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Plant growth promotion and biocontrol traits of *Vitis vinifera* “Glera” bacterial endophytes in the sustainable management of viticulture

Coordinator: Prof. Ildikò Szabò
Supervisor: Prof. Barbara Baldan

Ph.D. student: Filippo G. Zanella

Contents

Abstract 1

Chapter 1 5

Pantoea agglomerans (GL83) and *Bacillus licheniformis* (GL174) display an endophytic life-style after Glera apical cuttings inoculation 5

1. Introduction 6
2. Materials and Methods 8
   - 2.1 Bacteria strains and growth conditions 8
   - 2.2 Plant material 8
   - 2.3 Glera apical cuttings inoculation with GL83*::gfp2x* GL174*::gfp2x* 8
   - 2.4 Laser scanning confocal microscopy 9
   - 2.5 GL83*::gfp2x* and GL174*::gfp2x* enumeration on Glera apical cuttings 9
3. Results 10
   - 3.1 GL83*::gfp2x* and GL174*::gfp2x* colonize the *V.vinifera* cv. Glera inner tissues 10
   - 3.2 GL83*::gfp2x* and GL174*::gfp2x* enumeration through cultivable dependent method 13
4. Discussion 15
5. References 17

Chapter 2 21

Application of *Vitis vinifera* cv. Glera microbiota as potential bio-fertilizer in sustainable agriculture 21

1. Introduction 22
2. Materials and Methods 24
   - 2.1 Bacteria endophyte strains and compatibility assay 24
   - 2.2 Rooting cuttings production line 24
   - 2.3 Bacteria growth conditions and rooting cuttings inoculation 24
   - 2.4 Biofertilization experiment on 1-year-old grapevine plants 26
   - 2.5 Chlorophyll determination 26
   - 2.6 Statistical analysis 26
3. Results 27
   - 3.1 Plant growth promoting bacterial strains selection 27
   - 3.2 Growth promotion evaluation of rooting cuttings inoculated during hydration step 28
   - 3.3 Growth promotion evaluation of rooting cuttings inoculated at root level 30
   - 3.4 GL83 and GL174 strains as Glera rooting cuttings bio-fertilizer candidates 32

3.5 Bio-fertilization effects of GL83 and GL174 on 1-year-old grapevine plant growth and physiology 35
4. Discussion 36
5. References 39
Chapter 3 41
Pantoea agglomerans (GL83) confers to Glera rooting cuttings tolerance to drought stress 41
1. Introduction 42
2. Materials and Methods 44
2.1 Handling of bacteria and growth conditions 44
2.2 Handling Glera rooting cuttings 44
2.3 Glera rooting cuttings inoculation and induction of drought stress 44
2.4 Glera rooting cuttings growth promotion under greenhouse conditions 45
2.5 Relative Water Content determination 45
2.6 Chlorophyll content and water content determination 45
2.7 Statistical analysis 46
3. Results 46
3.1 GL83 increases grapevine root biomass under drought stress 46
3.2 GL83 inoculation effects on grapevine plant physiology under drought stress 47
4. Discussion 48
5. References 51
Chapter 4 55
Bacillus lipopeptides: characterization, plant perception and biocontrol properties 55
1. Introduction 56
2. Materials and Methods 58
2.1 Bacterial strains and growth conditions 58
2.2 *In vivo* antagonism assay 59
2.3 Cell viability assay 60
2.4 *Wild type* and *aequorin-expressing Arabidopsis thaliana* cell cultures and growth conditions 60
2.5 Calcium measurements 60
2.6 Primers design 61
2.7 RNA extraction and first strand cDNA systhesis 61
2.8 Semi-quantitative (RT)-PCR 61
2.9 Relative expression quantification 62
2.10 UPLC-MS analysis 62

2.11 Pumilacidin semi-purification 63

2.12 *In vitro* antagonism assays 63

3. Results 64

3.1 *In vivo* biocontrol effects of GL174 against *Botrytis cinerea* 64

3.2 *Bacillus* LP families induced no cytotoxic effects on *Arabidopsis* cell suspension cultures 65

3.3 Involvement of cytosolic Ca^{2+} elevations in cell suspension cultures and plant LPs perception 66

3.4 LPs effects on defence gene expression 68

3.5 Glera *Bacillus* endophytes produce surfactin, fengycin and pumilacidin 69

3.6 Pumilacidin antibacterial properties 70

4. Discussion 71

5. References 76

Conclusions 83

References 85

Abstract

This thesis describes the results obtained during the three years PhD course which was focused on plant-bacteria interactions, in particular V*itis vinifera* cultivar Glera and endophytes, previously isolated from the same plant species.

The project aimed to investigate the possible beneficial effects that four selected endophyte strains, belonging to *Pantoea* and *Bacillus genera*, can give to Glera rooting cuttings and their potential utility as environmental-friendly alternative approach in plant growth promotion, stress alleviation, and biocontrol. The project was prompted to reduce the extensive use of agro-chemicals in agriculture and to promote a sustainable management of viticulture.

As a first step, *P. agglomerans* (GL83) and *B. licheniformis* (GL174) selected for their valuable plant growth promoting traits were GFP-tagged and used to inoculate in vitro Glera apical cuttings in order to confirm their endophytic nature. Exploiting laser scanning confocal microscopy and a cultivation dependent method the colonization of stem endosphere of Glera apical cuttings after 20 and 30 days post inoculation (dpi) was demonstrated. The analysis was then extended at 45, 52 and 60 dpi in Glera cuttings which were in the meantime transferred in hydroponic non-sterile medium. Both strains were mostly visualized inside the xylem vessels of the stem until 60 dpi confirming the strains endophytic attitude and persistence. Then, the four strains were directly applied in the field by inoculating rootstocks (Kober 5BB) and Glera buds with each bacteria suspension (GL83, GL174, GL189, GL452 and the consortium of these four) both during the hydration step of rooting cuttings production line and by administering the bacterial suspensions at the root level, after plants have developed roots. Then, both the morphological and physiological plant parameters were recorded sampling plants at different time-point of their development.

GL83 and GL174 were the most promising grapevine bio-fertilizer strains, especially when rootstocks and buds were inoculated during the hydration step of rooting cuttings production line. Inoculated plants presented improved leaf physiological parameters such as photosynthesis rate and transpiration flux

linked to an increase of root and shoot fresh biomasses compared to the control plants. Moreover, an experiment aimed to simulate a fertilization procedure using 1-year-old Glera grapevine plants, which were bio-fertilized once a week for seven consecutive weeks with GL83 and GL174 confirmed these strains as promising bio-fertilizer candidates. Furthermore, GL83 was able to relieve the effects of drought stress in inoculated Glera rooting cuttings. In fact, stressed-inoculated plants presented enhanced physiological parameters compared to stressed-control plants and, in addition, GL83 plant growth promoting traits seemed to be water stress dependent.

Another issue investigated during these three PhD years was the role of *Bacillus* cyclic lipopeptides (LPs) families as elicitors of plant defence responses, through induced systemic resistance (ISR) pathway.

LPs are amphiphilic compounds produced by some beneficial bacterial strains freely living in the soil or undergoing mutualistic interactions with plants. The natural functions of LPs suggest their role in antagonism toward other microorganisms, like plant pathogens, and their involvement in motility and attachment to surfaces encouraging bacteria biofilm formation and development.

So far, the mechanisms underlying the induced physiological responses in host plants, upon the exposure to these compounds remain unclear. Challenging *Arabidopsis* cell suspension cultures with the commercial *Bacillus* LPs families (surfactin, fengycin and iturin) both the early cell response and the later events like defence-gene expression were evaluated. Through Evans blue test no cell death increase was detected challenging *Arabidopsis* cell culture with three different concentrations of the LPs families. The administration of the highest concentration of surfactin (50 µg/ml) to *A. thaliana* cell cultures expressing the Ca^{2+}-sensitive photoprotein aequorin in the cytosol evoked a cytosolic Ca^{2+} transient, suggesting that the perception of surfactin could be mediated by Ca^{2+}, which is one of the main important intracellular messenger that generate a wide range of different spatial and temporal signals depending on the stimulus perceived.

Then, to evaluate the possible long-term effects generated upon the perception of LPs, a semi-quantitative PCR defence-gene expression analysis was performed at 2 and 6 hours after LPs cell culture-exposure. Lipoxygenase 1 (LOX1), Pathogen-related protein 1 (PR1), Phenylalanine ammonia lyase (PAL1), Non-expressor of Pathogenesis-Related genes 1 (NPR1) and Mitogen-Activated Protein Kinase 3 (MAPK3) were the main genes investigated which are considered reliable markers of ISR and systemic acquired resistance (SAR).

The results showed that ~3-fold increase of LOX1 and PAL1 expression was recorded in treated samples respect to controls. A ~2-fold increase in the NPR1 gene expression was observed comparing samples at 2 h with control. No differences in PR1 and MAPK3 gene expression were detected between samples and controls. The up-regulation of the LOX1 and NPR1 could suggest that surfactin elicits the activation of jasmonate-mediated pathway that could culminate in ISR. Instead, up-regulation of PAL1 without PR1 expression may indicate an initial SAR triggering, afterwards inhibited by some steps of ISR pathway.

A UPLC-MS analysis performed at the Gembloux Agro-Bio Tech (University of Liège, Belgium) revealed that some selected Glera Bacillus endophytes produce surfactin and fengycin. Moreover, a quite uncommon type of surfactin called pumilacidin was detected and semi-purified. An *in vitro* antagonism assays against some plant pathogen bacteria showed that pumilacidin had a quite strong antibacterial effect. This is a preliminary result and other tests against, for example, infectious fungi such as *B. cinerea* need to be performed. Nevertheless, this is an important starting point for developing new sustainable practices with potential application in disease control.

In conclusion, among grapevine cultivable endophytic strains, GL83 and GL174 showed to be promising candidates as bio-fertilizer enhancing both the grapevine growth and health: GL83 was able to promote growth and to confer an increased tolerance to drought stress whereas GL174 seemed to have a protecting action against some fungal grapevine pathogens.

The possibility of endophyte exploitation to help plants to cope with abiotic as well as biotic environmental stresses, interfering with plant morphology and

physiology or priming their systemic responses against pathogens, opens new scenarios for an environmentally friendly shift in agricultural practices with much lower impact of synthetic agro-chemicals.

Results obtained from this work are aligned with the huge amount of studies, grown exponentially in the last years, in which the endophytes abilities and properties are often assayed in a single plant species or within groups of closely related plant genotypes. In addition, investigations are usually performed using microbial species that are relatively easy to cultivate leaving out that plant biome is always characterized by wide and complex interactions. The plant phenotype is determined not only by the plant responses to the environment but it is also orchestrated by the associated microbiota, the responses of the microbiota to the environment, and the complex interactions between individuals in the endosphere microbial plant community. Future exciting challenges, based on classical approaches and new valuable technologies (i.e. next-generation sequencing) applied to greenhouse and field conditions would allow to explore and characterize the contributions of genetic and metabolic elements involved in the interactions between host plants and endophytes, providing new ecological and evolutionary insights and a better knowledge of the plant-microbiome-environment relationships.

Chapter 1

Pantoea agglomerans **(GL83) and** ***Bacillus licheniformis*** **(GL174) display an endophytic life-style after Glera apical cuttings inoculation**

In this chapter the abilities of two strains, previously isolated from grapevine leaves, *Pantoea agglomerans* (GL83) and *Bacillus licheniformis* (GL174) to colonize, survive and proliferate inside *Vitis vinifera* cv. Glera tissues were evaluated.

In this study we used the two Glera endophyte strains GL83::*gfp2x* and GL174::*gfp2x* genetically transformed with the GFP coding cassette.

Using laser scanning confocal microscopy and cultivable dependent method we demonstrated the colonization of Glera cuttings until 60 days post inoculation.

Glera apical cuttings were *in vitro* inoculated and monitored not only during their growth in gnotobiotic condition but also in a hydroponic non-sterile cultivation system.

1. Introduction

All plants have been studied so far are reported to be colonized by bacteria as well as by fungi. Rhizosphere, which is the narrow layer of soil stuck to plant roots, contains a huge number of different microorganisms and it is directly influenced by plant root exudates. Amongst the rhizobacterial communities inhabiting the soil, plant growth promoting bacteria (PGPB) are plant beneficial microorganisms that have been studying for decades. Endophytes represents a subgroup of PGPB and they can be defined as microorganisms that can spend part or the entire of their life cycle inside plant tissues without causing any sign of damage to their plant hosts (Ryan *et. al.*, 2008; Compant *et. al.*, 2010; Hardoim *et al.*, 2015).

Endophytes can colonize all plant organs such as roots, stems, leaves and sometimes also fruits generally located between cells and within intercellular spaces. They can enter into plant tissues from roots and as soon as they reach the plant vascular system they can spread systematically inside the whole plant body (Hallmann *et al.*, 2001; Hardoim *et. al.*, 2008; Compant *et. al.*, 2011).

It is well known that during these associations plants and bacteria interact to give each other benefits. Endophytes can enhance plant nutrient availability providing plant essential nutrients such as nitrogen and phosphorus; they can also act as biocontrol agents producing several secondary metabolites so they can help plant in keeping pathogen under control. Endophytes benefit from this association because plants ensure them a useful niche, protection from environmental stresses and a constant supply of nutrients in absence of competitors (Whipps, 2001; Bulgarelli *et. al.*, 2013; Brader *et. al.*, 2014).

Endophytes can be isolated from surface-sterilized plant organs through cultivable dependent methods but now it is also possible to visualize them thank to fluorescent probes combined with sophisticated microscopy techniques.

The generation of GFP-tagged endophyte strains represents so far the most powerful tool to visualize in real time alive bacteria directly into plant tissues. These techniques allowed scientists both to precisely determine the bacteria localization and to predict the population density (Compant *et al.*, 2008; Compant *et al.*, 2011; Hurek *et al.*, 2011; Torres *et al.*, 2013).

Compant and colleagues demonstrated that *Vitis vinifera* cv. Chardonnay was colonized by *Burkholderia phytofirmans* PsJN::*gfp*2x; they found it in stems, roots and leaves demonstrating that is it possible to use GFP tagged strains to follow grapevine colonization. Using microscopic analysis by in situ fluorescence hybridization of resin-embedded samples they demonstrated that also flowers berries and seeds of grapevine plants are colonized by various endophytic bacterial genera (Compant *et al*., 2008 and 2011).
It has also been demonstrated that *Vitis vinifera* cv. Glera hosts an endophytic bacterial community which was then characterized both from taxonomical and biochemical points of view (Baldan *et al*., 2014; 2015).

In this work the endophytic attitude and persistence of GL83 and GL174 in Glera apical cuttings and plantlets were evaluated using the homologous GL83::*gfp*2x and GL174::*gfp*2x tagged strains, already available in the laboratory. After Glera apical cuttings inoculation, the endophytic attitude and persistence were evaluated after 20 and 30 days post inoculation (dpi) in gnotobiotic conditions; then cuttings were transferred in hydroponic solution and sampled after 45, 52 and 60 dpi. The fluorescent strains were detected and enumerated during each time point through laser scanning confocal microscopy observations and cultivable dependent methods, respectively.

2. Materials and Methods

2.1 Bacteria strains and growth conditions

GL83 and GL174 were previously isolated from surface-sterilized leaves of *Vitis vinifera* cv. Glera (Baldan *et al*., 2014).

Thank to a previous work both strains were transformed with a plasmid pUT*gfp*2x which contains a mini-Tn5 transposon coding for Kanamycin resistance and GFP protein (Elbertagi *et al*., 2001). Bacteria were grown in Nutrient Broth (Fluka) supplemented with Kanamycin 30 mg/L at 28°C for 48 hours on shaking.

2.2 Plant material

All the experiments were performed using *Vitis vinifera* L. cv. Glera clone 10 (ISV-ESAV 10, biotype Balbi) cultivated *in vitro* and in hydroponic medium. Plants were *in vitro* propagated in plastic boxes (ECO2BOX, Duchefa) containing 80 ml of ½ Murashige & Skoog (MS) medium without sugar. Plants in hydroponic condition were cultivated in cylindrical plastic boxes OS140BOX (Duchefa) with 400 ml of hydroponic solution [KH_2PO_4 0.5 mM; K_2SO_4 0.5 mM; $Ca(NO_3)_2$ $4H_2O$ 2 mM; $MgSO_4$ 0.65 mM; H_3BO_3 0.5 µM; $CuSO_4$ $5H_2O$ 0.05 µM; $ZnSO_4$ $7H_2O$ 0.05 µM; $(NH_4)_6Mo_7O_{24}$ $4H_2O$ 0.02 µM; $MnSO_4$ H_2O 0.05 µM; FeEDDHA 10 µM]. All plants were kept in a green-house chamber (25°C, photoperiod 16/8 h).

2.3 Glera apical cuttings inoculation with GL83*::gfp2x* GL174*::gfp2x*

To prove the endophytic nature of the selected strains, Glera apical cuttings were inoculated and analysed through laser scanning confocal microscopy. Both strains were grown in 5 ml of Nutrient Broth added with 30 mg/L of Kanamycin overnight. They were then centrifuged and re-suspended in 5 ml of 10 mM $MgSO_4$. Once the optical densities were assessed both strains were diluted in physiological solution to reach a cell density of 10^6 cell/ml.

A drop of each strain (5 µl) was placed on the surface of solid ½ MS poured in a plastic box. Then in correspondence of each drop an apical Glera cutting was planted into the medium. The negative control was inoculated with the same volume of 10 mM $MgSO_4$.

To verify both the colonization and the persistence into the plant tissues apical cuttings were sampled at 20 days post inoculation (dpi) and after 30 dpi.

For each sampling time a control plant was analysed.

After 30 dpi the remaining plants were transferred in hydroponic solution and sampled after 45, 52 and 60 dpi with the corresponding control plants.

2.4 Laser scanning confocal microscopy

To visualize and localize the selected strains into the plant tissues, laser scanning confocal microscopy (LSCM) was performed after 20 and 30 dpi on Glera apical cuttings and after 45, 52 and 60 dpi in cuttings transferred into the hydroponic solution.

Samples were collected and surface sterilized 30 seconds in 70 % Ethanol and subsequently in 1.5 % NaClO and rinsed with sterile water three times for 10 minutes. Stems and roots were sliced longitudinally with a blade and leaf fragments were directly observed. Plant material was mounted on a slide with 50 % glycerol and covered with a coverslip. Confocal laser scanning microscopy observation was performed with Leica SP5 system (Leica) using an excitation laser of 488 nm (Argon laser) and collecting the emission band 515-560 nm for the GFP fluorescence and 695-765 nm for the chlorophyll fluorescence.

2.5 GL83*::gfp2x* and GL174*::gfp2x* enumeration on Glera apical cuttings

Glera apical cuttings of each sampling time were subdivided into root, stem and shoot. Each plant organ was superficially sterilized 30 seconds in 70 % Ethanol and subsequently in 1.5 % NaClO. Afterwards, the samples were rinsed with sterile water at least seven times to avoid any traces of the sterilizing solutions. Samples were grounded in a mortar in 2 ml of 10 mM $MgSO_4$.

Appropriate dilutions were plated onto Nutrient Agar supplemented with 30 mg/L of Kanamycin and after two days fluorescent colonies were enumerated.

3. Results

3.1 GL83*::gfp2x* and GL174*::gfp2x* colonize the *V.vinifera* cv. Glera inner tissues

Firstly, the health of inoculated apical cuttings was evaluated. None of the plants presented any symptoms of infection during all the experiments (**Fig.1**).

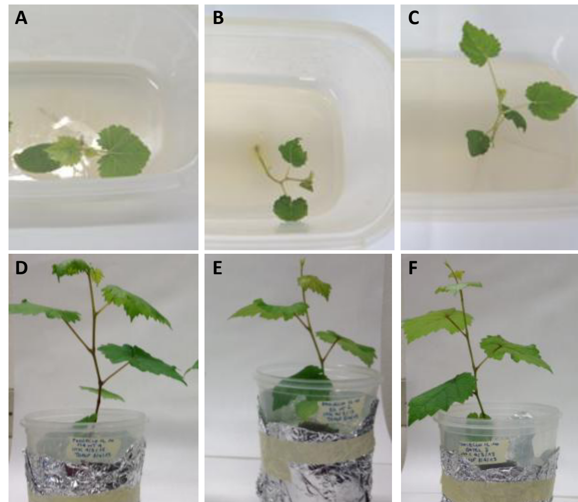

Fig.1. Representative pictures of Glera apical cuttings. **A**,**B**,**C**, Glera apical cuttings not inoculated, inoculated with GL83::*gfp*2x and inoculated with GL174::*gfp*2x at 20 days post inoculation respectively. **D**,**E**,**F**, Glera apical cuttings transferred in hydroponic medium not inoculated, inoculated with GL83::*gfp*2x and inoculated with GL174::*gfp*2x at 45 days post inoculation respectively.

The ability of GL83::*gfp*2x and GL174::*gfp*2x to colonize, survive and proliferate inside plant tissues was confirmed by LSCM and cultivable dependent method.

Moreover, looking forward for the future application of these selected strains in agriculture their persistence inside plant tissues was also evaluated in non-sterile hydroponic condition.

After 20 and 30 days post inoculation (dpi) fluorescent bacteria were mainly visualized by LSCM into the stem of Glera apical cuttings between the intercellular spaces and inside the vascular system (**Fig.2,3**).

After 30 dpi Glera apical cuttings were transferred into non-sterile hydroponic medium (**Fig.1**) and the persistence of both strains was evaluated after 45, 52 and 60 dpi. At 45 dpi LCSM analysis localized both strains inside root tissues (**Fig.4**) and into the central cylinder probably along the xylem vascular vessels demonstrating the ability of both strains to swarm and colonize plant root tissues.

After 52 and 60 dpi bacteria maintained the same plant colonization pattern, in fact they were still detected not far from plant vascular system and probably into the root cortex (**Fig.5,6**). Bacteria were never visualized inside leaves at all the considered time points.

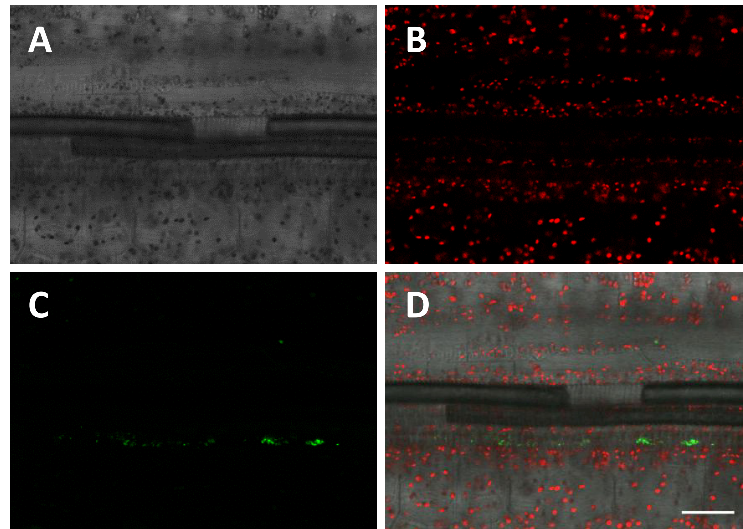

Fig.2. LSCM of stem slice of Glera cuttings inoculated with GL83::*gfp*2x at 20 days post inoculation. **A**, bright field; **B**, chlorophyll fluorescence; **C**, GFP fluorescence; **D**, merge of bright field, chlorophyll and GFP fluorescence. Scale bar= 50μm.

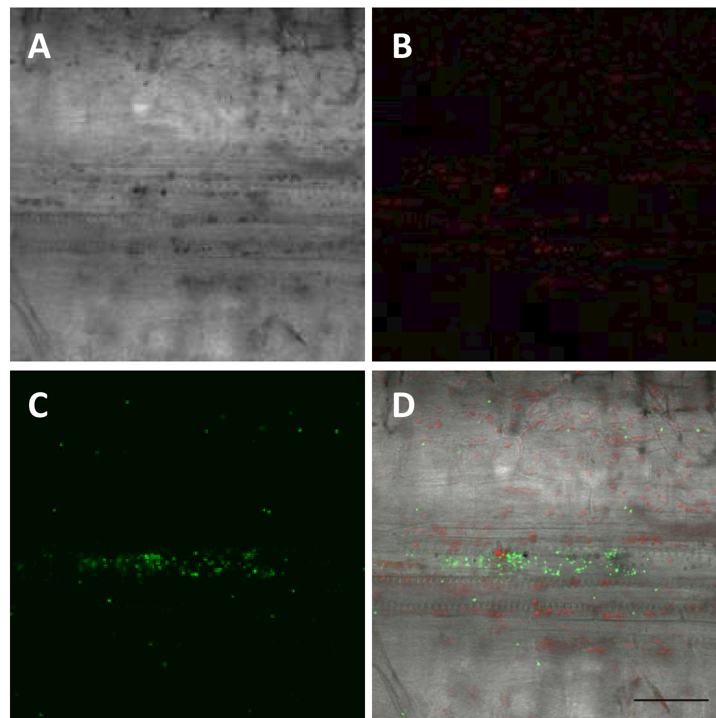

Fig.3. LSCM of stem slice of Glera cuttings inoculated with GL174::*gfp*2x at 20 days post inoculation. **A**, bright field; **B**, chlorophyll fluorescence; **C**, GFP fluorescence; **D**, merge of bright field, chlorophyll and GFP fluorescence. Scale bar= 50μm.

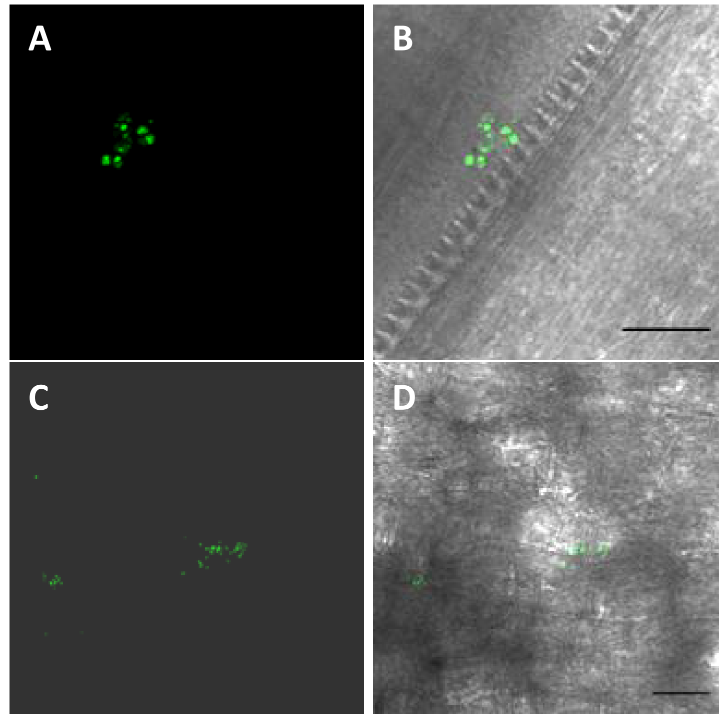

Fig.4. A,B LSCM of root slice of Glera cuttings inoculated with GL83::*gfp*2x at 45 days post inoculation. **A**, chlorophyll fluorescence; **B**, merge of bright field and chlorophyll fluorescence overlay. Scale bar= 50µm. **C,D** LSCM of root slice of Glera cuttings inoculated with GL174::*gfp*2x at 45 days post inoculation. **C**, GFP fluorescence; **D**, merge of bright field and chlorophyll fluorescence overlay. Scale bar= 20µm.

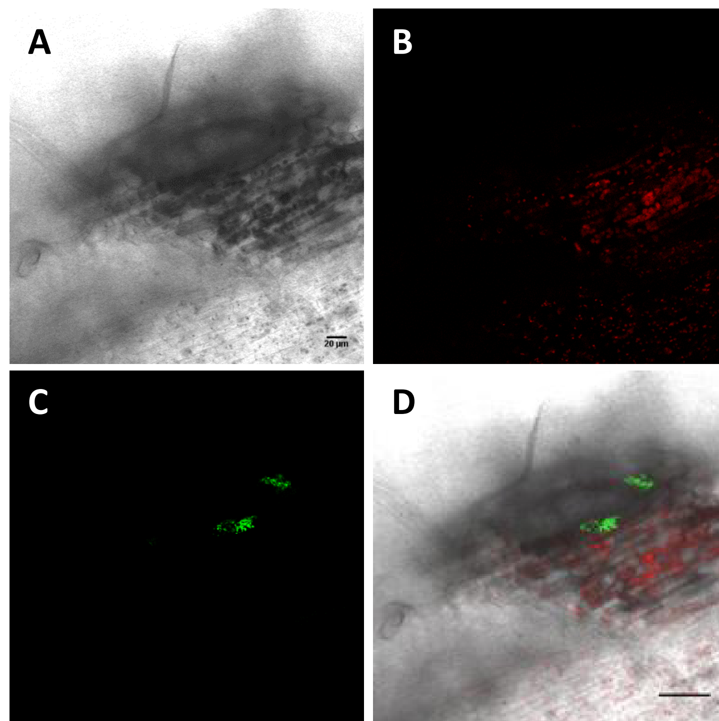

Fig.5. LSCM of stem slice of Glera cuttings inoculated with GL174::*gfp*2x at 52 days post inoculation. **A**, bright field; **B**, chlorophyll fluorescence; **C**, GFP fluorescence; **D**, merge of bright field, chlorophyll and GFP fluorescence. Scale bar= 20µm.

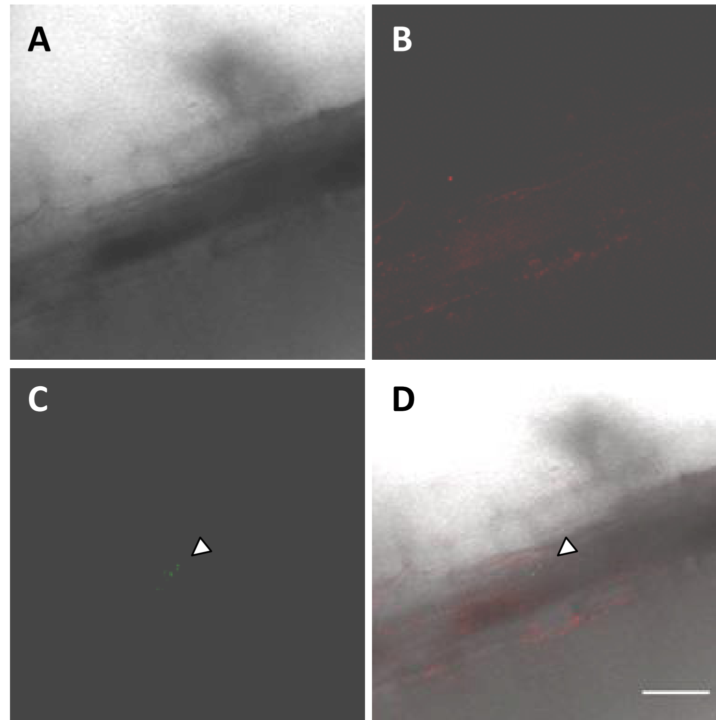

Fig.6. LSCM of stem slice of Glera cuttings inoculated with GL174::*gfp*2x at 60 days post inoculation. **A**, bright field; **B**, chlorophyll fluorescence; **C**, GFP fluorescence; **D**, merge of bright field, chlorophyll and GFP fluorescence. Scale bar= 50µm.

3.2 GL83*::gfp2x* and GL174*::gfp2x* enumeration through cultivable dependent method

To confirm the LSCM analysis both fluorescent strains were enumerated, through cultivable dependent method, at each sampling time (**Fig.7**)

The enumeration of fluorescent colonies was performed plating 100 µl of the appropriate dilutions onto Nutrient Agar 1.5% supplemented with 30 mg/L of Kanamycin.

GL83::*gfp*2x and GL174::*gfp*2x were found both in the stem and in the root of cuttings with some different concentrations at each sampling time from 20 to 60 dpi confirming the LSCM analysis.

In particular, the GL83::*gfp*2x colony concentration reached a maximum at 30 dpi (7,64 ± 0,16 log10 CFU/gr FW) while in cuttings transferred into hydroponic conditions it stabilized around 6,28 ± 0,14 log10 CFU/gr FW.

GL174::*gfp*2x colonies reached a peak at 20 dpi (6,34 ± 0,49 log10 CFU/gr FW) and then decreased up to (5,66 ± 0,21 log10 CFU/gr FW) in hydroponic medium.

Both the strains efficiently colonize stem and root and generally the number of colonies counted within the stem was lower than those in the root at each sampling time and for both the inoculated cuttings.

No bacteria were ever recorded from leaves of both the inoculated cuttings.

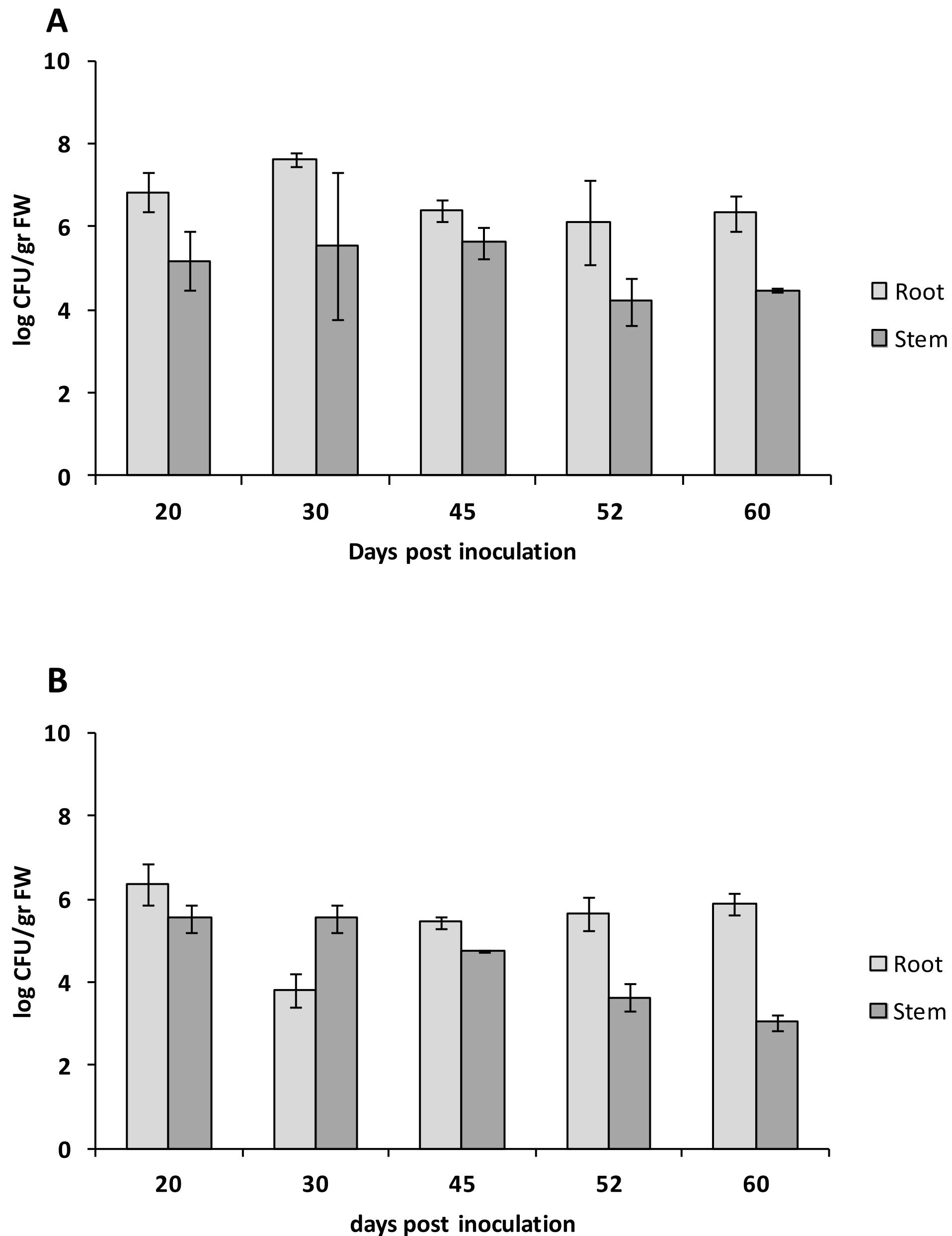

Fig.7. Colony forming unit (CFU) obtained from stems and roots of inoculated cuttings with at 20, 30, 45, 52, and 60 days post inoculation. A, log10 CFU from stems and roots of plants inoculated with GL83::gfp2x. B, log10 CFU from stems and roots of plants inoculated with GL174::gfp2x. Values are means ± S.D, n=3

4. Discussion

It is known that all plants have been studied so far host some bacterial endophytic communities (Ryan *et al*., 2008). Endophytes have been defined as microorganisms that can spend part or their entire life-cycle inside plant tissues and they can be also isolated from surface-sterilized plant organs using cultivable dependent methods. Endophytes can also be classified as obligate, facultative, opportunistic or passenger according to their life cycle strategies (Hardoim *et al*., 2008). Anyway, a true competent endophyte has to be able to re-infect the plant from which it was isolated and it has to be re-isolated.

In order to determine the endophytic life-style of GL83 and GL174 as true competent Glera endophytes we used the two GFP tagged strains (GL83::*gfp*2x and GL174::*gfp*2x) assuming that even the wild-type strains maintain the same endophytic behaviour in Glera.

Direct LSCM confirmed the true endophytic life-style of both the strains examined, they were visualized in stem and root of inoculated cuttings and they were also re-isolated and enumerated through cultivable dependent method.

After 20 and 30 dpi apical cuttings have not developed yet the roots, hence GL83::*gfp*2x and GL174::*gfp*2x were mainly found in central cylinder of the cutting stems. After 30 dpi cuttings were transferred in hydroponic medium. The analysis were repeated after 45 dpi, when cuttings have started to develop a sizable roots and both strains were also visualized between cortex cells of roots tissues. It is clear that both strains were able to exploit the vascular system of growing plant to move and colonize the new plant tissues.

At 52 and 60 dpi fluorescent spots were still detected in the central portion of inoculated plant stems and inside root tissues.

No fluorescent bacteria were visualized by LSCM or found by cultivable method inside the leaves of cuttings. Colonization process is time consuming and probably they need more time to reach the plant aerial part and to adapt a different environment such leaves. This is in agreement with previous studies which demonstrated that endophytic populations decrease from underground parts to the above-ground parts (Hallman *et al*., 2001; Compant *et al*., 2011).

It could be due to the resolution power of LSCM, only the bacteria population forming microcolonies can be detected by LSCM as little as green spots; when the number of tagged-bacteria falls below the threshold they cannot be visualize anymore. In this contest other approaches such as fluorescence in situ hybridization could be useful in microscopic visualization of endophyte plant invaded tissues (Compant *et al*., 2011).

Concerning the inoculation/colonization process a simplified but useful system was adopted, using apical cuttings bacteria can directly enter the plant tissues without any physical barriers, then their endophytic attitudes and persistence in plant were demonstrated with multi-disciplinary approaches. Inoculation method aside, previously *in vitro* biochemical test showed that GL83 is able to secrete hydrolytic enzymes such as endoglucanase. This is an important feature that allows the strain to get in loosening the plant tissues and overcoming the physical barriers represented by plant cell walls facilitating its colonization process. On the contrary GL174 did not score positive in the cellulose assay but it was demonstrated that it is able to produce lipopeptides (LPs) (Favaro *et al*., 2016). The natural functions of *Bacillus* LPs families are antagonism toward other microorganisms, motility and attachment to surfaces. In particular surfactin members stand out to be bio-surfactant and emulsifying agents and for their role in biofilm formation and colonization (Ongena *et al*., 2008; Raaijmakers e*t al*., 2010). Both these traits are useful to the colonization and invasion process following the apical cuttings inoculation.

In conclusion these results confirm that GFP tagged bacteria are a reliable and powerful tool to study and visualise the plant colonization pattern; recently GFP-tagged bacteria were used to investigate the colonization patter of poplar tree and periwinkle by bacterial endophytes, demonstrating the technique effectiveness in several others plant species (Torres *et al*., 2013; Germaine *et al*., 2017). The inoculation method used could be also considered a good way to inoculate *in vitro* cuttings. These experiments represent the first step in a sustainable management of agriculture which aims to encourage the introduction of beneficial endophytes in viticulture management to reduce the

extensive use of harmful agro-chemicals as very recently demonstrated by (Rolli *et al*., 2017).

Considering our previous assumptions, we proved that GL83 and GL174 are true competent Glera endophytes. Moreover, considering both their endophytic attitudes and their plant growth promoting traits new scenarios will be opened for the utilization of these two strains directly in the viticulture practices.

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Chapter 2

Application of *Vitis vinifera* cv. Glera microbiota as potential bio-fertilizer in sustainable agriculture

This chapter describes the results obtained from different field experiments performed during two consecutive years. These experiments aimed to confirm in the field some plant growth promoting abilities of four bacterial endophyte strains, *Pantoea agglomerans* (GL83), *Bacillus licheniformis* (GL174), *Bacillus subtilis* (GL189) and *Bacillus subtilis* (GL452), previously isolated from surface-sterilized leaves of *Vitis vinifera* cv. Glera and selected for their PGP traits tested in vitro. The experimental design was to inoculate the *V. vinifera* cv. Glera with the selected bacteria during two different steps of rooting cuttings production line.

In the first experimental design rootstocks (Kober 5BB) and buds of *V. vinifera* cv. Glera were inoculated during the hydration step while in the second one rooting cuttings were inoculated at root level.

Both the morphological and physiological plant parameters were monitored.

The results show that GL83 and the GL174 are able to promote plant growth enhancing the plant physiological parameters. Inoculation of rooting cuttings during their production processes makes them stronger and it increases their economic value. The idea behind these field trials was to create a bio-fertilizer which could support both grapevine growth and health, promoting a sustainable agriculture and reducing the extensive use of agrochemicals.

With this aim, 1-year-old *V. vinifera* cv. Glera plants were fertilized once a week for seven weeks with GL83 and GL174; the results confirm that these two strains are good candidates to be applied as bio-fertilizer in agriculture.

1. Introduction

The ability of plants to form association with beneficial microorganisms is thought to be one of the critical innovations leading to colonization of land by plants (Delaux *et al*., 2015). Soil microbial communities represent the greatest reservoir of biological diversity known in the world so far (Berendsen *et al*., 2012). The rhizosphere is the narrow layer of soil surrounding plant roots where most plant-microorganism interactions are established. Rhizobacterial communities encompass all the biological diversity in term of microbes, some of them can be neutral or deleterious while other microbes can support their plant partners. These latters are defined as Plant Growth Promoting Bacteria (PGPB); they belong to a beneficial and heterogeneous group of microorganisms that can be found in close association with plant roots. They can mostly act outside plant roots or attached to the root surface forming biofilm and microcolonies (de Souza *et al*., 2015). Some PGPB defined endophytes, can passively or actively colonize plant inner tissues spending the entire or part of their life cycle inside the host plant (Ryan *et al*., 2008; Compant *et al*., 2010).

It is well known that several bacterial endophyte strains can promote both plant growth and health. They can directly stimulate plant growth enhancing plant nutrient availability and producing phytohormones such as auxin which is able to shape plant root system architecture (Vacheron *et al*., 2013; Baldan *et al*., 2015).

Nitrogen and phosphorus are two of the most important inorganic compounds for plant nutrition and they are the major limiting nutrients in the soil. PGPB can mainly improve plant nutrients availability releasing into the soil NH_3.

Moreover, microorganisms able to improve plant phosphorus uptake can effectively contribute to plant growth. Mineral phosphorus solubilization is achieved by the secretion of organic acids or phosphatases that release orthophosphate from soil inorganic phosphorus pool (Vessey *et al*., 2003; Lucy *et al*., 2004; Glick B., 2012; Sharma *et al*., 2013; Bulgarelli *et al*., 2013; Owen *et al*., 2015).

PGPB can also act as biocontrol agents producing a wide range of secondary metabolites such as antibiotic, lipopeptides and volatile compounds able both to stimulate plant growth and to prevent or minimize the deleterious effects of

several plant diseases (Bulgarelli *et al*., 2013; Cawoy *et al*., 2015; Park *et al*., 2015).

Some endophytes can be also effective in cleaning up soils from fertilizers and chemicals, extensively used in agriculture to increase plant growth and yield and to limit the incidence of diseases. (Ryan *et al*., 2008).

In the last decades more and more attention has been given to the application of PGPB in agriculture as bio-fertilizers especially on viticulture in the North-East of Italy, where the high economic importance of viticulture has led an increase in research efforts in finding new bio-fertilizers which support a more sustainable vineyards management.

Unfortunately, during the last decades the use of agro-chemicals to maximize the grapevine yield or to prevent diseases have considerably increased, along with their environmental impact. In fact, an exacerbate soil fertilization can lead to nitrate and phosphate accumulation that can contaminate surface and ground water. Phosphate and nitrogen run-off can cause respectively eutrophication and oxygen starvation both in marine and in river waters leading to a severe reduction of the local fauna (Yang *et al*., 2009).

Hence, it is necessary to support sustainable agriculture which aims to reduce the extensive use of chemicals and to increase agricultural productivity by alternative practices, such as the application of plant growth-promoting bacteria (Glick B. 2014). PGPB can represent the new frontier of plant and soil fertilization, contributing to reduce the amount of chemical fertilizers released into the environment.

This work aims to evaluate the positive effects that selected endophyte bacteria previously isolated (Baldan *et al*., 2014) have on *Vitis vinifera* cv. Glera rooting cuttings and to investigate their potential applications in field cultivation.

2. Materials and Methods

2.1 Bacteria endophyte strains and compatibility assay

Pantoea agglomerans (GL83), *Bacillus licheniformis* (GL174) and two different strains of *Bacillus subtilis* (GL189) and (GL452) were selected for the field experiments. The capability of the selected strains to growth ensemble was evaluated streaking them in the same Petri dish on Nutrient Agar (Fluka) 1.5% making them get in contact in the middle. The test was first performed streaking each pair of strains separately and then streaking all together. After three days the growth of each strain was evaluated.

2.2 Rooting cuttings production line

The **Fig.1** shortly describes the rooting cuttings production line, which is the starting point to develop the field experiments. The water hydration (idratazione) step is a procedure that aim to reactivate the woody part of rootstocks and buds. Then grafting process (innesto) is made through a special machine which is devised to graft every single bud into a rootstock creating plantlets. The process during which these plantlets develop roots and shoot is called forcing (forzatura). Plants are covered with sawdust and the forcing takes place in greenhouse at 30° for 20 days. At the end of this procedure, rooting cuttings are planted (invasatura) in paper pots.

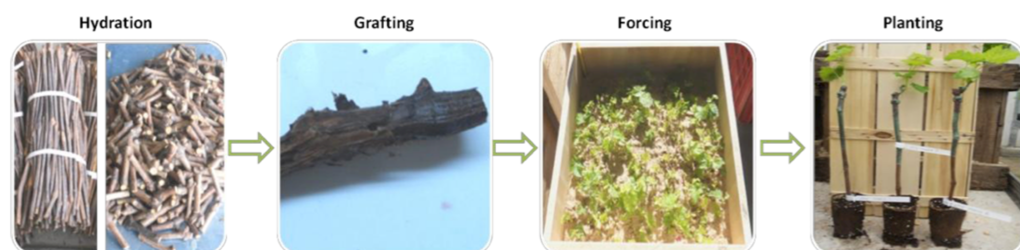

Fig.1. Rooting cuttings production line.

2.3 Bacteria growth conditions and rooting cuttings inoculation

The strains were twice streaked on Nutrient Agar 1.5% plates to check both their purity and bacterial growth. Each single strain was transferred in 500 ml of Nutrient Broth (Fluka) prepared in 3 L Erlenmeyer flask and incubated at 28°C on

a shaker for 48 hours. In order to create a consortium the four strains were in liquid media grown together in the same conditions mentioned above.

The bacterial pellets were then collected by centrifugation (6000 g, 10 min) and re-suspended in 1L of 10 mM $MgSO_4$ physiological solution. Each OD $_{(600nm)}$ were then measured.

The first in field experimental design was thought to use rootstocks (Kober 5BB) and *Vitis vinifera* cv. Glera buds which were inoculated with the different selected plant growth promoting endophytic bacterial strains during the hydration step of the rooting cuttings production line.

Rootstocks and buds were subdivided in six different groups, each group was hydrated for 48 hours respectively with GL83, GL174, GL189, GL452 and the consortium of strains in 20L of 10 mM $MgSO_4$ containing each bacteria suspension adjusted to 10^6 CFU/ml. The control group was hydrated in 20L of 10 mM $MgSO_4$. Rootstocks and buds of each group were then grafted and subjected to the routine practices which are forcing and subsequently planting rooting cuttings in pots.

The second *in field* experimental design was based on inoculation of rooting cuttings at the root level after the forcing process. It was performed starting from a new set of rootstocks (Kober 5BB) and *Vitis vinifera* cv. Glera buds which were previously subjected to a classical water hydration for 48 hours and a forcing process in sawdust. Then, plants were subdivided in six groups, and plant roots of each group was soaked for 48 hours in 5L of 10 mM $MgSO_4$ containing respectively bacterial suspensions of GL83, GL174, GL189, GL452 and the consortium adjusted to 10^6 CFU/ml. The roots of control group was soaked in 5L of 10 mM $MgSO_4$. After that plants were planted in 7 cm paper pots.

All the pots were placed in a greenhouse at 30/20°C (day/night temperature), with the natural circadian rhythm. To monitor the plant physiological parameters, gas exchange measurements were taken on fully expanded grapevine leaves with a portable device (CIRAS-1, PP System, USA) at three different time points during their growth. Net photosynthesis, transpiration flux, stomatal conductance and internal carbon dioxide content were assessed. Moreover, the shoot and the roots fresh biomasses were measured.

2.4 Biofertilization experiment on 1-year-old grapevine plants

Bacterial strains used for this experiment were GL83 and GL174. The strains were prepared as described above. 1-year-old grapevine plants were potted in antispiralization plastic square pots (13 cm side-length) in universal soil (GRAMOFLOR) mixed with perlite.

Potted plants were kept in green-house at 20-30°C (night/day temperature) from April to June. In this experiment sixty 1-year-old grapevine plants were randomly separated to create three different groups containing each twenty plants.

The three groups of plants were adapted to the imposed conditions for two weeks before being watered with 100 ml of GL83 or GL174 at final concentration of 10^7 CFU/ml or 10 mM $MgSO_4$ physiological solution once a week for seven weeks.

2.5 Chlorophyll determination

The fully expanded grapevine leaves used to determine the physiological parameters through the gas exchange analyzer were detached. Chlorophyll was extracted to link it to the leaves physiological data. From each leaf a little circle was cut off and added with 1 ml of N,N-Dimethylformamide (DMF), samples were stored at 4°C per 48 hours to allow the pigments extraction.

The total chlorophyll content was calculated as [Chla]:$(12 \cdot A_{664} – 3{,}11 \cdot A_{647})$ V/W and [Chlb]:$(20{,}78 \cdot A_{647} – 4{,}88 A_{664})$ V/W (V represents the volume of DMF, W represents the sample fresh weight) and was reported as mg Chl. per g FW, as described by Wellburn, 1994).

2.6 Statistical analysis

To perform the statistical analysis of the first year in field experiment the non-parametric two-sample Wilcoxon test was applied because of the limited size of samples and the huge parameter variability of rooting cuttings. Significant differences P*value ≤ 0.05, P**value ≤ 0.01, P***value ≤ 0.001. Instead, since the samples size was enhanced, to analyze the data belong to the experiments performed only with the most promising strains GL83 and GL174 and the bio-fertilization test on the1-year-old grapevine the Student t-test was applied.

3. Results

3.1 Plant growth promoting bacterial strains selection

The bacterial strains chosen for these field experiments belong to a collection of 381 culturable endophyte strains isolated from surface-sterilizes leaves of grapevine plants located in six different vineyards into the Conegliano-Valdobbiadene area, North-East of Italy (Baldan *et al.*, 2014). Four strains were chosen GL83, GL174, GL189, GL452 for their potential plant growth promoting abilities such as indol-acetic acid (IAA) and ammonium ions production and inorganic phosphorus solubilisation (**Table1**).

Selected strain	Taxonomic group	Surfactin synthetase	Fengycin synthetase	Mycosubtilin synthetase	Siderophore production	CMC	IAA production	Phosphate solubilization	Ammonia production
GL83	**P. agglomerans**	-	-	-	++	+	++	+	+
GL174	**B. licheniformis**	+	-	+	-	-	-	-	+
GL189	**B. subtilis**	-	+	-	-	-	-	+	+
GL452	**B. subtilis**	+	+	+	+	-	-	+	+

Table1. The selected strains isolated from surface-sterilized tissues leaves of *Vitis vinifera* cv. Glera (Baldan *et al.*, 2014). The strains were characterized both from the taxonomical and biochemical points of view. Plant growth promotion and biocontrol abilities attributed to the selected strains. "+" positive for the test, "-" negative for the test.

In order to build up the consortium the strains compatibility was checked streaking and getting them in contact in the same Petri dish. The **Fig.2** shows that none of the strain growth resulted affect by the presence of the others.

Fig.2. Compatibility assay. The picture shows the ability of each strain to growth in the same environment in presence of the others. Scale bar 1 cm.

3.2 Growth promotion evaluation of rooting cuttings inoculated during hydration step

In order to evaluate the plant growth promoting ability in the field the plant roots biomass and the shoot biomass were monitored at 70, 85 and 100 days after the inoculation during the hydration step of rooting cutting production line. No significant differences were monitored in shoot and root biomasses except for shoot biomass of plant inoculated with GL83 after 85 days post inoculation. It is worth to mention that there was an increase in root biomass of plants inoculated with GL83 even if there was no statistical significance (**Fig.3**).

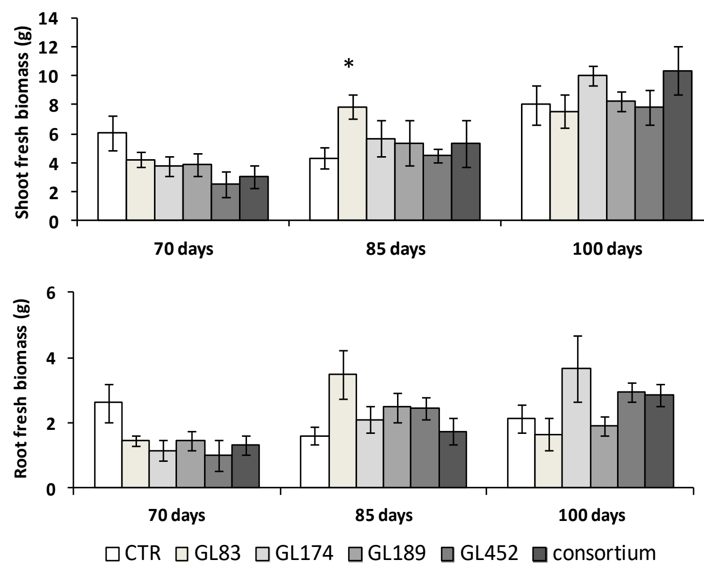

Fig.3. Plant growth promotion effects evaluated on shoot and root biomasses of Glera rooting cuttings inoculated with the selected strains during hydration step. Data subjected to statistical analysis using non-parametric Wilcoxon test. Significant differences reported as P*value ≤ 0.05. Values are means ± S.E, n=5.

The plant physiological parameters were also determined as important markers of plant health taking gas exchange measurements on fully expanded leaves before sacrificing the plants (**Fig.4**). What stands out is that the plants inoculated with GL83 presented an augmented leaves physiological parameters in terms of net photosynthesis, transpiration flux and stomatal conductance during their development. Net photosynthesis was also found higher in plant inoculated with GL189 compared to control plants. Chlorophyll content of plants inoculated with GL83 confirms the enhanced leaves physiological state (**Fig.4**).

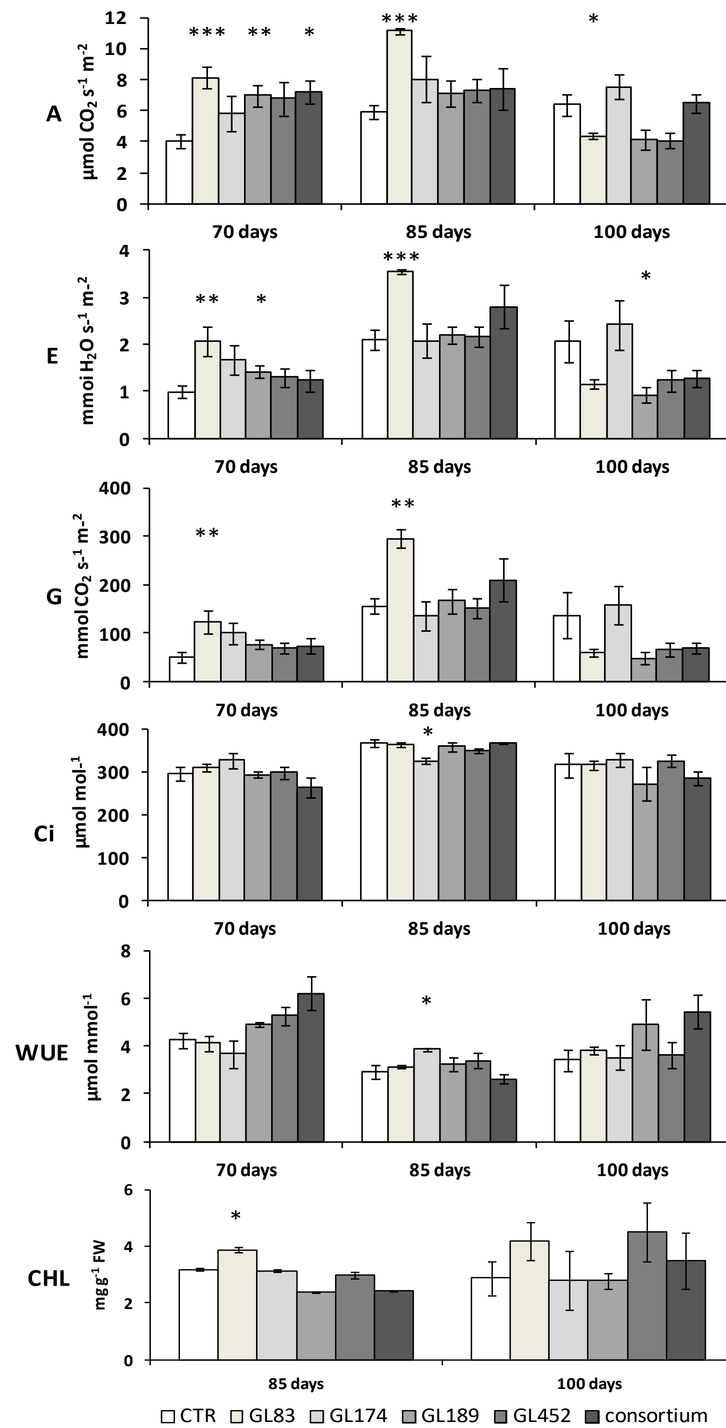

Fig.4. Plant physiological parameters evaluated through gas exchange measurement on fully expanded leaves of Glera rooting cuttings inoculated with the selected strains during hydration step. **A**, net photosynthesis; **E**, transpiration; **G**, stomatal conductance; **Ci**, internal carbon dioxide; **WUE**, water use efficiency; **CHL**, total chlorophyll content. Data analyzed using non-parametric Wilcoxon test. Significant differences reported as P*value ≤ 0.05; P**value ≤ 0.01; P***value ≤ 0.001. Values are means ± S.E, n=5.

3.3 Growth promotion evaluation of rooting cuttings inoculated at root level

Plant root and shoot biomasses were evaluated at 50, 65 and 80 days after the inoculation at the root level (**Fig.5**). The results showed no significant differences in plant growth parameters, the most promising strains seem to be GL83 and GL174 even if there were no statistical significances in growth parameters between inoculated and un-inoculated control plants.

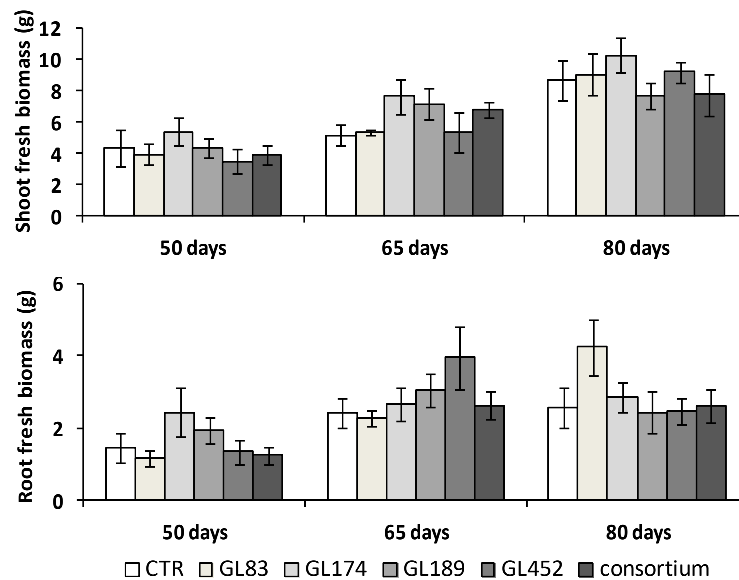

Fig.5. Plant growth promotion effects evaluated on shoot and root biomasses of Glera rooting cuttings inoculated at the root level. **A**, shoot fresh biomass; **B**, root fresh biomass. Data subjected to statistical analysis using non-parametric Wilcoxon test. Significant differences reported as P*value ≤ 0.05; P**value ≤ 0.01; P***value ≤ 0.001. Values are means ± S.E, n=5.

No significant differences were also detected in plant physiological parameters, except at 80 days post inoculation, in which almost of the strains seemed to improve net photosynthesis (**Fig.6**).

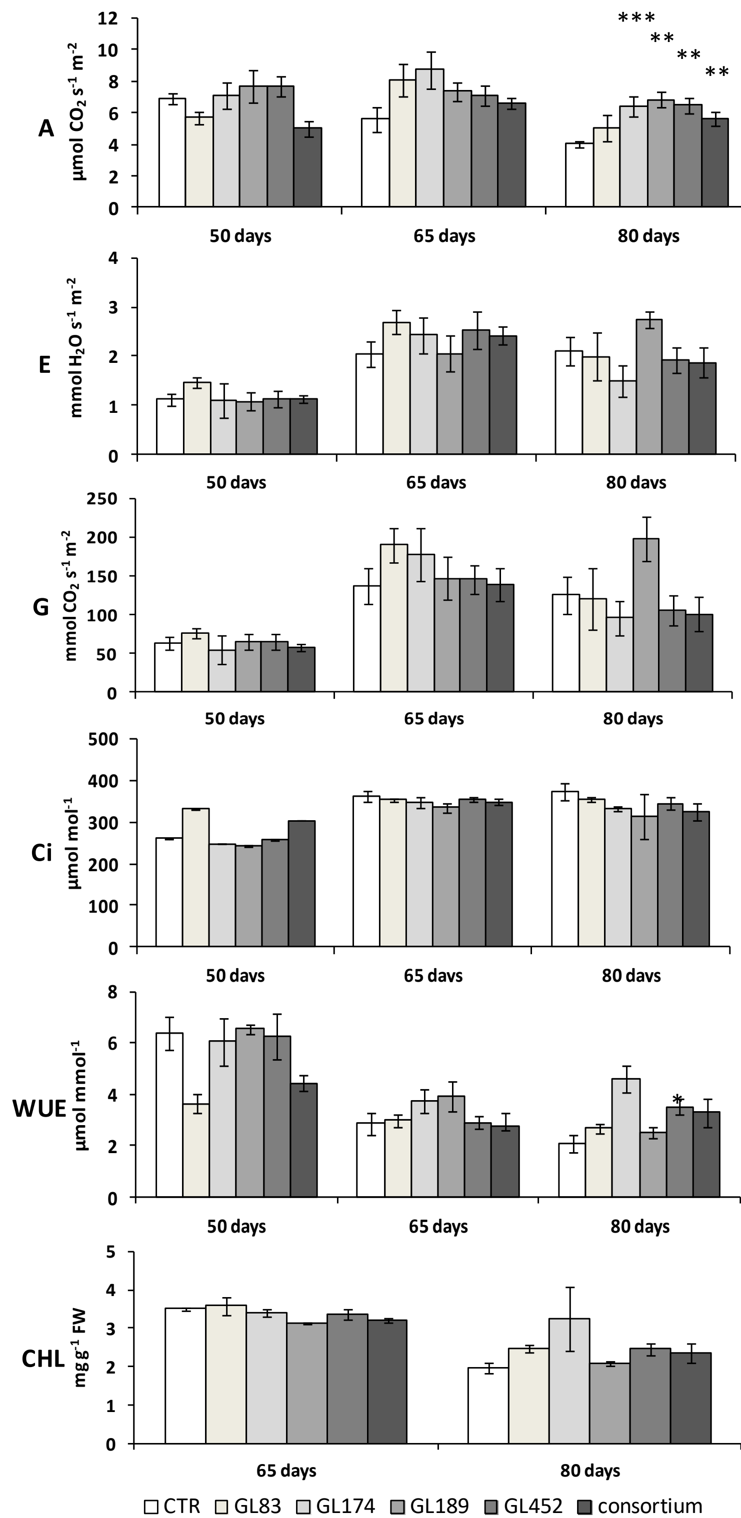

Fig.6. Plant physiological status evaluated through gas exchange measurement on fully expanded leaves of Glera rooting cuttings inoculated at the root level. **A**, net photosynthesis; **E**, transpiration; **G**, stomatal conductance; **Ci**, internal carbon dioxide; **WUE**, water use efficiency; **CHL**, total chlorophyll content. Data analyzed using non-parametric Wilcoxon test. Significant differences reported as P*value ≤ 0.05; P**value ≤ 0.01; P***value ≤ 0.001. Values are means ± S.E, n=5.

3.4 GL83 and GL174 strains as Glera rooting cuttings bio-fertilizer candidates

To validate the results obtained in the first large experiments both the field trials were repeated. Both experiments were performed using the most promising strains (GL83 and GL174) increasing plants number in order to achieve a stronger statistical significance.

Both plant growth and physiological plant status were assessed on Glera rooting cuttings inoculated during hydration (**Fig.7**) and Glera rooting cuttings inoculated at the root level (**Fig.8**).

Plants inoculated with GL83 and GL174 during the hydration step of rooting cuttings production line showed an higher shoot and root biomasses at 100 and 115 days post inoculation compared to the control plants (**Fig.7**). They displayed an higher net photosynthesis too suggesting a plant growth promoting activities exerted by both the selected strains.

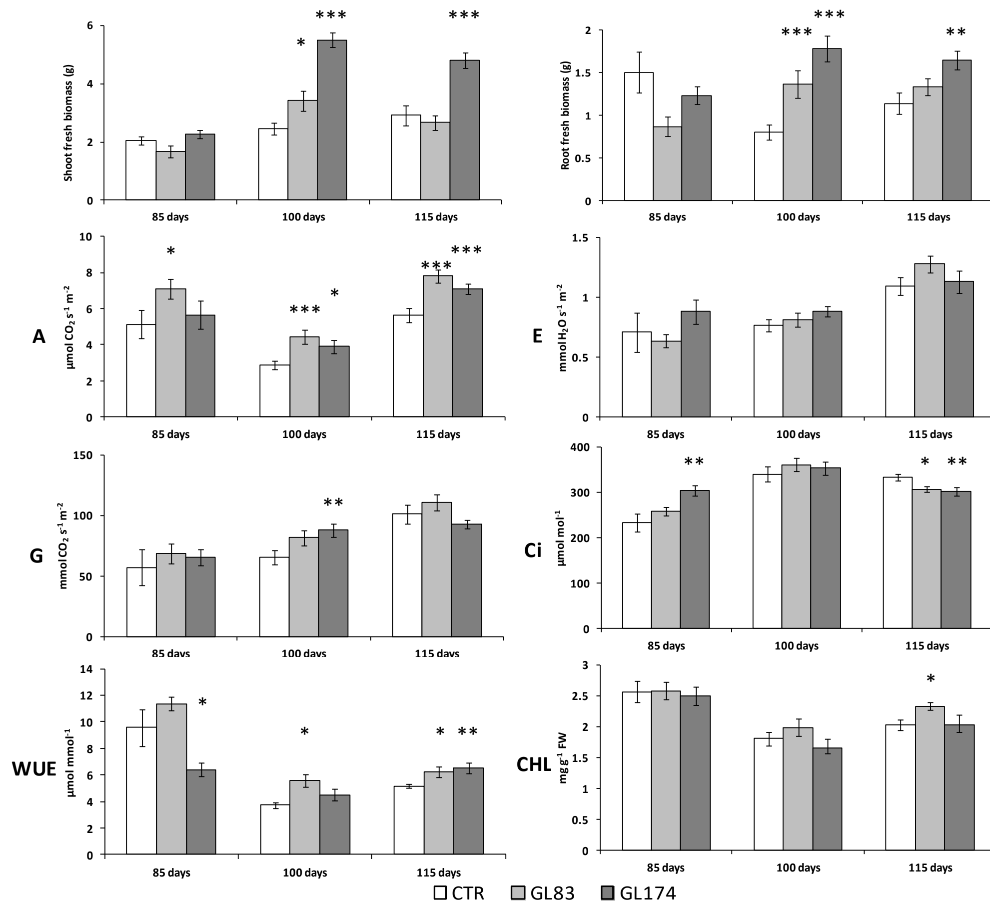

Fig.7. Plant growth promotion effects and plant physiological parameters of Glera rooting cuttings inoculated during hydration. **A**, net photosynthesis; **E**, transpiration; **G**, stomatal conductance; **Ci**, internal carbon dioxide; **WUE**, water use efficiency; **CHL**, total chlorophyll content. Data analyzed using Student t-test. Significant differences reported as P*value ≤ 0.05; P**value ≤ 0.01; P***value ≤ 0.001.
Values are means ± S.E, n=10.

Plants inoculated with GL83 and GL174 at the root level showed an higher shoot and root biomasses compared to the control plants only at 65 days post inoculation (**Fig.9**). The plant physiological parameters of inoculated plants did not display several significant differences compared to the control once, but it is worth to notice that there was an expected correlation between growth parameters and physiological one. The plants with an augmented shoot and root weight display also an enhanced leaf physiological status in terms of photosynthesis, transpiration and stomatal conductance (**Fig.8**).

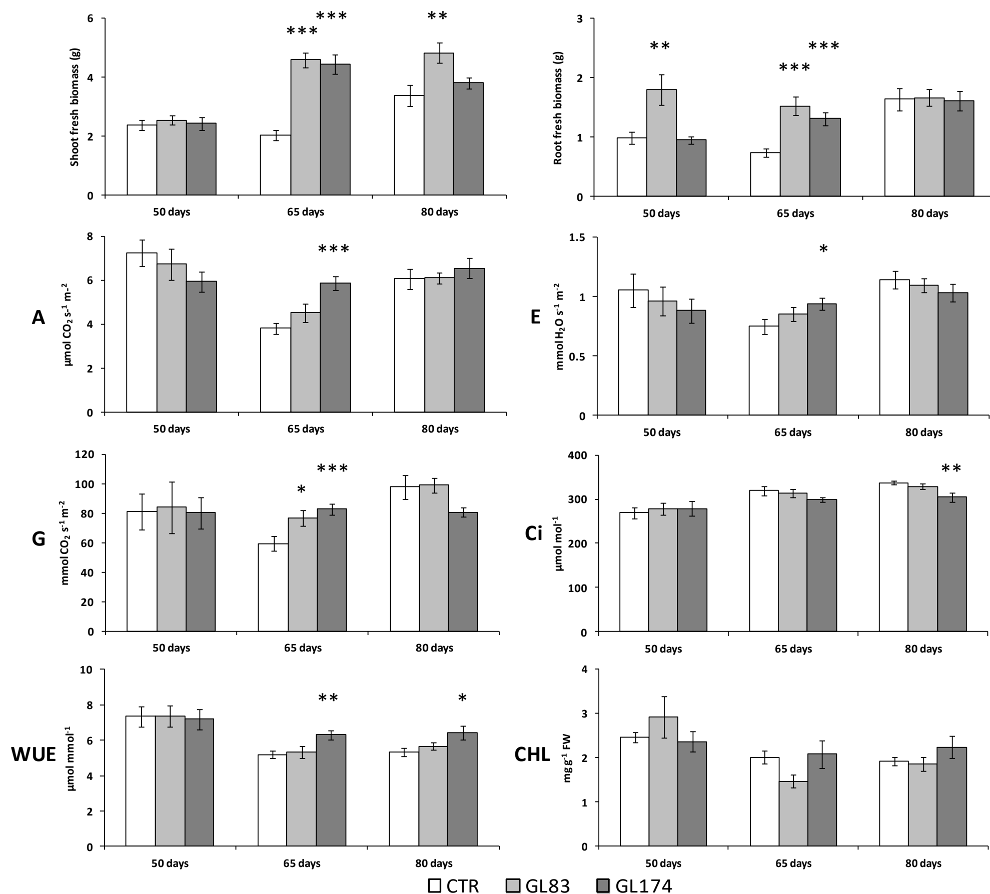

Fig.8. Plant growth promotion effects and plant physiological parameters of Glera rooting cuttings inoculated at the root level. **A**, net photosynthesis; **E**, transpiration; **G**, stomatal conductance; **Ci**, internal carbon dioxide; **WUE**, water use efficiency; **CHL**, total chlorophyll content. Data analyzed using Student t-test. Significant differences reported as P*value ≤ 0.05; P**value ≤ 0.01; P***value ≤ 0.001. Values are means ± S.E, n=10.

Fig.9. Representative image of Glera rooting cuttings inoculated at the root level GL83 and GL174 can efficiently improve shoot biomass. From left to right: Un-inoculated control, inoculated with GL83, inoculated with GL174, respectively.

3.5 Bio-fertilization effects of GL83 and GL174 on 1-year-old grapevine plant growth and physiology

To evaluate the potential application of the selected strains as bio-fertilizers, 1-year-old grapevine plants were fertilized once a week for seven consecutive weeks with GL83 and GL174.

The plant growth parameters and the plant physiological status were monitored (**Fig.10**). No differences were registered in the shoot biomass while a strong increase in root fresh biomass were detected in plants fertilized with both of the strains. Both strains enhanced the biomass of plant roots allowing plants to better looking for water and nutrients.

Physiological plant parameters were recorded through gas exchange measurements on the fourth leaf of grapevine plants with a portable photosynthesis system (CIRAS-1, PP System, USA). Plants fertilized with both the strains showed an increase in photosynthesis rate and in transpiration flux compared to the non-fertilized control plants even if no variations were recorded in internal carbon dioxide content. A slightly increase in stomatal conductance was detected, but the statistical analysis did not record significance differences. The water use efficiency did not score significant variations (**Fig.10B**).

No significant differences were monitored in total chlorophyll content in fertilized plants compared to the controls (**Fig.10C**).

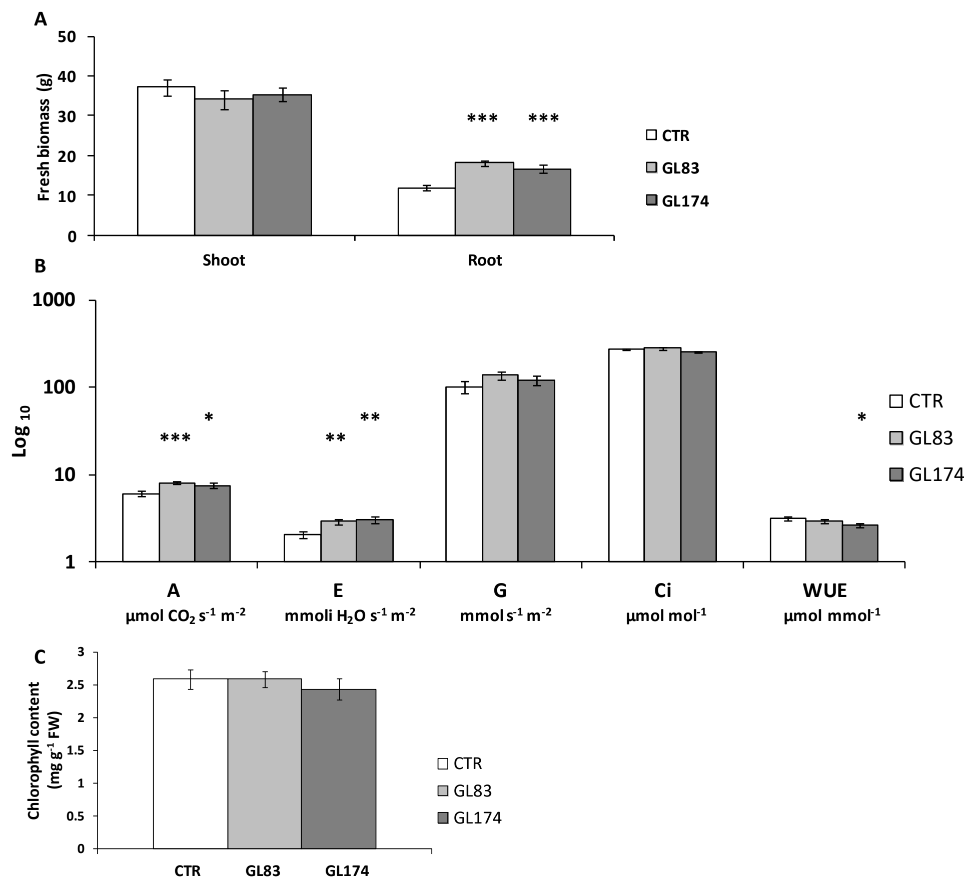

Fig.10. Plant growth evaluation and plant physiological parameters of 1-year old grapevine plants fertilized with GL83 and GL174 compared with non-fertilized control plants. **A**, shoot and root fresh biomass; **B**, physiological leaves parameters; **C**, total chlorophyll content. Data analyzed using Student t-test. Significant differences reported as P*value ≤ 0.05; P**value ≤ 0.01; P***value ≤ 0.001. Values are means ± S.E, n=20.

4. Discussion

Studies of plant-endophyte interactions are commonly based on *in vitro* or ideal conditions for growth of host plant. Microbial behaviour might change when host plants are grown under less optimized conditions and more data coming from different experimental conditions would be required for newer insight into functioning, ecology and interactions of microbes in plants (Hardoim *et al*., 2015). Indeed, to better understand the role and the possible beneficial effects of bacterial endophyte strains interacting with plants the set up of field trials is essential. To monitor in field the behaviour of the two partners during the interaction and possibly to identify the mechanisms underlined their beneficial relationship, strains were directly applied in the field by inoculating rootstocks (Kober 5BB) and Glera buds with each bacteria suspension (GL83, GL174, GL189,

GL452 and the consortium of these four) during the hydration step of rooting cuttings production line, and by administering the bacterial suspensions at the root level, after plants have developed roots. At first a large field trial was designed to use four strains. It was a useful screening to determine the most promising strains. In the second field trial we focused our attention on GL83 and GL174 which showed the best potential applications.

These field experiments represent the end-point of an experimental approach for the selection of promising plant growth promoting strains that may sustain plant growth and health. Moreover, we proposed two inoculation methods during two different steps of the rooting cuttings production line.

The morphological and physiological plant parameters, recorded sampling plants at different time-point of their development, revealed, among the tested strains, that *Pantoea agglomerans* (GL83) and *Bacillus licheniformis* (GL174) are the most promising grapevine bio-fertilizer strains, especially when rootstocks and buds were inoculated during the hydration step of rooting cuttings production line. Inoculated plants presented a higher photosynthesis rate coupled to an increase of root and shoot fresh biomasses compared to the control plants. Plants inoculated during hydration could better benefit of the action of bacteria because they directly entered the inner woody part of rooting cuttings and started to exert their beneficial activity in proximity to the shoot, making plants stronger thus increasing their economic value. Moreover, an experiment aimed to simulate a fertilization procedure using 1-year-old Glera grapevine plants, which were bio-fertilized once a week for seven consecutive weeks with GL83 and GL174, confirmed these strains as promising bio-fertilizer candidates.

The mechanisms underlying these results are yet to be discovered but some hypothesis can be formulated.

Grapevine as all terrestrial plants depends on its root system for the uptake of both water and nutrients. Uptake is related to root absorptive surface area, which is influenced by root length, thickness and density.

Bacteria can modify the plant root system architecture changing the key hormones balance ratio (Vacheron *et al*., 2013). In a previous work (Baldan *et al*., 2015) it was demonstrated that the *V. vinifera* cv. Glera endophyte GL83 is able

to produce auxin, one of the most important plant hormone involved in all developmental plant stages, and able to shape the *Arabidopsis thaliana* root system. High level of exogenous auxin leads to an increase in lateral roots formation and root hairs number coupled to primary root growth reduction.

This trait could be conserved also in the field resulting in an alteration of grapevine root morphology that causes the increase in root fresh weight observed in inoculated rooting cuttings.

Endophytes can also influence plant nutrition making nutrients, such as nitrogen and phosphorus bio-available (Glick B., 2012; Bulgarelli *et al.*, 2013; de Souza *et al.*, 2015). Nitrogen, phosphorus and iron are the most important limiting factors in plant nutrition and development and they are also important signal molecules. These signals trigger molecular mechanisms that can modify cell division rate or cell differentiation with profound impact on root physiology by changing gene transcription and metabolite biosynthesis and as a consequence on all plant physiological status (Cruz-ramı *et al.*, 2003).

In conclusion, the field trials showed that GL83 and GL174 are the most promising grapevine bio-fertilizer strains especially when rootstocks and buds were inoculated during the hydration step of rooting cuttings production line which appeared the best of the two inoculation methods.

Plants inoculated during hydration could better benefit of the action of bacteria because they can directly enter the inner woody part of rooting cuttings and start to exert their activity in proximity to the shoot.

In the very early phases of development of grapevine, bacterial inoculants can make the plant stronger and leading to an increase in their economical value.

Regarding the efforts for reducing the amount of agro-chemicals in agriculture, our strains exerted a promising actions in enhancing plant growth and fitness, as confirmed in 1-year-old grapevine bio-fertilization assay.

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Chapter 3

Pantoea agglomerans (GL83) confers to Glera rooting cuttings tolerance to drought stress

In this chapter the ability of *Pantoea agglomerans* (GL83) to relieve the effects of drought stress was investigated. The results showed that Glera rooting cuttings inoculated with GL83 are less affected by drought.

Moreover, when rooting cuttings were subjected to a second inoculation resulted almost unaffected by drought. These results demonstrate that GL83 is a promising strain candidate as bio-fertilizer not only for its plant growth promoting abilities but also in relieving effects caused by drought.

1. Introduction

Climate change, especially global warming, is one of the most important and serious issues affecting crops all over the world. Global warming is going to increase the frequency and the amplitude of the drought lands in the future leading to a possible food reduction worldwide (Naveed *et al*., 2014). In Europe most of these areas are located around the Mediterranean basin.

Grapevine is a crop cultivated on about seven million hectares (FAOSTAT, 2013) and several arable lands, in which it is cultivated, are located in suffering regions or going to suffer problems related to drought (Gomez del Campo *et al*., 2002; Flexas *et al*., 2010).

Grapevine is one of the best crop tolerant to drought and it is well known that drought is exploited in most wine making areas to improve the quality and the flavour of grapes and consequently of the obtained wine. However, extreme environmental conditions with prolonged dry periods and high temperatures can severely affect grapevine physiology as well as the yield and quality of the grape that can be significantly reduced.

Irrigation supports grapevine growth and health during dry seasons and ensure wine yield and quality (Rolli *et al*., 2015). The aquifer exploitation, linked to the fact that in viticulture the use of chemicals is exponentially increasing, needs to develop sustainable agricultural practices to maintain the productivity and quality saving water and reducing the amount of fertilizers applied to the soil.

In fact, during the last decades more and more attention has been given to plant-microorganisms interactions and more and more bacterial strains have been standing out to have not only beneficial plant growth effects but also the ability to confer tolerance to several plant abiotic stresses such as drought, high salinity and nutrient deficiency (Yang *et al*., 2009; Dimkpa *et al*., 2009).

It has been reported that *Bacillus amyloliquefaciens 5113* and *Azospirillum brasilense NO40* are beneficial bacteria in controlling drought stress in wheat and *Burkholderia phytofirmans PsJN* and *Enterobacter sp. FD17* can increase drought stress resilience of maize (Kasim *et al*., 2013; Naveed *et al*., 2014). Moreover, it has been assessed that the microbiome associated to tomato, grapevine and pepper plants enhances plant growth and plant tolerance under

drought stress conditions (Mayak *et al*., 2004; Marasco *et al*., 2012; Rolli *et al*., 2015). Different mechanisms have been proposed for beneficial plant microbes-induced drought tolerance. Under drought conditions microorganisms can elicited several plant responses such as the modification of the root system architecture increasing the number of root hairs and/or stimulating the root cell division. It allows plant to improve the uptake of water and nutrients (Michiels *et al*., 1989 Vacheron *et al*., 2013).

In addition, some PGPB can modify the level of stress-related hormones like ethylene and/or abscissic acid which production is well known to be enhanced under drought stress. However, microbial ACC-deaminase enzyme can contribute to inhibit the ethylene synthesis reducing the level of ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) (Glick, 2005; Grover *et al*., 2011).

However, a detailed understanding of the mechanisms through which some microorganisms can confer tolerance to drought and to various plant stresses is still missing; further studies and a deeper knowledge on plant-bacteria interactions can give the possibility to shed light on the microbes' role in supporting plant to cope with stresses for a future microbes application in sustainable agriculture.

Viticulture has a great economic impact in the North-East of Italy and several rural areas base their profit on the production and cultivation of grapevine. For this reason in the recent years the research for new sustainable agricultural practices is increasing.

Here it is shown the ability of plant growth-promoting bacteria GL83, previously isolated from grapevine cv. Glera (Baldan *et al*., 2014) to relieve the effects of drought stress on Glera rooting cuttings.

2. Materials and Methods

2.1 Handling of bacteria and growth conditions

The strain used in the present work was *Pantoea agglomerans* (GL83).
It belongs to a collection of 377 strains isolated from surface-sterilized leaves of *Vitis vinifera* cv. Glera (Baldan *et al.*, 2014). It was stored at -80°C in 30% glycerol. It was first cultivated in Nutrient Agar 1.5% (Fluka) at 28°C and then cultivated in Nutrient Broth (Fluka) at 28°C on shaking.

2.2 Handling Glera rooting cuttings

In this experiment Glera rooting cuttings and Glera rooting cuttings previously-inoculated with GL83 (Materials and Methods, **Chapter2**) were managed.
All plants were pruned and four nodes on stems were left. Plants were then immediately planted in little plastic pots (12 cm diameter) filled with the same amount of a blend of commercial soils (0.5% Gramoflor, 0.25% Irish Peat, 0.25% Universal commercial soil and pearlite). Pots were placed in a green-house at 30/20°C (day/night temperature), with the environmental circadian rhythm. Plants were then acclimated, maintained in green-house and watered twice a week for three weeks. These procedures were necessary to standardize the experimental conditions.
The experiment was designed to obtain for each group (Glera rooting cuttings and Glera rooting cuttings previously-inoculated with GL83) an irrigated or drought stressed-controls, and irrigated or drought-stressed cuttings inoculated with GL83.

2.3 Glera rooting cuttings inoculation and induction of drought stress

After the acclimatization time all the plant root systems were completely dipped in water until the soil reached the full water field capacity. The following day plants were inoculated with 100 ml of 10^7 CFU/ml bacterial suspension in 10 mM $MgSO_4$. The control plants were watered only with $MgSO_4$. Then plants subjected to drought stress were not watered at all for 15 days, on the contrary control plants every 5 days.

2.4 Glera rooting cuttings growth promotion under greenhouse conditions

At the end of the 15-day drought stress the plant physiological status was evaluated recording gas exchange measurements on fully expanded leaves of grapevine plants with a portable photosynthesis system (CIRAS-1, PP System, USA). Net CO_2 assimilation rate, stomatal conductance, transpiration flux and internal carbon dioxide content were assessed at a CO_2 concentration of 400 μmol mol, 50% relative humidity, 28-30°C temperature, 200 ml/min flow rate and a photon flux density of 1000 μmol $(m^2)^{-1}$ s^{-1}. The instrument was stabilized according to the manufacturer guidelines. The WUE was calculated as net photosynthesis/transpiration flux. After gas exchange measurements, all plants were harvested and both root and shoot biomasses were weighted.

2.5 Relative Water Content determination

At the end of the experiment the third leaf of each plant was harvested to determine the relative water content. The leaf fresh weight (FW) and dry weight (DW) were measured. The fully turgid weight (FTW) defined as the weight of each leaf kept in 100% humidity in the dark at 4 ◦C for 48 h was also recorded. The relative water content (RWC) was calculated: RWC = (FW−DW)/(FTW–DW).

2.6 Chlorophyll content and water content determination

The leaf used to determine the physiological parameters through the gas exchange analyzer was detached. Chlorophyll was extracted and five biological replicates were collected and analyzed. Samples added with 1 ml of N,N-Dimethylformamide (DMF) were stored at 4°C per 48 hours to allow the pigments extraction. The total chlorophyll content was calculated as [Chla]:(12·A664–3,11·A647) V/W and [Chlb]:(20,78·A647–4,88A664) V/W (V represents the volume of DMF, W represents the sample fresh weight) and was reported as mg Chl. per g FW, as described by Wellburn (1994).

The water content (WC) was determined as [(FW-DW)/FW]*100. FW and DW represent the leaf fresh weight and the drought weight respectively.

2.7 Statistical analysis

The mean values of all the measured parameters were individually compared with their internal control. Both the growth parameter and physiological data were subjected to statistical analysis using the Student t-test. Differences were considered significant for P*value ≤ 0.05, P**value ≤ 0.01, P***value ≤ 0.001.

3. Results

3.1 GL83 increases grapevine root biomass under drought stress

To evaluate the ability of GL83 in relieving the effects of drought stress, root and shoot biomasses of plant exposed or not exposed to drought stress were weighted. To measure precisely the plant root weight, avoiding the different amount of water between treatments, root dry biomass was evaluated. The results showed no significant differences in shoot fresh biomass and in root dry biomass comparing not-stressed plants with inoculated not-stressed plants (**Fig.1A,B**); no differences in shoot fresh biomass were detected in stressed-plants (**Fig.1A**). While, the stressed-previously inoculated plants and stressed-plants subjected to double inoculation displayed an enhanced dry root biomasses respect to stressed-control plants (**Fig.1B,C**).

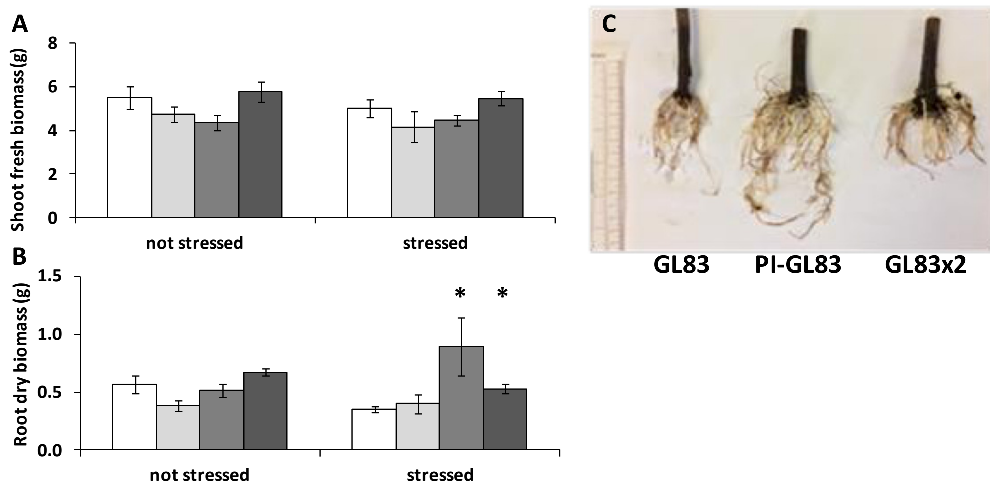

Fig.1. GL83 improves grapevine resistance to drought. **A**, shot fresh biomass. **B**, root dry biomass. **C**, representative image showing the differences in root system between stressed-inoculated plants. CTR: un-inoculated plants, GL83: plant inoculated with GL83 before the drought stress, PI-GL83: plants previously inoculated with GL83, GL83x2: plants subjected to double GL83 inoculation. Data analyzed using the Student t-test. Significance reported as *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001. n=8.

3.2 GL83 inoculation effects on grapevine plant physiology under drought stress

To evaluate how GL83 can relieve the effects of water stress, a gas exchange measurements on fully expanded leaves were taken with a portable photosynthesis apparatus (CIRAS-1, PP-SYSTEM, USA). Net CO_2 assimilation rate, transpiration flux, stomatal conductance and internal CO_2 content were assessed. The results showed that stressed plants when inoculated with GL83 presented higher photosynthesis rate, transpiration flux and stomatal conductance compared with the relative control plants. In particular, GL83 seems to be more active in improving tolerance to drought when plants were inoculated just before the stress administration (**Fig.2A,E,G**). Moreover, inoculated plants displayed a slight but significant reduction of internal CO_2 content, which is in agreement with an enhanced amount in CO_2 assimilated (**Fig.2Ci**). Instead, no differences were recorded in any of these parameters between not-stressed control plants and not-stressed inoculated plants (**Fig.2**). In order to better understand the impact of drought stress the WUE was calculated, determined as A/E, and total chlorophyll content were evaluated. A slight increase in WUE in stressed-inoculated plants was assessed, even if no statistical significance was shown. No significant differences were recorded in chlorophyll content both in not-stressed plants and in inoculated-stressed plants (**Fig.2WUE,CHL**).

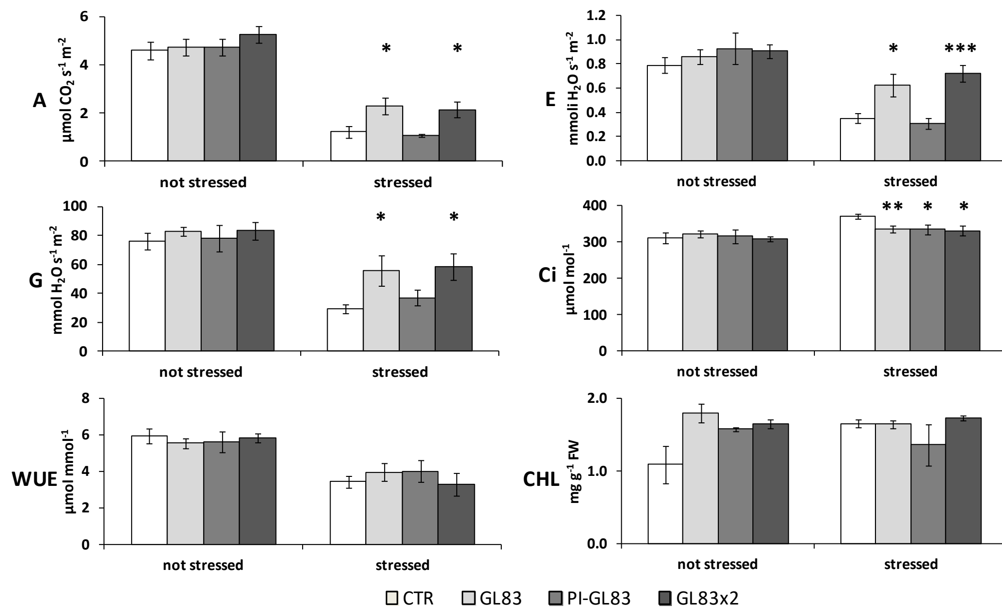

Fig.2. GL83 improves grapevine plant tolerance to drought stress. **A**, net photosynthesis; **E**, transpiration; **G**, stomatal conductance; **Ci**, internal carbon dioxide; **WUE**, water use efficiency; **CHL**, total chlorophyll content. Data analyzed using t-Student test. Significant differences reported as P*value ≤ 0.05; P**value ≤ 0.01; P***value ≤ 0.001. Values are means ± S.E, n=8.

4. Discussion

Nowadays water stress is one of the main environmental issues affecting crops. In the last decades the amplitude and frequency of drought lands are increasing with deleterious consequences in crop productivity and sometimes in food supply (Naveed *et al.*, 2014). Therefore, the scientific community is making numerous efforts in looking for solutions to protect crops from this serious problem; the development of engineered stress-resistant crops has been taken in consideration but it is an expensive and time-consuming approach. In recent years plant-microorganism interactions have given encouraging results in controlling environmental plant stresses and some beneficial plant-associated microorganisms were reported to help plant against biotic and abiotic stresses (Dimkpa *et al.*, 2009; Compant *et al.*, 2010). Plant-associated bacteria belonging to genera *Bacillus*, *Pseudomonas*, *Pantoea*, *Burkholderia*, *Azospirillum* and many others have been described to confer tolerance to plant in different stressful environments. There are several microbial mechanisms known to drive bacterial-elicited stress tolerance in plants: hormones production, ability to release volatile organic compounds (VOCs) and ACC-deaminase activity (Grover

et al., 2011). The volatile compound 2R,3r-butanediol emitted by *Pseudomonas chlororaphis* O6 plays an important role in the induction of drought tolerance in *Arabidopsis* (Ryu *et al*., 2004; Cho *et al*., 2008).

Another PGPR strain, *Achromobacter piechaudii* ARV8, which produced 1-aminocyclopropane-1-carboxylate (ACC) deaminase, confers drought and salt tolerance in pepper and tomato plants (Mayak *et al*., 2004).

It is known that PGPR-indole acetic acid (IAA) producers cause a re-configuration of plant root architectural system leading to an increase in plant uptake of nutrient and enhancing plant health during stress conditions (Egamberdieva *et al*., 2009; Vacheron *et al*., 2013; Owen *et al*., 2015). Concerning the latter, GL83 is able to produce IAA as demonstrated in the colorimetric test described by Patten and Glick (2002). GL83 can modify the *Arabidopsis* root system architecture in terms of length, thickness and in number of root hairs formation; *Arabidopsis* plants grown in presence of GL83 presented shorter but bigger roots with dramatic increase in root hair formation compared to the control plants (Baldan *et al*., 2015). This trait could exert a positive function in inoculated grapevine plants during moderate drought stress, in fact plant with strong and jointed root apparatus can search for water and nutrients more efficiently; moreover, shorter roots can help plant to better save water.

Little is still known concerning the contribution of root-associated microbes to plant adaptation to drought, especially in grapevine. Salomon and colleagues demonstrated that some rhizosphere-associated bacteria can diminish the water loss which is correlated to an dramatic increase in ABA stimulation-synthesis in *in vitro* cultured *V. vinifera* cv. Malbec (Salomon *et al*., 2014).

Moreover, Rolli and colleagues have started to investigate the role and the potential applications in the field of grapevine root-associated microbes. They demonstrated that some grapevine rootstocks-associated microbes were able to increase shoot and leaf biomasses and photosynthetic activity of drought-challenged grapevine plants. They also shown that microorganisms can be used in agriculture as bio-fertilizers to ameliorate plant growth and tolerance to water stress (Rolli *et al*., 2015; 2017).

The results reported in this study describe the abilities of *Pantoea agglomerans* (GL83), a bacterial endophyte isolated from *V. vinifera* cv. Glera tissues, to relieve the drought stress effects in Glera rooting cuttings.

GL83 was administered both to Glera rooting cuttings already inoculated (they had been inoculated at the root level; see **Chapter2**) and to the Glera cuttings just before the drought stress administration. In order to test the GL83 abilities in priming Glera plants to react more efficiently to drought stress conditions. Our results showed that plants subjected to a double inoculation with GL83 presented an higher root biomass under drought stress compared to the stressed un-inoculated controls (**Fig.1**). This observation was also correlated to the leaf physiological status in terms of net photosynthesis, transpiration and stomatal conductance which were higher in respect to the controls, demonstrating that bacteria able to boost the plant root system have positive effects also on plant health (**Fig.2**). It is worth to notice that stressed-plants inoculated with GL83 just before stress administration showed the same pattern only in terms of leaf physiological status, during the temporal window analyzed. These data are in agreement to what have been observed by Rolli and colleagues who described that the PGP promotion of some isolates is a water stress-dependent trait (Rolli *et al.*, 2015). In fact, stressed-plants previously inoculated but not subjected to the second inoculation presented only an enhanced root biomass compared to the stressed-control plants.

Concerning the not-stressed Glera cuttings no differences were detected between any parameters assessed (**Fig.1,2**); it would have been expected to find some PGP activities, such as an improving in shoot/root biomass and/or in physiological leaves parameters, similar to what previously described in **Chapter2**. A possible explanation could regard the time passed from the first inoculation, in fact we did not find PGP effects after 65-80 days post inoculation (see Chapter 2) and plants used in this experiment had been inoculated at the root level 110 days before and it is possible that the GL83 effectiveness could be decline in a time dependent manner. Therefore, it is necessary to search for more effective inoculation strategies that can assure a longer bacterial persistence both inside plant tissues and in the rhizosphere.

No differences were found in water and chlorophyll contents neither in not-stressed plants nor in stressed one; on the contrary inoculated-stressed plants presented a higher physiological status with this equal water content, which could appear a contradiction; however, recent literature concerning plant–microbe interactions during drought stress is focused on osmotic tolerant bacteria; some salt tolerant bacteria were able to delay wilting symptoms or to produce molecules such as proline, phenolics compounds and EPS with osmoregulation properties and with the ability to retain water in the soil or active in scavenging reactive oxygen species (Chen *et al*., 2007; Bano & Fatima, 2009; Sandhya, *et al*., 2009; 2010; Llamas *et al*., 2012; Chakraborty *et al*., 2013). These are a few of the possible bacterial features described in literature useful to relieve the effects of drought stress on plants; in the future more biochemical tests, followed by field trials, on GL83 will be needed to definitively characterize this Glera endophyte strain. In the meantime, this work reports the potential application of GL83 not only as plant growth promoting bacteria but also as effectors able to confer grapevine tolerance to drought.

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Chapter 4

Bacillus lipopeptides: characterization, plant perception and biocontrol properties

This chapter reports the biocontrol properties of GL174, a cyclic lipopeptides (LPs)-producing endophyte strain of Glera grapevine, in *in vivo* assays against the pathogenic fungus *Botrytis cinerea*. To shed light on the mechanisms through which LPs can act as elicitors of plant defence responses, the *Arabidopsis tha*liana cell suspension cultures were challenged with commercial *Bacillus* LPs families, plant-LPs perception and the later events of defence-gene expression were investigated.

Finally, the characterization by UPLC-MS analysis of some Glera *Bacillus* endophyte strains for the production of LPs revealed the presence of pumilacidin, which was tested in *in vitro* antibacterial assays.

1. Introduction

Lipopeptides (LPs) are amphiphilic molecules produced by several soil microorganisms composed by an oligopeptide linked to a fatty acid chain.

LPs are synthesized non-ribosomally via big multi-enzymes complexes leading to a large diversity in terms of aminoacid sequence, length and branching of the fatty acid chain.

In the context of biocontrol of plant diseases the bacterial LPs producers, *Pseudomonas* and *Bacillus* genera have received the greatest attention (Ongena *et al*., 2008; Raaijmakers *et al*., 2010). The *Bacillus* LPs families –surfactin, fengycin and iturin- were the first molecules studied for their potential antagonisms activities against several plant pathogens.

Surfactin members' family are powerful bio-surfactant agents with emulsifying properties and at elevate concentration they can lead to a complete membrane integrity disruption. Surfactins display antibacterial, antiviral and haemolitic effects but not antifungal properties. Their amphiphilic nature suggests that anchor themselves to lipid layers, thus interfering with biological membrane integrity in a dose-dependent manner (Henry *et al*., 2011).

Iturin and fengycin families have a quite strong antifungal activity against a wide range of yeast and fungi but limited antibacterial and antiviral actions.

It is thought that their activities are mainly based on osmotic membrane perturbation which leads to an enhancement of membrane permeability.

It is worth to mention that LPs usually act in synergistic manner as suggested by numerous studies (Maget-Dana e*t al*., 1992; Ongena *et al*., 2008; Cawoy *et al*., 2015).

The natural functions of LPs from the bacteria side suggest their role in antagonism toward other microorganisms and their involvement in motility and attachment to surfaces encouraging the biofilm formation and development.

LPs play an important role in plant tissues colonization, as they contribute to motility of the microbes by reducing surface tension and thereby decreasing the viscosity of the liquid surface, allowing a more efficient chemotaxis toward root exudates (Raaijmakers *et al*., 2010; Pauwelyn *et al*., 2013).

Furthermore, increasing evidences suggest that LPs, in particular surfactin and fengycin can act as elicitors of plant defence responses (Ongena *et al*., 2005a Ongena *et al*., 2005b; Pérez-García *et al*., 2011; Cawoy *et al*., 2015; Farace *et al*., 2015). They are ascribed to trigger induced systemic resistance (ISR), a mechanism by which beneficial microbes in the rhizosphere prime the entire plant body for enhanced defence responses against pathogens. ISR-primed plants are ready to react faster and stronger upon a subsequent pathogen invasions (Pieterse *et al*., 2014). ISR response is different from the quite well known systemic acquired resistance (SAR), a systemic induced disease response triggered upon infection by necrotizing pathogen. SAR state is characterized by an early increase in synthesis of salicylic acid which is the signal molecule that activate genes encoding pathogenesis-related (PR) proteins (Ryals *et al*., 1996; Van Loon *et. al*., 1998; Pieterse *et al*., 2014).

ISR state requires responsiveness to plant hormone Jasmonic-acid (JA) and Ethylene (ET). ISR primed state is based on an enhance sensitization of JA/ET-responsive genes because the colonization of *Arabidopsis* roots by ISR-inducing microbes does not increase the production of these hormones nor the induction of the defence responsive genes. For this reason ISR is not easily detected in unchallenged plants. However, recently the R2R3-type MYB transcription factor gene MYB72 was identified as one of the significantly induced gene in *Arabidopsis* roots colonized by *Pseudomonas simiae* WCS417r. Knockout *myb72* mutants of *Arabidopsis* are impaired in triggering ISR against several foliar plant pathogens, indicating that this gene is a root-specific transcription factor playing a pivotal role for the onset of ISR (Pieterse *et al*., 2000; Van der Ent *et al*., 2008; Pieterse *et al*., 2014).

In the last decade LPs were shown to act as possible elicitors of plant defence responses. This finds its origin in the early observations that some beneficial microbes, especially *Pseudomonas spp*. and *Bacillus spp*. are able to stimulate considerably the plant defence systems. Ongena and co-workers showed that surfactin and fengycin over-expressing strains possess a clearly higher host defence-stimulation action, compared to the wild type bacteria (Ongena *et al*., 2007). It was demonstrated that changes occur in the patterns of defence genes

expression, upon the treatment with LPs, and/or the increased activity of defence correlated enzymes, like lipoxygenase (LOX) and phenylalanine ammonia lyase (PAL) (Ongena *et al*, 2007; Jourdan *et al*, 2009; Farace *et al*, 2015).

Although several biochemical studies on LPs have already been conducted on their mode of action and their ecological role has been assessed, several questions about their interactions with plants are still unanswered.

In most of the plant-microbe interactions, the very early events in the recognition of microbial elicitors are protein phosphorylation and activation of plasma membrane proteins, which then mobilize or generate second messengers, like free calcium, reactive oxygen species and nitric oxide all of them able to change the transcriptome defence profile (Garcia-brugger *et al*., 2006).

In this work the biocontrol activity of LPs-producing strain GL174, a cultivable endophyte strain of Glera grapevine, was demonstrated in *in vivo* assays. Then, the attention was focused on the mechanisms through which LPs trigger defence responses in plant. So, to shed light on plant-LPs interaction both the early signaling events and the later responses in changing the defence-gene expression were evaluated, upon the *Bacillus* LPs administration to Arabidopsis.

2. Materials and Methods

2.1 Bacterial strains and growth conditions

The bacterial endophyte strains (GL41, GL189, GL412 and GL452) used in this experiment were isolated from surface sterilized tissues leaves of *Vitis vinifera* cv. Glera (Baldan *et al*., 2014). All these strains were used in LPs characterization by UPLC-MS and they belong to genus *Bacillus.* They were streaked on Luria medium and then colonies were used to inoculate 50 ml of rich medium optimized for LPs production Opt medium (Jacques *et. al*., 1999) on shaking 120 rpm at 30°C. Instead GL174, already characterized for LPs production (Favaro *et al*., 2016), was cultivated routinely in Nutrient Broth or Nutrient Agar (Difco) at 28°C and then it was directly applied on Glera cuttings in *in vivo* antagonism test against the pathogenic fungus *Botrytis cinerea*.

2.2 *In vivo* antagonism assay

The biocontrol activity of GL174 against *Botrytis cinerea* was tested using two sets of Glera plants: the first set was represented by 60 day-old plants in soil pots and the second one by 60 day-old plants inoculated with GL174 when propagated as apical cuttings (see Chapter 1 for detailed protocol); to mime the endophyte colonization and check their direct effect, the abaxial sides of leaves were infiltrated with a 10^3 cells/mL of bacteria suspension through a syringe without needle. On these two groups of plants, the antagonism tests were performed on detached leaves (Zhang *et al*., 2016) and on leaves *in planta*.

In the first test, detached fully expanded leaves from plants, both those infiltrated with GL174 and those from previously GL174-inoculated plants, were placed on wet paper and were challenged by placing a mycelial plug (diameter 10 mm) of *Botrytis cinerea*, collected from a 7-day-old PDA plate, on the middle of the leaves. The trays with the challenged leaves were covered to keep a high relative humidity for the fungus development.

In the second test, leaves of the plants, both those infiltrated with GL174 and those from previously GL174-inoculated plants, were challenged *in planta* with mycelial plugs as described above. Plants were kept in plastic bags in a growth chamber.

Non-treated leaves were included in all the experimental conditions: not infiltrated leaves and leaves from not inoculated plants were challenged with the fungus. Negative controls were also set providing sterile medium plugs to the leaves in order to check any detrimental effect of the inoculation method independently from the fungus.

The effects of the fungus infection on every set of treated leaves were evaluated after 1 week of infection collecting pictures of the leaves and measuring the surface of the brown lesions by means of the software ImageJ. The mean of the values recorded on bacterized leaves was compared with the mean of the surface values obtained by non treated leaves. Five plants were used in each treatment: for each treatment three/four leaves were used and data are expressed as damaged area (cm^2) and asterisks indicate statistically significant differences among treatments (T student test; $p=0.05$).

2.3 Cell viability assay

To evaluate the possible cytotoxicity of LPs cell viability assay was performed using Evans blue dye (Sigma-Aldrich). The test was performed on *wild type Arabidopsis thaliana* cell culture treated with different concentrations (10 – 20 – 50 µg/ml) of commercial LPs families (mycosubtilin represents iturin family). Aliquots of 500µl of cell cultures in exponential growth phase were uniformly sampled then 0.5% of Evans blue dye was added and samples were laid on a shaker for 15 minutes. Then, the unloaded dye was discarded with distilled water. Cells were then disrupted by adding a solution of methanol 50% and SDS 0.1%, at 55°C for 30 minutes. The stained solutions were then collected, properly diluted and the absorbance was spectrophotometrically evaluated at 600 nm. The measurements were taken at 2, 6 and 24 hours after the cell cultures LPs exposure.

2.4 *Wild type* and *aequorin-expressing Arabidopsis thaliana* cell cultures and growth conditions

To perform the experiments both the *wild type* and the stably cytosolic aequorin-expressing *Arabidopsis thaliana* Col-0 ecotype cell lines were used. Cell cultures were chosen because of their homogeneous cell populations rapidly proliferating *in vitro* and able to uniformly respond to a wide array of physiological signals. (Moscatiello *et al*., 2013). Cell cultures were weekly renewed transferring 1 packaged cell volume in 20ml of fresh Murashige-Skoog (Murashige T. & Skoog F. 1962) medium (Duchefa) containing 3% sucrose, pH.5.5, 0.5 µg/ml 2,4-dichlorophenoxyacetic acid (2,4-D), 0.25 µg/ml 6-Benzylaminopurine (BAP). Cell cultures were maintained at 24°C with a 16/8 h light/dark cycle under an illumination rate of 110 µmol photons m^{-2} s^{-1} on a rotary shaker at 80 rpm.

2.5 Calcium measurements

Cell suspension culture stably-expressing the Ca^{2+}-sensitive photoprotein aequorin in exponential-growth phase (4-day-old) was reconstituted overnight with 5 µM coelenterazine (Prolume Ldt, Pinetop, AZ, USA).

The following day, after extensive washing, 50 µl of cells were laid in the luminometer chamber and challenged with different concentrations of an equal volume of a 2-fold concentrated LP families.
Ca^{2+} dynamics were recorded for a total of 1800 seconds before the injection of 100 µl of the discharge solution (30% ethanol, 1 M $CaCl_2$).

2.6 Primers design

Primer pairs were using the Primer-Blast interface, which exploit the Primer3 software and the BLAST alignment algorithm. The primers used to obtain gene amplicons (~150-200b.p.) were: PAL1 (At2g37040) 5'-TGTGAAGGTGGAGCTATCGG-3', 5'-CCGGCGTTAAGGAATCTAATAAGT-3', LOX1 (At1g55020) 5'-TCCTTGAACCTCACTTCCGT-3', 5'-ACGGCCATCCCTCTCTTTTT-3', PR1 (At2g14610) 5'-AGGTGCTCTTGTTCTTCCCT-3', 5'-GCCCACCAGAGTGTATGAGT-3', NPR1 (At1g64280) 5'-GCAATGGAGATCGCCGAAAT-3', 5'-TCCCGAGTTCCACGGTTTTA-3', MAPK3 (At3g45640) 5'-ATGCGAAAAGATACATCCGGCAACT-3', 5'-TCATCATTCGGGTCGTGCAATTTAG-3'.

2.7 RNA extraction and first strand cDNA systhesis

To analyze the expression of specific genes total RNA of treated (50µg/ml) and untreated (control and EtOH 0.5%) *Arabidopsis* cell culture were extracted using the manufacture guideline of RNaesy (Qiagen). An aliquot of the total RNA extracted (5 µg) was added with 50 ng of random decamers, dNTPs 0.5 mM and RNase-free water up to 13 µl. The mixture was denatured at 65°C for 5 min. A cDNA synthesis mix was prepared with Superscript III reverse-transcriptase (Invitrogen, 10 units/µl), DTT 5 mM, First Strand Buffer 1X and RNase-free water up to 7 µl. The mix was then added to the solution containing the denatured RNA in a total volume of 20 µl. The following program was applied: 50°C for 1h, 70°C for 15 min.

2.8 Semi-quantitative (RT)-PCR

The relative gene expression between control samples and treated ones was measured by the QuantumRNA kit (Life Technologies). It contains the universal 18S primers pair and competimers mixture, as internal standard, to allow the

semi-quantitative analysis of the expressed genes in a multiplex PCR reaction, where 18S and target gene were simultaneously amplified.

Reverse transcription (RT)-PCR reactions were performed with 5 µl of diluted first-strand cDNA as a template in a 50 µl PCR reaction solution. The PCR reaction was prepared mixing buffer 1X, MgCl2 0.75 mM, DNTPs mix 0.2 mM, forward and reverse primers 0.4 µM, 4µl of 1:9 universal 18S primers pair/competimers mix, Taq polymerase 0.02 units/µl (Invitrogen) and Nuclease-free water according to the final volume. The thermocycler was programmed with the following parameters: initial denaturation 95°C for 3 min; 18-35 cycles -30 s at 95°C, 30 s at 59-63°C, 30 s at 72°C; 5 min at 72°C. The amplification products were visualized on 1.5% agarose gel.

2.9 Relative expression quantification

The images of electrophoresis agarose gels were taken using QuantityOne software (Bio-Rad). Densitometric analysis of bands were performed using ImageJ software to calculate relative pixel intensities. Areas of the peaks were measured and expressed in percentage values. Data are the mean of three independent experiments, statistical considerations were based on the R software data treatment, using the R commander library. Student t-test was performed in the comparison between the means of different sets of data. Statistical significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

2.10 UPLC-MS analysis

Bacillus LPs production was evaluated after 48 hours. After 48 hours 1 ml of each bacterial (GL41, GL189, GL412 and GL452) suspension was collected and centrifuged 10 minutes at 15000 rpm to avoid bacterial pellet. Supernatants were collected and filtered through 0.2 µm filter. Samples were analyzed by reverse phase UPLC-MS (UPLC, Waters, Acquity class H) coupled with a single quadrupole MS (SQDetector, Water, Acquity) on a Acquity UPLC BEH C18 2.1x50mm, 1.7µm column.

A method based on acetonitrile (ACN) gradients plus Formic acid 0.1% was used to detect simultaneously all the three LP families. Elution started with 30% ACN (flow rate of 0.60 ml min^{-1}). After 2.43 min, ACN percentage was brought up to

95% and held until 5.20 min. The column was then stabilized in ACN 30% for 1.7 min. Compounds were identified on the basis of their retention times compared with authentic standards (98% purity, Lipofabrik society, Villeneuve d'Asc, France) and the masses detected in the SQDetector. Ionization and source conditions were set as follows: source temperature, 130°C; desolvation temperature, 400°C; nitrogen flow, 1000 l h^{-1} and cone voltage, 120 V.

2.11 Pumilacidin semi-purification

A reasonable amount of pumilacidin was extracted from *Bacillus* strain 41 (the major producer) grown in two 1L Erlenmeyer flasks each containing 250ml of Opt medium (Jacques *et al*., 1999). The strain was grown 48 hours on shaking 160 rpm at 30°C. After having checked the presence of pumilacidin through UPLC-MS the two bacterial cultures were joined and centrifuged at 4700g for 15 min and the supernatant was collected.

Pumilacidin was semi-purified using C18 cartridges reactivated with 100ml of ACN and 100ml of distilled water. The column was loaded with the supernatant and the elution was discarded. The column was then washed with 50% of ACN:WATER and finally pumilacidin was eluted from the column with 100% ACN.

2.12 *In vitro* antagonism assays

Pumilacidin antibacterial properties was evaluated in microwell plate *in vitro* antagonisms tests against two plant bacterial pathogens *Erwinia carotovora* and *Xantomonas campestris*. Plant pathogen suspension cultures were previously grown in LB medium overnight on shaking.

Four pumilacidin concentrations (10 – 20 – 50 – 100 µg/ml) were spotted into the microwell plate. It was kept in an oven at 60 degree for 1 hour to allow the ACN to completely dry. Then, 200 µl of each well pathogen-diluted cultures (OD 0.01) were added. Control wells contained 200 µl of sterile LB medium or 200 µl of diluted pathogen culture without pumilacidin.

Each treatment was repeated at least three times. The microplate was laid in the spectromass in agitation at 25°C for 24 hours, the instrument reads the OD in each well every 30 min at 600 nm.

3. Results

3.1 *In vivo* biocontrol effects of GL174 against *Botrytis cinerea*

The antagonism test on detached and *in planta* grapevine leaves revealed the biocontrol attitude of the examined strain: GL174 reduces the *Botrytis cinerea* mycelium growth on grapevine leaves.

The detached leaves subsequently infiltrated with the GL174 strain showed a significant reduction of the necrotic pathogen-induced area (**Fig.1A**). Whereas the fungus effect on detached leaves from two month-old GL174 inoculated plants was comparable with that observed on leaves from un-inoculated plants (**Fig.1B**). *In planta* both GL174 infiltrated leaves and leaves from two month-old GL174 inoculated plants displayed a sharp reduction of area with symptoms compared to not treated plants (**Fig.1C,D**).

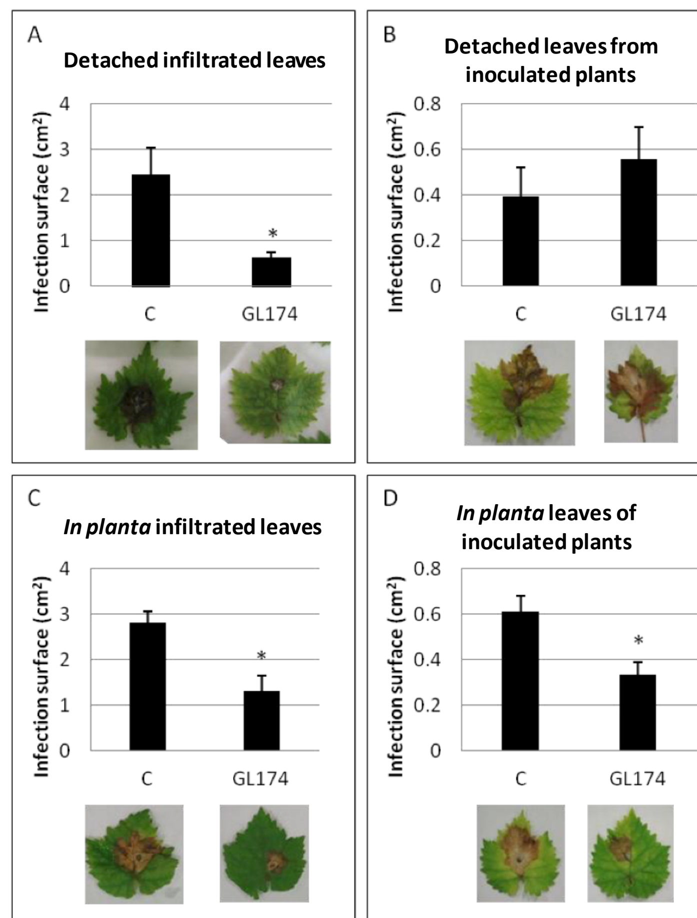

Fig.1. *In vivo* biocontrol assays. **A, B**, *in vivo* biocontrol assay on detached Glera leaves. **A**, leaves detached and then infiltrated with GL174; **B**, detached leaves from inoculated Glera plants. **C, D,** *in vivo* assay on *in planta* Glera leaves. **C,** leaves infiltrated with GL174; **D,** leaves of inoculated Glera plants.

3.2 *Bacillus* LP families induced no cytotoxic effects on *Arabidopsis* cell suspension cultures

The possible cytotoxic effect, in terms of cell death, of each commercial *Bacillus* LPs families was monitored using Evans blue test. Three different concentrations (10 µg/ml, 20 µg/ml, 50 µg/ml) of each commercial *Bacillus* LPs families were administered to *Arabidopsis* cell suspension cultures in exponential growth phase and the percentages of cell death were recorded at 2, 6 and 24 hours after the exposure (**Fig.2**). In general, the results showed that none the *Bacillus* LPs families are able to exert a cytotoxic effect. The highest average value of cell death, about 19.5% ± 7% was found for surfactin treatment (50 µg/ml) and it was observed at the 6 hours after exposure (**Fig.2A**). *Arabidopsis* cell cultures treated with ethanol 0.005% exhibited about 11.5% ± 1.1% of cell death (**Fig.2A**). Controls with the DMSO 0.01% treatment displayed a cell death of 14.8% ± 1.8% (**Fig.2B,C**). Untreated cultures were also evaluated and compared to the controls treated with ethanol or DMSO to assess a possible cytotoxic effect due to the solvent itself. The degree of cell death of the untreated cultures was around 11% ± 1% at 2, 6 and 24 hours indicating no significant differences between treated and untreated controls (data not shown).

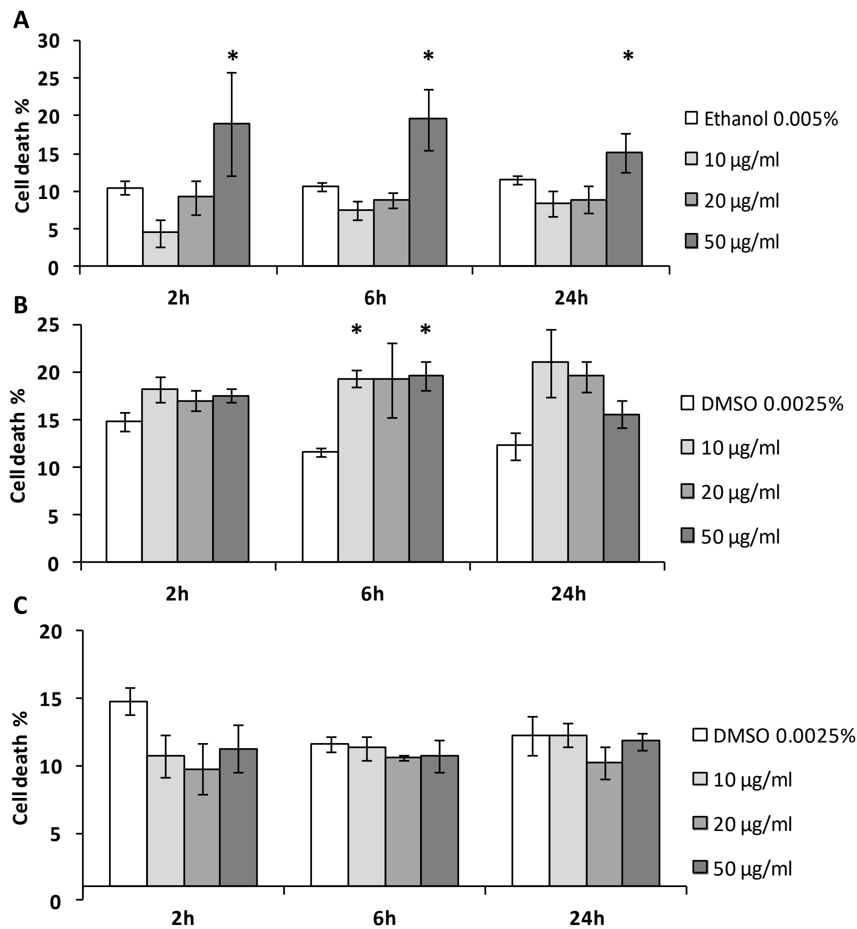

Fig.2. Viability assays of *Arabidopsis* cell cultures treated with commercial LPs families. Treatments at 10-20-50 µg/ml monitored at 2, 6 and 24 hours after exposure. **A**, surfactin treatment; **B**, fengycin treatment; **C**, mycosubtilin treatment. The values are representative of at least three independent experiments. Statistical significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

3.3 Involvement of cytosolic Ca^{2+} elevations in cell suspension cultures and plant LPs perception

Arabidopsis cell suspension cultures stably expressing the bioluminescent Ca^{2+} reporter aequorin targeted to the cytosol were used in Ca^{2+} measurement assays. Cell cultures were challenged with the three different *Bacillus* LPs families at different concentrations (10 - 20 - 50 µg/ml) for 30 min (**Fig.3**).

A clear dose-dependent elevations in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) were evoked after *Arabidopsis* cell cultures exposure to 10-50 µg/ml of surfactin. The highest elevation of $[Ca^{2+}]_{cyt}$ was found with the treatment at a final concentration of 50 µg/ml reaching a Ca^{2+} peak of about 1.2 µM (**Fig.3A**).

The peak was reached after about 5 min upon surfactin administration and the level did not completely fall back to the basal level within the selected time span.

Instead, Arabidopsis cell cultures exposed to fengycin and mycosubtilin did not display any elevation of $[Ca^{2+}]_{cyt}$ with any of the tested concentrations. Control cells challenged with ethanol 0.005%, and DMSO 0.01% in the case of fengycin and mycosubtilin did not evidence any detectable $[Ca^{2+}]_{cyt}$ elevation (**Fig.3B,C**).

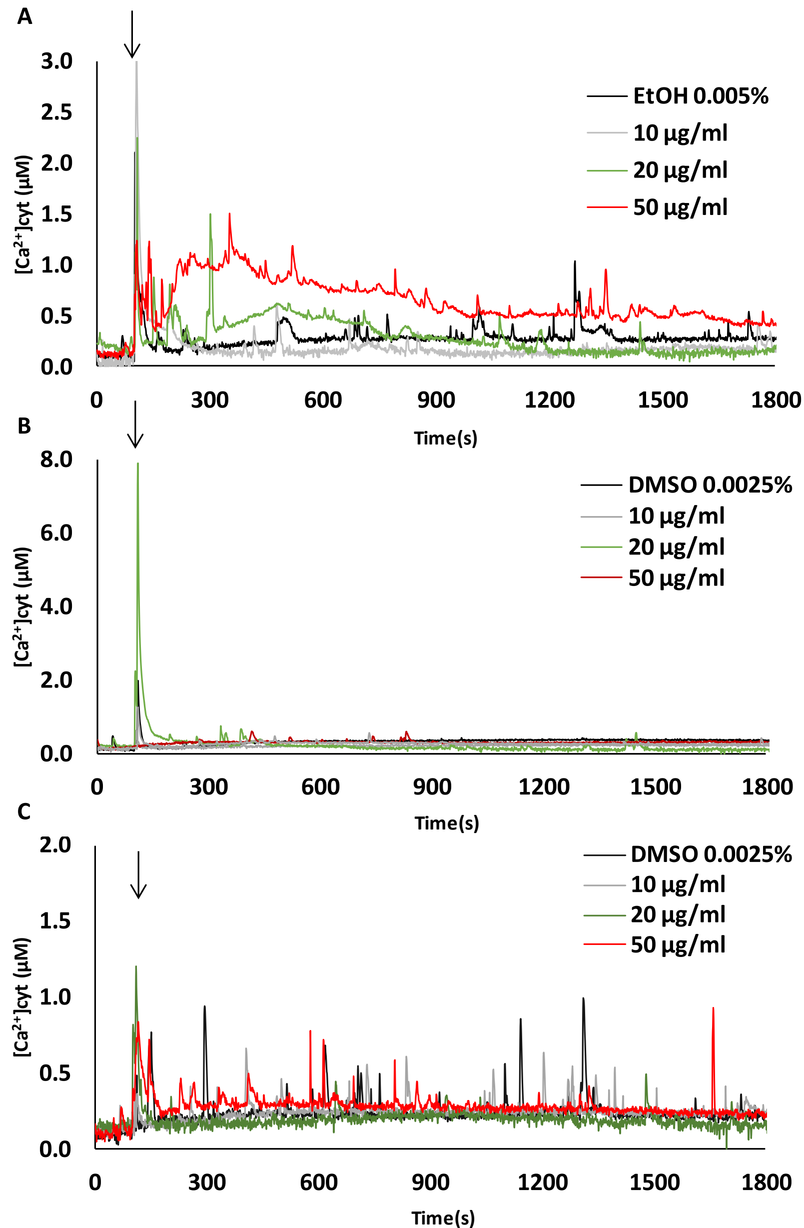

Fig.3. Monitoring of cytosolic Ca^{2+} concentrations in *Arabidopsis* cells expressing aequorin. **A**, surfactin treatment. **B**, fengycin treatment. **C**, mycosubtilin treatment. LPs families were injected after 100 s (arrows). Each trace is representative of three to six independent experiments. In each graph: black trace (control), grey trace (10 µg/ml), green trace (20 µg/ml), red trace (50 µg/ml).

3.4 LPs effects on defence gene expression

To study the possible induction of defence gene-expression resulting from Ca^{2+} transient evoked after the cell cultures exposure with surfactin (50 µg/ml) a RT-PCR was performed using the QuantumRNA Universal 18S Internal Standards Kit (Ambion Ltd., UK). RT-PCR was used to assess the relative amounts of defence-associated transcripts belonging to different metabolic pathways involved in plant immunity and defence responses, comparing untreated cells with those challenged with surfactin for 2 and 6 hours; moreover, a comparison to control samples treated with ethanol was carried out (**Fig.4** and **Fig.5**).

The histograms show that there is a consistent difference for each gene in relative expression between controls (both untreated and ethanol-treated) and the surfactin-treated samples. More than a 3-fold increase was recorded for LOX1 and PAL1 expression in treated samples; LOX1 expression remained at a high level both at 2 and 6 hours after treatment (**Fig.4A, B**).

A ~2-fold increase in the NPR1 gene expression in observed when comparing the treatment at 2h with the other samples, while it decreased when analyzed at 6h after cell cultures exposure (**Fig.4C**).

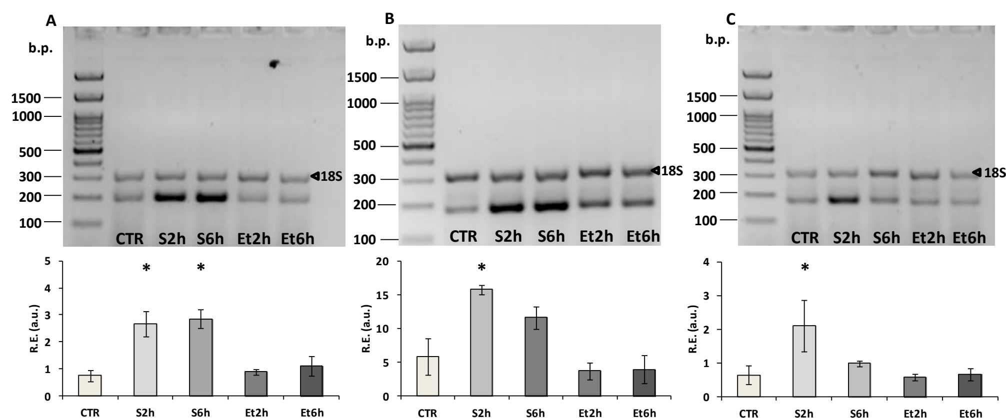

Fig.4. RT-PCR gene expression analysis correlated with the relative analysis of gene expression. **A**, LOX1 gene. **B**, PAL1 gene. **C**, NPR1 gene. A 18S gene was used as internal standard. For each gel: CTR, untreated cell culture; S2h and S6h, cells cultures treated with 50µg/ml of surfactin and sampled respectively after 2 and 6 hours after exposure; Et2h and Et6h, cells cultures treated with EtOH 0.005% and sampled respectively after 2 and 6 hours after exposure. Gels and graphs are representative of three independent experiments. Statistically significant differences (*p<0.05, Student's t- test).

The other two genes included in the analysis were PR1 as a reliable marker of SAR and MAPK3 which is involved in regulation of gene expression in response to bacteria and several abiotic stresses (Pedley & Martin, 2005; Beckers *et al.*, 2009). No significant differences were monitored on MAPK3 gene expression, even if a slight increase was detected in MAPK3 at 2h after cell cultures exposure (**Fig.5A**).

While, the gene expression pattern of PR1 remained unaltered for the entire the temporal window analyzed, suggesting that surfactin might not be involved in the activation of SAR (**Fig.5B**).

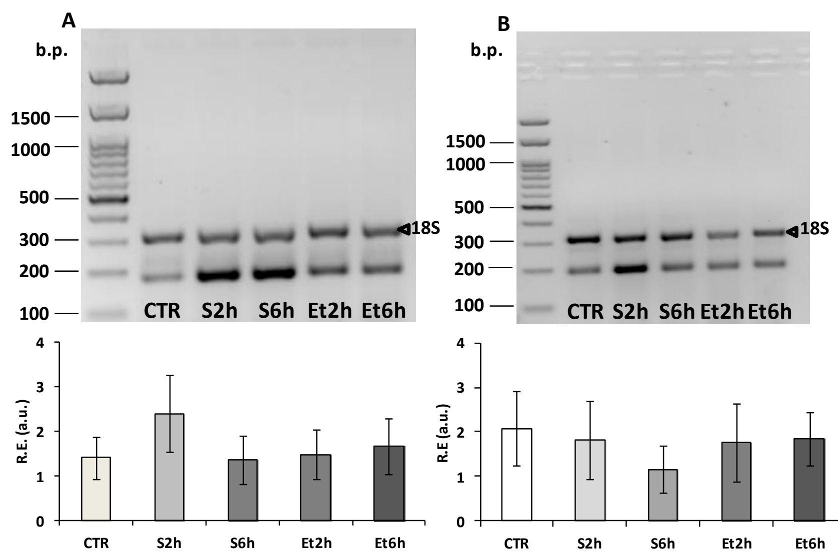

Fig.5. RT-PCR gene expression analysis correlated with the relative analysis of gene expression. **A**, MAPK3 gene. **B**, PR1 gene. A 18S gene was used as internal standard. . For each gel: CTR, untreated cell culture; S2h and S6h, cells cultures treated with 50µg/ml of surfactin and sampled respectively after 2 and 6 hours after exposure; Et2h and Et6h, cells cultures treated with EtOH 0.005% and sampled respectively after 2 and 6 hours after exposure. Gels and graphs are representative of three independent experiments.
Statistically significant differences (*p<0.05, Student's t- test).

3.5 Glera *Bacillus* endophytes produce surfactin, fengycin and pumilacidin

The UPLC coupled with MS analysis revealed that two Glera *Bacillus* endophytes (GL189 and GL452) are able to produce molecules belonging to surfactin and fengycin LPs families and the other two *Bacillus* strains analyzed (GL41 and GL412) produce pumilacidin. The different LPs families were identified on the basis of their retention times compared with purified standards. The different surfactins and fengycins signatures detected in the culture medium of selected

Glera *Bacillus* endophytes are shown in **Fig.6A**; while in the **Fig.6B** is shown the signatures of pumilacidin.

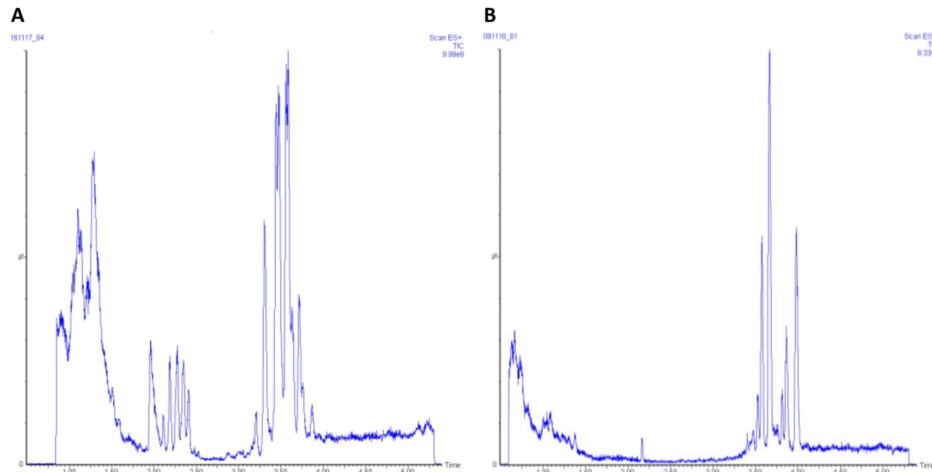

Fig.6. Representative graphs of UPLC analysis of the culture medium of *Bacillus* Glera endophytes. **A**, UPLC of GL189; **B**, UPLC of GL41. GL412 and GL452 showed similar LPs signatures.

3.6 Pumilacidin antibacterial properties

Semi-purified pumilacidin was tested *in vitro* antagonisms test against two crop-infectious bacterial pathogens *Erwinia carotovora* and *Xantomonas campestris*. The **Fig.7** shows the two bacterial growth curves monitored for 24 hours in which it is worth to notice that the higher the pumilacidin concentration the bigger is the inhibition of bacterial growth. In particular, pumilacidin seems to exert its antimicrobial activity against *X. campestris*; the pathogen growth is slightly retarded applying pumilacidin 20 µg/ml, while it is almost completely blocked with 50 µg/ml, and finally 100 µg/ml definitely prevents the bacterial growth (**Fig.7B**).

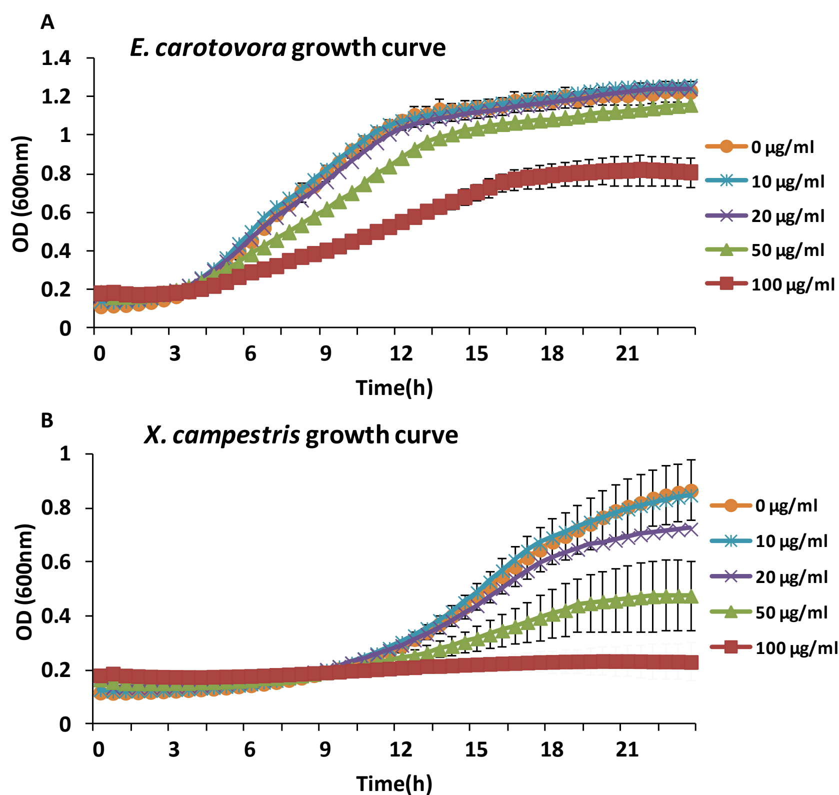

Fig.7 Pumilacidin antibacterial effect against two important crops infectious bacteria. **A**, *Erwinia carotovora*. **B**, X*antomonas campestris*. Growth curves in absence and in presence of four different concentrations of semi-purified pumilacidin. Statistically significant differences ($p<0.05$, Student's t- test).

4. Discussion

Several microorganisms are reported to be biocontrol agents acting as pathogen antagonists in the soil or inside plants. The mechanisms underlying this effect are not completely clear but LPs are standing out as antimicrobial and antifungal molecules which act directly against pathogens or stimulating plant defence responses. Here, first of all the biocontrol activity of LPs-producing GL174, a culturable endophyte strain of *V. vinifera* cv. Glera was analyzed.

GL174 has already taxonomically and biochemically characterized, focusing on its LPs products in a previous work, and its biocontrol activity was already *in vitro* tested, with promising results, against the infectious fungus *Botrytis cinerea* (Favaro *et al*., 2016).

To confirm that antifungal trait and to evaluate a possible future application in grapevine cultivation, the GL174 biocontrol properties were here *in vivo* tested against *B. cinerea*, one of the most important fungal pathogens that severely

damage vineyards in the Glera cultivation area. Detached leaves experiments demonstrated that GL174 is effective against the pathogen when the leaves were infiltrated directly. Detached leaves from inoculated plant did not show any sign of protection. Ge and co-workers found protective effect against *B. cinerea* when detached tomato leaves were previously sprayed with *B. methylotrophicus* NKG-1 (Ge *et al*., 2016). On the contrary, in the *in planta* experiments, both the bacteria inoculation methods resulted effective for the plant protection. These differences in the protective effects seem to indicate that the biocontrol could be exert locally by the direct bacteria action in the leaves, but also a systemically protection, given by plant induced defence responses against the gray mold, need to occur. These results are in agreement with Ongena and colleagues who found a protective effect against *B. cinerea* in *in vivo* tomato and bean plants inoculated at the root level with *Bacillus* LPs-producing strains (Ongena *et al*., 2007). The *in vivo* 'condition' seems to play a crucial role in the activation of plant defence responses as reported by Magnin-Robert who demonstrated that the severity of symptoms caused by *B. cinerea* on grapevine leaves is reduced drenching the vineyard soil with biocontrol grapevine associate bacteria (Magnin-Robert *et al*., 2007).

Moreover, molecules of the surfactin family are effectively recognized by plants eliciting an induced plant systemic response (ISR) that leads to increased pathogen tolerance (Ongena *et al.,* 2008; Falardeau *et al.,* 2013; Farace *et al.,* 2015). Surfactins and lichenysins also have a strong bio-surfactant action, and help bacteria to colonize and form biofilm as well as to improve cell movements.

On the other hand, this effect indirectly impairs colonization by other microorganisms such as pathogens (Leclère *et al.,* 2006). In this work the biocontrol properties of GL174 not only *in vitro* but also *in planta* were detected and these findings can pave the way to new sustainable scenarios, which can be considered a valid environmentally-friendly alternatives to the use of pesticides in the biological control of grapevine disease.

Many questions remain still unanswered such as: how LPs are perceived by plant? Which mechanisms, triggered by LPs inside plant cell, did induce the defence responses?

Effects of diverse *Bacillus* LPs families on *Arabidopsis thaliana* cell cultures were investigated. Plant suspension cell cultures are widely used in plant biology as a convenient tool to study a wide range of phenomena, bypassing the structural complexity of the plant organism *in toto*. The homogeneity of an *in vitro* cell population, large availability of material, high cell growth rate and optimal reproducibility of conditions, make suspension-cultured cells suitable to analyze complex physiological processes at cellular and molecular levels. As a first step a viability assays performed using Evans blue dye revealed no citotoxic effects treating *Arabidopsis* cell cultures with fengycin or mycosubtilin, confirming what found by Farace *et al*., 2015. Surfactin instead, at the highest concentration (50 µg/ml), caused a slight increase in *Arabidopsis* cell death. Aequorin-transformed plant cell suspension cultures have been shown to be a valuable tool to analyze calcium-based signaling pathways during plant-microbe interactions (Navazio *et al*., 2007). Thus, the analysis of early cell responses/perception to LPs, investigated through the elevation of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) was performed. Calcium has a pivotal role in generating a wide range of different spatial and temporal signals depending on the stimulus perceived (Berridge *et al*., 2000). Many responses to external biotic and abiotic stimuli were shown to be mediated by transient changes in $[Ca^{2+}]_{cyt}$. Using an *Arabidopsis* cell line stably expressing the bioluminescent Ca^{2+} reporter aequorin demonstrated the involvement of Ca^{2+}_{cyt} in surfactin perception. It could be due to their structures, in fact they can associate and tightly anchor into lipid membranes and can thus interfere with lipid integrity in a dose-dependent manner (Ongena *et al*., 2008; Henry *et al*., 2011). Surfactin has been reported to induce a strong alkalinisation response and an oxidative burst, which are other early events in signal transduction, both in grapevine and in tobacco cell suspension cultures (Henry *et al*., 2011; Farace *et al*., 2015). Instead, so far the involvement of Ca^{2+}_{cyt} in the LPs perception has not been clarified. Our results suggest that the perception of surfactin could be mediated by an elevation of $[Ca^{2+}_{cyt}]$, which could then trigger a signaling cascade activating several effectors proteins able to modify the metabolic pathways and gene expression to carry out specific responses adapted to the stimulus perceived (Berridge *et al*., 2000; Dodd *et al*., 2010). Concerning

the other two LPs families, fengycin and mycosubtilin, no changes in [Ca^{2+}_{cyt}] were recorded with any LPs concentrations tested, as reported in Farace *et al*., 2015. Here it is considered only the cytosolic compartment but for many microbial associate molecular patterns (MAMPs) the involvement of nuclear calcium signalling was demonstrated (Garcia-Brugger *et al*., 2006; Dodd *et al.*, 2010). Moreover, other intracellular compartments such as chloroplasts were demonstrated to be a primary site for the biosynthesis and transmission of pro-defence signals during plant immune responses; they were also seen to accumulate Ca^{2+} in the stroma in response to *flg22* (Nomura *et al*., 2012; Serrano *et al*., 2016). Chloroplasts are also sites for the production of salicylic acid and jasmonic acid, central mediators of plant immunity (Robert-Seilaniantz *et al*., 2011; Nomura *et al*., 2012; Fu & Dong, 2013; Pieterse *et al*., 2014). Since the molecular links between chloroplasts and the cytoplasmic/nuclear immune system remain largely unknown, it could be worth to look at Ca^{2+} transients in these organelles in response to LPs.

LPs are also reported as key players in the induction for plant immunity triggered by beneficial microorganisms (Raaijmakers *et al*., 2010; Falardeau *et al*., 2013); thus the later events based on defence gene expression were also evaluated.

Bacillus LPs were demonstrated to be involved in eliciting plant defence; in particular fengycin and iturin are directly active on several plant fungal aggressors, while surfactin displays antimicrobial activity and the major role in triggering defence-systemic responses (Ongena *et al*., 2007; Jourdan *et al*., 2009; Falardeau *et al*., 2013; Cawoy *et al*., 2015; Ma *et al*., 2016). The most studies conducted so far have been based on the effects of LPs on pathogens both *in vitro* and *in vivo* and on the activation of some defence genes, but many questions remain unanswered. To elucidate if plants respond to LPs through induced systemic resistance or through systemic acquired resistance, specific defence gene expression, representing valid markers for the different metabolic pathways involved in plant immunity and defence responses, was considered. Pathogen-Related protein 1 (PR1) and phenylalanine ammonia lyase (PAL1) are two marker genes for systemic acquired resistance mediated by salicylic acid; while lipoxygenase (LOX1) the first enzymes acting in the defence pathway that

leads to the formation of the compounds oxylipins, acting against a variety of pathogens and herbivores and involved in the production of the hormone jasmonic acid which has a central role to mediate induced systemic resistance. Non-expressor of Pathogenesis-Related genes 1 (NPR1) is a key player in systemic resistance signalling (Bannenberg *et al*., 2009; Pieterse *et al*., 2014). Mitogen-Activated Protein Kinase 3 (MAPK3) is up-regulated in primed cells and strongly activated upon pathogen attacks (Beckers *et al*., 2009).
The preliminary results here presented showed that upon surfactin administration an increase in LOX1, PAL1 and NPR1 expression occurred, while no differences in gene expression were observed for PR1 and MAPK3 (**Fig.4,5**).
This result demonstrated the activation of a systemic resistance in the plant. Moreover, LOX1 and NPR1 are both involved in the jasmonate-mediated ISR, while PR1 is a central marker of the salicylate-dependent SAR, hence the results suggest that the ISR could be the preferential way followed by Arabidopsis plants after surfactin exposure. However, it is possible that these two responses could act in synergistic manner because also PAL1 was found up-regulated at 2 hours after the surfactin administration (**Fig.4B**), which leads to an additively enhanced defensive capacity (Van Wees *et al*., 2000) or less probably, there could be the suppression of SAR pathway when plant recognized there were no pathogen attacks. Further investigations, possibly conducted *in planta*, are anyway required to shed light on the plant defence gene signature induced by LPs and on physiological plant responses.

A characterization by UPLC-MS analysis to detect the possible LPs produced by some *Bacillus* endophytes strains with potential biocontrol activity on grapevine was performed. Two out of four strains analyzed produce surfactin and fengycin compounds but two other strains (GL41 and GL412) produce pumilacidin compounds which have higher molecular weight than surfactin. Pumilacidin compounds were firstly described by Naruse *et. al*. (1990), little information is nowadays available in literature about pumilacidin compouds, but it is reported they have antimicrobial activity against multi-drug resistance bacteria, antiviral against *Herpex virus* strain HSV-1 and can suppress the mobility of *Vibrio alginolyticus* responsible from human marine infections (Fahim & Hussein, 2017;

Xiu *et al*., 2017). It is also reported the potential applications of several microbial LPs and Bacillus secondary metabolites (mainly surfactin and iturin) in the field of biomedical sciences (Singh & Cameotra, 2004; Rodrigues *et al*., 2006; Sansinenea & Ortiz, 2011).

In this work the antimicrobial activity of pumilacidin was in *vitro* tested against two important crop pathogens *Erwinia carotovora and Xanthomonas campestris* showing a quite strong antibacterial effects, in particular preventing *X. campestris* growth also a low concentration. This is a preliminary result and other tests against, for example, infectious fungi such as *B. cinerea* need to be performed, but this is an important starting point for developing new sustainable practices with potential application in plant and crops protection.

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Conclusions

All plants have been studied so far are reported to be colonized by several microbial communities comprising bacterial, archaeal, fungal, and protistic taxa. Some of them display an endophytic lifestyle playing a pivotal role in plant development, growth, fitness and establishment (Hardoim *et al*., 2015). Endophytes can enhance plant growth making nitrogen and phosphorus more available to plant or synthesizing plant hormones able to interfere with their host development; they can also act as biocontrol agents producing several metabolites with direct antifungal and antibacterial properties or improving the competition with pathogen for the same nice. On the other hand plant provides endophytes a constant source of food and a preferential nice to live and proliferate (Hardoim *et al*., 2008; 2015; Compant *et al*., 2010).

The present work aimed to evaluate the contribution of plant-endophyte bacterial microorganisms to help *Vitis vinifera* growth and to cope with, in a dynamic environment, drought stress and fungal pathogen challenges.

Among grapevine cultivable endophytic strains, *Pantoea agglomerans* (GL83) and *Bacillus licheniformis* (GL174) were selected, GFP-tagged and successively applied directly in the field as bio-fertilizers.

Results presented in **Chapter1** confirm the endophytic nature, the colonization pattern and the persistence of both strains inside inner tissues of Glera apical cuttings. Through laser scanning confocal microscopy and a cultivable dependent method both strains were visualized and enumerated.

They were then successfully applied directly in rooting cuttings production line and with this goal **Chapter2** describes the two new Glera inoculation methods applied. Moreover, the field trials performed reveal that GL83 and GL174 have to be considered promising candidates as bio-fertilizers, they enhance both the rooting cuttings growth and health, in particular when rootstocks and buds were inoculated during the hydration step.

Chapter3 reports that GL83 is also able to confer Glera rooting cuttings an increased tolerance to drought stress. The capacity to produce the plant hormone auxin could be the GL83 distinctive trait involved not only in plant

growth promotion but also in relieving the effects caused by drought stress (Dimkpa *et al*., 2009; Vacheron *et al*., 2013; Baldan *et al*., 2015). Moreover, rooting cuttings subjected to a second inoculation before the stress administration display almost not to be affected to drought.

Instead, GL174 can protect grapevine plant against some fungal grapevine pathogens such as *Botrytis cinerea*, one of the most infectious fungal pathogen affecting vineyards. The *in vivo* antagonism tests described in **Chapter4** reveal that grapevine plants inoculated with GL174 present significantly less necrotic lesions when infected with *B. cinerea*. It demonstrates that the ability of GL174 to produce LPs such as lychenisin and surfactin is maintained also *in vivo* and that it has a pivotal role in fighting pathogens (Favaro *et al*., 2016).

LPs in particular surfactin is also able to activate plant defence responses (Ongena *et al*., 2007). In **Chapter4**, using the model system *Arabidopsis thaliana* cell suspension cultures, the early phases of *Bacillus* LPs perception and the later events, such as defence gene expression were investigated using reliable markers for induced systemic resistance (ISR) or systemic acquired resistance (SAR) (Pieterse *et al*., 2014). The results, even if preliminary, suggest that the ISR could be the preferential way followed by plants.

Moreover, a characterization by UPLC-MS analysis confirm that some Glera *Bacillus* endophytes are able to produce LPs and one in particular called pumilacidin display *in vitro* a quite strong antibacterial effect.

In conclusion, the inoculation methods described in **Chapter2** provide a new but smart and simple ways to introduce beneficial bacteria into newborn grapevine plants which can both promote their growth and help them to cope with abiotic as well as biotic stresses. It is also worth to notice that the biological control is considered an valid alternative to the extensive use of pesticides and *Bacillus*-LPs-producers could be exploited in protecting vineyards from the most common fungal and bacterial pathogens contributing to reduce the amount of pesticides released.

So, the possibility of endophyte exploitation to help plants to cope with abiotic as well as biotic environmental stresses, interfering with plant morphology and physiology or priming their systemic responses against pathogens, opens new

scenarios for an environmentally friendly shift in agricultural practices such as improving the environment health and reducing the impact of synthetic agro-chemicals.

Results obtained from this work are aligned with the huge amount of studies, grown exponentially in the last years, in which the endophytes abilities and properties are often assayed in a single plant species or within groups of closely related plant genotypes. In addition, investigations are usually performed using microbial species that are relatively easy to cultivate leaving out that plant biome is always characterized by wide and complex interactions. The plant phenotype is determined not only by the plant responses to the environment but it is also orchestrated by the associated microbiota, the responses of the microbiota to the environment, and the complex interactions between individuals in the endosphere microbial plant community.

Future exciting challenges, based on classical approaches and new valuable technologies (i.e. next-generation sequencing) applied to greenhouse and field conditions will be to explore and characterize the contributions of genetic and metabolic elements involved in the interactions between host plants and endophytes, to provide new ecological and evolutionary insights and a better knowledge of the plant-microbiome-environment relationships.

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