IDH showed differences between genotypes (Tab. 4). The enzyme PEPC catalyzes the incorporation of bicarbonate into a C_3 organic acid, phosphoenolpyruvate (PEP), generating oxalacetate, which is converted to malate by malate dehydrogenase (Lance and Rustin 1984). This process is an important component of the pH-stat mechanism (Davies 1973). Increases on PEPC activity in roots has been reported in many model plants submitted to short period Fe-shortage and is a typical biochemical response mechanism to Fe-deficiency. In roots of *Cucumis sativus* it has been observed that Fe-deficiency induced a stimulus in the V_{max} of PEPC, without influencing the *Km* of the enzyme, indicating that probably Fe-deficiency increases the concentration of the enzyme in roots (De Nisi and Zocchi, 2000). In grapevine, Jimenez et al. (2007) reported an increase in roots PEPCase activity in the Fe-chlorosis tolerant genotype Cabernet Sauvignon submitted to a short period of Fe-depletion (7 days), whereas a lower increase has been observed in the sensitive cv Gloire de Montpellier. Data show a reduction in the activity of PEPC and MDH enzymes in roots of 101-14 and SO4 rootstocks submitted to 32 days of Fe-shortage, revealing a high susceptibility to a prolonged Fe-deficiency in the substrate. In contrast, Fe-deficiency did not cause any reduction of PEPC and MDH enzymes activity, suggesting a relatively higher tolerance of 110 Richter to prolonged Fe-deficiency. These results suggest that the duration and severity of Fe-deficiency stress can modulate the intensity of some biochemical

responses at root level. For example, a decrease in the activity of some root enzymes as a response to a prolonged Fe-deficiency (50 days) has been observed only in Fe chlorosis susceptible rootstocks of quince and pear species (Tagliavini et al., 1995).

The strong decreases in leaf chlorophyll content recorded in 101-14 and SO4 genotypes submitted to a prolonged Fe-deficiency (Tab. 1), influenced the root activity of PEPC and TCA enzymes. In a metabolically active tissue, the elevated requirement of glucose is in part maintained by translocation of photosynthate from the shoot (Zocchi, 2006). Without sufficient chlorophyll, leaves are unable to produce enough photosynthates (Miller et al. 1984), and a lower amount of glucose may be available for root metabolism. In Fe-deficient chlorotic leaves of *Beta vulgaris*, an increase of glucose-6-phosphate dehydrogenase (G6PDH) - a cytosolic enzyme in the pentose phosphate pathway- has been observed after 10 days of Fe-depletion (Lopez-Millán et al., 2001). The decreases in the activity of PEPC and consequently of MDH enzymes in Fe-deficient roots of 101-14 and SO4 genotypes, possibly derived from a limited synthesis of phosphoenolpyruvate (PEP) -the substrate of PEPC- provided from glycolysis, due to a strong reduction of glucose/photosynthetates production in leaves caused by a extremely severe Fe-deficiency.

At the end of the experiment, significant interactions between Fe level and genotype were recorded in citric and malic acids concentration in roots. Iron deficiency increased citric acid concentration in roots of three genotypes. A highly pronounced accumulation has been recorded in 101-14 genotype (27-fold), followed by 110 Richter and SO4 (5-fold and 2-fold respectively) (Tab. 6). Prolonged Fe-deficiency increased the concentration of malic acid in roots of 101-14 rootstock, whereas for 110 Richter and SO4 the malic acid concentration in roots did not change according to Fe level. The strong accumulation of citric acid and, in lower intensity, of malic acid recorded in roots of 101-14 –Fe vines contrasts with the low activity of PEPC and MDH enzymes. Some authors have verified the inhibition of PEPC enzyme by L-malate (Wong and Davies, 1973; Chollet, et al., 1996; López-Millán et al., 2000). In addition, it has been reported the inhibitor-effect of citric acid on the activity of PEPC (Wong and Davies, 1973). Consequently, it is possible that the high accumulation of organic acids in roots submitted to a prolonged Fe-deficiency in 101-14 genotype, contributed to slowdown the activity of PEPC and MDH due to an inhibitory effect caused by these acids. In addition, the strong accumulation of malic and particularly of citric acids,

recorded in 101-14 -Fe roots, suggests a scarce ability of this rootstock to detoxify the organic acid excess by degradation, xylem loading or exudation into the rhizosphere. In 110 Richter rootstock, the increases of citric acid concentration (Tab. 6) and the activity of PEPC and MDH enzymes (Tab. 4) in roots of Fe-deficient plants indicate that the organic acids metabolism was still active after a prolonged Fe-shortage. The moderate accumulation of citric acid and tendentially lower level of malate induced by Fe-deficiency in roots of 110 Richter indicate a marked capability to avoid an excessive accumulation of organic acids in roots. Regarding SO4 genotype, a different behavior has been found. Similary to 101-14 genptype, lower V_{max} of PEPC and MDH activity were recorded in SO4 Fe-deficient plants, clearly indicating a slowing down of the organic acids metabolism. In contrast, organic acids data suggest a high capability of SO4 rootstock to avoid high levels of organic acids in roots. These results indicate that SO4 submitted to a severe Fe-deficiency behaves as 110 Richter for certain tolerance responses to Fe chlorosis (avoiding a high accumulation of organic acids in roots), and simultaneously behaves as 101-14 displaying susceptibility responses to Fe-depletion (slowdown the activity of PEPC and MDH activity in roots). These physiological observations are in line with the intermediate level of Fe-deficiency symptoms exhibited in leaf chlorophyll content and plants biomass production as compared with 110 Richter and 101-14 genotypes. Additional physiological response mechanisms to a prolonged Fe-deficiency related to the reduction capacity of roots and exudation of organic compounds for different genotypes could contribute to explaining the different Fe chlorosis tolerance of these grapevine rootstocks.

4.5. Conclusions

This work investigated physiological responses to a prolonged Fe-deficiency stress in three grapevine rootstocks. The severity of Fe-deficiency chlorosis symptoms observed in the experiment is in line with the general behavior exhibited by these genotypes under field conditions. The Fe chlorosis susceptible rootstock 101-14 (*Vitis riparia x Vitis rupestris*) reacts to a prolonged Fe-deficiency reducing the activity of PEPC and MDH in roots. Noteworthy, it accumulates high levels of citric acid at root level, indicating a low capacity

to utilizing, transporting and/or exudating organic acids in the rhizosfere. In contrast, 110 Richter rootstock (*Vitis berlandieri x Vitis rupestris*) is capable to maintain an active metabolism of organic acids in roots, accumulating them to a lesser extent than 101-14. Similarly to 101-14, SO4 genotype displays a strong decrease of mechanisms associated to Fe chlorosis tolerance (PEPC and MDH enzyme activities). Nevertheless it is able to avoid excessive accumulation of citric acid at root level, similar as 110 Richter rootstock. This behavior reflects an intermediate response to Fe-deficiency of this hybrid (*Vitis berlandieri x Vitis riparia*). Overall, data show that root PEPC and MDH enzymes activities represent an important tool for screening Fe chlorosis tolerance between genotypes. However, the high levels of organic acid accumulation recorded in 101-14 and SO4 genotypes after a prolonged exposure to Fe-deficiency suggest caution when using this parameter for screening Fe chlorosis tolerance, because this trait may be a symptom of the scarce capability of roots to utilizing, transporting or exudating these compounds, rather than a reliable indicator of Fe chlorosis tolerance.

4.6. Literature cited

Bavaresco, L., E. Giachino, and S. Pezzutto. 2003. Grapevine rootstock effects on lime-induced chlorosis, nutrient uptake, and source-sink relationships. Journal of Plant nutrition, 26: 1451-1465.

Bavaresco, L. and Poni, S. 2003. Effect of calcareous soil on photosynthesis rate, mineral nutrition, and source-sink ratio of table grape. Journal of Plant Nutrition, 26 (10&11): 2123-2135.

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72: 248-254.

Brancadoro, L., Rabotti, G., Scienza, A. and Zocchi, G. 1995. Mechanisms of Fe-efficiency in roots of *Vitis* spp. in response to iron deficiency stress. Plant and Soil, 171: 229-234.

Campbell, W.H. 1999. Nitrate reductase structure, function and regulation: bridging the gap between biochemistry and physiology. Annual Review of Plant Physiology and Plant Molecular Biology, 50: 277-303.

Chollet, R., Vidal, J. and O'Leary, M.H. 1996. Phosphoenolpyruvate carboxylase: a ubiquitous, highly regulated enzyme in plants. Annual Review of Plant Physiology and Plant Molecular Biology, 47: 273–298.

Davies, D.D. 1973. Control of and by pH. Symposium of the Society for Experimental Biology, 27: 513–529.

De Carolis, E. and De Luca, V. 1994. 2-Oxoglutarate-dependent dioxygenase and related enzymes: Biochemical characterization. Phytochemistry, 36: 1093–1107.

Dell'Orto, M., Brancadoro, L., Scienza, A. and Zocchi, G. 2000. Use of biochemical parameters to select grapevine genotypes resistant to iron-chlorosis. Journal of Plant Nutrition, 23(11&12): 1767-1775.

De Nisi, P. and Zocchi, G. 2000. Phosphoenolpyruvate carboxylase in cucumber (Cucumis sativus L.) roots under iron deficiency: activity and kinetic characterization. Journal of Experimental Botany, 51(352): 1903-1909.

Goldberg, D.M. and Ellis, G. 1974. Isocitrate dehydrogenase. *In*: Bergmeyer HU (ed) Methods of enzymatic analysis. Verlag Chemie/Academic Press, New York, EEUU, pp 183–189.

Jimenez, S., Gogorcena, Y., Hévin, C., Rombolà, A.D., and Ollat, N. 2007. Nitrogen nutrition influences some biochemical responses to iron deficiency in tolerant and sensitive genotypes of Vitis. Plant and Soil, 290: 343-355.

Ksouri, R., M'rah, S., Gharsalli, M. and Lachaâl, M. 2006. Biochemical responses to true and bicarbonate-induced iron deficiency in grapevine genotypes. Journal of Plant Nutrition, 29: 305–315.

López-Millán, A.F., Morales, F., Abadía, A. and Abadía, J. 2001. Changes induced by Fe deficiency and Fe resupply in the organic acid metabolism of sugar beet (*Beta vulgaris*) leaves. Physiologia Plantarum, 112: 31–38.

Neumann, G. 2006. Root exudates and organic composition of plant roots. In: Luster J, Finlay R (eds) Handbook of methods used in rhizosphere research. Swiss Federal Research Institute WSL, Birmensdorf, 536 pp.

Mengel, K. 1994. Iron availability in plants tissues - Iron chlorosis on calcareous soils. Plant and Soil, 165: 275–283.

Mengel, K. and Kirkby, E.A. 2001. Principles of plant nutrition. Kluwer Academic Publishers, 5th Ed., Dordrecht, The Netherlands, 833 pp.

Miller, G.W., Pushnik, J.C. and Welkie, G.W. 1984. Iron chlorosis, a world wide problem: The relation of chlorophyll biosynthesis to iron. Journal of Plant Nutrition, 7: 1–22.

Ollat, N., Laborde, B., Neveux, M., Diakou-Verdin, P., Renaud, C. and Moing, A. 2003. Organic acid metabolism in roots of various grapevine (*Vitis*) rootstocks submitted to iron deficiency and bicarbonate nutrition. Journal of Plant Nutrition, 26(10&11): 2165–2176.

Rombolà, A.D. and Tagliavini, M. 2006. Iron nutrition of fruit tree crops. *In* Iron Nutrition in Plants and Rhizospheric Microorganisms (Eds, J, Abadía and L, Barton), Springer, Berlin, Germany, pp. 61-83.

Rombolà, A.D., Cremonini, M.A., Lucchi, A., Sorrenti, G., Placucci, G., Marangoni, B. 2002a. Leaching of soil-applied synthetic Fe chelates (Fe-EDDHA) in orchard ecosystem. *In:* Abstracts XI International Symposium on Iron Nutrition and Interaction in Plants, Udine, Italy.

Rombolà, A.D., Brüggemann, W., López-Millán, A.F., Tagliavini, M., Abadía, J., Marangoni, B. and Moog, P.R. 2002b. Biochemical responses to iron deficiency in kiwifruit (*Actinidia deliciosa*). Tree Physiology, 22: 869–875.

Rouze, P. and Caboche, M. 1992. Nitrate reduction in higher plants: molecular approaches to function and regulation. *In* Inducible Plant Proteins. JL Wray (Ed). Cambridge, MA: Cambridge University Press. pp. 45-77.

Smith, F. 1974. Malate dehydrogenase. *In*: Bergmeyer HU (Ed) Methods of enzymatic analysis. Verlag Chemie/Academic Press, New York, EEUU, pp 163–175.

Srere, P.A. 1967. Citrate synthase. In: Colowick SP, Kaplan NO (eds) Methods in enzymology. Academic Press, New York, pp 3–11.

Tagliavini, M. and Rombolà, A.D. 2001. Iron deficiency and chlorosis in orchard and vineyard ecosystems. European Journal of Agronomy, 15: 71-92.

Tagliavini, M., Rombolà, A.D. and Marangoni, B. 1995. Response to Fe-deficiency stress of pear and quince genotypes. Journal of Plant Nutrition, 18(11): 2465-2482.

Vance, C.P., Stade, S., Maxwell, C.A. 1983. Alfalfa root nodule carbon dioxide fixation. I: association with nitrogen fixation and incorporation into amino acids. Plant Physiology, 72: 469–473.

Wong, K.F. and Davies D.D. Regulation of Phosphoenolpyruvate Carboxylase of *Zea mays* by Metabolites. Journal of Biochemistry, 131: 451-458.

Zocchi, G. 2006. Metabolic changes in iron-stressed dicotyledonous plants. *In* Iron Nutrition in Plants and Rhizospheric Microorganisms (Eds, J, Abadía and L, Barton), Springer, Berlin, Germany, pp. 359-370.

4.7. Figures and tables

Table 1. Time course of chlorophyll content (SPAD index) determined during the experiment in the first expanded apical leaf for three grapevine genotypes (101-14; 110 Richter; SO4) grown in a nutrient solution containing 0 µM and 10 µM of Fe-EDDHA. Data are means of six replicates.

	Days of treatment										
	7	11	14	18		21		26		29	
				+ Fe	- Fe	+ Fe	- Fe	+ Fe	- Fe	+ Fe	- Fe
Genotype (G)											
101-14	14,6	16,8	16,9	23,9	6,5	24,8	0,9	25,1	0,4	26,3	0,1
110 Richter	14,9	16,3	16,3	19,1	11,6	21,2	7,6	22,4	6,5	21,9	6,1
SO4	15,9	17,6	15,7	22,0	4,5	22,7	3,0	22,6	1,8	21,3	1,7
Significance	n.s.	n.s.	n.s.								
Iron (Fe)											
+ Fe	16,8	19,1	19,5								
- Fe	13,5	14,7	13,0								
Significance	**	**	***								
G x Fe interaction	n.s.	n.s.	n.s.	***		***		*		**	
SEM				1,14		1,02		1,60		1,55	

[1]Abbreviation and symbols: ns, *, **, *** = not significant and significant at p≤0.05, p≤0.01 and p≤0.001 levels, respectively.
[2] SEM = standard error of the interaction means.

Table 2. Time course of the length of the main shoot (cm $plant^{-1}$) determined during the experiment for three grapevine genotypes (101-14; 110 Richter; SO4) grown in a nutrient solution containing 0 µM and 10 µM of Fe-EDDHA. Data are means of six replicates.

	Days of treatment								
	7	11	14	18	21	26		29	
						+ Fe	- Fe	+ Fe	- Fe
Genotype (G)									
101-14	9,9 a	11,8 a	13,9 a	20,3 a	22,7 a	54,8	13,5	67,5	13,8
110 Richter	7,9 b	9,1 b	10,5 b	13,0 b	15,2 b	24,5	13,6	29,4	14,3
SO4	6,8 c	8,2 b	9,6 b	11,8 b	14,3 b	30,6	10,7	38,1	10,9
Significance	***	***	***	*	**				
Iron (Fe)									
+ Fe	8,2	10,1	12,4	18,9	23,0				
- Fe	8,2	9,4	10,2	11,2	11,8				
Significance	n.s.	n.s.	*	**	***				
G x Fe interaction	n.s.	n.s.	n.s.	n.s.	n.s.	**		**	
SEM						4,63		5,80	

[1] Abbreviation and symbols: ns, *, **, *** = not significant and significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ levels, respectively. Means followed by the same letter in each column were not significantly different as per the SNK test.
[2] SEM = standard error of the interaction means.

Table 3. Biomass yield of organs (g plant^{-1} DW) determined at the end of experiment for three grapevine genotypes (101-14; 110 Richter; SO4) grown in a nutrient solution containing 0 µM and 10 µM of Fe-EDDHA. Data are means of six replicates.

	Roots		Shoot		Leaves		Total	
	+ Fe	- Fe	+ Fe	- Fe	+ Fe	- Fe	+ Fe	- Fe
Genotype (G)								
101-14	0,98	0,37	1,78	0,20	2,03	0,46	4,8	1,0
110 Richter	0,44	0,31	0,30	0,09	0,45	0,21	1,2	0,6
SO4	0,50	0,34	0,65	0,16	0,92	0,30	2,1	0,8
Significance								
Iron (Fe)								
+ Fe								
- Fe								
Significance								
G x Fe interaction	**		***		**		**	
SEM	0,08		0,14		0,16		0,37	

[1] Abbreviation and symbols: **, *** = significant at p≤0.01 and p≤0.001 levels, respectively.
[2] SEM = standard error of the interaction means.

Figure 1. Changes in the pH of the medium (10-l containers with 6 plants in each) during the period between 21 and 24 days of experiment for three grapevine genotypes (101-14; 110 Richter; SO4) grown in a nutrient solution containing 0 µM and 10 µM of Fe-EDDHA. The nutrient solution was not renewed during thus pH monitoring.

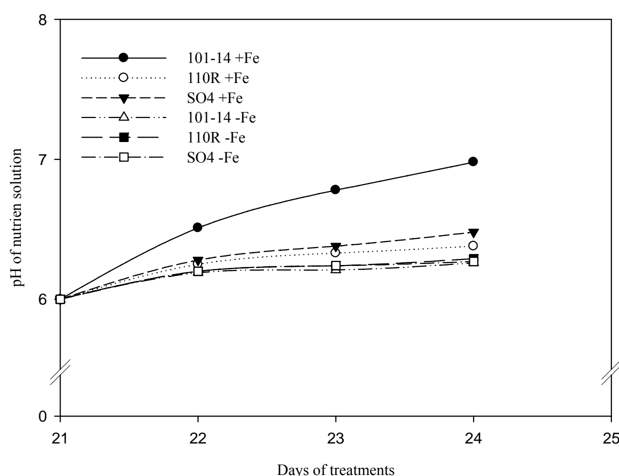


Table 4. Activities (nmol mg^{-1} protein min^{-1}) of PEPC, MDH, CS, $NADP^+$-IDH and protein concentration (mg g^{-1} FW) measured in root tip extracts for three grapevine genotypes (101-14; 110 Richter; SO4) grown in a nutrient solution containing 0 µM and 10 µM of Fe-EDDHA. Data are means of six replicates.

	PEPC		MDH		CS	$NADP^+$-IDH	Protein
Genotype (G)	+Fe	-Fe	+Fe	-Fe			
101-14	10,6	3,4	367,4	233,7	7,2	4,0 a	40,0
110 Richter	3,0	3,5	252,6	283,5	6,1	0,7 c	37,3
SO4	9,8	1,9	373,2	199,7	6,9	1,7 b	38,0
Significance	**		*		n.s.	***	n.s.
Iron (Fe)							
+ Fe					6,7	1,9	38,5
- Fe					6,7	2,4	38,4
Significance					n.s.	n.s.	n.s.
B x Fe interaction	*		**		n.s.	n.s.	n.s.
SEM[2]	1,15		37,5				

[1] Abbreviation and symbols: ns, *, ** = not significant and significant at $p \leq 0.05$ and $p \leq 0.01$ levels, respectively. Means followed by the same letter in each column were not significantly different as per the SNK test.

[2] SEM = standard error of the interaction means.

Table 5. Kinetic parameters *Km* (mM of $NaHCO_3^-$) and V_{max} (nmol mg^{-1} protein min^{-1}) of phosphoenolpyruvate carboxylase activity (PEPC) in extracts for three grapevine genotypes (101-14; 110 Richter; SO4) grown in a nutrient solution containing 0 µM and 10 µM of Fe-EDDHA. Data are means of three replicates.

	Km	V_{max}	
		+Fe	-Fe
Genotype (G)			
101-14	0,03	8,1	4,4
110 Richter	0,05	3,5	3,7
SO4	0,05	8,7	3,3
Significance	n.s.		
Iron (Fe)			
+ Fe	0,04		
- Fe	0,04		
Significance	n.s.		
B x Fe interaction	n.s.	*	
SEM		0,98	

Abbreviation and symbols: n.s., * = not significant and significant at p≤0.05 level.

Table 6. Organic acids in root tissue (mg g-1 FW) determined at the end of experiment for three grapevine genotypes (101-14; 110 Richter; SO4) grown in a nutrient solution containing 0 µM and 10 µM of Fe-EDDHA. Data are means of six replicates.

	Citrate		Malate		Ascorbate	Total	
	+ Fe	- Fe	+ Fe	- Fe		+ Fe	- Fe
Genotype (G)							
101-14	0,03	0,81	0,61	0,94	0,0067	0,65	1,76
110 Richter	0,07	0,35	0,60	0,39	0,0012	0,68	0,73
SO4	0,21	0,47	0,60	0,44	0,0004	0,81	0,91
Significance					n.s.		
Iron (Fe)							
+ Fe					0,003		
- Fe					0,003		
Significance					n.s.		
G x Fe interaction	***		*		n.s.	***	
SEM	0,045		0,110			0,114	

[1] Abbreviation and symbols: *, *** = significant at p≤0.05 and p≤0.001 levels, respectively.
[2] SEM = standard error of the interaction means.

CHAPTER V

EFFECTIVENESS AND PHYSIOLOGICAL IMPLICATIONS OF SUSTAINABLE MANAGEMENT TECHNIQUES FOR PREVENTING IRON CHLOROSIS IN GRAPEVINE

5.1. Introduction

The concepts of healthiness of agricultural products and sustainability of the production processes represent two main pillars for the development of modern viticulture. This implies that yield- and quality-related aspects should be environmentally-friendly and satisfactory profitable for growers. Iron (Fe) chlorosis is on of the main nutritional deficiency of woody plants, including grape (Tagliavini and Rombolà, 2001). It can cause a dramatic reduction of orchard economical life cycle as well as fruit yield and quality losses (Rombolà and Tagliavini, 2006). Grapevine is a Strategy I plant, typical of dicots and non graminaceuos monocots, therefore, under low external Fe availability it is able to reduce Fe at root level by ferric chelate reductse (FCR) enzyme and releasing protons into the rhizosphere (Rombolà and Tagliavini, 2006; Jimenez et al., 2007; Dell'Orto et al., 2000; Brancadoro et al., 1995). In addition, tolerant genotypes of grapevine and other species may increase the activity of phosphoenolpyruvate carboxylase (PEPC) enzyme for accumulating organic acids in roots (particularly citric acid) (Ollat et al., 2003; Rombolà et al., 2002a; Rombolà and Tagliavini, 2006; Jimenez et al., 2007). Therefore, the activity of FCR and PEPC and organic acids accumulation in roots have been used as biochemical markers for screening tolerant genotypes in several species (Abadía et al., 2002; Brancadoro et al., 1995; Gogorcena et al., 2004; Rombolà et al., 2002a).
The photosynthetic apparatus of plants represents one of the most Fe-enriched cellular systems (Spiller and Terry, 1980). Iron deficiency-mediated reductions in photosynthesis have been reported for many plant species (Terry and Abadía, 1986; Abadía, 1992; Morales

et al. 2000; Larbi et al., 2006). In Fe chlorotic leaves, reductions in photosynthesis have been attributed to a decrease in Photosystem II efficiency due to closure of Photosystem II reaction centers and decreases of the intrinsic Photosystem II efficiency (Morales et al., 2000). In addition, light absorption and Rubisco carboxylation efficiencies were down-regulated in response to Fe deficiency (Larbi et al., 2006). However, less information is available on the extent to which Fe chlorosis reduces photosynthesis under field conditions, in which photosynthetic photon flux density (PPFD) fluctuations are one of the main causes of the variation in leaf photosynthetic rates and photosynthetic leaf performance (Larbi et el., 2006).

The prevention/cure of Fe chlorosis with Fe-chelates is a very effective and widespread agronomical practice in vineyards and orchards. Nevertheless Fe-chelates are expensive, require repeated applications and due to their high stability and solubility increase the risk of leaching of metals and chelating agents in the deep soil layers and in the water table (Rombolà et al., 2002b; Rombolà e Tagliavini, 2006). Such environmental consequences strongly suggest the adoption of alternative strategies for managing iron nutrition in vineyards according to soil and plants parameters able to improve the efficiency of Fe acquisition (Tagliavini et al., 2000).

Graminaceus species are able to release into the rizosphere high affinity Fe(III) chelating compounds (phytosiderophores), solubilizing Fe and taking it up by roots as intact Fe-phytosiderophore complexes (Ma and Nomoto, 1996). It has been shown that intercropping of kiwifruit and citrus with graminaceous species prevents Fe chlorosis in co-cultivated plant as effectively as synthetic Fe chelates (Rombolà et al., 2003; Ammari and Rombolà 2010), due to their abilities of solubilizing soil Fe through phytosiderophore secretion (Cesco and Rombolà, 2007; Cesco et al., 2006; Ma et al., 2003; Ueno et al., 2007). Iron chlorosis prevention on intercropped plants depends on the effectiveness of graminaceous species used to release phytosiderophores (Ammari and Rombolà 2010). It has been reported that phytosiderophores secretion in *Festuca rubra* -an efficient species for phytosiderophores secretion- is characterized by a daily rhythm and is influenced by temperature at root level (Ma et al. 2003). Recent studies have shown that the presence of graminaceous species may stimulate, in co-cultivated species, the expression of *AhFRO1* and *AhIRT1* genes encoding proteins responsible for Fe absorption and such changes have

been mainly attributes to soil Fe level, cultivation conditions and phenological phase (Ding et al., 2009; Ding et al., 2010). The above mentioned evidences indicate that vineyards adopting intercropping with grasses properly selected may represent a sustainable tool for preventing Fe chlorosis avoiding or reducing Fe-chelates.

Most plants can take up of both ammonium (NH_4^+) and/or nitrate (NO_3^-) ions for metabolic functions. The take up of these two nitrogen (N) forms is controlled by genotype, plant development, physiological status, and by soil properties such as texture, structure, water content and pH (Loulakakis and Roubelakis-Angelakis 2001). In calcareous soils, NO_3^- is the prevalent form of inorganic nitrogen (N) in the soil solution, and NH_4^+ is rapidly nitrified. Nitrate uptake is normally mediated by two-proton co-transport, which can increase the pH in the rhizosphere or neutralize the protons released by roots to increase Fe solubility in the soil or in the apoplast (Kosegarten et al., 2004; Nikolic and Römheld, 2002). In this sense, NO_3^- behaves similarly to bicarbonate or some biological buffers, preventing the presence of acid microsites in the rizosphere (Lucena, 2000). Consequently, the presence of NO_3^- in the soil contributes to induce Fe-deficiency chlorosis in plants cultivated in lime soils (Mengel et al., 1994). In contrast, NH_4^+ uptake induce an acidification of the rhizosphere, due to the excretion of protons via the H^+-ATPase, favoring the reduction of Fe(III). In grapevine, positive effects of NH_4^+ supply in the nutrient solution for Fe chlorosis prevention have been reported (Jimenez et al., 2007). In order to improve the effectiveness of NH_4^+ to prevent/cure Fe chlorosis in plants, it is important to develop effective strategies for maintaining medium-low concentration of NH_4^+ in the soil, slowing down the oxidation of NH_4^+ to NO_3^- (nitrification process). In this context, the employment of nitrification inhibitors applied to the soil could be an interesting approach for optimizing N and Fe nutrition simultaneously. Some authors have been reported the effectiveness of 3,4-Dimethylpyrazole phosphate (DMPP) as nitrification inhibitor (Zerulla et al., 2001; Irigoyen et al., 2003). The utilize of DMPP at rates of 0,5-1,5 kg ha^{-1} are sufficient to achieve optimal nitrification inhibition in the substrate, reducing significantly NO_3^- leaching and N_2O emission, and improving crops yield (Zerulla et al., 2001). It has been observed that the effectiveness of DMPP may be drastically reduced in soils with extremely high temperatures (30 °C). This factor should be properly taken into account in warm climates (Irigoyen et al., 2003).

Management techniques for Fe chlorosis prevention based on N nutrition and intercropping with graminaceous species are expected to play an essential role in grapevine cultivation due to their implications on Fe nutrition and environmental impact. Therefore it would be of high scientific importance the establishment of experiments aimed to study the effects of these management techniques on physiological responses to Fe-deficiency at root and leaf levels.

5.2. Materials and Methods

5.2.1. Vegetal material, growth conditions and treatments

A biennial experiment (2010-2011) was conducted at the Experimental Station of the Agriculture Faculty of Bologna University. In summer 2010, grapevines cv Cabernet Sauvignon grafted on Fe chlorosis susceptible genotype *Vitis riparia* were acclimated under shady nets for 3 weeks and pruned maintaining one main shoot of 10-20 cm per plant. Plants were transferred to 33 l plastic pots (one plant per pot) filled with a calcareous soil (Tab. 1), and placed under a structure covered with plastic material. For avoiding excessively high temperatures in the soil, the pots were covered with aluminum film.

The treatments tested were: 1) Control (bare soil); 2) Soil-applied Fe-EDDHA chelate; 3) Leaf-applied Fe-EDTA chelate; 4) Intercropping with *Festuca rubra*; 5) Soil-applied NH_4^+; and 6) Soil-applied NH_4^+ + nitrification inhibitor. The experimental design was completely randomized, and each treatment was applied to seven plants. The nitrification inhibition was maintained during the experiment with the inhibitor 3,4-dimethylpirazole-phosphate (DMPP). With the aim to determine the effectiveness of the nitrification inhibitor, additional pots (three replicates) without plant were treated with NO_3^-, NH_4^+ and NH_4^+ + DMPP.

Iron-chelates were applied according to the leaf chlorophyll content, maintaining an intensive green color of leaves (SPAD index > 25). In 2010 and 2011 seasons, doses of 100 ml of 6% Fe-EDDHA 4,8% o-o solution (1 g Fe l^{-1}) were applied to the pots 3 and 2 times respectively. The solution of Fe-EDTA 2 mM was sprayed to the canopy 9 and 6 times on

2010 and 2011 seasons, respectively. Graminaceous species (*Festuca rubra*) was sown in pots one day after transplanting with a density of 20.000 seeds m^{-2}. Ammonium-fertilized vines received 100 ml of $(NH_4)_2SO_4$ (1 g of N l^{-1}) or 100 ml of $(NH_4)_2SO_4$ (1 g N l^{-1}) + DMPP (1% to N) solutions added weekly until reaching 1,5 g of N per vine during season. The N supplied in the treatments with NH_4^+ was balanced with a solution of $Ca(NO_3)_2$ (1 g of N l^{-1}) in the other treatments (control, Fe-EDDHA, Fe-EDTA and intercropping). In intercropped pots, an additional supply of N and water (20% total) has been added. The pots were daily irrigated with microdrip emitters (one per pot) maintaining a constant level of soil humidity, close to field water capacity (40% saturation). Weeds were manually removed and plant diseases protection was regularly carried out. In 2011 season, an additional supply of potassium (2 g of K as K_2SO_4) was added to intercropping pots, for overcoming visible potassium deficiency symptoms in leaves. Vines were grown in these conditions from 7 july 2010 to 13 july 2011. On February 2011, vines were pruned maintaining one spur with three buds per vine.

5.2.2. Leaf chlorophyll content and plants growth

Leaf chlorophyll content was periodically monitored during the experiment on five points of the first completely expanded leaf by the portable chlorophyll meter SPAD MINOLTA 502 (Osaka, Japan). In 2010 season, after leaf abscission, the dry biomass of shoots, leaves and pruning were recorded. At the end of the experiment (july 2011), plants were divided into roots, main shoot and leaves for dry biomass determinations and following analysis.

5.2.3. Nitrate and ammonium concentration in the soil

At the end of the experiment, soil samples were collected from each pot at 15 cm depth. Nitrate- and ammonium-N fractions were extracted by a solution of KCl 2 M. Samples were centrifuged, and the supernatant solution was collected and stored at -20 °C until analysis. Nitrate- and ammonium-N concentration in the soil extracts were determined by UV-spectrophotometric auto analyzer (Auto Analyzer AA-3; Bran + Luebbe, Norderstadt, Germany). Both NH_4^+ and NO_3^- absorbance were converted to concentration by

comparison with a calibration curve determined with standard solutions.

5.2.4. Enzyme assays and protein concentration in roots

At the end of the experiment, root tip (20–30 mm long) samples were collected from each plant, rinsed in deionized water, weighed, deep-frozen in liquid nitrogen, and kept at –80°C for enzyme activity analysis. The activity of phosphoenolpyruvate carboxylase (PEPC), malate dehydrogenase (MDH), citrate synthase (CS), and isocitrate dehydrogenase ($NADP^+$-IDH) were determined. The extraction of enzymes was performed as described by Jimenez et al. (2007).
Phosphoenolpyruvate carboxylase was determined by coupling its activity to malate dehydrogenase- catalyzed NADH oxidation (Vance et al., 1983). MDH activity was determined by monitoring the increase in absorbance at 340 nm, due to the enzymatic reduction of NAD^+ (Smith, 1974). CS activity was assayed by monitoring the reduction of acetyl CoA to CoA with DTNB at 412 nm (Srere, 1967). $NADP^+$-IDH was assayed as described by Goldberg and Ellis (1974), by monitoring the reduction of $NADP^+$ at 340 nm. Protein concentration was determinate by the Bradford method, using BSA as standard (Bradford, 1976). Data obtained in enzyme assays were referred to protein concentration of roots.

5.2.5. Organic acids concentration in roots

The organic acids concentration was determined in roots samples of each plant collected at the end of the experiment according to Neumann G. (2006). Frozen samples of root tips were submerges in a pre-cooled (4 °C) mortar with liquid nitrogen. After evaporation, the tissue was grind and homogenized with a pestle. For extraction and deproteinization, 5% H_3PO_4 was utilized. Organic acids were quantified by HPLC with 250 x 4 mm LiChrospher 5 um RP-18 column (SUPELCO INC., PA 16823-0048 USA). HPLC elution buffer was 18 mM KH_2PO_4, pH 2,1 adjusted with H_3PO_4. Samples were read at 210 nm for 40 minutes (Neumann G., 2006).

5.2.6. Mineral elements concentration in leaves

Total leaves per vine were collected at the end of the experiment. Leaf blades were separated from petiole, oven-dried at 60 °C to constant weight, and then were grinded at 0,2 mm and homogenized by a mill. Representative samples (300 mg) were mineralized by microwave ETHOS (MLS GmbH, Leutkirch, Germany) with 8 ml of HNO_3 65% and 2 ml of H_2O_2 30% at 180 °C for 20 minutes. After cooling, digested samples were diluted in 25 ml with ultrapure water. With this solution, microelements (Fe, Mn, Cu and Zn) concentrations were determined by an atomic absorption spectrometer (Varian, Spetr AA-200, Varian Inc., Victoria, Australia). For macroelements analysis, 5 ml of solution utilized for microelements determination were diluted with 1 ml of Cl_3La and 200 µl of CsCl in 25 ml with ultrapure water, and then were analyzed with atomic absorption spectrometer. For determined total N concentration, leaf samples were weighted in mini-capsules (2 mg) and analyzed using an elemental analyzer (EA 1110 Thermo Scientific). Nitrogen concentration in the samples was calculated with the area of the N peak, using acetanilide as a standard.

5.2.7. Leaf gas exchange and chlorophyll fluorescence measurements

On 2010 and 2011 seasons, simultaneous measurements of leaf CO_2 gas exchange and chlorophyll fluorescence were determined on three plants per treatment using an infrared gas analyzer (LI-Cor 6400 IRGA with an integrated 6400-40 leaf chamber fluorometer, Li-Cor, Inc., Lincoln, NE, USA). During the experiment, measures were performed on the first mature leaf inserted in the middle third of the shoot. Leaves were placed into the leaf cuvette of a Licor 6400 photosynthesis system and were illuminated by the Licor 6400 LED light source providing a photosynthetic photon flux density (PPFD) around 1000 mmol $m^{-2}s^{-1}$. Net photosynthesis (A_n) was measured when foliar CO_2 uptake was steady. During the experiment, gas-exchange measurements including CO_2 assimilation rate and stomatal conductance (g_s) were conducted in the morning between 9:00 and 12:00 pm. The leaf chamber was configured to track temperature (°C), humidity (%), and illumination conditions of the growth chamber. CO_2 levels were fixed at 380 ppm within the leaf chamber corresponding to the average concentration in the growth chamber. Chlorophyll

fluorescence has been recorded simultaneously at gas-exchange measurements. Fluorescence parameters were set following recommended values published in the LICOR 6400 manual (LICOR Biosciences, Inc., Lincoln, NE) for light-adapted leaves. Saturation pulses of approximately 8000 µmol photons m^{-2} s^{-1} with a 0,8-s duration were applied in order to saturate the PS II reaction centers for estimating the maximum fluorescence (Fm'). Additionally, the "dark pulse" routine was performed in order to estimate the yield of fluorescence in absence of an actinic (photosynthetic) light (Fo'). Comparison of these values with the steady-state yield of fluorescence in the light (Fs), gives information about the efficiency of photochemical quenching and by extension, the performance of PSII. This is accomplished by preferentially exciting PSI with far-red light, causing electrons to drain from PSII (see Licor manual for further details). The following fluorescence parameters for light-adapted leaves were calculated using the equations proposed by Genty et al. (1989) and review by Maxwell and Jhonson (2000):
The efficiency of Photosystem II photochemistry was calculated as:

$$\Phi_{PSII} = (Fm' - Fs)/Fm'$$

The quantum yield of PSII (Φ_{PSII}), was used to calculate linear electron transport rate (ETR), that represent the photosynthetic capacity *in vivo,* with the follow equation:

$$ETR = \Phi_{PSII} \text{ x } PFDa \text{ x } (0,5)$$

Where PFDa is absorbed light (µmol photon m^{-2} s^{-1}) (measured using an integrating sphere) and 0,5 is the factor that accounts for the partitioning of energy between PSII and PSI. Also, has been calculated the efficiency of excitation energy capture by open PSII reaction centers (Fv'/Fm'), and the expression was given by the equation:

$$Fv'/Fm' = (Fm' - Fo')/Fm'$$

5.2.8. Statistics

Comparison of means and analysis of variance between treatments were done by SAS software (SAS Institute, Cary, NC). Analyses were performed on raw data to maximize variance homogeneity. If significant differences were founded by the F-test, means were compared using the Newman and Keuls test ($p \leq 0{,}05$).

5.3. Results

5.3.1. Leaf chlorophyll content and plants growth

In the first season (2010), since fifteen days from treatments imposition plants started displaying Fe chlorosis symptoms with different intensity according to treatments (Fig. 1). Applications of Fe-EDDHA increased leaf chlorophyll content during the season (Fig. 1). Until 35 days, intercropped vines presented the lowest leaf chlorophyll content, however Fe chlorosis symptoms progressively disappeared in the following period (Fig. 1). Control vines exhibited Fe chlorosis symptoms after 49 days, showing the lowest SPAD values at the end of the season (Fig. 1). Vines treated with Fe-EDTA, NH_4^+ and NH_4^+ + DMPP presented intermediate leaf chlorophyll content (Fig. 1). During 2011 season, plants treated with Fe-EDDHA showed highest chlorophyll content, maintaining this trend until the end of the experiment (Fig. 2), whereas control vines showed the lowest values. The others treatments displayed intermediate values (Fig. 1).
In 2010 season, treatments did not modify leaf area and dry biomass of shoots (data not reported). In the second season (2011), Fe-EDTA significantly increased leaf area and biomass as compared to control (1,5-fold in both parameters), whereas intercropping strongly decreased leaf area (Tab. 2). The other strategies for Fe chlorosis prevention did not modify leaf area of vines (Tab. 2). Intercropping decreased the biomass of roots (-1,6-fold), shoots (-2-fold), leaves (-1,9-fold) and plant (-1,7-fold). Iron-EDDHA, NH_4^+ and NH_4^+ + DMPP applications did not induce statistical differences in leaf area and dry weight of organs as compared to control vines (Tab. 2).

5.3.2. Nitrate- and ammonium-N concentration in the soil

At the end of the experiment, significant differences in the soil N-NO_3^- concentration were recorded, whereas N-NH_4^+ concentration did not differ between treatments (Tab. 3). Soil collected from intercropping pots showed the largest decrease in N-NO_3^- concentration as compared to control (-3,9-fold), followed by Fe-EDTA (-2,4-fold), and NH_4^+ + DMPP (-1,9-fold) (Tab. 3). In pots without plants, fertilization with NH_4^+ + DMPP increased N-NH_4^+ concentration in the soil as compared to the application of NH_4^+ alone (data not reported). Consequently, DMPP supply effectively inhibited nitrifications processes.

5.3.3. Enzyme assays and protein concentration in roots

The activity of PEPC and some key enzymes of Krebs cycle have been determined in the root tip extracts. At the end of the experiment, roots from control plants showed higher activity of PEPC enzyme than the other treatments. Treatments did not influence the activity of MDH, CS, NADP -IDH enzymes and the protein concentration in roots (Tab. 4).

5.3.4. Organic acids concentration in roots

At the end of the experiment, the major organic acids present in root extracts were malic, followed by citric and ascorbic acids (Tab. 5). Significant differences between treatments were recorded. Iron-EDDHA and NH_4^+ + DMPP applications decreased citric acid concentration in roots as compared to control (Tab. 5). Malic and ascorbic acids concentration in roots did not show differences between treatments (Tab. 5).

5.3.5. Mineral elements concentration in leaves

Nitrogen (N) concentrations in leaves did not present significant differences between treatments (Tab. 6). The supply of NH_4^+ and NH_4^+ + DMPP decreased the leaf phosphorous (P) concentration respect to the control (Tab. 6). Intercropping and Fe-EDDHA increased potassium (K) concentration respect to control vines (Tab. 6). Iron-EDDHA decreased both calcium (Ca) and magnesium (Mg) concentrations in the leaves (Tab. 6), whereas applications of NH_4^+ + DMPP increased magnesium (Mg) concentration respect to control

(Tab. 6). Both Fe-chelates (EDDHA and EDTA) increased Fe concentration (Tab. 7), whereas Fe-EDDHA treatment decreased manganese (Mn) status in vines (Tab. 7). Iron-EDTA decreased zinc (Zn) concentration in leaves as compared to vines treated with NH_4^+ (Tab. 7). Copper (Cu) concentration did not show significant differences between treatments (Tab. 7).

5.3.6. Leaf gas exchange and chlorophyll fluorescence

During the experiment, gases exchanges and fluorescence parameters were determined in the first completely mature leaf. In the first season (2010), treatments did not modify net photosynthesis and stomatal conductance in leaves, whereas intercropping decreased Fv'/Fm', ΦPSII and ETR values as compared to control (Tab. 8). In the second season (2011), leaves of intercropped vines showed higher net photosynthesis than vines treated with Fe-EDTA, and the others treatments showed intermediate values (Tab. 9). In addition, intercropping with *Festuca rubra* increased stomatal conductance of leaves as compared to control vines. Treatments did not influence fluorescence parameters (Tab. 9), however the highest value of ETR (31% higher as compared to control) were recorded in intercropped plants (Tab. 9).

5.4. Discussion

During the first season (2010), treatments modified leaf chlorophyll content (Fig. 1) without changing plant biomass as compared to control (data not reported). Control vines exhibited Fe chlorosis symptoms after 49 days, showing the lowest leaf chlorophyll content at the end of the season (Fig. 1). Application of Fe-EDDHA effectively increased leaf chlorophyll content (Fig. 1). The decrease of leaf chlorophyll content induced by intercropping until 35 days after treatment imposition is ascrivible to the competition for Fe (and other nutrients) by growing *Festuca rubra* plants (Fig. 1). However, initial leaf yellowing showed by intercropped vines progressively disappeared in the following period, and higher values were recorded at the end of the season (Fig. 1). In general, leaves of

grapevines treated with NH_4^+ (alone or with DMPP) presented values tendentially higher than control and lower than Fe-EDDHA (Fig. 1). In the second season (2011), control vines exhibited symptoms of Fe-deficiency. The application of NH_4^+ alone did not induce a significant increase in leaf chlorophyll content, whereas the addition of DMPP to NH_4^+ supply resulted in a clear improvement of leaf chlorophyll content. Beneficial effects of NH_4^+ on the uptake of Fe (and other nutrients) counteracting the adverse effects of NO_3^-, have been reported (Houdusse et al., 2007). In *Beta macrocarpa*, total replacement of NO_3^- by NH_4^+ decreased leaf NO_3^-concentration and increased water use efficiency (Hessini et al., 2009). However, the application of NH_4^+ was associtated with a reduction in plant growth as compared with plants fed with mixed N forms (NO_3^-, NH_4^+ and urea) (Houdusse et al., 2007). These evidences suggest the importance of maintaining limited concentrations of NH_4^+ along with other forms of N in the soil, for improving plant Fe nutrition. The high leaf chlorophyll content recorded during the second season in intercropped vines shows that this soil management system effectively prevented Fe chlorosis (Fig. 2). However, the presence of graminaceous sepecies strongly reduced leaf area and plant biomass in intercropped vines (Tab. 2). Both similar (Inal et al., 2007) and contrasting (Zuo et al., 2000) results has been reported in peanut (*Arachis hypogaea* L.) intercropped with maize, suggesting that the effect of intercropping on the growth of co-cultivated crop may be variable according to the experimental conditions (water and nutrients availability and sowing density of cover-crop). In a pot experiment conducted in citrumelo "Swingle", intercropping with graminaceous did not reduce dry biomass of co-cultivated plants (Ammari and Rombolà, 2010). In grapevine, Bavaresco et al. (2010) did not observed a significant reduction of shoot length in potted vines intercropped with *Fesctuca ovina* after 4 years of cultivation on calcareous soil. In a six-year trial conducted in a non-irrigated vineyard, cover-crop constantly reduced vine vigor and yield (Palliotti et al., 2007). Intrieri et al. (2005) observed a strong reduction in the vigor and yield of grapevines, induced by a full sward composed by grasses species. A possible cause for the growth reduction in intercropped plants observed in this experiment, is the competition for mineral elements and water uptake, exerted by graminaceous species in spite of the additional (20%) water and N supply. This hypothesis is supported by the lower concentration of N-NO_3^- recorded in the soil covered with the intercropping system (Tab. 3). In addition, root growth

restriction caused by space limitations in pots, clearly exacerbates the competition for these essential sources between species. It is possible that the sowing density of *Festuca rubra* employed in this experiment resulted quite high for vines, and/or the supplement addition of water and N was not enough for satisfying plants needs. In some studies performed by Arneth (1979) and Müller et al. (1984), shoot growth of the vines was severely inhibited despite continuous water and nitrogen supply, suggesting possible allelopathic interferences (Lopes et al., 2004). Noteworthy, intercropping can be adapted to many climates and soils, and the influence on grapevine growth may be an economical and effective tool for containing the vigor. Consequently, in vineyards where the soils are deep and soil moisture is excessive, the competition exerted by cover crops on grapevine for water and nutrients may bring agronomic advantages. These evidences suggest that the employ of intercropping as a management technique requires grasses properly selected and managed according to crop and environment parameters.

Iron-deficiency increased the activity of PEPC in roots (Tab. 4), whereas the activity of some TCA key enzymes (MDH, CS, $NADP^+$-IDH), and protein concentration did not present differences between treatments (Tab. 4). Phosphoenolpyruvate carboxylase enzyme catalyzes the incorporation of bicarbonate into a C_3 organic acid, phosphoenolpyruvate (PEP), generating oxalacetate, which is converted to malate by malate dehydrogenase (Lance and Rustin 1984). This process is an important component of the pH-stat mechanism inside the cell (Davies 1973). Increases in root PEPC activity induced by Fe-deficiency have been largely reported for several model plants grown in hydroponic system, and it has been proposed by some authors as a biochemical marker of Fe-deficiency status in tolerant species (Rombolà et al., 2002a; Rombolà and Tagliavini, 2006; Jimenez et al., 2007). In grapevine, an increases in PEPC activity in roots has been verified in the Fe chlorosis tolerant genotype Cabernet Sauvignon (*Vitis vinifera)* and, in lower intensity, in the Fe-chlorosis susceptible Gloire de Montpellier (*Vitis riparia*) grown in hydroponic culture (Jimenez et al., 2007). However, to the best of my knowledge the activities of these enzymes have been scarcely reported in experiments conducted in soil conditions. This work indicates that the Fe chlorosis susceptible *Vitis riparia* rootstock is able to activate tolerance mechanisms to Fe-deficiency also under soil conditions. The activity of root PEPC -considered a biochemical marker of Fe status- in co-cultivated and NH_4^+-fed plants

was similar to that recorded in plants supplied with Fe, suggesting that both sustainable treatments improved Fe nutrition.

Increases in organic acid concentrations in roots of Fe-deficient plants are fairly ubiquitous, and occur both in Strategy I and Strategy II plant species (Abadía et al., 2002). The major organic acids present in root extracts of *Vitis riparia* rootstock were malate, followed by citrate and ascorbate (Tab. 5). Citric acid has an important role in iron absorption and xylem transport of Fe in plants, and citrate metabolism may be associated to proton extrusion and to Fe(III) reduction activity (Ollat et al., 2003). Results of this experiment show a lower concentration of citrate in roots of vines treated with Fe-EDDHA and NH_4^+ + DMPP (Tab. 5), supporting the effectiveness of these treatments on Fe-deficiency prevention. Surprisingly, foliar applications of Fe-EDTA did not decrease the concentration of citric acid in the roots, and Fe-EDTA-treated vines exhibited a behavior similar to Fe-deficient plants (Tab. 5). This evidence may arise from a reduced translocation of Fe applied as Fe-EDTA from leaves to roots, inducing an increase of citric acid concentration in roots as a response to Fe-deficiency. Fernández et al. (2008) reported a limited Fe translocation from treated to untreated areas within the same leaf, indicating a low Fe mobility into the tissue. Limited phloem translocations from shoots to roots may be also exacerbated by graft incompatibility between different genotypes, due to a lower continuity of functional phloem between the two internodes (Espen et al., 2005). In grapevine, incompatibility between commercial varieties and rootstock has been reported (Kocsis and Bakonyi, 1994; Ungurenau, 1995).

The management strategies for Fe-chlorosis prevention assessed in this work did not influenced the leaf N concentration of vines. This result indicates that the form of N (NO_3^- or NH_4^+) applied to the pots, did not change significantly the N plant status. In contrast, Fe-EDDHA increased K and decreased Ca concentration in leaves (Tab. 6), as reported in grapevine by Bavaresco et al. (2010). The higher leaf K concentration recorded in intercropped vines (Tab. 6) is presumably due to the additional supply of 2 g of K per pot applied in 2011 season for overcoming leaf K-deficiency symptoms. The application of Fe-EDDHA decreased the concentration of Mn in the leaves (Tab. 7). Similar effects have been reported in plants treated with Fe-chelates (Fernandez et al., 2008; Nadal et al., 2009; Rodriguez-Lucena et al., 2010) and intercropped with graminaceous species (Zuo and

Zhang, 2008). The mechanism of Fe and Mn uptake requires the reducing capacity of roots to accumulate Mn^{2+} and Fe^{2+} ions in the rhizosphere. Zuo and Zhang (2008) reported that the higher Mn concentration in Fe-deficient plants may be caused by the enhanced in the reducing capacity of Fe-deficient roots.

In the first season, intercropping decreased the fluorescence derived parameters (Fv'/Fm', ΦPSII and ETR) and tendentially the net photosynthesis of leaves (A_n) (Tab. 8). Reductions in photosynthesis rate in Fe-deficient chlorotic leaves, have been attributed to a decrease in Photosystem II efficiency due to closure of Photosystem II reaction centers and decreases of the intrinsic Photosystem II efficiency (Morales et al., 2000). Leaf Fv'/Fm' represent the efficiency of light harvesting by open reaction centers of PSII (Genty et al., 1989). The lower leaf chlorophyll content recorded in intercropped vines (Fig. 1) may in part explain the lower light harvesting efficiency observed. In addition, ΦPSII measures the proportion of light absorbed by chlorophyll associated with PSII that is used in photochemistry, while ETR is an indicator of overall photosynthetic capacity (Maxwell and Jhonson, 2000). Therefore, results of this experiment indicate that the establishment of intercropping induced in vines photochemical changes consequent to a temporary Fe-deficiency stress, decreasing leaf photosynthetic capacity independently of stomatal conductance (g_s).

Interestingly, in the second season intercropping increased g_s and tendentially on A_n and ETR as compared to control. These results indicate a strong recovery of leaf photosynthetic performance in intercropped vines. In the second season, intercropped vines were characterized by leaves with low area (Tab. 2), and high chlorophyll content (Fig. 2). These characteristics, probably induced by stress conditions, were associated to high leaf thickness. Niinemets (1999) reported an increase in photosynthetic capacity associated with a higher thickness of the leaf. This effect may be attributed to an accumulation of photosynthetically competent tissues per unit of leaf area, caused by the decreases in the fraction of leaf epidermis and mechanical tissues, such as sclerenchyma, with a proportional increasing of leaf mesophyll tissues (Niinemets, 1999). Changes in leaf morphology, induced by intercropping, deserving further specific observations (eg. TEM, SEM), appear as an adaptive mechanism for maintaining a sufficient source of photosynthates in vines with reduced leaf area and biomass yield.

5.5. Conclusions

Intercropping with *Festuca rubra* induces positive effects on chlorophyll content and leaf photosynthesis associated to changes of leaf morphology. In addition, intercropping reduces the activity of PEPC in roots, similary to Fe-chelate supply. Applications of NH_4^+ with a nitrification inhibitor prevents efficiently Fe-deficiency, increases chlorophyll content, and induces root biochemical responses (enzymes activity and organic acids concentration) as Fe-EDDHA. In contrast, without the addition of nitrification inhibitors, the effectiveness of NH_4^+ supply on Fe chlorosis prevention resulted significantly lower. Iron-chelates represents the most efficient treatment, however apart economical and environmental implications, supply of Fe-EDDHA impairs Mn nutrition.

Results obtained in this work evidenced the importance of developing researches addressed to improve management techniques based on intercropping systems and NH_4^+ supply for preventing Fe chlorosis in vineyards. In future researches, special attention should be devoted to selecting Fe-efficient graminaceous species with low water and developing natural nitrifications inhibitors.

5.6. Literature cited

Abadía, J. 1992. Leaf responses to Fe deficiency. A Review. J Plant Nutr 15:1699–1713.

Abadía, J., Lopez-Millan, A.F., Rombolà, A. and Abadía, A. 2002. Organic acids and Fe deficiency: a review. Plant and Soil, 241:75-86.

Ammari, T., Rombolà, A.D. 2010. Overcoming iron deficiency chlorosis in citrus through intercropping with perennial grass species. In: Acta Horticulturae, 19-23(868): 327-330.

Arneth, A.G. 1979. Untersuchungen über die eignung von dürreresistenten pflanzen und von mulchschichten im weinbau. Diss. Univ. Gießen.

Bavaresco, L., van Zeller, M.I., Civardi, S., Gatti, M. and Ferrari, F. 2010. Effects of traditional and new methods on overcoming lime-induced chlorosis of grapevine. American Journal of Enology and Viticulture, 61(2): 186-190.

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72: 248-254.

Brancadoro, L., Rabotti, G., Scienza, A. and Zocchi, G. 1995. Mechanisms of Fe-efficiency in roots of *Vitis* spp. in response to iron deficiency stress. Plant and Soil, 171: 229-234.

Cesco, S., Rombolà, A. 2007. Nutrient management at the rhizosphere through intercropping. In: Robert M. Goodmann. Encyclopedia of Plant and Crop Science, London: Taylor & Francis, p. 1 – 3. ISBN/ISSN: 978-0-8247-0944-0

Cesco, S., Rombolà, A.D., Tagliavini, M., Varanini, Z. and Pinton, R. 2006. Phytosiderophores released by graminaceous species promote ^{59}Fe-uptake in citrus. Plant and Soil, 287: 223–233.

Davies, D.D. 1973. Control of and by pH. Symposia of the Society of Experimantal Biology, 27: 513–529.

Dell'Orto, M., Brancadoro, L., Scienza, A. and Zocchi, G. 2000. Use of biochemical parameters to select grapevine genotypes resistant to iron-chlorosis. Journal of Plant Nutrition, 23(11&12), 1767-1775.

Ding, H., Duan, L., Wu, H., Yang, R., Ling, H., Li, W-X. and Zhang, F. 2009. Regulation of *Ah*FRO1, an Fe(III)-chelate reductase of peanut, during iron deficiency stress and intercropping with maize. Physiologia Plantarum, 136: 274–283.

Ding, H., Duan, L., Li, J., Yan, H., Zhao, M., Zhang, F and Li, W-X. 2010. Cloning and functional analysis of the peanut iron transporter *Ah*IRT1 during iron deficiency stress and intercropping with maize. Journal of Plant Physiology, 167: 996–1002.

Espen, L., Cocucci, M. and Sacchi, G.A. 2005. Differentiation and functional connection of vascular elements in compatible and incompatible pear/quince internode micrografts. Tree Physiology, 25: 1419–1425.

Fernández, V., Del Río, V., Pumariño, L., Igartua, E., Abadía, J. and Abadía, A. 2008. Foliar fertilization of peach (*Prunus persica* (L.) Batsch) with different iron formulations: Effects on re-greening, iron concentration and mineral composition in treated and untreated leaf surfaces. Scientia Horticulturae, 117: 241–248.

Genty, B., Briantais, J.M. and Baker, N.R. 1989. The relationship between quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochimica et Biophysica acta, 990: 87-92.

Gogorcena, Y., Abadía, J., Abadía, A. 2004. A new technique for screening iron-efficient genotypes in peach rootstocks elicitation of root ferric chelate reductase by manipulation of external iron concentrations. Journal of Plant Nutrition, 27: 1701– 15.

Goldberg, D.M. and Ellis, G. 1974. Isocitrate dehydrogenase. In: Bergmeyer HU (ed) Methods of enzymatic analysis. Verlag Chemie/Academic Press, New York, pp 183–189.

Hessini, K., Lachaâl, M., Cruz, C. and Soltani, A. 2009. Role of ammonium to limit nitrate accumulation and to increase water economy in wild swiss chard. Journal of Plant Nutrition, 32: 821–836.

Houdusse, F., Garnica, M. and García-Mina, J.M. 2007. Nitrogen fertiliser source effects on the growth and mineral nutrition of pepper (*Capsicum annuum* L.) and wheat (*Triticum aestivum* L.). Journal of the Science of Food and Agriculture, 87:2099–2105.

Inal, A., Gunes, A., Zhang, F. and Cakmak, I. 2007. Peanut/maize intercropping induced changes in rhizosphere and nutrient concentrations in shoots. Plant Physiology and Biochemistry, 45: 350-356.

Intrieri, C., Filippetti, I., Lia, G. and Colucci, E. 2005. Intra-row vineyard spacing and soil management. Long-term field trial results with cv. Pignoletto. Winery and Vineyard, January/February 2005: 76-87.

Irigoyen, I., Muro, J., Azpilikueta, M., Aparicio-Tejo P. and Lamsfus, C. 2003. Ammonium oxidation kinetics in the presence of nitrification inhibitors DCD and DMPP at various temperatures. Australian Journal of Soil Research, 41(6): 1177-1183.

Jimenez, S., Gogorcena, Y., Hévin, C., Rombolà, A.D., and Ollat, N. 2007. Nitrogen nutrition influences some biochemical responses to iron deficiency in tolerant and sensitive genotypes of Vitis. Plant and Soil, 290: 343-355.

Kocsis, L. and Bakonyi, L. 1994. The evaluation of the rootstock wood-fruiting wood interaction in hotroom callusing. Kerteszeti Tudomany-Horticultural Science, 26(2): 61–64.

Kosegarten, H., Hoffmann, B., Roco, E., Grolig, F., Glüsenkamp, K.-H. and Mengel, K. 2004. Apoplastic pH and FeIII reduction in young sunflower (*Helianthus annuus*) roots. Physiologia Plantarum, 122: 95–106.

Lance, C. and P. Rustin. 1984. The central role of malate in plant metabolism. Physiologie Vegetale, 22: 625–641.

Larbi, A., Abadía, A., Abadía, J.and Morales, F. 2006. Down co-regulation of light absorption, photochemistry, and carboxylation in Fe-deficient plants growing in different environments. Photosynthesis Research, 89: 113–126.

Lopes, C., Monteiro, A., Rückert, F.E., Gruber, B., Steinberg, B. and Schultz, H.R. 2004. Transpiration of grapevines and co-habitating cover crop and weed species in a vineyard. A "snapshot" at diurnal trends. Vitis, 43 (2): 111–117.

Loulakakis, K.A. and Roubelakis-Angelakis, K.A. 2001. Nitrogen assimilation in grapevine. In: Roubelakis-Angelakis KA (ed) Molecular biology and biotechnology of the grapevine. Kluwer Academic Publishers, Dordrecht/Boston/London, pp 59–85.

Lucena, J.J. 2000. Effects of bicarbonate, nitrate and other environmental factors on iron deficiency chlorosis. A review. Jouranl of Plant Nutrition, 23(11&12): 1591-1606.

Nadal, P., Hernández-Apaolaza, L. and Lucena, J.J. 2009. Effectiveness of N,N′-Bis(2-hydroxy-5-methylbenzyl) ethylenediamine-N,N′-diacetic acid (HJB) to supply iron to dicot plants. Plant and Soil, 325: 65-77.

Neumann, G. 2006. Root exudates and organic composition of plant roots. In: Luster J, Finlay R (eds) Handbook of methods used in rhizosphere research. Swiss Federal Research Institute WSL, Birmensdorf, 536 pp.

Nikolic, M. and Römheld, V. 2002. Does high bicarbonate supply to roots change availability of iron in the leaf apoplast? Plant and Soil, 241: 67–74.

Niinements, Ülo. 1999. Components of leaf dry mass per area – thickness and density – alter leaf photosynthetic capacity in reverse directions in woody plants. New Phytologist, 144: 35-47.

Ma, J.F. and Nomoto, K. 1996. Effective regulation of iron acquisition in graminaceous plants. The role of mugineic acids as phytosiderophores. Physiologia Plantarum, 97: 609-617.

Ma, J.F., Ueno, H., Ueno, D., Rombolà, A., Iwashita, T. 2003. Characterization of phytosiderophores secretion in *Festuca rubra*. Plant and Soil, 256: 131-137.

Maxwell, K. and Jhonson, G. 2000. Chlorophyll fluorescence – a practical guide. Journal of Experimental Botany, 51(345): 659-668.

Mengel, K., Planker, R and Hoffmann, B., 1994. Relationship between leaf apoplast pH and iron chlorosis of sunflower (*Helianthus annuus* L.) Journal of Plant Nutrition, 17: 1053-1065.

Morales, F., Belkhodja, R., Abadía, A. and Abadía, J. 2000. Photosystem II efficiency and mechanisms of energy dissipation in iron-deficient, field-grown pear trees (*Pyrus communis* L.). Photosynthesis Research, 63: 9–21

Müller, W., Rühl, E. and Gebbing, H. 1984. Untersuchungen über die wechselwirkung zwischen rebe und begrünungspflanzen. WeinWiss, 39: 3-15.

Ollat, N., Laborde, B., Neveux, M., Diakou-Verdin, P., Renaud, C. and Moing, A. 2003. Organic acid metabolism in roots of various grapevine (*Vitis*) rootstocks submitted to iron deficiency and bicarbonate nutrition. Journal of plant nutrition, 26(10 & 11): 2165–2176.

Palliotti, A., Cartechini, A., Petoumenou, D., Silvestroni, O., Mattioli, S. and Berrios, J.G. 2007. Long-term effects of seeded cover-crop on vegetative characteristics, yield and grape and wine composition of 'Grechetto' grapevines in central Italy. Acta Horticulturae (ISHS), 754: 515-521.

Rodríguez-Lucena, P., Ropero, E., Hernández-Apaolaza, L. and Lucena, J.J. 2010. Iron supply to soybean plants through the foliar application of IDHA/Fe^{3+}: effect of plant nutritional status and adjuvants. Journal of the Science of Food and Agriculture, 90: 2633-2640.

Rombolà, A., Baldi, E., Franceschi, A., Ammari, T., Minguez, J. and Tagliavini, M. 2003. Prevenzione della clorosi ferrica dell'actinidia (*Actinidia deliciosa*) mediante consociazione temporanea con specie Graminacee. In: Atti del convegno nazionale "Actinidia, la novità frutticola del XX secolo". November 21, Verona, Italy.

Rombolà, A.D., Brüggemann, W., López-Millán, A.F., Tagliavini, M., Abadía, J., Marangoni, B. and Moog, P.R. 2002a. Biochemical responses to iron deficiency in kiwifruit (*Actinidia deliciosa*). Tree Physiology, 22: 869–875.

Rombolà, A.D., Cremonini, M.A., Lucchi, A., Sorrenti, G., Placucci, G., Marangoni, B. 2002b. Leaching of soil-applied synthetic Fe chelates (Fe-EDDHA) in orchard ecosystem. *In Abstracts XI International Symposium on Iron Nutrition and Interaction in Plants*, Udine, Italy.

Rombolà, A.D. and Tagliavini, M. 2006. Iron nutrition of fruit tree crops. *In* Iron Nutrition in Plants and Rhizospheric Microorganisms (Eds, J, Abadía and L, Barton), Springer, Berlin, Germany, pp. 61-83.

Smith, F. 1974. Malate dehydrogenase. In: Bergmeyer HU (ed) Methods of enzymatic analysis. Verlag Chemie/Academic Press, New York, pp 163-175.

Spiller, S. and Terry, N. 1980. Limiting factors in photosynthesis. II. Iron stress diminishes photochemical capacity by reducing the number of photosynthetic units. Plant Physiology, 65: 121-125.

Srere, P.A. 1967. Citrate synthase. In: Colowick SP, Kaplan NO (eds) Methods in enzymology. Academic Press, New York, EEUU, pp 3–11.

Tagliavini, M., Abadia, J., Rombolà, A.D., Abadia, A., Tsipoudiris, C., Marangoni, B., 2000. Agronomic means for the control of iron chlorosis in deciduous fruit plants. Journal of Plant Nutrition, 23(11-12): 2007-2022.

Tagliavini, M. and Rombolà, A.D. 2001. Iron deficiency and chlorosis in orchard and vineyard ecosystems. European Journal of Agronomy, 15: 71-92.

Terry, N. and Abadía, J. 1986. Function of iron in chloroplasts. Jouranl of Plant Nutrition, 9: 609–646.

Ueno, D., Rombolà, A.D., Iwashita, T., Nomoto, K. and Ma, J.F. 2007. Identification of two novel phytosiderophores secreted by perennial grasses. New Phytologist, 174: 304–310.

Ungurenau, S. 1995. The affinity of new winegrape cvs to different rootstocks. Revista Pomicultura, Viticultura si vinificatia in Moldova, (7–8): 31–33.

Vance, C.P., Stade, S., Maxwell, C.A. 1983. Alfalfa root nodule carbon dioxide fixation. I: association with nitrogen fixation and incorporation into amino acids. Plant Physiology, 72: 469–473.

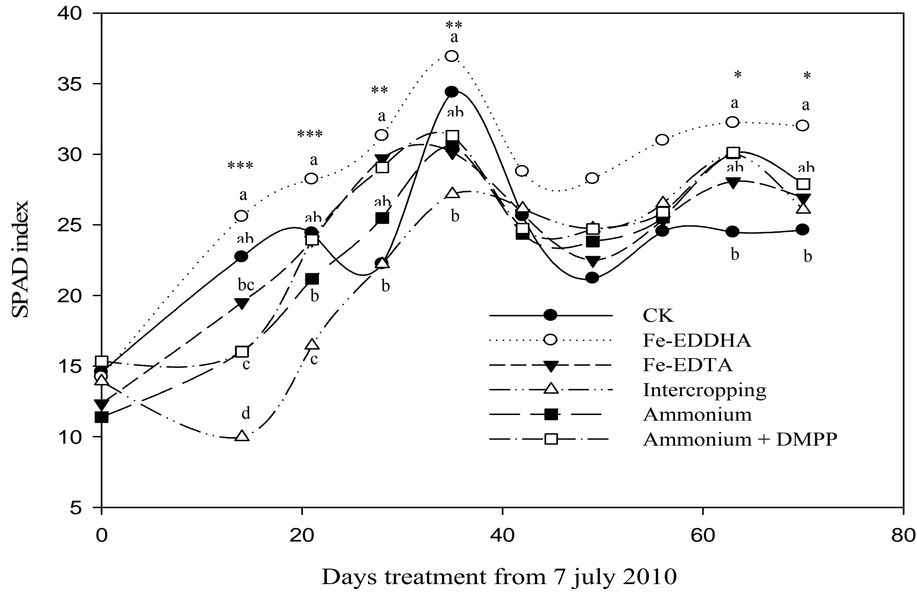
Zerulla, W., Barth, T., Dressel, J., Erhardt, K., von Locquenghien, K.H., Pasda, G., Rädle, M. and Wissemeier, A.H. 2001. 3,4-Dimethylpyrazole phosphate (DMPP) – a new nitrification inhibitor for agriculture and horticulture. An Introduction. Biology and Fertility of Soils, 34: 79–84.

Zuo, Y. and Zhang, F. 2008. Effect of peanut mixed cropping with gramineous species on micronutrient concentrations and iron chlorosis of peanut plants grown in a calcareous soil. Plant and Soil, 306: 23–36.

Zuo, Y., Zhang, F., Li, X. and Cao, Y. 2000. Studies on the improvement in iron nutrition of peanut by intercropping with maize on a calcareous soil. Plant and Soil, 220: 13–25.

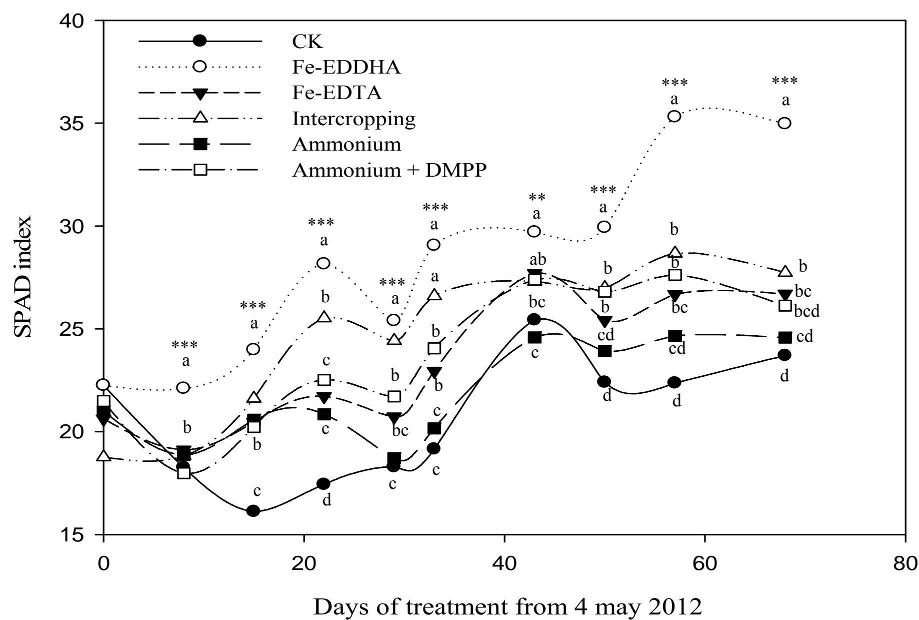
5.7. Figures and tables

Figure 1. Time course of chlorophyll content (SPAD index) determined during 2010 season in the first expanded apical leaf for treatments. Data are means of seven replicates.



Abbreviation and symbols: *, **, *** = significant at p≤0,05, significant at p≤0,01 and significant at p≤0,001 level. Different letters in each day indicate that data were significantly different as per the SNK test.

Figure 2. Time course of chlorophyll content (SPAD index) determined during 2011 season in the first expanded apical leaf for treatments. Data are means of seven replicates.



Abbreviation and symbols: **, *** = significant at p≤0,01 and significant at p≤0,001 level. Different letters in each day indicate that data were significantly different as per the SNK test.

Table 1. Chemical and physical parameters of soil.

Parameter	Unit	Value
pH in H_2O	-	8,39
Total carbonates ($CaCO_3$)	%	78
Active lime ($CaCO_3$)	%	19,2
Organic matter	%	0,54
total nitrogen (N)	‰	0,39
Phosphorous assimilable (P)	ppm	3
Potassium exchangeable (K)	ppm	195
Sodium exchangeable (Na)	ppm	186
Calcium exchangeable (Ca)	ppm	2611
Magnesium exchangeable (Mg)	ppm	47
Iron assimilable (Fe)	ppm	2,68
Manganese assimilable (Mn)	ppm	1
Zinc assimilable (Zn)	ppm	0,51
Copper assimilable (Cu)	ppm	2,2
Boron assimilable (B)	ppm	0,29
Relation C/N	-	8,03
Cation exchange capacity (CEC)	meg/100 g	14,72
Soil texture		
Sand	%	24
Lime	%	53
Clay	%	23

Table 2. Leaf area (m^2) and biomass yield of organs (g $plant^{-1}$ DW) for treatments determined at the end of experiment. Data are means of seven replicates.

Treatments	Leaf area (m^2)	Roots (g)	Trunk (g)	Shoots (g)	Leaves (g)	Total (g)
Control	0,18 b	24,5 ab	15,4	10,7 ab	12,8 b	63,5 ab
Fe-EDDHA	0,23 ab	26,8 ab	15,9	14,3 a	16,6 ab	73,5 ab
Fe-EDTA	0,27 a	31,3 a	16,7	15,8 a	19,1 a	82,9 a
Intercropping	0,11 c	15,0 c	11,0	5,1 b	6,8 c	37,9 c
Ammonium	0,19 b	22,1 b	13,4	10,1 ab	12,9 b	58,6 b
Ammonium + DMPP	0,18 b	23,3 ab	16,4	9,5 ab	12,4 b	61,6 ab
Significance	***	**	n.s.	**	***	**

Abbreviation and symbols: ns, **, *** = not significant, significant at $p \leq 0,01$ and significant at $p \leq 0,001$ level.
Means followed by the same letter in each column were not significantly different as per the SNK test.

Table 3. Nitrate and ammonium concentration (mg kg^{-1}) in soil samples collected from treated pots at the end of the experiment. Data are means of seven replicates.

Treatments	NO_3^- (mg kg^{-1})	NH_4^+ (mg kg^{-1})
Control	6,6 a	0,055
Fe-EDDHA	5,2 ab	0,055
Fe-EDTA	2,8 bc	0,003
Intercropping	1,7 c	0,038
Ammonium	4,6 abc	0,003
Ammonium + DMPP	3,5 bc	0,003
Significance	**	n.s.

Abbreviation and symbols: ** = significant at $p \leq 0.01$ level.
Means followed by the same letter in each column were not significantly different as per the SNK test.

Table 4. Activities (nmol mg^{-1} protein min^{-1}) of phosphoenolpyruvate carboxylase (PEPC), malate dehydrogenase (MDH), citrate synthase (CS), isocitrate dehydrogenase NADP^{+}-dependent (NADP^{+}-IDH) and total proteins (mg g^{-1} FW) measured in root tip extracts collected at the end of the experiment (july 2011). Data are means of seven replicates.

Treatments	PEPC	MDH	CS	NADP^{+}-IDH	Proteins
CK	27,3 a	556,0	32,3	13,1	29,9
Fe-EDDHA	16,4 b	535,8	26,9	9,2	32,2
Fe-EDTA	17,4 b	489,0	30,3	12,8	30,5
Intercropping	11,9 b	473,3	25,8	7,4	29,6
Ammonium	16,5 b	504,6	33,7	8,6	35,2
Ammonium + DMPP	12,0 b	453,2	19,3	9,2	41,7
Significance	**	*n.s.*	*n.s.*	*n.s.*	*n.s.*

Abbreviation and symbols: ns, ** = not significant, significant at p≤0,01, Means followed by the same letter in each column were not significantly different as per the SNK test.

Table 5. Organic acids concentration (mg g^{-1} PF) in root tissues sampled at the end of the experiment (july 2011). Data are means of seven replicates.

Treatments	Citric	Malic	Ascorbic	Total
CK	0,60ab	2,4 ab	0,05 ab	3,1 ab
Fe-EDDHA	0,39 c	2,1 ab	0,05 ab	2,5 b
Fe-EDTA	0,69 a	2,7 a	0,06 ab	3,5 a
Intercropping	0,49 bc	1,7 b	0,07 a	2,3 b
Ammonium	0,46 bc	1,9 b	0,03 b	2,4 b
Ammonium + DMPP	0,41 c	2,2 ab	0,03 b	2,6 b
Significance	**	**	*	**

Abbreviation and symbols: ns, ** = not significant, significant at p≤0.01. Means followed by the same letter in each column were not significantly different as per the SNK test.

Table 6. Macroelements (N, P, K, Ca and Mg) concentration in leaves (%) determined at the end of the experiment (july 2011). Data are means of seven replicates.

Treatments	N (%)	P (%)	K (%)	Ca (%)	Mg (%)
Control	2,1	0,21 a	0,59 b	2,3 ab	0,97 b
Fe-EDDHA	2,2	0,15 abc	0,73 a	1,6 c	0,61 c
Fe-EDTA	2,0	0,15 abc	0,59 b	2,1 b	0,99 b
Intercropping	2,3	0,18 ab	0,74 a	2,2 b	0,90 b
Ammonium	2,1	0,12 bc	0,54 b	2,4 ab	0,96 b
Ammonium + DMPP	2,1	0,09 c	0,56 b	2,5 a	1,09 a
Significance	n.s.	**	***	***	***

Abbreviation and symbols: *** = significant at $p \leq 0.001$ level.
Means followed by the same letter in each column were not significantly different as per the SNK test.

Table 7. Microelements (Fe, Mn, Cu and Zn) concentration in leaves ($mg\ kg^{-1}$) determined at the end of the experiment (july 2011). Data are means of seven replicates.

Treatments	Fe ($mg\ kg^{-1}$)	Mn ($mg\ kg^{-1}$)	Cu ($mg\ kg^{-1}$)	Zn ($mg\ kg^{-1}$)
Control	120,3 b	325,6 a	9,1	50,2 ab
Fe-EDDHA	238,0 a	240,5 b	9,7	46,4 ab
Fe-EDTA	303,4 a	272,9 ab	8,0	39,5 b
Intercropping	110,4 b	334,9 a	9,7	50,7 ab
Ammonium	105,2 b	352,3 a	10,1	54,7 a
Ammonium + DMPP	122,2 b	339,3 a	9,4	52,7 ab
Significance	***	**	n.s.	*

Abbreviation and symbols: ns, *, **, *** = not significant, significant at $p \leq 0.05$, significant at $p \leq 0.01$ and significant at $p \leq 0.001$ level, respectively. Means followed by the same letter in each column were not significantly different as per the SNK test.

Table 8. Net photosynthesis, A_n (µmol CO2 m^{-2} s^{-1}); stomatal conductance, g_s (mol H_2O m^{-2} s^{-1}); efficiency of excitation capture by open PSII reaction centers, Fv'/Fm' (relative units); efficiency of PSII photochemistry, Φ_{PSII} (relative units) and linear electron transport rate, ETR (relative units), measured in the first three mature leaves from the middle third shoot in 2010 season.

Treatments	A_n	g_s	Fv'/Fm'	Φ PSII	ETR
Control	7,7	0,24	0,47 a	0,19 ab	79,1 ab
Fe-EDDHA	6,8	0,27	0,47 a	0,19 ab	79,0 ab
Fe-EDTA	8,0	0,29	0,43 a	0,21 a	89,0 a
Intercropping	5,8	0,28	0,34 b	0,13 c	54,2 c
Ammonium	7,8	0,25	0,43 a	0,18 ab	78,3 ab
Ammonium + DMPP	6,5	0,27	0,42 a	0,15 bc	64,1 bc
Significance	n.s.	n.s.	***	**	*

Abbreviation and symbols: ns, *, **, *** = not significant, significant at $p \leq 0.05$, significant at $p \leq 0.01$ and significant at $p \leq 0.001$ level. Means followed by the same letter in each column were not significantly different as per the SNK test.

Table 9. Net photosynthesis, A_n (µmol CO2 m^{-2} s^{-1}); stomatal conductance, g_s (mol H_2O m^{-2} s^{-1}); efficiency of excitation capture by open PSII reaction centers, Fv'/Fm' (relative units); efficiency of PSII photochemistry, Φ_{PSII} (relative units); linear electron transport rate and ETR (relative units), measured in the first three mature leaves from the middle third shoot, in 2011 season.

Treatments	A_n	g_s	Fv'/Fm'	Φ PSII	ETR
Control	7,9 ab	0,10 b	0,53	0,20	86,9
Fe-EDDHA	7,8 ab	0,09 b	0,51	0,24	99,9
Fe-EDTA	7,0 b	0,10 b	0,51	0,20	86,7
Intercropping	11,2 a	0,19 a	0,53	0,27	114,2
Ammonium	8,2 ab	0,14 b	0,56	0,22	94,8
Ammonium + DMPP	8,3 ab	0,13 b	0,49	0,23	95,7
Significance	*	***	n.s.	n.s.	n.s.

Abbreviation and symbols: ns, *, *** = not significant, significant at $p \leq 0.05$ and significant at $p \leq 0.001$ level. Means followed by the same letter in each column were not significantly different as per the SNK test.

CHAPTER VI

GENERAL DISCUSSION AND CONCLUSIONS

This Doctoral Thesis studied fundamental and applied aspects of Fe nutrition in grapevine and assessed sustainable management techniques for Fe chlorosis prevention in vineyard ecosystems. Different experiments, conducted under controlled conditions, characterized response mechanisms to Fe-deficiency through agronomical, physiological and biochemical methodological approaches.

In Chapters III and IV the Fe chlorosis tolerance, in terms of physiological and biochemical mechanisms of Fe acquisition, have been studied in different genotypes. The Fe chlorosis tolerant genotype 140 Ruggeri (*Vitis berlandieri x Vitis rupestris*) strongly reacts to Fe-deficiency activating several response mechanisms. Under Fe-deficiency conditions, 140 Ruggeri genotype decreases the pH of the nutrient solution for increasing Fe availability in the substrate. In addition, 140 Ruggeri is able to increasing carbon fixation in roots by a higher activity of PEPCase and TCA enzymes at roots level leading to enhanced concentration of organic acids -overall citrate- in roots and xylem sap. However, when grapevine genotypes are submitted to a prolonged Fe-deficiency, a different behavior is exhibited according to the rootstock. Results obtained in this work evidenced that the duration and severity of Fe-deficiency stress modulate the intensity of biochemical responses at root level. The Fe chlorosis susceptible rootstock 101-14 (*Vitis riparia x Vitis rupestris*) and SO4 genotype (*Vitis berlandieri x Vitis riparia*) submitted to Fe-deficiency, showed a reduction in the activity of PEPC and MDH in roots. In contrast, 110 Richter rootstock (*Vitis berlandieri x Vitis rupestris*) is capable to maintain a high activity of these enzymes in roots. Interestingly, this work also indicates that the Fe chlorosis susceptible *Vitis riparia* rootstock is able to activate tolerance mechanisms to Fe-deficiency under soil conditions increasing the activity of PEPC in roots, an aspect scarcely reported in previous experiments conducted in soil conditions.

This investigation shows that root PEPC and TCA enzymes activities represent an important tool for screening Fe chlorosis tolerance in grapevine genotypes. Moreover, in

the Doctoral thesis it has been discussed that the different Fe chlorosis tolerance degree of *Vitis* hybrids is in part due to a differential ability of genotypes to synthesizing, transporting/exudating or utilizing organic acids in the roots as a response mechanism to Fe-deficiency. However, the high levels of organic acid accumulation recorded in 101-14 and SO4 genotypes after a prolonged exposure to Fe-deficiency suggest caution when using this parameter for screening Fe chlorosis tolerance, because this trait appear as a consequence of the scarce capability of roots to utilizing, transporting or exudating these compounds, rather than a reliable indicator of Fe chlorosis tolerance. In fact, the Fe chlorosis sensitive 101-14 genotype grown under Fe-shortage conditions accumulates high levels of citric acid at root level, indicating a low capacity to utilizing, transporting and/or exudating organic acids in the rhizosphere. In contrast, 110 Richter and SO4 rootstocks accumulate them to a lesser extent than 101-14. Additional physiological response mechanisms (FCR, exudation of phenols, etc) to a prolonged Fe-deficiency related to the reduction capacity of roots and exudation of organic compounds for different genotypes could contribute to explaining the different Fe chlorosis tolerance of these rootstocks.

The high variability observed in the Fe chlorosis tolerance for different genotypes is in line with the information reported in literature and the general behavior exhibited in the field by these rootstocks. Surely the genetic component of these hybrids is a preponderant factor modulating the response to Fe-deficiency. *Vitis berlandieri* species is classified as Fe chlorosis tolerant, whereas *Vitis riparia* is characterized by a high susceptibility to Fe chlorosis. *Vitis rupestris* genotype is classified as Fe chlorosis slightly susceptible. This work strongly suggests that 140 Ruggeri and 110 Richter rootstocks inherited the high tolerance to lime soils from *Vitis berlandieri*. In contrast, 101-14 inherited the lower capability to react under Fe-deficiency conditions from *Vitis riparia.* Noteworthy, the intermediate behavior that SO4 showed under a prolonged Fe-deficiency conditions suggests that this genotype exhibes characters from both ascendants species of *Vitis*. In this sense, this genotype probably inherited the enhanced ability to avoid excessive accumulation of organic acids in roots from *Vitis berlandieri* and the low capability to maintain an active organic acids synthesis under a prolonged Fe-deficiency from *Vitis riparia*.

The methodological approach adopted in this investigation has allowed to demonstrating that the presence of bicarbonate in the nutrient solution can modulate several Fe-deficiency response mechanisms, shifting the activity of PEPC and TCA cycle enzymes and the accumulation/translocation of organic acids in roots. It has been demonstrated that 140 Ruggeri genotype behaves as a calcicole plant, increasing the root biomass and accumulating malic acid in roots under high concentrations of bicarbonate in the substrate. This behavior could play a strong role in plant tolerance to bicarbonate excess. In addition, results obtained in the pH of xylem sap indicate that this genotype is able to load bicarbonate into the xylem vessels and transport it to the leaves enhancing the leaf chlorophyll content. This suggests a possible role of bicarbonate in chlorophyll degradation processes. Further researches will be needed to clarify the mechanisms employed by 140 Ruggeri rootstock for utilizing bicarbonate in the leaves, and the possible effects of grafting.

It is well known that the application of Fe chelates does not represent a sustainable management technique to prevent or cure Fe chlorosis in vineyards due to their costs and the environmental risks associated with their use. In the Chapter V of this Doctoral thesis, the effectiveness and physiological implications of alternative means for controlling Fe-deficiency chlorosis have been assessed in order to naturally enhance Fe availability in soil and plant. The investigation conducted in soil conditions, demonstrated that intercropping with *Festuca rubra* induces positive effects on chlorophyll content and improves the leaf photosynthesis inducing changes in leaf morphology. In addition, intercropping reduces the activity of PEPC in roots as Fe-chelate treatments. However, the adoption of this technique requires grasses properly selected and managed according to the crop and environment parameters. Applications of NH_4^+ with a nitrification inhibitor prevents efficiently Fe chlorosis, increases leaf chlorophyll content, and induces biochemical responses (enzymes activity and organic acids concentration) at root level similar to Fe-EDDHA applications. Without inhibiting soil nitrification processes, the effectiveness of NH_4^+ supply on Fe chlorosis prevention resulted significantly lower. In further researches, it will be particularly interesting to develop nitrification inhibitors from natural sources. Iron-chelates are the most efficient treatment, nevertheless, apart the economical and environmental implications, supply of Fe-EDDHA impairs Mn nutrition. In addition, the application of Fe-

EDTA to the canopy performed in this study indicate that the translocation of Fe from the leaves to the roots is restricted, possibly due to a scarce mobility of Fe from the leaves and/or to a phloem discontinuity originated by graft incompatibility phenomena.

In conclusion, the specific aspects intertwined in this investigation highlight the complexity of Fe physiology and the fine metabolic tuning of grapevine genotypes to Fe availability and soil-related environmental factors. The experimental evidences reveal the need to carry out future researches on Fe nutrition maintaining a continous flow of knowledge between theoretical and agronomical perspectives for fully supporting the efforts devoted to convert science into practice.

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