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CHANGES IN PLANT METABOLISM INDUCED BY DIOXYGENASE INHIBITORS AND THEIR EFFECT ON THE EPIPHYTIC MICROBIAL COMMUNITY AND FIRE BLIGHT (*ERWINIA AMYLOVORA*) CONTROL

Alterazioni del metabolismo della pianta indotte dagli inibitori delle diossigenasi e loro influenza sulla biocenosi microbica epifitica e sull'infezione da Colpo di Fuoco Batterico (*Erwinia amylovora*).

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Ai miei genitori e a mia sorella
a cui tanto devo

CONTENTS

PREFACE

Fire blight, caused by the gram negative bacterium *Erwinia amylovora*, is one of the most destructive bacterial diseases of *Pomaceous* plants. Therefore, the development of reliable methods to control this disease is desperately needed.
This research investigated the possibility to interfere, by altering plant metabolism, on the interactions occurring between *Erwinia amylovora*, the host plant and the epiphytic microbial community in order to obtain a more effective control of fire blight. Prohexadione-calcium and trinexapac-ethyl, two dioxygenase inhibitors, were chosen as a chemical tool to influence plant metabolism. These compounds inhibit the 2-oxoglutarate-dependent dioxygenases and, therefore, they greatly influence plant metabolism. Moreover, dioxygenase inhibitors were found to enhance plant resistance to a wide range of pathogens. In particular, dioxygenase inhibitors application seems a promising method to control fire blight. From cited literature, it is assumed that these compounds increase plant defence mainly by a transient alteration of flavonoids metabolism. We tried to demonstrate, that the reduction of susceptibility to disease could be partially due to an indirect influence on the microbial community established on plant surface.
The possibility to influence the interactions occurring in the epiphytic microbial community is particularly interesting, in fact, the relationships among different bacterial populations on plant surface is a key factor for a more effective biological control of plant diseases.
Furthermore, we evaluated the possibility to combine the application of dioxygenase inhibitors with biological control in order to develop an integrate strategy for control of fire blight.

The first step for this study was the isolation of a pathogenic strain of *E. amylovora*. In addition, we isolated different epiphytic bacteria, which respond to general requirements for biological control agents.
Successively, the effect of dioxygenase inhibitors treatment on microbial community was investigated on different plant organs (stigmas, nectaries and leaves). An increase in epiphytic microbial population was found. Further experiments were performed with aim to explain this effect. In particular, changes in sugar content of nectar were observed. These changes, decreasing the osmotic potential of nectar, might allow a more consistent growth of epiphytic bacteria on blossoms. On leaves were found similar differences as well.
As far as the interactions between *E. amylovora* and host plant, they were deeply investigated by advanced microscopical analysis. The influence of dioxygenase inhibitors and SAR inducers application on the infection process and migration of pathogen inside different plant tissues was studied. These microscopical techniques, combined with the use of *gpf*-labelled *E. amylovora*, allowed the development of a bioassay method for resistance inducers efficacy screening.
The final part of the work demonstrated that the reduction of disease susceptibility observed in plants treated with prohexadione-calcium is mainly due to the accumulation of a novel phytoalexins: luteoforol. This 3-deoxyflavonoid was proven to have a strong antimicrobial activity.

Keywords: fire blight, induced resistance, biological control, dioxygenase inhibitors, confocale laser scanning microscope, *gfp*-labelled bacteria, prohexadione-calcium, luteoforol.

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INTRODUCTION

FIRE BLIGHT

1. INTRODUCTION

Erwinia amylovora, a gram negative bacterium, is the causative agent of fire blight (Burrill 1883). This bacterium infects most of the plants belonging to family of *Rosaceae* and in particularly to the subfamily of *Pomoideae* such as *Cotoneater*, *Crateagus* and *Pyracantha*, even if, economically, the most important host plants are apple and pears (Eden-Green and Billing, 1974).
The bacterium penetrates in the plant trough flowers, but it can also enter via wounds and trough natural openings in the plant cuticle. *E. amylovora* can invade the whole tree solely by internal progression through the host tissues; thus, a single infection can potentially kill a tree (Vanneste, 1995). This aspect renders fire blight the most devastating bacterial disease of apples and pears. Production of bacterial exudate on the surface of infected tissues is the most characteristic symptom of fire blight (Bennet and Billing, 1980b). Other typical symptoms are the wilting and the consequent necrosis of the infected tissues. The symptomatology of fire blight is rather complicate with different symptoms in relation to different plant parts (blossom blight, shoot blight, overwintering cankers…).
The severity of fire blight is dependent on the host plant and on the environmental conditions. Generally, pears are more susceptible than apple (Eastgate, 2000). High humidity and warm temperature are favourable to the disease development. The blossoms are particularly sensitive to infection, even if in high susceptible host plants, the bacterium can infect also mature tissues (Eden-Green and Billing, 1974).
The economic impact of fire blight is rather high, in fact a severe outbreak can disrupt the production for several years, moreover it

limits the areas where most susceptible and economically interesting apple and pear varieties can be grown. Furthermore, its economical importance is likely to increase for several reasons. Firstly, fire blight is still spreading geographically into new apple- and pear- growing areas (Vanneste, 2000). Secondly, with the exception of streptomycin, there is no registered product that can effectively control fire blight. Finally, today, apple plantings are mainly constituted by susceptible cultivars grafted on susceptible rootstock planted in high-density orchards (Longstroth, 2001). These changes, together with the ever-increasing developments of streptomycin resistant strains of *E. amylovora*, stimulate the research of reliable strategies to control fire blight.

2. THE PATHOGEN: *ERWINIA AMYLOVORA*

2.1. Biology and metabolism

According to the *Bergey's Manual of Systematic Bacteriology*, 8th edition (Krieg and Holt, 1984), *Erwinia amylovora* is a Gram-negative bacteria belonging to the class of *Enterobacteriaceae*. It was the first bacterium identified as a plant pathogen (Burrill, 1883). The bacterial cell is a rod with 2-7 perithricous flagella and it may be surrounded by an exopolysaccharide capsule (EPS), which is visible with the electron microscope (Bennet and Billing, 1978) (fig.1). *E. amylovora* moves using these flagella and the motility seems important during the infection process. In fact, Bayot and Ries (1986) demonstrate that apple blossoms sprayed with a suspension of motile *E. amylovora* cell developed a higher incidence than blossom sprayed with non-

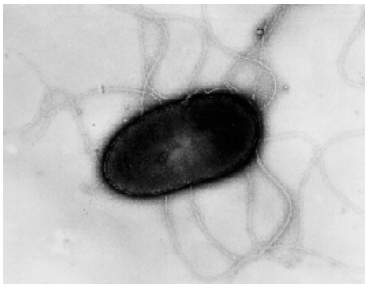

Figure 1: *E. amylovora* cell (TEM 100000 x)

motile cells. Moreover, the *E. amylovora* shows a positive chemiotaxis for aspartate and for fumarate, malate, maleate, malonate, oxoacetate and succinate (Raymundo and Reis 1980b). It is particularly interesting that these organic acids are present in the nectar from apple flowers. Consequently, it seems that *E. amylovora* could be attracted to the nectar and move toward the nectar cup in which there are the nectarhodes: the main sites of penetration for the pathogen inside the plant tissues. The motility is strictly related with the environmental conditions. Even the synthesis of flagella is related with the temperature and it has an optimum of 18-25°C (Raymundo and Reis 1980a, 1981).

The motility is higher in presence of an abundant carbon source, at pH 6.9 and in presence of chelanting agents such as ethylenediaminetetra-acetic acid (EDTA) (Raymundo and Reis 1980a, 1981). Even if the movement of *E. amylovora* is possible in anaerobic conditions in presence of an abundant suitable source of carbon, it has never been observed in the intercellular space of infected plant tissues.

The bacterial capsule is made mainly by two different kind of polysaccharides: amilovoran and levan. Amylovoran is composed of galactose, glucose, mannose and uronic acid, whereas levan is a polyfructose. Several studies suggest an important role of the EPS during the pathogenesis process (Bennet and Billing 1978, 1980a; Ayers *et al.* 1979; Goodman *et al.,* 1987; Geider *et al*. 1990-1992).

E. amylovora is able to grow between 3-5° C and 37° C, but the optimal growing temperature is 25-27° C (Billing et al., 1961); whereas, for natural infection, the optimal temperature occurs between 18° C and 30° C (Billing, 1992).

E. amylovora is a facultative anaerobe bacterium. Neither in aerobic nor in anaerobic conditions it produces gas form glucose, but only acid. Moreover, it is a weak fermentative bacterium (Holt *et al.* 1994).

In vitro, *E. amylovora* shows a high capability to fit to any differences in the nutritional status of the environment (Farrago and Gibbins, 1975). The glucose metabolism, in anaerobic conditions, results, as end-products, mainly in ethanol and carbon dioxide with

small amounts of lactic acid, acetic acid, succinic acid, formic acid, acetoin and 2,3-butanediol (Sutton and Starr 1959-60).
As far as nitrogen metabolism, *E. amylovora* differs from most of the *Erterobacteriacae*, since it does not reduce nitrate to nitrite.
E. amylovora can use the aspartate as nitrogen source, interestingly the aspartate represents the 58% of total amino acids in apple shoots (Lewis and Tolbert, 1964). Moreover, for growth *E. amylovora* needs nicotinic acid (Starr and Mandel 1950) and no other growth factors are needed. Only some strains, cured of pEA29 plasmid need also thiamine as growth factor. In fact, this plasmid, almost ubiquitous in *E. amylovora* strains(Falkenstein *et al.* 1989, Laurent *et al.* 1989), is involved in thiamine metabolism (Bennet and Billing, 1978; Laurent *et al.* 1989; Bereswill *et al*. 1992). This plasmid seems also play a quantitative role in pathogenicity (Laurent *et al.* 1989).
E. amylovora secretes different kinds of extracellular enzymes: a β-glucosidase, proteases and some hydrolytic enzymes.
E. amylovora shows only a weak β-glucosidase activity (Hildebrant and Schroth, 1965). This enzyme catalyses the reaction from arbutin, a compounds common in pears, to glucose and hydroquinone and the end-product is toxic to several bacteria (Hildebrant and Schroth, 1963) and to *E. amylovora* as well (Berg and Gibbins, 1983).
The presence of exogenous β-glucoside, such arbutin in pear tissues, could act, after transformation in hydroquinone as a plant defence mechanism. A similar role is supposed for phloridzin in apple tissues (Gibbins 1972).
Furthermore, *E. amylovora*, as the most part of "necrogens" bacteria, is able to produce a detectable amount of hydrolytic enzymes (Seemuller and Beer 1976). Nevertheless, no cellulolytic, pectolytic or xylolitic activity was detected. The role of these enzymes in the infection process has not been yet established. *E. amylovora* produces also two neutral proteases. These proteases have been isolated from the ooze and from infected plant tissues (Seemuller and Beer 1977). Finally, *E. amylovora* produces also two different molecules putatively identified as toxic factors.
The first molecule, which is 6-thioguanine, does not show any toxic effect on pear cell cultures (Feistner and Staub, 1986). The second is

(L)-2,5-dihydrophenylalanine (DHP) that is a real necrotoxin (Feistner, 1988). The mode of action of DHP is not completely clear. It can act killing directly the plant cells or it can block the hypersentitive reaction (HR). According to Schwartz *et al.*, (1991), the toxicity of DHP is due to its inhibition of the shikimic acid pathway in plant cells

Since not all the *E. amylovora* strains produce this toxin, it seems an incidental virulence factor more than a key factor necessary for pathogenesis (Geider *et al.,* 90).

2.2. Plasmids

None of the several plasmids found in *E. amylovora* (Marçais *et al.*1990; Laurent *et al.*, 1989; Panopoulos, 1978; Merckaert *et al.*, 1982) seem strictly involved in pathogenicity. Only one plasmid of ca. 30 Kb, named pEA29, is almost ubiquitous in all *E. amylovora* strains (Marçais *et al.*1990, Laurent *et al.*, 1989; Merckaert *et al.*, 1982; Vanneste *et al.*, 1985; Falkenstein *et al.*, 1988). This plasmid can not be transmitted by conjugation (Verdonck *et al.*, 1987) and it is particularly stable and resistant to the classical physical or chemical methods for curing (Laurent *et al.*, 1989).

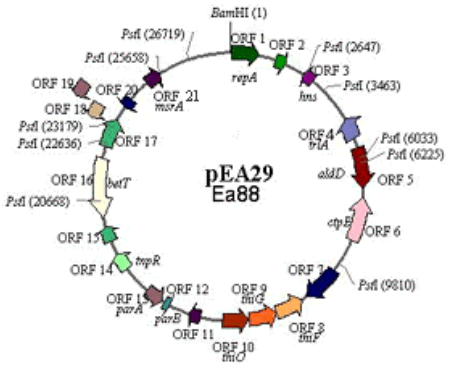

Figure 2: the structure of the ubiquitous plasmid pEA29
(from www.plantpathology.msu.edu)

In *E. amylovora*, the resistance to streptomycin is, in most of cases, chromosomally encoded (Panopoulos, 1978, Chiou and Jones, 1991; Thomson *et al.*, 1993; Schroth *et al.*, 1979; Minsavage *et al.*, 1990; Vanneste and Yu, 1993) even if, it could be plasmid encoded as well (Chiou and Jones, 1991). In fact, the resistance to streptomycin can be encoded by a 34 Kb plasmid, known as pEA34 (Chiou and Jones, 1991). The plasmid pEA34 carries the genes *strA* and *strB* which are

part of a transposon called *Tn5393* (Chiou and Jones, 1993). The streptomycin resistance genes are homologous to the ones isolated in several other bacterial species (Chiou and Jones, 1993; Norelli *et al.*, 1991).

2.3. Outer membrane and capsule of *Erwinia amylovora*

Outer membrane of *E. amylovora* is formed by lipopolysaccharide (LPS). LPS is characteristic of all the gram-negative bacteria. Since it is part of the outer membrane (fig. 3), it might be involved in the plant-microbe interaction (Vanneste, 1995). In addition, *E. amylovora* outer membrane in protected by a capsule, which is formed mainly by two exopolysaccharides (EPS): the homopolymer levan and the heteopolymer amylovoran.

Most of the structural genes involved in EPS synthesis are located in the *ams* region of bacterial chromosome (Burget and Geider, 1995). Numerous evidences suggest that the *ams* gene cluster is regulated in response to environmental stimuli (Burgert and Geider, 1995, Burgert and Geider, 1997, Ilan *et al.*, 1999).

EPS capsule may allow the bacteria to elude the plant defence mechanisms and it seem involved in obtainment of nutrients from the plant cells (Belleman *et al.* 1990, Coplin e Cook, 1990) even if, as demonstrated by Brisset and Paulin (1992), *E. amylovora* strains EPS deficient have still the ability to induce electrolyte leakage from plant cells. This capacity is a key step in pathogenesis, in fact, it allows the bacteria to multiply inside the plant environment.

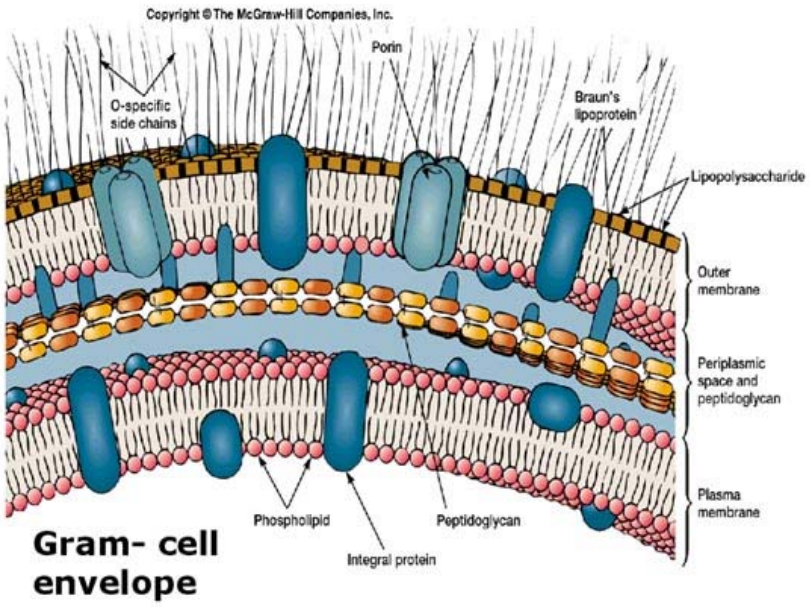

Figure 3: general structure of the cell envelope of a Gram-negative bacterium (from www.arches.uga.edu)

Levan is a β-2,6-linked polyfructan synthesised from sucrose by the extracellular enzyme levan sucrase (Gross *et al.*, 1992). Thus, levan is produced only when sucrose is present. Mutant strains unable to produce levan result as virulent as the wild strains, thus levan does not seem a key factor for pathogenicity (Eden-Green and Billing, 1974). Moreover, bacteria isolated from infected plant tissues do not usually present levan in the capsule (Bennet and Billing, 1980b; Eden-Green and Knee, 1974).

The synthesis of levan from sucrose may allow the bacteria to survive and multiply in the nectar solution: the transformation of sucrose in levan may result in a reduction of the osmotic potential to a suitable level for bacterial growth (Gross *et al.*, 1992). Some Authors (Geier and Geider, 1993) observed that strains levan deficient are only affected in virulence.

Amylovoran is an acidic etheropolysaccharide primarily containing galactose and glucuronic acid (Nimtz *et al.,* 1996; Smith *et al.,* 1990). The molecular weigh is around 10^6 Da and it contains approximately 1000 sugar subunits per molecule (Jumel *et al.,*1997).

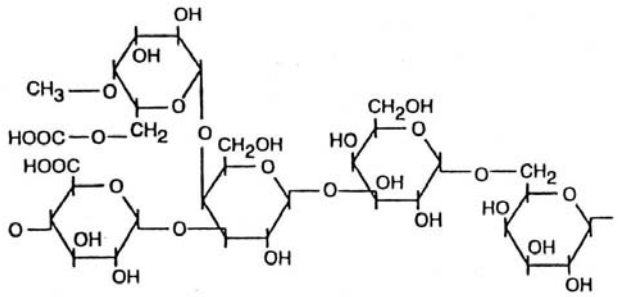

Figure 4: structure of amylovoran as determined by Smith *et al.*, 1990. Galactose, glucuronic-acid, acetate and pyruvate are in a molar ratio of 4:1:1:1. Though no glucose residues have been presented, amylovoran most probably contains some glucose (from Vanneste, 1995).

The ability to produce amylovoran is a key factor for the pathogenesis: strains amylovoran deficient are not pathogenic (Steinberger and Beer, 1998; Berhrad *et al.*, 1993). In fact, they do not multiply in plant tissues (Belleman and Geider, 1992) and they do not move in the xylem (Bog *et al.*, 1998).

Amylovoran could be tightly linked to the bacterial cell, forming the capsule, or freely released into the environment, producing the typical bacterial slime (Politis and Goodman, 1980). On artificial medium, containing sugar or sugar-alcohol, *E. amylovora* produces both capsulated and non- capsulated cells. Whereas, bacteria isolated from infected plants are usually embedded in EPS capsule.

As already mentioned, amylovoran, even if is not itself toxic, is required for pathogenicity (Eden-Green and Knee 1974, Sjulin and Beer, 1977). In fact, amylovoran-formed capsule is required for symptoms expression (Belleman and Geider, 1992; Ayers *et al.*, 1979; Vanneste *et al.*, 1990; Steinberg and Beer, 1988). Furthermore, amylovoran is involved in the pathogenesis also as slime: strains able to produce a high amount of slime result more virulent than strains

producing less amylovoran (Ayers *et al.*, 1979).
Several functions for amylovoran have been proposed. Firstly, it may act as a physical barrier avoiding the agglutination inside plant tissues (Romeiro *et al.*, 1981a-b). In fact, in comparison with the capsulated cell, the non-capsulated ones are agglutinated more frequently by malin, which is a small protein found in apple tissues (Romeiro *et al.*, 1981b). Malin seems to interact with the LPS that, in non-capsulated cells, is exposed on the bacterial surface (Romeiro *et al.*, 1981 a). This phenomenon could explain the absence of non-capsulated cells in plant tissues.
Secondly, amylovoran may be involved in the migration inside plant tissues. In fact, it can absorb water and swell up, pushing the bacteria inside the plant tissues through the path of less resistance (Eden-Green and Billing, 1974). This would explain the mass migration of bacteria in the cortical tissues (Vanneste, 1995). Also the production of exudates on the surface of the infected plants can be due to this mechanism (Schouten 1988-89).
Thirdly, the wilting of shoots seems due to the disruption of the water flux in the xylem due to *E. amylovora* accumulation (Goodman *et al.,* 1987). The localization in xylematic vessels can produce bacterial aggregates that stuck in the vessel obstructing the water flux (Sjulin and Beer, 1977; Goodman *et al.,* 1987). Occlusion of the xylem is a consequence both of bacterial multiplication (with a increased bacterial density) and EPS interaction with xylogucan and pectine, which are linked to plant cell walls (Goodman *et al.,* 1987). After the fissuring xylematic vessel walls, the bacteria can be forced in the parenchyma and finally they are extruded on plant surface.
These droplets of the exudate are important in the diffusion of bacteria and in epidemiology of fire blight (Bennet and Billing, 1978; Ayers *et al.*, 1979).

2.4. Virulence factors of *Erwinia amylovora*

Differently from other necrogenic bacteria, *Erwinia amylovora* does not secret any pectinolytic enzymes (Seemuller and Beer, 1976). Moreover, it does not produce any important toxin, nevertheless, two main factors are certainly involved in pathogenesis: the EPS (Bennet and Billin, 1980a) and the hypersensitive response proteins encoded by the *hrp* gene cluster (Steinberg and Beer, 1988).
Several factors, even if not directly involved in pathogenesis, are required for host tissues colonisation. These factors permit *E. amylovora* to overcome the lack of nutrients in the plant apoplast allowing the rapid bacterial multiplication necessary to permeate plant defences (Eastgate *et al.,* 1997).

2.4.1. EPS and amylovoran

The functions of EPS have been already mentioned in section 2.3. The effective role of EPS during the infection process has not been completely understood.
EPS may play several functions:

- probably it is involved in the trick out of plant defences by masking the bacterial cell surface elicitors.
- It has been supposed to be an external storage system for water (Langlotz and Geider, unpublished) and energy
- It could be responsible for the plant cells collapse and tissue distruption (Vanneste 1995)
- EPS, after hydratation and reaction with some plant compounds, can act a pressure that facilitates bacterial migration and extrusion of the bacteria in plant parenchyma.

2.4.2. *Hrp* genes

The *hrp* genes are common is several erwinias (Coplin *et al.* 1992; Laby and Beer 1992; Bauer *et al.*, 1994; Cui *et al.*, 1996; Nizan *et al.* 1997) and some Authors supposed that they are basic components of erwinias pathogenicity (Kim and Beer, 2000).
Hrp genes are located in a ca. 20-25 Kb region of DNA. The cluster consists in 8 complementation groups involved in the production and secretion of a HR elicitor protein known as harpin (Wei and Beer,

1993). The proteins encoded by these genes are necessary to induce both the hypersensitive reaction in non-host plants and symptoms development in susceptible plants (Beer *et al.*, 1991).

Insert 1: Hypersensitive Reaction (HR)

Most of the gram negative bacteria, when infiltrate in the intercellular space of a non-host plant, give a hypersensitive reaction (Klement 1982; Goodman Novacky 1994). The HR is a rapid localized defence response characterized by the collapse and death of cells in the plant tissue surrounding the infection site. The reaction is due to rapid K^+/H^+ exchange leading to the cell death and consequent release of toxic compounds. Macroscopically this reactions leads to withered area at the infiltration site.

HR to occur needs a high number of bacteria (at least $5x10^6$ cfu/ml), even if a single bacterium can induce HR on a single plant cell (Turner and Novacky, 1974). The contact between the bacterial and the plant cells is needed (Holliday *et al,.* 1981).

Several studies proved that the same genes involved in the HR are needed to develop the symptomatology in host plants (Lindergren *et al.,* 1986).

The proteins encoded by the *hrp* genes are involved in the production and secretion of harpin.

Some of them encode for proteins necessary for a type III secretion pathway and several of them have been characterized in *E. amylovora*.

This secretion pathway is used to export the virulence-associate molecules, such as harpin, directly into the plant cell. The type III secretion apparatus consists in a pilus-like structure extruded on the bacterial surface (Bogdanove *et al.*, 1996).

Some of the *hrp* genes characterized in *E. amylovora* are reported in the following list:

- *HrpV* probably encodes for an inner membrane component of the type III apparatus and it is essential for its functions (Alfano and Collmer, 1997; Wei and Beer, 1993).

- *HrpC, J* and *T* encode for proteins forming the outmembrane apparatus of the type III secretion. In particular, hrpC seems involved in the translocation of molecules across the outer membrane. It probably acts by assembling a multimeric channel trough the bacterial membrane (Alfano and Collmer, 1997; Kim *et al.* 1997). HrpJ may act as an extracellular sensor important in a contact-dependent expression and secretion of virulence factors (Bogdanove *et al.*, 1996).
- *HrpL* encodes for a regulatory protein needed for the expression of the other *hrp* loci (Wei and Beer; 1995). HrpL belongs to the ECF (**e**xtra**c**ytoplasmatic **f**unctions) subfamily of eubacterial σ factors. This factor is able to recognize the *hrp* boxes, which are conserved promoter sequences. As a σ factors, the *HrpL* bound with the RNA polymerase and induces the expression of the sequences promoted by the *hrp* boxes. *HrpL* is regulated, in response to the environmental stimuli, via the σ^{54}/hrpS system (Frederick *et al.*, 1993; Wei and Beer 1995). HrpS is a σ^{54}-dependent enhancer protein, which modulates the expression of *hrpL*. Moreover, two other regulatory proteins, HrpX and HrpY, activate expression of *hrpL* (Wei *et al.*, 2000). It has been suggested activation, via phospsorylation, of HrpY by HrpX that is a sensor kinase associated to the cell membrane. According to this model, HrpS is a positive regulator of *HrpL* transcription, whereas HrpX and Y modulate the expression levels (Wei *et al.*, 2000).
- *HrpN* encodes for harpin, which is necessary to induce the HR in incompatible plants (Wei *et al*. 1992a).

Harpin, which is a 37 kDa, glycine-rich, heat stable protein that lacks in cysteine, is encoded by *hrpN*. Since Harpin is responsible of HR in non-host plant (Wei et al., 1992a-b), it elicits the rapid K^+/H^+ exchange (Popham *et al.,* 1995) and the production of active oxygen species (Baker *et al.*, 1993), which lead to disruption of mitochondrial functions with the consequent programmed cell death (Xie and Chen, 2000). Even if the role of harpin during the HR is clear, it has not been established its function in the pathogenesis. In

fact, *hrpN*⁻ mutants, in some extent, can still produce disease symptoms (Barney, 1995).
Also an other group of genes is involved in pathogenicity and in host-pathogen specificity. These genes grouped in the *dsp* (disease specific) locus are required for the pathogenesis, but not for the HR (Vanneste 1995). Bogdanove *et al.* (1998b) suggested that the *dsp* genes are associated with the *hrp* and coregualted by hrpL. The two proteins encoded by the genes at the *dsp* locus are dspA/E and dspB/F and they are both required for pathogenicity (Gaudriault *et al.*, 1997). DspA/E is secreted by the type III secretion system Bogdanove *et al.* (1998a), whereas DspB/F is supposed to act as a chaperon ensuring an efficient secretion of dspA/B (Gaudriault *et al.*, 1997). Probably DspA/E is involved in the disruption of host cell functions.

2.5. Host Specificity

E. amylovora is a highly homogenous species, in which no subgroups based on host range, could be distinguished (Vanneste, 1995); thus, strains isolated from different host plants do not present any substantial difference. Moreover, any *E. amylovora* isolate is able to induce disease symptoms in all fire blight host plants.
E. amylovora infects the members of *Pomeoidea* tribe, even if some exceptions are known. For example, the *Rubus* plants (raspberry and blackberry), which do not belong to the *Pomoideae*, can be infected by some *E. amylovora* strains (Starr *et al.*, 1951; Ries and Otterbacher, 1977; Leheman 1933; Folsom 1947; Heimann and Worf 1985). Neither the genetic, nor the biochemical bases for these differences in host specificity are known (Vanneste, 1995).
Under high artificial inoculation, *E. amylovora* can induce symptoms also on other atypical host plants, such as strawberry and roses (Pierstorff 1931; Koldewey, 2002).

3. DISEASE CYCLE

The disease cycle is exemplified in figure 5. The overwintering cankers are the most probable origin of inoculum to start the spring cycle. From them the bacteria spread to open flowers (Thomson, 2000). The bacterium may be disseminated by rain, insects and also birds (Meijneke, 1974; Seidel *et al.*, 1994). The arrival of the pathogen on flowers allows a rapid multiplication and dispersion to other flowers. Also in secondary dissemination, both rain and insect play an important role (Thomson, 2000). Successively, the bacterium can infect plants also through wounds and natural opening on the plant surface.

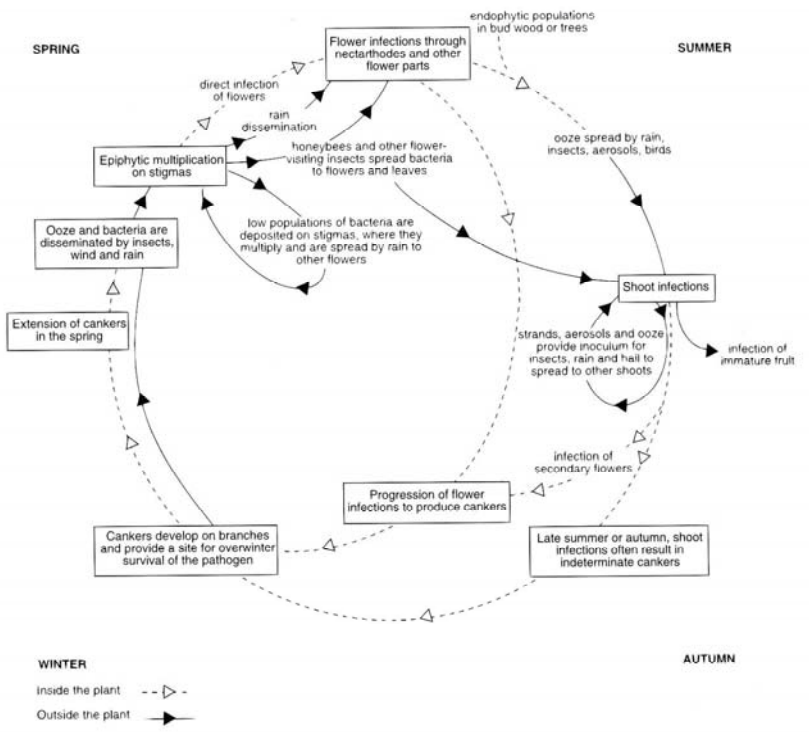

Figure 5: disease cycle (from Thomson 2000, In: Vanneste (ed.) Fire blight. The disease and its causative agent *Erwinia amylovora*. CABI publishing.

3.1. Infection

Erwinia amylovora, as most of the plant pathogenic bacteria, is unable to penetrate in host plants through the unwounded cutinised tissues. Thus, the bacterium exploits for penetration the wounds on the aerial part of the plant and all the natural openings in the cuticle, such as stomata and hydathoids.

As far as *E. amylovora*, stomata and hydathoids do not represent important sites for the penetration, whereas the nectarhodes are the main sites of infection.

In fact, the primary and most important infection sites are the blossoms (Eden-Green and Billing, 1974; Mazzucchi, 1992; Wilson and Lindow, 1993; Johnson and Stockwell, 1998).

As suggested by Thomson (1986), the penetration of the bacterium probably follows different key steps (fig. 6):

1. the bacterium reach the stigma transported by insect, rainfall, and pollen.
2. successively, it multiplies on the stigmatic surface and reaches a high population level. During this phase *E. amylovora* grows as an epiphyte. In fact, the stigmatic surface is humid and rich of nutrients, thus it is the only aerial part of the plant that allows the epiphytic multiplication of *E. amylovora*. This fact can explain why it is possible find a high population of bacteria also on healthy flowers (Miller and Schroth, 1972). Using a scanning electron microscope, (Hattingh *et al.* 1986; Wilson *et al.* 1990b) established that, on the stigma, bacteria are localized mainly among the secretory papillae.
3. then the bacterium moves along the stigmatic pedicel (Thomson, 1986) to the nectaries. Several factors are involved in this process. According to Thomson (1986), rain is the main factor responsible for *E. amylovora* movement to nectaries where infection could take place. Wilson *et al.* (1989b) showed how, under high humidity, the abundant stigmatic secretion can carry the bacteria to nectaries. Furthermore, the bacterial motility seems facilitate the

movement along the stigmatic pedicel (Bayot *et al.,* 1986). These Authors demonstrated that flowers sprayed with motile *E. amylovora* strain showed a higher incidence than the ones sprayed with non-motile mutants. According to Raymundo *et al.* (1980a), *E. amylovora* has also a positive chemotaxis to some of the organic acid, such as malic acid and tartaric acid, present in the apple nectar. Only few Authors (Pierstorff, 1931; Rosen 1936) suggested that the bacterium penetrates in the stigma and moves inside it to the nectaries.

4. Finally, the bacterium penetrates through the nectarhodes (Wilson *et al.*, 1990b). The nectarhodes are stomate-like openings, from where the nectar is secreted. Therefore, they allow direct access to plant internal tissues. Several studies suggest that in the nectaries the readily availability of nutrients increases the *E. amylovora* growth (Wilson *et al.*, 1989). This further bacterial multiplication in the nectaries seems needed for infection (Pierstorff 1931, Rosen, 1936; Hildedrand, 1937; Thomson, 1986; Wilson *et al.*, 1990b; Campbell *et al.*, 1991; Ivanoff and Kiett, 1941). In pear, the nectaries are full exposed and easily reachable for bacteria, whereas in apple, they are protected by trichomes and thus, they are much less accessible (Rosen, 1936). Finally, even if most of the studies (Baker, 1971; Wilson *et al.,* 1989 a-b, Thomson, 1986) showed that the main penetration sites for *E. amylovora* are the nectarhodes, different Authors (Rosen, 1936; Hildebrand, 1937; Wilson *et al.*, 19898a) suggested that the pathogen could enter through other parts of the flower. Hence, *E. amylovora* is probably able to exploit all the weakness present in the tissues of a particular flower (Vanneste, 1995).

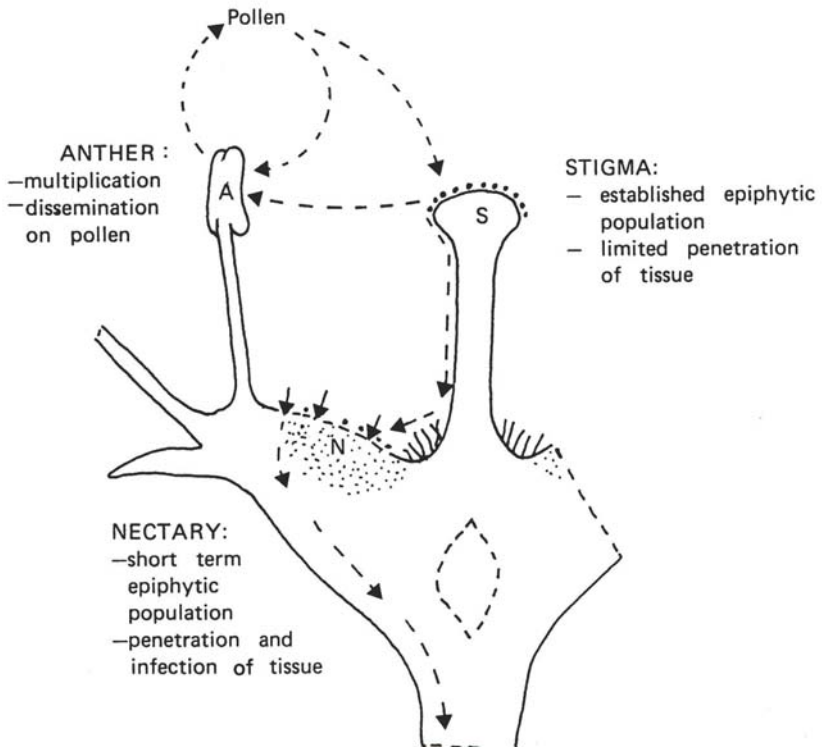

Figure 6: blossom infection (from Sigee, 1993, Bacterial Plant Pathology: Cell and Molecular Aspects. Cambridge University press).

E. amylovora can colonize also the anthers, but infection through the pedicel seems to be unlikely (Wilson *et al.*, 1989a). Nevertheless, the colonization of anthers, leading to infected pollen, could be important for pathogen dissemination (Wilson *et al.*, 1989a).
Several environmental factors influence the infection of blossoms: particularly important are temperature and humidity.
The temperature greatly influences the disease development (Thomson *et al.*, 1982). Primarily, a warm temperature enhances the bacterial multiplication (Zoller and Sisevich, 1979; Thomson *et al.*, 1982). Secondly, warm weather conditions allow a more intense activity of pollinating insects resulting in faster bacterial

dissemination from colonized flowers to non-colonized ones (Thomson *et al.*, 1975; Nuclo *et al.*, 1998; Hildebrand and Phillips, 1936; Johnson *et al.*, 1993; Keitt and Ivanoff, 1941; Matteson *et al.*, 1997; Pierstorff and Lamb, 1934; Van Laere *et al*, '81). In particular, honeybees (*Apis mellifera*) are active only at temperature higher than 12-14°C (Vicens and Bosch, 2000).

Therefore, a high temperature (T > 15°C) during the blooming period, promoting both the bacterial multiplication and the pollinators activity, represents a risk factor for the infection.

In general, a high humidity is favourable to the infection, because it allows a longer and more abundant colonization of the plant surface. Furthermore, the presence of water is necessary for the migration of bacteria from stigma to nectaries and for the growth in the nectar (Billing, 1976; Smith, 1990; Smith, 1996; Steiner, 1990; Thomson *et al.*, 1982). Indeed, water decreases the osmotic potential of the nectar to a suitable level for bacterial survival (Pusey, 1999).

It is particularly interesting that, during the secondary bloom, which usually happens between mid- spring and early summer, both a high temperature and humidity occurs. Considering these conditions, it is possible to understand why plants are more susceptible in that period (Covey, 1988; Schroth *et al.*, 1974). Nevertheless, the relative importance of the factors that make the secondary bloom so favourable for the disease development has not yet completely understood (Johnson and Stockwell 1998).

Figure 7: bacterial cells (b) in the intercellular spaces (i) surrounding the stigmatic papillae (c) at 24h after inoculation. (x2230) (from Wilson *et al.*, 1989b)

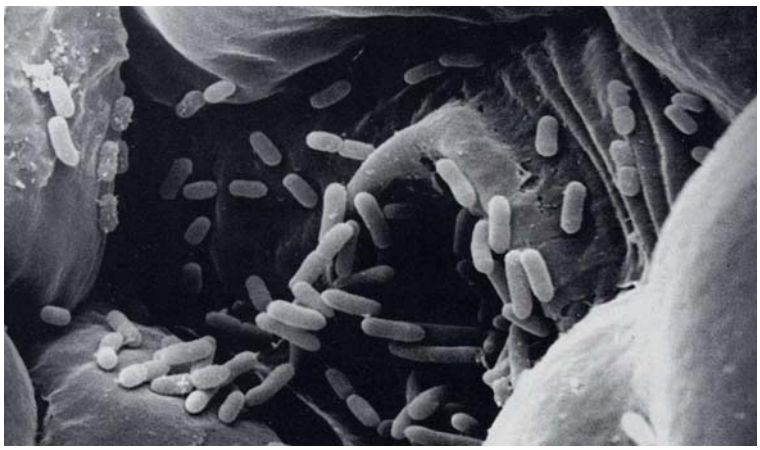

Figure 8: bacteria between the nectarhodal guard cell 24h after inoculation (x7500) (from Wilson *et al.*, 1990b)

3.2. Migration inside the plant tissues and symptoms development

How *E. amylovora* moves inside the plant is still debated. Some Authors (Eden-Green and Billing, 1974; Hokenhull, 1974) suggest a migration through the cortical parenchyma, other (Suhayada 1981; Goodman and White 1981) indicate a movement mainly localized in the xylem. These contrasting results may be explained with a different way of inoculation. When the inoculation is made cutting a leaf of a fresh tissues and infecting the cut edge, bacteria multiply in the xylem (Crosse *et al.*, 1972; Eden-Green and Billing 1974; Hockenhull 1979). On the contrary, when the inoculation is performed applying bacteria to the petioles, they move both in the cortical parenchyma and in the xylem (Huag and Goodman, 1976).

During the interaction of a necrogen bacterium with a compatible plant, one of the early events is the electrolytes leakage from the plant tissues (Vanneste, 1995). The electrolytes leakage is a fundamental step of infection process, since it allows the multiplication of the pathogen inside the plant (Youle and Cooper, 1987). In fact, this event releases nutrients from plant cells to the apoplast rendering them available for bacterial multiplication. As far as *E. amylovora*, both virulent and non-virulent strains induce this phenomenon when infiltrated in apple leaves (Burkowicz and Goodman, 1969). Moreover, both strains cause disorganization of the cellular membrane and organelles of apple leaf cells.

Harpin seems involved in the mechanism that leads to the electrolytes leakage, even if the way in which harpin acts is not yet completely understood.

Non-capsulated strains are still able to induce electrolytes leakage (Hignett and Roberts, 1985), but they don't kill the plant cells (Mansfiled *et al.*, 1995). Thus, the cell death does not seem a consequence of the electrolytes leakage.

According to Hignett and Roberts (1985), the high virulence of some strains could be explained with a more intense electrolytes leakage, which leads to a more rapid increase of bacterial population. The symptoms development, which occurs only when the bacterial population reach a high level, is consequent to the infection

(Goodman *et al.*, 1990, Klement *et al.*, 1966).
The macroscopical symptoms are mainly due to cell collapse and tissue decompartmentalization (Eden-Green and Billing, 1974). In particular, the wilting of the infected plant tissues may be an effect of two different mechanisms. On one side, amylovoran plugs the xylem vessels disrupting the water flux in the plant; on the other side, harpin induces electrolytes leakage by altering cell membrane integrity (Sjulin and Beer, 1978).
An other peculiar symptom of fire blight is the production of ooze on the surface of infected plant parts.
The exudate is constituted by bacterial cell embedded with EPS. EPS role in symptom development as been previously discussed.

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Figure 9: overwintering canker
Figure 10: shoot blight
Figure 11: blossom blight

CONTROL OF FIRE BLIGHT

1. CHEMICAL CONTROL

The chemical control acts both eliminating or inactivating the plant pathogenic bacteria before the penetration in the host tissues and rendering the plant surface unfavourable for the establishment of new infections (Psallidas and Tsiantos, 2000).
As far as fire blight, the control strategies have to achieve the following results:

1. destroy the primary source of inoculum, e.g. the overwintering cankers and the alternative hosts
2. protect the most susceptible parts of the plant, such as the blossoms, stomata, nectarhodes, lenticels and wounds.

Thus, to obtain the maximum of protection the plant should be treated in three different periods (Psallidas and Tsiantos, 2000): when the plant is dormant, during the blooming and, finally, during the vegetative growth. Since risk of phytotoxicity is low during dormancy, it is recommended the use of high concentrations of chemicals to enhance their activity and persistence (Psallidas and Tsiantos, 2000). On the other hand, during the blooming period, for the chemical applications lowest effective concentrations should be used, in fact buds, blossoms and young leaves are particularly susceptible to phytotoxicity.
Finally, chemical applications during summer have the aim to prevent both infection of the wounded plant parts and secondary blossoms, which are frequent in some pear variety.
Almost all the bactericides available against fire blight are preventive and not curative (Psallidas and Tsiantos, 2000). Few exceptions are known, streptomycin, for example, seems to have, in a certain extent, a curative action. Moreover, almost none of them, excluded some

exceptions, such as fosetyl-Al and BTH, can penetrate inside the plant tissues and thus have a systemic effect. Thus, they are effective to prevent fire blight infection, but they are inactive when the disease is already established.
Nevertheless, some chemicals, without a direct bactericidal activity can have systemic action. Some of them, such as benzothiadiazole (BTH) or harpin, belong to the class of SAR inducers, others, such Prohexadione-Ca and Trinexapac-ethyl, are biregulators characterized by the ability to increase plant resistance against pathogens. The main problem of these compounds is that they should be applied several days before the risk period to allow the plant to build up its defences.

1.1. Efficacy of chemical control

Today, since none of the antibacterial treatments is at the same time totally effective, environmental safe, non-phytotoxic and systemic, it is not yet available a completely reliable chemical method to control fire blight (Psallidas and Tsiantos, 2000).
Moreover, the chemical treatments present other weak points. In fact, the bactericides should be sprayed before the inoculum reaches the susceptible plant. Then, they should be effective during all the period in which the pathogen is present. Thus they have to be sprayed several times during the year according to a reliable prediction model. Moreover, the efficacy of a treatment is influenced by several factors:

- The environmental conditions, such as high humidity, temperature, rain or hail, can drastically decrease the efficacy of the chemicals used.
- The time of spraying, that can determine if a chemical will be effective or not.
- The method of application that influences the covertures of all the susceptible plant parts.
- The physiological state of the host plant at the time of treatment
- The plant species and cultivars

- The inoculum consistence: higher is the pathogen population lesser is the effectiveness of the chemical applied (Koistra and de Gruyter, 1984; Tsiantos and Psallidas, 1996a).

Since all these factors are involved in determining the efficacy of a chemicals, the efficiency of a certain compound results very difficult to assess, in field conditions experiments.
Finally, to increase the efficacy of chemical treatments, it is necessary combine them with other control methods (for instance cultural measures, proper irrigation, fertilization and pruning) in an integrated management programme (Psallidas and Tsiantos, 2000).

1.2. Current methodologies of chemical control

According to Van der Zwet and Keil (1979), the chemical tested against fire blight may be grouped in 4 categories:

1. Copper compounds
2. Antibiotics
3. Carbamates
4. Other compounds

More recently, different kinds of compounds have been developed. Among them, SAR inducers and bioregulators seem the more promising. This dissertation will focus on these compounds in the chapters “Growth Regulators” and “SAR Inducers”.
Since their low efficacy, the carbamates will not be treated in this dissertation.

1.3. Copper compounds

The active ingredient of these compounds is the copper ion, which is extremely toxic both to bacterial cell and to all plant life (Psallidas and Tsiantos, 2000). Since its high toxicity, copper solutions can not be used per se as a foliar pesticide. Thus, the copper is usually sprayed either in an insoluble form, such as copper hydroxide, copper oxychloride or cuprous oxide, or as $CuSO_4$ mixed with lime $Ca(OH)_2$ to form the Bordeaux mixture. The effectiveness of the Bordeaux mixture as a bactericide is greatly affected by the ingredients proportion used. In particular, the composition of the dry

deposit, formed on leaf surface after treatment, seems the main factor that determines the efficacy of the mixture (Gremlyn, 1990). Some of the major disadvantages of the Bordeaux mixture are the difficulty in the preparation and the residual phytoxicity towards some host plants, such as pears (Martin and Woodcock, 1983).
On the other hand, the insoluble copper compounds have the advantage to be easier to prepare and to apply than the Bordeaux mixture. The formulations, to be effective, have to release the toxic copper ion. According to Psallidas and Tsiantos (2000), the formation of soluble copper ion from an insoluble structure could be due to the reaction with:

- CO_2 or ammonium salt dissolved in the rainwater
- Microbial secretion on the plant surface
- Plant secretion form healthy or wounded surface

The copper formulations commercially available and most frequently used are: ammoniacal copper sulphate, copper hydroxide, copper oxide and copper oxychloride.
All these copper formulations were more effective than Bordeaux mixture in controlling fire blight, even if they resulted phytotoxic for blossoms and young leaves at the dosages recommended for pathogen control (Psallidas and Tsiantos, 2000). Finally, even if resistance to copper has not been yet detected, it is widely spread among other phytopathogenic bacteria (Cooksey, 1990).

1.4. Antibiotics

Antibiotics are organic compounds produced by microorganisms and able to inhibit or kill other microorganisms. *E. amylovora* is sensible, *in vitro*, to several antibiotics (Rudolph, 1946; Morgan and Goodman 1955; Martinec and Kocur, 1964). Nevertheless, only few of them (streptomycin, oxitetracycline

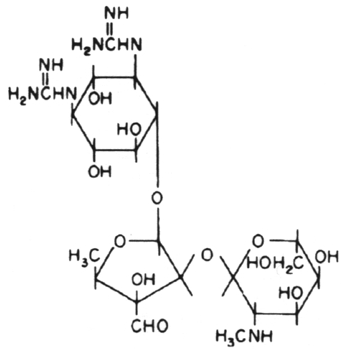


Figure 12: structure of streptomycin.

and kasugamycin) are valuable for practical field application (Ark, 1949). Among them, streptomycin is the most effective against fire blight.

Streptomycin was initially isolated by Schatz *et al.*, (1944) from *Sterptomyces griseus*. Streptomycin belongs to the class of aminoglycoside antibacterial compounds. Also amikacin, gentamicin, tobramycin**,** kanamycin and netilmicin belong to the same class of compounds. These compounds inhibit the protein synthesis in bacterial cells by affecting ribosome. In fact, all the aminoglycosides irreversibly bind to specific proteins of the ribosomal 30S subunit. Thus they interfere with the initiation complex and they cause the misreading of mRNA, which produces, by insertion of incorrect amino acids into the polypeptide, nonfunctional or toxic peptides (Gottliebe and Show, 1970). Finally they break up polysomes into nonfunctional monosomes.

Streptomycin is also toxic to the plants: it inhibits the chlorophyll synthesis, thus high concentration of this antibiotic may lead to chlorosis and death of the plant.

Streptomycin is usually foliar applied. In fact, even if it is easily up taken by the roots, its concentration inside the plant tissues is too low to be effective against bacterial pathogen (Anderson and Nienow, 1947).

Several experimental studies proved the efficacy of streptomycin in controlling fire blight (Heuberger and Poulos, 1953; Ark and Scott, 1954; van der Zwet and Keil, 1979). According to Van der Zewt and Keil (1979), a concentration of 100-150 ppm applied 3-5 times during the blooming period is enough to achieve good results. However, the effect of streptomycin is not long lasting: the plants sprayed with the antibiotic result protected from fire blight for 4 days, whereas injured plants are protected only within 6h from application (Van der Zwet and Keil, 1972).

An other problem connected with streptomycin is the built up of resistance among sensitive bacteria. For this reason the use of streptomycin is prohibited in several countries. Streptomycin resistant strains of *E. amylovora* were firstly reported in California in 1972 (Miller and Schroth 1972; Moller *et al.*, 1972). After these first

reports *E. amylovora* streptomycin resistant strains have been found in several countries (Coyer and Convey, 1975; Chiou and Jones, 1991; El-Goorani *et al.*, 1989; Thomson *et al.*, 1993).
Bacteria can avoid the lethal effect of streptomycin in three different ways:
altering the ribosomal proteins, producing enzymes able to modify and inactivate streptomycin or preventing the streptomycin access to the target site (Amyes and Gemmell, 1992). In *E. amylovora*, resistance to streptomycin could be due both to a mutation on the chromosomal DNA and to an acquired plasmid or transposon. As far as the mutation of the chromosomal DNA, since streptomycin binds to a single site on the ribosomal 30S subunit, resistance is conferred by a single base pair mutation at codon 43 of the *rpsL* gene, which results in a lysine to arginine conversion in ribosomal protein S12 (Jones *et al.*, 1996).
Trasposon Tn5393, which carries the gene *strA* and *strB* and, therefore, confers streptomycin resistance, is widely distributed among gram-negative bacteria isolated from apple orchards (Minsavage *et al.*, 1990; Jones *et al.*, 1991; Norelli *et al.*, 1991; Burr *et al.*, 1993). Moreover, Chiou and Jones (1991) demonstrated that this plasmid has a high frequency of transfer between the donor and recipient strains. Thus, streptomycin resistance among phytopatogenic gram-negative bacteria is an ever increasing problem that reduces the possibility to use this antibiotic.

1.5. Other compounds

Flumequin. It is a non-antibiotic, non-sulphamide bactericide active against both gram-positive and gram-negative bacteria. Its chemical name is (1H-5H)-dihydro-6,7-fluoro-9-methyl-5oxo-1-benzo (I,j)-quinolizin carboxilic acid-2, and it is commercially known as Fire Stop™ and Fructil™. It interferes with the DNA gyrase, thus blocking DNA replication (Psallidas and Tsiantos, 2000). This chemical has given promising results and seems having no phytotoxic effect (Psallidas and Tsiantos, 2000).
Oxolinic acid. It is a syntethic bactericide belonging to the family of quinoline. Its chemical name is 5-ethyl-5,8-dihydro-8-oxo-(1,3)-

dioxolo-(4,5g) quinoline-7-carboxilic acid and it is commercially known as Starner™. Its mode of action has not been yet understood completely. It does not seem to be phytotoxic. Some results indicate that this compound can have also a curative action against fire blight (Tsiantos and Psallidas, 1993 a, b, 1996b).

Fosetyl-aluminium (fosetyl-Al). It is a systemic fungicide commercially known as Alliette™. This compounds seems to act by inducing plant resistance more than with a direct antimicrobial effect (Farih *et al*., 1981; Guest, 1984). The plant can absorb this compound both by leaves and roots and, successively, it moves systemically inside the plant enhancing the defence mechanisms (Guest, 1986). More recently it has been hypothesised that fosetyl-Al have a double mechanism: on one hand, it act directly against microrganisms slowing down their growth, on the other hand, it stimulates the plant defences allowing the overwhelming of the pathogen (Fenn and Goffey 1984; Guest 1986, Chase 1993).

However, Alliette™ does not seem effective in controlling fire blight (Norelli and Aldwinckle, 1993; Clarke et al., 1993; Tsiantons and Psallidas, 1993 a, b, 1996a) even if the results from different laboratories are contrasting.

Also plant extracts and essential oils have been used against fire blight. Some of them have a direct bactericidal effect (Mosch *et al.*, 1989; Vanneste 1996). Whereas others, such the extract from leaves of *Reynutria sachalinensis*, *Hedera helix*, *Viscum album* and *Alchemilla vulgaris*, could induce plant resistance mechanisms (Mosch *et al.*, 1993 and 1996).

Bioregulators and SAR inducers will be treated in the in the relative chapters.

2. BIOLOGICAL CONTROL

The biological control is a manipulation of the biotic community with the aim to influence the pathogen population using other non-pathogenic organisms. The manipulation of the biotic community is

an extremely complicate process in which a high number of variables is involved. Thus, the results are often difficult to predict.
The objective of the biological control is not to eradicate the pathogen population, but to reduce its population under the risk threshold. Hence, it has the aim to prevent the host infection by the pathogen, but, after infection occurred, it usually has no curative efficacy. Biological control agents are effective and they can provide for the deficiencies of reliable chemical control methods. Furthermore, they are harmless to human beings and animals, and, in some cases, they could be cheaper than pesticides and highly effective throughout the crop growth period (Nakkeeran *et al.*, 2002). The microbial antagonists act through antibiosis, secretion of volatile toxic metabolites, mycolytic enzymes production, parasitism and competition for space and nutrients.
The mode of action of biological control agents can be grouped in three main categories:

- competition for space and nutrients
- antibiosis
- parasitism/ predation

As far as fire blight, production of antibiotics and competitive exclusion for sites and nutrients are considered to be the principal mechanisms used by bacterial antagonists to control *E. amylovora* (Wilson and Lindow, 1993; Vanneste 1996; Johnson and Stockwell, 2000).

2.1. Competition

All the ecological requirements of a species constitute its ecological niche. When two species share overlapping ecological niches, they may be forced into competition for the common resources of those niches. More deeply the two niches overlap, more intense is the competition. This interspecific competition is a density-dependent check on the growth of one or both populations.

Among different organisms competing together, the winner would be the one able to grow at the minimum level of the limiting resources (Armstrong and McGehee, 1980).
Theoretically, the population with a competitive advantage would lead the others to extinction. Unlikely, in natural habitats, one species will have a competitive advantage in all the parts of the habitat, thus the less effective competitor is usually not driven to extinction (Gause 1934a,b).
Some of the biological control agents compete with the pathogen for the limiting resources of the environments. The microorganisms belonging to this category should colonize rapidly the environmental niche and reach the environmental carrying capacity, thus avoiding a further colonization by the pathogen.
The main disadvantage of this biological control mechanism, it is that both the competing populations are negatively affected by competition (fig. 13).

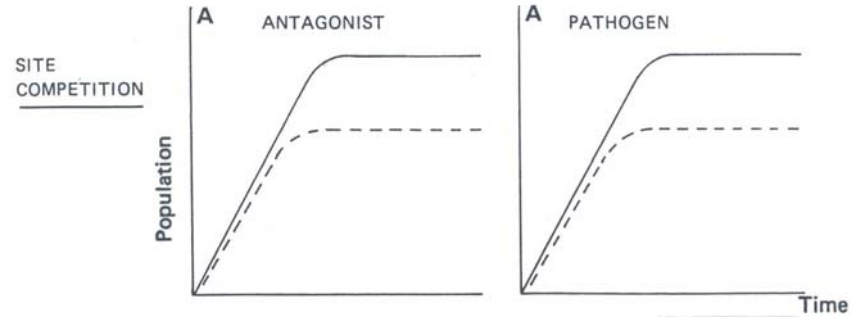


Figure 13: population dynamics during competition between two species. Solid lines and dotted lines represent, respectively, population growth in absence or presence of the other microorganism.

Theoretically, the best competitor against *E. amylovora* would be an avirulent strain of the pathogen with a competitive advantage in comparison to the wild pathogenic strains.
Pseudomonas fluorescens is a typical example of a biological control agent able to reduce pathogen population by competition.
A specific case of competition for environmental resources is the

competition for iron. The competition for iron could be the mode of action of some biological control agents. Thus the production of high effective siderophores may represent a competitive advantage.

2.2. Antibiosis

Some biological control agents, such as *Pantoea agglomerans*, produce antibiotics able to inhibit or to kill *E. amylovora*.

A particular kind of antibiosis occurs when an antagonistic organism does not produce any kind of toxic compounds by itself, but it modifies the plant environment thus leading to the production and release of toxic substances. For example, the antagonist may trigger out the production of phytoalexins or other plant defence compounds.

Also in this case, the antagonist, to successfully inhibit the pathogen, need to share the same habitat. In fact, the antibiotic compounds have usually a low diffusibility in the environment and they can be easily degraded. In comparison to competition, the main advantage of

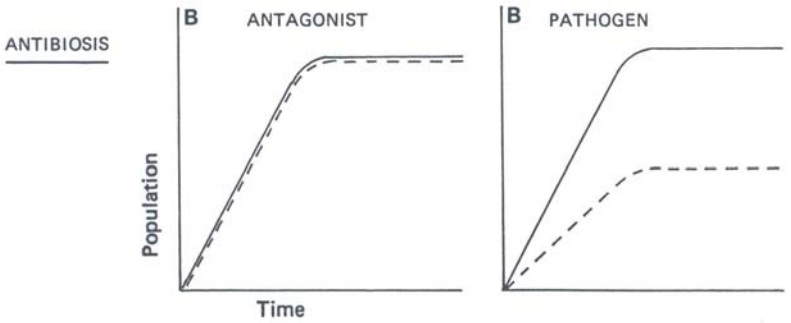


Figure 14: population dynamics during competition between a antibiotic-producing antagonist and a plant pathogenic microorganism. Solid lines and dotted lines represent, respectively, population growth in absence or presence of the other microorganism.

antibiosis is that the antagonist population is not negatively affected by the presence of the pathogen (fig. 14).

2.3. Parasitism/predation

Predation is defined by one population having negative effects on another's growth, whereas the one negatively impacted has a positive impact on the first one's growth. In other words, the antagonist uses the pathogen population as a resource to grow, thus, in this type of relationship, one organism benefits whereas the other organism is harmed. According to this definition of predation, also parasitism and grazing activity belong to the same category.

Bacteripophages are a typical example of biological control agents able to reduce the pathogen population by predations. Bacteriophages, also known as phages, are obligate intracellular parasites. In fact, after injection of their genetic material inside the bacterial cell, they multiply using the host biosynthetic machinery. Thereafter, the release of new synthesised phage particles causes the lysis of the bacterial cell.

This ecological interaction follows the rules exemplified by the mathematical model of Lotka-Volterra (Volterra, 1926). Thus, the predator population is influenced by the population density of the prey and if the prey will fade away so the predator will do (fig. 15).

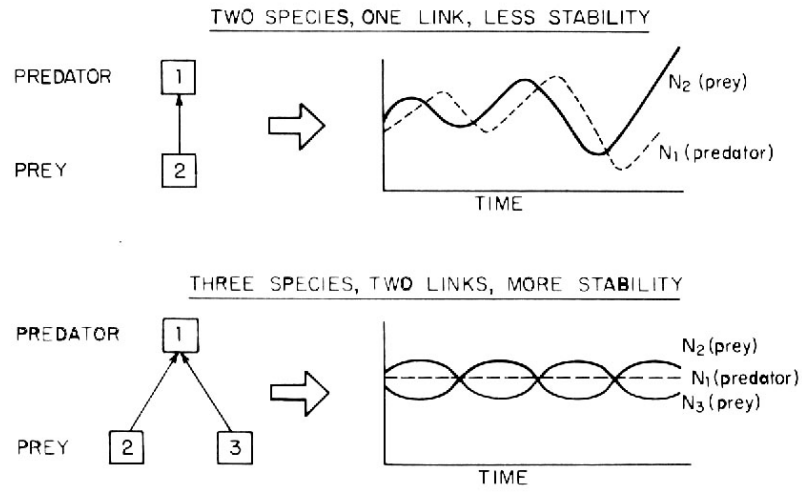


Figure 15: predator population dynamics is presence of one or two suitable preys.

2.4. Biological control of fire blight

Several kinds of antagonists have been evaluated for fire blight control: gram-negative bacteria, non virulent strains of *E. amylovora* (Tharaud *et al.*, 1997), yeasts (Mercier and Lindow, 1996), gram-positive bacteria (Jock *et al.*, 2002) and bacteriophages specific to *E. amylovora* (Ritchie and Klos, 1977; Palmer *et al.*, 1997).

The biological control to be effective has to interfere with the infection process. As suggested by Maxsons-Stein (2002), the best strategy for control fire blight is to prevent infection during the blooming period before wind-driven rain and pollinating insects spread the disease throughout the orchard. The first step of the infection is the pathogen multiplication on the stigma. Biological control aims to prevent this key step of pathogenesis. In fact, an effective interaction on stigma and hypanthial surface prevents the floral infection and thus the further damage to the plant. Therefore a better understanding of the biology of the pathogen and of its behaviour on the stigma is necessary to optimise biological control methods. Moreover, suppression of floral infections reduces the inoculum of *E. amylovora* available for other phase and cycle of disease, including shoot infections during the same season and floral infections in the following seasons (Johnson and Stockwell, 2000).

2.5. Biological interaction on stigmatic surface

The stigmatic surface is the site where the biological control agents must interact with the pathogen to successfully reduce the disease incidence (Hattingh *et al.*, 1986; Thomson 1986; Wilson *et al.*, 1989b, Vanneste 1995).

The stigmatic surface is characterized by particular epidermal cells known as stigmatic papillae. Among these papillae there are large intercellular spaces where bacteria can reside (Johnson and Stockwell, 2000). Since the stigma is rich of nourishing exudates, it is one of the few parts of the plant that allows epiphytical growth of *E. amylovora* (Wilson *et al.*, 1989b).

At the first opening of blossoms, stigmas are almost sterile (McLaughlin *et al.*, 1992). Thereafter, the exposed stigmas are soon colonised by several microorganisms.

The biological control of fire blight, reducing *E. amylovora* population on the stigma reaches two main aims.
The first is the decrease of pathogen population on the stigma: less is the pathogen population, less the probability to successfully infect the flower (Hirano and Upper, 1983; Johnson *et al.*, 1993b)
Secondly, it causes a reduction of the inoculum available to be spread from a flower to an other by rain (van der Zwet and Keil, 1979) and pollinating insects (Pierstorff and Lamb, 1934; Hildebrand and Phillips, 1936; Keit and Ivanoff 1941; Van Laere *et al.*, 1981).
A requirement for a successful biological control is the establishment of a large population of antagonistic organisms on the stigma before its colonization by *E. amylovora* (Wilson *et al.*, 1992; Johnson *et al.*, 1993b; Wilson and Lindow, 1993). A second requirement is the colonization of most of the flowers in orchards by a large population of the antagonistic organisms (Johnson *et al.*, 1993b; Lindow *et al.*, 1996). The biological control agent population should almost reach the carrying capacity of the apple and pear stigma that has been estimated to be around 10^6 cfu/ml (Wilson *et al.*, 1992; Wilson and Lindow, 1993). Thus to be effective the antagonistic population on stigma should range between 10^5 -10^6 cfu/ml.
During the second step of infection, after the stigmatic multiplication, the pathogen, washed by rain or heavy dew, reaches the nectaries trough which it penetrates. Therefore, to enhance biological control effectiveness the microbial antagonist should be able to rapidly colonise also the nectar cup surface.
Fire blight is a good candidate for biological control because the bacterial antagonists need to persist on the nutrient-rich, stigmatic surface for only about a week to suppress blossom infection effectively (Johnson and Stockwell, 2000).

2.6. Current biocontrol methodologies

Some of the bacterial antagonists useful to control fire blight are already commercially available. In particular, Pseudomonas *fluorescens Pf*A506 is commercialised in the USA market as BlightBan©, whereas in New Zealand, a strain of *Pantoea agglomerans* (P10C) is commercialised with the name of Blossom

Bless©. Several efforts have been made also to register the use of *Pantoea agglomerans Eh*C9-1 as a biological control agent in USA.
The bacteria selected for this purpose are resistant to several of the chemical treatments applied in commercial orchards, so they are not negatively affected by them and, in many cases, they can be applied together with other treatments and even with antibiotics.
The contemporary spray of biological control agents and antibiotics presents two main advantages.
First, the antibiotics reduce the pathogen population on the stigma. Then, they reduce the natural bacterial population on flowers and they free the stigmatic niche, thus allowing a more rapid colonization by the antagonist.
Today, a novel interest for the antagonistic bacteria is rising, since the pathogen developed resistances against some of the most effective chemical, such as streptomycin, used to control the fire blight disease (Loper *et al.*, 1991; McManus e Jones, 1994; Stockwell *et al.*, 1996).

2.6.1. *Pseudomonas fluorescens* A506

*Pf*A506 is available on the USA market since 1996 (BlightBan™ A506, Plant Health Technologies; Boise, Idaho). The application of this bacterium in orchard could results in a 40-60% reduction in incidence of fire blight on blossoms (Johnson *et al.* 1993b; Lindow *et al.*, 1996). Thus, its efficacy is comparable with that obtained using chemical agents. Furthermore, its application can suppress the severity of frost injuries caused by ice nucleation-active strains of *Pseudomonas syringae* (Lindow *et al.*, 1996; Johnson and Stockwell, 2000).
This bacterium is a good colonizer of apple and pear stigma especially during early spring when the temperature ranges between 10-12° C (Johnson *et al.*, 1993b; Stockwell *et al.*, 1996a).
Under field conditions, the population established on flowers is usually rather high and it is around 10^5-10^6 cfu/blossom, in addition, the percentage of colonized flowers ranges between the 50-70% of the treated blossoms (Stockwell *et al.*, 1992, 1998). The remaining

flowers are not colonized by the antagonistic bacteria and thus they remain unprotected and vulnerable to infection.
As already mentioned, *Pf*A506 antagonizes *E. amylovora* simply by competition for sites and nutrients (Hattings *et al.*, 1986; Vanneste 1996; Wilson *et al.*, 1992; Wilson and Lindow, 1993). In fact, *Pf*A506 can inhibit *E. amylovora* growth on the stigma only if it is inoculated several hours (24-72h) before the pathogen, whereas it is ineffective if co-inoculated with the pathogen (Wilson and Lindow, 1993). *Pf*A506 population exceeds, 70-80 hours after inoculation, the carrying capacity of the stigma (10^6 cfu). Thus *Pf*A506 suppress the pathogen by a pre-emptive sequestration of sites and resources on the stigma (Wilson and Lindow, 1993). Moreover, *Pf*A506 colonizes also the nectaries, thus reducing the probability of a successful infection by *E. amylovora* (Wilson and Lindow, 1993). *Pf*A506 does not seem to produce, *in vivo*, any kind of antibiotic or toxic compounds.
Nevertheless, other mechanisms of inhibition have been proposed for *P. fluorescens.* Among them, the production of siderophore and the stimulation of plant defence are particularly interesting. Wilson and Lindow (1993) observed that pear blossoms (cv. Comice) reddened after treatment with *P. fluoresecens* A506. According to these Authors, the reddening could be due to the production by *P. fluorescens* of a β-glucosidase, which catalyses the transformation of arbutin in new phenolic compounds able to inhibit the pathogen. In addition, when *P. fluoresecens* A506 is applied on plant kept in a grow chamber, the bacteria reduce the nectar secretion (Wilson and Lindow, 1993). This reduction in nectar secretion may successively increase its sugar concentration thus rendering its osmotic potential to high for *E. amylovora* survival (Wilson and Lindow, 1993). In this case, the application of the antagonist reduces the fire blight incidence suppressing *E. amylovora* just before the penetration through the nectarhodes (Wilson and Lindow, 1993). In spite of all these possible influences on plant metabolism, the treatment with *P. fluorescens* A506 does not affect either fruit production or flower attractiveness for pollinating insects (Wilson and Lindow, 1993).

2.6.2. *Pantoea agglomerans* (Gavini *et al.*, 1989) - formerly *Erwinia herbicola* (Löhnis, 1911)

Also different strains of *Pantoea agglomerans* have been widely investigated as possible biological control agents against fire blight. In particular, the strain *Eh*C9-1 (Ishimaru *et al.*, 1988) has been tested for long time in USA. It is an excellent colonizer of apple and pear stigma even more effective than *P. fluorescens*. Several studies demonstrated that the application of *Eh*C9-1 could result in a 50-80% reduction in fire blight incidence on blossoms (Johnson *et al.*, 1993b). Therefore, the protective level reached with *Eh*C9-1 is comparable to that obtained with streptomycin and usually exceeds the level of control provided by *Pf* A506 (Johnson and Stockwell, 2000).

Similarly to *Pf*A506, *Eh*C9-1 population on blossoms reach 10^4-10^6 cfu (Stockwell *et al.*, 1992, 1996a, 1998; Johnson *et al.* 1993b) and the 40-70% of treated blossom have a detectable *Eh*C9-1 population (Stockwell *et al.*, 1992, 1998).

Several other strains of *P. agglomerans*, such as *Eh252* (Vanneste *et al.*, 1992), *Eh*318 (Wright and Beer, 1996), *Eh*112Y (Wodzinski *et al.*, 1994), *Eh*1087 (Kearns and Hale, 1996), *EhHl9N13* (Wilson *et al.*, 1990a) and *Eh*325 (Pusey, 1997), have been tested with promising results as biological control agents against fire blight. Moreover, P10C, a *P. agglomerans* strain, has been register for use in New Zealand (Vanneste *et al.*, 2002b).

The efficacy of all *P. agglomerans* strains is related to production of antibiotics able to inhibit *E. amylovora*. For example *Eh*252 produces a putative microcin (Vanneste and *et al.*, 2002a), whereas *Eh*C9-1 produces two different β-lactams antibiotics called herbicolin O and herbicolin I (Ishimaru *et al.*, 1988). Herbicolin O is effective against a wide range of bacterial genera, whereas, herbicolin I is toxic to *E. amylovora*, *Bacillus cereus* and *Staphylococcus aureus* (Ishimaru *et al.*, 1988).

Even if antibiotic production is important for the inhibition of the pathogen multiplication, it is not, per se, sufficient to completely antagonize *E. amylovora*: competition for site and nutrient also

contributes to the overall effectiveness of biological control (Johnson and Stockwell, 2000).

2.6.3. Bacterial mixtures

To maximize the effectiveness of fire blight biological control, the use of bacterial mixtures of antagonists have been investigated in several laboratories (Stockwell *et al.*, 1992; Vanneste and Yu, 1996; Nuclo *et al.*, 1998).
The aims of these mixtures are:

- combine different mechanisms of biocontrol (antibiosis, exclusive competition…)
- expand the range of environmental conditions suitable for multiplication of the antagonists
- enhance the total antagonistic population
- increase the number and type of ecological niches colonized by antagonists

Using a mixture that follows these criteria it is, theoretically, possible achieve a large and stable community of bacterial antagonists on the plant surface.
A mixture of *EhC9-1* and *Pf*A506 responds to the criteria previously listed. In fact, these two bacteria have different and complementary mechanisms of antagonisms (Ishimaru *et al.*, 1988; Wilson and Lindow, 1993), they have different maximal temperatures for growth *in vitro* (27°C for *Pf*A506 and 37°C for *Eh*C9-1), they differ in tolerance to desiccation stress and UV radiation[1] and in ability to utilize various carbon and nitrogen sources (Wilson and Lindow, 1994). Moreover, *Pf*A506 used alone is a good colonizer of apple and pears flower especially during period characterized by frequent rainfall and moderate daytime temperature (10-12°C). Whereas, during period with limited rainfall and warm daytime temperature (16-22°C), *Eh*C9-1 colonizes the majority of flowers.
For these reasons, the application of a mixture of these two bacteria might result in a constant coverage of the plant surface almost independently of the climatic conditions. When applied as a mixture

[1] [1] V.O. Stockwell unpublished data reported in Johnson and Stockwell, 2000

the percentage of treated flowers colonized by at lest one of the antagonistic bacteria ranges between the 80-90% (Stockwell *et al.*, 1992).
Thus the application of a mixture of bacterial antagonists reaches the following purposes: a higher and well-established population of antagonistic bacteria on flower, and a higher percentage of flowers colonized by biocontrol agent (Johnson and Stockwell, 2000). Nevertheless, the application of a mixture of antagonistic bacteria does not achieve a greater reduction of fire blight incidence, than the application of a single biocontrol agent (Vanneste and Yu, 1996). Several hypotheses have been proposed to explain this unexpected result that has not yet completely understood. It is possible that the interspecific competition between the bacteria applied as biocontrol agents vanishes any possible synergic effect. For example the antibiotic production typical of *P. agglomerans* strains could negatively affect *Pf*A506 population, whereas, the latter bacterium could reduce *P. agglomerans* growth by pre-emptive sequestration of mutually required growth-limiting resources.

2.7. Selection of a biological control agent

A biological control agent has to responds to several criteria: it should be a good epiphyte in a wide range of environmental conditions, it should share the same epiphytic niche of the pathogen, and, of course, it should be harmless for plants, animals and human. Several screening tests, such as media-based test and immature pear fruits test, have been used to assess the effectiveness of a possible bacterial antagonist (Wrather *et al.*, 1973; Beer and Rundle 1983; Isenbeck and Schultz, 1985; Ishimaru *et al.*, 1988; Nicholson *et al.*, 1990; Vanneste *et al.*, 1992; Kearn and Mahanty, 1993). Unfortunately, these tests are mainly suitable for antibiotic producing bacteria, whereas they might underestimate the efficacy of bacteria, such as *Pf*A506, which inhibits *E. amylovora* by competition (Mercier and Lindow, 1996). Thus, testing the effectiveness of antagonistic bacteria directly on blossoms is considered by most researchers a more reliable method (Andrews, 1985; Mercier and Lindow, 1996; Pusey, 1997).

To verify the efficacy of antagonistic strains, detached flowers with the pedicel placed into vials containing a 10-25% sucrose solution (Pusey, 1997) can be used or alternatively it is possible use detached flowering branches.
In any case the blossoms used for the test have to be closed until the experiment is performed, thus the stigma remains almost sterile till the experimental inoculation.
Finally, the effectiveness of a possible biological control agent should be evaluated in orchard conditions. In fact, plants grown in greenhouse, or in controlled environment, usually support a higher epiphytic population than plants grown under field conditions (O'Brien and Lindow, 1989; Loper and Lindow, 1993; Beattie and Lindow, 1994). In fact, under field conditions, several factors, such as physiology and growth of plants, weather conditions and competition with indigenous microorganisms, may influence the establishment of a consistent population of the antagonist and therefore the efficacy of biological control.

2.8. Delivery of bacterial antagonists

The populations of bacterial antagonists artificially introduced in orchards are relatively resilient (Lindow *et al.*, 1996; Stockwell et *al.*, 1996a). Moreover, they can partially self-sustain by spreading naturally from blossom to blossom (Johnson *et al.*, 1993b; Lindow *et al.*, 1996; Nuclo *et al.*, 1998).
The initial establishment of a consistent and widely spread population of bacterial antagonists is the key factor that determines the efficacy of further biological control. Among the several factors, which influence the initial establishment of artificially introduced bacterial populations, the methods of preparation and application of inoculum, the insect activity, the bloom stage and the daytime temperature play a primary role (Johnson and Stockwell, 2000). As far as inoculum preparation, Stockwell *et al.* (1998) demonstrated that inoculum consisting of cells of *Pf*A506 and *Eh*C9-1 that had been freeze-dried and resuspended in water established epiphytic population in blossoms more consistently (large populations on a

greater proportion of flowers) than suspension of cells prepared from cultures actively growing on artificial medium.
A possible explanation of this observation has been proposed by Stockwell *et al.* (1998). They suggested that the freeze-drying process, exposing bacteria to freezing and desiccation stresses, selects a subset of cells with a higher fitness to the unreceptive environment of plant surface.
For delivery of bacterial antagonists, different methods have been proposed. The bacteria can be sprayed likewise chemical treatments, but they can be also dispersed by natural vectors, such as pollinating insects. Indeed, the dissemination by honeybees has been proposed by different laboratories as a method to implement the biological control in commercial orchards (Thomson *et al.*, 1992; Johnson *et al.*, 1993a; Vanneste, 1996). For this purpose a pollen insert, fulfilled with the bacterial inoculum, is placed at the entrance of the beehive (fig. 16).

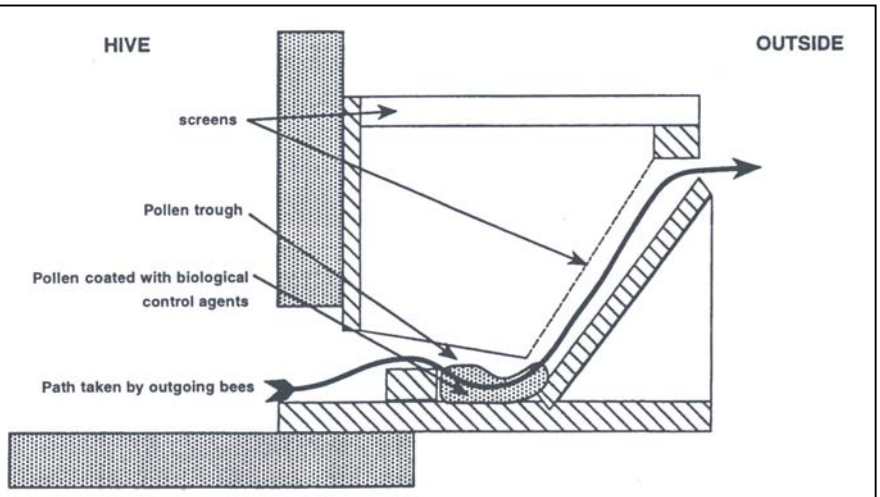


Figure 16: cross section of a pollen insert (from Vanneste. (1996). *Biocontrol News and Information 17: 67N-78N*).

Thus all the insects became contaminated by the bacterial inoculum as they exit from the beehive. The inoculum can consist in either lyophilised bacterial cells (Johnson et al., 1993a) or pollen grains

coated with the biological control agent (Thomson *et al.*, 1992). In the latter case, the dissemination of pollen could also enhances the flower pollination and thus the fruit production in the orchard. The delivery of antagonistic bacteria by honeybees could improve the secondary dissemination of bacteria from colonized blossoms to non-colonized ones, thus allowing the establishment of a consistent population on a great proportion of flowers (Nuclo *et al.*, 1998). Nevertheless, the orchard sprayers are more effective and reliable, for the initial establishment of bacterial antagonists, than dissemination via pollinator insects (Johnson *et al.*, 1993a). In fact, the dissemination of bacterial antagonists by honeybees is influenced by several environmental factors, such as temperature and presence of other flowering plants; these factors introduce a high variability in the pre-emptive establishment of biological control agents on flowers.
The time of application of bacterial antagonists is also important. The application at early bloom provides more time for the bacteria to grow to large populations. Conversely it protects only the small proportion of early-opened blossoms. Thus, a multiple application, at 25% and 90% bloom, covering both early-and late-opening blossoms, seems to provide the best protective effect (Johnson and Stockwell, 2000).

2.9. Contemporary use of bacterial antagonists and antibiotics

According to Lindow *et al.* (1996) and Stockwell *et al.* (1996a), biological control agents, to be effective, should be integrated with conventional orchard management strategies. Thus, their populations should remain consistent also after the use of other control methods, such as the chemical applications, which are usually employed in commercial orchards. Several Authors proved the compatibility of bacterial antagonists and traditional control methods (Lindow *et al.*, 1996; Stockwell *et al.*, 1996a; Vanneste *et al.*, 2001b).
For example, the application of streptomycin together with resistant bacterial antagonists may even increase the antagonists populations

on blossoms by suppressing indigenous bacterial epiphytes sensitive to the antibiotic, which compete with (Stockwell *et al.*, 1996a).
Therefore, biological control of fire blight should be viewed as a complementary disease control strategy, where the benefits from its use will be most significant when integrated with orchard sanitation and the application of antibiotics during periods of high infection risk (Johnson and Stockwell, 2000).

2.10. Contemporary use of bacterial antagonists and bacteriophages

Billing (1960) found a difference in the bacteriophage sensitivity between virulent and avirulent strains of *E. amylovora*. The susceptibility was related with the presence of the bacterial capsule. In 1985, Billing demonstrated that the LPS could be responsible for the bacteriophage susceptibility. Therefore, the absence of EPS capsule in some *E. amylovora* strains, exposing the LPS membrane, could increase the susceptibility to bacteriophage infection.
In 1973, Erskine considered the possible use of bacteriophages as biological control agent against fire blight. He isolated a specific phage, named S1, able to lyse both *E. amylovora* and a yellow *amylovora*-like saprophyte bacteria classified as Y. Moreover, this bacteria showed and inhibitory activity against the virulent strain of *E. amylovora*. It is likely that this yellow saprophytic bacterium was a *Pantoea agglomerans* strain susceptible to the same bacteriophage of *E. amylovora*. Similarly, to other bacteriophages, also this phage presented two different life cycles: the lytic and the lysogenic cycle. During the lytic cycle, new phage particles are produced within the infected bacterium. Thereafter, viruses usually lyse the infected host bacterium in order to be released. Instead, in the lysogenic life cycle, the phages can incorporate their DNA into the bacterial chromosome thus becoming noninfectious prophages. Hence, in a lysogenic cycle the viral genome is replicated along with the host DNA. Bacteriophages capable of a lysogenic life cycle are termed temperate phages, whereas bacterial cells carrying prophages are called lysogenic cells. Several environmental stimuli, such as ultraviolet light, can cause the reversion to a lytic cycle and then the

destruction of the bacterial host cell. Sometimes temperate viruses become lytic spontaneously.
Erskine observed that the phage released by the lysogenic form of Y, named Y (S1), were able to lyse the *E. amylovora* cells and showed a greater lysogenic ability for Y.
Erskine proposed that the release of phage particles from the lysogenic form of the yellow bacteria was responsible of a modulation of the disease severity and incidence by controlling the *E. amylovora* population sensitive to the S1 phage. Finally, the lysogenic form of *E. amylovora* resulted less virulent that the uninfected one. According to this result, it is likely that the presence S1 could select ipo-virulent strains of *E. amylovora*. This, together with the use of bacterial antagonists such as *Pantoea agglomerans* might represent an effective method to control fire blight. The use of bacteriophage as biological control organism was proposed also by Chatterjee and Gibbins (1971).

GROWTH RETARDANTS

1. INTRODUCTION

The plant growth retardants are compounds, which are used to control vegetative growth in a desired way without changing developmental patterns or being phytotoxic.
Their principal aim is the reduction of unwanted shoot elongation without lowering plant productivity.
They reduce the shoot growth by inhibiting cells elongation and division. In fact, they antagonize the functions of gibberellins and auxins; the plant hormones that are primarily involved in shoot elongation. However, it is possible control shoot growth using compounds other than growth retardants, such as chemicals with a low herbicidal activity. In this case, even if any phytotoxicity can be shown, a reduction in plant productivity has to be expected (Rademacher, 2000). The plant growth regulators can be applied for several purposes. For example, their application keeps fruit trees more compact, thus allowing high-density plantation. Moreover, they enhance canopy compactness, thus increasing light and air penetration in the canopy, which could lead to higher photosynthesis and to a better chemical spray penetration. Finally, they reduce pruning costs and they produce a better ratio between vegetative growth and fruit production (Fletcher *et al.*, 1999; Giafagna, 1995; Nickell, 1983; Rademacher, 1991; Schott and Walter, 1991). Since plant organs compete for nutrients, a reduction of shoot growth might enhance the amount of water and photo-assimilates available for the growth of other plant parts such as fruits (Costa *et al.*, 1986) and roots (De *et al.*, 1982). Growth retardants influence the hormone balance in plants; therefore they have, directly or indirectly, numerous effects on plant metabolism. Some of these effects will be discussed in the following paragraphs.

2. CLASSES OF PLANT GROWTH RETARDANTS

The growth retardants can be classified in two main classes: ethylene-releasing compounds and inhibitors of GA biosynthesis. Only the latter class will be discussed in this dissertation, this class can be further divide in four groups:

2.1. Onium-Type compounds. These compounds are characterized by a positively charged ammonium, posphonium or sulphonium group. They block the biosynthesis of GAs before the *ent*-kaurene. Among these compounds the chlormequat chloride (CCC) (Tolbert, 1960a-b) and the mepiquat chloride are the most well known. They both have a quaternary ammonium group.

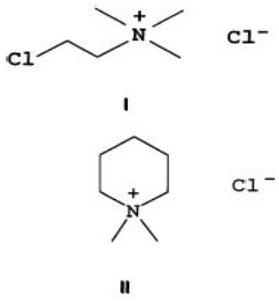


Figure 16: Onium-type plant growth retardants: Chlormequat chloride (I) and Mepiquat chloride (II). From Rademacher 2000.

2.2. Compounds with a N-containing heterocycle. Several growth retardants comprise a nitrogen-containing heterocycle. These compounds inhibit the monooxygenases, which catalyse the oxidative steps from *ent*-kaurene to *ent*-kaurenoic acid (Graebe, 1987; Miki *et al.*, 1990). Also triazole-type compounds belong to this class of growth retardants. Among them, Paclobutrazol (Lever *et al.*, 1982) and the closely related uniconazole-P (Izumi *et al.*, 1984; Izumi *et al.*, 1985) are highly active and have found practical uses in rice, fruit tress and ornamentals. Some of the triazole-type compounds, such as triadimenol and triadimefon

(Buchenauer and Röhner, 1981), are fungicides presenting some growth regulating effect as a side activity.

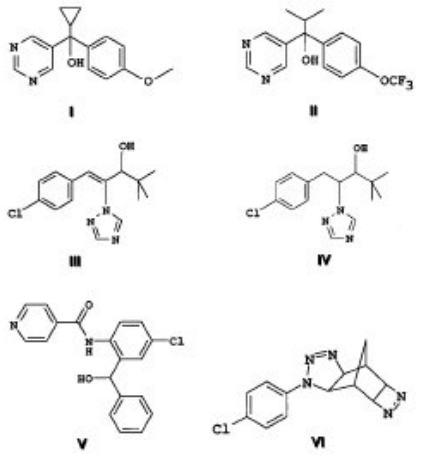


Figure 16: Plant growth retardants with an N-containing heterocycle:

- I acymidol
- II flurprimidol
- III uniconazole-P
- IV paclobutrazol
- V inanbenfide
- VI tetcyclacis

From Rademacher, 2000.

Also the paclobutrazol has a fungicidal side activity that is probably due to its effect on sterol formation (Sugavanam, 1984).

2.3. Structural mimic of 2-oxoglutaric acid. These compounds inhibit the formation of active GAs by mimicking the 2-oxoglutaric acid that is the co-substrate of dioxigenases that catalyse late steps of GA biosynthesis. Acylcyclohexanediones, such as Prohexadione-calcium (ProCa) and trinexapac-ethyl (TrixE), belong to this class of compounds. Also daminozide is a structural mimic of 2-oxoglutaric acid, but it is active only toward few plant species, such as apple and chrysanthemums (Rademacher, 1993; Brown *et al.*, 1997). Moreover, it has a high toxicity against animal (Brown and Casida, 1988). This dissertation will focus mainly on prohexadione-calcium and trinexapac-ethyl.

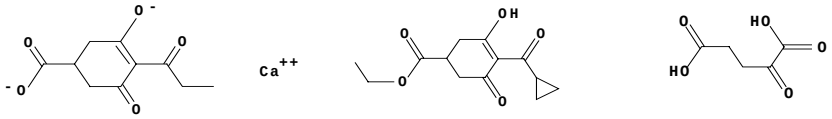


Figure 17: Structural mimic of 2-oxoglutaric acid from the left: ProCa, TrixE and oxoglutaric acid.

2.4. 16,17- Dihydro-GA_5 and related structures. These compounds represent the most recent group of growth retardants. Several different structures, mostly GA_5 derivates, have been described to reduce shoot elongation in graminaceous plants (Evans *et al.*, 1994; Mander *et al.*, 1998a-b; Mander *et al.*, 1995; King *et al.*, 1997). These compounds inhibit the dioxygenases, and particularly the 3β-hydroxilases, which catalyse the late steps of GA metabolism (Foster *et al.*, 1997; Junttila *et al.*, 1997; Tagaki *et al.*, 1994). These compounds seem to compete with the natural GA substrate, such as GA_{20}, for the respective enzymatic sites (Tagaki *et al.*, 1994). Their effect on GA metabolism is similar to the one obtained with acylcyclohexanediones (Junttila *et al.*, 1997; Foster *et al.*, 1997). *Exo*-16,17- dihydro-GA_5-13-acetate is the most active growth retardants ever known for graminaceous plants (Rademacher *et al.*, 1999), although it is virtually inactive in reducing shoot elongation in any other plant species tested (Rademacher *et al.*, 1999).

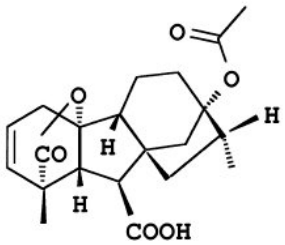


Figure18: 16,17-Dihydro-GAs exo-16,17-Dihydro-GA5-13-acetate (from Rademacher, 2000).

To understand the mode of action of the growth retardants, it is necessary briefly introduce the GA biosynthesis.

Insert 2: Gibberellin Biosynthesis

GAs are diterpenoids and consist of 19 or 20 carbon atoms. At the present, 125 different GAs are known to occur in higher plants and/or GA-producing fungi (Rademacher, 2000). GAs have several biological effects: they influence stem elongation, flower induction, anther development, seed and pericarp growth and seed germination by inducing hydrolytic enzymes. Furthermore, they mediate environmental stimuli, which modify the flux through the GA-biosynthetic pathway. Regulation of GA biosynthesis is therefore of fundamental importance to plant development and adaptation to the environment (Hedden and Kamiya, 1997).

The biosynthesis of GAs can de divided into three stages according to the nature of enzymes involved and to the corresponding localization in the cell:

1. Proplastidial phase: the terpene cyclases
2. Endoplasmatic reticum phase: the monooxigenases
3. Cytosolic phase: the dioxigenases

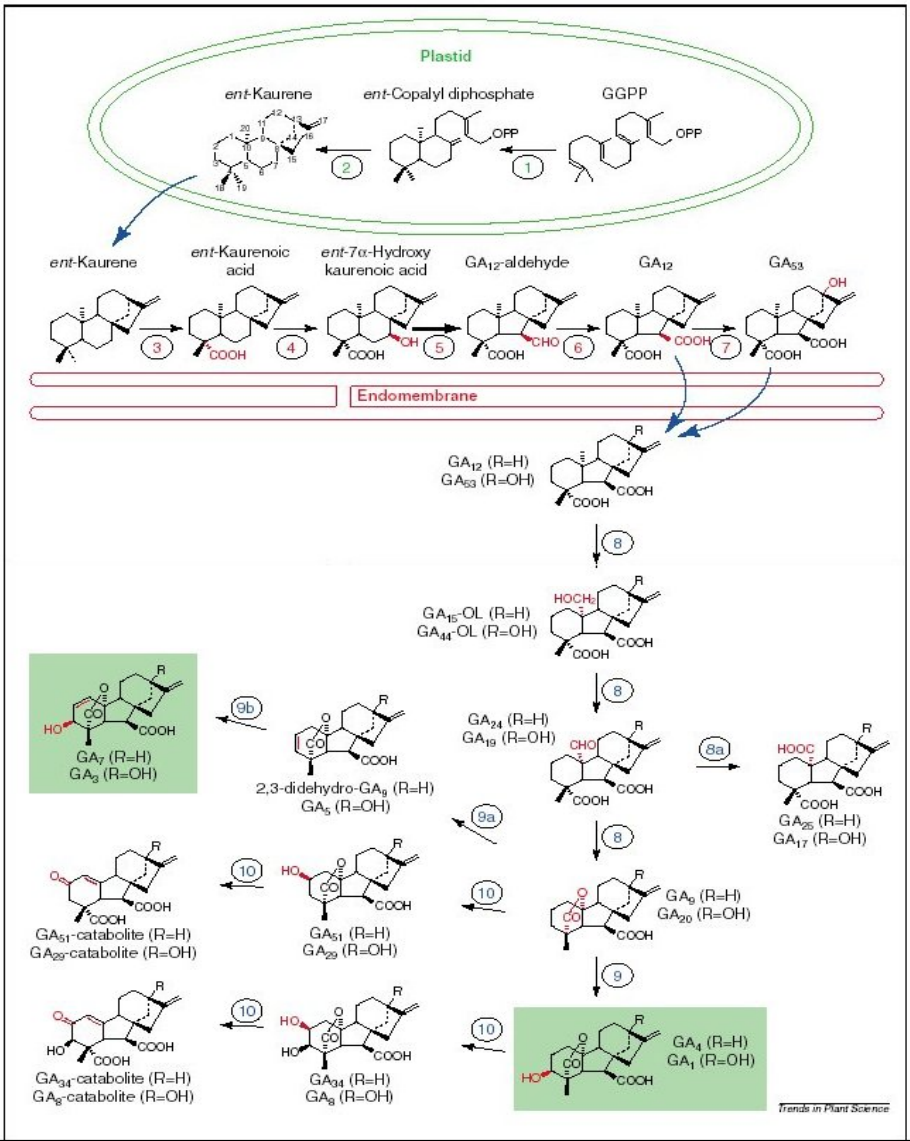


Figure 19: GAs biosynthesis. From Hedden and Phillips, 2000

Plastidial phase: formation of *ent*-Kaurene

GAs are exclusively formed from C_5 compound isopentenil diphosphate (IPP). The IPP is synthesised both from mevalonic acid (MVA) (Chappell, 1995) in cytosol and via non-mevalonate pathway in plastids (Lichtenthaler, 1999; Rohemer, 1999). In this pathway, D-glyceraldheide 3-phoshate plus pyruvate yields 1-deoxy-D-xylulose 5 phosphate, which is converted into IPP. GAs precursors are formed primarily via 1-deoxy-D-xylulose 5-phosphate pathway in plastids of green tissues although this has not yet been conclusively demonstrated (Rademacher, 2000).
IPP is transformed into dimethylallyl-PP by an isomerase, successively three molecules of IPP are sequentially added to this compound to form geranyl diphosphate (GPP), farnesyl diphosphate (FPP) and the C_{20} geranylgeranyl diphosphate (GGPP). Finally, the GGPP is cyclized via copalyl diphoshate (CPP) to *ent*-kaurene. The *ent*-kaurene is primarily produced in rapidly dividing cells (Aach *et al.*, 1997).

The endoplasmatic reticulum phase: oxidation of *ent*-kaurene to GA_{12}-Aldehyde

The reactions of this phase are catalysed by monooxigenase, which require O_2 and NADPH for activity and involve cytochrome P450. *ent*-Kaurene is oxidized stepwise at C-19 via *ent*-kaurenol and *ent*-kaurenal to *ent*-kaurenoic acid. Successively, *ent*-kaurenoic acid is hydroxylated to *ent*-7α-hydroxykaurenoic acid. After an oxidative ring contraction with extrusion of C-7, GA_{12}-aldehyde, which is the first intermediate specific for GAs, is formed (Rademacher, 2000).

The cytosolic phase: further oxidation of GA12-Aldehyde to different GAs

Most of the reactions during this phase are catalysed by soluble dioxygenases, which require 2-oxoglutarate as co-substrate and Fe^{II} and ascorbate as co-factors for activity. Hydroxylations at position 13 and 12α may involve both dioxygenases and monooxygenases. GA_{12}, the first GA, is obtained from GA_{12}-aldehyde by the oxidation,

at position 7, of the aldehyde function into a carboxylic acid group. In higher plants, the GA_{12} is commonly transformed, through the 13-hydroxylation pathway, in GA_{53}. Successively, a stepwise oxidation of C-20, and the lactone formation from C-19, with the loss of C-20 as CO_2 is catalysed by the multifunctional GA 20-oxidase. These reactions lead to GA_{20}, a C_{19}-GA (Rademacher, 2000). Considerable biological activity can be found only among C_{19}-GA, which are further hydroxylated at position 3β, e.g. GA_1, which is formed from GA_{20} (Rademacher, 2000). Whereas, the hydroxylation at position 2β (e.g. conversion of GA_1 into GA_8) drastically reduces biological activity. These steps, together with further oxidative reactions and conjugation with sugars, have the function of terminating the mission of a GA (Rademacher, 2000).
Several kinds or isoenzymes of GA 20-oxidase, 3β-hydroxilase and 2β-hydroxilase exist in plant tissues. Moreover, since their specificity of substrate is relatively low, it is probable that they have overlapping activities (Rademacher, 2000).

Dioxygenases involved in GA biosynthesis

The enzymes involved in the third stage of the pathway are soluble oxidases that use 2-oxoglutarate as a co-substrate (Hedden and Kamiya, 1997). These 2-oxoglutarate-dependent dioxygenases belong to the family of nonheme Fe-containing enzymes (De Carolis and De Luca, 1994; Prescott, 1993; Proebsting *et al.*, 1992). These enzymes are inhibited by acylcyclohexanediones.

3. ACYLCYCLOHEXANEDIONES

3.1. Effect on gibberellin biosynthesis

As mentioned previously, prohexadione-calcium (ProCa) (Nakayama *et al.*, 1990a) and trinexapac-ethyl (TrixE) (Adams *et al.*, 1992) belong to this class of growth retardants. These two acylcyclohexanediones have structural similarity to 2-oxoglutaric

acid, therefore, they inhibit the formation of active GAs by mimicking 2-oxoglutaric acid, which is the co-substrate of dioxigenases that catalyse late steps of GA biosynthesis (Griggs *et al.*, 1991; Nakayama *et al.*, 1991a). Virtually all higher plants react with a reduced shoot growth after treatment (Rademacher, 2000).

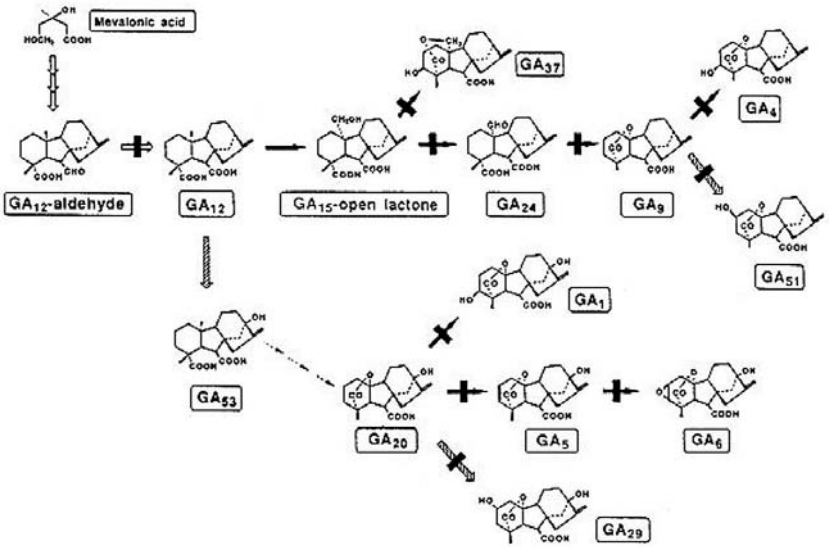


Figure 20: GAs biosynthesis steps inhibited by acylcyclohexanediones (from Nakayama *et al.*, 1990b)

Several studies with cell-free preparations demonstrated that acylcyclohexanediones block most of the steps after GA_{12}-aldehyde formation (Graebe *et al.*, 1992; Griggs *et al.*, 1991; Hedden, 1991; Kamiya *et al.*, 1992; Nakayama *et al.*, 1990b, Nakayama *et al.*, 1991a; Rademacher et al. 1992). They inhibit both 3β-hydroxilases, which are involved, for example, in the formation of active GA_1 from inactive GA_{20}, and 2β-hydroxilases, which catalyse the conversion of GA_1 into inactive GA_8 (Grossmann *et al.*, 1989; Nakayama *et al.*, 1991a). In fact, the application of acylcyclohexanediones results in lowered levels of the biologically active GA_1 and its metabolite GA_8, which is accompanied by an increased concentration of GA_{20} and earlier precursors of GA_1 (Adams *et al.*, 1992; Brown *et al.*, 1997;

Junttila *et al.*, 1997; Kamiya *et al.*, 1992; Lee *et al.*, 1998; Nakayama *et al.*, 1991a; Rademacher et al. 1992; Zeevaart *et al.*, 1993).
Since, the application of GAs, which need to be metabolically activated, does not reverse the growth retardant effect of acylcyclohexanediones, it has been suggested that also the late stages of GA formation are blocked by these compounds (Junttila *et al.*, 1991; Nakayama *et al.*, 1991b; Zeevaart *et al.*, 1993).
In some cases, simultaneous application of active GAs, such as GA_1, and acylcyclohexanediones can results in an increased GA-like activity since the acylcyclohexanediones protect the exogenous GAs from inactivation by 2β-hydroxilases (Nakayama *et al.*, 1990a; Sponsel and Reid, 1992).
As far as acylcyclohexanediones structure, the presence of a free carboxylic acid group enhances the similarity to 2-oxoglutaric acid, thus it results in higher activity as compared to the corresponding methyl or ethyl ester. Also longer acyl side-chains lead to increased inhibitory activity as compared to shorter ones, but too long chains cause phytotoxicity. Therefore, substitutes such as ethyl or cyclopropil, as in ProCa and TrixE respectively, appear to be optimal for practical uses (Rademacher, 2000).
Even if acylcyclohexanediones with a free carboxylic acid group are more active, the ones with an esteric group in their structure are often more easily up taken by leaves than the ionised forms (Rademacher, 2000). Successively, in the plant cell, the acid can be formed again by saponification. Under practical conditions, ProCa (a salt of an acid) and TrixE (an ester) have a comparable effect on graminaceous plants. However, on dicotyledonous plants, ProCa generally gives better results than TrixE (Rademacher, 2000). This can indicate that TrixE is easily saponificated into its active acidic form in grasses, whereas this process is not as pronounced in dicotyledonous plants.

3.2. Effect on the levels of other phytohormones

Acylcyclohexanediones seem to have a side effect on other plant hormones. For example, ProCa and TrixE reduce ethylene levels in sunflower cell suspensions and in leaf disks of wheat (Grossmann, 1992). Moreover, in shoots of wheat and oilseed rape, ProCa

application increases the concentrations of cytokinins and abscissic acid (ABA), whereas it does not affect indole-3-acetic acid (IAA) contents (Grossmann *et al.*, 1994). The effect on cytokinins and ABA seems indirect. In fact, after the application of different growth retardants, the assimilates are generally shifted into the roots, which are the major site of cytokinins biosynthesis. Thus, a stimulation of root growth may lead to an increased formation of cytokinins, which are successively exported to shoots (Fletcher and Arnold, 1986). On the contrary, the effect on ethylene seems a direct interaction of acylcyclohexanediones with ACC oxidase. This enzyme, that catalyses the conversion of aminocyclopropanecarboxylic acid (ACC) to ethylene, is a dioxygenase that requires ascorbic acid as a co-substrate. 2-Oxoglutaric acid and other compounds with similar structure (e.g. acylcyclohexanediones) may inhibit this enzymes mimicking the ascorbic acid (Iturriagagoitia-Bueno *et al.*, 1996).

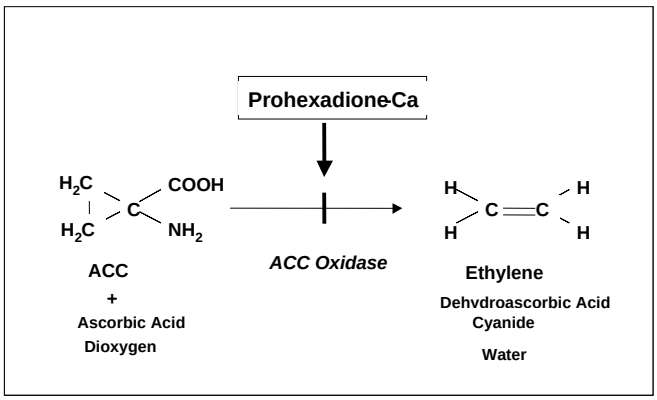


Figure 21: Conversion of Aminocyclopropanecarboxylic acid (ACC) into Ethylene

Indeed, ProCa, on enzyme system from ripe pears, inhibits the ACC oxidase at an I_{50} of approximately 10^{-5} M (Rademacher *et al.*, 1998).

3.3. Effect on flavonoid metabolism

Since the biosynthesis of flavonoids and other phenylpropanoids comprises steps that are catalysed by cytochrome P450-dependent monooxygenases and by 2-oxoglutarate-dependent dioxygenases (2-ODDs), it may be affected by growth retardants belonging to both compounds with a N-containig heterocycle and structural mimic of 2-oxoglutaric acid (Forkmann and Heller, 1999; Weisshaar and Jenkins, 1998).

High dosages of acylcyclohexanediones (ProCa and TrixE) inhibit anthocyanidines formation in flowers and other parts of higher plants (Rademacher *et al.*, 1992). Most probably, these growth retardants inhibit the 2-oxoglutarate-dependent dioxygenases and, in particular the flavanon 3-hydroxylase (FHT), which are involved in anthocyanins biosynthesis (Rademacher et al. 1992). These changes in flavonoids and other phenylpropanoids metabolism seem related to the decrease of susceptibility to pathogen observed in treated plants. In fact, plants treated with ProCa or TrixE are significantly less affected by several diseases, such as fire blight (*Erwinia amylovora*) (Fernando and Jones, 1999; Momol *et al.*, 1999; Costa *et al.*, 2000-2001; Winkler 1997; Yoder *et al.*, 1999) or apple scab (*Venturia inaequalis*) (Costa *et al.,* 2001; Spinelli *et al.,* 2002). Moreover, the application of ProCa may also reduce the grey mould (*Botrytis cinerea*) incidence on grapevine (Rademacher, 1999 unpublished data).

3.4. Prohexadione-Ca

Prohexadione-Ca (calcium, 3-oxido-4-propionyl-5-oxo-cyclohexene carboxylate) is a bioregulator that has been developed by BASF Corporation and Kumiai Chemical Industry Company, Ltd. for controlling vegetative growth in apple (Winkler, 1997). ProCa is a promising tool to control vegetative growth on apple (Owens and Stover, 1999; Unrath 1999) and pear (Elfving, 1999). In addition to its beneficial effects in controlling shoot growth and optimising fruit production, it is effective in decreasing host susceptibility to diseases. According to Guak *et al.*, (2001), the treatment with ProCa increases the concentration of non-structural carbohydrates (TNC) in

all the plant parts treated without altering the allocation pattern. This increment is mainly due to increased levels of starch rather than soluble sugars.
The effects due to ProCa application on shoot growth, non-structural sugars concentration and N allocation can be reverted by foliar application of GA_{4+7}.
Treatments with ProCa result in a reduction in number of leaves and therefore of total leaf area of the canopy (Guak *et al.*, 2001). According to these Authors, the total leaf area was reduced of 18%. In addition, in treated leaves the dry matter per unit of leaf area increased after treatment. Also the root dry weight is increased, therefore suggesting a modification in the sinks competition for nutrients. As already mentioned, ProCa increase the ratio of TNC to N in leaves and stem. This effect, together with the increase of carbohydrate content, could leads to enhanced cold hardiness, bloom and fruit set (Guak *et al.*, 2001). Moreover, also growth patterns of apple can be manipulated by altering the supply of gibberellins (Guak *et al.*, 2001).
ProCa is absorbed via green tissues and translocated acropetally within the plant. In fact, foliar absorption is the only significant means of plant up take. ProCa uptake is generally complete within eight hours following the application (Evans *et al.,* 1997). Vegetative growth control is manifested by reduction in internodal length. Treatments are most effective at spray concentration of 125 to 250 ppm when new vegetative growth is between 10-20 cm. Biological activity continues for three to four weeks, at which time a second treatment may be desirable. Thus, ProCa does not persist in the plant or effect vegetative growth the following season (Evans *et al.,* 1997).
The optimum rate of ProCa required to provide effective control of vegetative growth depends on tree vigor, age, training system, crop load and other factors that influence vegetative growth (Evans *et al.,* 1997). According to the same Authors, earlier applications (petal fall) have provided more effective control of vegetative growth than later applications (second cover).
Moreover, ProCa has a very low toxicity to mammalian and a low propensity for crop residues (Winkler, 1997).

Acute rat toxicity	LD_{50}
Inhalation	> 4.21 mg/kg
Dermal	> 2000 mg/kg
Oral	> 5000 mg/kg

Table 1: toxicity of ProCa on rats. (from BAS 125 10W technical data sheet)

There is no other hazard associated with the compound and no health risk for user or consumer is indicated. In chronic toxicity, no carcinogenic, mutagenic or teratogenic effects were observed. ProCa rapidly dissipates in soil by microbial community and causes no detrimental ecological effects. In fact, in soil it is rapidly metabolised to CO_2 and its half-life is less than one day. Since ProCa reduces canopy density, its use reduces the tree row spray volumes of other pesticides up to 25% (Winkler, 1997). Thus, ProCa fits exceptionally well into the Integrated Pest Management (IPM) programs approved by the US Environmental Protection Agency (EPA) (Winkler, 1997).

Test method	Test subject	Results
LC_{50} - 96 h	Fish (*Oncharynchus mykiss*)	> 100 mg/l
EC_{50} - 48 h	Water flea (*Daphia magna*)	> 100 mg/l
EC_{50} - 120 h	Algae (*Selenastrum capricornutum*)	> 100 mg/l
LC_{50} - 14 days	Earthworm (*Eisenia foetida*)	> 100 mg/l

Table 2: ProCa ecological effects: it is no harmful to fish, algae and earthworm (from BAS 125 10W technical data sheet)

As mentioned before, ProCa reduces host susceptibility to different plant diseases such as fire blight and apple scab. Since ProCa has no direct antimicrobial activity (Römmelt *et al.*, 1999), several mechanisms have been proposed to explain the reduction of host susceptibility. ProCa may protect the plant from pathogens by a more rapid hardening off of new shoots and leaves (Winkler, 1997). Moreover, the reduction of canopy density may lead to a different microclimatic conditions (less humidity, more air circulation)

unfavourable to plant pathogens. Nevertheless, ProCa seems to reduce plant susceptibility mainly by altering flavonoids metabolism. These compounds, in fact, are involved plant defence mechanisms (Römmelt *et al.*, 1999). For example, in apple plants, scab resistance is correlated with flavan-3-ol content in plant tissues (Treutter and Feucht, 1990; Mayr *et al.*, 1997). It is also known that plant tissues in proximity to the infection site, are rich in flavonoids (Feucht *et al.*, 1998). In particular, flavonoids accumulate, together with other phenolic compounds, in apple shoots infected by *E. amylovora* (Römmelt *et al.*, 1999-2000). Finally, several phenolic compounds have an antimicrobial activity. The effect of ProCa on flavonoids biosynthesis it probably due to the inhibition of the 2-ODDs, involved in flavonoids biosynthesis.

3.4.1. Influence of ProCa on flavonoids biosynthesis

According to Römmelt *et al.*, (2000), the treatment with ProCa induces, on apple leaves, the biosynthesis of distinct flavonoids, which usually do not occur: two eriodictyol derivatives (eriodictyol 7-glucoside and eriodictyol 3′-glucoside) and luteoliflavan (3-deoxycatechin). The content of these compounds reaches the highest level in the youngest apple leaves within 12 to 21 days after treatment (Römmelt *et al.*, 1999). The formation of eriodictyolderivatives and luteoliflavan could be explained by a possible inhibition of FHT (flavanone 3-hydroxylase), which is a 2-oxoglutarate dependent dioxygenase (Heller and Forkmann. 1993). FHT catalyses the hydroxylation of flavanones to dihydroflavonols (Heller and Forkmann, 1993), therefore ProCa interferes also with the synthesis of naturally formed flavonols, catechins, proanthocyanidins and anthocyanidins. In fact, also inhibition of anthocyanins formation by ProCa has been described (Rademacher, 1993).

Moreover, ProCa treatment stimulates the accumulation of simple phenols, phenolic acids, flavan-3-ols as epicatechin, and of some procyanidins. After ProCa treatment, the presence of new biosynthesis 3-deoxyflavonoids, naturally absent in apple leaves, indicates that an alternative pathway is induced. This pathway leads

to the synthesis of flavan 4-ols and derived compounds, which might be responsible of the enhanced resistance of treated plants to pathogens. The identification of 3-deoxyanthocyanins as phytoalexins in *Sorghum* (Lo Sze-Chung *et al.*, 1999) corroborates this hypothesis.

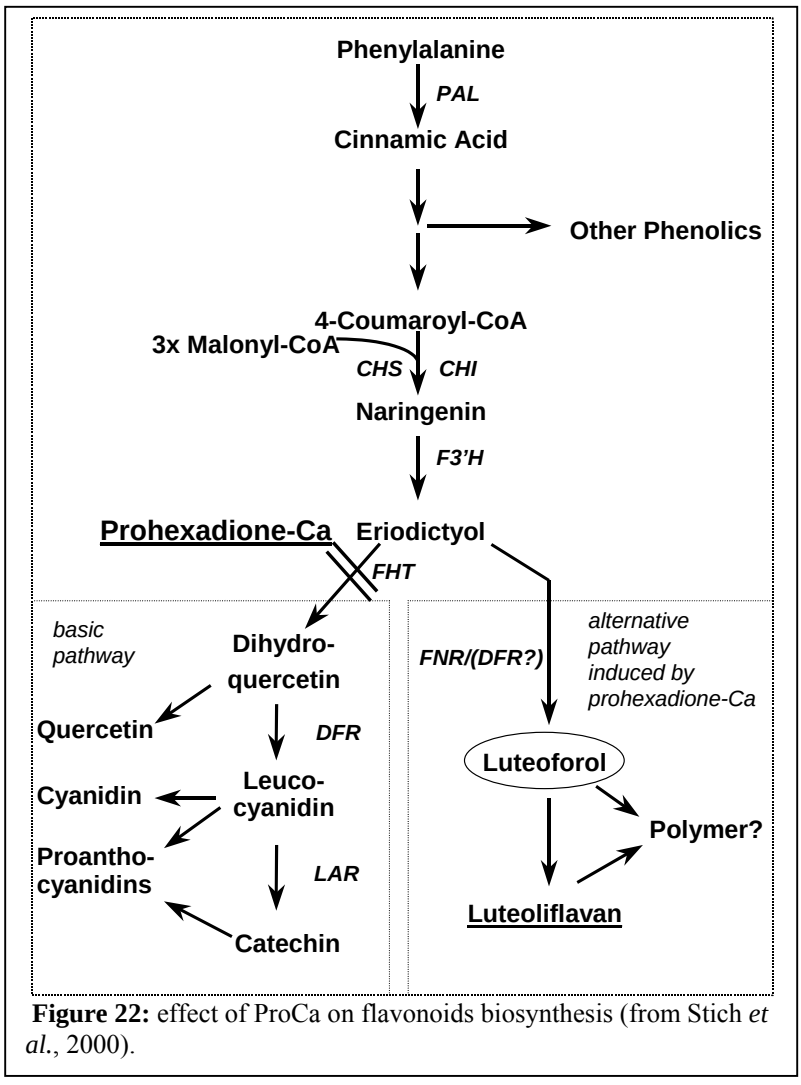


Figure 22: effect of ProCa on flavonoids biosynthesis (from Stich *et al.*, 2000).

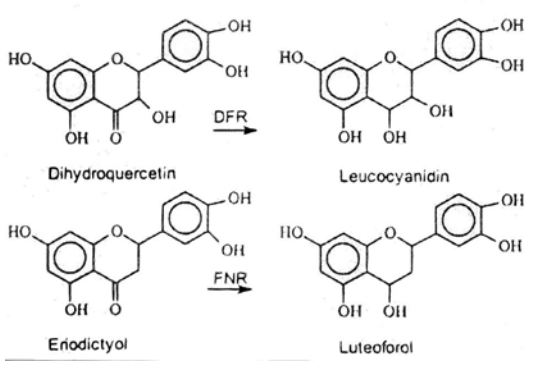


Figure 23: Reaction generally catalysed by FNR and luteoforol structure (form Stich *et al.*, 2000)

Insert 3: a general overview of flavonoids

Flavonoids belong to plant secondary metabolites, and they can be found in all higher plants and also in some mosses (Forkmann and Heller, 1999). More than 4000 different flavonoids have been isolated and identified from thousands of plant species (Forkamann and Martens, 2001). Flavonoids have numerous biological functions. For example, the flower coloration that is involved in attraction of pollinators is due to flavonoids such as anthocyanins. The flavonoids are also responsible of the coloration of other plants parts such as fruits: in this case the colour due to flavonoid pigments attract animal, therefore facilitating seeds distributions. Moreover, the flavonoid pigments are involved in protection against UV light.
Flavonoids are also involved in plant-microbe interactions. For example, they are implicated in plant defence mechanisms. In fact, several phytoalexins, such as pterocarpans, which structurally derive from 3-deoxyanthocyanins and from 5-deoxyanthocyanins, belong to the class of flavonoids. Moreover, flavonoids are present in root

exudates and they modulate interactions between host plant and symbiotic bacteria and fungi in the soil. Some flavonoids and other phenolic compounds probably stimulate the growth of various arbuscular mycorrhizal fungal species while inhibiting others (Bécard *et al.*, 1992; Poulin, *et al.*, 1993). Therefore, flavonoid derivatives can influence the initial stages of the fungal life cycle and micorrhizal symbiosis establishment.
Finally, they are involved in fertility and germination of pollen and in regulating plant growth and enzyme activity. Fore example, in some cases, flavonoids act as auxin transport inhibitors (Jacobs *et al.*, 1988) and for that reason they alter the hormone balance in the plant parts.

Biosynthesis of Flavonoids

The flavonoids biosynthetic pathway is showed in fig. 24. The reactions to the anthocyanins are catalysed by chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (FHT), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS) and flavonoids 3-O-glucosyltransferase (FGT). The other flavonoid classes are derived from intermediates in anthocyanidin formation, and the respective reactions are catalysed by leucoanthocyanidin reductase (LAR), flavone synthase I or II (FNS I, FNS II), flavonol synthase (FLS) and isoflavone synthase (IFS). Proanthocyanidins are most probably synthesised by condensing leucoanthocyanidins (flavan 3,4-diols) with flavan 3-ols. 5-Deoxyflavonoids are synthesised through the combined action of CHS and chalcone ketide reductase (CHKR). The formation of 3-deoxyflavonoids is initiated by flavanone 4-reductase (FNR) through a reaction similar to that of DFR. Flavonoids can be extensively modified by hydroxylation, methylation of hydroxyl groups, glycosylation, acylation, and a number of other reactions. Flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′,5′-hydroxylase (F3′5′H) are of particular interest; these enzymes catalyse the introduction of B-ring hydroxyl groups at the appropriate positions to provide the precursors for forming many different flavonoids, including luteolin and tricetin (flavones), quercetin and myricetin (flavonols), and cyanidin and delphinidin

(anthocyanidins). The main substrates for B-ring hydroxylation are naringenin and dihydrokaempferol (Fig. 24).

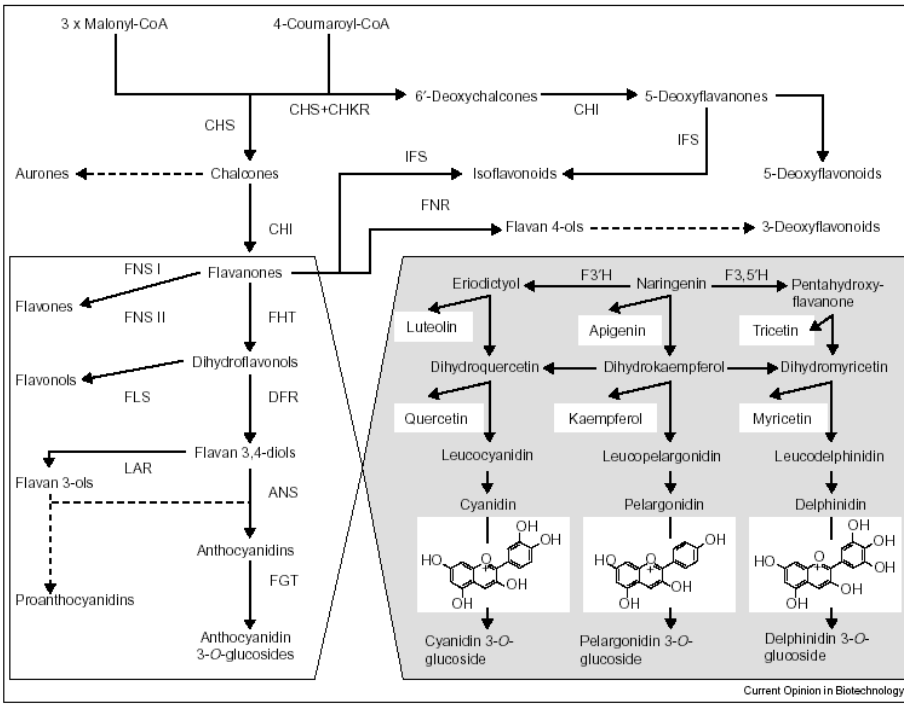


Figure 24: Scheme of the individual steps in the flavonoid pathway leading to the most important classes and the hydroxylation of ring B (shaded region shows the respective hydroxylated compounds from some flavonoid classes in the left box). ANS, anthocyanidin synthase; CHI, chalcone isomerase; CHKR, chalcone ketide reductase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; FGT, flavonoid 3-O-glucosyltransferase; FHT, flavanone 3-hydroxylase; FLS, flavonol synthase; FNR, flavanone 4-reductase; FNS, flavone synthase;
IFS, isoflavone synthase; LAR, leucoanthocyanidin reductase (from Forkmann and Martens, 2001).

2-ODDs involved in flavonoids metabolism: a possible target for acylcyclohexanediones

These enzymes catalyse different reactions, such as the hydroxylation, desaturation, epoxidation or cyclization of substrates. Their activities depend on ferrous iron and molecular oxygen, which is reduced during catalysis by two electrons provided by decarboxylation of the co-substrate (Prescott, 1993; DeCarolis and DeLuca, 1994; Prescott and John, 1996). Intermolecular dioxygenases catalyse numerous reactions in plants, e.g. in the formation of gibberellins (Hedden and Graebe, 1982) and of secondary metabolites such as various flavonoids (Forkmann *et al.*, 1980; Britsch *et al.*, 1981; Lukačin *et al.*, 2000). Different 2-oxoglutarate-dependent dioxygenases have been identified in flavonoid biosynthesis, which include the widely distributed anthocyanidin synthase (ANS) (Menssen *et al.*, 1990), flavanone 3b-hydroxylase (FHT) (Forkmann *et al.*, 1980; Britsch et al., 1981; Lukačin *et al.*, 2000) and flavonol synthase (FLS) (Britsch *et al.*, 1981; Holton *et al.*, 1993). These enzymes might be a possibly influenced by ProCa.

Flavonoid phytoalexins

Phytoalexins are chemically diverse (Bailey and Mansfield, 1982; Hammerschmidt and Schultz 1996). Several of them belong to simple phenylpropanoid derivatives, flavonoid- and isoflavonoid-derived phytoalexins, sesquiterpenes, and polyketides; representative structures are found in figure 25. Phytoalexins may be biosynthetically derived from one or several primary biosynthetic pathways. The isoflavonoids are the best understood class of phytoalexins in term of biosynthesis, enzymology and molecular biology. Furthermore, their role in disease resistance has been addressed trough many studies correlating timing and localization of their appearance with inhibition of microbial ingress (Rhodes, 1985).

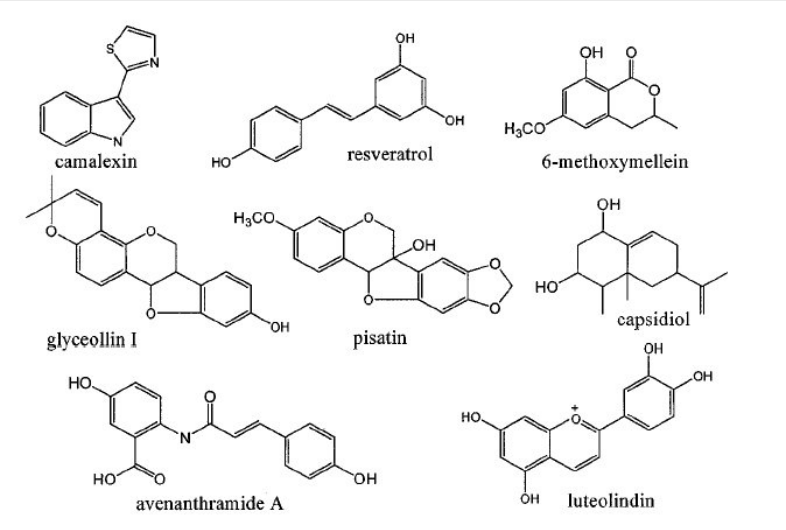


Figure 25: representative phytoalexins structures (from Hammershmidt, 1999)

3.5. Trinexapac-ethyl

Trinexapac-ethyl [4-(cyclopropyl-a-hydroxymethylene)-3,5-dioxo-cyclohexanecarboxylic acid ethylester] is used to regulate the growth of many types of grasses.

Since, TrixE is a foliar growth retardant, it must be applied to emerged plants to be effective. In fact, like ProCa, TrixE is rapidly foliar absorbed by plants.

TrixE has a toxicity and an ecological effect similar to the ones of ProCa (see tables 1 and 2). Very few data are available on the effect of TrixE in reducing plant susceptibility toward diseases (Burpee, 1998; Golembiewski and Danneberger 1998; Costa *et al.*, 2001). TrixE has not been studied as widely as ProCa, nevertheless, since TrixE shows several structural similarities to ProCa, many of the features that have been described for ProCa are valid also for TrixE.

On creeping bentgrass (*Agrostis palustris*) TrixE reduces the severity of *Sclerotina homoeocarpa* infection (Golembiewski and Danneberger 1998). Moreover, on apple, the application of TrixE might prevent scab (*Venturia inaequalis*) infection (Costa *et al.*, 2001), but, to be effective, both on shoot control and disease suppression, TrixE should be used at higher dosages than ProCa (Costa *et al.*, 2001; Wertheim S.J. 1993; Rademacher unpublished data). The reason for TrixE having less biological activity in fruit trees lies, most probably, in the fact that it has to be saponified into its acid form. This reaction is very efficient in graminaceous plants but not in fruit trees and many other dicots (Rademacher, 2000). The use of high dosages leads to several problems: the reduction in the numbers of blossoms, and therefore of fruits, in the following season is the major of them (Rademacher 2000).

SAR INDUCERS

1. Benzothiadiazole (BTH)[1]

BTH, also known as acibenzolar-S-methyl, is a SAR inducer and it is commercialised by Syngenta as Actigard 50W™, Blockade™ and Plant Activator™ for leafy vegetables. BTH can be absorbed both by green tissues and roots. This compound has no direct effect on pathogens, instead it stimulates plant natural defences by triggering out the Systemic Acquired Resistance (SAR). In most plants, low levels of the pathogen inoculum naturally trigger a resistance response.

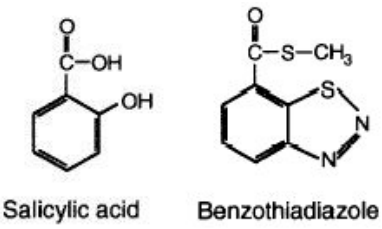


Figure 26: molecular structure of salicylic acid (SA) and BTH (from Wendehenne *et al.*, 1998, modified)

BTH does not belong to growth retardants; therefore it seems to have no affects the plant growth (Anfoka, 2000). Nevertheless, Godard *et al.,* (1999) observed a growth reduction affecting seedlings height of cauliflower plants treated with BTH; in addition this reduction was dose-dependent.

Similarly to ProCa and TrixE, also BTH showed a very low toxicity. As far as the risks for human health, EPA has concluded that there are no risks of concern from BTH use. There was no significant acute toxicity in a battery of acute toxicity studies and no dermal sensitivity was detected. Furthermore, in mutagenicity studies on rats and mice, BTH was found negative for carcinogenicity. As far as the

1 In this dissertation I will refer to BHT also with its former commercial name BION® from NOVARTIS company.

environmental risks, BTH does not seem to cause detrimental ecological effects. In fact, BTH readily degrades under environmental conditions by abiotic and biotic processes. Biotically, BTH rapidly degraded aerobically on soil (5hours) and water (less than one day), and was somewhat slower in water anaerobically indicating a biphasic pattern of decline (4 days followed by 95 days). Moreover, it is practically non-toxic to terrestrial animals on an acute and subacute basis, practically non-toxic to insects, moderately or highly toxic to freshwater and estuarine aquatic animal on an acute basis. Due to its low toxicity and low environmental risks (see table 1 and 2), BTH fits completely into IPM programs approved by EPA.
To understand BTH mode of action it is necessary introduce briefly the
systemic acquired resistance and its role in plant defence mechanisms.

Insert 4: SAR

After infection, plants react to pathogen activating several defence mechanisms. In resistant plant, the hypersensitive response (HR), that is a rapid localized defence response, leads to the collapse and death of cells in the plant tissue surrounding the infection site, thus blocking further development of the infection process. In addition, the distal uninfected parts of the plant usually develop systemic acquired resistance (SAR) which results in enhanced long-lasting resistance against pathogens (Ryals *et al.*, 1996). SAR confers quantitative protection against a broad spectrum of microorganisms in a manner comparable to immunization in mammals, although underlying mechanisms differ (Sticher *et al.*, 1997).
Both HR and SAR trigger out the enhanced expression of genes involved in plant defence that encode for phytoalexin biosynthetic enzymes, proteinase inhibitors, peroxidase, antiviral factors, hydrolytic enzymes and pathogenesis-related (PR) proteins (Wendehenne *et al.*, 1998).
Several efforts have been made in the identification of signals involved in initiation and regulation of HR and SAR (Bent, A.F.,

1996; Hammond and Jones 1996; Durner *et al.*, 1997). One of these factors, salicylic acid (SA), plays a key role in the induction and maintenance of plant resistance against pathogens (Hammond and Jones 1996; Durner *et al.*, 1997). It inhibits catalase and ascorbate peroxidase, which are two of major H_2O_2-sacavenging enzymes (Conrath *et al.*, 1995; Durner and Klessing, 1995). Thus, it leads, in infected tissues, to an accumulation of H_2O_2, which might activate, jointly with changes in the cellular redox state, the defence response, and the PR genes induction (Conrath *et al.*, 1995; Durner and Klessing, 1996; Chen *et al.*, 1993). This mechanism seems not to act in uninfected tissues in which SA level is too low to effectively inhibit catalase or APX (By *et al.*, 1995; Summermatter *et al.*, 1995). When applied exogenously to tobacco plants, SA induces PR genes and enhances plant resistance to tobacco mosaic virus (TMV) (White, 1979). Moreover, it induces the same set of genes that are systemically activated by TMV infection (Ward *et al.*, 1991). Finally, the development of host resistance correlates with an increasing of endogenous SA in plant tissues (Malamy and Klessing, 1992; Métraux *et al.*, 1990).

BTH is a structural analogous of SA (fig. 26). Thus, it has been suggested that BTH might activate the SAR pathway by mimicking the endogenous SA signal.

The resistance induction obtained with BTH is associated with activation of several pathogenesis-related protein genes (Görlach *et al.*, 1996; Lawton *et al.*, 1996; Friedrich *et al.*, 1996) and phenolics compounds as well as β-glucoside residues (Benhamou and Bélarnger, 1998). In tobacco plants, Wendehenne *et al.* (1998) demonstrated that BTH is effective in inhibition of both APX and catalase. Moreover, BTH is more efficient than SA in inhibition of catalase and induction of defence genes expression. BTH was shown to activate plant defences in different plant species against viral, bacterial and fungal pathogens (Friedrich *et al.*, 1996; Görlach *et al.*, 1996; Lawton *et al.*, 1996; Anfoka, 2000; Godard *et al.,* 1999; Maxson-Stein *et al.*, 2002).

The application of BTH could reduce both incidence and severity of disease (Anfoka, 2000; Maxson-Stein *et al.*, 2002). In some cases, after the application of BTH on tobacco plants, a reduction of disease up to 80% was observed (Friedrich *et al.*, 1996). The maximum induction of resistance occurs few days (2-4) after treatment with BTH (Godard *et al.,* 1999, Thomson *et al.*, 1999). Nevertheless, the protection obtained with BTH is durable up to 15-30 days after treatment (Godard *et al.,* 1999).

BTH has been also tested for controlling fire blight disease in apple and pear (Maxson-Stein *et al.*, 2002; Brisset *et al.*, 2000; Thomson *et al.*, 1999).

Maxson-Stein *et al.* (2002) reported that the level of PR-1 and PR-8 in apple seedlings was increased 10-fold in 2-7 days after treatments, whereas the level of PR-2 increased 100 folds. According to the same Authors, under field conditions, apple trees treated with BHT at weekly interval from pink bottom stage showed a significantly decrease on fire blight incidence both from natural infection and following artificial inoculation with the pathogen. However, its effect was less consistent than streptomycin applied weekly, which resulted in 95% of control of natural infections. Moreover, the disease severity decreased with increasing dosages of BTH.

Finally, it is possible combine streptomycin and BTH to achieve better results in controlling fire blight incidence and severity (Maxson-Stein *et al.*, 2002).

The Authors observed a reduction of fire blight incidence in flower under natural infection. In addition, they found that a weekly application of BTH three times before and three times after artificial inoculation significantly reduced fire blight incidence also on shoots.

Syngenta Corporation, on the base of experiences on leafy vegetables, recommends dosages of BTH ranging from 37.5 mg a.i./l to 75 mg a.i./l. Nevertheless, as suggested by Brisset *et al.* (2000), to control fire blight on apple higher dosages might be necessary. The most effective dosages tested range from 150 mg a.i./l to 300 mg a.i./l. Moreover, several applications both before and after the inoculation may be required (Brisset *et al.*, 2000; Maxson-Stein *et al.,* 2002).

Even if on herbaceous plants the protective effect of BTH has been described as durable at least two weeks, apple trees, under field conditions, sprayed with biweekly application intervals showed a higher incidence than plants treated with weekly intervals.
For all these reasons, BTH should be considered as a supplement rather than as a replacement for the bactericides currently used to control fire blight (Maxson-Stein *et al.*, 2002).

AIM OF THE STUDY

AIM OF THE STUDY

The primary aim of this study was to investigate the possibility to interfere both on host-pathogen relationship and epiphytic microbial community by altering plant metabolism. More in details, this research intended to elucidate how the modifications induced in plant metabolism influence *Erwinia amylovora* infection and the antagonistic bacteria used to control fire blight.

Two growth retardants, prohexadione-calcium and trinexapac-ethyl, were chosen as a chemical tool to influence plant metabolism. These compounds inhibit the 2-oxoglutarate-dependent dioxygenases (2-ODDs), which are enzymes involved in several metabolic pathways. In fact, some of the key enzymes of the gibberellin and other phytohormones metabolism are 2-ODDs and they also play an important role in flavonoids metabolism. This research focused also on elucidation of the mechanisms underlying the reduction of plant susceptibility observed after dioxygenase inhibitors applications. From cited literature, it is assumed that these compounds increase plant defence mainly by a transient alteration of flavonoids metabolism. This research had the aim to demonstrate that the reduction of susceptibility to disease could be partially due to an indirect influence on the microbial community established on plant surface. In fact, the possibility to interfere on the interrelations occurring among different bacterial populations on plant surface is particularly interesting since it is key factor for a more effective biological control of plant diseases. Therefore, the effect of dioxygenase inhibitors treatment on microbial community was investigated on different plant organs (stigmas, nectaries and leaves).

Furthermore, to explain their effect on epiphytic microbial population, the composition of the nectar and the solutions secreted by the plant both on flower and leaf surface were studied. These

secretions can be an important nutritional resource for bacterial growth.

In addition, this research intended to evaluate, as a practical application, the possibility to combine the use of dioxygenase inhibitors with biological control in order to develop an integrate strategy for fire blight control.

To study the influence of induced modifications in plant metabolism on the multiplication and migration of *Erwinia amylovora* inside plant tissues, the infection process was deeply investigated by means of advanced microscopical analysis combined with molecular biology methodologies. The influence of dioxygenase inhibitors and SAR inducers application on *E. amylovora* colonization of host tissues was studied and correlated with the histological accumulation of phenolic compounds, which can act as antimicrobial substances. In addition, this research aimed to evaluate these techniques as a novel bioassay method for screening of resistance inducers efficacy.

The final part of the work intended to demonstrate that the reduction of disease susceptibility observed in apple plants treated with prohexadione-calcium is mainly due to the accumulation of a novel phytoalexins: luteoforol. This 3-deoxyflavonoid, which, in apple, is exclusively biosynthesised after treatment, was proven to be a highly active antimicrobial compound.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. ISOLATION, IDENTIFICATION AND SELECTION OF A VIRULENT *ERWINIA AMYLOVORA* STRAIN

The first step necessary for all the successive experiments was the selection of local virulent strain of *E. amyolovora*.
Infected pear shoots were collected from a commercial orchard in Altedo (Ferrara district). The infected plant material was incubated in a humid camber at 20°C, until droplets of bacterial exudates occurred on shoot surface. These droplets were sampled with a sterile needle and diluted in sterile $MgSO_4$ (10mM pH 7.3); 50 µl aliquots were successively plated on Miller and Schroth medium and on CCT medium (See Annex). Both these media are selective for *E. amylovora*. The putative *E. amylovora* colonies were picked up and spread on fresh prepared Luria Agar. After 24 hours incubation at 27° C, single colonies were picked up with a sterile toothpick and identified according to Bereswill *et al.*, (1992) protocol. The isolate obtained in this way was named **EaDCA289/01**. To confirm the identification, a molecular classification based on amplification of 16SrDNA was performed using the primers designed by Weisburg *et al.*, (1991). According to this methodology, 16S ribosomal DNA was amplified by PCR and sequenced. Successively, the sequences were compared with corresponding fragments available in Genbank (NCBI).
Finally, the virulence of EaDCA289/01 was tested on immature pear fruits and on apple seedlings.

1.1. Bereswill *et al.*, (1992) protocol:

Two oligomers derived from plasmid pEA29 were used to amplify a 0.9 kb fragment. This fragment, specific for *E. amylovora*, was successively detected by separation on agarose gel. The two 17-mer oligonucteotides chosen as primers have the following sequences:
Primer A: 5′-CGGTTTTTAACGCTGGG
Primer B: 5′-GGGCAAATACTCGGATT
PCR assay: the PCR was carried out in a total volume of 50 µl containing (final concentration):

- 25pmol of primer A
- 25pmol of primer B
- 0.5 U of *Tth* DNA polymerase
- 0.2 mM of dNTPS
- 16 mM of ammonium sulfate
- 67 mM of Tris-HCl (pH 8.8)
- 1,5 mM of $MgCl_2$
- 10 mM of 2-mercatoethanol
- 160 µg/ml of bovine serum albumin
- 5% dimethyl sulfoxide
- 1% Tween 20

It is not necessary extract bacterial DNA, in fact Tween 20 is used to enhance the lyses of bacterial cell, which were put directly in the PCR tubes.

Denaturation: 93° C - first cycle 2 minutes

Denaturation: 93° C - 1 minute
Annealing: 52° C - 2 minutes
Polymerisation: 72° C- 2 minutes
} 35 cycles

Polymerisation: 72° C- 2 minutes

The PCR products were separated on 1.5 % agarose gel (100V for 1.5-2h), stained with ethidium bromide, and observed under UV light (302 nm).

1.2. Amplification of 16SrDNA using primers designed by Weisburg *et al.*, (1991):

Primer fD1 (forward): 5′-AGAGTTTGATCCTGGCTCAG
Primer rP2 (reverse): 5′-ACGGCTACCTTGTTACGACTT
Primer fD2 (forward): 5′-AGAGTTTGATCATGGCTCAG
(Of course, it is possible combine only forward primers with reverse ones)

PCR assay: the PCR was carried out in a total volume of 30 µl containing:

- 0.4 µl of forward primer (5 pM/µl)
- 0.4 µl of reverse primer (5 pM/µl)
- 0.4 µl dNTPS (10mM)
- 0.1 µl of TAQ (5 U/µl) (Thermoprime Plus - ABgenes)
- 3 µl ReddyMix Reaction Buffer (10x concentration - ABgenes)
- 1.8 µl of $MgCl_2$ 25mM
- 23,9 µl of MilliQ
- 0.2 µl of a cloudy bacterial suspension (~ 10^9 cfu/ml)

Denaturation: 94 °C - first cycle 3 minutes

Denaturation: 94 °C - 1 minute
Annealing: 60 °C - 1 minute
Polymerisation: 72° C- 1 minute
} 35 cycles

Polymerisation: 72 °C- 7 minutes

PCR products were separated on 0.7% agarose gel (147V for 1-1.30h), stained with ethidium bromide, and observed under UV light (302 nm).
The expected 1.6 Kb band was cut from the gel, and the DNA eluted and extracted using QUIAquick Gel Extraction Kit (Quiagen). The

DNA fragment was successively sequenced and compared with corresponding sequences available in Genbank (NCBI).

2. ISOLATION, IDENTIFICATION AND SELECTION OF BACTERIAL ANTAGONISTS AGAINST FIRE BLIGHT

Flowers and leaves from several apple and pear cultivars were sampled in Bologna District, Germany and France.
Single flowers and leaves were put in 1.5 ml eppendorf tubes filled with 1ml of sterile $MgSO_4$ (10mM pH 7.3). The tubes were vortexed for 10 seconds. Successively, 25 µl aliquots were picked up and dropped on Petri dishes filled with Luria Agar amended with cycloheximide (50 mg/L). On each quadrant of plate, 4 drops, from different plant samples, were placed. After 24h incubation at 27° C, each morphologically different colony was picked up and spread on a freshly prepared LA plate. A further 24h incubation and spread was needed to purify the bacteria.
Bacteria, from pure cultures obtained as described, were tested as possible biological control agents against fire blight.
The bacteria were submitted to the following test:

- Inhibition test against *E. amylovora*. The bacteria negative to this test were discarded.
- HR test on tobacco leaves. The bacteria positive to this test were discarded.
- Immature pear fruit test
- Control of fire blight on detached flowers and branches.

Finally, the bacteria selected by these test were identified by amplification of 16SrDNA and fragments sequencing

2.1. Inhibition test against *E. amylovora*

For the experiments plate of Ceria 132 minimal medium were prepared. Each plate was overlaid with a thin layer (2-3mm) of Ceria 132 minimal medium infiltrated with a pure culture of *E. amylovora* - Ea1540. After solidification, a loop of each possible antagonists

Figure 1: inhibition halo produced by *Eh* 252 on a plate infiltrated with *E. amylovora (*from Vanneste)

was placed on the agar surface. For the inoculation, the bacteria were picked up from 24h-old cultures on Luria agar. As positive control a strain of *Pantoea agglomerans*, named P10C, was used; as negative control Ea1540, the same strain infiltrated in the Ceria 132, was used.

After, a 24-48 hours incubation at 27° C, the plates were observed to assess the presence of an inhibition halo around the antagonistic bacteria. Almost all the isolates negative to this test were discarded. Among them, only bacteria characterized by a very fast growth were used for the following tests. They were successively plated on KB medium for detection of fluorescent Pseudomonads.

2.2. HR test on tobacco leaves

The bacteria positive to the inhibition test were good candidates for biological control of fire blight. This test had the aim to check if these bacteria were phytopatogenic or not. In fact, phytopathogenic bacteria induce HR on non-host plants.

Fresh cultures of bacteria were used to prepare a cloudy water suspension. The bacterial concentration of the suspension was assessed with a visual method to be approximately higher than 10^8 cfu/ml.

To infiltrate the bacteria into plant tissues a syringe without needle was used. The bacterial suspension was infiltrated on the inferior surface of tobacco leaf lamina. As positive control Ea1540 was used, as negative water. The development of a hypersensitive reaction was assessed 2-3 days after infiltration. All bacteria positive to this test were supposed to be phytopathogenic and therefore discarded.

Bacteria negative to the HR test were stored in glycerol stock. To prepare glycerol stock, bacteria, collected from 24 hours-old pure cultures, grown on Luria agar, were used.

2.3. Immature pear fruit test (IPF Test)

Immature pear fruits were surface sterilized by immersion for 5 minutes in a bleach solution containing 3% active chloride. The fruits were successively rinsed in water until all trace of hypochloride disappeared.

Only cores of 5 mm diameter were used for the experiment. The cores were placed in empty Petri dishes. A 10 µl drop of a $MgSO_4$ (10 mM) suspension of antagonistic bacteria was pipetted on the top of the cores. Soon after a 10 µl drop of a $MgSO_4$ (10 mM) suspension of Ea1540 was added.

Different ratio pathogen/antagonist were tested: 1:1, 1:10, 1:100. Three replicates of 10 fruit cores per treatments were used.

The minimal pathogen concentration used was $3x10^6$ cfu/ml.

After inoculation, the infected cores were incubated at 27°C in a disinfected humid chamber for 5 to 7 days. Production of exudate on core surface was assessed daily.

Figure 2: immature pear fruit cores used for IMP test. This test is useful to check the virulence of *E. amylovora* strains, the efficacy of biological control agents or antimicrobial activity of chemical for controlling fire blight.

2.4. Control of fire blight on detached flowers

Apple blossoms at popcorn stage were collected from the orchard. Single flower, still closed, were placed with the pedicel in a 1.5 ml eppendorf filled with water an incubated, in a disinfected chamber at 20°C, until opening. The opened flowers were sprayed with an aqueous suspension of antagonistic bacteria, which contained approximately 1 x 10^8 cfu/ml. Successively, *E. amylovora* - Ea8865, was sprayed (ca. 5 x 10^6 cfu/ml). Water was sprayed as negative control.

2.5. Control of fire blight on detached flowering branches

This experiment was performed likewise the one on single detached flowers, the only difference was that the plant material was constituted by detached flowering braches. Using this plant material, the experiment can be performed for longer period. During the first 24 hours after inoculation the branches were closed in transparent plastic bags to increase humidity and favour the infection.

Finally, some of the most promising bacteria selected by these tests were identified by amplification and sequencing of the 16SrDNA.

3. EFFICACY OF DIOXYGENASE INHIBITORS IN REDUCING SHOOT BLIGHT INCIDENCE (SECONDARY INFECTION)

The experiments were performed in an experimental orchard of HortResearch Ruakura Research Center (Hamilton - New Zealand) Apple plants cv. Braedburn were hand sprayed with TrixE 500 ppm until run off. TrixE was sprayed as Moddus that is a commercial formulation containing 250 g/L of active principle. Fifteen days after treatment, experimental inoculation was performed. Five to ten shoots per plant were inoculated with *E. amylovora* Ea8865 by cutting the apex of the three youngest leaves with scissors dipped in a suspension containing 1.2 x 10^7 bacterial cell/ml. Deionised water was used as control.
Symptoms development was monitored at 7, 21, 27, 37, 56 days after inoculation. Both incidence and severity were assessed.
Note: A similar experiment was designed to assess the efficacy of ProCa. But, because of the changes in New Zealand pesticide regulation, it was not possible to perform the experiment. Since in Italy it is not allowed fire blight experimental inoculation in orchard condition, data on ProCa effect are missing.

4. EFFICACY OF DIOXYGENASE INHIBITORS IN REDUCING SHOOT GROWTH

The same apple plants (cv. Braedburn) used in the previous experiment were pruned to cut off infected shoots and treated once again with TrixE 500 ppm until ruff off. Apple plants of cv. Pink lady were treated as well. The shoot growth was monitored at 1, 14, 21, 60 and 120 days after treatment.

5. EFFICACY OF DIOXYGENASE INHIBITORS IN REDUCING BLOSSOM BLIGHT INCIDENCE (PRIMARY INFECTION)

All these experiments were performed on apple plant materials cv. Royal Gala.
At pink button stage, Royal Gala trees were treated with TrixE 500 ppm until run off. The efficacy of TrixE was assessed on detached flower, detached flowering braches and entire trees. Inoculation was performed spraying a water suspension of Ea8865 containing ca. 5 x 10^5 cfu/ml. Entire trees were inoculated 6 days after treatments, single flowers at 14 days, and finally, branches at 21days.
An other set of experiments was performed to assess ProCa efficacy in controlling blossom blight on pear under controlled conditions. Potted scions of different pear cultivars (Abbé fetèl, Tosca and Williams) were sprayed until run off with a ProCa solution (250ppm). The artificial inoculation was performed 12 days after treatment by spraying an *E. amylovora* suspensions containing 1.8 x 10^7 viable cells/ml of EaDCA289/01. Number of infected blossoms and infected blossoms developing in shoot infections was assed from 2 to 8 days after inoculation.

6. EFFECT OF DIOXYGENASE INHIBITORS APPLICATION ON APPLE AND PEAR NECTAR COMPOSITION

The experiments were conducted for two consecutive years, in the experimental orchard of Bologna University. Apple plants (cv. Golden deliciuos) and pear plants (cv. Abbé fetèl) were treated at pink button stage, with 250 ppm of ProCa until run off. For each treatment, 3 randomised replicates of 8 trees each were considered. After 15 days, the nectar was collected from the nectaries using a glass pipette especially designed for the nectar sampling.

The nectar was collected in two different days with similar weather conditions: moderate maximal temperature (18-22) and humidity (60-70%). To minimize the possible differences due to differential

secretion of nectar during the day, nectar was collected from control and treated plants contemporary.
Daily production of nectar, total sugar concentration, ratio between sugars and dry matter were determined by gaschromatographic and rifractometric techniques.
Moreover, the penolic compounds content was assessed by HPLC.

Figure 3: pipette especially designed for collecting nectar. Pipette design has the aim to minimize the dilution of nectar with humidity presents in human breath.

6.1. Determination methods of nectar sugar content by gaschromatography (GC) (Bagdanov *et al*., 1997)

A nectar aliquot of 100 µl was added to 4 ml of distilled water and mixed with 40 µl a 10% mannitol solution (internal standard from Fluka).
100 µl of the solution was transfered to a conica-bottomed test tube and dried out in a current of nitrogen at 50°C temperature.
Successively 200 µl of oxime reagent (pyridine solution containing 12 mg/ml hydroxylamine hydrochloride) were added and the tube sealed with a screw-on plug. After mixing, the tube was heated at 70-75 °C for 30 min. When the sample cooled to room temperature, 100 µl of hexamethyldisilazane and 10 µl of trifluoroacetic acid were added, then the tubes were well mixed and let stand for 30 minutes.

Afterwards the sample was centrifuged at 5000 rpm for several second.
For the GC analysis 0.6 – 1 µl of sample were injected.
The equipment used for analysis was a gaschromatograph fitted with an SE 52 capillary column (or equivalent) and a flame ionisation detector. The fused silica gel capillary column was 25 m, 0.32 mm id, 0.1-0.15 µm film thickness. A carrier gas flow of 4 ml/min of hydrogen was used.

6.2. Determination of nectar phenolic compounds content by HPLC

Nectar samples were lyophilised and 100 mg dry weight were exhaustively extracted with 500 ml methanol containing the internal standard (flavon 0.1 mg/ml in methanol pro analysis) in a cooled water bath during sonification. The crude extract was centrifuged (10000 g for 10 minutes at -10 °C), the supernatant collected and directly ready for HPLC analysis.
Separation and identification was performed following the HPLC/CRD technique previously described by Treutter, 1989 and Treutter *et al.*, 1994. The HPLC equipment consisted of two pumps (mod. 422, Kontron Instrument) and a gradient programmer (mod. M800, Kontron Instrument). A reverse phase column (25 cm long, 4 mm internal diameter) prepacked with Shandon Hypersil ODS (3 mm diameter) was employed. Solvent system was composed by 5% formic acid (A) and methanol (B) with a flow rate of 0,5 ml/min.
Gradient profile was the following: 0-5 min., isocratic, 5% B in A; 5-10 min., 5-10% B in A; 10-15 min., isocratic, 10% B in A; 15-35 min., 10-15% B in A; 35-55 min, isocratic, 15% B in A; 55-70 min., 15-20% B in A; 70-80 min., isocratic, 20% B in A; 80-95 min., 20-25% B in A; 95-125 min., 25-30% B in A; 125-145 min, 30-40% B in A; 145-160 min., 40-50% B in A; 160-175 min., 50-90% B in A; 175-195 min., isocratic, 90% B in A; 195-210 min., 90-5% B in A; 210-235 min., isocratic, 5% B in A.
After column, absorbance determination was performed at 280 nm with an HPLC detector (mod. 432 Kontron Instrument) and single compounds UV spectra were detected among 250 and 400 nm with a

Diode Array Detector (Hewlett Packard mod. 1040 A HPLC Detection System).
Post column derivatisation for flavanols quantification (Treutter, 1989) was performed by using the selective reagent 4-dimethylaminocinnamaldehyde (DMACA) mixed with the eluent containing phenols in a stainless steel T-connection. Absorbance of blue coloured reaction products was detected with another HPLC detector (mod. 432 Kontron Instrument) settled at 640 nm.

Note: during the second year the effect of TrixE was tested. Unfortunately, due to the adverse weather conditions, it was not possible sample enough nectar for the analysis.

7. Effect of Nectar Sugar Composition on the Bacterial Growth

The differences in sugar composition of nectar from treated and untreated flower were tested on the growth of different bacterial species, both antagonistic and plant pathogenic. The bacteria used for this experiment are listed in table 1.
Artificial nectar was prepared according to the data collected in the previous experiments.

Bacterial strains	Organism	Type	Isolation
Ea DCA	*Erwinia amylovora*	Plant pathogenic	F. Spinelli
P10C	*Pantoea agglomerans*	Epiphytic	J.L. Vanneste
Eh DCA	*Pantoea agglomerans*	Epiphytic	C.A. Ishimaru
Pf A506	*Pseudomonas fluorescens*	Epiphytic	S.E. Lindow
Microbacterium sp.	*Microbacterium sp.*	Epiphytic	F. Spinelli

Table 1: Bacterial species used to test the influence of sugar nectar composition

The different sugars were dissolved in phosphate buffer 50 mM pH 7.3. Due to the high sugar content, the solutions were sterilized by filtration. Each bacterial strain was cultivated in 30 ml glass vials containing 5ml of artificial nectar; 10 µl of an overnight culture were use as inoculum for the nectar solution. The bacterial cultures were incubated for 5 days at 27°C with moderate shaking.
The bacterial population was assessed at 0, 4, 24, 48, 72, 96 hours after inoculation by tenfold sequential dilutions. The experiment was repeated 4 times.

7.1. Serial dilutions method to assess the bacterial growth

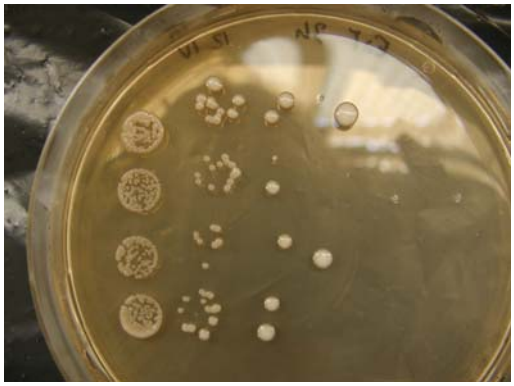


Figure 4: tenfold dilutions plated on LA plate.

This method is a particularly useful to asses bacterial population.
The concentration of bacteria in a culture could be assessed in this way: pick up 100 µl aliquot of bacterial suspension and mix it with 900 µl of sterile $MgSO_4$ 10 mM. Repeat this operation with the solution obtained: each step is a tenfold dilution. In this way repeating the dilution 5 times the final concentration obtained is 105 times less concentrated than the initial suspension.
Four 10 µl drops per dilutions can be pipetted on the dry surface of an appropriate agar medium. This is a further 100 times dilution (10 µl drop from 1 ml). After a 24-48h incubation it is possible count the single colonies per dilutions. To calculate the concentration of the initial bacterial suspension, it is necessary multiply the average number of colonies counted per the 100 (first dilution) and, finally per 10 to the dilution number. For example, in the case shown in fig 4, the average of colonies at the second dilution is 16.5. This number

has to be multiplied per 10 to the dilution number (10^2). Since the first dilution, as mentioned, is a 100 times dilution, the number obtained has to be multiplied again for 10^2. Thus the initial concentration of viable bacteria was 16.5 x 10^4, that is 1.65 x 10^5.

8. Effect of Dioxygenase Inhibitors Application on Flowers and Nectar Attractiveness to Honeybees (*Apis mellifera*)

Since pollinating insects show preference to different types of nectar in relation to the sugar concentration, sugar ratio and presence of volatile compounds, this experiment had the aim to verify if the changes found in nectar composition, after treatment with ProCa and TrixE, might affect the attractiveness of flowers.
The experiment was performed in the experimental orchard of Bologna University. Golden deliciuos apple plants were treated, at pink button stage, with ProCa 250 ppm, TrixE 500 ppm or with water as control. After 15 days the number of honeybees visiting treated and untreated trees was counted. The count of honeybees was performed each hour, from 12:30pm to 3:30pm, during three consecutive days characterized by moderate temperature (18-22°C) and humidity (60-70%). In addition, an other experiment was performed using flowering braches collected from the same trees and put in water containing pots, which were placed 7 m far from 5 beehives.

9. Effect of Dioxygenase Inhibitors Application on the Natural Microbial Community on Apple and Pear Blossoms

The experiment was performed in an experimental orchard of HortResearch Ruakura Research Center (Hamilton - New Zealand). Apple plants cv. Royal Gala were treated, at pink button stage, with 500 ppm of TrixE or water, as control, until run off. Treated and

untreated flowers were sampled at 0, 24, 72 and 120 hours after treatment.
From each flower the stigma and the nectar cup were collected aseptically. Successively, they were washed separately in 1 ml of $MgSO_4$ 10 mM. Four aliquots (25 µl) of the washing solution were spread on Luria Agar plates amended with cycloheximide (50 mg/L). The plates were incubated for 24-48 hours at 27°C.
Total number of colonies was assessed. In addition, the colonies were separated in relation to colony morphology. Using these data the biodiversity index of Shannon-Weiner was calculated.
A similar experiment was performed in the experimental orchard of Bologna University. In this case, pear plants (cv. Abbé fetèl) were sprayed with ProCa 250 ppm or water, as negative control, until run off. Changes in the natural bacterial community were determined both on stigma and on the nectaries 20 days after treatment.

9.1. Shannon-Weiner index

This index of diversity is derived from the information theory. It considers the number of species in the environment and their relative abundance. Therefore, this index does not contemplate the ecological importance of the single species. Shannon-Winner index can be expressed by the following mathematical formula:

$$H' = \Sigma_{j=1,s} [(n_j /N) \log (n_j /N)]$$

In this formula:

- s: number of species in the community
- n_j: individuals of a single species
- N: Σn_j total number of individuals

The theoretical maximum for H' is log(s). The minimum value (when N>>s) is log[N / (N-s)].

Note: base conversion with Logarithms
To convert from known log bases to any other log base use:

$$\log_b = \log_e (x)/\log_e (b)$$

where b is the base value, loge is the natural logarithm, and x is the value to be transformed. For example to take a log base 2 you would use:

$$\log_2 = \log_e (x)/\log_e (2)$$

10. Effect of Dioxygenase Inhibitors Application on *E. amylovora*, *P. agglomerans* and *P. fluorescens* Population on Apple Blossoms

The experiment was performed in an experimental orchard of HortResearch Ruakura Research Center (Hamilton - New Zealand). Apple plants cv. Royal Gala were treated, at pink button stage, with TrixE 500 ppm or water, as control, until run off. Each treatment consisted of 16 trees. These trees were divided in 4 groups consisting in 4 plants each. Six day after treatments, 30 freshly opened blossoms per tree were marked with water resistant paint. Only flowers with the anthers not jet matured were chosen. The 4 groups of trees were sprayed with different bacteria or with water as control. The inoculation was performed using a hand sprayer. Particular attention was paid to the treatment of marked blossoms. The bacterial strains used in this experiment are reported in table 2.

Strain	Organism	Antibiotic resistance	Inoculum concentration
Ea8865	*Erwinia amylovora*	Rif	$1.6x10^7$ cfu/ml
P10C	*Pantoea agglomerans*	Strep + Rif	$3.66x10^7$ cfu/ml
*Pf*A506	*Pseudomonas fluorescens*	Strep	$4.33x10^4$ cfu/ml

Table 2: bacterial species used for artificial inoculation of apple blossoms in field conditions.

Ea8865 inoculum was prepared diluting in tap water overnight culture. Also P10C was prepared in the way. Whereas, *Pf*A506 inoculum was prepared from freeze-dried cells, following the commercial package instructions.
The bacterial population on the stigma was assessed at 0.5 h, 3h and 1, 2, 3, 4, 5 days after artificial inoculation. Before the inoculation was checked the natural microbial community on the treated and control stigmas. To assess the bacterial population stigmas were collected aseptically from flowers and washed in 1 ml of sterile $MgSO_4$ 10 mM. Tenfold sequential dilutions were made and plated in the appropriate agar medium amended with cycloheximide (50 mg/L):
Ea8865 was plated on Luria Agar amended with rifampicin (100 μg/mg), P10C on Luria agar plus rifampicin and streptomycin both 100 μg/mg, finally *Pf*A506 was plated on Luria agar plus streptomycin 100 μg/mg. Using these selective plates was possible assess only population of artificially introduced bacteria.
The washing solution from water treated stigmas was plated on standard Luria agar, and on Luria agar amended with all the antibiotic combinations. In this way was possible check the natural occurrence of antibiotic resistant microorganisms.

11. Effect of Dioxygenase Inhibitors Application on Natural Microbial Community on Apple Leaves

This experiment was substantially similar to the experiment described in paragraph 9. and it was performed in an experimental orchard of HortResearch Ruakura Research Center (Hamilton - New Zealand). Apple plants cv. Royal Gala and Pink Lady were treated with 500ppm of TrixE or water, as control, until run off. Treated and untreated leaves were sampled at 0, 24, 72 hours after treatment.
To assess the consistence of the epiphytic microbial community leaf imprinting, both of the superior and inferior leaf lamina, were taken. The prints were taken on Luria agar plates amended with 50 mg/L of

cycloheximide. Total number of colonies was assessed. In addition, bacterial colonies were separated in relation to their colony morphology. Using these data the biodiversity index of Shannon-Weiner was calculated.

11.1. Leaf imprinting

Leaf imprinting is a simple technique to assess the epiphytic microbial population.
This method could be applied also to other plant parts such as stigma and nectaries. Leaves were sampled using a disinfected forceps and placed in single plastic bags. The leaf surface is then press on the surface of dry agar. After incubation it is possible count the number of colonies and assess the biodiversity inside the microbial community.

12. Effect of Dioxygenase Inhibitors Application on *E. amylovora*, *P. agglomerans* and *P. fluorescens* Population on Apple Leaves

This experiment was similar to the experiment described in paragraph 10. Apple plants cv. Pink Lady were treated, at pink button stage, with TrixE 500 ppm or water, as control, until run off. Each treatment consisted of 16 trees. These trees were divided in 4 groups consisting in 4 plants each. The 4 groups of trees were sprayed with different bacteria or with water as control. The inoculation was performed using a hand sprayer. The bacterial strains used in this experiment are reported in table 2.
Inoculum, for all the bacteria used, was prepared diluting in tap water overnight culture. Bacterial population on leaves was assessed at 0.5, 8, 24, 48, 72 and 96 hours after artificial inoculation. Before inoculation, the natural microbial community on treated and control leaves was also checked. To assess the bacterial population leaf imprinting technique was used. Ea8865 was plated on Luria Agar amended with rifampicin (100 μg/mg) and cycloheximide (50 mg/L),

P10C on Luria agar plus cycloheximide (50 mg/L), rifampicin and streptomycin both 100 µg/mg, finally *Pf*A506 was plated on Luria agar plus cycloheximide (50 mg/L) and streptomycin 100 µg/mg. Using these selective medium plates was possible assessing only the population of artificially introduced bacteria.

13. SUGAR ON LEAVES

This experiment had the aim to explain the differences found in the epiphytical community between treated and untreated leaves.
For the experiment apple plants cv. Royal Gala were treated with ProCa 250 ppm, TrixE 500 ppm and water as control.
After 15 day several leaves were randomly sampled. To assess the sugar content on leaves 20 gr of fresh leaves were washed in 20 ml of extraction solution. This solution was prepared by mixing 80 % of absolute ethanol with 20% of imidazole (0.1 M pH 7). The washing solution was centrifuged at 8000 rpm for 1 minute and the supernatant transferred in a new vial. After the addition of 500 µl of silanising agent plus internal standard, the samples were dried out under a current of air at room temperature. The silanising agent consisted in: 9 parts of pyridine + 3 parts of hexamethyldisilazane + 1 part of trimethylchlorosilane.
The internal standard, consisting in 1 mg of phenil β-glucoside, was added to each sample. For each treatment, total leaf area, and dry weight were determined as well. The GC methodologies used have been described in paragraph 6.1.

14. EFFECT OF TRIXE ON BACTERIAL ENDOPHYTIC POPULATION IN APPLE TISSUES

Also this experiment was performed in an experimental orchard of HortResearch Ruakura Research Center (Hamilton - New Zealand). Apple plants cv. Pink Lady and Braeburn were treated with 500 ppm

of TrixE or water, as control, until run off. After 15 days, treated and untreated leaves and flowers were sampled. As far as flowers, only nectar cup and stigma were considered.
The cut edge of pedicel, both for leaves and flowers was sealed with paraffin. The plant material was surface sterilized. For sterilization the plant material was immersed 1 minute in ethanol 95%, 3 minutes in bleach solution (14% free chloride), 30 seconds ethanol 95% and, finally, rinsed several times in sterile deionised water (Johnston, 1998). To verify if the sterilization method was effective, leaves artificially sprayed with P10C were analysed. After sterilization, no. P10C colonies ware detected on selective medium. Moreover, from sterilized leaves and flowers no bacterial colonies was detected also on standard Luria agar plates.
Finally, to verify if the sterilization affects also bacterial endophytic population a qualitative test was performed. Leaves were artificially infected with Ea8865. After surface sterilization, it was still possible find Ea8865 from the internal leaf tissues. A comparable sterilization was obtained also by 5 minutes immersion in bleach solution (3% free chloride) followed by several rinse in sterile water. After surface sterilization, the plant material was grounded in the plastic bag with the addition of 2ml of $MgSO_4$ 10 mM. Immediately after, 1ml of juice was picked up, placed in a 1.5 ml eppendorf, and centrifuged at 5000 rpm for 30 seconds. Approximately all the supernatant was transferred to a clean tube and centrifuged again at 8000 rpm for 3 minute. The supernatant was discarded and the pellet resuspended in 200 µl of sterile $MgSO_4$ 10 mM. This suspension was diluted and plated on Luria agar amended with cycloheximide (50 mg/L).
The bacteria isolated were putatively identified by colony morphology. The most frequently isolated ones were identified by amplification and sequencing of 16SrDNA or alternatively with Biolog GN2 MicroPlate™. This Biolog system is designed for identification and characterization of a very wide range of aerobic gram-negative bacteria. The Biolog GN2 MicroPlate performs 95 discrete tests simultaneously and gives a characteristic reaction pattern called a “metabolic fingerprint”. The metabolic fingerprint

patterns are compared and identified using the MicroLog™ database software.

15. MICROSCOPICAL INVESTIGATION I: EFFECT OF DIOXYGENASE INHIBITORS ON PRIMARY INFECTION OF BLOSSOMS

Different kinds of techniques were used for these investigations.
The morphology of apple, pear, *Pyracantha* sp. and *Crataegus* sp. stigma was investigated with a fluorescent stereomicroscope. Further analyses were conducted using a scanning electron microscope (SEM). Finally, the infection process was investigated by merging two powerful techniques: confocal laser scanning microscopy and the *gfp*-labelling of *E. amylovora*. In this it way was possible observing the pathogen migration on thick, viable plant tissues without any kind of manipulation of the samples. The main aim of these experiments was to check if dioxygenase inhibitors application could affect the infection process and in particular the migration of bacteria from the stigmatic surface to the nectaries. Furthermore, the interaction on stigma between the antagonists and the pathogen was investigated. Finally, the accumulation of phenolic compounds in relation to infection was studied. The blossoms were sprayed with ProCa 250 ppm. After 9 days, artificial inoculation was performed by spraying an *E. amylovora* suspension containing approximately $5x10^6$ cfu/ml. The strains used were *gfp*-Ea 1/79, *rfp*-Ea1/79; *gfp*-Ea 286 and *rfp*-Ea286. The inoculated blossoms were observed from 1 to 5 days after artificial inoculations.

15.1. *gfp*- and *rfp*-labelled bacteria: transformation by electroporation

The competent cells were prepared by removal of ions through several rounds of rinsing with cool-sterile water. 1 ml overnight culture was transferred in 1.5 ml sterile eppendorf. Cells were collected by centrifugation at 8000 g for 5 min, washed several times

with large volume of bidistilled sterile water and, finally, resuspended in 100 µl of sterile water. The competent cells were used immediately. For electroporation, 1 - 2 µl of plasmid DNA (pfdC1Z´-gfp or pfdC1Z´-rfp) was added to the bacterial suspension and mixed. The suspension was then transferred to a 2 mm electroporation cuvette (Eurogenetics, Belgium) and pulsed at 800 Ohms, 2.5 kV, 2.5 µFD with a BioRad gene pulser. After electroporation, 1 ml of SOC medium was added instantly, incubated 60 min at 28 °C with slight shaking and then spread the selective agar plate with proper dilution. Actively growing cultures were observed under fluorescence microscope to confirm expression of the inserted plasmids.

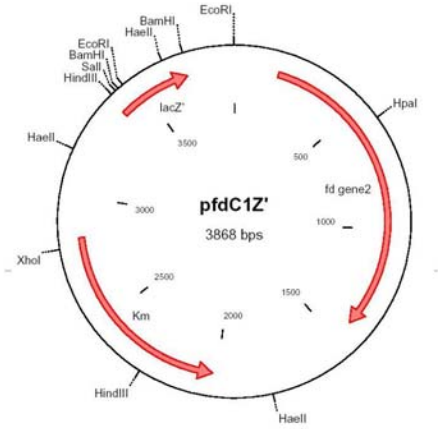


Figure 5: plasmid pfdC1Z'; *gfp* gene was inserted in lacZ´ linker. The plasmid confers kanamicin resistance

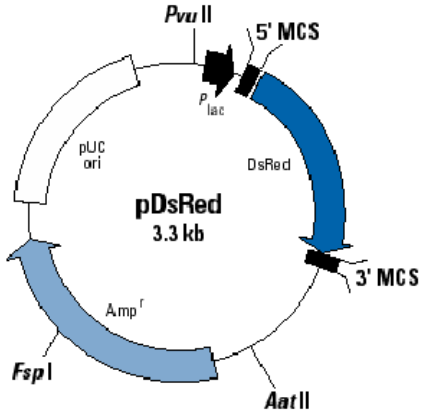


Figure 6: plasmid pfdC1Z'; It was commercially available at Clontech. The plasmid confers rifampicin resistance

15.2. Scanning electron microscope equipment (SEM)

Sample collected for scanning electron microscope (SEM) analysis were fixed in 5 % glutaraldeyde and dehydrated in 100% ethanol. After critical-point drying and gold-sputter coating, samples were analysed by SEM Philips 501B

15.3. Confocal laser scanning microscope equipment (CSLM)

Optical section were obtained with a Confocal Laser Scanning Microscope (CLSM)(Microradiance, Bio-Rad Instruments, Henel, UK) mounted on Nikon Optiphot microscope and equipped with an Argon laser. A 60x objective and the BHS (GHS) filterset were used for imaging. All images were collected using a stepper motor to make Z-series.

15.4. Fluorescence microscope

For observation, a Zeiss Axiophot optical microscope (Neofluar 100x oil immersion objective), with BHS (GHS) filterset was used. BHS: excitation light λ 543 nm, emission λ 555-700 nm; GHS: excitation light λ 488 nm, emission λ 500-535 nm.

16. MICROSCOPICAL INVESTIGATION II: EFFECT OF DIOXYGENASE INHIBITORS AND SAR INDUCER ON *E. AMYLOVORA* MIGRATION INSIDE THE PLANT TISSUES

For these experiments *gfp*-labelled Ea 1/79 was used. Plant tissues were observed both with fluoresce microscope and CSLM.
Pathogen migration was correlated with phenolic compounds accumulation in plant tissues. Phenolic compounds and/or flavonoid accumulation was detected, in fresh viable plant tissues, exploiting the autofluorescence of these compounds or using specific staining techniques based on Neu's reagent, vanillin HCl, ammonia and Wilson reagent. These methodologies are described in detail in Annex part 3.

16.1. Effect on apple plants

ProCa and Bion (BTH content 50%) efficacy was also tested by monitoring symptoms development with labelled *E. amylovora*. The bacterial strain used for inoculation was *gfp*-Ea1/79 ($5.5x10^7$ cfu/ml)

Apple seedlings (cv. Golden Delicious) were treated according to the experimental design reported in the table 3:

Treatment	Concentration	Days before inoculation
ProCa	25 ppm	14
ProCa	50 ppm	14
ProCa	100 ppm	14
Bion	120 mg/20ml	4

Table 3: treatments tested on apple seedlings.

Bion was applied dissolving 120 mg of commercial product in 20 ml of water, and then watering plants with this solution. Two different kind of inoculation were performed: by trespassing leaf lamina between nervations with an infected needle, or by cutting with infected scissors the apical tip of the leaves.
Four and five days after artificial inoculation, migration of *E. amylovora* was followed, using a fluorescence microscope, both in parechymatic and xylematic tissues.
To measure migration an empirical scale was developed and the assessment was performed by three independent observers.
Furthermore, the method was tested as a reliable tool to follow bacterial migration not only in leaf tissues but also on other plant tissues.

16.2. Effect on pear plants

Micropropagated pear plantlets (cv. Abbé fetèl) 20-25 cm tall were treated according to the schedule reported in the following table:

Chemical	Concentration	Days before inoculation
Water		11
ProCa	25 ppm	11
ProCa	50 ppm	11
ProCa	125 ppm	11
ProCa	250 ppm	11
TriXE	500 ppm	11
Bion	6mg/ml (20mlxplant)	4
H_2O_2	0,2%	2
H_2O_2	1%	2

Table 4: treatments tested on pear micropropagated plants

The experiment was performed in greenhouse under controlled climatic conditions. The plants were inoculated with an aqueous suspension of *gfp*-labelled Ea1/79 containing $1{,}28x10^7$ cfu/ml. The inoculation was performed cutting the apical part of all the leaves in each single plant. The migration rate in xylem and parenchyma was assessed 10 days after the inoculation with a fluorescence microscope.
The experiments with H_2O_2 was performed separately. The plants treated with H_2O_2 were inoculated with an aqueous suspension of *gfp*-labelled Ea1/79 containing $3{,}9x10^7$ cfu/ml. The migration rate in xylem and parenchyma was assessed 5 days after the inoculation with a fluorescence microscope.

17. ANTIMICROBIAL ACTIVITY OF LUTEOFOROL

Four groups of experiments were performed. Since luteoforol could not be isolated from the leaves, it was chemically synthesised by Dr. Rheinheimer (BASF AG laboratory, Ludwigshafen, Germany) according to the methodologies published by Stick and Forkmann, (1988) and Bate-Smith, (1969). Successively it was quantified using a calibration line obtained by addition of a defined quantity of (^{14}C)-labelled eriodictyol to a luteoforol synthesis (Bate-Smith and Rasper, 1969). The activity of luteoforol was compared with luteolinidin,

which a phytoalexin of *Sorghum bicolor*. Luteolinidin (standard for HPLC analysis) was bought from Extrasynthese© (Genay, France).In the first group of experiments, the activity of luteoforol and luteolinidin against different *E. amylovora* strains and antagonistic bacteria involved in fire blight disease was tested.
Initially, the activity of luteoforol (1mM) and luteolinidin (10 mM) was assessed using an inhibition test on solid M9 minimal medium. Then the antimicrobial effect of different concentrations of luteoforol (from 1mM to 0,01 mM) and luteolinidin (from 10 mM to 0,1 mM) were tested against several strains of *E. amylovora* and some bacteria used as biological control agents as well. The luteoforol solvent (ethyl acetate) and the luteolinidin solvent (methanol) were also tested. The experiments were performed on liquid minimal medium M9. The bacterial population was determined at 0h, 4h, 24h, 48h, 72h, and 96h after the inoculation.
In the second group, the effect of luteoforol and luteolinidin against several plant pathogenic fungi was tested. The experiments were performed in tests similar to those used for the bacteria. Fungi were cultivated on *Aspergillus* minimal medium. The spore germination was checked daily with the aid of a stereomicroscope.
The third group consisted in testing the efficacy of luteoforol and luteolinidin to control the fire blight symptoms development in the IPF (Immature Pear Fruit) test.
Finally, the effect of luteoforol, luteolinidin and their solvents on micropropagated plantlets was tested.

17.1. Inhibition test on solid medium

This experiments was a screening test to assess the efficacy of luteoforol and luteolinidin to inhibit bacterial growth. The bacteria used in the experiment are listed in table 5.
Plates with M9 minimal medium were prepared for the experiments and overlaid with a thin layer (2-3 mm) of minimal medium infiltrated with a pure bacterial culture. Sterile disks of filter paper, imbibed with 10µl of the tested compounds were placed on the agar surface.

BACTERIAL STRAINS	ORGANISMS	TYPE	ISOLATION
Ea 1/79	*Erwinia amylovora*	Plant pathogenic	K. Geider
Ea 286	*Erwinia amylovora*	Plant pathogenic	K. Geider
Ea 1540	*Erwinia amylovora*	Plant pathogenic	D.V. Dye
Ea 8865	*Erwinia amylovora*	Plant pathogenic	S. V. Thomson
Ea DCA289/01	*Erwinia amylovora*	Plant pathogenic	F. Spinelli
Eh 252	*Pantoea agglomerans*	Epiphytic	S. Beer
Eh C9-1	*Pantoea agglomerans*	Epiphytic	C.A. Ishimaru
Eh DCA269/01	*Pantoea agglomerans*	Epiphytic .	F. Spinelli
Pf A506	*Pseudomonas fluorescens*	Epiphytic	S.E. Lindow
MicDCA210/01	*Microbacteriaceae* str..	Epiphytic	F. Spinelli
Streptococcus mutans	*Streptococcus sobrinus*	Human pathogenic	DMSZ

Table 5: bacterial species against which luteoforol was tested

The chemicals used were: streptomycin sulphate (200 ppm) as positive control, sterile water as negative control, luteolinidin 10 mM, methanol (luteolinidin solvent), luteoforol 1mM and ethyl acetate (luteoforol solvent). The plates were incubated for 24-48h at 27° C. Thereafter, the presence of inhibition haloes was assessed.

17.2. Biological effect of luteoforol against some *Erwinia amylovora* strains and Epiphytic bacteria

Different concentrations of luteoforol, luteolinidin and their solvents were tested in liquid cultures of several bacterial strains. The bacterial strains used were the same as in the inhibition test on solid medium.

To test the effect of the examined compounds, the bacterial strains were cultured in 96-well microtiter plates. The *Streptococcus* strain, one of the causative agents of human dental caries, was cultured on CasoY at 37° C. The remaining bacterial strains were grown in M9 liquid minimal medium maintained at 27° C.

The final volume in each well was 150 µl. Each well was inoculated with an aliquot from an overnight culture on LB of the respective bacterial species. The compounds tested were:

Luteoforol	1mM
	0,1mM
	0,01 mM
Luteolinidin	10 mM
	1mM
	0,1 mM
Ethyl-acetate 1	Same volume used to reach in the final sol. the luteoforol 1mM
Ethyl-acetate 2	Same volume used to reach in the final sol. the luteoforol 0,1mM
Ethyl-acetate 3	Same volume used to reach in the final sol. the luteoforol 0,01mM
Methanol 1	Same volume used to reach in the final sol. the luteolinidin 10 mM
Methanol 2	Same volume used to reach in the final sol. the luteolinidin 1 mM
Methanol 3	Same volume used to reach in the final sol. the luteolinidin 0,1 mM
Water	Used as negative control

Table 6: chemical tested for they ability to inhibit bacterial growth and fungal spore germination.

The bacterial population was assessed at 0h, 4h, 24h, 48h, 72h and 96h after inoculation. For the population assessment, 15 l of bacterial culture was used to make serial dilutions. Each dilution was plated on LA medium and incubated at 27 °C. After 24-48 h the number of bacterial colonies was counted.
The experiment was repeated twice.

17.3. Biological effect of luteoforol and luteolinidin on the spore germination of some phytopathogenic fungi

This experiment was performed in a similar way to those with bacteria. All the fungi were incubated in 96 well microtiter plates, spore germination and development of mycelium were assessed. Each well was filled with *Asperigillus* minimal medium and with one of the tested compounds. The concentrations of the compounds tested were the same as those used with the bacteria. The plates, wrapped in aluminium foil, were incubated at room temperature. Spore germination and mycelial growth were assessed daily with the aid of a stereomicroscope.

The fungi used in this experiments are reported in the following table:

ORGANISM	SPORE CONCENTRATION
Alternaria solani	$2x10^4$
Alternaria brassicicola	$2x10^4$
Botrytis cinerea	$2x10^3$
Colletoctricum lagenarium	$2x10^4$
Fusarium culmorum	$2x10^3$
Phytophthora infestans	$2x10^4$
Pyricularia orizae	$2x10^4$
Venturia inaequalis	$2x10^4$

Table 7: fungal species against which luteoforol was tested

The germination of sporangia and zoospores of *Phytophthora infestans* was assessed.
The experiment was repeated twice.

17.4. IPF test

Immature pear fruits of cv. Conference were used for the test. The fruits were surfaced sterilised for 5 minutes in a bleach solution, which contained 3% active chlorine. The fruits were then rinsed several times in sterile water. 20 fruit cores per chemical were used in the experiment. The chemicals tested were: luteoforol 1mM and luteolinidin 10mM; ethyl acetate, methanol and water acted as controls. Each core was inoculated with a 10 l drop of a 1,75 x 10^4 cfu/ml culture of Ea 1/79. Immediately after inoculation, the chemical solution was added as a 10 l drop. The cores were incubated in a humid chamber at 27° C for one week. Symptom development was assessed daily.
A similar experiment was also performed in Bologna. In this case, a 1-day old luteoforol solution (50 ppm) was used. A 10 µl drop of a luteoforol solution was added to the immature pear fruit cores previously inoculated with 10 µl of a bacterial suspension of Ea1/79 containing 4.2 x 10^7 cfu/ml.

RESULTS

RESULTS

1. ISOLATION, IDENTIFICATION AND SELECTION OF A VIRULENT *ERWINIA AMYLOVORA* STRAIN

The putative *E. amylovora* colonies isolated on MS and CCT medium were transferred on Luria agar plates. Using the Bereswill *et al.*, (1992) protocol an *E. amylovora* strain was successfully isolated. The isolate obtained in this way was named **EaDCA289/01**. The identification was confirmed by amplification and sequencing of 16S ribosomal DNA.
Finally, the virulence of EaDCA 289/01 was successfully tested on immature pear fruits and on apple and pear seedlings.
Using an IPT test, this strain was compared for virulence with Ea1540 and Ea8865, two *E. amylovora* strains from New Zealand (table 1).

	N° of core with exudates					
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Control	0	0	0	0	0	0
EaDCA 289/01	0	3	10	10	10	10
Ea1540	0	0	10	10	10	10
Ea8865	0	0	9	10	10	10

Table 1: virulence of EaDCA289/01 in comparison to Ea1540 and Ea8865. For the inoculation a 10 µl drop of a $MgSO_4$ (10 mM) suspension containing $3.5x10^6$ cfu/ml of each pathogenic strain was pipetted on the top of the cores.

2. ISOLATION, IDENTIFICATION AND SELECTION OF BACTERIAL ANTAGONISTS AGAINST FIRE BLIGHT

Several bacterial strains have been isolated and tested as biological control agents against fire blight. Among them, only the two most promising were successfully identified.
One of them is an antibiotic-producing *Pantoea agglomerans* strain, named **EhDCA269/01**. The other one is a gram positive bacterium belonging to *Microbacteriacea* family and named **MicDCA210/01**.It does not seem to produce antibiotics. The 16SrRNA sequences and the BLAST report is showed in the followings boxes.

***Pantoea agglomerans* EhDCA269/01**
16SrRNA Sequence:

```
TTAAGCTACCTACTTCTTTTGCAACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTA
TTCACCGTGGCATTCTGATCCACGATTACTAGCGATTCCGACTTCACGGAGTCGAGTTGCAGACTCCGATCC
GGACTACGACGCACTTTGTGAGGTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTTGTATGCGCCATTGTAGCA
CGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTATCACCGGC
AGTCTCCTTTGAGTTCCCGACCGAATCGCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACC
CAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGTCTCACGGTTCCCGAAGGCACCAAGGCAT
CTCTGCCAAATTCCGTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCC
ACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGGCGTACTCCCCAGGCGGTCGACTT
AACGCGTTAGCCTCCGGAAGCCACTCCTCAAAGGGAACAAACCTCCAAGTCGACATCGTTTACGGCGTGGAC
TAACCAGGGTATCTAAATCCT
```

Sequences producing significant alignment	Organism	Score (bits)	E Value
gi|4582193|emb|AJ233423.1|PAG233423	*Pantoea agglomerans*	1237	0.0
gi|4754827|gb|AF130895.1|AF130895	*Pantoea agglomerans*	1237	0.0

Summary of BLAST report:

- gi|4582193|emb|AJ233423.1|PAG233423 *P. agglomerans* 16S rRNA gene (strain DSM 3493)
  Length = 1473

Score = 1237 bits (624), Expect = 0.0
Identities = 658/664 (99%), Gaps = 4/664 (0%)
Strand = Plus / Minus

- gi|4754827|gb|AF130895.1|AF130895 Pantoea agglomerans strain new*16 16S ribosomal RNA gene, partial sequence
  Length = 1485

Score = 1237 bits (624), Expect = 0.0
Identities = 658/664 (99%), Gaps = 4/664 (0%)
Strand = Plus / Minus

***Microbacterium* sp. MicDCA210/01**
16SrRNA Sequence:

TTCCACAAGGGTTAGGCCACCGGCTTCGGGTGTTACCGACTTTCATGACTTGACGGGCGGTGTGTACAAGGCC
CGGGAACGTATTCACCGCAGCGTTGCTGATCTGCGATTACTAGCGACTCCGACTTCATGAGGTCGAGTTGCAG
ACCTCAATCCGAACTGAGACCGGCTTTTTGGGATTCGCTCCACCTTGCGGTATTGCAGCCCTTTGTACCGGCC
ATTGTAGCATGCGTGAAGCCCAAGACATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGAGTTG
ACCCCGGCAGTCTCCTATGAGTTCCCACCATTACGTGCTGGCAACATAGAACGAGGGTTGCGCTCGTTGCGGG
ACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTTTACGAGTGTCCAAAGAGTTG
ACTATTTCTAGCCCGTTCTCGTATATGTCAAGCCTTGGGTAAGGTTCTTCGCGTTGCATCGAATTAATCCGCA
TGCTCCGCCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTTAAGCCTTGCGGCCGTACTCCCCAGGCGGG
GCGCTTAATGCGTTAGCTGCGACACGGAAA

Sequences producing significant alignment	Organism	Score (bits)	E Value
gi|11275332|dbj|AB028941.1|	Microbacteriaceae str.	1300	0.0
gi|515021|emb|X77450.1|CA16SR	*C. aquaticum*	1279	0.0

Summary of BLAST report:

- gi|11275332|dbj|AB028941.1| Microbacteriaceae str. DB103 16S rRNA gene

  Length = 1410

Score = 1300 bits (656), Expect = 0.0
Identities = 761/788 (96%), Gaps = 8/788 (1%)
Strand = Plus / Minus

- gi|515021|emb|X77450.1|CA16SR *C. aquaticum* (DSM 20146) 16S rRNA gene

  Length = 1475

Score = 1279 bits (645), Expect = 0.0
Identities = 758/788 (96%), Gaps = 8/788 (1%)
Strand = Plus / Minus

All the bacterial isolates able to inhibit *E. amylovora* on minimal medium were stored glycerol stock for further test and for identification. In the following figures (1-3), the efficacy of EhDCA269/01 in reducing fire blight symptoms development in IPF test is shown. The results achieved with EhDCA269/01 were compared with the ones obtained with P10C, a *P. agglomerans* strain registered in New Zealand as fire blight control agent.

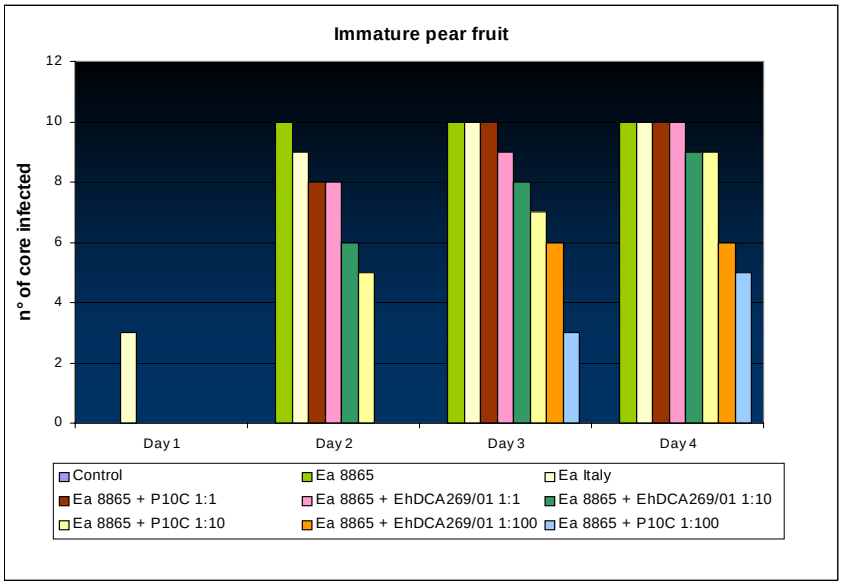


Figure 1: EhDCA269/01 inhibits Ea8865 in IPT test with an efficacy comparable P10C effect. The IPF were inoculated with a pathogen suspension containing $3.5x10^6$ cfu/ml. The minimal concentration for both the antagonists was $2.2x10^6$ cfu/ml.

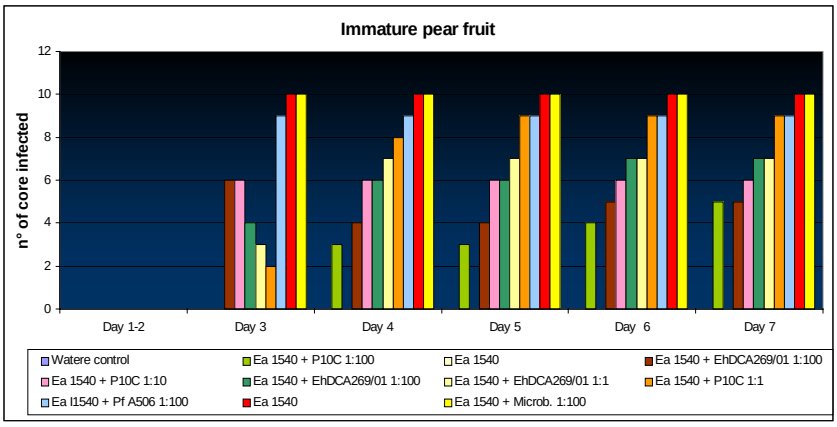


Figure 2: effect of EhDCA 269/01 and MicDCA 210/01 on Ea1540. Their efficacy is compared with P10C effect. The IPF were inoculated with a pathogen suspension containing $4.3x10^6$ cfu/ml. The minimal concentration for each antagonists ranged between $2.1\text{-}2.8x10^6$ cfu/ml.

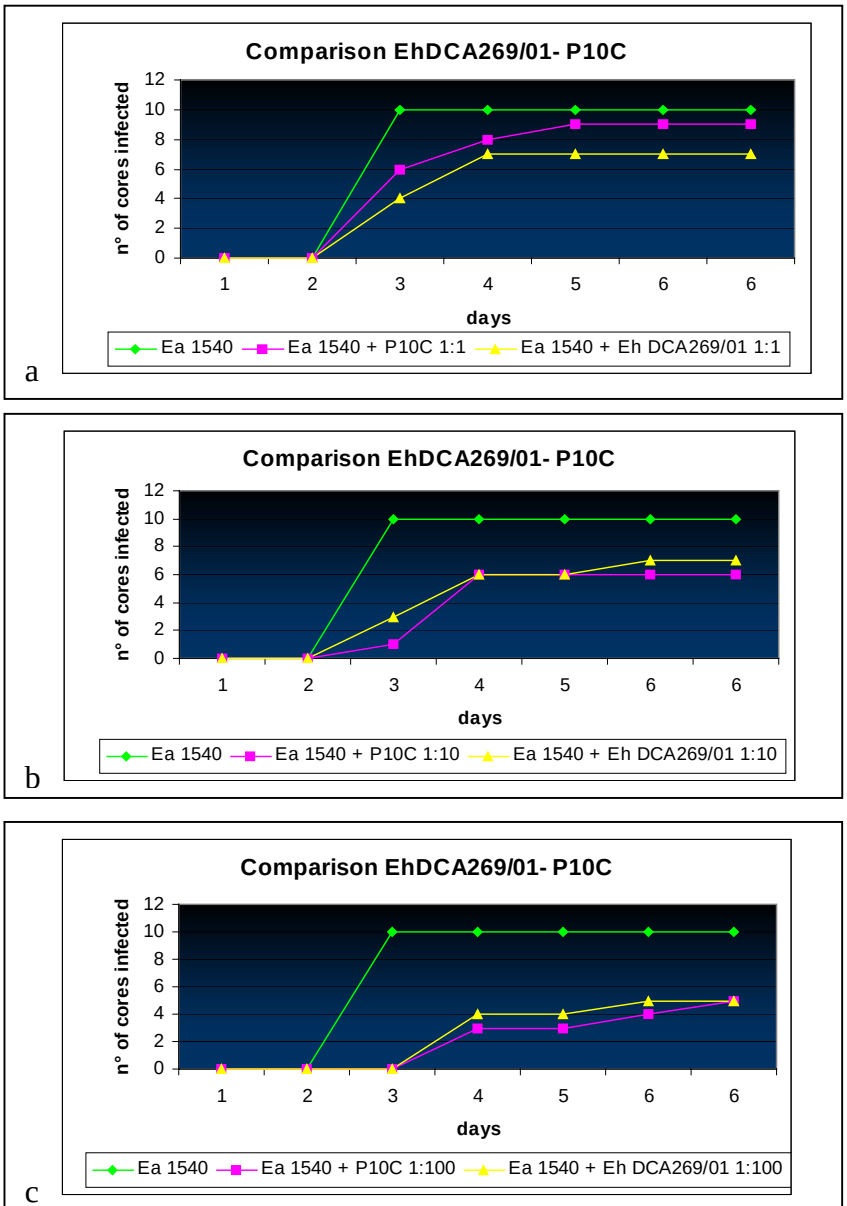


Figure 3: comparison between P10C and EhDCA269/01 in reducing symptoms development in immature pear fruits inoculated with Ea1540. A: Ea/Eh ratio 1:1; B: Ea/Eh ratio 1:10; C: Ea/Eh ratio 1:100. The IPF were inoculated with a pathogen suspension containing $4.3x10^6$ cfu/ml. The minimal concentration for both the *P. agglomerans* strains ranged between $2.1\text{-}2.8x10^6$ cfu/ml.

3. EFFICACY OF DIOXYGENASE INHIBITORS IN REDUCING SHOOT BLIGHT INCIDENCE (SECONDARY INFECTION)

Under field conditions, TrixE, applied on at a dosage of 500 ppm and 15 day before artificial inoculation, reduced both fire blight incidence and severity. The results are reported in figure 4 and 5. The treatment reduced the incidence of almost 50%, in comparison to control. Moreover, this effect was long lasting, since the incidence reduction was still detectable almost 2 month after inoculation. Severity has been calculated as the ratio between shoot necrosis length and total shoot length:

Severity = (Ln/Lt)x100

Ln: necrosis length
Lt: shoot length

Therefore, the severity increase, observed in treated shoots 27 days after inoculation, might be explained with a reduction in total shoot length due to TrixE growth retardant effect (see experiment 4).

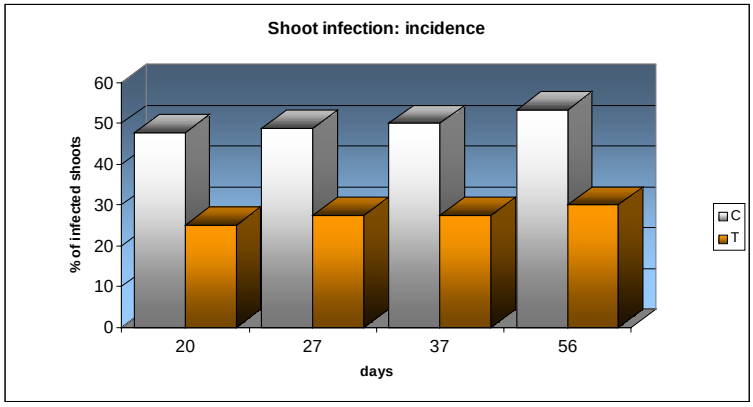


Figure 4: fire blight incidence reduced by TrixE (500ppm) application. Experiment performed in orchard conditions on Braedburn trees artificially inoculated with Ea8865 (1.2 x 10^7 bacterial cfu/ml). The differences observed are significant according to SNK ($p<0.05$)

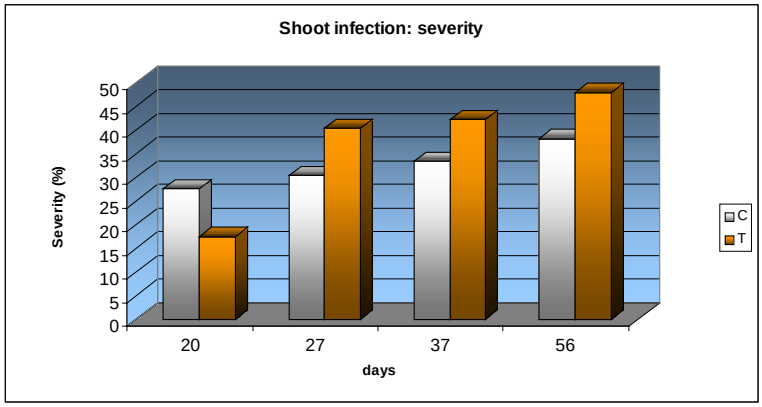


Figure 5: Effect of TrixE application (500ppm) on shoot blight severity. Experiment performed in orchard conditions on Braedburn trees artificially inoculated with Ea8865 (1.2 x 10^7 bacterial cfu/ml). No significant difference.

4. EFFICACY OF DIOXYGENASE INHIBITORS IN REDUCING SHOOT GROWTH.

The application of TrixE reduced shoot growth both on Braeburn and Pink Lady plants. Since Braeburn trees were the same used in the previous experiments, they were treated two times with TrixE 500 ppm. The residual effect of the first TrixE application could explain the initial difference in shoot length observed between treated and control plants.

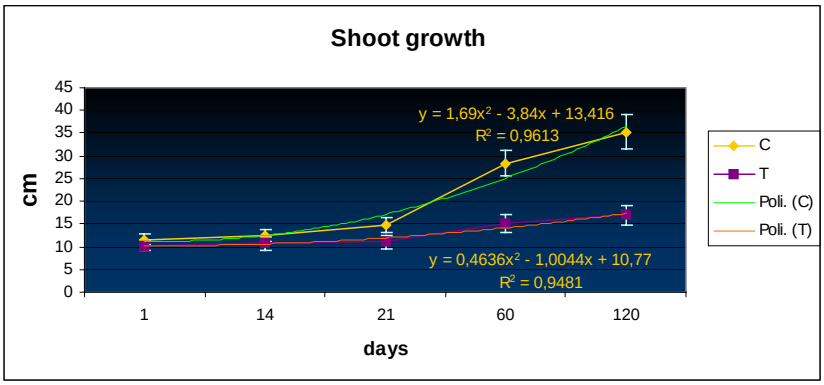


Figure 6: effect of TrixE application on shoot growth of Pink Lady trees. The differences observed are significant according to SNK ($p<0.05$)

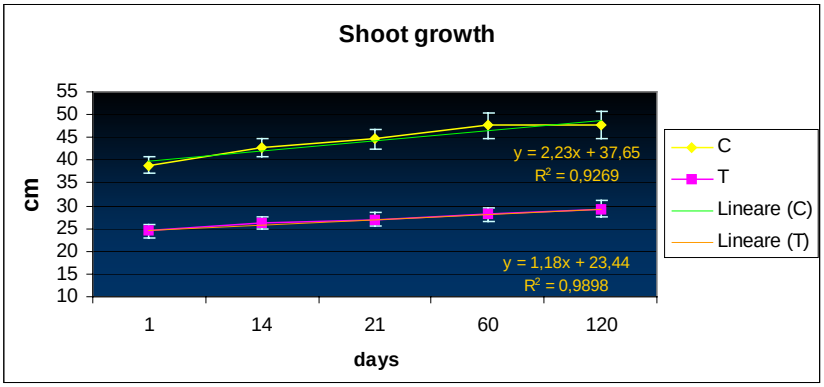


Figure 7: effect of TrixE application on shoot growth of Braedburn trees. The differences observed are significant according to SNK ($p<0.05$)

5. EFFICACY OF DIOXYGENASE INHIBITORS IN REDUCING BLOSSOM BLIGHT INCIDENCE (PRIMARY INFECTION)

All these experiments were performed on apple plant materials cv. Royal Gala.

TrixE application reduced fire blight incidence both in controlled and field conditions. On flowering branches the incidence reduction, in comparison to control was about 50% (fig. 8). On detached flowers, TrixE was ineffective, and treated flowers showed a slightly higher incidence than control ones (fig. 9).

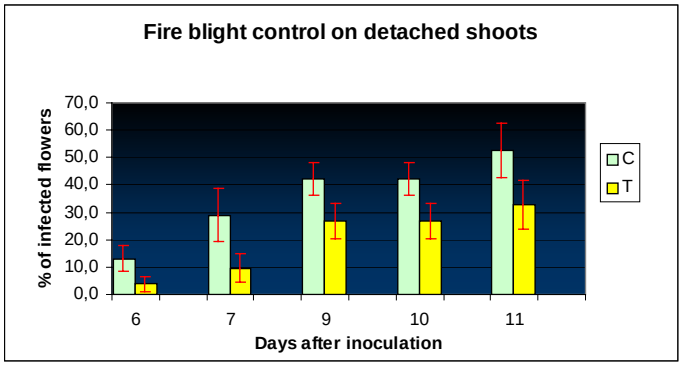


Figure 8: effect of TrixE on blossom blight incidence on flowering braches. Differences are significant at day 9-10 according to Duncan's test ($P<0.05$). Standard error is shown.

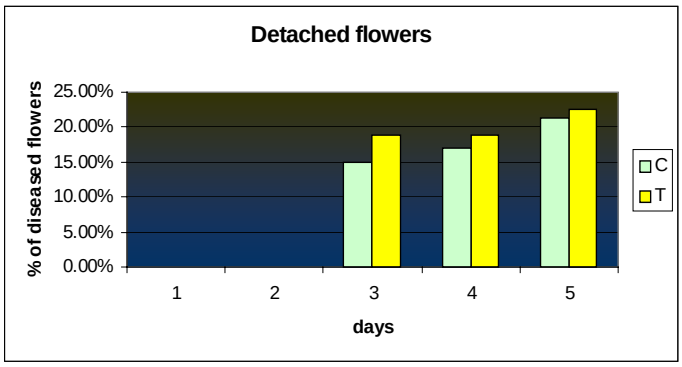


Figure 9: effect of TrixE on blossom blight incidence on detached flowers. No significant differences were observed.

TrixE treatment reduced the blossom blight incidence also under field conditions (fig. 10).

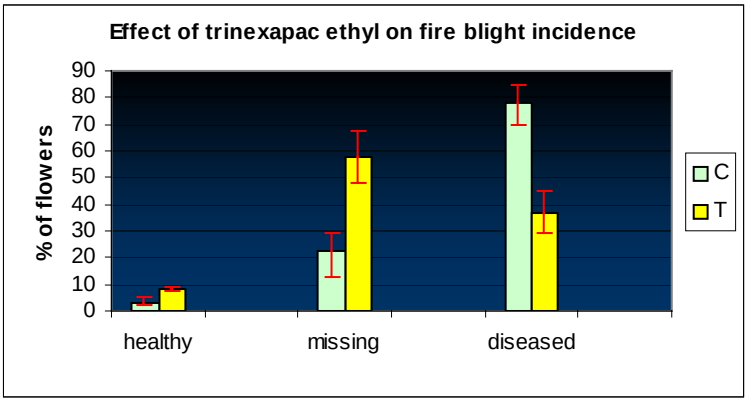


Figure 10: effect of TrixE on blossom blight on apple trees under filed conditions. Differences found missed and diseased flowers are significant according to SNK test ($P<0.05$). Standard error is shown.

Moreover, the percentage of healthy flowers in treated plant was three times higher than in control. Finally, in treated plants more flowers fell down than in control.

The efficacy of ProCa in reducing blossom blight was tested on different pear cultivars (Abbé fetèl, Tosca and Williams). ProCa application reduced blossom blight, but the reduction was not statistically significant on Abbé fetèl and Williams scions. On the contrary, a statistically significant reduction of incidence was observed on Tosca plants 8 days after inoculation (fig. 11-12). The number of infected blossoms, which successively developed shoot necrosis, was assessed 6-8 days after inoculation (fig 13). Both on Abbé fetèl and Tosca scions the treatment reduced significantly shoot blight incidence consequent to flower inoculation.

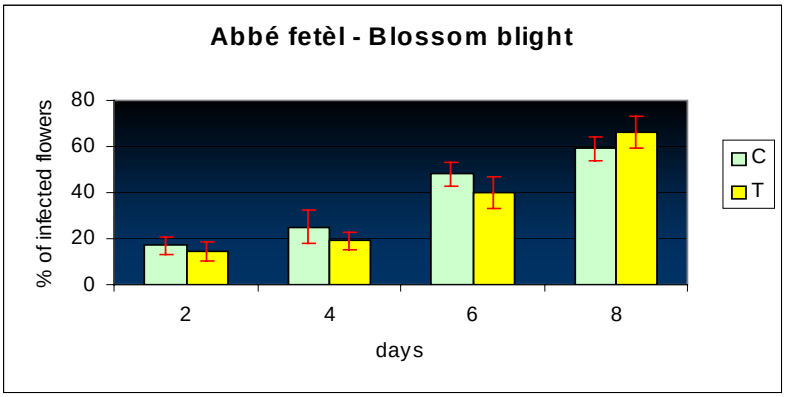


Figure 11: effect of ProCa on blossom blight (Abbé fetèl). Means separation according to according to SNK test (P<0.05).

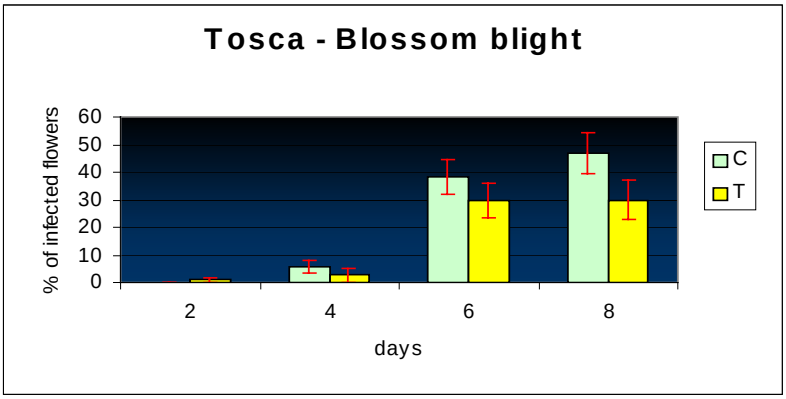


Figure 12: effect of ProCa on blossom blight (Tosca). Means separation according to according to SNK test (P<0.05).

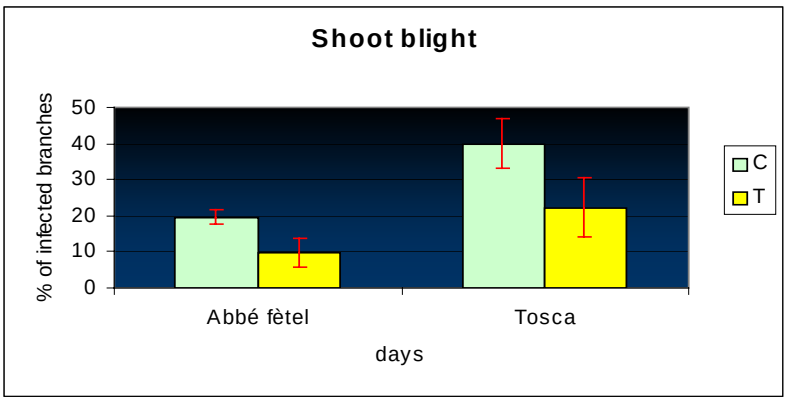


Figure 13: effect of ProCa on shoot blight after flower inoculation. Means separation according to SNK test (P<0.05).

6. EFFECT OF DIOXYGENASE INHIBITORS APPLICATION ON APPLE AND PEAR NECTAR COMPOSITION

Even if apple and pear nectar is mainly composed by sucrose, fructose and glucose, the relative ratio of these sugars differs between the two species. Pear nectar is much less concentrated than apple one. Sugar composition of apple and pear nectar is shown in table 2. Both in apple and pear the ProCa application reduced the nectar sugar content significantly. Moreover, ProCa treatment enhanced the daily production of nectar per flower.

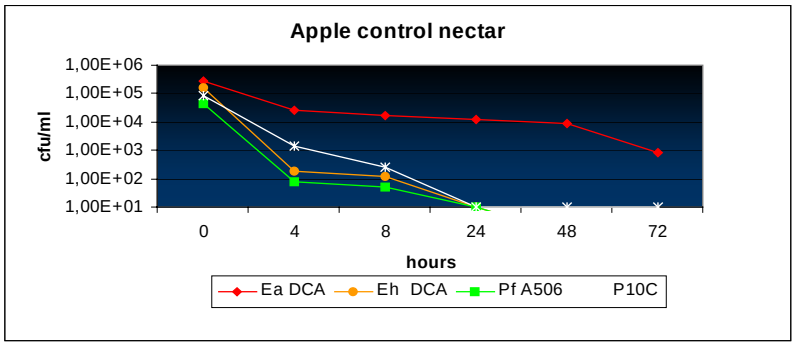
	Fructose %	Glucose %	Sucrose %	TOTAL %	N° flowers/100 µl nectar
Pear T.	5,8821 a	6,6300 a	0,8355 a	13,348 a	197
Pear C.	7,7298 a	8,6431 b	1,0109 a	17,556 b	273
Apple T.	6,3811 a	6,6756 a	27,059 a	40,116 a	331
Apple C.	9,2564 b	9,5769 b	30,895 b	50,338 b	442

Table 2: effect of ProCa on sugar concentration of apple and pear nectar. The sugar concentration is expressed as a percentage on the total weigh of nectar. Means separation by Duncan's test ($P \leq 0,05$).

As far as, the determination of phenolic compounds the nectar form treated and control flowers, the extraction and detection resulted ineffective.

7. EFFECT OF NECTAR SUGAR COMPOSITION ON THE BACTERIAL GROWTH

Artificial nectar, prepared according to the analytical data obtained in the previous experiment, was used to cultivate *E. amylovora* and some bacterial antagonists.
Since its lower sugars concentration, the treated nectar had a lower osmotic potential than control nectar. Therefore, it was more suitable for bacterial growth.



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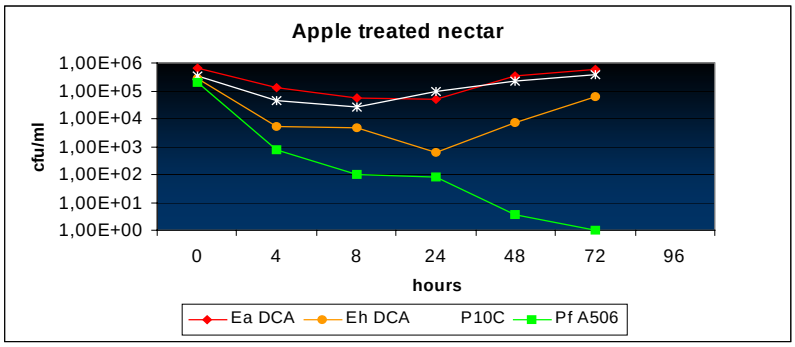

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Figure 14-15: effect of apple nectar on the bacterial growth. In fig. 11 is shown the effect on nectar from control flowers, whereas, fig. 12 shows the effect of nectar from flowers treated with ProCa 250ppm

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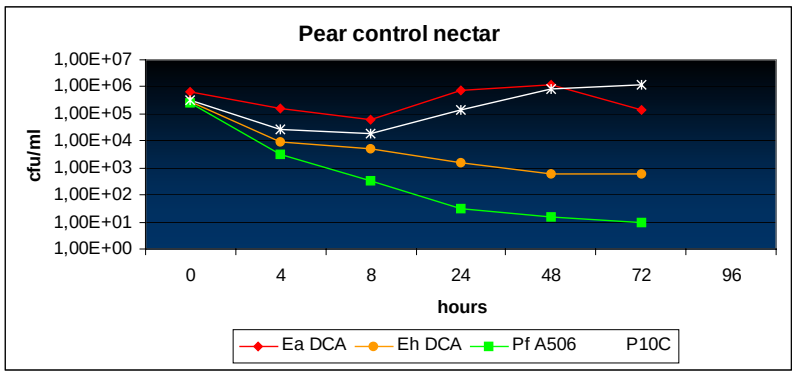

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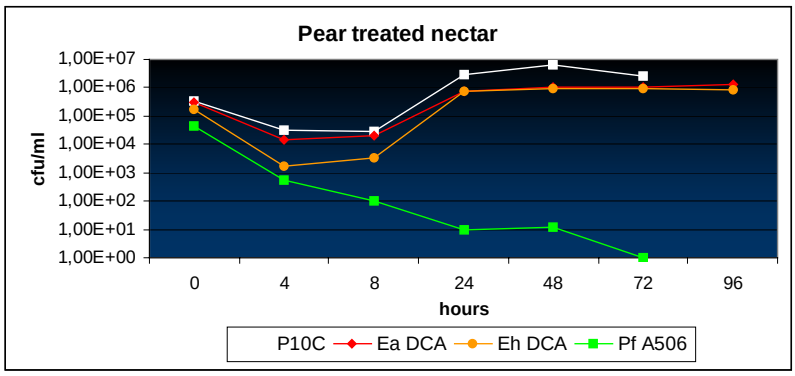


Figure 16-17: effect of pear nectar on the bacterial growth. In fig. 13 is shown the effect on nectar from control flowers, whereas, fig. 14 shows the effect of nectar from flowers treated with ProCa 250ppm

EaDCA289/01, the *E. amylovora* strain used in this experiment, was less sensitive than the bacterial antagonists to the nectar sugar concentration. Whereas, *Pf*A506 resulted the most sensitive bacterium. When cultivated in apple nectar from control flowers, the population of all the bacterial antagonists tested decreased rapidly, and it was not detectable 24 hours after inoculation. In apple treated nectar, *P. agglomerans* (EhDCA269/01 an P10C) populations stabilized to a consistent level (~10^5 cfu/ml). *Pf*A506 population decreased and it was not detectable anymore 72 hours after

inoculation. Therefore, these bacteria can survive longer in the treated nectar and they might even multiply.
The pear nectar is less concentrated than the apple one. Therefore, its effect on the bacterial growth is less strong. Nevertheless, the nectar from treated flowers supported a higher bacterial population than the one from control flowers. In treated pear nectar, EhDCA269/01 and P10C multiplied, whereas they just survive in control nectar. As far as EaDCA289/01, it can multiply indifferently on treated or control pear nectar.
The effect of treated and control nectar was tested also on MicDCA210/01 and on a not-identified white bacterium, which was very often isolated from apple blossoms. The results obtained with these two bacteria are substantially similar to the ones obtained with the other epiphytic bacteria previously tested and they are not shown in this dissertation. As far as the white bacteria, which will be mentioned also in following experiments, none of our several efforts of identification succeeded.

8. EFFECT OF DIOXYGENASE INHIBITORS APPLICATION ON FLOWERS AND NECTAR ATTRACTIVENESS TO HONEYBEES (*APIS MELLIFERA*)

ProCa and TrixE treatment did not affect honeybee's preference for apple flowers. Therefore, the differences in nectar sugar content did not influence honeybees feeding choice. The data were collected from12:30pm and 3:30pm, during three consecutive days characterized by moderate temperature (18-22°C) and humidity (60-70%).

Day time	12:30/13:30	13:30/14:30	14:30/15:30
ProCa	21	25	23
TrixE	25	20	22
Control	27	20	28

Table 3: number of honeybees visiting treated and control flowers at three different hours. The values shown in the table are the means of three different days. According to SNK test ($P<0.05$), no significant difference was detected

No data are available on the experiment in which flowering detached branches were used. In fact, no bees visited the branches during observations time.

9. Effect of Dioxygenase Inhibitors Application on the Natural Microbial Community on Apple and Pear Blossoms

Apple and pear blossoms, were respectively, treated with TrixE 500ppm or ProCa 250ppm. The experiment on pear blossoms was carried out in Italy. The natural epiphytic population on stigma and on nectaries was assessed (fig. 18-19).

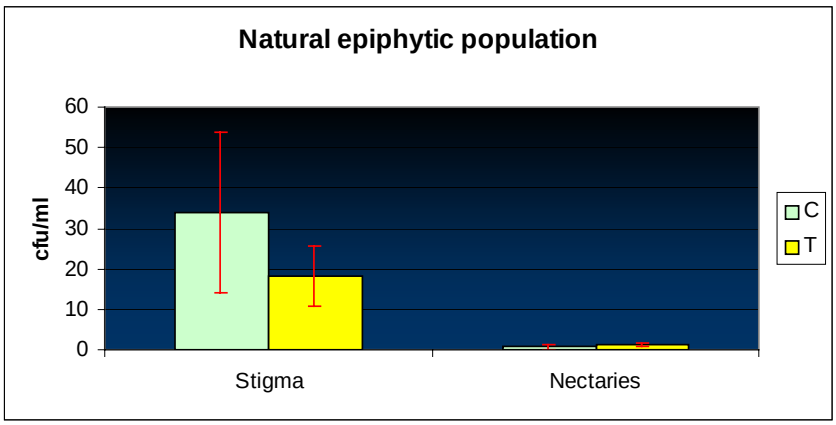


Figure 18: natural epiphytic community on pear blossoms (Bologna district).

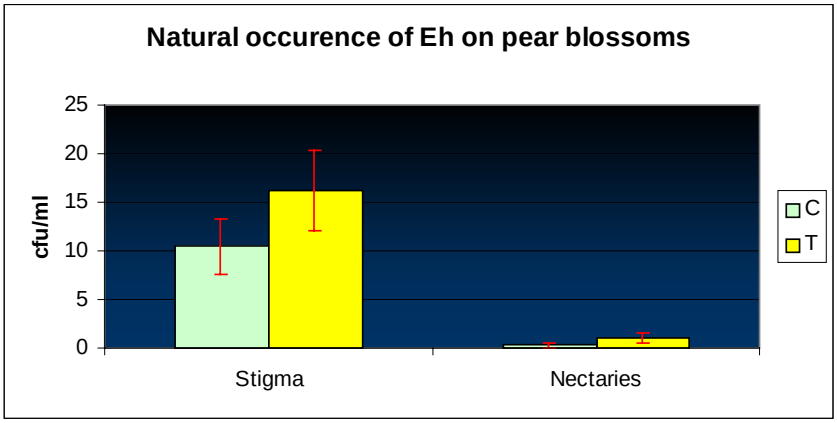


Figure 19: natural occurrence of *P. agglomerans* on pear flowers.

In these graphics the average of epiphytic population per flower is shown. The untreated stigmas supported more consistent microbial community than treated ones.
Nevertheless, the putative autochthonous *P. agglomerans* population on treated flower was higher than on control ones (fig.19). Moreover, in treated blossom, *P. agglomerans* is the predominant bacterium, and its population represent the 84-87% of all the microbial community. On the contrary, in control flowers an unidentified white bacterium it is the most frequently isolated bacterium (54-63% of all the microbial community). *P. agglomerans* colonies were putatively identified by colony morphology on Miller and Schroth medium. In any case, according to SNK test ($P<0.05$), no statistical difference was found between treated and control blossom. These results and the biodiversity index of Shannon-Weiner (H') are reported in table 4.

Control Stigma	**Total number of bacteria**	**H'**
P. agglomerans	416	0.81
White bacterium	863	
Others	74	
Treated Stigma		
P. agglomerans	649	0.44
White bacterium	79	
Others	16	
Control nectaries		
P. agglomerans	12	0.69
White bacterium	14	
Others	0	
Treated nectaries		
P. agglomerans	43	0.43
White bacterium	8	
Others	0	

Table 4: total number of bacteria isolated from treated and control pear blossoms. The Shannon-Weiner index has been calculated. Nectaries supported a very low epiphytic population

A similar experiment was performed in New Zealand on apple flowers treated with TrixE 500ppm. The data collected in this experiment are reported in figure 20-21.

The data shown refer to the average number of total epiphytic population found on treated and control stigmas. The average was calculated from the values of total microbial population on 25 different flowers per treatment.

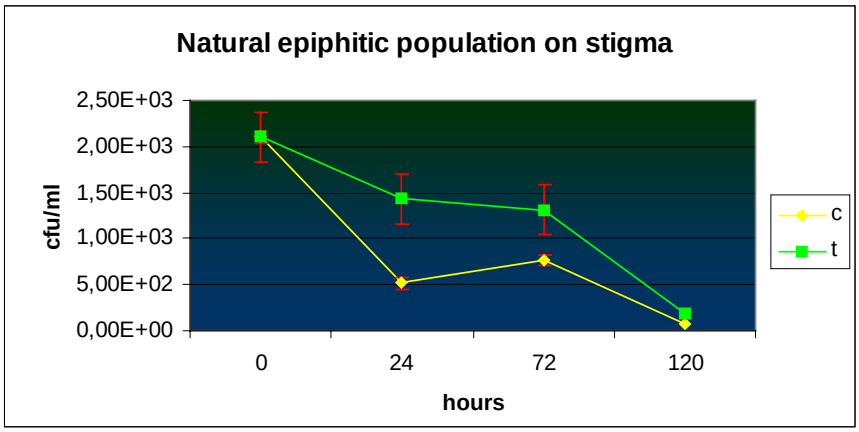


Figure 20: average number of total epiphytic bacteria on apple stigma. Standard error is shown. Means separation according to SNK test ($p<0.05$)

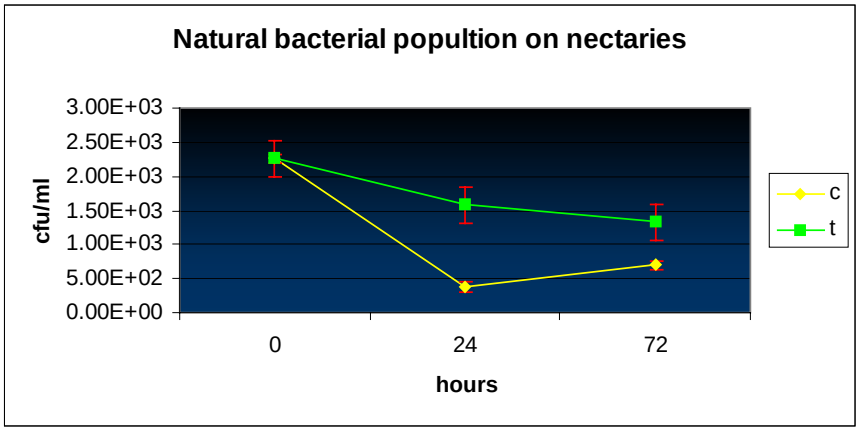


Figure 21: average number of total epiphytic bacteria on apple nectaries. Standard error is shown. Means separation according to SNK test ($p<0.05$)

Both treated stigmas and nectaries supported a higher bacterial population than control ones. No differences were found on the population of *P. agglomerans*-like bacteria. The same trees used to assess the natural bacterial population on blossoms, were inoculated, 120 hours after treatment, with Ea8865, *Pf*A506 and P10C to perform experiment n. 10. Therefore, the microbial community on stigmas at 120 hours (fig. 20) was assessed just before the artificial inoculation. The values reported result from sum of colony counting

on Luria agar plates amended with rifampicin, streptomycin and rifampicin plus streptomycin. Thus, these values might be underestimated.
Finally, the biodiversity on treated and control flowers did not show any difference (table 5)

Stigma	Hours	Biodiversity (H')	Nectary	Hours	Biodiversity (H')
Control	0	0.9649	Control	0	1.1667
Treated	0	1.1161	Treated	0	0.704
Control	24	0.8924	Control	24	0.5738
Treated	24	0.8676	Treated	24	1.0093
Control	72	1.1665	Control	72	0.724
Treated	72	1.1314	Treated	72	0.7433

Table 5: biodiversity of the microbial epiphytic community on apple stigmas and nectaries.

10. Effect of Dioxygenase Inhibitors Application on *E. amylovora*, *P. agglomerans* and *P. fluorescens* Population on Apple Blossoms

The microbial population on apple stigmas was assessed at 0, 3, 24, 48, 72, 96, 120 hours after artificial inoculation. For each treatment the microbial population of 12 flowers were examined. As far as Ea8865, not significant differences have been found between treated and untreated stigmas (fig. 22).

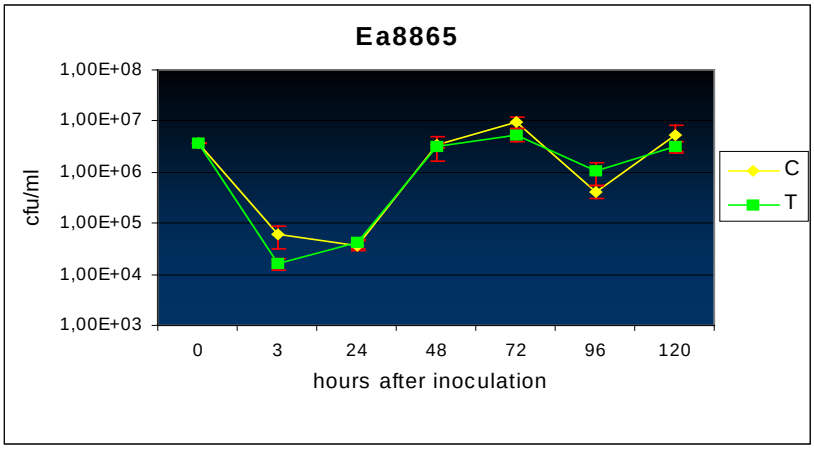


Figure 22: Ea8865 population on treated and control stigmas. No significant differences were found

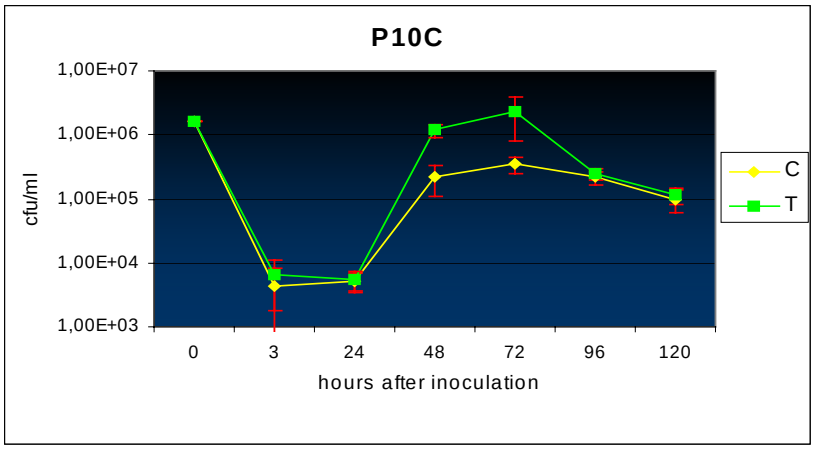


Figure 23: P10C population on treated control stigmas. The differences at 48 and 72 hours are significant according to Duncan's test ($P < 0.05$)

*Pf*A506 population was not detectable 24 hours after artificial inoculation. P10C population on treated stigmas was higher than on control ones, and, at 48 and 72 hours after inoculation this diversity was, according to Duncan's test, statistically significant for $P < 0.05$. Water treated blossoms were used as control. The natural epiphytic population on them was assessed by plating the washing solution of stigmas on Luria agar plates amended with rifampicin, streptomycin and rifampicin plus streptomycin.
No significant differences in *P. agglomerans*-like and in *E. amylovora*-like bacteria were detected between treated and control blossoms. Nevertheless, a white bacterium was often isolated (Experiment n. 7). This bacterium resulted streptomycin and rifampicin resistant. Its colony morphology resembled E. amylovora, but it did not produce HR on tobacco. The population of this bacterium was assessed both on water and *Pf*A506 treated plant (fig. 21). Also in this case, treated stigmas supported a higher bacterial population than control ones.

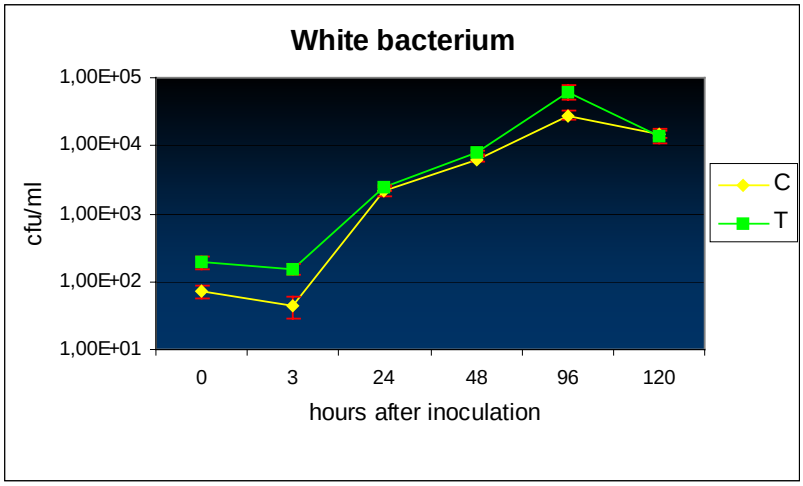


Figure 24: natural population of an unidentified white bacterium on treated and control stigmas. Means separations according SNK test ($P<0.05$)

11. EFFECT OF DIOXYGENASE INHIBITORS APPLICATION ON NATURAL MICROBIAL COMMUNITY ON APPLE LEAVES

The aim of this experiment was to test if the differences, found in the natural microbial community on blossoms, occurred also on leaves. The microbial population was assessed, by leaf imprinting, both for the superior and inferior leaf page. Both in treated and control leaves no significant differences were found between the superior and inferior leaf page. The natural epiphytic population on leaves showed such a high degree of variability that no clear statement could be drawn. Nevertheless, the biodiversity Index of Shannon-Weiner was calculated at 3 days after treatment (table 6).

Pink Lady	**(H')**	**Braedburn**	**(H')**
Inferior			
Control	1.3874	Control	1.0790
Treated	0.3883	Treated	1.4424
Superior			
Control	1.1467	Control	0.3883
Treated	1.2710	Treated	1.4597

Table 6: Shannon-Weiner Index calculated for inferior and superior leaf page.

12. EFFECT OF DIOXYGENASE INHIBITORS APPLICATION ON *E. AMYLOVORA*, *P. AGGLOMERANS* AND *P. FLUORESCENS* POPULATION ON APPLE LEAVES

Similarly to treated blossoms, also treated leaves supported a higher population of all the bacteria sprayed. As shown in the following graphics, on treated leaves the bacteria survived for a longer time and with a higher population than on control ones.

Among the bacteria tested P10C was found the best epiphyte: in comparison to Ea8865 and *Pf*A506, it reached the highest and more resistant population. In fact, P10C population was still consistent 72 hours after inoculation, whereas Ea8865 population decreased rapidly to a very low level in the first 48 hours. *Pf*A506 showed a poor ability to colonize leaf surface: its population did no reached a high level and it declined so rapidly that 7 hour after treatment it was almost undetectable. In all cases, the bacteria tested simply survive on leaves and they do not multiply. P10C, *Pf*A506 and Ea8865 populations are reported in figure 25, 26 and 27 respectively.

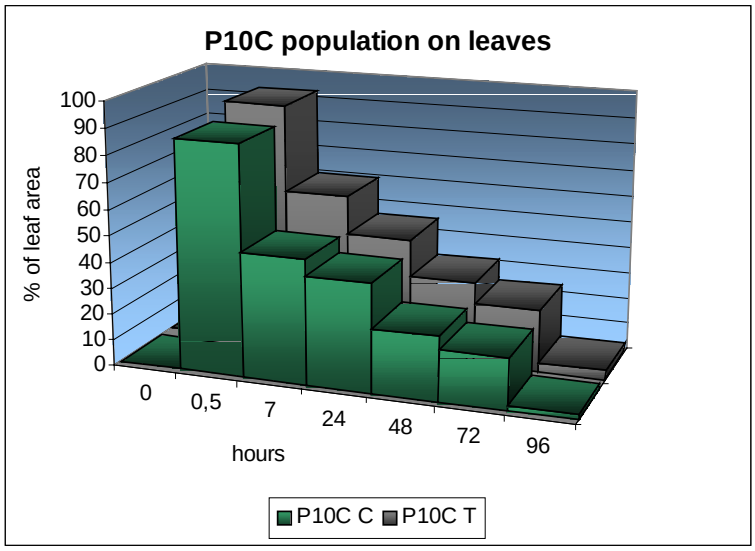


Figure 25: P10C population on apple leaves treated with TrixE 500ppm.

The bacterial population on leaves is expressed as the percentage of leaf covered by bacterial colonies. The colonies counting was always performed after 48 hours-incubation to minimize the possible differences due to a longer and more intense bacterial growth. Water treated leaves were used as control. The natural epiphytic population on them was assessed by leaf imprinting on Luria agar plates amended with rifampicin, streptomycin and rifampicin plus streptomycin. No statistically significant differences in *P.*

agglomerans-like, *P. fluorescens*-like and in *E. amylovora*-like bacteria were detected between treated and control leaves.

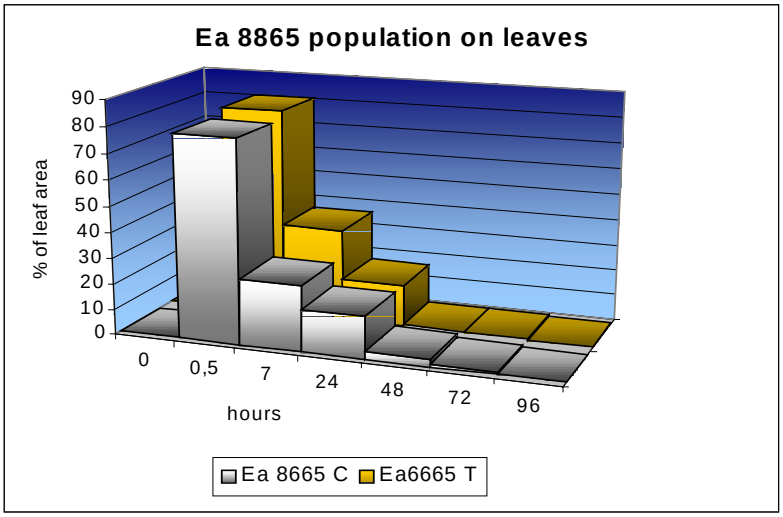


Figure 26: Ea8865 population on apple leaves treated with TrixE 500ppm.

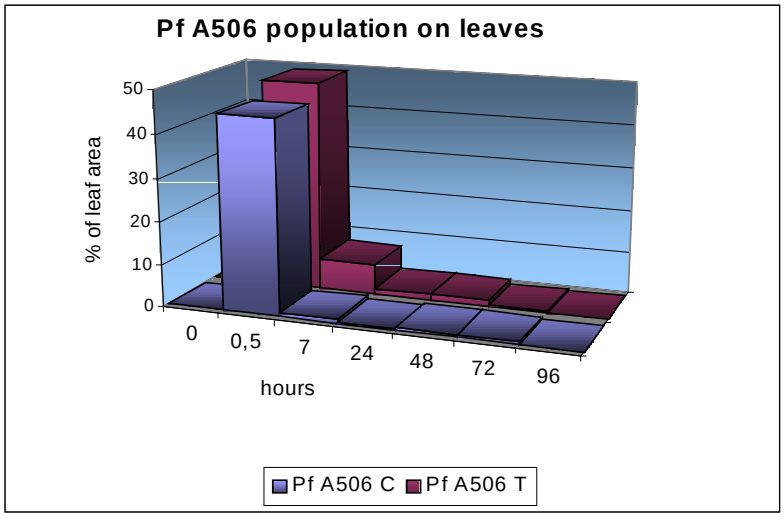
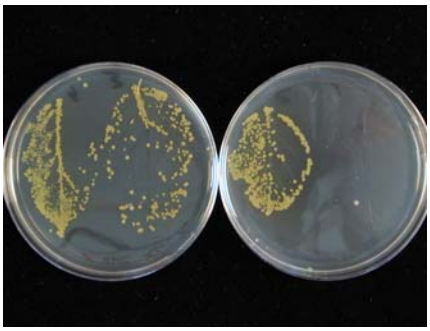


Figure 27: Ea8865 population on apple leaves treated with TrixE 500ppm.



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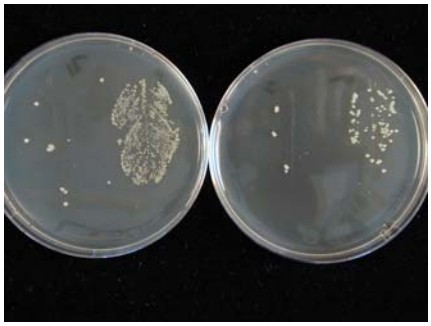

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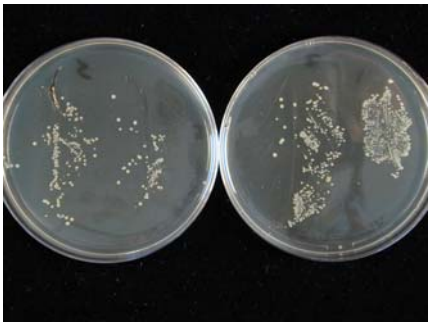

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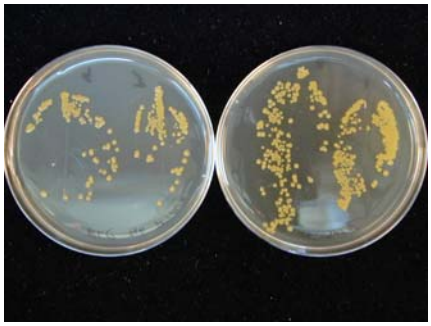

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Figure 28: control leaf imprinting 7h after inoculation with P10C
Figure 29: treated leaf imprinting 7h after inoculation with P10C
Figure 30: control leaf imprinting 7h after inoculation with Ea8865
Figure 31: treated leaf imprinting 7h after inoculation with Ea8865
Figure 32: treated leaf imprinting 24h after inoculation with P10C

13. SUGAR ON LEAVES

This experiment had the aim to explain the differences observed in the epiphytical community of treated and untreated leaves.
Sugar were extracted from leaf surface and analysed by GC. The results found are mainly qualitative. Therefore it was not possible compare the sugar content found on treated and control leaves.

	ProCa	TrixE	Control
succinic acid	3,807391	1,679644	11,11369
malic acid	5,767077	2,01891	24,36195
citric acid	1,842105	0	0
quinic acid	0,593505	0,122358	0
xylitol	0,431131	0	0
a-xylose	0,268757	0	0
b-xylose	1,690929	0	0
sucrose	0,475	0	0
fructose	4,591265	1,234705	5,452436
α-glucose	2,110862	1,334816	10,48724
β-glucose	6,304591	7,658509	51,11369
sorbitol + mannitol	1,483763	0,51168	0
mannose	15,77268	6,920467	44,64037
maltose	1,50056	1,484983	0

Table 7: organic acids and sugars on apple leaf surface treated with water, TrixE or ProCa.

Nevertheless, we found that the sugars present on leaf surface are mainly constituted by mannose, α-and β-glucose. Moreover, the amount of sugars on the surface of water treated leaves seems higher than in leaves sprayed with ProCa and TrixE. This difference is mainly due to a higher level of β-glucose. Finally, all the investigate compounds were found on ProCa treated leaves, whereas, several of them were undetectable on control leaves. Also on TrixE treated

leaves an higher number of compounds were detected in comparison to control.

14. EFFECT OF TRIXE ON BACTERIAL ENDOPHYTIC POPULATION IN APPLE TISSUES

An effective method to determine endophytic microorganisms was developed. This method confers a complete surface sterilization of plant materials (flowers and leaves), but it does not affect the bacterial population inside plant tissues.
Using this method was possible a qualitative determination of endophytic microorganisms in apple tissues. The bacteria were divided, according to colony morphology, in different groups. No significant difference in the consistence of endophytic microbial population was detected between treated tissues and control ones. The average number of microorganisms inside apple leaf tissues ranges between 8 to 41 cfu per gram of dry matter. The bacteria most frequently isolated were tested as potential biocontrol agents. Among them, different isolates presented a morphology similar to *P. agglomerans*. All the *P. agglomerans*-like bacteria resulted positive in the inhibition test against *E. amylovora*. These bacteria were stored in glycerol-stock for further test and identification. As far as the other endophytic bacteria, it was not possible the identification by amplification and sequencing of the 16S ribosomal DNA. Therefore, they were processed with the Gram stain. Then, Biolog System was used to identify the unknown bacteria. Also this method was ineffective.

15. MICROSCOPICAL INVESTIGATION I: EFFECT OF DIOXYGENASE INHIBITORS ON PRIMARY INFECTION OF BLOSSOMS

Using *gfp*-labelled *E. amylovora* and CLSM investigate the infection process on blossoms without altering specimens by staining or sectioning was possible. In addition, the SEM observation allowed the discovery of a particular anatomical feature of stigma of plants belonging to *Pomoideae* subfamily.
Observing pear, apple, *Pyracantha* sp. and *Crataegus* sp. stigmas, a channel, originating from the distal part of the stigma and continuing along the pedicel to the nectar cup, was observed. Moreover, the epidermis of this channel is constituted by stigmatic papillae.

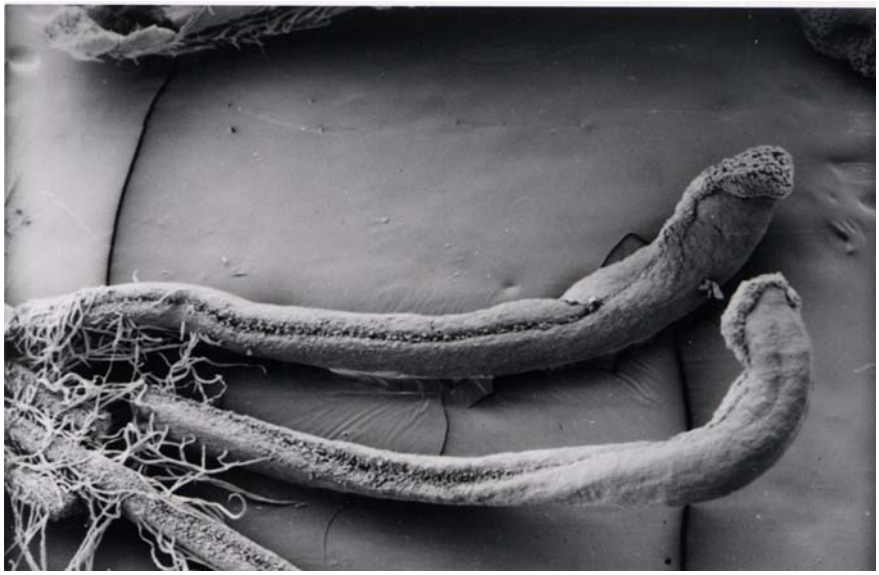


Figure 33: apple stigma. It is possible observe a channel all along the pedicel. This channel is covered with stigmatic papillae and originates from stigma. (SEM 28x)

Figure 34: particular of stigma. It is clear how the channel originate from stigma. (SEM 50x)

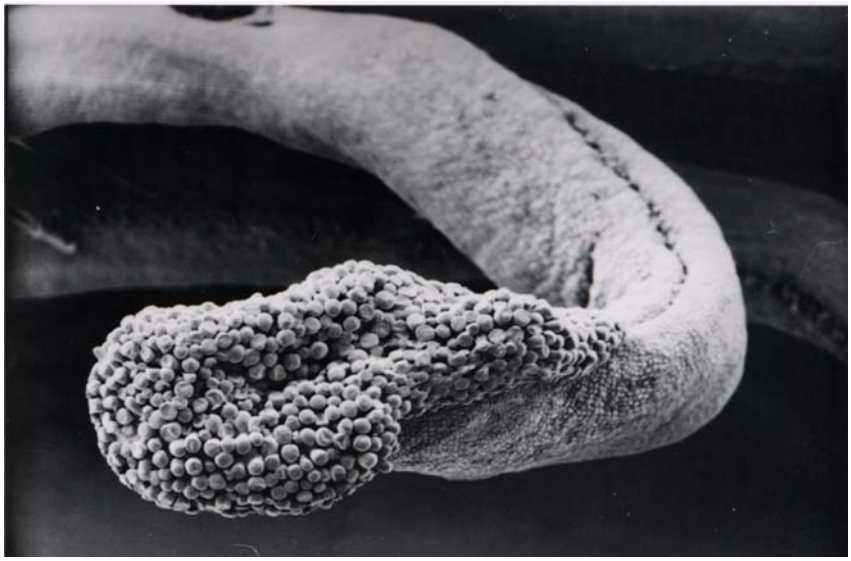


Figure 35: particular of stigma. It is clear how the channel originate from stigma. The head of stigma, covered with papillae, is "U" shaped likewise the channel (SEM 84x)

Figure 36: particular of the channel with the stigmatic papillae. (SEM 168x)

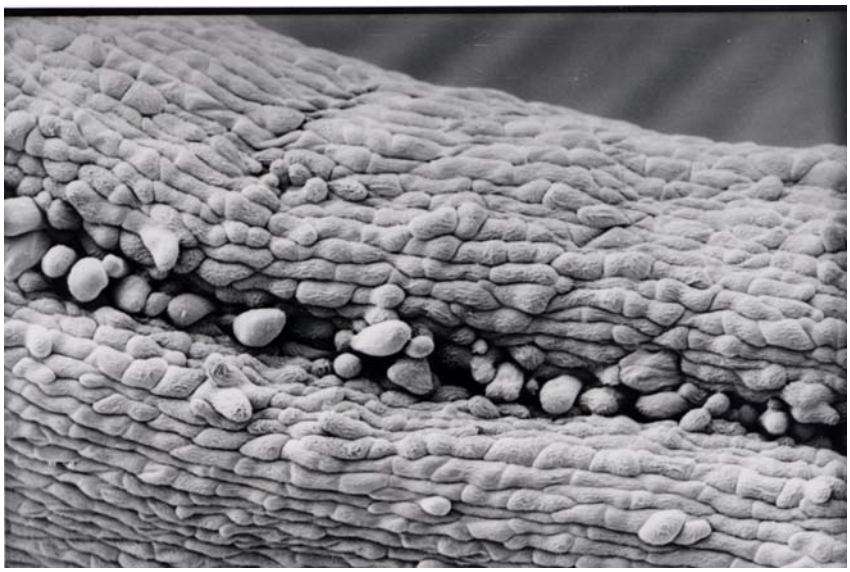


Figure 37: particular of the channel with the stigmatic papillae. (SEM 336x)

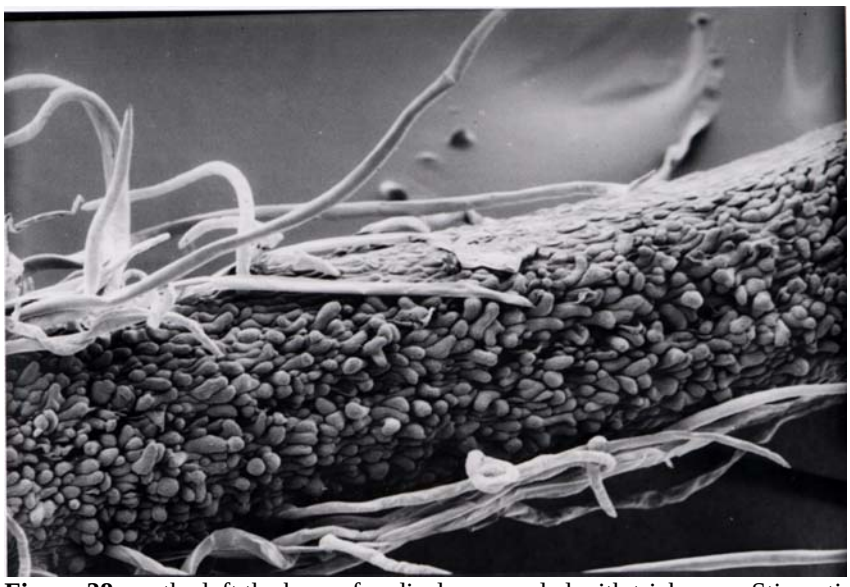


Figure 38: on the left the base of pedicel surrounded with trichomes. Stigmatic papillae from the channel are evident (SEM 168x).

This channel was found in all the plant material observed: apple ((fig.30-38, 40), pear (fig. 39), *Phyracantha* sp. and *Crataegus* sp.(fig. 41-44).

Furthermore, observing the migration of *gfp*-labelled *E. amylovora*, it was possible prove that the bacterium preferentially exploits this channel during the movement from the stigmatic surface to the nectaries (fig. 35, 36, 37, 38, 39, 40).

Neither, treatment with ProCa, nor with TrixE negatively affects the pathogen migration along the stigma.

Similarly, the colonization of stigma by *E. amylovora* and *P. agglomerans* was investigated. Bacteria localized mainly among the papillae (fig. 41-43).

The penetration of *E. amylovora* through the nectarhodes and the infection of nectaries was investigated (fig 44-47). *E. amylovora* was found moving mainly in parenchyma.

Finally, using different techniques for staining phenolic compounds, accumulation of these substances in the tissues surrounding the infection sites was detected. Nevertheless, no differences between treated and control blossoms were found.
Using Neu's reagent, tissues stained with a green-yellow colour were putatively identified as flavonoids-rich tissues.

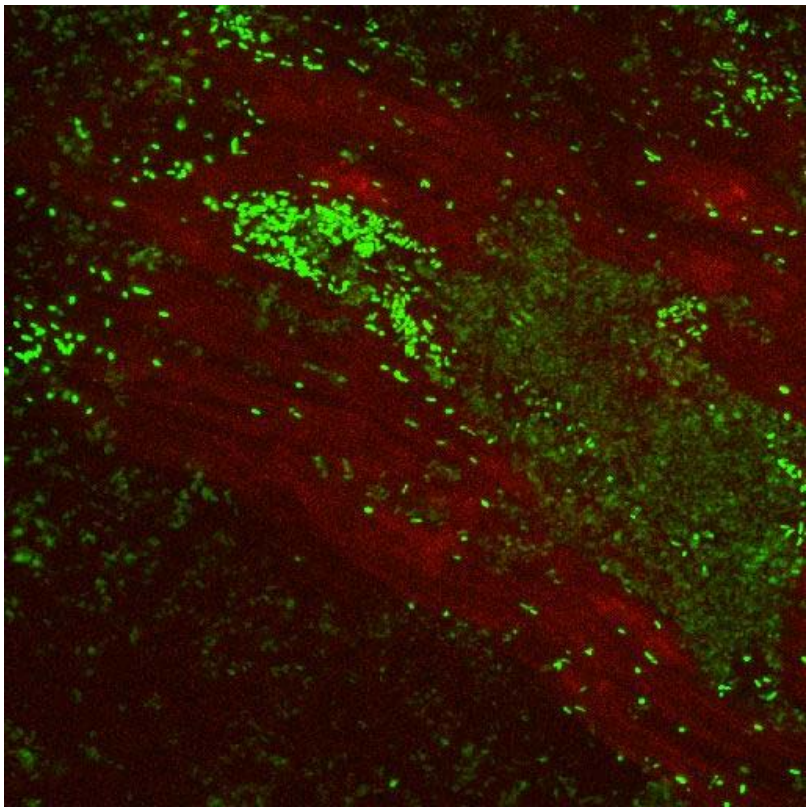


Figure 39: pear stigma heavily infected. A great number of bacteria (green) is visible in the stigmatic channel (red: plant tissues) (CLSM x1000).

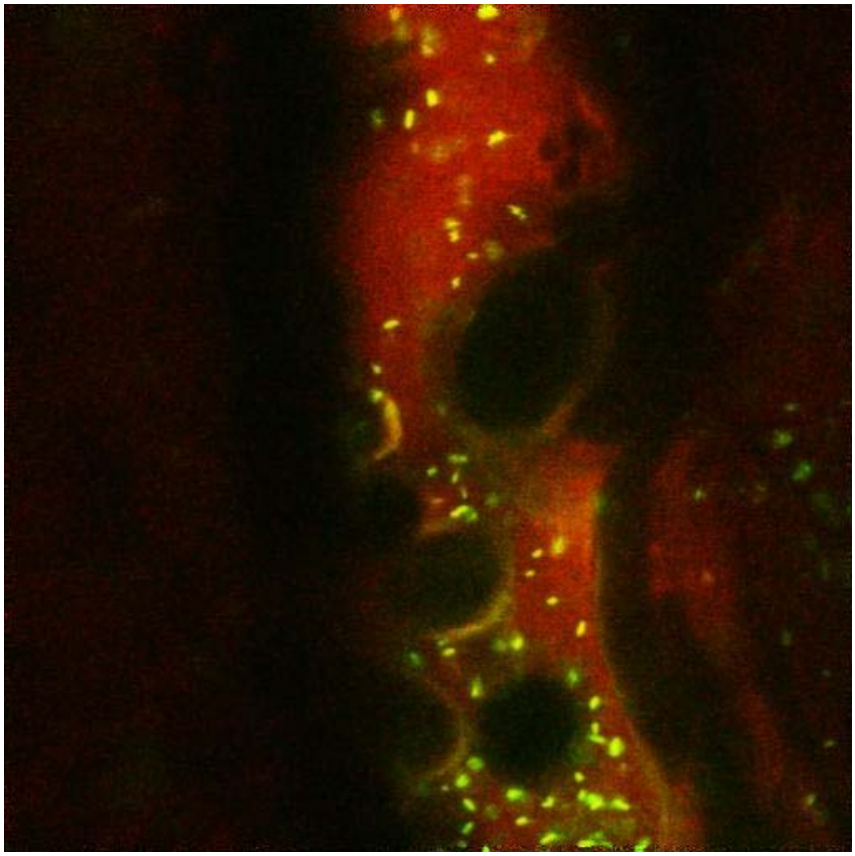


Figure 40: pedicel of apple stigma. It is evident the channels, several stigmatic papillae and the bacteria (green) moving inside the channel (CLSM 1500x).

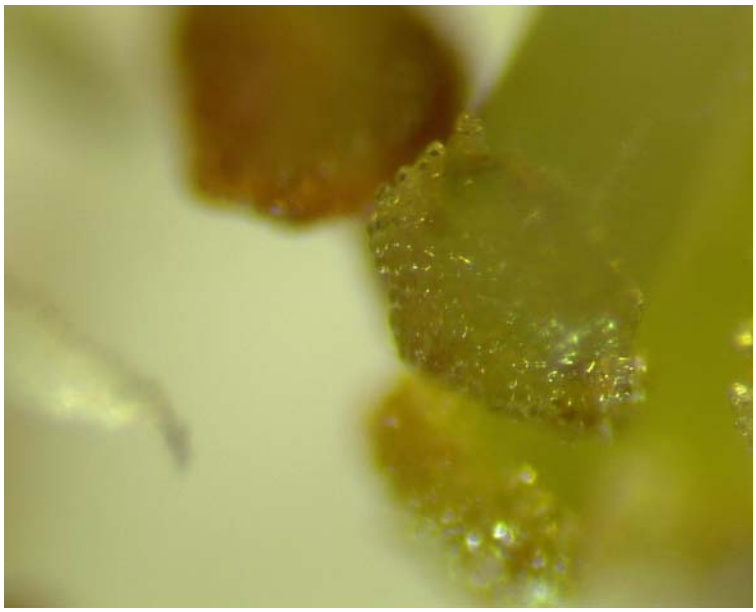


Figure 41: *Crataegus* sp. stigmas observed with a stereomicroscope (x 60). The arrow indicates the stigmatic channel.

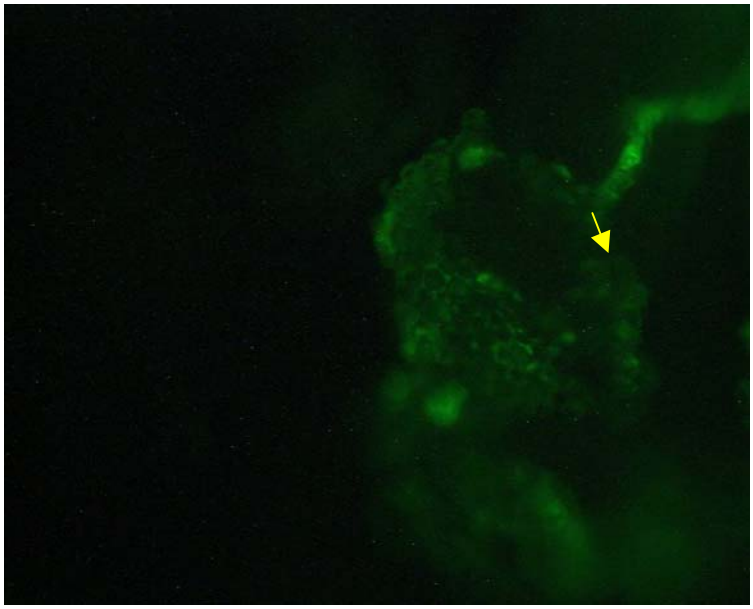


Figure 42: *Crataegus* sp. stigmas observed with a fluorescence (GHS filter set) stereomicroscope (x 60). In green the bacteria moving inside the channel

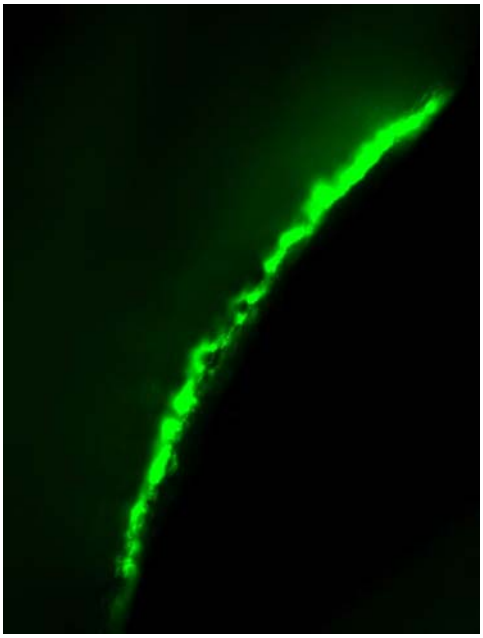


Figure 43: *E. amylovora* moving mainly inside the channel from *Crataegus* sp. stigma to nectar cup. Fluorescence stereomicroscope (170x)

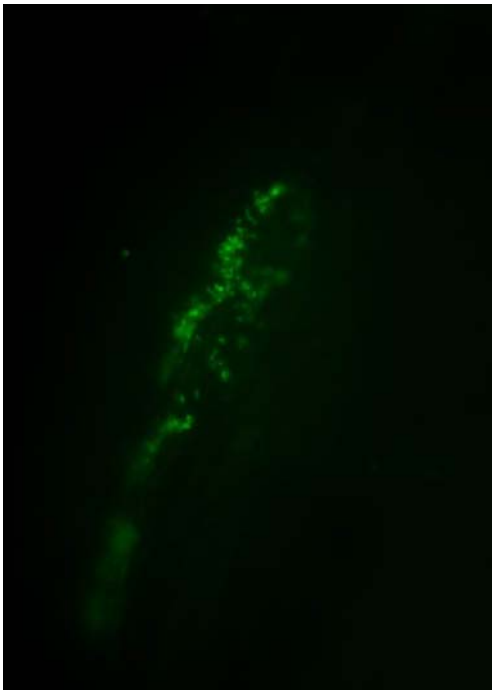


Figure 44: *Crataegus* sp. stigmas observed with a fluorescence (GHS filter set) stereomicroscope (x 60). In green the bacteria moving inside the channel (170x)

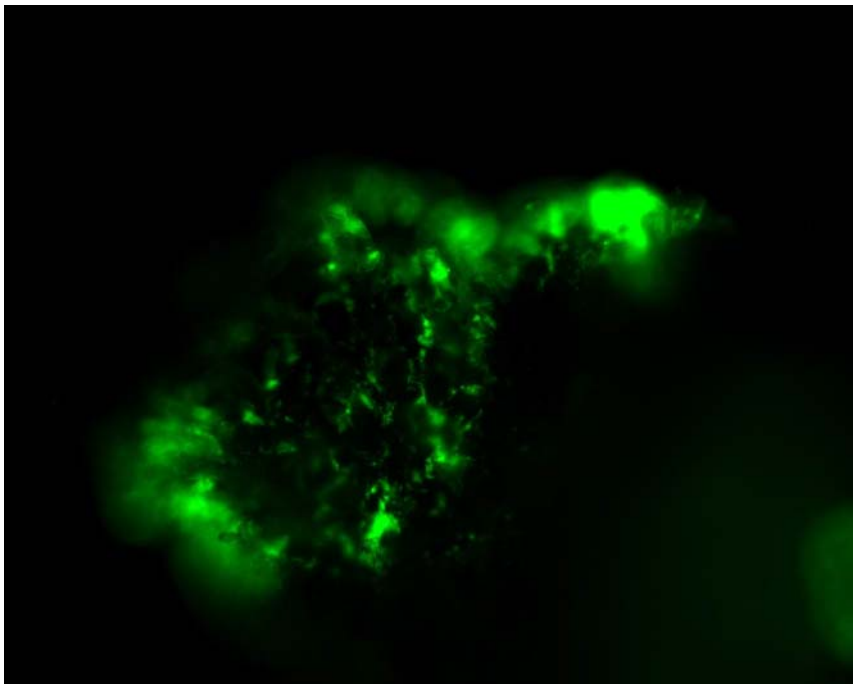


Figure 45: *E. amylovora* multiplying among the stigmatic papillae of a *Crataegus* sp. stigma (120x)

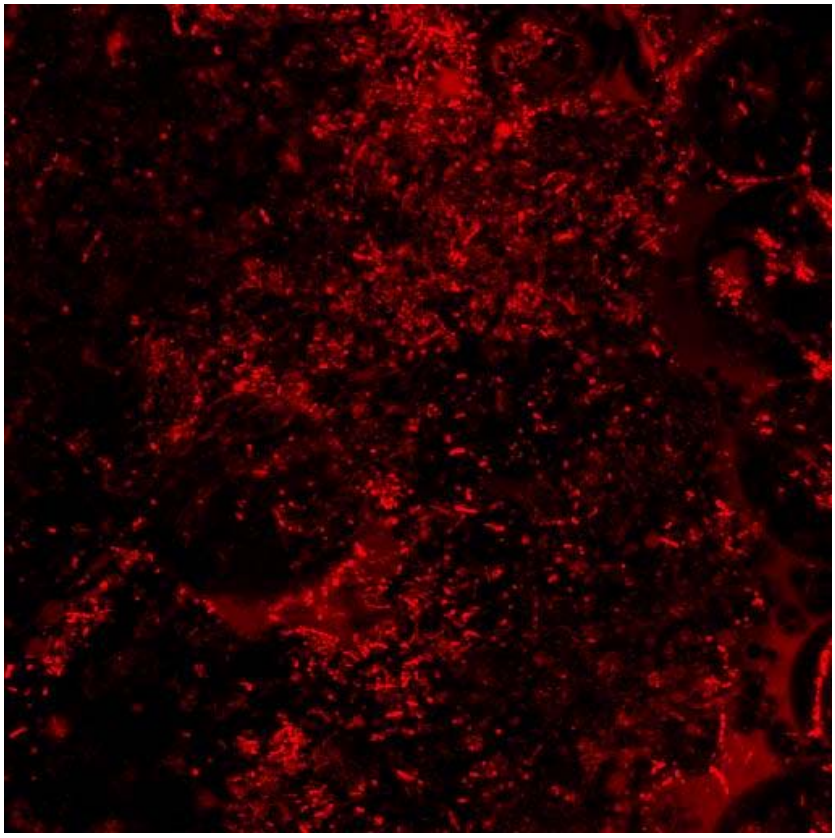


Figure 46 *E. amylovora* multiplying among the stigmatic papillae of a *Pyracantha* sp. stigma (1500x).

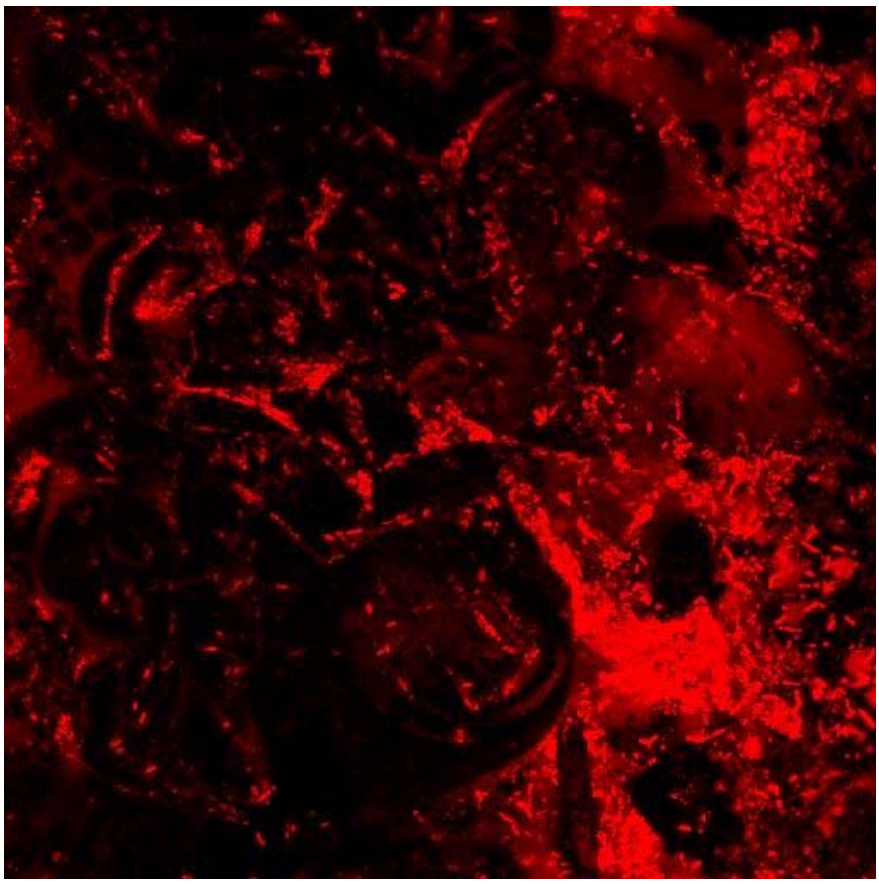
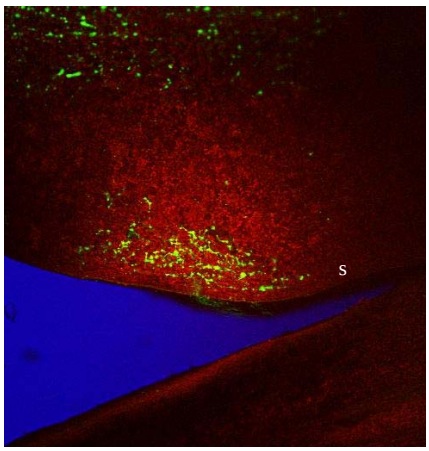


Figure 47 *P. agglomerans* multiplying among the stigmatic papillae of a *Pyracantha* sp. stigma (1550x)



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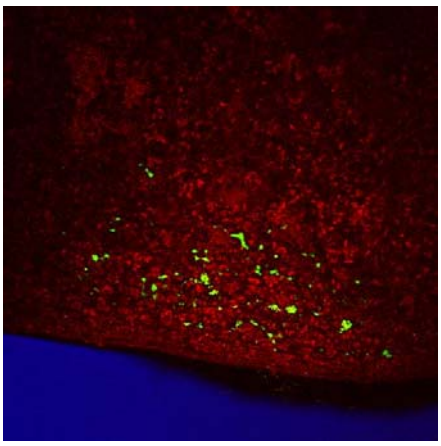

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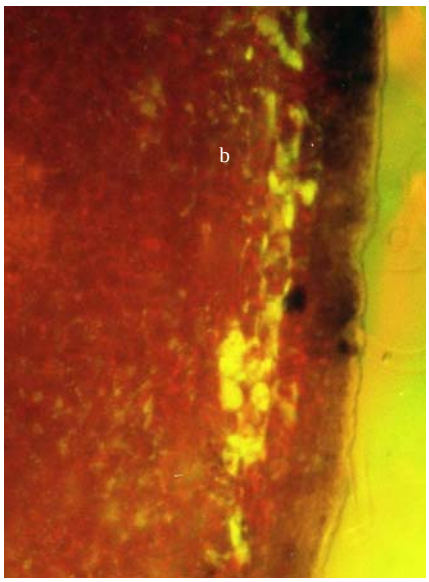

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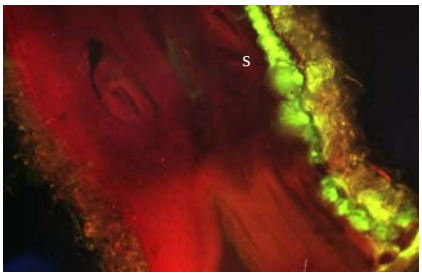

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Figure 48-51: infection of nectaries. *E. amylovora* (green) localised in parechymatic tissues. In yellow (fig. 47) phenolic compounds accumulation . The specimen was treated with Neu's reagent. p: stigmatic pedicel; b: bacteria. Respectively 100, 200, 400, 50x

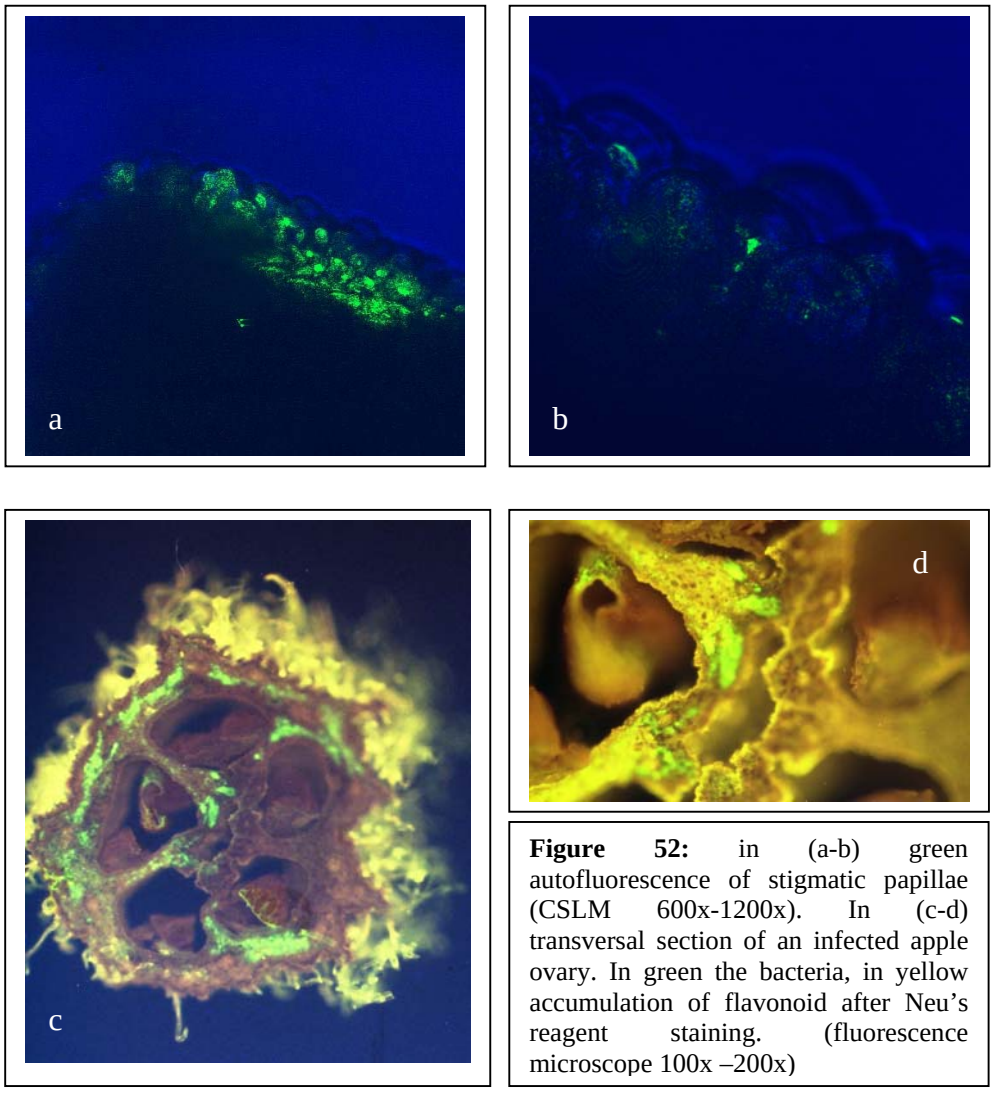


Figure 52: in (a-b) green autofluorescence of stigmatic papillae (CSLM 600x-1200x). In (c-d) transversal section of an infected apple ovary. In green the bacteria, in yellow accumulation of flavonoid after Neu's reagent staining. (fluorescence microscope 100x –200x)

16. MICROSCOPICAL INVESTIGATION II: EFFECT OF DIOXYGENASE INHIBITORS AND SAR INDUCER ON *E. AMYLOVORA* MIGRATION INSIDE PLANT TISSUES

Two sets of experiments were performed to test the efficacy of bioregulators and SAR inducers as inhibitors of *E. amylovora* migration inside plant tissues: one on apple plants and the other on pears.

16.1. Effect on apple plants

Apple seedlings (cv. Golden Delicious) were treated with ProCa (25, 50 and 100 ppm) and with Bion respectively two weeks and 4 days before artificial inoculation. The inoculation was performed with *E. amylovora* GFP labelled in order to be detectable with a florescence microscope. In figure 53-54 are reported symptoms development, measured with an empirical scale (disease rate) in xylematic and parenchymatic tissues. Symptoms development was monitored by analysing plants 4 days after artificial inoculation.

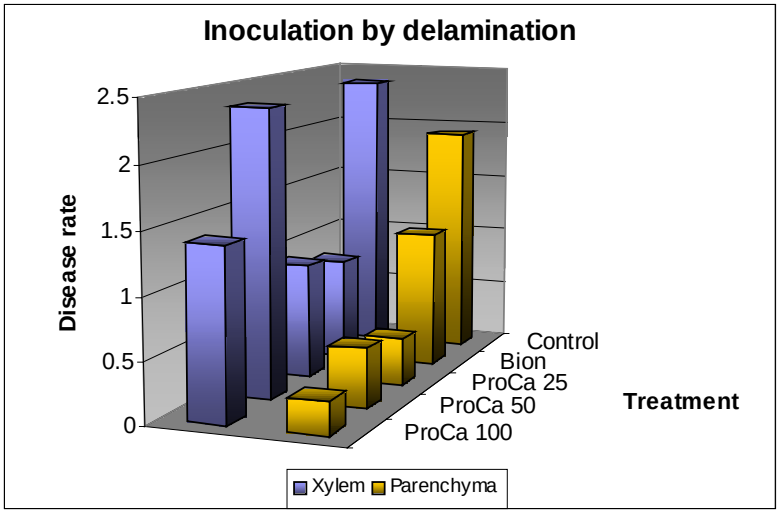


Figure 53: *E. amylovora* migration inside leaf tissues. The migration is expressed as a Disease Rate visually assessed.

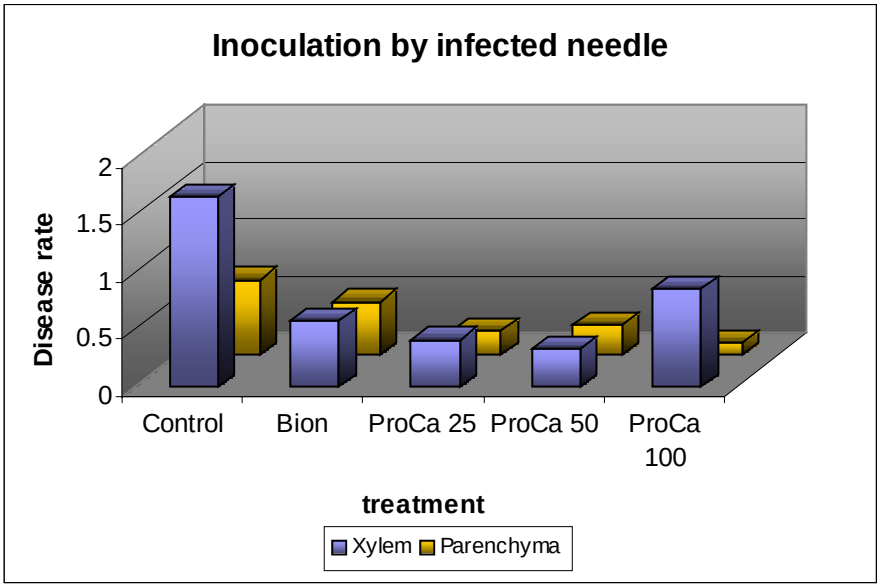


Figure 54: *E. amylovora* migration inside leaf tissues. The migration is expressed as a Disease Rate visually assessed.

With both kinds of inoculation (apical delamination with infected scissors and puncture with a contaminated needle) treatments were able to limit bacterial diffusion, especially in parenchymatic tissue. As regarding to ProCa treatments, the effects were not clearly related to concentration. When inoculation was performed by apical delamination, a statistically significant reduction of *E. amylovora* migration inside both xylem and parenchyma was observed in relation to treatments (table 9).

Xylem			Parenchyma		
	Migration			Migration	
Control	2.37	a	Control	1.925	a
Bion	0.85	b	Bion	1.14	b
ProCa25	0.95	b	ProCa25	0.39	b
ProCa50	2.35	a	ProCa50	0.49	b
ProCa100	1.39	b	ProCa100	0.267	b

Table 8: migration of *E. amylovora* after inoculation by delamination. Means separation according to NSK test for P<0.05.

16.2. Effect on pear plants

Also on Williams micropropagated plants, ProCa treatment reduced *E. amylovora* migration inside leaf tissues. Also in this experiment, the effect of ProCa seems not strictly dependent on the concentration (figure 55).

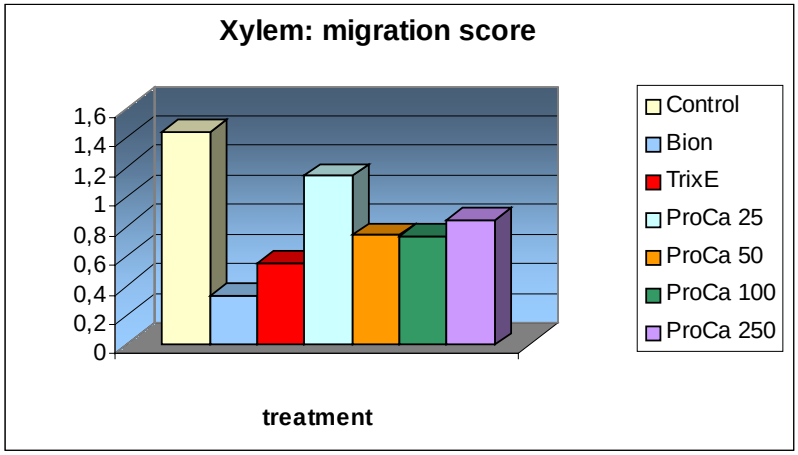


Figure 55: migration of the *gfp*-labelled Ea 1/79 in the leaf parenchyma in relation to different treatments. The migration rate was assessed 10days after inoculation.

A quite low dose (50ppm) didn't show any relevant difference in comparison with the highest dosage. Bion, a SAR inducers, showed a greater effect than any ProCa concentrations, whereas the TrixE, used at high dosage (500ppm), was as effective as ProCa.

The differences observed in the migration rate were processed by SNK test (table8). In this experiment bacterial migration inside parenchyma was observed rarely. In any case, neither bioregulators, nor SAR inducers effected *E. amylovora* movement inside parenchyma.

A separate experiment, using plants treated with H_2O_2, was performed (fig. 56). Treatments with H_2O_2 significantly inhibits the migration of Ea in the leaf tissues. No phytotoxic effect after H_2O_2 treatment was observed.

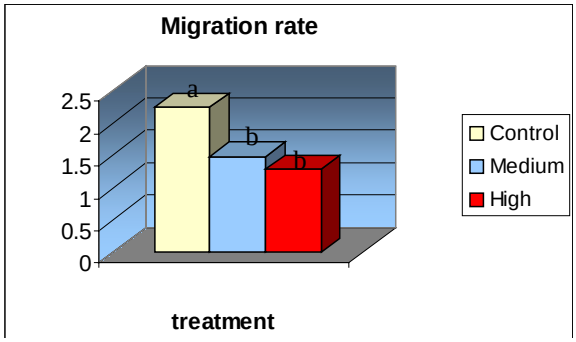


Figure 56: Reduction of the bacterial migration in the leaf tissue after treatment with H_2O_2 . Medium= 0,2%, High= 1%. The migration rate was assessed 5 days after inoculation. Means separation by SNK test (P<0.05)

	Migration score	Means separation
Control	1,4325	a
Bion	0,33	c
TrixE	0,555	b
ProCa 25	1,14	a
ProCa 50	0,74	b
ProCa 100	0,733333333	bc
ProCa 250	0,846666667	b
Control	2,235	a
H_2O_2 Medium	1,48	b
H_2O_2 Hight	1,29	b

Table 9: migration score in xylem calculated in relation to treatment. Means separation according to NSK test (P<0.05)

Therefore, both in apple and pear, ProCa and TrixE were able to inhibit the pathogen migration inside plant tissues. In addition, their

efficacy was comparable to results obtained with SAR inducers (BTH and H_2O_2).
Furthermore, it was demonstrated that this method is a simple and effective tool to test the efficacy of chemicals in reducing disease progression of fire blight.

Using this method was also possible investigate how *E. amylovora* migrates inside different plant organs, such as leaves and shoots, without altering sample by fixation and staining (fig 57-58-61). Different infection pathway were observed: inside the leaf, after infection by apical delamination, *E. amylovora* moved indifferently inside xylem (fig 57 b-c-d; 58 a-b) or parenchyma (Fig. 57 a-e). Whereas, in shoots, the pathogen moved preferentially inside xylematic vessels (fig. 58c) even if, in heavly infected shoots also the cortical parenchyma is colonized (fig. 57d). Migration from xylem to parenchyma were also observed. Occlusion of xylematic vessels by bacterial accumulation was noted. The accumulation of phenolic compounds in infected plant tissues was investigated (fig. 59-60).
Infected tissues, observed after treatments with Neu's reagent presented a yellow-green fluorescence which indicate the presence of flanonoids. The same sections, stained with vanillin-HCl (specific for condensed tannins), showed a brown-red colouration (Fig. 61). Nevertheless, no clear differences between treated plants and control were observed.

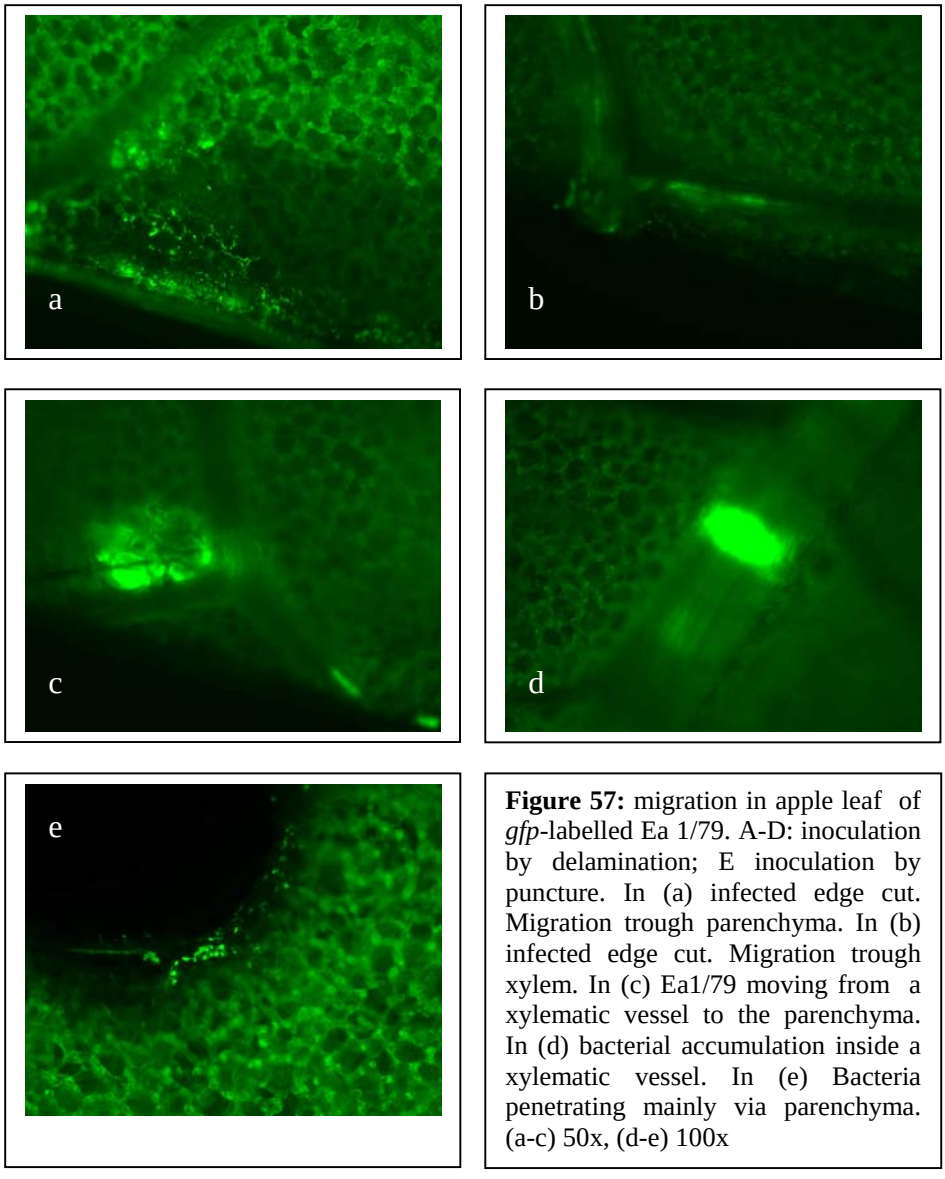


Figure 57: migration in apple leaf of *gfp*-labelled Ea 1/79. A-D: inoculation by delamination; E inoculation by puncture. In (a) infected edge cut. Migration trough parenchyma. In (b) infected edge cut. Migration trough xylem. In (c) Ea1/79 moving from a xylematic vessel to the parenchyma. In (d) bacterial accumulation inside a xylematic vessel. In (e) Bacteria penetrating mainly via parenchyma. (a-c) 50x, (d-e) 100x

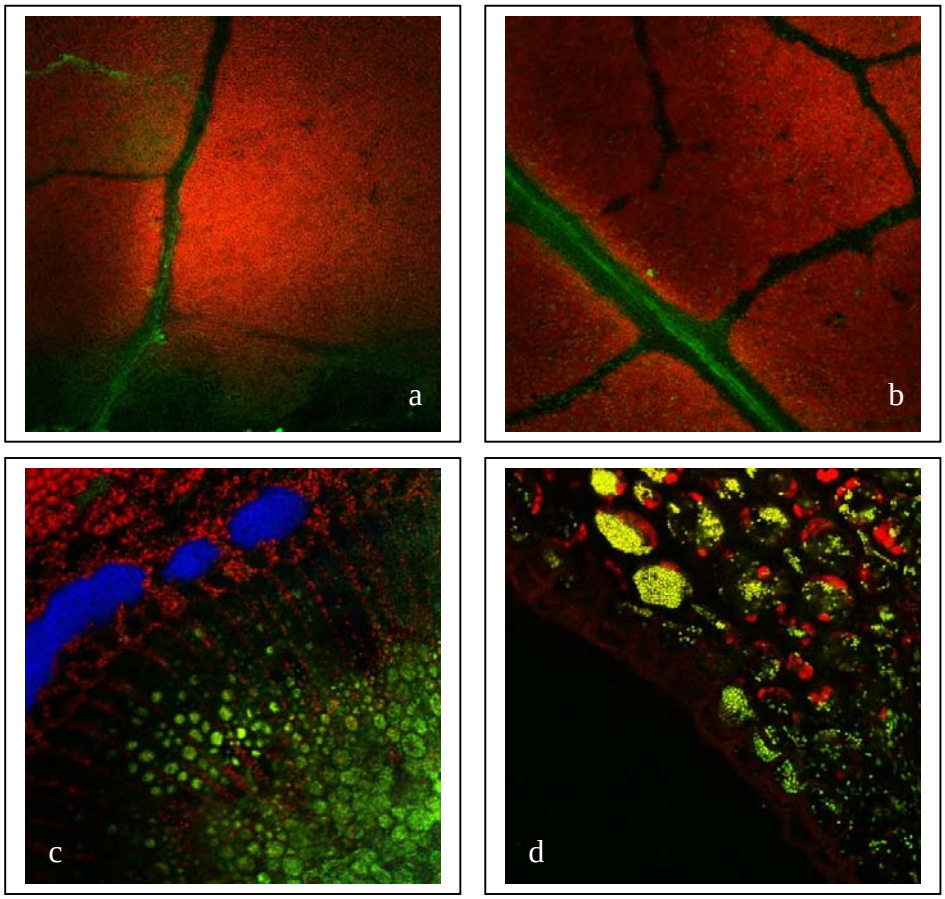


Figure 58: migration inside leaf and stem. In (a) and (b) the bacteria (green) moving inside a xylematic vessel are evident. As in fig. 57, the superior leaf page of viable leaves was observed. In (a) also a collateral vein is infected an the bacteria are moving from xylem to parenchyma. In (c) and (d) transversal sections of infected pear stem. The bacteria are migrating mainly in medullary xylematic vessels. Some of them (c) are completely full of bacterial cells. In (c) the blue coloured tissue is sclerenchyma In (d) red corpuscles are chloroplasts, in this sections bacteria full filled cells of cortical parechyma. a-b CSLM (50x and 150x) c-d fluorescence microscope (50x and 150x)

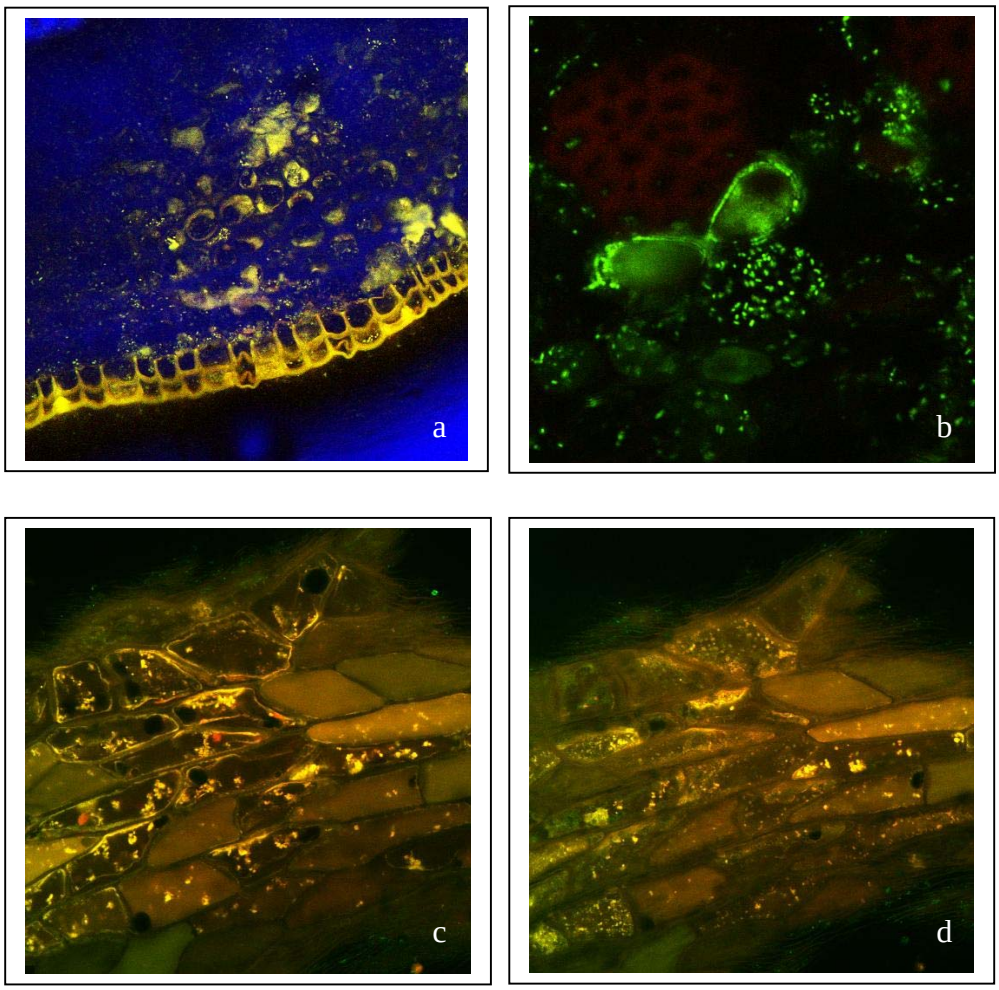
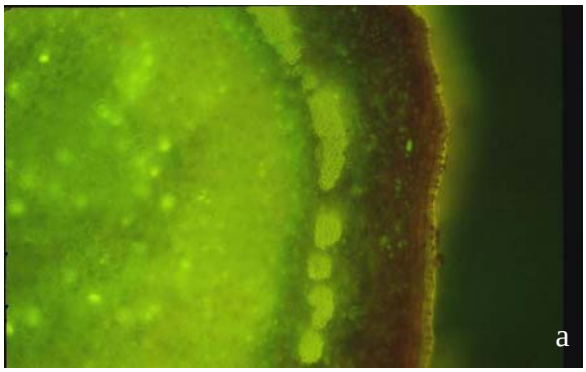
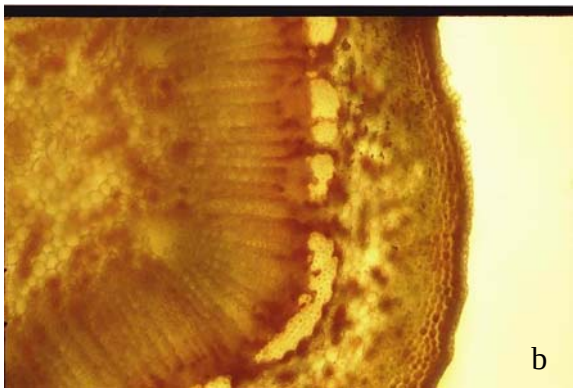


Figure 59: (a) transversal section of infected pear shoot. Cells of cortical parenchyma and epidermis were yellow coloured after Neu's reagent treatment (CSLM, BHS filterset 100x). (b) Infected parechymatic cell infected with (CSLM, GHS filter set 430x). Surrounding cell presents a green fluorescence mainly localize in proximity ofr the cell wall. (c-d) infected cells from a pear leaf (CSLM, BHS filter set 430x). Cells present a yellow colouration after treatment with Neu's reagent. Also in this case the colouration is mainly localize near cell walls. The section shown in (c) is 4 µm beside the section shown in (d)





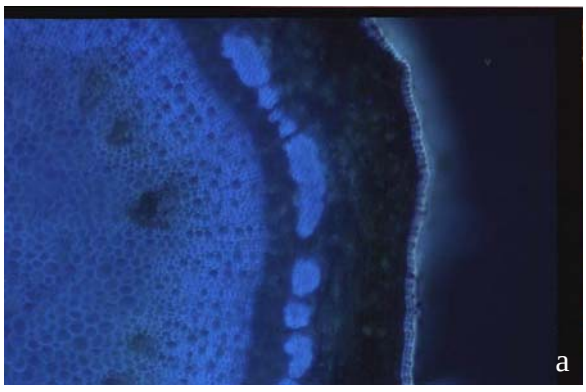


Figure 60:
transversal sections of pear shoot observed using a fluorescent microscope (33x). In (a) using a GFP filterset it is possible detect the bacteria (green) localized manly in medullary xylem.
In (b) the same section after Vanillin-HCl stain. The red-brown colour is in elation to the presence of condensed tannins. Finally in (c) the same section is observed under UV light: the blue colour is due mainly to lignin and other cell wall bound phenols (such as ferulic acid).

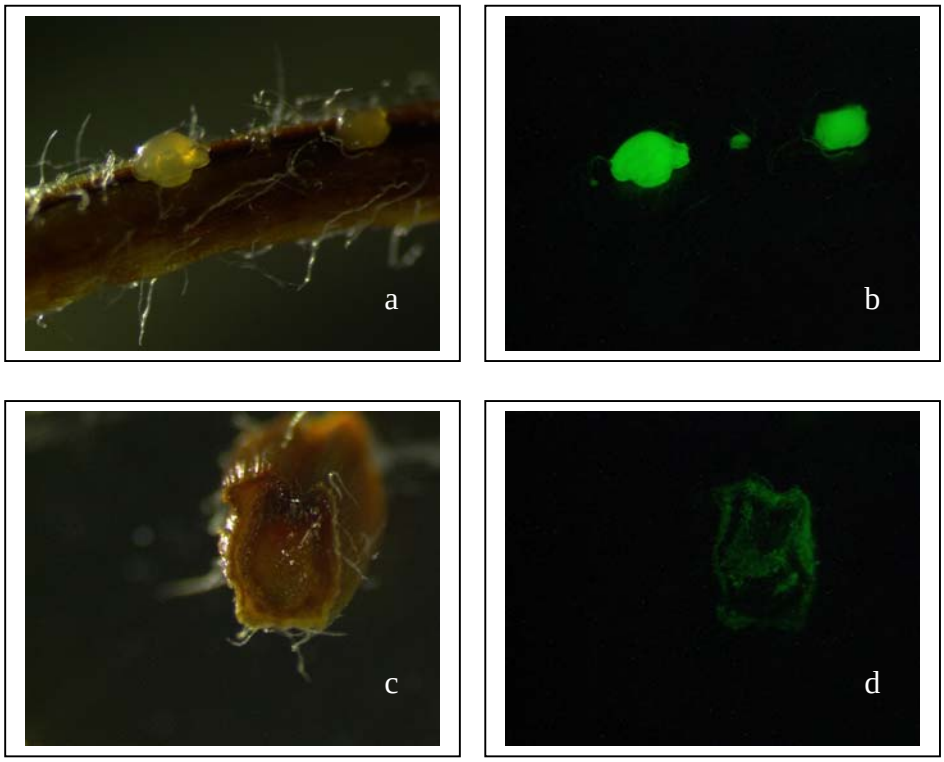
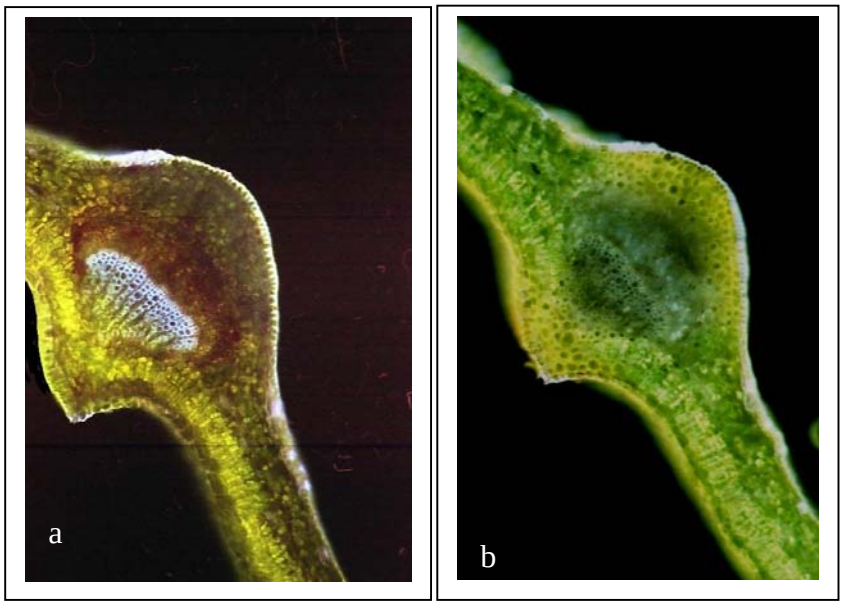


Figure 61: In (a) bacterial exudate extruded on the stem surface, the same exudate observed in (b) with a GFP filter set. Several day after infection *gfp*-labelled Ea1/79 retained the ability to produce GFP. In (c) an transversal hand-section of a apple stem. When observed using a fluorescence microscope (d) it is possible detect the bacteria (green colour) localized mainly in central xylem and cortical parenchyma. (a-b: 8x; c-d: 6x)



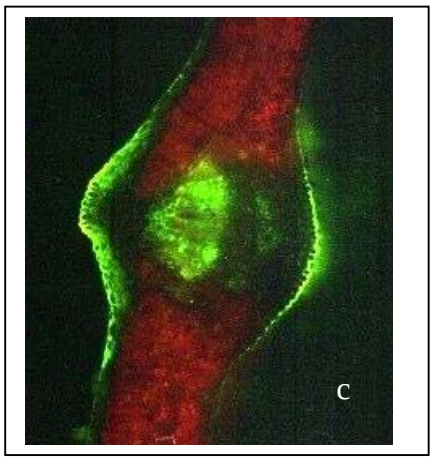


Figure 62: In (a) transversal section of infected pear leaf stained with Vanillin-HCl. The brown red coloration is probably due condensed tannins accumulation. In (b) the same leaf was treated with Neu's reagent to stain flavonoids. In (c) the fluorescent bacteria (green) are mainly localized in the xylem and under the epidermis layers (fluorescence microscope 100x).

17. ANTIMICROBIAL ACTIVITY OF LUTEOFOROL

17.1. Inhibition test on solid medium

The inhibition test on M9 minimal medium plates was used as a screening assay to asses the efficacy of luteoforol and luteolinidin. The application of luteoforol resulted in a faint inhibition halo. Luteolinidin was not effective in this kind of test. This result could be due to a low diffusion of luteoforol in the agar medium (fig. 63). Neither methanol nor ethyl acetate gave an inhibition halo. Furthermore, different bacterial strains showed different sensibility to luteoforol. The greatest effect was observed in the plates inoculated with Ea 1540.

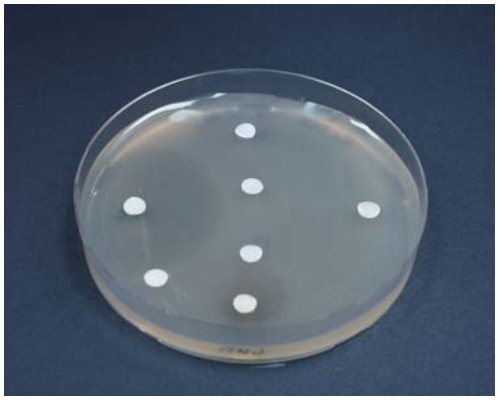
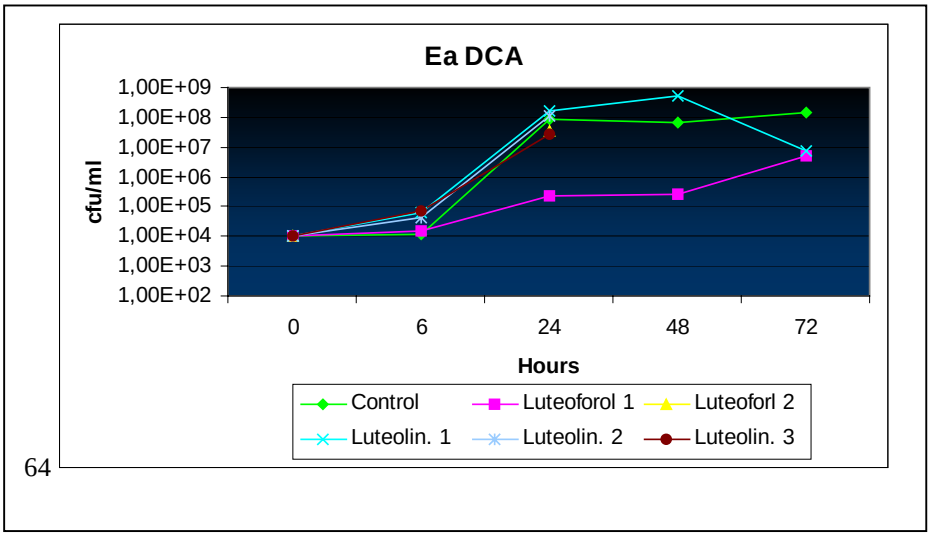


Figure 63: Inhibition test on minimal medium: on the left the positive control (Streptomycin 200ppm), on the bottom the luteoforol (1mM) on the right luteolinidin (10mM) and on the upper part the negative control (water). The other disks were imbibed with methanol and ethyl acetate. The plate had been inoculated with *E. amvlovora* DCA 289/01

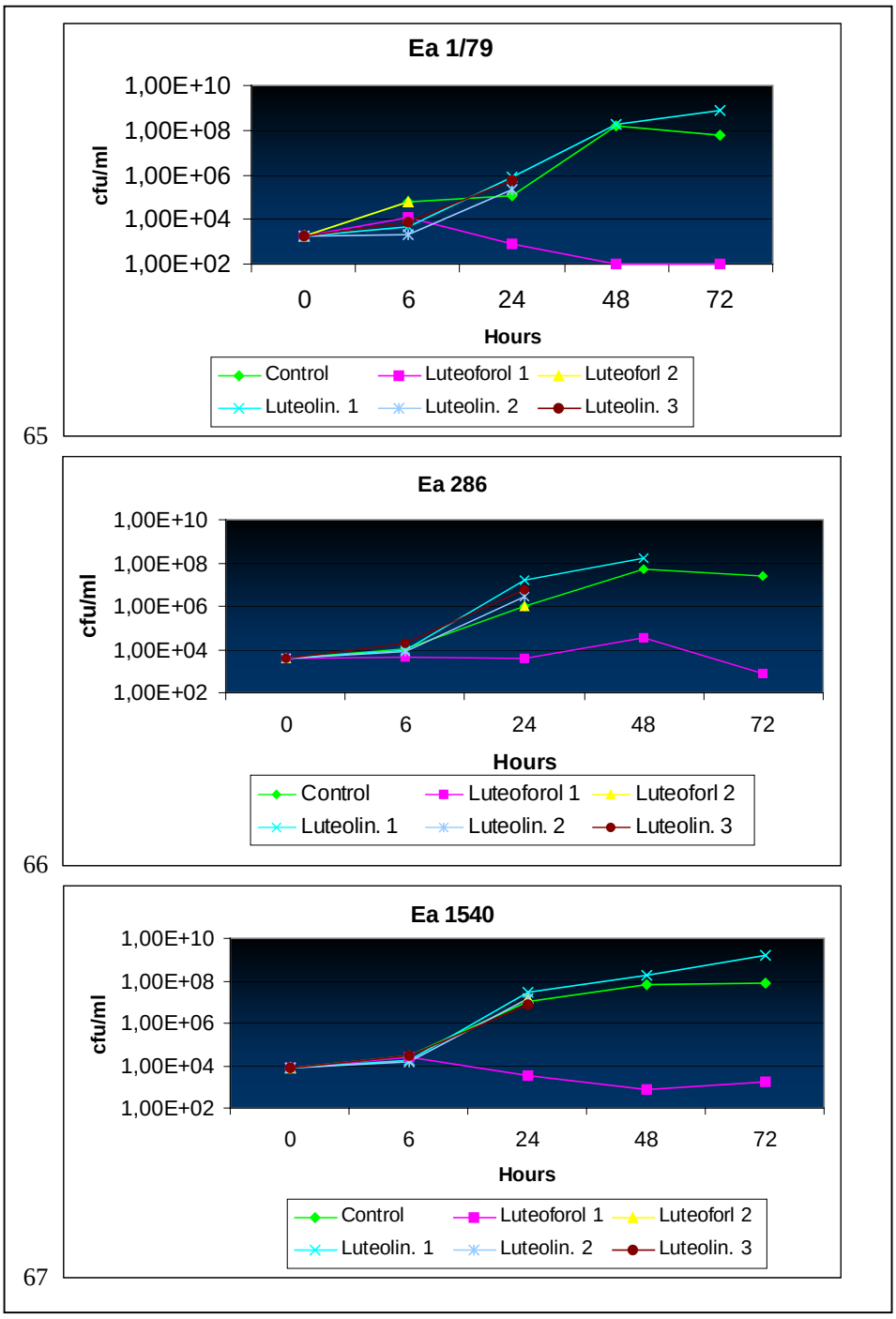
17.2. Biological effect of luteoforol against some *Erwinia amylovora* strains and some epiphytic bacteria

Four kinds of experiments were performed. In the first, the activity of different concentrations of luteoforol (from 1mM to 0,01mM) and luteolinidin (from 10mM to 0,1mM) were tested against several strains of *Erwinia amylovora* and against some bacteria used as biological control agents. Luteoforol was tested against: *Pantoea agglomerans* (Eh – different strains), *Pseudomonas fluorescens* A506 (*Pf*A506) and *Microbacteracea* str. The luteoforol solvent (ethyl-acetate) and the luteolinidin solvent (methanol) were also tested (fig.67-71). The experiments were performed in minimal

liquid medium M9. The bacterial population was assessed at 0h, 6h, 24h, 48h, 72h, 96h after the inoculation. The results are reported in the following graphs (fig. 64-68). The experiments were repeated two times. Luteoforol showed a greater activity against bacteria than luteolinidin. The effect of luteoforol is concentration dependent, but at the lowest concentration, activity could be observed. In some cases, luteoforol reduced the bacterial population to a non-detectable level. Methanol (luteolinidin solvent), it did not show any effect on the bacteria multiplication. Ethyl-acetate (luteoforol solvent) gave a slightly inhibition when applied at the highest concentration, even if its effects is not comparable with the results obtained with luteoforol. Finally, we found that different strains of *E. amylovora* present different susceptibilities toward luteoforol.



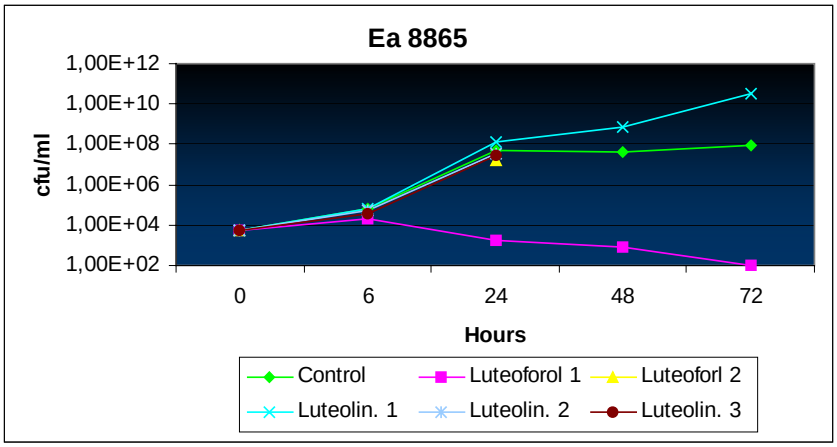
64


Ea 1/79
1,00E+10
1,00E+08
1,00E+06
1,00E+04
1,00E+02
cfu/ml
0
6
24
48
72
Hours
Control
Luteoforol 1
Luteoforl 2
Luteolin. 1
Luteolin. 2
Luteolin. 3
Ea 286
1,00E+10
1,00E+08
1,00E+06
1,00E+04
1,00E+02
cfu/ml
0
6
24
48
72
Hours
Control
Luteoforol 1
Luteoforl 2
Luteolin. 1
Luteolin. 2
Luteolin. 3
Ea 1540
1,00E+10
1,00E+08
1,00E+06
1,00E+04
1,00E+02
cfu/ml
0
6
24
48
72
Hours
Control
Luteoforol 1
Luteoforl 2
Luteolin. 1
Luteolin. 2
Luteolin. 3

65

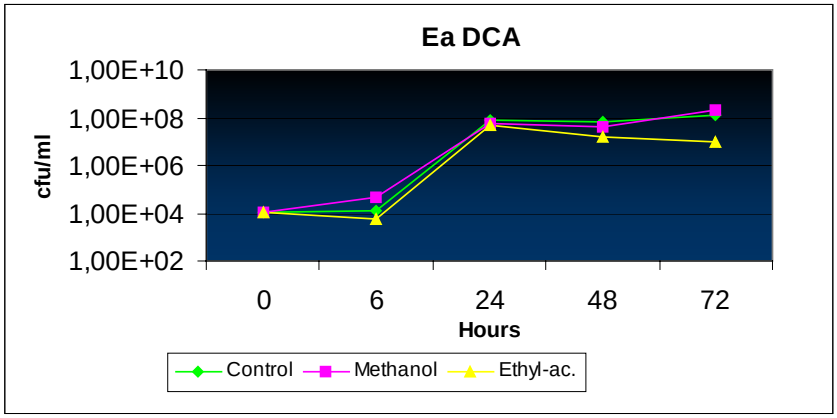
66

67

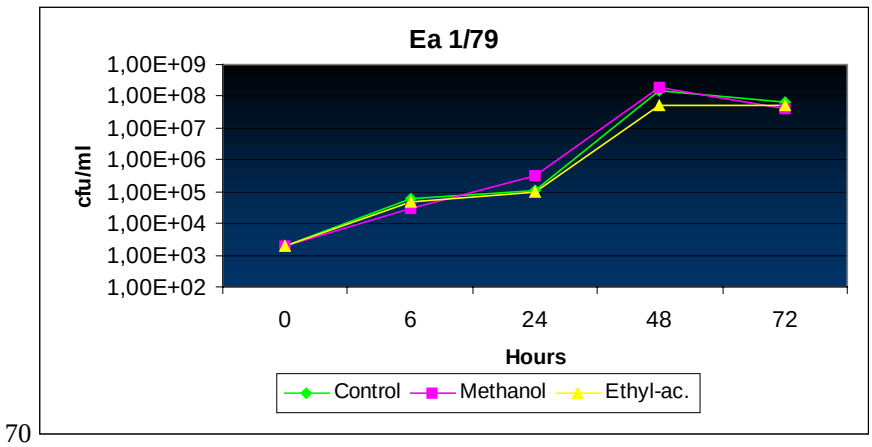


68

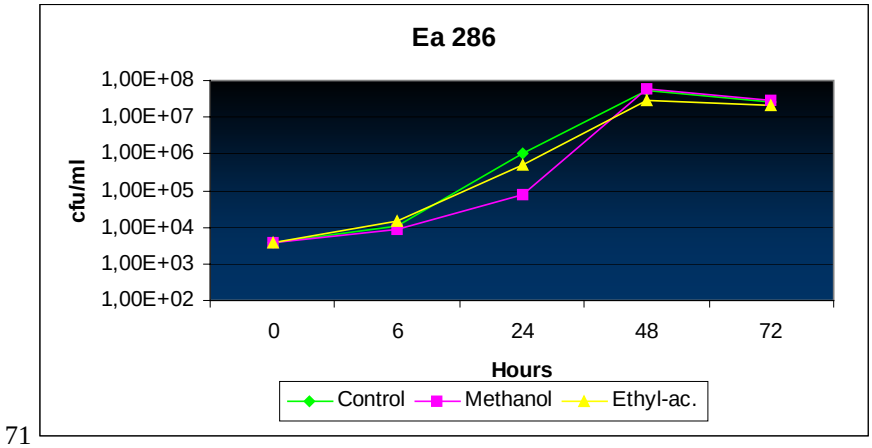
Figure 64-68: Effect of luteoforol and luteolinidin on different *E. amylovora* strains



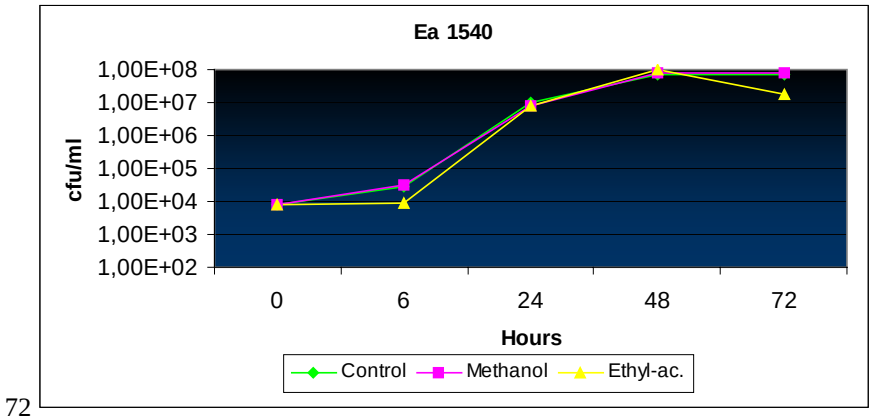
69


Ea 1/79
1,00E+09
1,00E+08
1,00E+07
1,00E+06
1,00E+05
1,00E+04
1,00E+03
1,00E+02
cfu/ml
0
6
24
48
72
Hours
Control
Methanol
Ethyl-ac.

70


Ea 286
1,00E+08
1,00E+07
1,00E+06
1,00E+05
1,00E+04
1,00E+03
1,00E+02
cfu/ml
0
6
24
48
72
Hours
Control
Methanol
Ethyl-ac.

71


Ea 1540
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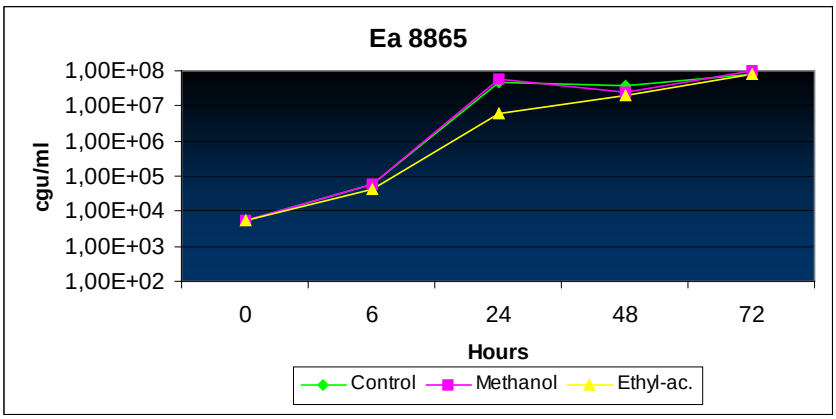
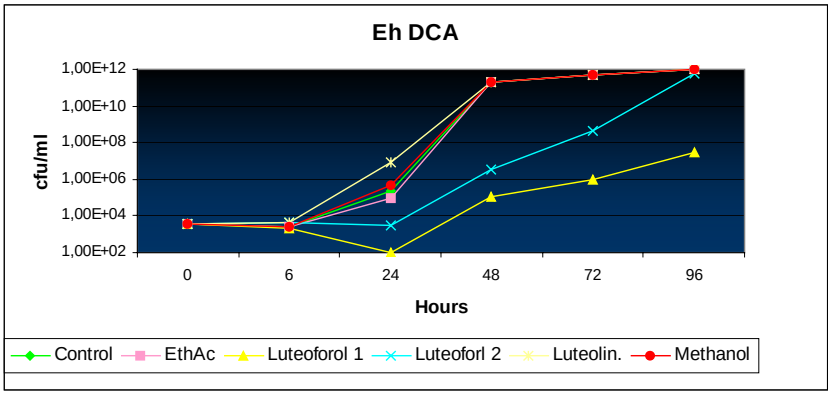
1,00E+07
1,00E+06
1,00E+05
1,00E+04
1,00E+03
1,00E+02
cfu/ml
0
6
24
48
72
Hours
Control
Methanol
Ethyl-ac.

72

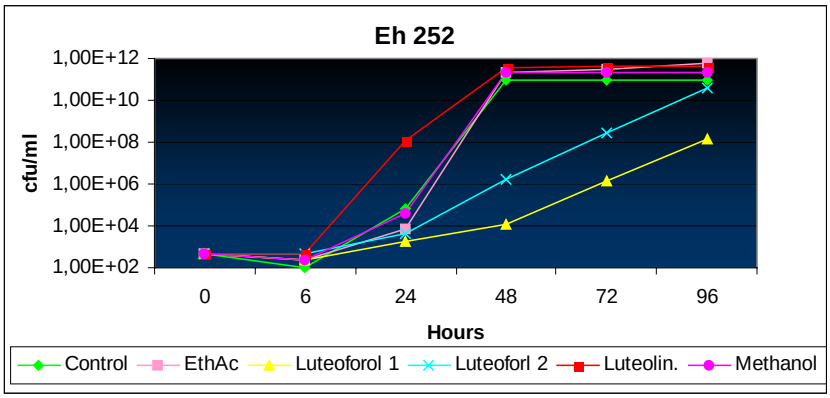


73

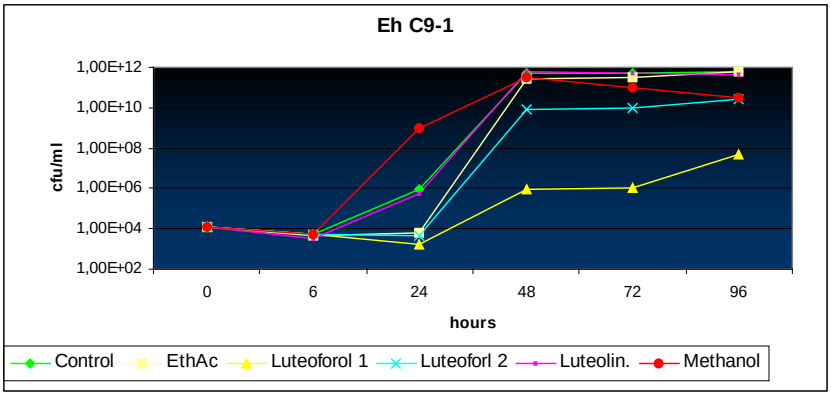


74

Figure 69-73: Effect of the highest concentration of lutoforol and luteolinid solvents (ethyl acetate and methanol respectively) on different E. amylovora strains.

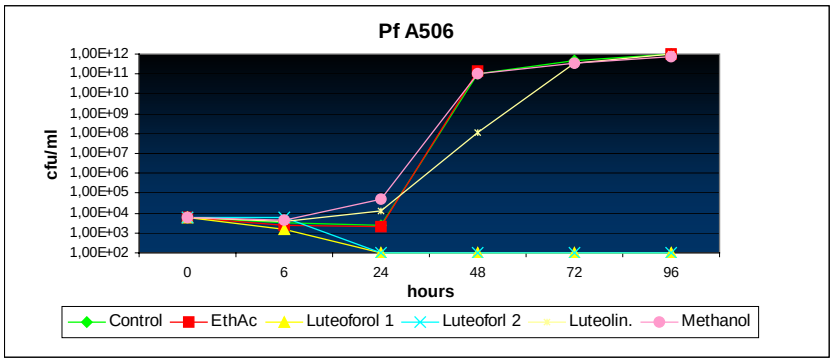

Eh 252
1,00E+12
1,00E+10
1,00E+08
1,00E+06
1,00E+04
1,00E+02
cfu/ml
0
6
24
48
72
96
Hours
Control
EthAc
Luteoforol 1
Luteoforl 2
Luteolin.
Methanol

75


Eh C9-1
1,00E+12
1,00E+10
1,00E+08
1,00E+06
1,00E+04
1,00E+02
cfu/ml
0
6
24
48
72
96
hours
Control
EthAc
Luteoforol 1
Luteoforl 2
Luteolin.
Methanol

76

77



78

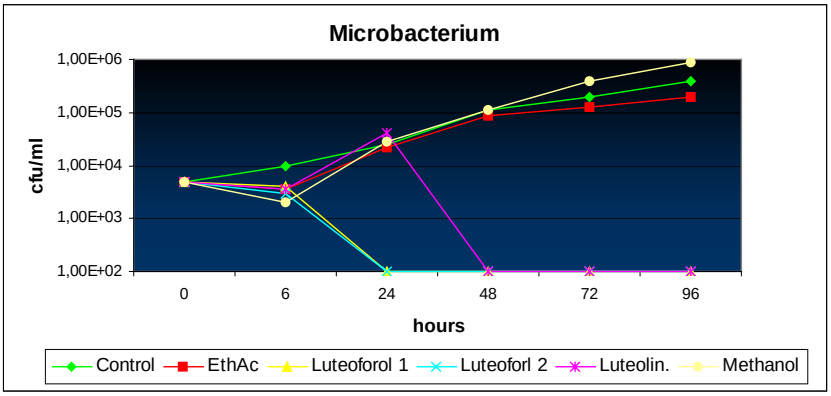


Figure 74-78: Effect of luteoforol, luteolinidin and their solvents on the bacterial growth of some biological control agents.

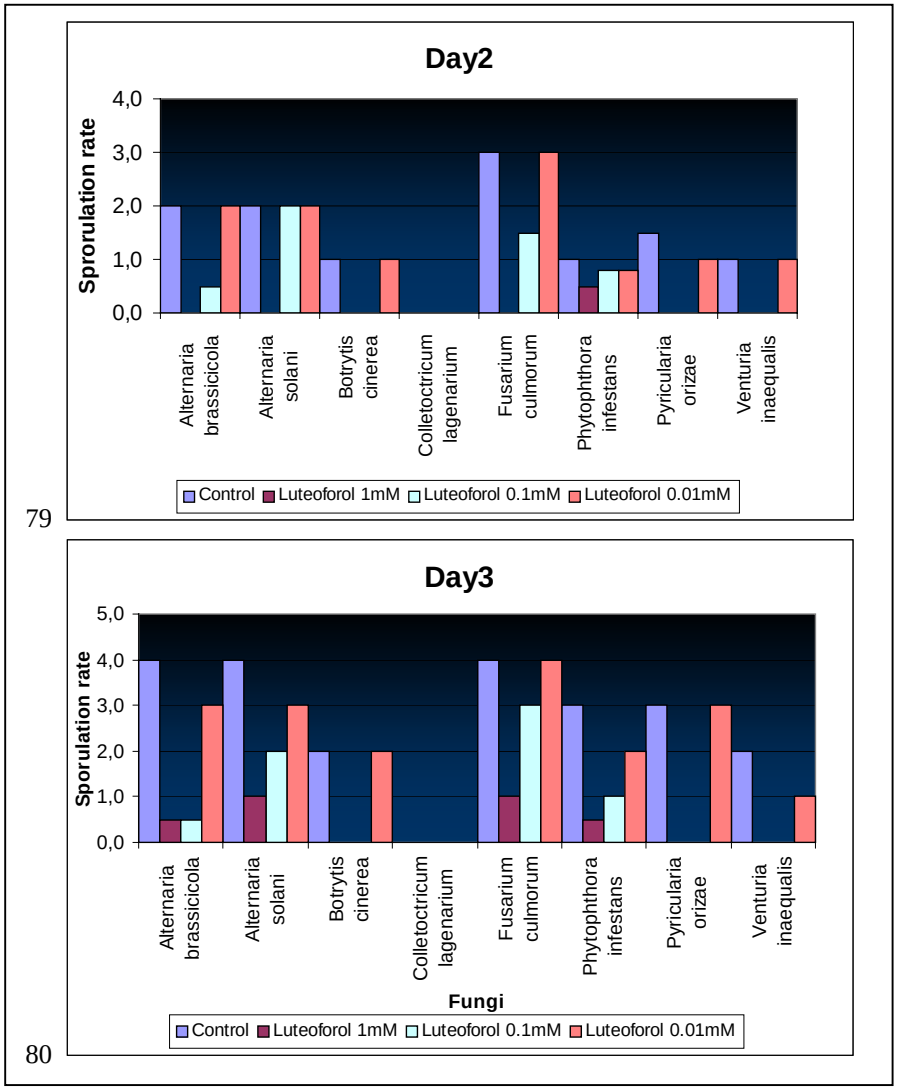
17.3. Biological effect of luteoforol and luteolinidin on the spore germination of some phytopathogenic fungi

The effect of luteoforol and luteolinid against several plant pathogenic fungi was tested in a second group of experiments. The experiments was performed likewise the tests against bacteria. The experiments were similar to those with bacteria. Fungi were cultivated on *Aspergillus* mimimal medium. Spore germination was monitored daily under a stereomicroscope. The experiments were repeated once. In the repetition, *V. inaequalis* did not germinate. The results are shown in the following figures.

When applied at the highest dosage (1 mM),the luteoforol inhibits completely the spore germination of all the fungi examined.

When applied at the highest dosage (1mM),the luteoforol inhibits completely the spore germination of all the fungi examined.

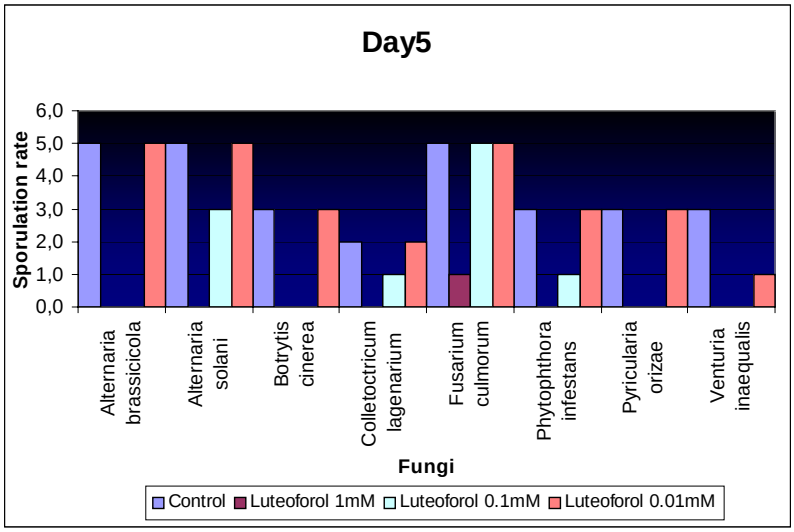
The methanol did not affect the spore germination, whereas a slight inhibition was observed with ethyl acetate at the highest concentration. Again, the efficacy of luteoforol was concentration dependent. Moreover, the luteoforol showed a greater effect than luteolinidin.


Day2
Sprorulation rate
4,0
3,0
2,0
1,0
0,0
Alternaria brassicicola
Alternaria solani
Botrytis cinerea
Colletoctricum lagenarium
Fusarium culmorum
Phytophthora infestans
Pyricularia orizae
Venturia inaequalis
Control
Luteoforol 1mM
Luteoforol 0.1mM
Luteoforol 0.01mM

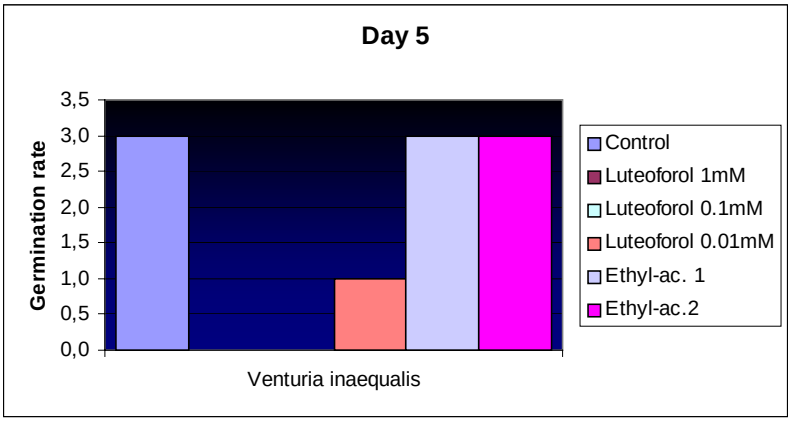
79

Day3
Sporulation rate
5,0
4,0
3,0
2,0
1,0
0,0
Alternaria brassicicola
Alternaria solani
Botrytis cinerea
Colletoctricum lagenarium
Fusarium culmorum
Phytophthora infestans
Pyricularia orizae
Venturia inaequalis
Fungi
Control
Luteoforol 1mM
Luteoforol 0.1mM
Luteoforol 0.01mM

80



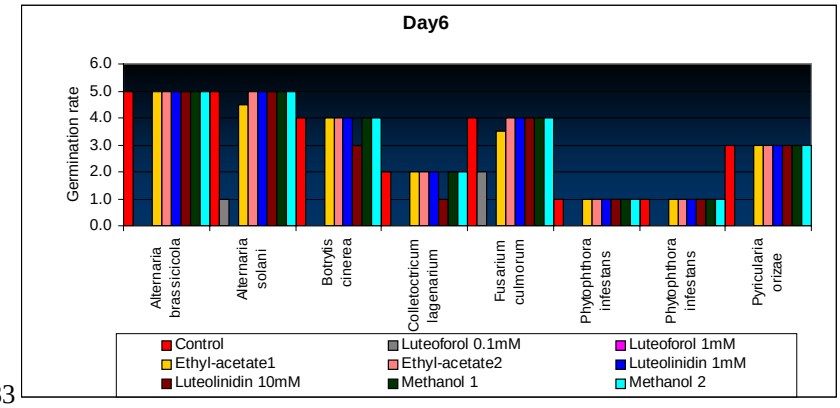
81



82

Figure 79-81: Effect of luteoforol on the spore germination of some phytopatogenic fungi. The data shown in the graph refer to the 2, 3, 5th day after treatment.

Figure 82: Paricular of day 5 -Effect of luteoforol on the spore germination of *Venturia inaequalis* the causative agent of the **Apple Scab**



83

Figure 83: Effect of luteoforol, luteolinidin and their solvents on the spore germination of some phytopatogenic fungi. The data shown in the graph refer to the 6th day after treatment. The second column of *Phytophthora* refers to the

17.4. IPF Test

The efficacy of luteoforol and luteolinidin to control fire blight symptom development was assessed in the IPF (Immature Pear Fruit) test. Neither the application of luteoforol nor luteolinidin was effective. The results on the IPF test are not conclusive: 5-6 days after inoculation, the pear cores were colonised by fungi and the experiment was therefore terminated.

In a previous experiment performed in Bologna, the efficacy of luteoforol in reducing incidence in IPF test was assessed using a 1-day old luteoforol. Since this compound is supposed to be highly instable, the results obtained are not conclusive. The result are shown in figure 84

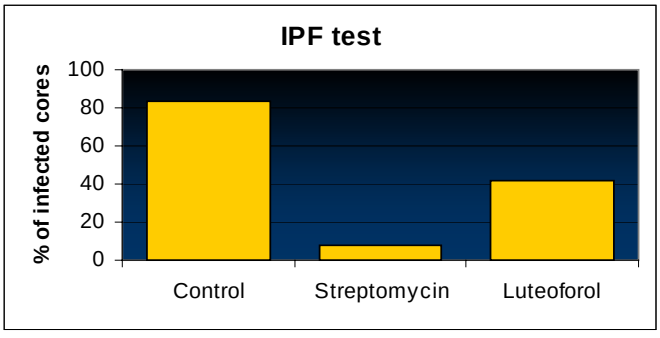


Figure 84: effect of luteoforol in reducing symptoms development in IPF test

17.5. Biological effect of luteoforol and luteolinidin on micropropagated plants

When applied to micropropagated plantlets, luteoforol resulted in a phytotoxic effect. None of the other compounds showed a similar effect. Luteoforol was applied at 1 mM. The treated plantlets died after 12h.

Figure 85: Effect of luteoforol (1mM) and luteolinidin (10 mM) 12 hours after application. Ethyl acetate and methanol were also tested.
Each vial contained 2-3 micropropagated pear plantlets (cv. Williams)

DISCUSSION

DISCUSSION

1. Dioxygenase inhibitors as a tool to decrease plant susceptibility to diseases.

According to literature cited, efficacy of dioxygenase inhibitors in reducing shoot blight was confirmed.

TrixE and ProCa reduced clearly shoot blight incidence. However, also a reduction in severity was observed. This effect is transient: fire blight severity, in treated plants, rapidly exceeds the one of control trees. These observations are probably imputable to the method used for severity calculation. In fact, severity is inversely proportional to shoot length. Since ProCa and TrixE are growth retardants, treated shoots grow slower than control ones, therefore a slower shoot growth leads to a more rapid severity increase. The shoot length reduction was also demonstrated in treated apple plants. On the two different cultivars tested (Braedburn and Pink Lady) TrixE showed a differential efficacy. In particular, on Pink Lady trees the growth retardant effect was very clear, whereas on Braedburn plants shoot control was not so strong. A possible explanation could be that Braedburn plants were previously treated with TrixE.

At the beginning of experiment, treated shoots were already shorter than control ones and during all the experiment these difference increased.

Dioxygenase inhibitors applications reduced blossom blight incidence as well. Nevertheless, on detached flowers, TrixE was ineffective. Since the incidence reduction is supposed to be related to an increase of plant defences after treatment, the ineffectiveness of TrixE could be explained by supposing that detached flowers do not have the possibility to react readily to infection. In fact, blossoms in these conditions simply survive. Detached flowering branches and entire trees were found a more reliable material to test the

effectiveness of dioxygenase inhibitors. In field conditions, on treated apple plants, a statistically significant increment in fallen blossoms was noted. Since a phytotoxic effect of ProCa and TrixE seems unlikely, a possible explanation could reside in their inhibitory effect on GAs biosynthesis. In apple, the fruit setting activity of GAs in well known: an increase of GAs level (mainly GA_3 and GA_{4-7}) enhances the number of blossoms that develop in fruits. In fact, in flowers, GAs delay the formation of the pedicel-stem abscission zone which results in the shedding of blossoms. Therefore, the application of acylcyclohexanediones, reducing GAs level on plant tissues, might increase flower abscission.

Nonetheless, application of dioxygenase inhibitors might also have a positive effect on blossoming. Indeed, acylcyclohexanediones are structural analogous of 2-oxoglutaric and ascorbic acid and, thus, they inhibit ACC oxidase and ethylene formation (Iturriagagoitia-Bueno *et al.*, 1996). It is well known that ethylene is involved in flower senescence and abscission processes (Bleecker and Kende 2000; Bartoli *et al.*, 1997).

However, thinning is an important problem in apple plants, hence, the increased number of fallen flowers in treated plants probably should not affect yield. In fact, on apple plants, dioxygenase inhibitors application at pink button stage does not reduce yield and it even enhances the average fruit production per tress (Costa et al., 2001).

The increased abscission of flowers after TrixE treatment might also be responsible of the reduced blossom blight incidence. According to this hypothesis, TrixE treatment, increasing plant reactivity to diseases, reduces blossom blight incidence simply inducing a rapid fall of infected flowers. In treated blossoms, the release of a toxic compound, as a response to pathogens (paragraph 4), might leads to plant cells death and to detachment of flowers. Moreover, in treated flowers, *E. amylovora* migration is supposed to be slower (paragraph 3). Consequently, the desegregation of plant tissues produced by infection might results in detachment of blossoms before the penetration of *E. amylovora* inside the branch tissues. The

combination of these factors could explain the reduction of fire blight incidence on shoot resulting from flower inoculation.

2. Plants treated with dioxygenase inhibitors support a more consistent microbial community.

Plants treated with dioxygenase inhibitors are less sensitive to fire blight infection. The transient modifications in flavonoids metabolism induced by treatment are the main factors implicated in reduction of susceptibility to *E. amylovora* (Römmelt *et al.*, 2000). Nevertheless, an effect on the epiphytic microbial community might also be involved. In fact, treated plants can sustain higher populations of both natural epiphytic bacteria and artificially introduced antagonists.

After treatment, a higher microbial population was found on different plant organs, such as stigmas, nectaries and leaves. The increased consistence of the beneficial bacterial community on blossom might play an important role on fire blight control. Moreover, the reduction in sugar concentration observed in treated nectar allows the multiplication of antagonists in nectaries. On the contrary, *E. amylovora* seem less affected by sugar concentration changes.

As mentioned previously, interaction among the bacterial antagonists and the pathogen occurs primarily on stigma. Thus, the stigma is the site for biological control of fire blight. Nevertheless, colonization of nectaries by antagonists might represent a further step for biological control (Pusey, 1999). Since bacterial antagonists can survive in treated nectar, they can colonize nectaries and consequently reduce the possibility of infection by *E. amylovora*.

Since acylcyclohexanediones have several effects on plant metabolism and not all of them have been deeply investigated, the reason for the sugar content reduction in treated flowers is still unknown. Nevertheless a possible explanation could be suggested. In fact, both ethylene and gibberellins seem involved in flower development and nectar secretion.

After application of gibberellin (1500 ppm) to *Cucumis sativus* flowers, Kamler and Tronickova (1980-1982) observed an increase sugar content of nectar. In addition, ethylene promotes mobilization

of substrates and an efflux of material from petals to the gynaecium, nectar and stem (Nichols and Ho, 1975). These Authors observed that levels of ethylene, which caused irreversible wilting of petals, also promoted an accelerated transfer of sucrose to the nectar. Therefore, it is likely that the application of acylcyclohexanediones, reducing both ethylene and gibberellins level in plant tissues, might decrease sugar content of nectar.

According to our results ProCa application decreases sugar concentration of nectar, but increases daily secretion. This increase might be due to a higher availability of water. In fact, after ProCa application, the assimilates are generally shifted into the roots, which are stimulate to grow (Fletcher and Arnold, 1986; Guak *et al.*, 2001).

Biological control agents can colonize also leaf surface. The colonization of leaves by bacterial antagonists is important for two main reasons: firstly, it can protect plants from secondary infection, and, secondly, it can facilitate the dissemination from colonized plant surface to non-colonized ones, thus allowing the establishment of a resistant and resilient population of antagonistic bacteria.

Treatments with ProCa result in a reduction of number of leaves and therefore of total leaf area of the canopy (Guak *et al.*, 2001). Therefore, treatment with dioxygenase inhibitors, altering the canopy structure (Winkler, 1997), can change microclimatic conditions inside foliage. These changes might influence the epiphytic microbial community. Even if treated leaves can sustain a higher epiphytic community, the differences observed between treated and untreated plants were not statistically significant.

Saprophytic bacteria can epiphytically multiply on leaves only in presence of organic nutrients. Consequently, the occurrence of sugars and other organic compounds on leaves was investigated.

The results obtained are mainly qualitative. The sugars present on leaf surface are mainly constituted by mannose and α-and β-glucose. Moreover, the amount of sugars on the surface of water treated leaves seems higher than in leaves sprayed with ProCa and TrixE. This difference is mainly due to a higher level of β-glucose. Finally, all the investigate compounds were found on ProCa treated leaves, whereas, several of them were undetectable on control leaves. Also

on TrixE treated leaves a higher number of compounds was detected in comparison to control.
Thus, dioxygenase treatments, seems to reduce the amount of sugar on leaf surface, but it increases the diversity in number of substances secreted. As already mentioned a decrease in sugar content was also observed on nectar. The metabolic reasons of these phenomena are still unknown. However, a possible explanation can be suggested.
According to Guak *et al.*, (2001), the treatment with ProCa increases the concentration of non-structural carbohydrates (TNC) in all the plant parts treated. Therefore, a higher availability of TNC might lead to secretion of sugars not usually present on leaf surface. Instead the lower amount of total sugars on leaf surface might be due to the accumulation of TNC as starch (Guak *et al.*, 2001).
Finally, an other hypothesis could be suggested: the reduction in sugar content observed in treated leaves might be due only to a higher epiphytic microbial population and thus, to an increased feeding activity. According to this hypothesis, the higher variability among organic compounds found on leaf surface might be essentially due to the metabolism of microbial community.
Even if plant organs treated with TrixE and ProCa can sustain a more consistent beneficial microbial community, no synergic effect was observed combining dioxygenase inhibitors treatment with bacterial antagonists. The experiments performed are not conclusive: a small number of plants was used under glasshouse conditions, which are more favourable to infection than field conditions. Moreover, under natural conditions the application of ProCa and TrixE increasing microbial population on all plant organs may enhance the spread of antagonists from colonized plants to non-colonised ones allowing the establishment of a more resistant and resilient beneficial microbial community. The possible positive effects of these factors can be pointed out only on long-term experiments.

3. Infection process and migration inside plant tissues: effect of dioxygenase inhibitors and SAR inducers.

The use of CLSM combined with *gfp*-labelled bacteria, is a powerful method to study the microscopical interaction between host plants

and bacterial pathogens. These techniques are especially interesting since plant tissues are not exposed to any kind of treatments or manipulations: no fixation, sectioning or stain is needed. Therefore, these methodologies allow in *vivo* observation of infection process, migration inside host tissues and disease development.

The first steps of infection process, such as the epiphytic multiplication of *E. amylovora* on the stigma and migration from stigma to nectaries seem not substantially influenced by dioxygenase inhibitors application.

On the contrary, dioxygenase inhibitors and SAR inducers greatly influence the migration inside plant tissues: underling the importance of their effect on plant defences.

Until the pathogens was localised on plant surface, where plant defences are ineffective, no influence due dioxygenase inhibitors application was detected: in fact, the activation of plant resistance mechanisms occurs only when the pathogen penetrates inside plant tissues and interacts directly with viable plant cells.

On stigmatic surface, *E. amylovora* seems to exploit the nutrients produced by stigmatic papillae. In very few cases, a desegregation of stigmatic tissues integrity, subsequent to E. *amylovora* colonization, was observed. Therefore, no electrolytes leakage on stigmatic cells is induced by *E. amylovora* and penetration through stigmatic tissues seems unlikely. In all *Pomaceous* plants investigated, a stigmatic channel was observed. It originates from stigma and reaches, dwelling along all stigmatic pedicel, the nectaries. *E. amylovora* migration toward nectaries preferentially occurs in this channel. It is noteworthy that the channel epidermis is completely constituted by stigmatic papillae, which provide a nutrient-rich and protected environment for the pathogen. The presence of bacteria within the channel, which deeply penetrates inside the pedicel, might mislead to the impression of an improbable bacterial infection through stigmatic tissues.

Since the channel is covered with stigmatic papillae, it allows the multiplication of bacterial antagonists, and thus, within the channel biological control agents and the pathogen might interact.

As far as the effect of dioxygenase inhibitors and SAR inducers in reducing bacterial migration inside green tissues, their efficacy was higher inside parenchyma than in xylem. This observation corroborates the hypothesis that their protective effect is mainly due to a strengthening of plant defences. In addition, secondary colonization of parenchyma by bacteria, which come out from xylematic vessels, is reduced in treated plants. Xylem, in fact, is constituted by death cells, whereas the parenchyma by viable and metabolic active cells, therefore, it is parenchyma that plant defence mechanisms occur.

A concentration dependent effect of ProCa was not observed. Since the plant material used was constituted by seedling or young and still herbaceous plants, the lowest dosage tested probably reached a saturating effect. As a result, the expected dose-response effect of ProCa was not observed.

The inoculation method greatly determines the migration pathway inside the leaves. After inoculation by puncture with an infected needle, bacteria move preferentially in xylem, whereas inoculation by apical delamination leads to bacterial migration almost indifferently through xylem or parenchyma. Thus, even if with the methodologies used the artificial influences were minimized, the observations performed were affected by inoculation methods.

As regarding to shoots infection, the pathogen migrates mainly in the medullar xylematic vessels. In heavily infected shoots the vessel are completely occluded by bacteria. This observations confirms the disruption of water flux due to bacterial accumulation in xylem as noted by Goodman *et al.,* (1987), Sjulin and Beer, (1977). However, *E. amylovora* was often observed also in cortical parenchyma. Even if bacteria move in the apoplast between intercellular spaces, the bacteria localize in the intercellular spaces, they were frequently noted inside cells.

Phenolic compounds accumulation was observed in the tissues surrounding infection. No differences were found among treated and control tissues. Probably, the methodologies used were not sensitive enough to visualize changes in flavonoids content between treated

and untreated tissues. These changes, in fact, are mainly qualitative (Andreotti, 2000) and not quantitative.
Finally, these methodologies can be used to perform a simple screening test to evaluate the efficacy of investigated compounds in increasing plant resistance against pathogens.

4. Luteoforol: a novel phytolexin induced by ProCa application is involved in increased resistance to *E. amylovora* infection.

Chemical analyses of the treated apple tissues showed the occurrence of flavonoids, which are not commonly formed in apple. In particular, the accumulation of the 3-deoxycatechin luteoliflavan was observed, which has never been detected in *Rosaceous* species so far (Röemmelt *et al.*, 1999). Luteoliflavan accumulation is imputable to FHT-inhibition by 2-Oxoglutarate analogues (ProCa and TrixE). Luteoliflavan, a 3-deoxycatechin, derives from eriodictyol, which is a substrate for FHT. 3-Deoxycatechins belong to the rare class of 3-deoxyflavonoids, which are found only in a few plant species (Styles and Ceska, 1975; Lo Sze-Chung *et al*., 1999; Stich and Forkmann, 1988; Ramesh *et al*., 2001). The most prominent 3-deoxyflavonoids are the 3-deoxyanthocyanidins, which are plant pigments (Stich and Forkmann, 1988). In *Sorghum bicolor*, however, they act as phytoalexins (Lo *et al*., 1999) and an extract from *Bridelia crenulata* showed inhibiting effects on human pathogenic bacteria (Ramesh *et al*., 2001). Luteoliflavan, the 3-deoxycatechin induced by ProCa treatment, and a number of constitutive as well as induced phenolic compounds such as flavanone 7-O-glucosides, phloretin derivatives, *p*-coumaroylglucose, caffeic acid, chlorogenic acid and 6''-*O*-*trans*-*p*-coumaroyl-eriodictyol 3'-O-glucosid did not show sufficient inhibitory effects *in vitro* to provide an explanation for the enhanced fire blight resistance. A more precise understanding of the novel biosynthetic pathway induced by ProCa showed that luteoliflavan is formed from luteoforol. This is a rather unstable intermediate and shows strong chemical reactivity which impedes analytical detection.
The experiments performed showed that luteoforol is the active compound responsible for the enhanced resistance after

prohexadione-Ca treatment. Due to the high efficacy, even the low concentrations of luteoforol present in the leaves after treatment may cause the effects observed.

Since luteoforol could not be isolated from the leaves, it was chemically synthesised (Bate-Smith, 1969) and tested *in vitro* for its effect against *E. amylovora* and, in general, against a large number of different bacterial and fungal pathogens. Even low concentrations (0.1mM to 1mM) of luteoforol showed strong antimicrobial effect, which was appreciably more pronounced than that observed with luteolinidin, the 3-deoxyanthocyanidin phytoalexin in *Sorghum bicolor*. Spore germination of *Venturia inaequalis* and other plant pathogenic fungi was inhibited by concentrations of 0.01 mM upwards. The effect observed was not just bacteriostatic or fungistatic, but luteoforol clearly revealed fungicidal and bactericidal activity. The broad toxic effect is an important feature since it should not easily be overcome by pathogen variants.

Unexpectedly, luteoforol was found phytotoxic at low dosages. Therefore, luteoforol is supposed to be compartmentalised inside plant cell organelles, such as vacuole, to avoid its toxic effect. During the infection, *E. amylovora* induces electrolytes leakage by altering the cell membrane integrity (Sjulin and Beer, 1978; Youle and Cooper, 1987). The subsequent cell collapse might release luteoforol. In the apoplast, luteoforol might act both against plant and bacterial cells. Thus, luteoforol is supposed to reduce further disease development firstly by killing directly pathogen cells and, secondarily, by mimicking a hypersensitive reaction.

Luteoforol is also very active against the bacterial antagonists tested. To combine ProCa treatment with biological control the selection of a luteoforol-resistant bacterial antagonists is not necessarily needed, since luteoforol is not secreted on plant surface. Nevertheless, we found that some antagonistic bacteria, such as *P. agglomerans*, can survive as endophyte. In this case, a luteoforol-resistant antagonist might enhance biological control effectiveness.

FINAL REMARKS

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Dioxygenase inhibitors application resulted a useful tool to reduce fire blight incidence both on blossoms and shoots. Incidence reduction obtained with ProCa and TrixE ranged between 50 to 66% in comparison to control. Furthermore, this research demonstrated that the decrease of plant susceptibility to disease is mainly due to the treatment-induced accumulation of luteoforol, which is a new phytoalexin of apple. In fact, this compounds has a very high antimicrobial activity and even at the low concentrations founded in apple leaves it can be active against pathogens.
In addition, the incidence reduction can be partially explained with the plant-mediated effect of dioxygenase inhibitors on epiphytic microbial populations. In fact, when ProCa and related compounds are applied, a higher beneficial microbial community can be found both on blossoms and leaves. Furthermore, after dioxygenase application a reduction in the sugar content of nectar was observed. It was demonstrated that the resulting lowered osmotic potential allows the growth of bacterial antagonists in the nectar and thus they can reduce *E. amylovora* colonization of nectar cup and penetration in host tissues.

As far as practical applications, it is likely that, combining dioxygenase inhibitors treatment with other control methods, higher reduction of fire blight incidence and severity would be achieved. In particular, the combination of ProCa and related compounds with environmentally safe control methods, such as biological control, seems a promising strategy. In fact, since ProCa showed a very low toxicity and environmental risks, it would completely fit in an integrated pest management system. Moreover, ProCa and TrixE application reduces canopy density and thus a reduced amount of

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other chemicals is needed. All these observations open a promising and environmental safe method for resistance induction in pomefruit trees against fire blight and other bacterial and fungal diseases. Moreover, dioxygenase inhibitors present some advantages in comparison to BTH and SAR inducers. In fact, they are plant growth retardants and they can be used also to control vegetative growth and fruit production in commercial orchards, thus allowing high-density plantation fields. Finally, since ProCa and TrixE stimulate plant resistance, their application likely prevents resistance development both in phytopathogenic fungi and bacteria.
Despite of all these considerations, our preliminary data on the combination of biological control and dioxygenase inhibitors did not confirm the hypothesis of their synergic effect. Therefore, further researches are needed both to investigate this hypothesis and the possible combination of dioxygenase inhibitors with other fire blight control strategies.

Moreover, this research deeply investigated how the infection process occurs and how it is influenced by plant metabolism. Several aspects of *E. amylovora* penetration and migration in host plant tissues were elucidated. Furthermore, the practical application of the techniques used for these investigations allowed the development of a new and reliable method for resistance inducers efficacy screening.

Further researches are also needed to investigate more deeply ProCa and TrixE influence on plant metabolism. Because of the observed influence of dioxygenase inhibitors on nectar and foliar secretions, similar effects have been hypothesised also for stigmatic secretions. Since we observed a general reduction in the amount of sugars secreted on nectar and on leaf surface, it likely that also the nutrient rich stigmatic secretion might be affected by dioxygenase application. These possible differences might explain the higher bacterial population found on treated stigmas. However, the mechanisms underlying all these phenomena are still unknown.
Moreover, it could be interesting a more precise quantitative determination of the organic compounds secreted on nectar cup and

leaves. It could also be interesting investigate the presence of phenolic compound in the nectar. In fact, since many of them have, in some extent, an antimicrobial activity, they can protect blossoms from fire blight infection. Finally also possible hysto-anatomical changes on leaves and blossom due to dioxygenase inhibitors application should be further studied.

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ANNEX

1. General Purpose Media and Reagents

***Aspergillus nidulans* minimal medium**

Solution A:

$NaNO_3$	6.00 g
KH_2PO_4	1.52 g
KCl	1.52 g
Distilled water	500.0 ml

Adjust pH to 6.5 with 2 N NaOH.

Solution B:

$MgSO_4$ x 7 H_2O	0.52 g
$FeSO_4$ x 7 H_2O	trace
$ZnSO_4$ x 7 H_2O	trace
Agar	15.0 g
Distilled water	250.0 ml

Solution C:

Glucose	10.0 g
Distilled water	200.0 ml

Sterilize solution C by filtration

Autoclave separately at 121°C for 15 min. Mix A, B and C before pouring plates.
(by DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany)

Ceria 132 minimal medium

Agar	18.0 g
20x Ceria 132 salts	50 ml
Distilled water	950 ml

Dissolve agar in water, sterilize at 121°C for 15 minutes. Cool the autoclaved medium to 50ºC and add 50 ml of 20x salts filter sterilized.

Ceria 132 salts (20x)

K_2HPO_4	35.1 g
KH_2PO_4	15.1 g
L-Asparagine	15.0 g
$(NH_4)_2\,SO_4$	10.0 g
Nicotinic Acid	2.50 g
Glucose	20.0 g
Na Citrate (tri-Na)	2.50 g
$MgSO_4x7H_20$	0.05 g
Distilled water	500 ml

Filter-sterilise and store at -20.

King's B medium (KB) (King *et al*. 1954)

Proteose peptone (Difco No.3/Oxoid L46)	20.0 g
K_2HPO_4	1.5 g
$MgSO_4$ x $7H_2O$	1.5g
Agar	15.0 g
Glycerol	10.0 ml
Distilled water	1.0 L

This general purpose medium is particularly useful for detecting fluorescent pseudomonads, but other bacteria also grow easily on it.
Autoclave dextrose separately from the other ingredients. Cool the autoclaved medium to 50ºC and mix well before pouring the plates so the calcium carbonate remains suspended. Xanthomonads produce large, yellow, domed, mucoid colonies on this medium, which can be used for general isolation from plant tissues.

Luria broth (LB)

Tryptone	10.0 g
Yeast extract	5.0 g
NaCl	10.0 g
Distilled water	1.0 L

Shake until the solutes have dissolved. Adjust the pH to 7.0 with 5N NaOH (~ 0.2 ml). Adjust the volume of the solution to 1 liter with deionised H_2O and autoclave for 15 minutes at 121 °C. Luria agar is LB plus 15 g of agar. From Sambrock *et al.*, 1989.

M9 minimal medium

Per liter:

To 750 ml of sterile deionised H_2O (cooled to 50°C or less), add:

5x M9 salts	200.0 ml
sterile deionised H_2O	to 1 liter
20% solution of glucose*	20.0 ml

(*or of an other appropriate carbon source)

If necessary, supplement the M9 medium with stock solution of the appropriate amino acids.

M9 salts (5x)

Na_2HPO_4 x 7 H_2O	64.0 g
KH_2PO_4	15.0 g
NaCl	2.5 g
NH_4Cl	5.0 g

M9 salts is made by dissolving these salts in deionised H_2O to a final volume of 1 liter. The salts solution is divided into 200 ml aliquots and sterilized by autoclaving for 15 minutes at 121°C. From Sambrock *et al.*, 1989

Nutrient agar (NA)

Beef extract	3.0 g
Peptone	5.0 g
Agar	15.0 g
Distilled water	1.0 L

Prepared formulations of NA are commercially available and usually preferred. Nutrient broth is NA without agar.
(by DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany)

Sodium Phosphate Buffer (50 mM, pH 7.3)

$Na_2HPO_4x12H_2O$	5.75 g
$Na_2HPO_4x2H_2O$	2.0 g
Distilled water	1 L

Calculated from ISCO (1982).

SOC medium (for electroporation)

Bacto tryptone	10 g
Bacto yeast extract	2.5 g
NaCl	292.1 mg
KCl	94.4 mg
$MgCl_2$	476.1 mg
$MgSO_4$	601.5 mg
Glucose	1.98 g
Distilled water	500 ml

Dissolve agar in water, sterilize at 121°C for 15 minutes.

2. Selective Media for *E. amylovora*

CCT medium (Ishimaru and Klos, 1984)

Sucrose	100 g
Sorbitol	10.0 g
1% acqueous solution of tergitol anionic 7	30.0 ml
Crystal violet (0.1% in absolute ethanol)	2.0 ml
Nutrient Agar (Difco)	23.0 g
Distilled water	970 ml

Autoclave for 15 minutes at 121 °C and cool at 50°C, then add 2ml of thallium nitrate (1% w/v in water) and 50 mg of cycloheximide. After poured in Petri dishes, CCT could be stored in the dark at 5° C for 2-3 weeks. Longer storage is not recommended since CCT can become toxic to *E. amylovora* upon prolonged storage.
Using this medium, *E. amylovora* colonies appear, after 3 days incubation, large (4.0-7.0 mm), with entire margins, pulvinate, light blue and opalescent.

Glycerol stock

Luria broth	0.85 ml
Glycerol 100%	0.2 ml

The bacteria in glycerol stock can be stored at –20°C or –70°C for a long period. To prepare glycerol stock 24h-old culture on solid medium should be used. Collect the bacteria from the agar surface and dissolve them in the Luria broth until the suspension become very concentrated (108-109 cfu/ml). Add the glycerol, mix the tube vigorously and store at –20°C or -70°C.

Miller and Schroth medium (MS) (Miller and Schroth, 1972)

Mannitol	10 g
Nicotinic acid	0.5 g
L-asparagine	3.0 g
K_2HPO_4	2.0 g
$MgSO_4 \times 7H_2O$	0.2 g
Sodium taurocholate	2.5 g
Tergitol anionic 7 (Sodium heptadecyl sulfate)	0.1 ml
Nitrilotriacetic acid (NTA) (2% acqueous solution[1])	10 ml
Bromytol blue (0.5 % aqueous solution)	9 ml
Neutral red (0.5 % aqueous solution)	2.5 ml
Agar	20 g
Distilled water	1.0 L
Thallium nitrate (1 % aqueous solution)	1.75 ml
Cycloheximide	50 mg

1: the NTA aqueous solution should be neutralized with ca. 0.73 g of KOH/g NTA

Add single compounds in the order listed. Adjust the medium to pH 7.2-7.3 with ca 5 ml of NaOH (1N). Autoclave for 15 minutes at 121 °C and cool at 50°C, then add 1.75 ml of thallium nitrate (1% w/v in water) and 50 mg of cycloheximide. The pH of the medium after autoclaving should be ca. 7.4. *Erwinia* genus shows, on this medium, reddish-orange coloured colonies. Colonies of Pseudomonads and other bacteria appear green to blue in colour. Observing the colonies morphology with a 10x dissecting microscope it is possible discern among the different *Erwinia* species. *E. amylovora* colonies are smooth, with dark orange centres, and have entire translucent margins

3. Staining Techniques

3.1. Gram Stain

Principle

Bacteria can be recognized as gram positive (blue-black/purple) if they retain the primary dye complex of crystal violet and iodine in the face of attempted decolourization, or as gram negative (pink) if decolourization occurs as shown by the cell accepting the counterstain safranin. Generally the mechanism of the Gram stain is: The fixed bacteria are stained with the triphenylmethane dye, crystal violet. Next the smear is flooded with Grams solution which oxidatively forms an insoluble complex with the crystal violet. The smear is then flooded with the organic solvent, acetone-alcohol. Depending on cell permeability the crystal violet-iodine complex will be washed from Gram negative bacteria in solvent but not from Gram positive bacteria. Upon counterstaining with safranin, organisms which had been discolorized by the ethanol (Gram negative) will stain pink. Gram positive organisms which retained the crystal violet will appear blue-black/purple microscopically.

Materials

- Crystal violet solution
- Grams Iodine solution
- Acetone alcohol
- Safranin solution

Procedure

1. Prepare the film on the slide and allow to air dry. Do not heat to dry film.
2. When film is dry, place slide on heating block for several minutes. Slide should be just warm to your hand. Do not overheat.
3. Allow slide to cool - this will happen quickly - in just a few seconds. Do not add stain to hot slide.
4. Flood slide with crystal violet - leave 1 minute.
5. Wash gently with water.
6. Flood slide with Grams Iodine - leave 1 minute.
7. Wash iodine from slide with acetone-alcohol mixture. Add a few more drops of acetone alcohol
   until no more colour comes from film - usually 30 seconds.
8. Wash gently with water.
9. Flood slide with safranin - leave 1 minute.
10. Wash gently with water. Clean back of slide with tissue and place slide in tray.

Precaution

1. At no time should the film (smear) be exposed to too much heat. When the specimen is still wet, heat causes coagulation of the protein resulting in heavy overstaining which cannot be removed by the decolourizer. A thick smear will also show more tendency to "lift off" during staining.
2. Rinsing the Grams Iodine off with the decolorizer gives more stability to the CV-GI complex and false over decolorizing will not take place.
3. Flooding a hot slide with crystal violet will cause the stain to precipitate and make decolourizing much more difficult.

Quality Control
It is recommended that controls be run concurrently with unknowns or at least run on a daily basis using known smears containing Gram positive and Gram negative bacteria.
[by Prof. Nam Sun Wang - Department of Chemical Engineering University of Maryland, and form Bartholomew and Finkelstein (1958)].

3.2 Staining techniques for flavonoids and phenolic compounds

Phenolic compounds can be visualized with the following staining method for are based mainly. Most of these techniques imply the observation with a fluorescence microscope.
The filter commonly used (UV, BLU and GREEN filter stets) are described in the NA methods and in Material and Methods, paragraph 15.

Autofluorescence
Flavonoids and phenolic compoud can be observed with fluorescence microscope without any king of specifical stain. These compound infact show a certain degree of autofluorescence (Laplaze *et al*., 1999).

Ammonium hydroxide
An increase in pH, obtained with 0.1 M ammonium hydroxide (pH 10.3) treatment, causes the ionisation of the phenolic OH groups, and a subsequent change in fluorescence colour to intense green.
It is possible distinguish between lignin and other phenols bound to cell wall. Lignin, in fact, fluoresces with a blue colour also after treatments with ammonium hydroxide, whereas the fluorescence of other phenols, such as ferulic acid, changes from blue to green.
Also a NH_4OH (0,5% w/v) could be used.

NA or Neu's Reagent
This reagents is obtained by dissolving 1% (w/v) diphenylboric acid 2-aminoethyl ester (Naturestoffreagenz A or NA) in absolute

methanol. The specimens were immersed in this solution for 1-5 minutes. After staining the section were mounted in glycerine-water (15% v/v) and observed with epifluorescence. This reagent induces a secondarye autofluorescence in flavonoids (Markham, 1982).
.
Two filter set were used:
The first (UV filter) with a 340- to 380- nm excitation and 425- nm barrier filter
The second (BLU filter) with a 450- to 490- nm excitation filter and a 515- nm barrier filter.
Flavonoids shows a weak orange fluorescence after excitation at 365 nm and a bright yellow-green fluorescence after excitation at 420 nm (Laplaze *et al*., 1999).

Wilson's reagent
The reagent is prepared by mixing (5:5 w/w) citric acid and boric acid (Prolabo) in 100 ml of absolute methanol. The sections should be immersed in Wilson's reagent for 15 minutes, successively mounted in glycerine-water and observed using a fluorescent microscope. The secondary fluorescence induced by this method is similar to the one obtained with NA.

Vanillin HCL (Sharkar and Howarth, 1976)
This staining techniques is used to visualize condensed tannins and catechins.
The reagent is prepared by mixing vanillin 10% (w/v) in a solution containing absolute ethanol and concentrated HCl (1:1). The specimens are mounted directly in this reagent an observed with a normal light microscope. Flavonols, catechins, and condensed tannins assume a brown-red colour.