SYMBIOTIC AGRICULTURE: INCREASING KNOWLEDGE ON THE MODE OF ACTION OF BENEFICIAL MICROORGANISMS

A thesis Submitted to the

University of Modena and Reggio Emilia

For the Degree of

Doctor of Philosophy

In the faculty of

AGRI-FOOD SCIENCES, TECHNOLOGIES AND BIO-TECHNOLOGIES

(Plant Pathology)

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Reggio Emilia, Italy, 2016-2019

Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revision, as accepted by my examiners.

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ABSTRACT

Bacteria that are beneficial to plants are considered to be plant growth-promoting bacteria (PGPB) and can facilitate plant growth by a number of direct and indirect mechanisms. Non-pathogenic, soil microbes that occupy the rhizosphere can influence plant growth and induce changes in the plant's physiological, chemical, metabolic, molecular activities, influencing plant-microbe interactions with abiotic and biotic stressors. Plants colonized by these microbes express unique plant phenotypes that show increased root and shoot mass, enhanced nutrient uptake, and stress mitigation. Additionally, the microbes may fix nitrogen and phosphate or produce siderophores for plant use. Among the plant-associated microbes, plant growth-promoting rhizobacteria (PGPR) are the most commonly used as inoculants for biofertilization. Plant growth-promoting rhizobacteria are non-pathogenic, free-living soil and root-inhabiting bacteria that colonize seeds, root tissue (endophytic/epiphytic), or the production of root exudates. In addition to these adaptations, PGPRs may utilize other mechanisms to facilitate plant growth including IAA synthesis, siderophore production, phosphate solubilization activity, ammonia production, and antifungal and antibacterial compounds production.

Plant growth-promoting bacterial endophytes employ similar plant growth promotion mechanisms to those used by rhizospheric PGPB. In fact, bacterial endophytes are PGPBs that go one step further and colonize the inside of the plant tissues and provide more efficient and prompted protection to their hosts compared to those that bind exclusively to the plant's rhizosphere. Therefore, it is likely that endophytic plant growth-promoting bacteria will be superior to similar non-endophytic bacterial strains in promoting plant growth under a wide range of environmental conditions.

In the present study, Chapter I describes a set of beneficial plant growth promoting strains were evaluated for their *in vitro* plant growth promoting traits and

antagonistic activity on various phytopathogenic bacteria and fungi. We identified plant growth-promoting endophytes within the bacterial groups as part of the core bacterial consortium. Among all the strains tested, two prospective isolates *Streptomyces* sp. strain SA51 and *Pseudomonas* sp. strain PT65 used in the present study were extensively characterized to evaluate their *in vitro* plant growth promoting (PGP) traits and their biocontrol activity. Here, we characterized both the strains SA51 and PT65 for their colonization ability, plant growth promotion and protection against tomato spot disease caused by *Xanthomonas vesicatoria* on tomato (*Solanum lycopersicum*) as model plant. In this study, direct inhibitory action against *X. vesicatoria* by the bacterized tomato plants showed significantly good plant growth, as compared to unbacterized controls. Protection against ***X. vesicatoria*** by the bacterized tomato plants was confirmed in the greenhouse: disease was reduced by approximately 96%. Additionally, plants bacterized by strain SA51 showed significant plant growth, particularly in aerial parts as compared to non-bacterized controls. Finally, benefit was seen in inoculated healthy plants in terms of a significant increase in dry weight and length of roots and shoots, as compared to the uninoculated controls. A GFP mutant of strain SA51 was produced to study its endophytic colonisation in tomato plants: results confirmed that SA51 was able to efficiently colonise tomato endophytically, from the roots to the leaves. Field experiments confirmed the ability of strain SA51 to act as plant growth promoting agent: such promoting activity was also reflected into an increase of fruit production by approximately 7%.

Furthermore, we performed whole genome sequence (WGS) analysis for the strain SA51, which provided in detailed properties of the strain metabolic profile using the Kyoto Encyclopedia of Genes and Genomes (KEGG), thus providing evidence for the presence of genes involved in the pathway for indole alkaloid biosynthesis and in iron transport and metabolism, together with genes coding for proteins acting in the regulation of iron homeostasis. At the same time, based on RAST annotations, we provided evidence for the presence of genes and operons related to

metal transporters and antibiotic biosynthesis, suggesting that SA51 could be involved in the biological control of plant pathogens and/or in the reshaping of the soil microbiota.

In chapter II, we have tested a set of microbes as microbial consortium (a set of prospective microbes) for biocontrol, biochemical and differential gene expression as compare to control (un-inoculated) in grapevine plants. Since plant growth promotion is a multigenic process under the influence of many factors, an understanding of these processes and the functions regulated may have profound implications. The gene expression changes, represented by different time points hours post inoculation (hpi) have been studied to gain insight into various genes responsible for pathogen related (PR) proteins, lytic enzymes, growth hormones and to maintain cell wall integrity assisted plant growth promotion of grapevine leaves. It was observed that the microbial consortium profusely induced the upregulation of grapevine genes involved in maintenance of biocontrol and plant growth promotion activity. Almost all the genes were downregulated initially after 0 hpi and 2 hpi, but later from 4 hpi genes like *ACC, CHS, PAL & PER* were significantly upregulated. Particularly, *PR11* and *PR12* genes were significantly upregulated after 4 hpi. In case of biochemical aspects microbial treatments has increased the quality in terms of pigmentation and stability towards oxidation. Microbial consortium also tested for the biocontrol activity of two diseases like Flavescence Dorée and Esca. In case of Flavescence Dorée, results were not satisfactory and no difference in disease progression and quantity was observed between treated and untreated plots. This can be explained with the lack of vector control. Whereas, in case of Esca, experiments showed a remarkable effect of sprays with the microbial consortium in slowering disease progression. Since grapevine is a multiannual crop – a vineyard may last over 30 years – a continuous disease slowering may have a positive impact in grapevine longevity and productivity

In summary, we were able to confirm the ability of single beneficial microbes and a microbial consortium to act as promoting factor for plant growth and health; this study was done *in vitro* and in *planta*.

ACKNOWLEDGEMENTS

I would like to acknowledge many people who helped me throughout the process of completion of my thesis in many ways. Without their help, it would have not been possible to successfully accomplish this degree.

First and foremost, I would like to thank my supervisor Prof. Emilio Stefani who has given me the opportunity to work in his laboratories by accepting me as graduate student. I deeply appreciate his tireless and dedicated guidance throughout my research period. His motivation will remain a great energy source for me in the future as well.

Many people with excellent technical expertise made our project possible from mere idea to workable and reproducible piece of work. For this, my greatest gratitude goes to Dr. Davide Giovanardi, for taking care of my experimental procedures and co-tutoring me for some time, Prof. Stefano Cassanelli, who helped me in designing qPCR experiments and analysing results, to Prof. Mauro Mandrioli, for the help with bioinformatics and timely guidance, and to my colleague Dr. Noel Mekam who helped me a lot in field studies and Irem Altin for her help in lab as a wonderful lab mate for being good friend.

I am grateful to my professors Dr. Sk.Z. Ali and Dr. V. Sandhya Rao (Agri Biotech Foundation, India) who has motivated me towards research and to pursue Ph.D., without their support and guidance this degree would not be possible.

I would also like to thank our Ph.D. School faculty and my other colleagues, my special thanks to Prof. Alessandro Ulrici, Prof. Andrea Pulvarenti, Prof. Luisa Antonella Volpelli, Prof. Maria Gullo and Dr. Luciana De Vero.

I would also thank CCS AOSTA Srl. company for providing funds for the Ph.D. programme and their great support.

I deeply appreciate and thank my external examiners, Dr. Jeffery B. Jones (University of Florida, USA) and Dr. Subramaniam Gopalakrishnan (ICRISAT, INDIA) for taking their precious time in reading and reviewing my thesis.

I would also like to thank my friends Nihar Sahu, Dr. Divya Dhanasekar, Dr. Mounika Nagabhairava, Bindu Prathyusha, Dr. Shrey Bodhankar, Manjari Shrivastava and Bindu Sunkar for being part of my journey to achieve doctoral degree.

Of course, without thanking my family this journey would not be possible.

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CHARACTERISATION OF A SET OF MICRORGANISMS AS MICROBIAL BIOCONTROL AGENTS

1. INTRODUCTION:

The world's population currently is ~7.7 billion people and is likely to increase to around 10 billion by 2050. Thus, in the next ten to twenty years, there will be a significant challenge to feed all the world's people, a problem that will likely increase with time. However, to do so it is necessary to greatly increase agricultural productivity in a sustainable and environmentally friendly manner. It is necessary to re-examine many of the existing approaches to agriculture that presently include the use of chemical fertilizers, herbicides, fungicides, and insecticides. Instead, sustainable agriculture will need to make much greater use of both transgenic plants (for example, see http://www.isaaa.org/inbrief/default.asp) and beneficial microorganisms (*e.g.*: plant growth-promoting bacteria, PGPB) **(Glick, 2012)**. High-input, resource-intensive farming systems, which have caused massive deforestation, water scarcities, soil depletion and high levels of greenhouse gas emissions, cannot deliver sustainable food and agricultural production. Needed are innovative systems that protect and enhance the natural resource base, while increasing productivity. Needed is a transformative process towards 'holistic' approaches, such as agro-ecology, agro-forestry, climate-smart agriculture and conservation agriculture, which also build upon indigenous and traditional knowledge. Technological improvements, along with drastic cuts in economy-wide and agricultural fossil fuel use, would help address climate change and the intensification of natural hazards, which affect all ecosystems and every aspect of human life. Greater international collaboration is needed to prevent emerging transboundary agriculture and food system threats, such as pests and diseases **(FAO, 2017)**.

Figure 1.1: World population data by country. Adapted from **(Roser et al., 2019)**

1.1. SYMBIOTIC AGRICULTURE: BENEFICIAL MICROORGANISMS FOR SUSTAINABLE AGRICULTURE:

Agriculture has been the largest source of wealth since civilization, earth being a planet with the population around 7.7 billion **(Figure 1.1)**, inhabiting 6.38 billion hectares of land **(World Population Data Sheet, 2016)** and about 1.3 billion people directly dependent on agriculture for their survival. Degradation of soil is something that cannot be neglected **(FAO, 2011)**. Estimation by the Food and Agriculture Organization (FAO) Food Balance Sheet 2004 has shown that 99.7% of food for the total population comes from the terrestrial environment alone. With about 79 million people being added to the world population every year, there has been a continuous increase in the demand for food and scarcity in the supply **(Alexandratos, 2003)**. For example, in India 60.6% of land is used for agricultural purposes by half of its population for growing several forms of cereals, vegetables, pulses and so forth. Agricultural productivity, water quality and climate change are greatly influenced by the effectual exchange of nutrients, energy and carbon between soil organic matters and plant system **(Lehmann and Kleber, 2015)**. The nature of soil has been regulated by several aspects such as organic carbon content, moisture, nitrogen (N), phosphorus (P) and potassium (K) (NPK) content and several other factors.

Direct manifestation of soil through microbes present in it and through leguminous plants as a holobiont relationship by the process of biomineralization and synergetic coevolution have been observed to have great potential in the improvement of soil quality and fertility **(Paredes and Lebeis, 2016)**. The soil physiology and performance are critically affected by plant associated microorganisms, which suggest that there might be a holobiont link in the coevolution and ecology of plants and animals **(Agler, 2016)**. It has also been evident that accumulating coevolution of soil microbes with plants is vital in response to transmute or extreme abiotic environments, thus resulting in the improvement of economic viability, soil fertility and environmental sustainability **(Khan et al., 2016; Compant et al., 2016)**. The coevolution between plants and microbes can be best explained considering plant growth promoting microbes, which show antagonistic and synergistic interactions with microorganisms, plant roots and soil, either directly or indirectly boosting the plant growth rate **(Rout and Callaway, 2012; Bhardwaj et al., 2014)**.

Plants have always been in a symbiotic relationship with several forms of soil microbes for their growth, development and other requirements. The symbiotic microorganisms inhabiting the rhizosphere of many plant species have diverse beneficial effects on the host plant **(Raza et al., 2016)**. These beneficial free-living soil bacteria are usually referred to as PGPR. In the current era, rhizobacteria possess a conspicuous impact on plants that can be a significant tool to defend the health of plants in an eco-friendly manner **(Akhtar et al., 2012)**. Applications of PGPR associations with different plant species have been investigated in certain cases such as oat, canola, soybean, potato, maize, pea, tomato, lentil, barley, wheat, radicchio, cucumber and other vegetable crops **(Gray and Smith, 2005; Anusha et al., 2019)**. They have been involved in various biotic and abiotic activities inside the soil ecosystem, making it dynamic for alimental turn over and sustainable for crop production by enhancing its physiological properties **(Gouda et al., 2018)**.

Bacteria with multiple plant growth promoting (PGP) traits located in the rhizosphere or root surface of plants can increase the growth and yield of crops **(Figure 1.2)**. Some of the known mechanisms by which PGPRs could be beneficial to the plants include: (i) bio-remediating contaminated soils by sequestering toxic heavy metal species and degrading xenobiotic compounds and improving soil structure (*e.g.*: by bacterial exopolysaccharides) **(Glick, 2010; Wang et al., 2019);** (ii) enzyme synthesis, *e.g.*: ACC (1-aminocyclopropane-1-carboxylate) deaminase, an enzyme involved in decreasing the level of stress-induced ethylene in the root of developing plants **(Glick, 2004; Belimov et al., 2019)**; (iii) providing ammonia or related nitrogenous supply to plants, through biological nitrogen fixation; (iv) production of siderophores; (v) generation of phytohormones (*e.g.*, ABA (abscisic acid), GA (gibberellic acid), auxin, *i.e.*, indole-3-acetic acid (IAA), and CK (cytokinins); (vi) control of plant pathogens by different mechanisms, like generation of extracellular enzymes hydrolysing the fungal cell wall, competition for nutrients (niches) within the rhizosphere, induction of systemic resistance (ISR) and the production of antibiotics and siderophores **(Compant et al., 2005)**; (vii) solubilization and mineralization of nutrients, particularly mineral phosphates; and (viii) improvement of abiotic stress tolerance **(Glick, 2014; Hayat et al., 2010)**.

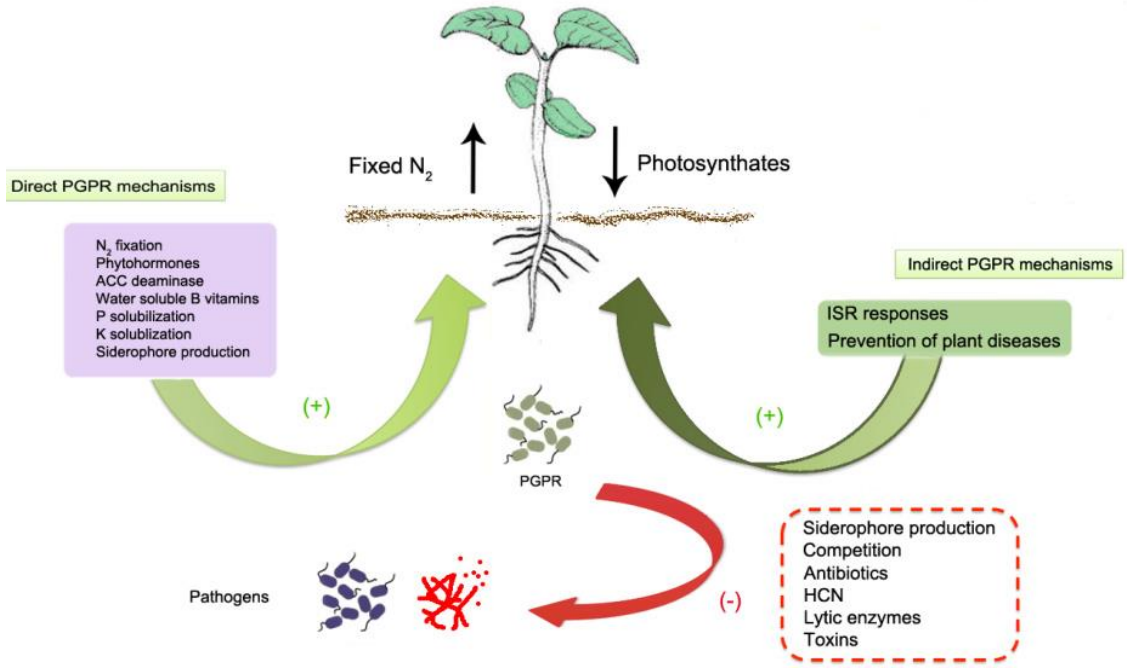


Figure 1.2. Plant growth promoting rhizobacteria and their traits to promote plant health. Adapted from **(García-Fraile et al., 2015)**.

1.2. UNDERSTANDING THE PLANT-MICROBE INTERACTIONS TO DEVELOP INNOVATIVE MICROBIALS:

Habitually not visible to the naked eye, interactions between plants and microorganisms occur in many ways and on many different levels. Virtually all organs of the plant interact with microorganisms at a certain stage of their life and this interaction is not necessarily negative for the plant. Indeed, there are plenty of interactions where the plant benefits either through direct or indirect effects of the associated microbes. In these interactions, plants serve as host (in cas of endophytes) for the microorganisms that may colonize apoplastic spaces, plant surface areas (epiphytes) or areas adjacent to the plant surface, *e.g.*, the rhizosoil, the soil in the vicinity of roots. In addition to a sheltered habitat and a future source of nutrients that are liberated upon plant death, many plants release compounds that attract and feed

the associated microbes. The associated microbes may in turn secrete compounds that favour plant growth, they may make the plant more resistant to abiotic or biotic stress, or they may defend the plant against more pathogenic microbes, or they promote plant growth directly or indirectly **(Mavrodi et al., 2017; Schirawski and Perlin, 2018)**.

In general, there are two possibilities to influence the antagonistic/plant growth-promoting potential: (1) by managing the indigenous microbial potential, *e.g.*, by introduction of organic or inorganic amendments **(Hallmann et al., 1999; Conn and Lazarovits, 2000)** and (2) by applying autochthonous microorganisms as biocontrol or plant growth-promoting agents **(Emmert and Handelsman, 1999; Whipps, 2001; Weller et al., 2002; Compant et al., 2005; Weller, 2007)**. Furthermore, diverse microbial inoculants are already on the market and, in recent years, the popularity of microbial inoculants increased substantially, as extensive and systematic research has enhanced their effectiveness and consistency **(Thakore, 2006)**. Recent surveys of both conventional and organic growers indicate an interest in using microbial inoculants, suggesting that the market potential of biocontrol products will increase in the coming years **(McSpadden Gardener and Fravel, 2002)**.

Plant-microbe interactions are mutualistic associations benefitting both the partners. For example, plants commonly react to root colonization by microbes by increasing the release of exudates **(Phillips et al., 2004)** or they produce several compounds that mimic Quorum Sensing (QS) signals that influence the bacterial communities **(Bauer and Mathesius, 2004)**. Kamilova et al. **(2006)** showed that the pathogen *Fusarium oxysporum* as well as the antagonist *P. fluorescens* WCS365 influence the composition of organic acids and sugars in tomato root exudates. Plant-associated microorganisms have been shown to activate plant host defense when the symbiotic interaction becomes unproductive **(Parniske et al., 1991)**. PGPR that elicits ISR in one plant species may not do so in another, again indicating specificity in the interaction between PGPR and plants. Substances involved in ISR are partly the same with those involved in microbial antagonisms and include the following: siderophores,

antibiotics, N-acyl-homoserine lactones, VOCs (*e.g.,* 2,3-butandiol). Whereas some PGPR activate defense-related gene expression, other examples appear to act solely through priming of effective resistance mechanisms, as reflected by earlier and stronger defense reaction once infection occurs **(Berg, 2009)**.

Microbial inoculants/formulations can be divided into different categories based on regulations on usage of these productionLegislation and regulations were depeds on their respective countries. In the US, the products must be registered by the United States Environmental Protection Agency, while in Europe, common EU rules are responsible (see http://www.rebeca.de). From a scientific point of view, we can differentiate between (1) biofertilizers, (2) plant strengtheners, (3) phytostimulants and (4) biopesticides **(Lugtenberg et al., 2002)**. While the first three categories are based on plant growth promoting agents (PGPAs), biopesticides contain Biological control agents (BCAs). A new but, maybe in the future, growing category is flavour-stimulating agents, which enhance fruit flavour like *Methylobacterium* in strawberry **(Zabetakis, 1997)**. In general, microbial inoculants are available as liquid-based formulations, water-dispersible granules, or wettable powders or pellets.

1.3. TOMATO: AN IMPORTANT CROP WORLDWIDE AND A MODEL PLANT:

The cultivated tomato, *Solanum lycopersicum* L. is the world's most highly consumed vegetable due to its status as a basic ingredient in a large variety of raw, cooked or processed foods and is the 2nd most cultivated crop next to potato worldwide. It belongs to the family *Solanaceae*, which comprises several other commercially important species. Tomato is grown worldwide for local use or as an export crop. In 2016, the global area cultivated with tomato was 5 million hectares, with a production of 177 million tonnes, the major tomato-producing countries being the People's Republic of China and India. Tomato can be grown in a variety of geographical zones in open fields, tunnels or in specialised greenhouses and the fruit can be harvested by manual or mechanical means. Under primitive conditions (*e.g.*

rejuvenation pruning, weeding, irrigation, frost protection), this crop plant can be perennial or semi-perennial, but commercially it is considered an annual crop **(Geisenberg and Stewart, 1986)**.

The *Solanaceae*, commonly known as the nightshade family, also includes other notable cultivated plants such as tobacco, chilli pepper, potato and eggplant. Tomato classification has been the subject of much discussion and the diversity of the genus has led to reassessment of earlier taxonomic treatments. Tomato was originally named *Solanum lycopersicum* by Linnaeus in 1753 and later regarded as *Lycopersicon lycopersicum* (L.) **(Valdes and Gray, 1998)**. Miller **(1768)** in The Gardener's Dictionary used *Lycopersicon esculentum*. Rick **(1979)** included nine species in the *Lycopersicon* genus. For a long time tomatoes were known as *L. esculentum*, but recent research has shown that they are part of the genus *Solanum* and are now again broadly referred to as *Solanum lycopersicum* **(Spooner et al., 1993; Bohs and Olmstead, 1997; Olmstead and Palmer, 1997; Knapp, 2002; Spooner et al., 2005, 2003; Peralta et al., 2008)**.

The commercially important tomato fruit can vary in colour, size and shape **(Vaughan and Geissler, 1997)**. The fruit contains a large quantity of water, vitamins and minerals, low amounts of proteins and fats, and some carbohydrates. It also contains carotenes, such as lycopene (which gives the fruit its predominantly red colour) and beta-Carotene (which gives the fruit its orange colour). Modern tomato cultivars produce fruits that contain up to 3% sugar of fresh fruit weight. It also contains tomatine, an alkaloid with fungicidal properties. The concentration of tomatine decreases as the fruit matures and tomatine concentration contributes to determining the taxonomy of the species. Thus, it can be useful in crop breeding for cultivated tomatoes **(OECD, 2008; Spooner et al., 1993)**.

Tomato is one of the best studied cultivated dicotyledonous plants at the molecular level and has been used as a model species for research into gene mapping, gene characterisation (*e.g.* plant growth promoting and plant pathogen resistance

genes) and gene transfer approaches. It is also useful to study other plant traits, such as fruit ripening, hormone functions and vitamin biosynthesis **(Gebhardt et al., 1991; Chetelat and Ji, 2006; Ji and Scott, 2006)**. Over the last decade the effect of plant growth promoting rhizobacteria (PGPR) has been extensively studied by various researchers around the world.

2. OBJECTIVES OF PRESENT STUDY

Micosat F is a commercial formulation product of CCS Aosta Srl. Company which is Italian based company whose primary objective is to develop microbial bioinoculants containing a set of bacterial strains, whose efficiency was not studied deeply (for additional information see: www.micosat.it). In the first part of the thesis we aimed to characterize each of these strains under *in vitro* and *in vivo* conditions. From the literature it was evident that PGPR bacteria were very efficient in plant growth promotion and biocontrol of various disease. To test this hypothesis, we mainly focussed on screening these strains for various PGP traits like IAA production, P-solubilization, Ammonia production, Siderophore and HCN production and also antagonistic activity against various phytopathogens of tomato crop. From these data the most prospective strain(s) will be tested under greenhouse conditions for its/their PGP and biocontrol activity. These strains will be further evaluated for endophytic colonization and field studies.

3. LITERATURE REVIEW

3.1. INTRODUCTION TO PLANT GROWTH-PROMOTING RHIZOBACTERIA (PGPR):

Agriculture contributes to a major share of national income and export earnings in many developing countries, while ensuring food security and employment. Sustainable agriculture is vitally important in today's world because it offers the potential to meet our future agricultural needs, something that conventional agriculture will not be able to do. Recently, there has been a great interest in eco-friendly and sustainable agriculture. In natural conditions, both plant above ground (phyllosphere) organs and its below ground termed as rhizosphere are colonized by bacteria, fungi, actinomycetes, protozoa and algae. Ninety five percent of all the colonizing microorganisms are bacteria **(Glick, 2012)**. PGPR are known to improve plant growth in many ways, when compared to synthetic fertilizers, insecticides and pesticides. The rhizospheric soil contains diverse types of PGPR communities, which exhibit beneficial effects on crop productivity. Several research investigations are conducted on the understanding of the diversity, dynamics and importance of soil PGPR communities and their beneficial and cooperative roles in agricultural productivity. Some common examples of PGPR genera exhibiting plant growth promoting activity are *Pseudomonas*, *Agrobacterium*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Rhizobium*, *Actinobacteria Mesorhizobium*, *Flavobacterium*, etc., **(Singh, 2013)**.

3.1.1. Pseudomonas spp.:

Pseudomonas species are ubiquitous bacteria in agricultural soils and have many traits that make them well suited as PGPR. Fluorescent pseudomonads are Gram negative, aerobic rods, motile with polar flagella and can produce water soluble yellow green pigment **(Palleroni et al., 1973)**. *Pseudomonas* taxonomy is still controversial: considering those groups containing species that may include antagonistic bacteria they are: the *Pseudomonas aeruginosa* group, containing several

species of clinical interest, therefore questionable as biocontrol agents to be used in agriculture (*e.g.*: *P. mendocina,* **Kaur et al. 2014**); the *P. chlororaphis* group, containing the species *P. chlororaphis* that proved to be active against tomato fungi **(Postma and Nijhuis, 2019)**; the most known *P. fluorescens* group (several authors published on it) and the *P. putida* group **(Anzai et al., 2000)**. They are well adapted to the rhizosphere and rhizoplane, have a fast growth rate in the rhizoplane and can utilize a large number of organic substrates **(Stolp and Godkari, 1981)** including root exudates **(Rovira and Davey, 1974)**. The worldwide interest in this group of rhizobacteria was sparked by the studies initiated at the University of California, Berkeley, USA during 1970s. Fluorescent pseudomonads exhibit diverse mechanisms of biocontrol which include antibiosis, HCN (Hydrogen Cyanide) production, siderophore production, competition for space and nutrients and induced systemic resistance **(Chen et al., 2000; Stanzin et al., 2017)**.

Among the group of PGPR, the genus *Pseudomonas* is strongly represented. This genus comprises over one hundred species of aerobic bacteria that belong to the γ subclass of the Proteobacteria **(Mulet et al., 2013)**. Although some *Pseudomonas* spp. are plant pathogens, many have been found to protect plants by antagonizing soil-borne pathogens through competition for nutrients, production of antimicrobial compounds, or by eliciting a systemic immune response that is effective against a broad spectrum of pathogens, called induced systemic resistance (ISR) **(Lugtenberg and Kamilova, 2009; Pieterse et al., 2014)**. Mutualistic root-colonizing *Pseudomonas* spp. emerged as important players in disease-suppressive soils **(Mendes et al., 2011; Weller, 2007)**, and served as model PGPR in research toward understanding how non-symbiotic root-associated bacteria protect plants against pests and diseases **(Haas and Défago, 2007; Roeland et al., 2015)**.

3.1.2. Streptomyces spp.:

Actinobacteria, and streptomycetes in particular, are known to constitute a large part of the rhizosphere microbiota. They may live saprophytically and endophytically in both natural and agricultural environments, where they may colonize the rhizosphere and different morphological parts of plant roots **(Saleem et al., 2016)**. Therefore, considering their plant growth-promoting activity, streptomycetes represent an excellent alternative for improving nutrient availability to crop plants and promoting innovation and sustainability in agricultural systems **(Figueiredo, 2010)**. Plant growth-promoting streptomycetes (PGPS) stimulate and enhance several direct and indirect biosynthetic pathways in plants, for example, inorganic phosphate solubilisation, biosynthesis of chelating compounds, phytohormones production, inhibition of plant pathogens and alleviation of various abiotic stresses **(Sousa and Olivares, 2016)**.

Actinobacteria, in general, may have a positive role in plant mineral nutrition. This is correlated to both nitrogen fixation and metal mobilizing ability involving mineral nutrients such as Fe, Zn, and Se. More recently, Viaene et al. **(2016)** highlighted the contribution of streptomycetes to plant growth and health. The plant has an important role in shaping its root microbiome through root exudate composition (chemotaxis) and nutritional interactions **(Bulgarelli et al., 2013; Massalha et al., 2017; Bais et al., 2006)**. Plant root exudates are a source of metabolic signals (such as flavonoids, strigolactones, and terpenoids) that can shape the microbial communities in the rhizosphere. The signals that attract streptomycetes into the rhizosphere are still unknown. From the rhizosphere, streptomycetes can enter roots and colonize root tissues and vessels from where they can be isolated and purified to identify them and describe their physiology and their microbe–microbe interactions **(Coombs and Franco, 2003)**.

Many scientific studies have focused on Actinobacteria and particularly streptomycetes, in stimulating the production of plant growth hormones, thus

enhancing plant growth. A study by Dochhil et al. **(2013)** described the evidence of plant growth-promoting activity and a higher percentage of seed germination due to the synthesis of higher concentrations (71 g/mL and 197 g/mL) of the plant growth hormone indole acetic acid (IAA) by two *Streptomyces* spp. strains isolated from *Centella asiatica*. In field trials, increased growth promotion and yield of cucumber was achieved by the application of *Streptomyces spiralis* alone, or in combination with other microbial "activators", such as *Actinoplanes campanulatus* or *Micromonospora chalcea*. Such experiments highlight the role of multiple microbes (or a microbial consortium) in productive crop systems **(EI-Tarabily et al., 2010; Golinska et al., 2015)**. In soil, most of the known actinomycetes belong to the genus, *Streptomyces,* and have been used for various agricultural purposes, mainly due to their production of antifungal and antibacterial metabolites and several plant growth-promoting (PGP) traits **(Goodfellow and Simpson, 1987; Suzuki et al., 2000)**. Indeed, more than 60% of known compounds with antimicrobial or plant growth-promoting activity originate from this genus **(Alam et al., 2012)**. In agricultural environments, *Streptomyces* species are an important group of soil bacteria because of their ample capacity to produce PGP substances, secondary metabolites (such as antibiotics) and enzymes **(Abd-Alla et al., 2013; Inbar et al., 2005)**.

3.1.3. Agrobacterium spp.:

Despite extensive literature on modes of action of PGPR's (especially in the *Proteobacteria*), the molecular features that define a PGPR remain elusive, because the PGPR status is not always well defined. First, PGPR may occupy different microbial habitats, as they range from saprophytic soil bacteria that colonize the rhizosphere to bacteria that can also colonize internal root tissues. This means that the distinction is not often simple, respectively with saprophytes without plant-beneficial effects (especially plant commensals) and with vertically inherited endophytes or plant endosymbionts. Second, several bacteria display alternate ecological niches, and at

times some may function as PGPR. For instance, certain tumor-inducing and non-inducing *Agrobacterium* strains, like *A. radiobacter,* have plant growth stimulation potential on non-host plants **(Walker et al., 2013)**, a property also found in an *Escherichia coli* gut commensals **(Walker et al., 2013)**. Third, the genes implicated in plant-beneficial functions range from genes directly conferring plant-beneficial properties, such as *nif* (nitrogen fixation) **(Bashan and de-Bashan, 2010)** or *phl* (phloroglucinol synthesis) **(Haas and Keel, 2003)**, to genes contributing to a variety of cell functions indirectly or secondarily including plant-beneficial ones, such as *pqq* (pyrroloquinoline quinone synthesis) **(Misra et al., 2012)**. Fourth, many PGPR strains are not yet recognized as such (as determination of PGPR status requires experimental assessment), and it is very likely that not all plant-beneficial traits and the corresponding genes have been identified. Fifth, the assessment of genes encoding plant-beneficial properties is commonly restrained to particular bacterial clades **(Ellis et al., 2000),** if not particular PGPR strains **(Couillerot et al., 2009; Haas and Keel, 2003)**, without a more general analysis of gene distribution across several bacterial clades **(Spaepen et al., 2007)**.

3.1.4. Bacillus spp.:

Diversified populations of aerobic endospore forming bacteria (AEFB), like species of *Bacillus*, occur in agricultural fields and contribute to crop productivity directly or indirectly. Physiological traits, such as multi-layered cell wall, stress resistant endospore formation and secretion of peptide antibiotics, peptide signal molecules and extracellular enzymes, are ubiquitous to these bacilli and contribute to their survival under adverse environmental conditions for extended periods of time. Multiple species of *Bacillus* and *Paenibacillus* are known to promote plant growth. The principal mechanisms of growth promotion include production of growth stimulating phytohormones, solubilization and mobilization of phosphate, siderophore production, antibiosis (*i.e.,* production of antibiotics) helps the plants indirectly by acting on plant pathogens, inhibition of plant ethylene synthesis and induction of

plant systemic resistance to pathogens **(Richardson et al., 2009; Idris et al., 2007; Gutierrez-Manero et al., 2001; Whipps 2001).** It is very likely that plant growth promotion by rhizosphere bacilli may be a result of combined action of two or more of these mechanisms.

Enhancement of plant growth by root-colonizing species of *Bacillus* and *Paenibacillus* is well documented and PGPR members of the genus *Bacillus* can provide a solution to the formulation problem encountered during the development of BCAs to be used as commercial products, due in part to their ability to form heat and desiccation-resistant spores **(Kloepper et al., 2004; Emmert and Handelsman, 1999)**. In the past few years, research has been directed more toward the induced systemic resistance (ISR), a process by which PGPR stimulate the defense mechanisms of host plants without causing apparent harm to the host. Choudhary and Johri **(2008)** have reviewed ISR by *Bacillus* spp. in relation to crop plants and emphasized on the mechanisms and possible applications of ISR in the biological control of pathogenic microbes. Various strains of *B. amyloliquefaciens*, *B. subtilis*, *B. pasteurii*, *B. cereus*, *B. pumilus*, *B. mycoides*, and *B. sphaericus* are known as potential elicitors of ISR and exhibit significant reduction in the incidence or severity of various diseases on diverse hosts **(Choudhary and Johri, 2008**; **Kloepper et al., 2004)**. It is believed that plants can acquire enhanced resistance to pathogens after being exposed to biotic stimuli provided by many PGPRs and this is known as rhizobacteria mediated ISR **(Choudhary et al., 2007)**.

3.2. Plant Growth Promoting Rhizobacteria (PGPR) and their Interactions

Different microbial genera are vital components of soils. They are involved in various biotic activities of the soil ecosystem to make it dynamic for nutrient turn over and sustainable for crop production **(Ahemad et al., 2009; Chandler et al., 2008)**.

3.2.1. Mechanisms used by PGPR:

PGPR can affect plant growth by various direct and indirect mechanisms **(Kloepper and Schroth, 1978; Glick et al., 1995; Cattelan et al., 1999; Gupta et al., 2000; Li et al., 2000; Hayat et al., 2010; Saraf et al. 2011; Minaxi et al., 2012; Kavamura et al., 2013; Ahemad and Kibret, 2014)**. These mechanisms can probably be active simultaneously or sequentially at different stages of plant growth **(Chaparro et al., 2013)**. Some examples of these mechanisms are represented in figure 3.

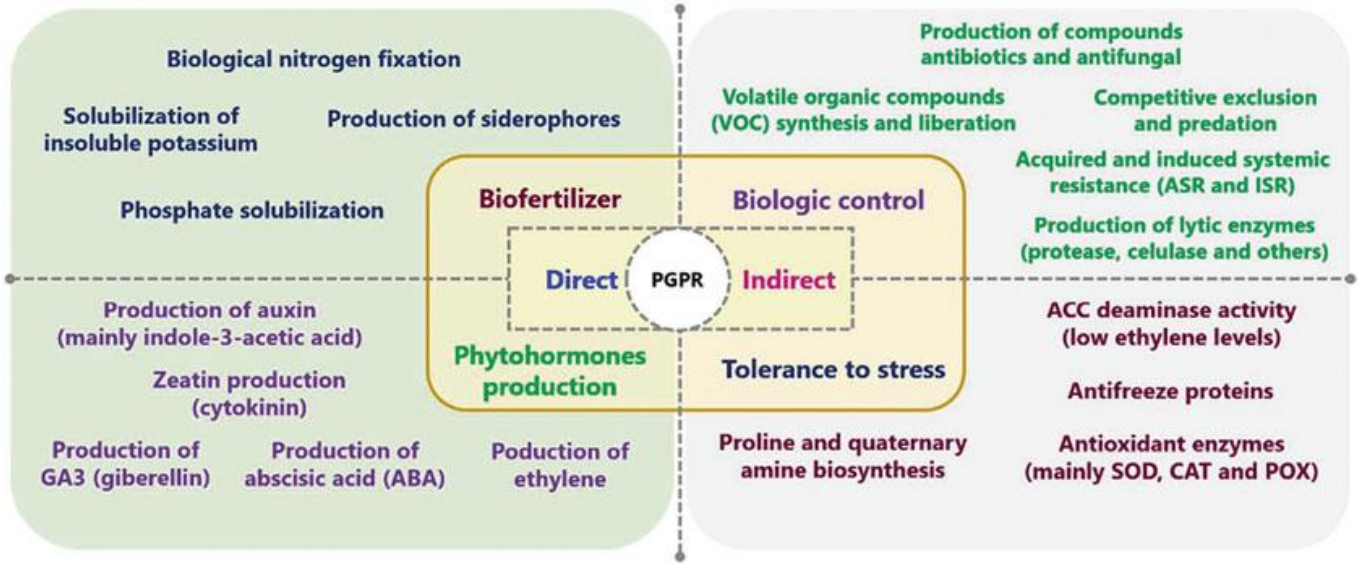


Figure 3.1. Mechanisms used by plant growth promoting rhizobacteria (PGPR). Adapted from **(Chauhan et al. 2015; Pii et al. 2015)**

3.2.2. Direct mechanisms of action

PGPR directly affects plant metabolism by providing nutrients, such as nitrogen, that are usually scarce in the rhizosphere, **(Ahmad et al., 2008; Babalola, 2010)**. The capture and subsequent release of nitrogen to plants is carried out by bacteria present in the rhizo- and endosphere through a diverse set of processes. PGPRs may convert nitrogen trapped in the molecular or atmospheric form (N_2) into biologically useful forms in a process known as biological nitrogen fixation (BNF). Only diazotrophic bacteria execute BNF, as the nitrogenase enzyme is present only in these organisms **(Bhattacharjee et al., 2008)**. Members of the genera *Anabaena*,

Azospirillum, *Azotobacter*, *Bacillus*, *Clostridium*, *Klebsiella*, *Nostoc*, *Paenibacillus* and *Rhodobacter* are examples of free-living diazotrophic bacteria that provide available nitrogen to several plants **(Grobelak et al., 2015)**.

a. Nitrogen Fixation:

Nitrogen is the nutrient that is required in the highest amount by plants, and its availability is a major factor that limits their development **(Courty et al., 2015)**. Globally, considerable attention has been given to the shortage of nitrogen in agricultural soils, which reduces plant yield capacity, and new technologies have been developed and tested to prevent the use of chemical fertilizers in cultivated areas **(Bhattacharjee et al., 2008; Figueiredo et al., 2013)**. The combination of species of *Anabaena*, a free-living diazotrophic bacterium that fixes nitrogen, and *Azolla* is a natural means of providing nitrogen to waterlogged rice plants **(Bhuvaneshwari and Kumar, 2013; Fosu-Mensah et al., 2015)**. In this case, the free-living diazotrophic *Anabaena* may be referred to as a "biofertilizer," *i.e.,* a beneficial microorganism that helps to maintain soil quality and plant health through its biological activity. Biofertilization of rice with *Anabaena* contributes high nitrogen amounts (up to 50 kg/ ha), reduces nitrogen loss *via* ammonia volatilization, and stimulates plant growth **(Bhuvaneshwari and Kumar, 2013)**.

b. Phosphate solubilisation:

In addition to nitrogen, phosphorus and potassium are important nutrients provided to plants by PGPR under nutrient-limited conditions **(Babalola, 2010; Sharma et al., 2013; Courty et al., 2015)**. The mechanisms involved in phosphorus uptake by PGPRs remain poorly understood **(Pii et al., 2015)**. Phosphorus is found in soil mainly in an organic form, principally phytate or insoluble inorganic phosphate, and is commonly found as calcium phosphate, hydroxyapatite, and/or rock phosphate **(Richardson et al., 2009)**. Most of the PGPRs act as phosphate solubilizers and convert inaccessible phosphorus into forms that can be absorbed by plants through phytase

action or the production of organic acids **(Sharma et al., 2013)**. Phytase (myoinositol hexakisphosphate phosphohydrolase) is an enzyme that is active in *Bacillus*, *Enterobacter*, *Klebsiella* and *Pseudomonas* **(Jorquera et al., 2011; Sharma et al., 2013; Vacheron et al., 2013)**. For this reason, these PGPRs are collectively referred to as phosphate solubilizing or phytase-producing bacteria (PPB). The capacity to mineralize phytate in combination with other PGPR qualities, *e.g.*, siderophore and phytohormone production, increases the potential use of PGPRs in soils with high organic phosphate contents **(Pii et al., 2015)**. Moreover, phosphate solubilizing PGPR that provide phosphates through the release of organic acids are important in modern agriculture (**Sharma et al., 2013)**. *Bacillus*, *Burkholderia*, *Erwinia*, *Paenibacillus*, *Pseudomonas*, *Rhizobium* and *Serratia* are described in literature as possessing phosphate solubilizing ability through the release of organic acids **(Öğüt et al., 2011)**. The release of organic acids, mainly acetate, oxalate, and citrate, by PGPRs enhances proton efflux and acidifies the rhizosphere; consequently, inorganic phosphate is solubilized from mineral sources **(Bhattacharyya and Jha, 2012)**. The use of phosphate solubilizing PGPRs is considered an environmentally friendly alternative to phosphorus supplementation and improves plant growth.

c. Phytohormones Production:

Auxins are important phytohormones, and the auxin indole-3-acetic acid (IAA) was shown to promote several growth and developmental events, such as cell division, elongation and differentiation **(Asgher et al., 2015)**. IAA is synthesized from and chemically similar to tryptophan. Ljung **(2013)** produced strong evidence favouring auxin-mediated growth and developmental control through alterations in gene expression patterns. Many reports were available depicting varied modulations in the synthesis, transport, metabolism and activity of auxins after plant exposure to stresses **(Ljung, 2013)**; however, plenty of research reports are available advocating the role of auxins in mediating and improving plant tolerance to abiotic stresses **(Kazan, 2013)**. Rice plants exhibited a significant decline in IAA after exposure to

salinity stress. In addition, this variation in IAA can induce growth modulation through an increase in other phytohormones, such as ABA, as reported by Iqbal and Ashraf **(2013)**. Jung and Park **(2011)** found a link among auxin signalling and salt stress which developed through auxin involvement in modulating the membrane bound transcription factor NTM2. Auxins have an important role, whether directly or indirectly, in promoting heavy metal tolerance, as Hu et al. **(2013)** observed that heavy metals have a negative effect on the biosynthesis of auxins. The toxic effect of lead (Pb) on sunflower plant growth was alleviated by the addition of a low concentration of IAA , which stimulated increases in root volume, surface area and diameter **(Fässler et al., 2010)**. IAA induced an increase in shoot biomass and increased Pb and Zn accumulation in plant tissue, indicating the potential of auxins to enhance the phytoextraction of metals. Iqbal and Ashraf **(2007)** have reported a significant mitigation of salt stress-induced hostile effects in wheat after seed priming with IAA, which resulted in ionic homeostasis and induction of SA biosynthesis. These studies indicate the existence of possible crosstalk between auxin and SA that mediates tolerance responses in plants. Salinity restricts the synthesis of IAA; however, the exogenous application of SA proved effective in mitigating hostile effects by causing significant alleviation of salinity-induced inhibition **(Fahad and Bano, 2012)**.

Cytokinins (CK), an important group of plant hormones are involved in maintaining cellular proliferation and differentiation and the prevention of senescence, therefore leading to the inhibition of premature leaf senescence **(Schmulling, 2002)**. However, under stress conditions, particularly water stress at the grain-filling stage, it was observed that stay-green genotypes have the potential to exhibit increased tolerance, which was ascribed to an increased concentration of cytokinin in the xylem sap **(Borrell et al., 2000)**. Zhang et al. **(2010)** demonstrated that cytokinin-over-expressing transgenic cassava exhibited greater tolerance to drought in comparison to wild-type plants. The genes involved in the biosynthesis of cytokinin have been over-expressed, and their role in stress tolerance has been validated **(Peleg**

and Blumwald, 2011). Reduced cytokinin leads to ABA-induced stomatal closure, thereby reducing carbon uptake and assimilation and under stressful conditions, the up-regulation of cytokinin oxidase may also reduce carbon metabolism; work on this topic can be fruitful in improving the plant growth and yield. Mohapatra et al. **(2011)** demonstrated that cytokinin improves grain filling. Currently, exogenous application of cytokinin is being employed to optimize the internal concentrations of cytokinin. It has also been documented that heavy metals, such as zinc and lead, severely hamper the seedling growth of chickpea through the inhibition of GA_3 and Z concentrations in plant tissue **(Atici et al., 2005)**. In an earlier report, the application of kinetin to chickpea stimulated plant growth and development under salt stress **(Bozcuk, 1981).**

Another important plant growth regulator is gibberellin, often referred to as gibberellic acid (GA3), which has a vital role in seed dormancy formation of floral organs and lateral shoot growth **(Olszewski et al., 2002)**. GA3 was found to stimulate plant growth and development under various abiotic stress conditions **(Ahmad, 2010)**. Enhanced plant water uptake and reduced stomatal resistance was observed in gibberellic acid-treated tomato plants under saline conditions **(Maggio et al., 2010)**. GA3 induces efficient uptake and ion partitioning within the plant system, leading to enhanced growth and maintaining the metabolism of plants under normal and stress conditions **(Iqbal and Ashraf, 2013)**. Under salt stress conditions, improved germination and growth due to gibberellic acid has been reported by several studies **(Tuna et al., 2008; Ahmad, 2010; Manjili et al., 2012)**. The synthesis of gibberellins can also be promoted through the application of other hormones, such as auxin **(Wolbang et al., 2004)**. Enhanced synthesis of gibberellic acid leads to enhanced ABA catabolism. Moreover, GA3 directly affects growth, yield and mineral nutrition as well as nitrogen metabolism. Khan et al. **(2004)** reported that increase in fruit yield, leaf area and nitrogen phosphorous and potassium uptake in tomato was due to the exogenous application of GA3. The endogenous application of GA3 resulted in amendment of

osmotic stress in plants and maintenance of tissue water content **(Ahmad, 2010)**. Such effects were observed in wheat **(Manjili et al., 2012)** and maize **(Tuna et al., 2008)**. In addition, gibberellic acid enhanced antioxidant enzyme activity by lowering the levels of reactive oxygen species (ROS) that contribute to better growth under stress **(Manjili et al., 2012)**. In addition, exogenous application of GA3 mitigates salinity-induced effects on germination and growth in *Arabidopsis thaliana* by mediating enhanced synthesis of SA, which causes increased activity of isochorismate synthase 1 **(Alonso-Ramirez et al., 2009)**.

Ethylene is an essential phytohormone for the normal growth and development of plants **(Khalid et al. 2006)**. This plant growth hormone is produced endogenously by approximately all plants and is also produced by different biotic and abiotic processes in soils and is important in inducing multifarious physiological changes in plants. Apart from being a plant growth regulator, ethylene has also been established as a stress hormone **(Saleem et al., 2007)**. Under stress conditions like those generated by salinity, drought, water logging, heavy metals and pathogenicity, simplified as biotic and abiotic conditions the endogenous level of ethylene is significantly increased which negatively affects the overall plant growth. For instance, the high concentration of ethylene induces defoliation and other cellular processes that may lead to reduced crop performance **(Saleem et al., 2007; Bhattacharyya and Jha, 2012)**. PGPRs, which possess the enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, facilitate plant growth and development by decreasing ethylene levels, inducing salt tolerance and reducing drought stress in plants **(Nadeem et al., 2007; Zahir et al., 2008)**. Currently, bacterial strains exhibiting ACC deaminase activity have been identified in a wide range of genera such as *Acinetobacter*, *Achromobacter*, *Agrobacterium*, *Alcaligenes*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Ralstonia*, *Serratia* and *Rhizobium* etc. **(Shaharoona et al., 2007a, b; Nadeem et al., 2007; Zahir et al., 2008; Zahir et al., 2009; Kang et al., 2010)**. Such rhizobacteria take up the ethylene precursor ACC and convert it into α-ketobutyrate

and NH3 **(Arshad et al., 2007)**. Several forms of stress are relieved by ACC deaminase producers, such as effects of phytopathogenic microorganisms (viruses, bacteria, and fungi etc.) and resistance to stress from polyaromatic hydrocarbons, heavy metals, radiation, wounding, insect predation, high salt concentration, draft, extremes of temperature, high light intensity and flooding **(Glick, 2012; Lugtenberg and Kamilova, 2009)**.

3.2.3. Indirect mechanisms of action:

Several PGPRs are known to reduce the effects of plant stresses by limiting phytopathogen-caused damage. This can occur, *e.g*. via local antagonism of soilborn pathogens, or by induction of systemic resistance against pathogens throughout the entire plant. Over the last decades, a great diversity of rhizospheric microorganisms has been described, characterized and in many cases tested for activity as biocontrol agents against soilborn pathogens. Such microorganisms can produce substances that may limit the damage caused by phytopathogens, *e.g*. by producing antibiotics, siderophores and a variety of enzymes. These microorganisms can also function as competitors of pathogens for colonization sites and nutrients. Nevertheless, biocontrol has not yet become widely applied, for several reasons: for instance, the efficiency of a biocontrol strain under field conditions is likely to be affected by several environmental conditions like pH, temperature, water content and interactions with other microorganisms. Also, some biocontrol agents that showed promising traits in initial experiments failed to be efficient rhizosphere colonizers under these limitations, and genetic, biochemical and physiological factors that contribute to the activity of biocontrol agents.

Iron is a vital nutrient for almost all forms of life. In the aerobic environment, iron occurs principally as Fe^{3+} and is likely to form insoluble hydroxides and oxyhydroxides, thus making it generally inaccessible to both plants and

microorganisms **(Rajkumar et al., 2010)**. Commonly, bacteria acquire iron by the secretion of low-molecular mass iron chelators referred to as siderophores, which have high association constants for complexing iron. Most of the siderophores are water-soluble and can be divided into extracellular siderophores and intracellular siderophores. Thus, siderophores act as solubilizing agents for iron from minerals or organic compounds under conditions of iron limitation **(Indiragandhi et al., 2008)**. Not only iron, siderophores also form stable complexes with other heavy metals that are of environmental concern, such as Al, Cd, Cu, Ga, In, Pb and Zn, as well as with radionuclides including U and Np **(Neubauer et al., 2000; Kiss and Farkas, 1998)**. Binding of the siderophore to a metal increases the soluble metal concentration **(Rajkumar et al.,2010)**.

Hence, bacterial siderophores help to alleviate the stresses imposed on plants by high soil levels of heavy metals. Plants assimilate iron from bacterial siderophores by means of different mechanisms, for instance, chelate and release of iron, the direct uptake of siderophore-Fe complexes, or by a ligand exchange reaction **(Schmidt, 1999)**. Numerous studies of the plant growth promotion in relation to siderophore-mediated Fe-uptake, as a result of siderophore producing rhizobacterial inoculations, have been reported **(Rajkumar et al., 2010)**. For example, Crowley and Kraemer **(2007)** revealed a siderophore mediated iron transport system in oat plants and inferred that siderophores produced by rhizosphere microorganisms deliver iron to oat, which has mechanisms for using Fe-siderophore complexes under iron-limited conditions. Similarly, the Fe-pyoverdine complex synthesized by *Pseudomonas fluorescens* C7 was taken up by *Arabidopsis thaliana* plants, leading to an increase of iron inside plant tissues and to improved plant growth **(Vansuyt et al., 2007)**.

One of the major mechanisms used by PGPR to control soil borne pathogens involves the production of cell wall-degrading enzymes **(Chet et al., 1990; Kobayashi et al., 2002)**. Cell wall-degrading enzymes such as β-1,3-glucanase, chitinase, cellulase and protease secreted by biocontrol strains of PGPR exert a direct inhibitory effect on

the hyphal growth of fungal pathogens by degrading their cell wall. Chitinase degrades chitin, an insoluble linear polymer of β-1, 4-N-acetyl-glucoseamine, which is the major component of the fungal cell wall. The β-1,3-glucanase synthesized by strains of *Paenibacillus* and *Streptomyces* spp. can easily degrade fungal cell walls of pathogenic *F. oxysporum* **(Compant et al., 2005)**. In a similar manner, *Burkholderia cepacia* synthesizes β-1,3-glucanase, which destroys the cell walls of the soil borne pathogens *R. solani*, *P. ultimum*, and *Sclerotium rolfsii* **(Compant et al., 2005)**. Potential biocontrol agents with chitinolytic activities include *Bacillus licheniformis*, *B. cereus*, *B. circulans*, *B. subtilis* and *B. thuringiensis* **(Sadfi et al., 2001)**. Among the Gram-negative bacteria, *Serratia marcescens*, *Enterobacter agglomerans*, *Pseudomonas aeruginosa* and *P. fluorescens* have been found to possess chitinolytic activities **(Neiendam-Nielsen and Sørensen, 1999)**. Cell wall-degrading enzymes of rhizobacteria affect the structural integrity of the walls of the target pathogen: **Budi et al., (2000)** and **Someya et al., (2000)** studied the chitinolytic and antifungal activities of a potent biocontrol strain of *Serratia marcescens* B2 against the soil borne pathogens *Rhizoctonia solani* and *Fusarium oxysporum*. The mycelia of the fungal pathogens co-inoculated with this strain showed various abnormalities such as partial swelling in the hyphae and at the tip, hyphal curling, or bursting of the hyphal tip. Examples of protection from phytopathogenic infection as a result of the activity of cell wall-degrading enzymes include control of *Sclerotium rolfsii* and *F. oxysporum* on beans **(Felse and Panda, 2000)**. Thus, the production of these enzymes by PGPR can categorize them as biocontrol agent against fungal pathogens.

Use of microbial antagonists against plant pathogens in agricultural crops has been proposed as an alternative to chemical pesticides. PGPRs belonging to *Bacillus*, *Pseudomonas* and *Streptomyces* species play an active role in the suppression of pathogenic micro-organisms producing antibiotics. These bacterial antagonists enforce suppression of plant pathogens by the secretion of extracellular metabolites that are inhibitory even at low concentration. Bacteria belonging to *Bacillus* genus

produce a wide variety of antibacterial and antifungal antibiotics. Some of these compounds including subtilin, subtilosin A, TasA and sublancin are well known and are derived from ribosomal origin, but others, such as bacilysin, chlorotetain, mycobacillin, rhizocticins, bacillaene, difficidin and lipopeptides belonging to the surfactin, iturin and fengycin families, are formed by non-ribosomal peptide synthetases (NRPSs) and/or polyketide synthases (PKS) **(Leclere et al., 2005)**. Antibiotics are also produced by strains of *Pseudomonas* where *P. fluorescens* and *P. aeruginosa* are thoroughly studied.

Interestingly, interactions of streptomycetes with plants lead to suppression of the innate plant responses to phytopathogens. Therefore, it is of great importance to choose and characterize single *Streptomyces* strains for possible use as microbial antagonists. This is conveniently done through extensive *in vitro* and *in planta* studies on the roles of their antibiotics and possible production of VOCs **(Citron et al., 2015)**. One of the most common metabolites in streptomycetes communities is geosmin, a bicyclic alcohol derivative of decalin that confers the typical "earthy" flavour to the substrates they colonize **(Gerber, N.N.; Lechevalier, 1965)**. Geosmin may be regarded as a volatile organic compound of microbial origin to which the human nose is extremely sensitive **(Polak and Provasi, 1992)**. Although geosmin has no known antibiotic activity and its adaptive significance is not yet known, this metabolite might have an important role in the biology of streptomycetes **(Rosenzweig, 2014):** indeed, it is a well-conserved trait and the gene responsible is highly conserved among *Streptomyces* spp. **(Hopwood, 2007)**. Geosmin enables bacteria to adapt to various environments, such as microbial communities or the host, ultimately influencing bacterial competition and cooperation **(Audrain et al., 2015)**. It also has ability to induce selective growth of geosmin-utilizing bacteria **(Ngoc et al., 2016)**.

3.3. Bacterial colonization studies

PGPR were first defined by Kloepper and Schroth **(1978)** to describe soil bacteria colonizing the roots of plants following inoculation onto seeds, and that

enhance plant growth. This implies a colonization process that includes the ability to (1) survive inoculation onto seed; (2) multiply in the spermosphere (region surrounding the seed) in response to seed exudates; (3) attach to the root surface; and (4) colonize the developing root system **(Kloepper, 1994)**. The ineffectiveness of PGPRs in the field has often been attributed to their inability to colonize plant roots **(Benizri et al., 2001; Bolemberg and Lutenberg, 2001)**. A variety of bacterial traits and specific genes contribute to root colonization, but only a few have been identified **(Lugtenberg et al., 2001)** and include motility, chemotaxis to seed and root exudates, production of pili or fimbriae, production of specific cell surface components, ability to use specific components of root exudates, protein secretion, and recently biofilm-forming ability of the microbes and quorum sensing **(Sharma et al., 2003)**. The generation of mutants altered in expression of these traits is aiding our understanding of the precise role each plays in the colonization process **(Persello-Cartieaux et al., 2003)**. Progress in the identification of new, previously uncharacterized genes is being made using nonbiased screening strategies that rely on gene fusion technologies. These strategies employ promoterless reporter transposons **(Roberts et al., 1999)** and *in vitro* expression technology **(Rainey, 1999)** to detect genes expressed during colonization.

An important aspect of colonization is the ability to compete with indigenous microorganisms present in the soil and rhizosphere of the developing plant. Understanding of the factors involved in these interactions has been hindered by inability to culture and characterize diverse members of the rhizosphere community and to determine how that community varies with plant species, plant age, location on the root and soil properties. Phenotypic and genotypic approaches are now available to characterize rhizobacterial community structure. Phenotypic methods that rely on the ability to culture microorganisms include standard plating methods on selective media, community level physiological profiles (CLPP) using the BIOLOG system **(Garland, 1996)**, phospholipid fatty acid (PLFA) **(Tunlid and White, 1992)** and

fatty acid methyl ester (FAME) profiling **(Germida et al., 1998)**. Culture-independent molecular techniques are based on direct extraction of DNA from soil and 16S-rRNA gene sequence analysis, bacterial artificial chromosome or expression cloning systems **(Rondon et al., 1999)**. These are providing new insights into the diversity of rhizosphere microbial communities, the heterogeneity of the root environment and the importance of environmental and biological factors in determining community structure **(Baudoin et al., 2002; Berg et al., 2002; Smalla et al., 2001)**. These approaches can also be used to determine the impact of inoculation of PGPR on the rhizosphere community **(Ciccillo et al., 2002; Steddom et al., 2002)**.

Various microbial inoculants are used to treat plant seeds and seedling roots to promote plant growth and protect plant health. Numerous factors, both biotic and abiotic, are known to influence the performance of inoculated bacteria under field conditions. These factors may influence inoculants survival, colonization and establishment in the rhizosphere; however, in many cases no assessment has been made to detect the presence and colonization of inoculated bacteria in the rhizosphere. This is primarily due to the absence of selection criteria for the inoculants to distinguish them from indigenous bacteria. Recent advances in molecular techniques have given hope for developing inoculants with specific markers to be included for detection and colonization in the rhizosphere and to assess their performance.

3.3.1. Root colonization:

Root exudates released into the soil environment from plants have been traditionally grouped into low- and high-molecular weight compounds. High-molecular weight compounds include polysaccharides, mucilage and proteins. Plant mucilages are released from the root cap, the primary cell wall between epidermal and sloughed root cap and epidermal cells (including root hairs). Lysates are released from roots during autolysis. Rhizospheric microorganisms also release microbial mucilages. Collectively, plant and microbial mucilages, microbial cells and their

products together with associated organic and mineral matter are referred to as mucigel **(Walker et al., 2003)**. Low-molecular organic compounds released by plant roots include ethylene, sugars, amino acids, vitamins, polysaccharides, and enzymes. Nutritional resources influence population structure and play a role in niche colonization and competition. The microbial population in and around roots includes bacteria, fungi, yeasts, and protozoa. Bacterial populations in the rhizosphere are predominantly Gram-negative short rods including species of *Pseudomonas*, *Flavobacterium* and *Alcaligenes*, etc. Some are free-living while others form symbiotic associations with plants. The interaction between microorganisms and roots may be beneficial, harmful, or neutral for the plant and sometimes the effect of microorganisms may vary as consequence of soil conditions **(Alexander, 1985; Lynch, 1990; Ahmad, 2006)**. Root colonizers may be pathogenic, symbiotic and plant growth-promoting microorganisms. Based on these activities, the plant-beneficial microorganisms can be classified as biofertilizers, phytostimulators, rhizoremediators and biopesticides. Despite their importance to plant growth, the molecular basis of colonization in these plant–microbe interactions are not completely understood. This is a key reason for the limited success of PGPRs in field conditions **(Ahmad et al., 2011)**.

3.3.2. Endophytic colonization:

Several bacteria deriving from the rhizosphere do not only colonize the rhizosphere and/or the rhizoplane, but can also enter plants and colonize internal tissues and many of them have shown plant growth-promoting effects **(Hallmann, 2001; Compant et al., 2005b, 2008a; Sessitsch et al., 2004; Hallmann and Berg, 2007)**. As early as 1887, Victor Galippe postulated that soil microorganisms can penetrate tissues of healthy plants and that the involved colonization mechanisms needed to be investigated **(Galippe, 1887)**. These early findings were, however, dismissed due to the general belief that microorganisms detected inside plants represent contaminants

obtained during the isolation process. Several recent studies confirm that plants host diverse endophytic communities **(Idris et al., 2004; Krechel et al., 2004)** and that endophytic bacteria mostly derive from the rhizosphere **(Hardoim et al., 2008)**. Endophytes represent a subgroup of the rhizobacterial communities, which could enter the endorhiza (the root interior) of their hosts once the rhizoplane is colonized **(Gray and Smith, 2005; Rosenblueth and MartínezRomero, 2006; Hallmann and Berg, 2007)**. In general, endophytes are more likely to show plant growth-promoting effects than bacteria exclusively colonizing the rhizosphere **(Conn et al., 1997; Chanway et al., 2000)**.

Following rhizosphere and rhizoplane colonization, some soil borne microorganisms can enter roots, and establish subpopulations ranging from 10^5-10^7 CFU g-1 of fresh weight **(Hallmann, 2001)**. This involves specific traits required for endophytic competence, *i.e.* the ability to successfully colonize the host plant. The penetration process does not necessarily involve active mechanisms and thus all rhizosphere bacteria can be expected to be endophytic at one stage of their life **(Hardoim et al., 2008)**. Passive penetration can take place at cracks, such as those occurring at root emergence sites or created by deleterious microorganisms, as well as by root tips. For certain bacteria specific adaptations have evolved, such as for nodulating bacteria or microbes, which have specific mechanisms for active penetration of the root system **(Hardoim et al., 2008)**. Although not frequently investigated, it is well known that endophytes may spread systemically inside the plant and colonize the root system and the aerial parts **(Hardoim et al., 2008)**, where their cultivable population densities may reach 10^3-10^4 CFU g-1 of fresh weight under natural conditions **(Hallmann, 2001)**. It is not clear, whether endophytes colonizing roots or above ground plant tissues have different effects on the plant or whether root colonization is enough for conferring beneficial effects. Some endophytes colonize nutrient-rich intercellular spaces of plant hosts using them to spread inside host plants **(Cavalcante and Dobereiner, 1988; Dong et al., 1994)**. Some systemic bacterial

colonizers can also use the lumen of xylem vessels to spread throughout the plant **(James et al., 2001; Compant et al., 2005b, 2008a)**. Lumen colonization of xylem vessels has been, however, more frequently reported as a route for spreading of endophytic bacteria to reach vegetative plant parts, probably because they are open conduits, whereas migration along intercellular spaces requires the secretion of active cell wall degrading enzymes. Although at the beginning of the 1990's it was strongly argued, that lumen xylem colonization is a property of phytopathogens **(McCully, 2001)**, it is nowadays known that non-phytopathogenic endophytes can spread inside plants in the same manner. Beneficial bacteria can pass from one xylem element to another using the perforated plates. The size of the plate holes allows the passage of bacteria without requiring specific, enzymatic activity **(Bartz, 2005)**. Bacterial flagella and/or the plant transpiration stream seem to further support their movements inside plants **(James et al., 2002; Compant et al., 2005b)**. However, only few endophytes can colonize aerial vegetative plants parts **(Hallmann, 2001),** as they must pass over several barriers as well as need to possess the physiological requirements to establish in different plant niches. Those migrating to the above ground parts are thus well adapted to this endophytic environment.

3.3.3. Green fluorescent protein tagging to visualize PGPR:

Initial studies using auto fluorescent proteins (in specific GFP) as markers for PGPR were used for localization studies. Most of these studies showed that PGPR and other microorganisms, such as phytopathogenic fungi, preferentially colonize the junctions between the root cells **(Bloemberg et al., 1997, 2000; Tombolini et al., 1999; Lagopodi et al., 2002; Bolwerk et al., 2003; Gamalero et al., 2005)**. Most steps in the symbiosis process between *Rhizobiaceae* and leguminous plants occur inside the root after entrance into the infection thread. The use of GFP allowed us to visualize the process of attachment, entrance and nodule occupancy in detail **(Gage et al., 1996)** making it even possible to determine the growth rate of the cells in the infection thread

(Gage et al., 1996). Stuurman et al. **(2000)** showed that GFP tagged *Rhizobium* bacteroids move in the root nodule. Since the root is sometimes too thick for successful imaging, due to loss of light in the deeper root parts, sectioning of the plant material can solve this. This is also used for the study of endophytes, as shown by several publications on *Herbaspirillum* spp. **(Elbeltagy et al., 2001)** and the pathogen invasion of *Xylella fastidiosa* **(Newman et al., 2003)**. When necessary, plant material can also be stored before visualization by fixation with paraformaldehyde, which leaves GFP intact for fluorescent studies **(Stuurman et al., 2000; Elbeltagy et al., 2001)**. Confocal laser scanning microscopy (CLSM) analysis of the colonization behaviour of gfp-tagged antagonistic strains can also provide important information on the sampling strategy required for monitoring inoculant strains and combine these with data on the kinetics of the endogenous microflora **(Gotz et al., 2006)**. Localization studies are also valuable for the analysis of the properties of mutant strains, for instance for their adhering abilities. For example, Biancotto et al. **(2001)** showed, by using mucoid mutant strains of *P. fluorescens* CHA0, that acidic extracellular polysaccharides (EPS) are an important factor in adhesion to roots and fungi **(Bloemberg, 2007)**.

3.4. Bacterial spot disease of tomato

The bacterial spot or scab disease is seed-borne and probably occurs wherever tomato was grown as extensively as field crop. The causal agent of the disease is *Xanthomonas campestris* pv. *vesicatoria* **(Doidge, 1920)**, which affect natural hosts like tomato (*Lycopersicon esculentum)*.

3.4.1. Importance and distribution of the disease

The disease occurs worldwide, it causes losses in USA, Australia, Argentina, India, Sudan, Nigeria, Egypt, Italy, Russia, Austria, Romania, and Yugoslavia **(Smith et al.,1988)**. It is an important disease of outdoor-growing crops causing considerable damage to the leaves and stems especially of seedlings, but it is most noticeable by its effects on the fruits. The disease is well developed in warm temperate climates **(Fahy and Persley, 1983; Lelliott and Stead, 1987)**.

3.4.2. Characterization of the bacterial leaf spot pathogen of tomato

The actual scientific name of the pathogen is *Xanthomonas vesicatoria* (ex-Doidge) which is synonym. of *Xanthomonas campestris* pv. *vesicatoria* (ex-Doidge). It is closely related to the species of the genus *Pseudomonas*. It is Gram negative, rod shape and belongs to the family Xanthomonadaceae **(Bradbury, 1984)**. As other *Xanthomonas* spp., their cells are 1.0-1.5 x 0.6-0.7μm in size with only one polar flagellum, straight or slightly curved, on the other hand, they never denitrifying nitrate. Colonies appear on the third day after cultivation, producing highly characteristic pale-yellow lens-shape colonies on nutrient broth agar, or dark yellow pigments (xanthomonadins) on GYC agar medium.

Xanthomonas species are plant pathogens, in which *Xanthomonas campestris* has many pathovars most of which are host specific **(Smith et al., 1988)**. *Xanthomonas vesicatoria* was before a pathovar of *Xanthomonas campestris* **(Elliott, 1951; Hayward and Waterston, 1964a)**. Three biotypes can be distinguished; One type only infects pepper, another one infects tomato, the third type attacks both **(Lovrekovich and Klement, 1965; Agrios, 1997)**. Strains originating from tomato and pepper behave differently on nutrient agar containing soluble starch. Pepper isolates do not hydrolyze starch, all tomato isolates strongly hydrolyze starch, except one group of isolates **(Király et al., 1974)**.

It has been differentiated into four groups (races) **(Cook and Stall, 1982)** later, Ritchie and Dittapongpitch **(1991)** described ten races based on pathogenicity to *Capsicum annuum* (pepper) cultivars. Also, pathological, biochemical, serological and phage sensitivity tests have proved that *Xanthomonas vesicatoria* is not a uniform species.

3.4.3. The disease progression

The pathogen survives over wintertime as a seed contaminant or in infected plant debris, in the soil and in other hosts, it can penetrate leaves through stomata and wounds and fruits through wounds and lenticels. The disease spreads by rain, insects,

wind, or direct contact of diseased plant parts. Infection of flower parts usually results in serious blossom drop. Optimum conditions for disease development are at a temperature of about 30 ^{0}C and relative humidity of about 80% **(Smith et al.,1988)**. Numerous spots on infected leaves may cause defoliation or make the leaves appear ragged. Spots on leaves appear earlier and in greater numbers at 28-30 ^{0}C **(Király et al., 1974; Agrios, 1997)**.

The pathogen penetrates the intercellular spaces through stomata. Multiplying bacteria cause blistering which in time results in the development of the bacteria through the cracks once again reach the surface and from here splashing rain, wind and insects carry bacteria to healthy plants **(Smith et al., 1988)**. The disease causes significant damage on fruits where brown spots appear. Symptoms are quite obvious on green or red fruits. In green fruits, first tiny dark green and brown-black round bulging spots appear. Later they spread and coalesce due to the attacked and lacerated epidermis and cuticle. The developing fruit may crack, providing the opportunity for attack by secondary organisms. Such fruits may rot while still on the plant **(Király et al., 1974; Agrios, 1997)**.***3.4.4.***

3.4.4. Symptoms of the disease

Bacterial spot disease on tomato is often characterized by lesions being small, brown to black spots usually with chlorotic margins that are most visible on the underside of leaves. In stems these spots are round or elongated. Spots may coalesce causing cankerous stem lesions suberized with time. These symptoms eventually result in leaf blight and premature abscission. In fruits, spots appear as slightly raised, corky scabs, usually irregular in shape, surrounded by water-soaked margins **(Fahy and Persley, 1983)**. Later in the season, spots become brown to dark, slightly sunken, with a rough, scab surface and the fruit epidermis rolled back. Spots that become irregularly circular with a yellow, translucent margin have brown to black, later parchment-like centers. Spots may coalesce and form irregular streaks along veins or

leaf margins. Edges and tips of leaves may become dead, dry and breakaway giving leaves a tattered appearance.

A **B**

Figure 3.2. Bacterial spot disease of Tomato caused by *Xanthomonas vesicatoria.* A: Tomato fruit infected with bacterial spot, irregular spots with water-soaked margins. B: Bacterial spot on tomato leaves. Image adopted from our field study during the year 2017-2018.

Heavily infected leaves turn yellow or brown and young leaves become distorted and die (Király et al., 1974; Smith et al., 1988; Lelliott and Stead, 1987; Agrios, 1997).

3.4.5. Control measures

The success of disease control measures depends on the use of pathogenic bacteria-free (certified) seeds and seedlings, resistant varieties, crop rotations and sprays with fixed copper fungicides in the field. Under reasonably dry weather, premixed Bordeaux mixture and Zineb are also used **(Agrios, 1997)**. Phosetyl Aluminum is considered to affect the pathogen indirectly and to induce natural resistance mechanism in treated ornamental plant species infected with bacterial spot and blight caused by *Xanthomonas campestris* **(Chase, 1987)**. Seed treatments or

dressings or hot water treatment (for tomato only), streptomycin spraying (where allowed), and 3 -4 years' rotations were also recommended **(Smith et al., 1988)**.

Biological control: The use of beneficial bacteria such as PGPRs as biological control agents of bacterial spot diseases was reported during the last decade and gave promising results. Certain *Pseudomonas fluorescens* strains have been isolated that colonized tomato and sweet pepper seeds and showed an antagonistic activity to *Xanthomonas vesicatoria* **Campbell et al., 1998; Amat and Larrinaga, 1992; Colin et al., 1984; Tzeng et al., 1994** have shown that different strains of *Pseudomonas fluorescens* have clear inhibitory effects on *Xanthomonas vesicatoria* and many other *Xanthomonas campestris* pathovars under *in vitro* conditions. Protozoa have been also used against some pathovars of *Xanthomonas campestris* in soil and have promising results **(Habte and Alxender, 1975)**.

4. MATERIALS AND METHODS:

4.1. Isolation and characterization of endophytes

4.1.1. Isolation of bacteria

Root samples from the healthy plants like grapevine, tomato, pepper and olive plants were collected from the study site which is located in multiple sites along with rhizosphere soil. For the isolation of endophytic bacteria, 2–5 *g* of fresh roots was washed under running tap water and surface sterilized in 0.53% NaOCl for 1 min. After washing three times with sterilized distilled water, the root samples were ground with a sterilized mortar and pestle. Serial dilutions were prepared from the ground roots, and 100 µl aliquots from each dilution of 1×10^{-6}, 1×10^{-7}, and 1×10^{-8} CFU mL^{-1} were spread on King's B agar, nutrient agar and ISP-2 plates and incubated for 2-7 days at 28 ± 2 °C. Morphologically distinct bacterial colonies were selected for further purifications. The purified isolates were preserved and further characterized for morphological and microscopic analysis as described by Islam et al. **(2016)**.

4.1.2. Gram staining/microscopic analysis

The bacterial isolates were differentiated through colony morphology, microscopic examination and biochemical test. Morphological characterization of the isolates was done by Gram's staining method as described by Beveridge **(2001)**. Further the bacterial isolates were identified according to the criteria given in Bergeys's manual of Determinative Bacteriology **(Holt et al., 1994)**.

4.1.3. Motility test

Strains were tested for (i) swimming, (ii) swarming and (iii) twitching motility. Swimming motility was assayed on nutrient media swim plates (peptone 1%, NaCl 0.5%, agar, 0.3%), which was spot inoculated with 1% of overnight culture (0.5 OD at

λ = 600 nm) into the centre of the agar. For swarming, swam plates were prepared using 0.5% of agar in nutrient broth supplemented with 0.5% glucose and inoculated with 1% culture. The plates were incubated at 28 °C for 24 h. Swimming motility was evaluated within semisolid agar medium and swarming motility was checked across the agar surface. For twitching motility, endophytic bacteria were stab inoculated into the bottom of the petri dish with agar medium (1% of agar in nutrient broth). Plates were incubated for 24 to 48 h at 28 °C and the motility diameter at the interface between the agar medium and petri dish surface was measured by staining with 0.1% (w/vol) crystal violet (1 min) **(Sandhya and Ali, 2018)**.

4.2. Plant growth promoting traits

4.2.1. Determination of indole acetic acid production

IAA production by bacterial strains was estimated based on the method of Gordon and Weber **(1951)**. Five-hundred microliters of bacterial suspensions from 24 h-old culture were inoculated into 50 ml of nutrient broth added with 0.1% DL-tryptophan and incubated at 30 ± 0.1 °C for 2 days in dark. After incubation, the bacterial cultures were centrifuged at 10,000 rpm for 10 min. Salkowski reagent (4 ml) was added to one ml of collected supernatant and incubated for 30 min: the development of pink colour was observed as an indication of IAA production. To quantify IAA, absorbance was taken at λ = 535 nm by using UV/Visible spectrophotometer. The IAA concentration was estimated with a standard curve of IAA.

4.2.2. Siderophore production assay

The bacterial isolates were qualitatively screened for siderophore production by spot inoculation on the surface of Chrome Azurole' S agar medium according to the method of Schwyn and Neilands, **(1987)** and incubated at 28 °C for 3 days. The development of orange haloes around the bacterial colonies after incubation was considered as an indication for siderophore production.

4.2.3. Hydrogen cyanide production

For hydrogen yyanide (HCN) production bacterial isolates were streaked on nutrient agar medium supplemented with 0.4% of glycine. The production of cyanide was detected by placing Whatman filter paper No.1 soaked in solution of 0.5% picric acid in 2% sodium carbonate under side of the petri dish lids. The development of orange colour after incubation of three days indicates HCN production **(Bakker and Schipper, 1987)**.

4.2.4. Qualitative estimation of phosphate solubilization

All of the bacterial isolates were screened qualitatively for phosphate solubilization on the Pikovskaya agar medium containing tri-calcium phosphate (TCP) as spot inoculation. All the bacterial isolates were spot inoculated onto these plates. The phosphate solubilization ability was analyzed by the formation of a halo zone around colonies after incubation at 28 °C for seven days. The phosphate solubilization ability was analyzed by the formation of a halo zone around the colonies, indicating solubilization of tricalcium phosphate **(Mehta and Nautiyal, 2001)** that was measured in millimetres. The halo zone was determined by the phosphate solubilization index (PSI). PSI was calculated by using the following formula.

$$PSI = \frac{\text{Colony diameter}}{\text{Halo diameter + Colony diameter}}$$

4.2.5. Determination of Ammonia production

The production of ammonia was determined by the method of Cappuccino and Sherman **(1992)**. According to this method, the bacterial strains were grown in test tubes having 10 ml of peptone water and incubated at 30 ± 0.1 °C for 2 days. After that,

Nessler's reagent (0.5 ml) was added into each tube and the development of yellow to brown colour indicating ammonia production was observed.

4.3. *In vitro* Antagonistic activity

4.3.1. Antibacterial activity

To test possible antibacterial activity, the following phytopathogenic bacteria were tested as indicator strains: *Clavibacter michiganensis* subsp. *michiganensis, Clavibacter michiganensis* subsp. *sepedonicus, Acidovorax citrulli, Ralstonia solanacearum* and *Xanthomonas vesicatoria* (these strains were present at Prof. Emilio Stefani lab, UNIMORE). For each putative bacterial antagonist, a 15 µl droplet of bacterial suspension at a concentration of 10^8 cfu/ml, spectrophotometrically adjusted, was pipetted onto nutrient agar in a Petri dish and then incubated for 48 hr at 27°C, to allow the growth of a macrocolony of the putative antagonist in the centre of the inoculated dish. Once the macrocolonies were grown, the Petri dishes were taken out from the incubator, opened inside a sterile hood and sprayed with a suspension of bacterial pathogen (i.., indicator) at a concentration of 10^6 cfu/ml. The Petri dishes were then closed and placed again into the incubator at the same temperature as above, for additional 2 days, to allow growth of the pathogen. The inhibition activity of the putative bacterial antagonists against test pathogen was measured calculating the average inhibition area (AIA) around the macrocolony as AIA= (R2*3.14) – (r2*3.14) all the experiments *in vitro* were done in triplicate **(Tontou et al., 2016)**.

4.3.2. Antifungal activity

For antifungal activity, following phytopathogenic fungi were considered: *Rhizoctonia solani, Sclerotinia sclerotiorum, Alternaria alternata, Fusarium oxysporum* f. sp. *lycopersici* and *Monilia laxa* (these strains were present at Prof. Emilio Stefani lab, UNIMORE). The test isolates were spot inoculated (15 µl of a suspension containing 10^8 cfu/ml, spectrophotometrically adjusted) at one side (1 cm from the edge) of the

potato dextrose agar (PDA) plates 48 h before pathogen inoculation. Five days old fungal discs (4 mm diameter), grown on potato dextrose agar (PDA) at 28 °C were placed at another side (1 cm from the edge) of PDA plates, opposite to test bacterial inoculation. Plates without the test isolate served as control. All plates were incubated at 30 ± 2 °C for 5 days. After incubation, the zone of inhibition (in mm) was measured and colony growth inhibition (%) was calculated by using the formula: $PI = (C - T)/C \times 100$, where PI is the percent inhibition, C is the colony growth of pathogen in control, and T is the colony growth of pathogen in dual culture. All isolates were tested in triplicate **(Shrivastava et al., 2017)**.

4.4. *In planta* studies on Tomato as model plant

4.4.1. Plant growth promotion

Pot experiments were carried out using tomato (*Solanum lycopersicum* L. var. Leader F1, ISI SEMENTI, Italy) as a test plant under green-house conditions. Seeds were surface sterilized with 0.1% $HgCl_2$ (Mercuric Chloride) followed by sterilizing in 70% ethanol for 1 min. The seed were then rinsed 5–6 times in sterile water and sown in plastic pots (surface-sterilized) filled with 300 g sterile vertisol soil. After seedling emergence, 1 ml of test bacterial culture (most prospective PGPRs SA51 & PT65) containing approximately 10^8 cfu/ml cells was applied to the soil around each seedling. Both inoculated and un-inoculated treatments were replicated 3 times with each replicate consisting in 12 plants, each one in a single pot. Soil moisture was maintained during the experiment by daily sprinkling with sterile distilled water. After five consecutive treatments (each treatment every 10 days) plants were measured for root and shoot length, and dry biomass was recorded according to standard protocols **(Vurukonda et al., 2016)**.

4.4.2. Biocontrol activity

The two most prospective beneficial microorganisms, *Streptomyces* sp., strain SA51 and *Pseudomonas* sp., strain PT65 were tested against *Xanthomonas vesicatoria* in

greenhouse conditions, individually and as co-inoculants. Twenty-one-day old tomato seedlings were transplanted to pots and antagonist(s) were inoculated by drenching the roots on each plant every 10 days (3 treatments, with a microbial suspension of 10^8 cfu/ml). Four weeks after transplanting, *X. vesicatoria* was spray-inoculated as foliar application at a concentration of 10^8 cfu/ml. Each inoculated plant was sealed in a polythene bag (PE) overnight, and the bags were removed early the next morning. The first disease ratings were done as symptoms appearing (~65-70 days old plants) from inoculation) and were carried out weekly for three weeks. Disease severity was evaluated using a descriptive scale ranging from 0 to 4: 0 = no symptom; 1 = 1-10 spots on 1-3 leaves; 2 = 11-30 spots on 4-10 leaves; 3 = more than 30 spots and some confluent necrosis on 5-20 leaves; 4= confluent necrosis on more than 20 leaves or branch desiccation. Data were collected and statistically evaluated **(Giovanardi et al., 2015).** For each treatment 12 plants were evaluated for disease severity and the experiment was repeated for three biological replicates. All the results were statistically evaluated and disease control efficacy for each treatment can be calculated using following formula: Disease control efficacy (%) = Control value – Treatment value / Control value **(Ahmet et al., 2018)**.

4.4.3. Endophytic colonisation

i. Gfp protein tagging

In order to describe the possible penetration into the host plant and the tomato tissue colonization pattern of beneficial microorganisms, *Streptomyces* sp., strain SA51 was chosen as a model. The strain was tagged with the *gfp* marker genes by transformation protocols described by **Ali et al. (2018).** Briefly, the wild-type strain SA51 was grown in ISP medium in 5 mL cultures at 28 °C, until the optical density at λ = 600 nm was 0.6. The bacterial cells were then pelleted by centrifugation (5000 rpm, 5 min, 4 °C), resuspended in 5 mL of cold sterile water, and centrifuged at 5000 rpm for 5 min. The supernatant was discarded, and the pellet was washed twice (5000 rpm

for 5 min) with 10% glycerol. Transformation was performed by electroporation (Gene Pulser Xcell™ electroporation system, Bio-Rad, USA) in an electroporation cuvette (0.2 cm) containing 100 μL of competent cells, plus 2 μL plasmid DNA (100 ng μL−1). The following pulse conditions were applied: 12.5 kV cm^{-1}, 25 μF, and 200 Ω **(Krzyzanowska et al., 2012a, b; Choi et al., 2006)**. After transformation, 1 mL of LB broth medium was added; the mixture was then incubated for 1 h at 28 °C and plated on to LB agar medium supplemented with kanamycin (50 μg/mL) and incubated again for 48 h at 28 °C. The identification and selection of clones carrying the *gfp* gene were carried out under UV light.

ii. Colonisation pattern

To validate the establishment and colonization of strain SA51 on different parts of tomato seedlings, endophytic colonization studies were performed using confocal laser scanning microscopy (CLSM). Tomato tissue specimens (root, stem, petiole and leaf) were prepared by cutting a piece of the tissue surface, around 1 cm in length, from different parts of each tissue with a sterile blade. All specimens were gently rinsed with sterile distilled water prior to immersing in phosphate-buffered saline (PBS) for microscopic observation. Confocal laser scanning microscopy (CLSM) was performed with an Axio Imager 2 system using a Carl Zeiss LSM 780 confocal microscope (Zeiss, Zaventem, Belgium). While GFP fluorescence was recorded by using an excitation laser of 488 nm (Argon laser) and collecting the emission of 500–600 nm, an excitation with NeHe laser of 561 nm was used and the emission band of 538–624 nm was collected. Images were acquired and reconstructed by Zen 2012 Software.

iii. PCR amplification

Specific primers for both the strains SA51 and PT65 were designed from their 16SrRNA sequence using NCBI primer designing tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to amplify the PCR product of size 560 bp and 620 bp for SA51 and PT65 respectively. The temperature for PCR

condition starts to pre-denaturation (94 °C, 5 minutes), denaturation (94 °C, 60 seconds), annealing (55 °C, 60 seconds), elongation (72 °C, 1 minute 30 seconds), post-elongation (72 °C, 5 minutes). Denaturation phase, primer attachment, and elongation were conducted for 35 cycles. Forty-five days after inoculation with bacterial strains plants were uprooted and thoroughly washed under tap water then surface sterilized with ethanol and then washed with sterile distilled water. Root samples of 5 g and stem samples of 8 g were surface sterilized and ground in 20 mL of sterile PBS buffer and then incubated on a rotary shaker at 120 rpm for 30 min. Bacterial pellet was collected by centrifugation at two different steps. In the first step to collect the plant material particulate the samples were centrifuged at 5000 rpm for 10 min at 4°C. The supernatant was discarded and again the pellet was resuspended in 5 mL of PBS buffer. In the second step bacterial pellet was collected by centrifugation at 10,000 rpm for 20 mins at 4 °C. The pellet was resuspended in 1 mL enzymatic lysis buffer and DNA was extracted using DNeasy plant mini kit (QIAGEN, Italy), later quantification of DNA assessed by using NanoDrop.

4.5. Field Studies:

The plant growth promotion experiments under field conditions were carried out in Cadriano experimental field (under containment conditions), which is located north of Bologna, Emilia Romagna, Italy during the years 2018-2019. The experiment was conducted in tomato cv. Rio Grande, a common commercial variety. Thirty-day old plants of approximately equal sizes were selected for this experiment. The experiment was set up in completely randomized block design. Each treatment block had a total of 40 plants in four replicates i.e., one replicate had 10 plants.

The selected bacterial strains were grown on nutrient broth (Sigma, Itlay) for 48 h (for bacteria) and 72-96 h (for streptomycetes) at 120 rpm continuous shaking at 30°C. The bacterial cells were centrifuged at 10,000 rpm for 10 min and the pellets were

diluted with sterile 0.856% Sodium Chloride (NaCl) to make a final concentration of 10^8–10^9 CFU ml^{-1}. The resulting bacterial cell suspensions (100 ml/plant) were used as a soil drench and foliar spray to treat tomato plants. Before transplanting in field, seedlings were root injured and were dipped in desired bacterial suspension for 30 min. Later, after 20 days of transplantation bacterial suspension was sprayed over the plant and also near the roots. Likewise, five consecutive treatments were given with 15 days' time gap between each treatment. The treatment details for PGP assay in nursery condition are as follows:

4.5.1. Treatment details:

T1: -Ve Control (plants inoculated with sterile distilled water without bacterial suspension.

T2: Strain SA51 treatment (test bacterial strain of present study)

T3: Commercial Microbial consortium (MICOSAT F) treatment (commercial product developed by CCS Aosta Srl., product is under evaluation)

T4: +Ve Control (plants treated with only pathogen)

4.5.2. Antagonistic Activity:

Antagonistic activity of strain SA51 was evaluated under field conditions against *X. vesicatoria* for the bacterial spot disease of tomato. When all treatments were done with antagonist and later pathogen was inoculated with *X. vesicatoria* on the following day. bacterial suspensions were used at a concentration of 10^8cfu ml^{-1} in sterile saline water. All inoculations were done at sunset, in order to maximize viability of bacteria cells following inoculation. Moreover, after pathogen treatment, plants were covered with a light and transparent plastic sheet, to allow the formation of a high humidity environment around the plants. The sheet was removed the following day before sunrise. Disease development was monitored weekly, starting one week after the pathogen inoculation (June, initial flowering stage), until late

August (harvest). Phytopathometry was done by measuring the number of leaves showing typical bacterial spots and the number of spots per leaf.

Figure 4.1. Tomato plants from field study at Cadriano experimental field station, Bologna, Italy during the year 2018-2019. Plants were treated with strain SA51: 1,2,3, & 4 are the replicates with ten plants each.

4.6. Molecular identification of strains

4.6.1. DNA amplification

Total genomic DNA was extracted according Chen and Kuo (1993) for Gram negative bacteria and for Gram positive bacteria DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Amplification of the 16S rRNA gene was performed using 16s rRNA gene universal primers 1525R (5′- AAGGAG GTGATCCAGCC-3′) and 27F (5′-AGAGTTTGATCCTGGCTCAG-3') (Vurukonda et al., 2016) for strain PT65 and primers strepB (5′-ACAAGCCCTGGAAACGGGGT-3') strepE (5′-CACCAGGAATTCCGATCT-3') (Ramazani et al., 2013) for strain SA51,

with target fragments of 1500 bp respectively. The amplification was carried out in a 25 µL volume. PCR amplifications were performed with 5 µL (5 x GoTaq Buffer), 1 µL forward primer, 1 µL reverse primer, 2 µL DNA template, 0.5 µL dNTP's, 1 U TaqPolymerase, 1.250 µL $MgCl_2$ and remaining volume added with nuclease-free water. The condition of PCR complied with the method of Marchesi et al. (1998). The temperatures for PCR condition started with pre-denaturation (94 °C, 5 minutes), followed by denaturation (94 °C, 60 seconds), annealing (55 °C, 60 seconds), elongation (72 °C, 1 minute 30 seconds), post-elongation (72 °C, 5 minutes). Denaturation phase, primer attachment, and elongation were conducted for 35 cycles. The PCR products were migrated on 1.2% of agarose gel by electrophoresis technique at 65 V for 40 minutes. The electrophoresis results were visualized using UV light after immersion using ethidium bromide dye for 20 minutes.

4.6.2. Sequencing

PCR products were visualized on a 1.2% agarose gel stained with ethidium bromide under UV light to confirm the presence of a ~1,500 bp band. PCR products were purified using PCR purification kit (QIAGEN, Hilden, Germany) prior to bi-directional sequencing using primers 27F and 1525R. Sanger sequences were generated at the at BIOFAB Laboratories (ROME, Italy).

4.6.3. Bioinformatics

DNA sequencing of the amplicons was performed and the nucleotide sequences of 16S rRNA genes were analyzed using BLAST online at http://blast.ncbi.nlm.nih.gov/Blast.cgi. **(Tamura et al. 2007)**.

4.7. Whole Genome Sequence (WGS) analysis of strain SA51

4.7.1. DNA Extraction

For WGS studies of strain SA51, starting with genomic DNA extraction, single colonies of *Streptomyces* sp. SA51 were grown in Tryptic Soy Broth (TSB) for 3 days at 28 °C. Genomic DNA was extracted and purified using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and its quantity and quality was checked using the NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Fischer Scientific, Waltham, USA), followed by gel electrophoresis.

4.7.2. Genome Sequence

DNA sequencing was performed using an Illumina HiSeq2000 sequencer. High-quality Illumina sequences libraries were prepared using the Nextera DNA Flex Library Prep Kit.

4.7.3. Bioinformatics

Genome assembly from pair-ended sequence reads has been done using the default parameters of the assembler module available in the *Geneious* software v 1.0 (www.geneious.com) that includes quality control, trimming and assembly steps using default parameters. Sequence alignment was done using the *clustalW* and the "*Map to a reference*" tools available in the *Geneious* software v 1.0 **(Vurukonda et al., 2019)**.

4.7.4. Data Submission

WGS data of assembled and raw reads of SA51 was submitted to NCBI by creating BioProject folder. The data is available on NCBI website (https://www.ncbi.nlm.nih.gov/home/submit/).

4.8. Statistical Analysis:

Data were statistically tested by analysis of variance (ANOVA) followed by Tukey's multiple comparison tests using MaxStats Lite latest software version. For plant experiments a total of forty replicates were arranged in four rows each of ten plants was employed to investigate an error in experimentation with two treatments (control: devoid of bacterial inoculation, inoculation: bacterized plants). For biocontrol activity each treatment was analyzed with 12 technical replicates of three individual biological replicates and the standard deviation was calculated and data expressed as the mean ± SD of 12 replicates.

5. RESULTS

5.1. Characterization of bacterial isolates

Microbial isolation from selected plants led to a collection of total 20 of different microorganism. Six isolates were chosen based on colony morphology, HR (Hypersensitivity Reaction on Tobacco leaves) test for further characterization. The morphological characteristics of the chosen bacterial isolates varied widely as shown in **(Table 5.1; Figure 5.1)**. All the isolates produced mostly round and conical shaped colonies whereas, in case of streptomycetes gram positive spore forming filamentour hypahe, the elevation was either raised or convex, had smooth or undulate margin with the colour ranging from white to pink. Microscopic and agar plate observations were performed to investigate characteristics of the isolates such as cell shape, colour, morphology and motility **(Table 5.1)**. All the isolates were motile, the gram-negative cells were mostly rod-shaped whereas Gram-positive bacteria such as *Streptomyces* were spore forming filamentous hyphae.

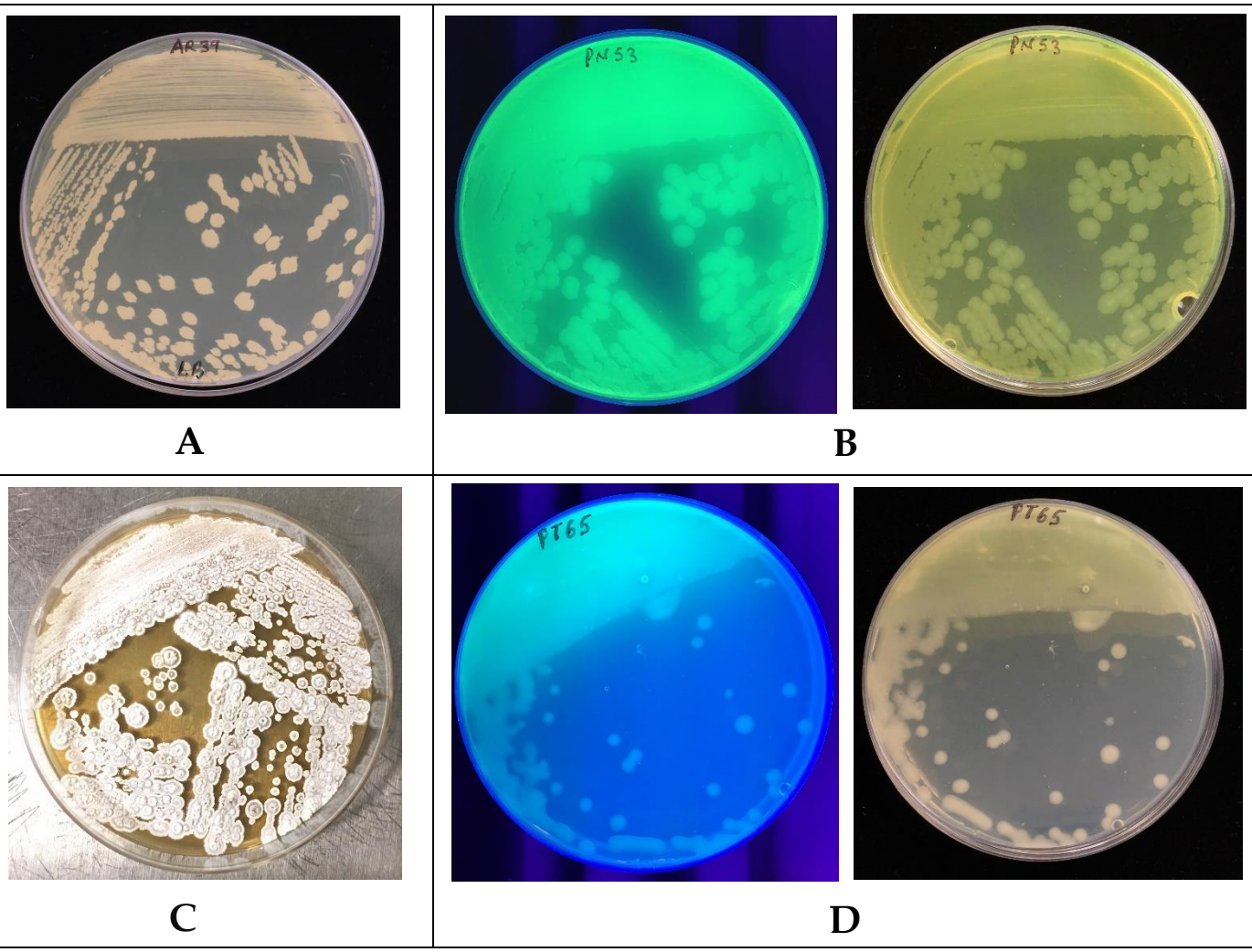


Figure 5.1. Colony morphology of the strains on their respective specific agar media. **A;** *Agrobacterium radiobacter* strain AR39 colonies on Luria Bertani (LB) agar medium. **B;** *Pseudomonas fluorescens* strain PN53 morphology, left side fluorescent colonies under UV light, right side yellow colonies on KING's B agar medium. **C;** *Streptomyces avermitilis* strain SA51 on International Streptomycetes Project (ISP) agar medium. **D;** *Pseudomonas fluorescens* strain PT65 morphology, left side fluorescent colonies under UV light, right side white, round and mucoid colonies on KING's B agar medium.

Table 5.1: Morphological and microscopic characteristics of isolates used in the present study

		ISOLATES					
		AR39	**PN53**	**PT65**	**SB14**	**SA51**	**SL81**
MORPHOLOGICAL	**Colour**	White ice, sometimes verging on salmon colour	Green fluorescent colonies under UV light	Yellow to green fluorescent colonies under UV light	Hazelnut	Antique pink / cream	Grey cream
	Colour Uniformity	No	Darker central area	No	yes	Darker central area	Hoops
	Surface Shape	Irregular	Lemon shaped	Round	Colonies my crack	Round	Conical
	Visual Aspect	Slightly glossy	Glossy	Glossy mucosa	dry	Cottony	Cottony
	Colony Thickness (mm)	< 1	1	>5	1	1	2
	Diameter (mm)	150	3	>5	3	6	6
	Edges	Very jagged	Smooth	Smooth	Tends to smooth	Smooth	Smooth
	Motility Swimming Swamming Twitching	 + + +	 + + -	 + + -	 - - -	 + + -	 - - -
MICROSCOPIC	**Vegetative form**	Very elongated sticks	Pastina/hair	Thin sticks	Hyphae	Hyphae	Hyphae
	Aggregation	In chains	No	No	NA	NA	NA

'+' – Positive; **'-'** – Negative result

5.2. Screening for Potential PGP

Plant growth promoting (PGP) properties of six strains were extensively studied for their PGP traits. All isolates produced IAA and ammonia and solubilized inorganic phosphorous. However, significant differences in these PGP traits were observed among the isolates **(Table 5.2)**. Isolate SA51 produced the highest amount of IAA (25.26±2.01µg/ml), closely followed by isolate PN53 (23.90±1.34) and PT65 (22.78±1.18). The amount of P solubilized was maximum in isolate AR39 (271), followed by PT65 (233) and SA51 (209). Hydrogen cyanide production was observed in only one isolate (PT65) and siderophore production was observed in three isolates *i.e.*, SA51, PT65 and PN53 **(Figure 5.2)**. Ammonia production was measured in all isolates, but quantitative variation was observed.

A **B** **C**

Figure 5.2. Plant growth promoting traits: A – Siderophore production by strain SA51; orange halo indicates positive reaction, B – HCN production by strain PT65; yellow coloured picric acid filter paper turns to orange color which is positive reaction, and C – Swimming motility of strain PN53 on Nutrient agar (0.3%) plates.

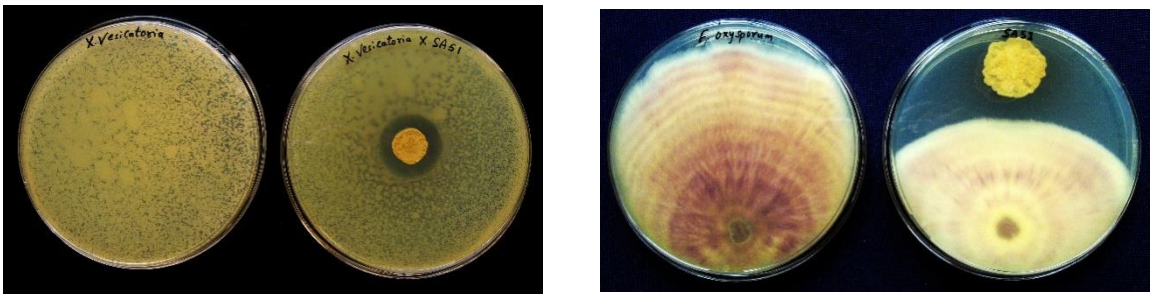
Table 5.2: Plant Growth Promoting (PGP) phenotypic traits of bacterial isolates

Test Bacteria	Ammonia Production	P-Solubilization Index	IAA production (µg/ml)	Siderophore activity	HCN production
SB14	+++	150	19.76±1.12	-	-
SA51	+++	209	25.26±2.01	+	-
SL81	++	188	20.54±1.45	-	-
PT65	++	233	22.78±1.18	+	+
PN53	++	200	23.90±1.34	+	-
AR39	+++	271	18.70±1.24	-	-

Numerical values are mean ± SD (n=3); IAA, Indole acetic acid; HCN, hydrogen cyanide; + positive; - negative.

5.3. Biocontrol Abilities of Bacterial Isolates

The biocontrol activity of the selected isolates against phytopathogens was checked by measuring the area of the inhibition halo. All the isolates were able to significantly inhibit the growth of phytopathogens *in vitro* **(Figures 5.3; 5.4 & 5.5)**: strain SA51 was particularly active against both bacterial and fungal pathogens, giving an inhibition halo up to 1,334 mm^2 against *Cmm* and most of the fungal strains were inhibited almost 50% which considered effective. In addition, some of the test bacteria were able to inhibit several phytopathogenic bacteria and fungi. Four of the antagonists SA51, PT65, PN53 and AR39 were able to significantly inhibit the growth of important phytopathogenic bacteria and fungi. Particularly, strain AR39 significantly inhibited fungal strains. In other cases, such as for isolates SL81 and SB14, no significant inhibitory effect on pathogens growth *in vitro* was observed. A quantitative comparison of the inhibitory effect of the six antagonists against different pathogens confirmed their activity. These results show that different species may have different levels of antagonistic activity and, also, among the same bacterial species.



A **B**

Figure 5.3. Antagonistic property of strain SA51: A – Antimicrobial activity against *X. vesicatoria* halozone indicates the inhibition of pathogen. B – Antifungal activity against *F. oxysporum.* Test bacterial secondary metabolites strongly dissolved in the agar media which inhibiting the fungal radial growth.

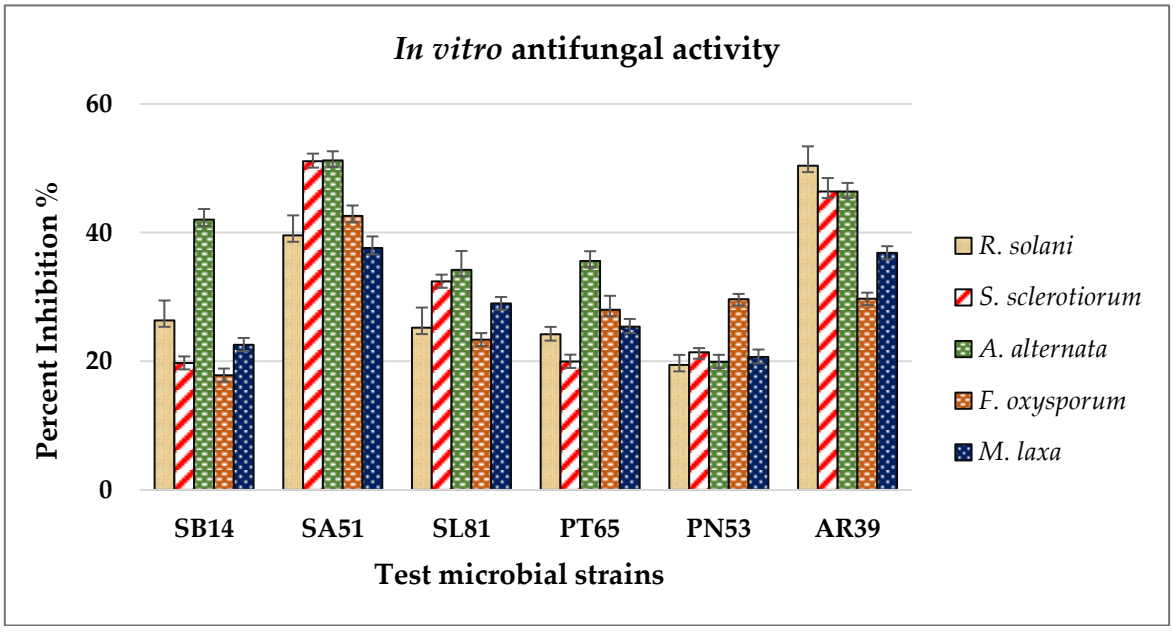


Figure 5.4. Antagonistic effects of test isolates against different phytopathogenic fungi as measured by inhibition of growth. Strain which shows the 50% inhibition were considered as the prospective. Error bars Mean of ± SD (n=3).

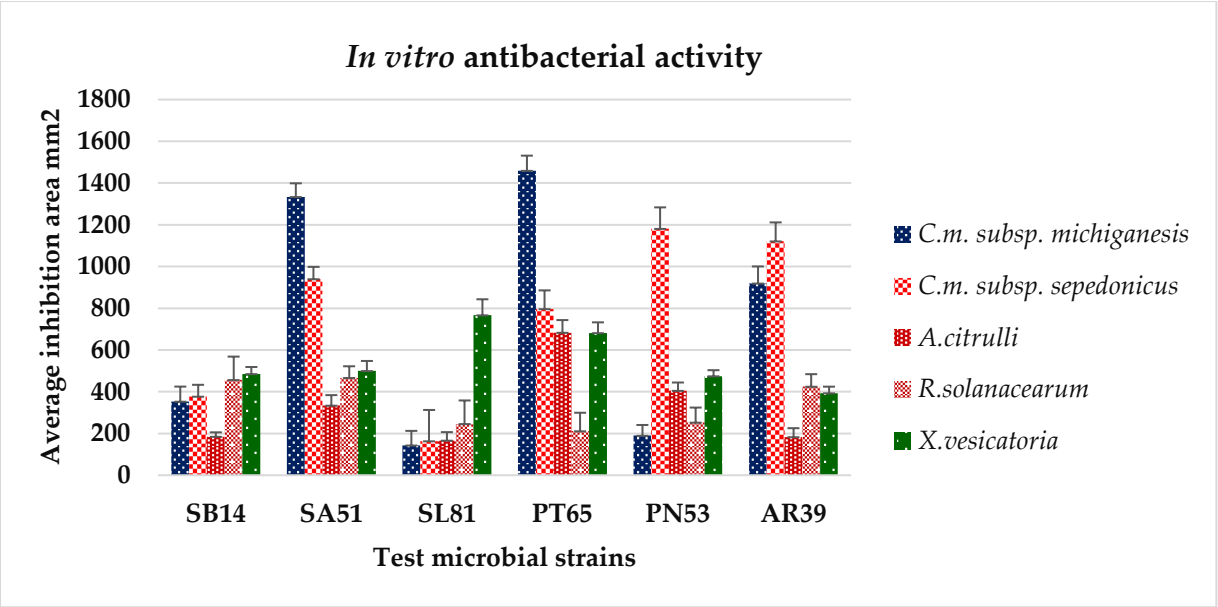


Figure 5.5. Antibacterial activity of test isolates against different phytopathogenic bacteria. Error bars Mean of ± SD (n=3).

5.4. Plant growth promoting activity:

The effect on tomato seedlings inoculation with two selected isolates, *Streptomyces* sp. strain SA51 and *Pseudomonas* sp. strain PT65, were studied under greenhouse conditions. Inoculation improved shoot, root length, and dry biomass of plants, with respect to values of the uninoculated plants that showed lower values **(Table 5.3)**. In addition, benefit was seen in inoculated healthy plants in terms of increase in dry weight and length of roots and shoots as compared to the uninoculated controls. Strain SA51 increased root and shoot lengths up to 31% and 34% respectively and also dry biomass to 90%, whereas strain PT65 increased root and shoot lengths to 18 & 16% also with 70% increase in dry biomass when compared to control plants **(Table 5.3; Figure 5.6)**

Table 5.3: ***In planta*** **PGP traits of tomato seedlings inoculated with selected bacteria**

Test Isolates	Root Length (cm)	Shoot Length (cm)	Root dry biomass (g)
CONTROL	15.5±2.1	32.06±10.11	0.61
SA51	20.35±1.27	43.04±13.18	1.16
PT65	18.3±0.89	37.37±9.5	1.07
SA51 + PT65	20.26±0.58	37.29±9.81	1.05

Numerical values are mean ± SD (n=10); cm, centimetre; g, gram.

Figure 5.6. Plant growth promoting activity of strain SA51 when inoculated on tomato seedlings. In the above figures it is evident that bacterial inoculation increased the plant growth in terms of shoots (right) and roots (left) when compared to untreated control plants.

5.5. Disease protection under green house conditions

Disease severity was initially recorded one week after visualisation of the first symptoms and continued for three consecutive weeks. Data were recorded and

disease progress observed. The number of plants affected from initial disease symptoms to the final necrotic stage was evaluated using disease indexed scale mentioned in materials and methods: isolates SA51 and PT65, singularly or in combination, were significantly effective in suppressing the disease **(Figure 5.7a)**. Disease severity was calculated using an index scale **(Figure 5.7b)** and statistical calculations were performed using the MaxStats software. whereas in bacterized plants disease was inhibited approximately 96% (SA51), 38% (PT65) and 85% (SA51+PT65 co-inoculation) compare to control plants.

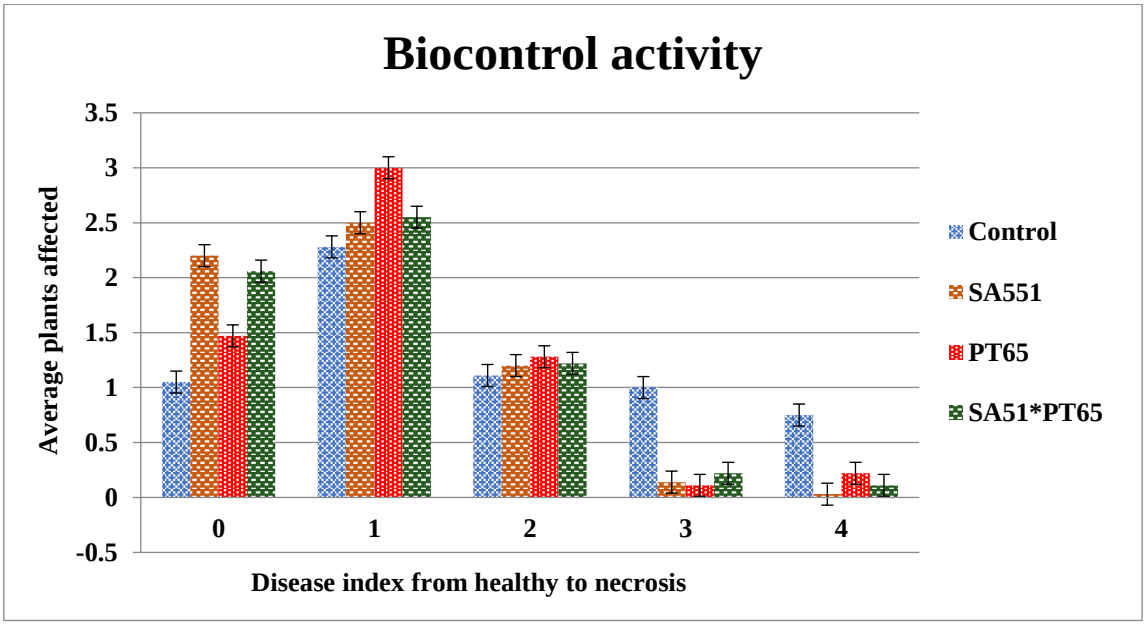


Figure 5.7a. Suppression of bacterial spot disease of tomato treated with sterile distilled water (SDW) as control (blue line), *Streptomyces* strain SA51 (brown line), *Pseudomonas* strain PT65 (red line) and both the strains SA51+PT65 (green line). Error bars Mean of ± SD (n=12).

Figure 5.7b. Bacterial spot disease index scale used to measure the disease severity of tomato plants under greenhouse conditions. 0-healthy leaf; 1-1 to 10 spots; 2-11 to 20 spots; 3-21 to 30 spots and 4-necrotic leaf.

5.6. PCR amplification of strains SA51 & PT65 and Endophytic colonization of tomato plants by strain SA51

Tomato plants treated with strains SA51 and PT65 were uprooted after 30 days, and genomic DNA was extracted and PCR amplification with specific primers for each strain confirmed positive bands with gel electrophoresis and whereas no bands in control plants **(Figure 5.8)**.

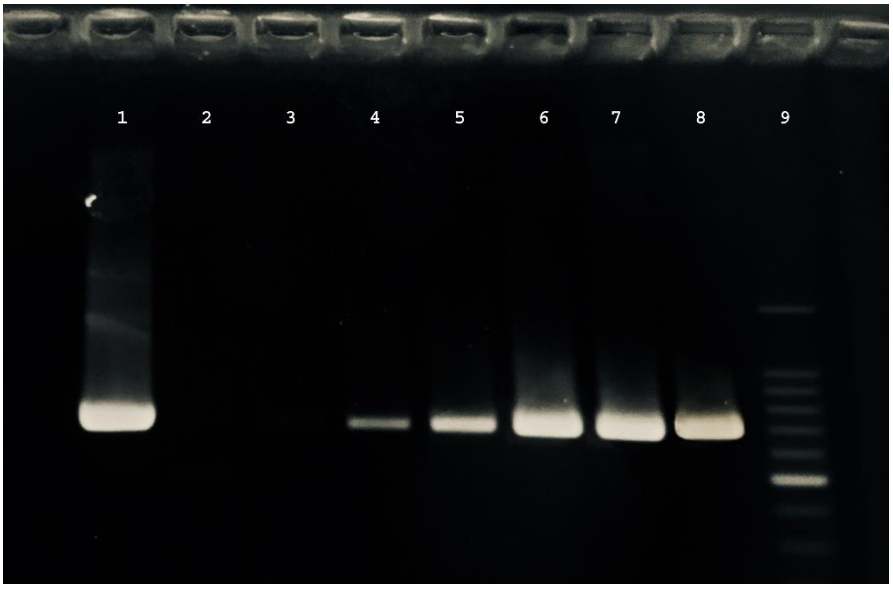


Figure 5.8. Endophytic PCR amplification of strain PT65: Lane 1 – +ve control of strain PT65, Lane 2 & 3 - root and stem sample of control plants, Lane 4 & 5 – root and stem samples of PT65 inoculated plants, Lane 6 & 7 – root and stem samples of SA51 inoculated plants, Lane 8 - +ve control of strain SA51, Lane 9 – 100 bp ladder.

Electroporation enabled us to introduce the *gfp* gene into *Streptomyces* sp. strain SA51 and to obtain constitutive GFP expression in the modified bacterial cells. To visualize root colonization 15 days after inoculation of tomato plant with the gfp. strain SA51, different sections of roots of the plant were prepared on slides and were viewed by CLSM. As shown in **Figure 5.9**, the fluorescent structures of strain SA51 indicated the presence of high numbers of bacterial cells in root tissues compared to other tissues, indicating the successful endophytic colonization strain SA51. The use of fluorescently tagged bacteria and CLSM enabled visualization that SA51 effectively colonized roots of *in vitro* propagated tomato plants under sterile conditions. The entire colonization process by an EGFP-tagged SA51 derivative was recorded, from the very earliest stages (bacteria adhesion to roots) to the colonization of the intercellular spaces of stem, leaves and petioles within the cortex of the differentiation

zone. The tomato root was rapidly colonized by EGFP-tagged SA51 cells, which were first attached to plant surface and later profusely colonized internal plants after 15 days: SA51 cells were randomly distributed along the internal root, stem, leaf and petiole sections.

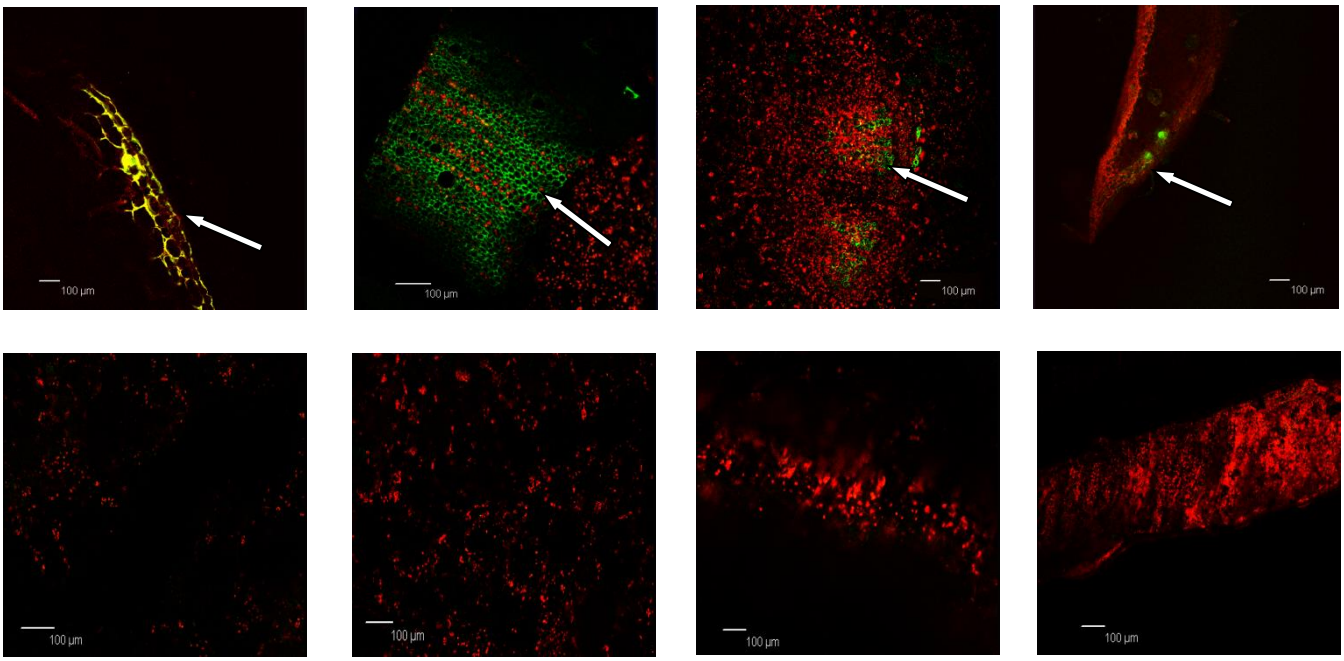


Figure 5.9. Endophytic colonization of strain SA51 in internal tissue of root, stem, petiole and leaf respectively from left to right of tomato plant. Top line SA51 strain and bottom-line control (un-inoculated) of tomato seedlings. After inoculation with SA51-*gfp* for 15 days, colonization was observed in roots, stems, petiole and leaves (left to right rescpectively) with SA51-*gfp* using confocal laser electronic microscope (CLSM). The bright green colour (Arrow indicates) were SA51-*gfp* colonies strongly colonized inside tissues. Bar represent the scale of measurement.

5.7. Plant growth promotion under field conditions

Under field condtions, SA51 significantly increased the production of marketable fruits (approx. +36%). A good influence on tomato productivity was also obtained using the microbial consortium (approx. +15%) **(Table 5.4)**. As regards protection from the tomato spot disease, there was no significant differences among

the treatments were observed. Indeed, the growing season was remarkably hotter than normal in the region, with almost no rain, thus inhibiting the development of the bacterial disease. In fact, we were not able to see any disease development or typical bacterial spots. Only the plants treated with pathogen (PC treatment) were some extent damaged results in reduced fruit production.

Table 5.4. Marketable production of tomato plants during the growing season 2018-2019.

Treatment	Marketable Production	
	Kg's / 40plants	Tons / Hectare
NC	37.94±1.92	18.97
PC	35.42±1.41	17.71
SA51	57.33±2.13	25.80
MICOSAT	43.37+0.61	21.68

NC: -ve control, PC: +ve control,

5.8. Identification and characterization of strains SA51 and PT65

The prospective isolates, selected based on *in vitro* PGP traits and biocontrol activity, were characterized based on morphological and molecular features. Microscopic studies revealed that the strain PT65 was Gram negative, motile, and rod-shaped bacteria. On King's B medium the isolate appeared as creamy, smooth, shiny, circular, convex colonies with bluish pigmentation under ultra-violet light, while strain SA51 was gram positive, with colonies appearing antique pink, round and cottony. Based on 16S rRNA gene sequence, blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis strain PT65 was identified as *Pseudomonas granadensis* and strain SA51 as *Streptomyces avermitilis* with sequence homology of 99 and 96%, respectively, with the strains present in NCBI GenBank..

5.9. WGS results of strain SA51:

Strain SA51 genome sequence alignment similar to that of *S. avermitilis* reference genome (NC_003155.5) available in NCBI GenBank. The total genome size of SA51 is 5,465,072 bp (including 792 assembled contigs and 2959 unassembled reads) with a GC content of 70.1%. The mean contig length was 2,832 bp, whereas the shorter and longer contigs were 707 bp and 23,079 bp respectively (N50 length: 3,517 bp) **(Table 5.5)**.

Table 5.5: Strain SA51 genome contigs assembly report

Statistics	Unassembled Reads	All Contigs	Contigs>=100 bp	Contigs <=1000 bp
Number of	2,959	792	792	74
Min Length (bp)	398	707	707	1,001
Median Length (bp)		2,179	2,325	2,179
Mean Length (bp)	1,088	2,832	2,832	2,969
Max Length (bp)	14,732	23,079	23,079	23,079
N50 Length (bp)		3,517	3,517	3,565
Number of Contigs >= N50		199	199	193
Length Sum (bp)	3,221,808	2,243,264	2,243,264	2,197,473

From the WGS analysis, we provided evidence for the presence of the complete *copZA* operon coding for the copper transport system, together with genes coding for nickel and cobalt transporters acting in concert with vitamin B12 biosynthesis systems and genes coding for various polyols ABC transporters and ATP-binding components that are involved in the uptake of selenate and selenite, thus suggesting a potential use of SA51 also for soil detoxification and bioremediation. Lastly, we provided evidence for the presence of genes for complete pathways involved in biosynthesis of tetracycline, penicillin, clavulanic acid, novobiocin, cephalosporin, streptomycin and 2-, 14- and

16-membered macrolides, suggesting that SA51 could be involved in the biological control of plant pathogens.

Table 5.6. Specific (highlighted with green color) and novel genes (highlighted with yellow color) identified in Strain SA51

Category	**Subcategory**	**Subsystem**	**Role**
Cofactors, Vitamins, Prosthetic Groups, Pigments	Folate and pterines	Folate Biosynthesis	Dihydrofolate reductase
Iron acquisition and metabolism	Siderophores	Siderophore assembly kit	Putative ABC iron siderophore transporter, fused permease and ATPase domains
Iron acquisition and metabolism	no subcategory	Encapsulating protein for DyP-type peroxidase and ferritin-like protein oligomers	Predicted dye decolorizing peroxidase (DyP), YfeX like subgroup
Iron acquisition and metabolism	no subcategory	Heme, hemin uptake and utilization systems in Gram Positives	Heme ABC transporter, cell surface heme and hemoprotein receptor HmuT
Membrane Transport	Cation transporters	Copper Transport System	Repressor CsoR of the copZA operon
Membrane Transport	Cation transporters	Copper transport and blue copper proteins	Copper binding protein, plastocyanin/azurin family
Stress Response	Detoxification	Uptake of selenate and selenite	Various polyols ABC transporter, ATP-binding component
Stress Response	Osmotic stress	Choline and Betaine Uptake and Betaine Biosynthesis	HTH-type transcriptional regulator BetI
Stress Response	Osmotic stress	Choline and Betaine Uptake and Betaine Biosynthesis	High-affinity choline uptake protein BetT
Virulence, Disease and Defense	Resistance to antibiotics and toxic compounds	Beta-lactamase	Beta-lactamase

6. DISCUSSION

DISCUSSION:

Plant–microbe interactions are critical to the integrity, function, and long-term sustainability of agro-ecosystems **(Nannipieri et al., 2003)**. Plant-associated beneficial bacterial communities sustain many important ecosystem processes, such as nutrient uptake, decomposition of organic matter and waste, nutrient availability, degradation of pesticides, maintain soil structure, and plant growth and health. An understanding of the plant-associated bacterial community is important, considering their potential significance in plant growth promotion, protection against biotic and abiotic stresses, source of novel biomolecules, and agents in bioremediation and determinants of soil and environmental heal**th (Choudhary and Johri, 2008; Lugtenberg and Kamilova, 2009; Nelson, 2004; Sessitsch et al., 2004)**.

In the present study, we observed that several strains naturally present in different crop plants as endophytes/epiphytes, such as AR39, PT65, PN53, SB14, SA51 and SL81, were able to produce IAA, siderophore, solubilize phosphate and HCN production. Siderophore production by bacteria can promote plant growth through enhanced direct iron availability to plants under iron deficient conditions or by inhibiting the availability of iron to plant pathogens **(Ahmad et al., 2008)**. In addition, phosphorus is one of the major macronutrients necessary for biological growth and development of plants. Phosphate solubilizing bacteria are capable of solubilizing the insoluble phosphate present in the soil, resulting in an increase in soil quality and also can enhance plant growth **(Rodriguez and Fraga, 1999)**. It is well understood that such properties have a crucial role in plant growth promotion and some researchers have previously reported these capabilities in other strains belonging to the same genus identified in this work. **Vurukonda et al. (2016)** reported *P. putida* strains with plant growth promotion and their activity was associated with the production of IAA, siderophores and the solubilization of phosphate. *P. putida* AKMP-7 with the ability for IAA and siderophore production significantly increased the root weight and enhance the growth of wheat plants under stress condition **(Ali et al., 2011)**. Recent reports state that endophytic bacteria have similar effects on maize plant with the

main mechanism of plant growth promotion **(Sandhya et al., 2017)**. Among the isolates only PT65 was able to produce HCN; some authors noted that some deleterious bacteria reduce plant yields through HCN production **(Bakker and Schippers, 1987)**. However, HCN production is a biological control mechanism by bacterial antagonists against various phytopathogens **(Kumar et al., 2012)**. Beneficial plant growth promoting bacteria produce such secondary metabolites, which are recognized as biological control agents against fungal and bacterial plant pathogens. Also, HCN can indirectly increase availability of phosphorus and iron to plants, resulting in increased plant growth promotion activity **(Rijavec and Lapanje, 2016)**.

PGPBs have been applied to a wide range of agricultural species for the purpose of growth enhancement, including increased rate of seed germination, crop yields, stress tolerance and disease control **(Kloepper et al. 1991, 1980; Gururani et al. 2012)**. For example, seed germination rate increases of 10–40% for canola, when seeds were coated with PGPR before planting, and plant weight of tuber-treated potatoes increased by 80% on average by midseason **(Kloepper and Schroth 1981)**. Yield increases between 10% and 20% with biofortification and it has been documented for several agricultural crops **(Kloepper et al., 1991; Dimkpa et al., 2008; 2009)**. To determine the plant growth promotion by bacteria, bacterial suspensions of SA51 and PT65 were applied to tomato seedlings as root inoculations. Four weeks after consecutive inoculation, growth parameters such as root, shoot length and root dry weight were measured. Interestingly, these strains increased the number of surviving plants in the soil without any fertilization. Plants treated with both the isolates were taller and broad leaves, when compared to the untreated control, with significantly enhanced dry weight of roots. These results indicate that both the isolates, particularly SA51, could be used to facilitate an effective plant growth promotion in tomato plants. Since this stain is very prospective in terms of growth promotion, biocontrol and profusely binding to internal plant tissues. Our present results were much likely similar with that of **Vurukonda et al. 2016, Prasad et al. 2016 and Sandhya et al. 2017**.

Antagonistic effects of endophytic bacteria *in vitro* by a dual culture test is extensively used as one of *in vitro* tests for preliminary screening of biological control agents **(Desai et al., 2002)**. According to the previous reports, dual culture tests showed that many *Bacillus* isolates from livestock manure composts and cotton-waste composts have antagonistic effects against the isolates of soilborne fungi, *F. oxysporum*, *P. capsici*, *R. solani* AG-4, and *S. sclerotiorum* **(Kim et al., 2008)**. Similarly, in this study, all six isolates were tested for antagonistic effects *in vitro* against pathogenic fungi and bacteria. Antagonistic effects were confirmed by the formation of inhibition zones between the antagonist and the pathogens. All six bacterial isolates reduced mycelial and bacterial growth of all the pathogens. Among them, only three isolates SA51, PT65 and AR39 showed highest agonistic activities. Isolate SA51 performed best in the inhibition of fungi and bacteria: therefore, this strain was extensively studied for its endophytic colonization by transforming it with green fluorescent labelled gene *gfp*. Cells of *Streptomyces sp.* SA51 proved to be very difficult to transform. This is not an unusual problem. Environmental isolates are more problematic to genetically modified laboratory strains, especially when it comes to Gram positive bacteria. Endophytic colonization is the primary requirement to ensure an intimate association with the plant and thus supporting endophytic efficacy for growth promotion **(Marasco et al., 2012)**. For the establishment and internal colonization of endophytes, surface characteristics of the bacteria and plant polymer degrading enzymes such as cellulases, chitinases, proteases and pectinases play a significant role **(Company et al., 2005)**. Though there was variability in the motility shown by all the isolates, strain SA51 significantly showed flagella mediated swimming and swarming motility for its easy movement into the plant tissue. To evaluate the ability of *Streptomyces* sp. strain SA51 to colonize internal parts of the tomato plants, endophytic colonization studies were performed. The results showed that plants were highly colonized by gfp-labelled *Streptomyces* sp. strain SA51, even after 15 days of inoculation, and showed profuse colonization in roots, collar and aerial parts of tomato plants. Colonization of *P. putida* and *Bacillus amyloliquefaciens* in chickpea rhizosphere has been visualized using gfp

labelling, and the synergistic effect of both the strains mitigated drought stress and induced growth promotion in chickpea **(Kumar et al., 2016)**. However, this technique has been used to monitor epi- and/or endophytic colonization of beneficial bacteria inside different plant hosts **(Gamalero et al., 2004; Chi et al., 2005; Götz et al., 2006)**.

While some PGPRs colonizing the rhizosphere can inhibit an infection process localized in the phyllosphere by a phenomenon named induced systemic resistance (ISR), endophytic PGPRs can also suppress pathogen infection and colonisation by other biological control mechanisms due to their systemic distribution internally. Endophytic bacteria in the present study (*Streptomyces* sp. SA51 and *Pseudomonas* sp. PT65) were used to inhibit *Xanthomonas vesicatoria* from causing infection in tomato and successful results have been obtained. Since SA51 and PT65 significantly inhibited the growth of *X. vesicatoria* (and other bacteria) in agar plates, thus indicating that some antibacterial molecule is produced and released in the medium, the same antibacterial substance may be produced in colonised plant tissue, thus explaining disease reduction in tomato. From the greenhouse data it was evident that protection against *X. vesicatoria* by the bacterized tomato plants **(Figure 5.7)**, antagonists were more aggressive as the disease progression at final stage (necrosis) disease was reduced which means at initial stages even on inoculated plants mild symptomatic spots were observed and after 3 weeks antagonists inhibited pathogen progress and in control plants disease was very aggressive and plants were significantly damaged These data are in agreement with the several other experiments conducted by Riley and Wertz **(2002);** Linares et al. **(2006);** Montesinos **(2007)**.

Additionally, many researchers have investigated the efficacy of different endophytic bacteria on a variety of pathosystems and obtained neutral or different levels of positive effects **(Kang et al., 2007; Muthukumar et al., 2010)**. **Romero et al. (2016)** determined the efficacy of different endophytes on tomato growth and some diseases at neutral or different levels. Similarly, *Streptomyces*, *Bacillus* and *Pseudomonas* species were determined to have different levels of antagonistic effect on bacterial spot disease and plant growth in tomato **(Naue et al., 2014)** and pepper plants **(Mirik et**

al., 2008). Bacterial inoculation with endophytes reduced the severity of bacterial spot disease in tomato plants: **Romero et al. (2016)** reported that some endophytic bacteria having antimicrobial activity against *P. syringae* pv. *tomato* in *in vitro* and the disease has been suppressed by some of them in tomato plant via this route. On the other hand, in our study, the isolate SA51 resulted more successful in suppressing disease and it is correlated with the inhibition of *X. vesicatoria* development *in vitro*. **Ribaudo et al. (2016)** identified that endophytic bacteria application reduced symptoms in tomato as a result of activating ethylene hormone genes and SI-ACS genes related to pathogenicity in the plant. In addition to *Ochrobactrum lupine* KUDC1013 limit disease caused by *X.a* pv. *vesicatoria* in pepper plants by stimulating plant resistance **(Hahm et al., 2012)**.

Plant growth physiological parameters varied according to individual bacterial inoculation, and the effect was observed to be positive. It was clear that bacterial inoculation has a significant effect on plant height in tomato plants (Table 3). Similarly, though many studies have taken the increase in plant height as an indicator of PGPR effects **(Kang et al., 2007; Muthukumar et al., 2010; Xia et al., 2015)**, **Huang et al. (2017)** proposed that this parameter alone was not enough to assess the PGPR effects. In our study, the root and shoot length along with root dry biomass in bacterized plants were observed to increase by 24-26% and 50% respectively. Similarly, **Xia et al. (2015)** stated that different endophytic bacteria increased growth parameters by 25% in tomato. This significant increase obtained in current study may be considered the result of changes in the plant nutrition and hormonal balances. This situation leads to the conclusion that the ACC deaminase enzyme produced by PGPRs may prevent harm by disintegrating the ACC that is the precursor of ethylene **(Penrose and Glick, 2003; Glick, 2014)**. However, we did not characterize the isolates for ACC deaminase activity. **Ribaudo et al. (2016)** explained that the effects of endophytic bacteria on tomato growth may be due to the IAA they produce. Similarly, **Khan et al. (2012)** showed that endophytic bacteria that produce IAA additionally have an advantage of nitrogen fixation ability to increase the growth, flowering and yield of many plants,

including tomato. In most of the findings, the endophytic *Pseudomonas* spp. **(Muthukumar et al., 2010)**, *Bacillus* spp. and *Serratia* spp. **(Amaresan et al., 2012)** were able to improve root and shoot growth via secondary metabolites production like IAA, siderophore and inorganic phosphate solvent enzymes, etc. These findings were much likely to our results, since we confirmed that strains SA51 and PT65 were successful in production of IAA, siderophore and phosphate solubilization ability **(Table 5.2)**: therefore, we consider that the increase observed in plant development parameters and tolerance may be due to these metabolites, these results coincide with the data from the field studies, where plants treated with SA51 were more prospective for producing highest amount of fruits, thus confirming recent results by **Ahmet et al. (2018)**. The identification of strain SA51 as *S. avermitilis* may suggest that our streptomycete has the capability of producing the unique avermectin, which is known to act as an antimicrobial and an insecticide **(Cheng et al., 2018; Choi et al., 2018; Rath et al., 2018)**. Similarly, **Riera et al. (2017)** group found that *P. granadensis* strains have the beneficial effects on plants by producing PGP traits like P-solubilization and siderophore production. While comparing both the strains SA51 and PT65, among both strain SA51 is most prospective in terms of biocontrol activity whether it is *in vitro* or *in vivo.* Due to which we have selected strain SA51 for further studies. To identify the genes involved in plant growth promotion by strain SA51 we used metabolic profile by KEGG pathway **(Kanehisa and Goto, 2000),** provided the presence of genes involved in the pathway for indole alkaloid biosynthesis and in the iron transport and metabolism, together with genes coding for proteins acting in the regulation of iron homeostasis. At the same time, based on RAST annotations, we provided evidence for the presence of genes and operons related to metal transporters and antibiotics biosynthesis, suggesting that SA51 could be involved in the biological control of plant pathogens and/or in the reshaping of the soil microbiota **(Vurukonda et al., 2019)**.

7. CONCLUSION:

Plant growth-promoting bacteria protect plants from a wide range of environmental changes and related stresses like biotic and abiotic. PGPB may also provide protection to plants *via* a number of other mechanisms including phytohormone production; siderophore production; p-solubilization, nitrogen fixation and resistance to various bacterial and fungal phytopathogens. A subset of these bacteria, *i.e.*, bacterial endophytes are plants' natural companions and can be isolated from the interior parts of various plant tissues. Since bacterial endophytes are better adapted to various host plants, they may be more beneficial than their counterpart, rhizospheric bacteria. Bacterial endophytes express more induced activity over rhizospheric-binding bacteria and transgenic plants in the same environmental conditions. Considering all the above parameters, the present research will improve the understanding on the use of microbial biocontrol agents and will implement innovative biocontrol strategies to bacterial and fungal diseases. In particular, *Streptomyces* sp. SA51 and *Pseudomonas* sp. PT65 were found to be most active for *in vitro* biocontrol activity. *In vitro* biocontrol activity has shown very significant results also related to possible growth promoting activity. Though both the strains were very effective in *in vitro*, it is very important to assess their endophytic colonization in different parts of the plant tissue. Since strain SA51 had the best possibility for use in enhancing growth and controlling disease, its endophytic colonization was confirmed by both PCR and gfp tagging protocols.

The present research will improve the knowledge on bacterial colonization and its beneficial effects on plants which confirms the symbiotic relationship of plants and microbes. Field experiments confirmed the ability of strain SA51 to act as plant growth promoting agent: such promoting activity was also reflected into an increase of fruit production by approximately 7%. Using such strains and microbial inoculants not only increase the crop yield also protects the environment from harmful chemicals.

We conclude from our work that using these types of microbes will be useful towards achieving sustainable agriculture practices.

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CHAPTER 2

EVALUATION OF A MICROBIAL CONSORTIUM FOR THE BIOCONTROL OF FLAVESCENCE DORÉE AND ESCA DISEASE AND DIFFERENTIAL GENE EXPRESSION ANALYSIS OF GRAPEVINE LEAVES

ABSTRACT:

Grapevine (*Vitis vinifera* L.) is one of the most important commercial fruit crops grown in Italy. It is considered one of the most widely cultivated crops in temperate, sub-tropical and tropical regions of the world. Grapevine cultivation offers a great economic potential due to its higher yield and monetary returns owing to the export. Grapevine is subjected to the various diseases, two among them are Flavescence dorée (FD) and Esca. In the present study, we aimed to evaluate the effect of biocontrol, biochemical and differential gene expression effects of commercial microbial consortium production MICOSAT F on grapevines. The study was carried out during the three successive seasons (2017, 2018 and 2019) at an organic vineyard (*Lambrusco di Sorbara*) orchard located in Campagnola, Reggio Emilia, Italy. *Candidatus* Phytoplasma vitis is causative agent of Flavescence dorée and fungi *Phaeoacremonium aleophilum* and *P. chlamydospora* are among the causative agents of Esca: both the diseases causes severe losses in yield quantity and quality. Application of microbial biocontrol agents in the present study was an attempt to control the diseases. From the three years field data it was observed that antimicrobial activity again FD was not so effective, whereas Esca disease progression gradually decreased year by year. Biochemical aspects of grapes were evaluated and the positive effect of MICOSAT F reflected on the higher stability of most and pigments content grape juice. Gene expression of grapevine leaves following MICOSAT F sprays was studied in controlled conditions. We observed that the microbial consortium profusely induced the upregulation of grapevine genes involved in maintenance of biocontrol and plant growth promotion activity. Almost all the genes were downregulated initially after 0 hpi and 2 hpi, but later from 4 hpi genes like *ACC, CHS, PAL & PER* were significantly upregulated. Particularly, *PR11* and *PR12* genes were significantly upregulated after 4 hpi. It was concluded that the microbial consortium named Micosat F is a trulty prospective microbial for the management of Esca, but its activity was not sufficiently effective to control FD.

1. INTRODUCTION

1.1 Global economic importance of grapevine crop and vine production

Grape (*Vitis vinifera* L.; *Vitaceae*) is a fruit, botanically a berry, of the deciduous woody vines of the flowering plant genus *Vitis*. The cultivation of the domesticated grape began 6000 - 8000 years ago in the Near East **(This et al., 2006)**. The earliest archaeological evidence for a dominant position of wine-making in human culture dates from 8000 years ago in Georgia **(Keys, 2003; McGovern, 2003)**, Ancient Egypt and the West Asia region including Syria and Palestine, where it has existed since the Canaanite era. Furthermore, ancient Egypt was supplied with Palestinian wine as early as Chalcolithic, the Early and Late Bronze Ages, and Egyptians from the 15th century BCE, described the wine of Canaan as being "more abundant than water" **(Broshi, 2001)**. However, most *Vitis* species are found in the temperate regions of the Northern Hemisphere in Asia, North America while a few are found in the tropics regions in the world. Also, grapevines are influenced by their surrounding environment with a seasonal variation in production from 30% - 32.5% **(Chloupek et al., 2004)**. Climate is one of the key factors in grape production **(Fraga et al., 2014)**, affecting the suitability of certain grape varieties to a region, as well as the type and quality of the wine produced. A few years ago, many researchers in the Mediterranean region studied the relationship between plant production (fruit trees), physiology, plant biology, biodiversity, phytosociological, plant communities and climate-bioclimate factors **(Ighbareyeh et al., 2014; 2014; 2014; 2015; Cano et al., 2014; Ighbareyeh et al., 2015)**. Biologically, most *Vitis* species contain 38 chromosomes (n = 19) **(Bennett and Leitch, 2012)**, while *Vitis rotundifolia* has 40 (n = 20). According to the Food and Agriculture Organization (FAO), 75,866 km^2 of the world are dedicated to grapes, of which 71% of world grape production is used for wine, 27% as fresh fruit, and 2% as dried fruit. Over the past few years, China, the United States, Turkey, South Africa, Argentina and some European countries such as Spain, Italy and France have been among the world's top grape producers **(FAO, 2012)**.

1.2 Microbial consortia: definition and use

Microbial consortia can survive diverse conditions through the formation of synergistic population-level structures, such as stromatolites, microbial mats and biofilms. Biotechnological applications are poised to capitalize on these unique interactions. However, current artificial co-cultures constructed for environmental and for agricultural benefits, including biosynthesis, agriculture and bioremediation, face many challenges to perform as well as natural consortia. Interkingdom microbial consortia tend to be more robust and have higher productivity compared with monocultures and intrakingdom consortia, but the control and design of these diverse artificial consortia have received limited attention. Further, feasible research techniques and instrumentation for comprehensive mechanistic insights have only recently been established for interkingdom microbial communities. In recent reviews, these recent advances in technology and current understanding of microbial interaction mechanisms involved in sustaining or developing interkingdom consortia for biotechnological applications attracted many researchers to develop the microbial consortium. Some of the interactions among members from different kingdoms follow similar mechanisms observed for intrakingdom microbial consortia. However, unique interactions in interkingdom consortia, including endosymbiosis or interkingdom-specific cell–cell interactions, provide improved mitigation to external stresses and inhibitory compounds. Furthermore, antagonistic interactions among interkingdom species can promote fitness, diversification and adaptation, along with the production of beneficial metabolites and enzymes for plant **(Zhang et al., 2018)**.

1.2.1. Biofortification

The term bio-fertiliser or 'microbial inoculant' is defined as a preparation containing live or latent cells of efficient strains of nitrogen fixing, phosphate solubilising or cellulolytic microorganisms used for application on soil or composting areas with the objective of increasing the number of such microorganisms and accelerate certain microbial process to augment the extent

of the availability of nutrients in a form which can be assimilated by plant **(NIIR Board, 2004)**. Unlike chemical fertilisers, bio-fertilisers prevent pollution and are environmentally-friendly. Living microorganisms used in the preparation of bio-fertilisers promote the adequate supply of nutrients to the host plants and ensure their proper development of growth and regulation in their physiology **(Mishra et al., 2013)**. In agricultural eco-systems, microorganisms have vital role in fixing/ solubilizing/mobilizing/nutrient recycling. These microorganisms occur in soils naturally, but their populations are often scanty. In order to increase the crop yield, the desired microbes from rhizosphere are isolated and artificially cultured in adequate count and mixed with suitable carriers or as they are in suitable combinations (Microbial consortium) by artificial culturing. These are known as biofertilizers or microbial inoculants **(Pindi and Satyanarayana, 2012)**.

Important examples of positive plant-microbe interactions associated to plant growth promotion include PGP rhizobacteria: non-pathogenic *Pseudomonas Bacillus, Azotobacter, Serratia, Azospirillum* and *Streptomyces* capable of improving nutrient availability in soil, plant nutrient uptake and assimilation, as well as supporting nitrogen cycling **(Raaijmakers et al., 2009; Berg et al., 2014; Lugtenberg, 2015)**. Many recent studies demonstrate the potential as plant biostimulants of microbial consortia, rhizobacteria, and rhizofungi, that function as agricultural probiotics **(de Vries and Wallenstein, 2017; Wallenstein, 2017; Kong et al., 2018; Woo and Pepe, 2018)**.

1.2.2. Bioremediation

Soil contamination with toxic compounds has become a great environmental concern in recent years because toxic compounds in soil and groundwater are a threat to both human health and nature **(Chen et al., 2015)**. In particular, an accidental oil spill leads to the release of large quantities of petroleum into the environment, and subsequently perturbs environmental ecosystems tremendously **(Khamforoush et al., 2013; Macaulay and Rees, 2014)**. Bioremediation, which relies on microbiological processes, has proven to be a non-disruptive, cost-effective, and highly efficient

approach to remove organic pollutants, particularly compared to other physico-chemical approaches **(Tyagi et al., 2011; Fathepure, 2014; Zhao et al., 2017)**. Many diverse microorganisms that can be used for the bioremediation of contaminated soil have been isolated from terrestrial habitats worldwide **(Jeon et al., 2003; Bardgett and van der Putten, 2014; Shekhar et al., 2015; Ismaeil et al., 2018; Roy et al., 2018)**. However, the accessibility and availability of biological resources obtained from abroad is currently limited due to stringent international law and national legislation. Therefore, many efforts are made to isolate, preserve, and characterize biological resources, to overcome these legal issues in all countries **(Overmann, 2015; Overmann and Scholz, 2017)**.

Most contaminated sites are generally contaminated with multiple pollutants rather than a single type, and harbour a variety of different environmental conditions for biological activity **(Andreoni and Gianfreda, 2007)**. Therefore, bioremediation using a single type of microorganism often results in failure, due to low biodegradability, adaptability, and viability of the applied microorganisms in a contaminated site with diverse environmental conditions **(Tyagi et al., 2011; Rayu et al., 2012; Herrero and Stuckey, 2015)**. To accomplish successful bioremediation, many issues, including the types of organic compound present, the use of appropriate biodegrading microorganisms and their biodegradation properties, and diverse environmental factors such as water content, temperature, pH, and heavy metal content should be addressed **(Gandolfi et al., 2010; Jeon and Madsen, 2013)**, but are not easily resolved due to their complexity. However, these limitations may be easily overcome by the application of a microbial consortium consisting of multiple strains with diverse biodegradation abilities and physiological properties that ensure survival in a contaminated site with diverse environmental conditions **(Gurav et al., 2017; Lee et al., 2018)**.

1.3. Grapevine and use of beneficial microorganisms

Grape is commercially cultivated in several areas globally. Commercial grapevine varieties have substantially differed in their nutrient efficiency, and there is experimental evidence that, in some cases, associative rhizobacteria are involved in

the expression of nutrient efficiency. Chemical fertilizers were used extensively to provide nutrients for a long time. This has resulted in pollution, reduced biodiversity in intensively farmed regions and environmental degradation, which is increasingly widespread and sometimes irreversible. Beneficial microorganisms, including nitrogen (N2)-fixing bacteria, provide minerals to plants and they directly correlated with efficient crop production. Bio-fertilizers could be considered as a kind of beneficial microorganisms, with the ability to mobilize nutrient elements from non-usable to usable form, thereby saving considerable amount of chemical fertilizers needed by the plants. PGPR have been suggested to be important for agriculture with the aim of improving nutrients availability for plants and have been increasingly used worldwide in sustainable agriculture as biological fertilizer **(Yildirim et al., 2011a)**. Some strains of PGPR have been reported to enhance nutrient uptake by plants. Earlier studies indicated that PGPR could improve growth, yield and nutrient uptake of vegetable crops such as tomato, lettuce and broccoli **(Turan et al., 2007; Yildirim et al., 2008; Yildirim et al., 2011b; Gunes et al., 2009)**.

Dense monoculture leads to soil exhaustion and increased pollution in viticulture. Because of overdose in fertilization and high mineralization rate, crucial amount of nitrogen from vineyards is dispersed in underground waters. New and environmental-friendly strategies are needed to decrease chemical fertilizer and pesticide use. Additional and alternative sources should be evaluated, and nutrient intake efficiency should be increased at plant nutrition. Thus, it is necessary to identify convenient rhizobacteria species to use in viticulture, which could be isolated from local country soils involving high-throughput studies. Improved biological fertilizer formulations that are convenient for agriculture application can help to strengthen the organic grape sector by increasing the quality and productivity of organic viticulture **(Cakmakci and Erdogan, 2012)**. While annual plants provide a lot of information about the activity of N2-fixing and P-solubilising soil microorganisms, there is very limited information about nitrogen-fixing and phosphate-solubilizing bacteria on perennial plants such as grapevine. The inoculation of grapevine explants with PGPR

isolates increases the physiologic activity and plant resistance to cold stress. *Burkholderia phytofirmans* and grapevine interaction experiments proved that bacteria can be transferred to the young fruits from roots or leaves after inoculation. On the other hand, it has been determined that *Burkholderia* sp. PsJN isolate excretes high amount of 1-aminocyclopropane-1-carboxylate (ACC) deaminase which decreases ethylene by cleaving it and helps in preventing effect and induces grapevine growth **(Compant et al., 2005a)**. Some endophytic bacteria can reach the root xylem and colonize on flower, fruit, upper parts and seeds of the grapevine, and also, they generate plant growth effects on other plants **(Compant et al., 2005b)**. Field studies have shown that some strains isolated from plant proliferation organs can be colonized especially on grapevine rhizosphere and endosphere **(Compant et al., 2010)**. It was reported that the widespread endophytic bacteria species on grapevine belong to *Pseudomonas*, *Enterobacter* and *Bacillus* species **(Lo Piccolo et al., 2010)**. In studies conducted in Turkey, the bacteria that can be cultured from wild grapevine soils and that are convenient to use as biological fertilizer on viticulture according to specialities as nitrogen fixation and phosphate solubilization specialty have been detected as isolates mostly belonging to *Bacillus*, *Pseudomonas*, *Paenibacillus*, *Brevibacillus* and *Stenotrophomonas* species, and *Pseudomonas putida*, *Pseudomonas fluorescens*, *Bacillus megaterium* and *Stenotrophomonas maltophilia* species **(Karagöz et al., 2012)**. Additionally, grapevine rhizospheres could be studied to determine the change in bacterial types according to population, geographic location, soil pH level and vegetation type on the research fields. It was stated that *Pseudomonas* and *Bacillus* species induced callus generation and growth **(Kose et al., 2005)**.

2. OBJECTIVES OF PRESENT STUDY

The present study focuses on the possible role of a microbial consortium, marketed with a commercial name Micosat F UNO (the product is under evaluation at different locations) (see also: www.micosat.it), to improve health of vineyards severely affected by two devastating diseases, like Flavescence Dorée and Esca disease

and, in the same time, increase the quality of grapes, juices and wines. In integrated management, both diseases are very difficult to control with pesticides and, in organic viticulture, they are limiting factors to viticulture.

Two approaches were planned: firstly, the application of Micosat F consortium (commercial product developed by CCS Aosta Srl., Italy. Consortium contains different type bacterial strains which we have studied in chapter I of present work, and this product is under evaluation) in vineyards to study the disease progression or suppression during three following seasons 2017, 2018, 2019; secondly, in another study, using same consortium product Micosat F a transcriptomic study in grapevine by sprayin microbial consortium under controlled conditions, to check possible expression of genes related to the induced systemic resistance at different time points like 0, 2, 4, 10 and 24 hour post inoculation (hpi).

3. LITERATURE REVIEW

3.1. Grapevine

Grape (*Vitis vinifera*) belongs to the family *Vitaceae*, which comprises about 60 inter-fertile wild *Vitis* species distributed in Asia, North America and Europe under subtropical, Mediterranean and continental–temperate climatic conditions. It is the single *Vitis vinifera* species that acquired significant economic interest over time; some other species, for example the North American *V. rupestris*, *V. riparia* or *V. berlandieri* are used as breeding rootstock due to their resistance against grapevine pests and pathogens, such as *Phylloxera* and mildews. Indeed, a great majority of cultivars widely cultivated for fruit, juice and mainly for wine, classified as *Vitis vinifera* L. subsp. *vinifera* (or *sativa*), derive from wild forms (*Vitis vinifera* L. subsp. *sylvestris* (Gmelin) Hegi) **(Rossetto et al., 2002; Sefc et al., 2003; Crespan, 2004; Jean-Frédéric et al., 2010)**. In the past 30 years the global wine industry has undergone a transformation. It has changed from an industry characterised by relatively small, traditionally oriented, family-owned enterprises and a European focus, to a much more cosmopolitan industry dominated by multinational corporations. As a result, there is now a stronger focus on quality assurance and consistency **(Aylward, 2005)**. More wine is now produced and consumed in countries that have little or no history of wine production and consumption, and grapes are being grown in some very challenging climates. However, the vine propagation industry has not experienced the same degree of change. It remains largely an industry dominated by small to medium-sized family businesses and cooperatives, and although the progress towards modernisation has enabled nurseries to increase production, the quality of planting material is not yet of a consistently high standard.

3.2. Main grapevine diseases

The commercial grape varieties belonging to *Vitis vinifera* are susceptible to several diseases. Around the world where grapevine is cultivated, plants are attacked by a number of pathogens that cause diseases, like bacterial diseases, fungal diseases, virus diseases, miscellaneous diseases and disorders, nematodes and insect, pests,

which reduce yields and quality of fruits. Among the diseases, downy mildew, powdery mildew, probably are important (although manageable), and they affect the green tender parts viz. young shoots, leaves, tendrils, flowers and berries; and can cause up to 100% crop loss.

3.2.1. Phytoplasmas and Grapevine Yellows

Phytoplasmas, the gram-negative bacterial pathogens inducing yellows and witches' broom type diseases on different crops and causing devastating yield losses, are worldwide distributed. Until 1967 these diseases were thought to be caused by viruses due to thesimilarity of symptoms with virus disease, transmissibility by insects and the inability to culture on artificial media. They were named after the disease symptoms they caused on the host plant. **Doi et al. (1967)** discovered variable shaped structures in ultrathin sections of the phloem of plants affected by these diseases. These agents had no rigid cell walls, they were surrounded by a single cell membrane, their shape pleomorphic and their size ranges were similar to those of mycoplasmas (80–800 nanometres). In 1994, the trivial term of phytoplasma was given to these organisms by the Phytoplasma Working Team at the 10th Congress of the International Organization of Mycoplasmology **(Hogenhout et al., 2008)**. Recent studies applying advanced molecular techniques provided possibility to further clarify the status of these pathogens and in 2004, a new taxonomic designation was created for them. It was proposed that phytoplasmas be placed within the novel genus '*Candidatus* (*Ca*.) Phytoplasma' **(IRCPM, 2004)**. Phytoplasmas are the causal agents of a complex of diseases called "Grapevine Yellows".

Grapevine yellows diseases:

Grapevine yellows (GY) is a term for all the phytoplasma diseases occurring on *Vitis vinifera* cultivars on different continents. **GY diseases include:**

– *Flavescence dorée* (FD), Palatinate grapevine yellows (PGY) and *Bois noir* (BN, described also as Black wood, Legno nero) in certain countries of Europe

– North American grapevine yellows (Virginia grapevine yellows, I and III, New York grapevine yellows and grapevine yellows in Canada)

– Australian grapevine yellows (in Australia and New Zealand and Buckland Valley grapevine yellows in Australia)

– Grapevine yellows diseases described in other regions including South Africa and Chile **(Martelli and Boudon Padieu, 2006)**.

Symptoms of GY may appear on several parts of the grapevine stock including shoots, leaves, flowers, bunches and canes. The first symptoms become visible on young leaves in June-July. The young diseased *V. vinifera* shoots are weak and the necrosis of their terminal buds is frequent. The shoots have fir-like appearance due to their zig-zag growth and shortened internodes; their leaves are pale and slightly rolling downwards; this rolling of leaves will become more evident during the vegetation. With passing of time the leaf symptoms grow stronger; their rolling becomes triangle-shape, which is typical for phytoplasma infection. Discoloration develops on the leaf blade. On white varieties, the pale chlorotic colour turns later yellow to golden and becomes necrotic, on the red varieties: reddish to purple colours may appear sectorial or on the entire leaf blade including the veins. Due to uneven lignification, the diseased shoots have a weeping appearance. The rubbery canes become susceptible to frost and die during cold winter. It is common that symptoms develop only on one shoot or branch of the plant. Infected flowers wither may die and fall down. GY phytoplasmas are transmitted in persistent mode by univoltine Hemiptera vectors: leafhopper (*Cixiidae*) and planthopper (*Fulgoridae*) species that feed in the phloem of the leaf veins. Phytoplasmas multiply in the body of the insects. Getting into the salivary gland and then in the saliva they become injected into the phloem of the plant when the insect vector feeds. Phytoplasmas are spread by their vectors only short distances within the vineyards and its vicinity. Phytoplasmas overwinter in grapevine plants. Long distance dissemination of GY phytoplasmas occurs via infected propagating material **(Martelli and Boudon-Padieu, 2006)**.

3.2.2. Flavescence dorée (FD)

Flavescence Dorée (FD) was the first GY disease described by Caudwell **(1957)** in France. Its causal agent, the Flavescene dorée phytoplasma of the 16SrV phylogenetic group, and the FD isolates belong to subgroups 16SrV-C and –D. Based on the newer classification, the species name is '*Candidatus* Phytoplasma vitis'. FD phytoplasma is on the A2 Quarantine list of European and Mediterranean Plant Protection Organisation **(EPPO, 2010)**, a regulated pest in the European Union (Council Directive 2000/29), in the countries of the North American Plant Protection Organization **(NAPPO, 2009)**, in South Africa and New Zealand, too. FD is known to occur in France, Italy, Portugal, Serbia, Slovenia, Spain, and Switzerland **(Martelli & Boudon-Padieu, 2006)**. Quite recently it has been reported also in Croatia and Austria **(Seruga Music et al., 2010; EPPO, 2010)**. FD is highly epidemic and can cause important crop losses. The quantity and quality of the crop of the infected vines are significantly reduced. In case of severe infection, the plants may decline in a few years after it became infected **(OEPP/EPPO, 1997)**. All *Vitis vinifera* varieties grown in France, Italy and Spain were found to be susceptible to FD but they showed various levels of sensitivity. It was observed that the highly susceptible varieties did not recover after infection. FD infected vines may recover in the second year if they were protected with insecticide sprayings from re-infection. In case of re-infection of plants after recovery, symptoms might appear only on a few shots. The highly sensitive varieties do not recover. FD phytoplasma is transmitted from grapevine to grapevine only by *Scaphoideus titanus* Ball (Homoptera, Cicadellidae), the American grapevine leafhopper in a persistent manner. It was introduced from North America into Europe by infested propagating material at the beginning of the 20th century and it became established in several countries: France, Italy, Spain, Portugal, Serbia, Slovenia, Switzerland, Croatia, Austria and Hungary **(Der et al., 2007; Kölber et al., 2011)**.

3.2.3. Esca disease

Esca is a devastating trunk disease of grapevine. Due to its complexity, Esca is frequently named a SYNDROME, therefore, a quite complex disease characterised by

a large set of symptoms and signs referring to more pathogens, that may act more or less independently or in different life stages on the host plant. Indeed, two forms of Esca are known: 1) Apoplexia, a very acute disease onset with a rapid death of the plant within a few weeks; and 2) Chronic Esca, a disease where symptoms appear and develop slowly, season after season, with a progressive decay and death of the host plant within a few years. Symptoms of Esca are visible on any aerial part of the grapevine: leaves develop marginal necrosis, slowly progressing towards the central part of the leaf; grapes show a reduced growth and desiccate before ripening; twigs and vines show reduced growth, blights and necrosis. The most typical symptoms are visible when the trunk is cut transversally: the wood appear brown to dark brown, necrotic and no more functional **(Surico et al., 2008)**.

The causal agents of Esca are several fungi, each one with different importance in disease pathogenensis. The most important are: *Phaeoacremonium aleophilum*, *Phaeomoniella chlamydospora* and *Fomitiporia mediterranea*. They are necrotrophic fungi and enter the host plant through wounds along the trunk, cordons or lignified vines (mechanical injuries or pruning wounds); these fungi cause the collapse of the vascular tissue of lignified tissues and, additionally, they may produce phytotoxins as well. Visible symptoms are the result of blocking the xylem vessels and of phytotoxins **(Surico et al., 2008)**.

3.3. Gene expression in grapevine following microbial application

When the first land plants appeared about 500 million years ago from a pioneer green algal ancestor, they had to face harsh terrestrial environmental conditions, including desiccation, UV radiation, and attack of microbial pathogens **(Kenrick and Crane, 1997)**. The emergence of the several metabolic, biochemical, growth promoting, and resistance pathways considered to be important adaptations that allow land plants to survive under these important stresses **(Ferrer et al., 2008)**. The need to discover alternative crop protection strategies that can be used to improve food safety and security, as well as for maintaining human health, has been the target

of many investigations in recent years **(Romanazzi et al., 2012; Burketova et al., 2015)**. In particular, studies have increasingly targeted exogenous molecules that induce defense responses **(Walters et al., 2013)**. In this context, investigations on how nontoxic products (Microbial Consortium) can control plant diseases through activation of plant defense responses were fascinating. The effectiveness of compounds that have been described as 'resistance inducers' has been tested according to different crop protection strategies. These have shown encouraging results for their use as alternatives to traditional chemicals such as pesticides including fungicides **(Burketova et al., 2015; Oliveira et al., 2016)**. Grapevine transcriptomics studies started over a decade ago, initially using microarrays but later, exploiting the sequenced genomes **(Jaillon et al., 2007; Velasco et al., 2007)** and the availability of high-throughput sequencing, also using RNA-Seq approaches. As systems biology becomes more prevailing in everyday analysis, one of the pressing aspect of analysis is how to integrate different sources of information into one coherent framework that can be interrogated in order to gain knowledge about the system as a whole **(Rhee et al., 2006)**.

4. MATERIALS AND METHODS

4.1. Microbial consortium and its preparation

Bacterial strains individually studied in chapter I were used to prepare the consortium to check their effectiveness as together. Before formulating these strains as consortium, we have checked for compatibility of these strains each other (results not included). Formulation of consortia was done by permutation combinations in which repetition was not allowed and order of the bacteria does not matter. Six bacterial strains (SB14, SL81, SA51, PT65, PN53 and AR39) were formulated by using the following by inoculating one loopful of individual bacterial isolates, in 500 ml of sterilized nutrient broth having 0.01% Tween-80. Inoculated broths were incubated in an orbital shaker at 35°C, 200 rpm for 16 hrs to obtain a high concentration of actively growing bacteria. Wettable powder (WP) form consortium was prepared according to commercial product standards such as MICOSAT F **(Mukred et al., 2008)**.

4.2. Treatments of vineyards

Two neighbouring vineyards located in the municipality of Campagnola Emilia (Italy) where chosen. The grapevine cultivar was Lambrusco Salamino, the most popular variety in that area. One of the vineyards (VY1) was severely affected by Flavescence Dorée, with a disease incidence over 60%, the second one (VY2) was affected by Esca, again with a disease index over 60%. Following experimental plot designs was prepared:

VY1: 4 x 2 rows, each containing 300 plants, for a total of 2.400 plants; one row treated with Micoasat F, and one row (in parallel) untreated. Treatments: 60 g of Micosat F per plant applied to the roots in wintertime, plus 6 sprays from May (20-30 cm length of vine sprouts) to early August (40-50 days before vintage). Disease monitoring was done monthly (three times), assessing disease incidence and severity, based on a disease index: 0 = healthy plant, 1 = mild symptoms; 2 = heavy symptoms.

VY2: Six blocks (replicates) of 5 plants each, randomly distributed in the vineyard (approx. 2,000 plants). Three treatments were planned: 1) Micosat F1, applied as

described above; 2) Curvit, an innovative "copper + nutrients" product. 3) No sprays against FD. Disease monitoring was done 5 times, from May (20-30 cm vine length) to late August (berries with initial changes in colour, *i.e.* veraison). Disease monitoring was done monthly (three times), assessing disease incidence and severity, based on a disease index: 0 = healthy plant, 1 = mild symptoms; 2 = heavy symptoms; 3 = dead plant. Disease quantity (Q) was calculated as the product of Incidence and Severity, according the formula: Q = I x S.

4.3. Biochemical analysis of grapes/wine

Biochemical analyses were done on grapes sampled in VY1, since FD may greatly affect quality of juice. During vintage, for each thesis 20 grape clusters were harvested and subject to biochemical analyses to assess the quality of juice, prior to fermentation into wine. Biochemical parameters evaluated were density at different wavelength, total flavonoids content, pigments, anthocians, purity, and productivity.

4.4. Differential Gene expression Studies

4.4.1. Experimental Design

Leaf Material: Experiments were carried out on leaves of grapevine cv. *Lambrusco Salamino.* Two-years old plants in excellent phytosanitary conditions were selected in a nursery for the experiments and kept 2 months under controlled conditions in a large greenhouse.

Treatments: The mRNA expression changes in the grapevine leaves were analysed after a foliar treatment with the commercial microbial consortium (MICOSAT F) sprayed at a concentration of 6 g/l on three plants. In parallel sterile distilled water was sprayed on other three plants as control. Samples were taken at 0, 2, 4, 10 and 24 h post treatment: each sample contained one leaf for each plant (three leaves per sample). Leaves were cut from the plant and immediately dipped in liquid nitrogen, prior to keep them refrigerated in dry ice and stored at −80 °C, until RNA extraction.

4.2.2. Primers and Reference Gene Selection:

Specific primer sets were designed using the Primer3 software (http://biotools.umassmededu/bioapps/primer3_www.cgi). From the literature by Dufour et al. **(2016)** different genes along with reference genes **(Table 4.1)** were selected to understand the gene changes under microbial inoculation. Major classes of pathogenesis related (PR) genes, plant growth promotion and cell wall integrity and hormone dependent metabolism involved in a broad spectrum of biological pathways were chosen to study **(Table 4.1)**.

Table 4.1: Primer sequences used for RT-qPCR

S.No.	Gene Name	Gene (Nº accession NCBI)	Sequence	T_m °C	Role	Amplicon Length (bp)
1	1-amino cyclopropane-1-carboxylic acid	*ACC* (AF424611)	FP: GAAGGCCTTTTACGGGTCTC RP: CCAGCATCAGTGTGTGCTCT	55	Growth promoter released upon induction of ethylene. Expressed in 4-8 h upon stress until 48 h	120
2	Thaumatin-like / osmotin	*PR5* (XM_002282928.1)	FP: GGAGGCAATGGTTTCCACCTTGGG RP: ACTTGGACGGGACCATAGAGGTTAG	60	Defence responsive gene expressed upon pathogen attack; expression was initiate at 4-6 hpi until 48 hpi	187
3	Chitinase type 1	*PR11* (XM_002270543.1)	FP: CTCCACTGCGCAAACCGTGGT RP: TTTGCGTTTTCGGAGGAAATCGTGA	55	Helps in defence mechanism, low level expression in control but upon pathogen treatment high level expression after 6 hpi up to 48-96 hpi.	159
4	Defensin	*PR12* (XM_002281153)	FP: GTGCAAGAACTGGGAGGGTGCC RP: GCAGAAGCATGCAACTCCCGGG	60	Protective role against insects, fungi and bacteria. Expression upon pathogen attack 24-72 hpi	70
5	Phenylalanine ammonialyse	*PAL* (X75967)	FP: ACAACAATGGACTGCCATCA RP: CACTTTCGACATGGTTGGTG	52	Defence related, expressed 6 hpi to 48 hpi max.	144
6	Chalcone Synthase	*CHS* (X75969.1)	FP: CCAACAATGGTGTCAGTTGC	52		159

			RP: CTCGGTCATGTGCTCACTGT		Committed enzyme in the pathways of flavonoid and anthocyanin compounds. Abundant in leaves.	
7	Lignin forming Peroxidase	*PER* (XM_002274762.1)	FP: TAAGCGCCACAAGAACACTG RP: GGACCTCCTTGTTGAGTCCA	52	Jasmonic acid pathway, PGPR mediated induced systemic resistance. 6-8 hpi	106
8	SA Methyl Transferase	*SAMT1* (XM_002262982.1)	FP: AATCCTTGCCCAAGTTCCAG RP: GAGACAACCATTGGAGACTG	53	Synthesized from salicylic acid, a phytohormone that contributes to plant pathogen defence and provides taste and scent to many fruits.	159
9	Pathogenesis Related	*PR1 (AY560589)*	FP: TGGTGTCGGCCCTATGACA RP: GGCCACCAGAGTGTTGCAT	55	Defence responsive, pathogenesis related	101
10	Pathogenesis Related	*PR3 (FJ596176)*	FP: TAGCCCTGGCGACAATCTTG RP: ATAGCTGTCCCCTGCATTGC	55	Defence responsive, pathogenesis related	125
11	Elongation factor	*EF1 (*AF176496)	FP: GAAGGTTGACCTCTCGGATG RP: AGAGCCTCTCCCTCAAAAGG	55	Reference gene	84
12	Tubulin Alpha	*TA* (XM_002285685.1)	FP: GTCGGCGCTGAAGGTGTGGA RP: GAGGTGGCGGGCAAACCCTC	60	Reference gene	112

4.4.3. RNA extraction

High quality total RNA was obtained from the fruit according to the protocol of Landi and Romanazzi **(2011)**. Briefly, 5 g of leaf samples including both control and treated were ground in liquid nitrogen, and 200 mg of the resulting leaf powders was randomly collected for RNA extraction. Manually prepared extraction buffer was added (1 mL; 100 mM Tris-HCl, pH 8.0, 25 mM EDTA, pH 8.0, 2% [w/v] CTAB, 2% [v/v] β-mercaptoethanol, 2.5 M NaCl, and 2% [w/v] soluble PVP-40), and the samples were incubated at 65 °C for 30 min, following centrifugation at 10,000 g for 10 mins at 4 ^{0}C. The supernatant was transferred to QIAshredder spin columns and further proceed according to manufactures protocol when using RNeasy Plant Mini Kit, QIAGEN, Italy. RNA integrity was verified by agarose gels that were stained using SYBRSafe (Invitrogen, Italy). RNA purity was assessed based on an absorbance ratio of 1.80 to 1.90 at 260/280 nm, using Nanodrop and 1.8 to 2.0 at 230/260 nm.

4.4.4. Reverse Transcription:

A total of 300 ng RNA was used for cDNA synthesis with reverse-transcription PCR, using GoTaq 2-step RT-qPCR system (Promega, Milan, Italy), according to the manufacturer's instructions. From each RNA extraction, cDNA synthesis was performed twice, and the products were mixed before the gene expression studies.

4.4.5. Real-time qPCR with ABI-7500 Real-Time thermocycler

The expression of the selected genes was assessed by using an ABI-7500 qPCR system (Applied Biosystems) with SYBR Green to detect dsDNA synthesis. For each reaction, 1 µL of each primer at 200 nM and 10 µL of GoTaq® qPCR Master Mix Plus for SYBR® Assay (Promega, Italy) including Hot start DNA polymerase, dNTP and MgCl2 and 2 µL of cDNAs, were used according to the manufacturer's instructions. Each PCR reaction was done in triplicate. PCR was performed at 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 s, 52-60 °C for 15 s and 72 °C for 45 s. Data were analyzed as the cycle of quantification (Cq), where the fluorescence signal of the

amplified DNA intersected with the background noise. For each gene and for each modality, a mean Cq value was obtained.

qRT-PCR was carried out using an ABI-7500 Real-Time thermocycler. Three technical replicates of each biological replicate were used for qRT-PCR analysis using GoTaq qPCR Master Mix (Promega, Italy). Experiments were performed on each of the target genes together with two reference genes (Elongation factor and Tubulin Alpha) for equal amplification efficiencies. Melting curve analysis was performed and gel electrophoresis of the final product confirmed single amplicons. Negative control reactions using with RNA were run to check the absence of genomic DNA. To determine relative fold differences for each sample in each treatment, the CT value for each gene was normalized to the CT value for the reference gene using LINreg and REST software's and was calculated relative to a control using the 2-ΔΔCT method **(Livak and Schmittgen, 2001)**.

4.5. STATISTICAL ANALYSIS

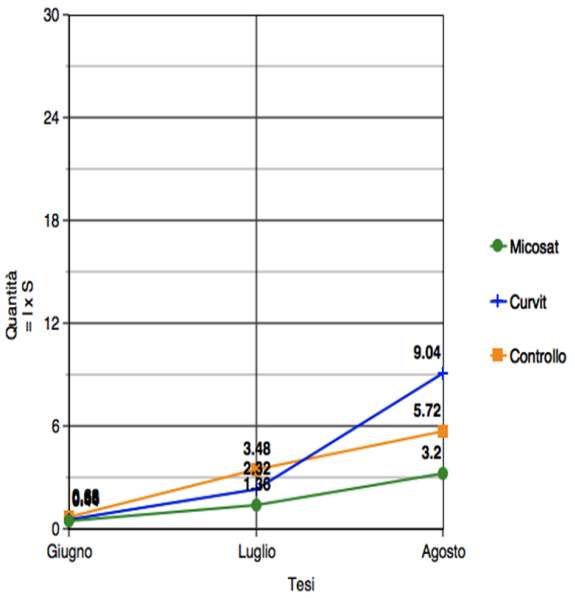
Data were statistically tested by REST 2009 and LINreg software. Each treatment was analyzed with three of each biological replicate analysed as three technical replicate and the standard deviation was calculated and data expressed as the mean ± SEM of three replicates.

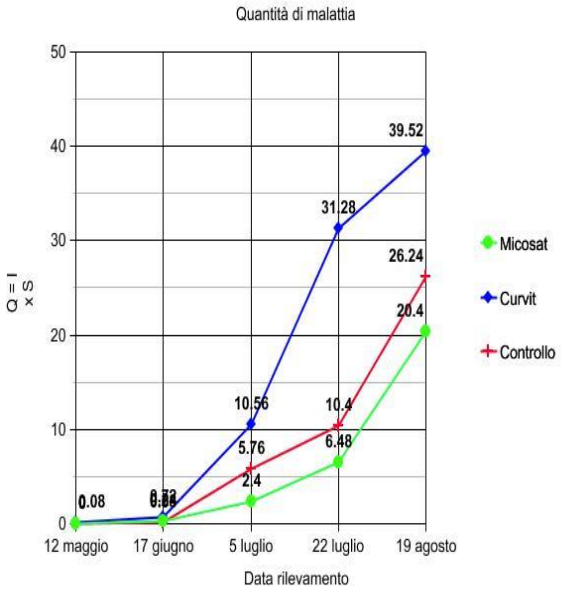
5. RESULTS

5.1. Biocontrol and biochemical activity:

The development of Flavescence Dorée in the experimental vineyard was monitored along three seasons. Both in treated and in the control plots the disease increased by 10-12% each year; no significant difference was observed among the plots, so that, at the end of the three-year experiment, in all plot's disease incidence reached 90-95%.

The development of Esca disease in the second experimental vineyard along three season showed significative differences among the three treatments **(Figure 5.1 a, b, c)**.





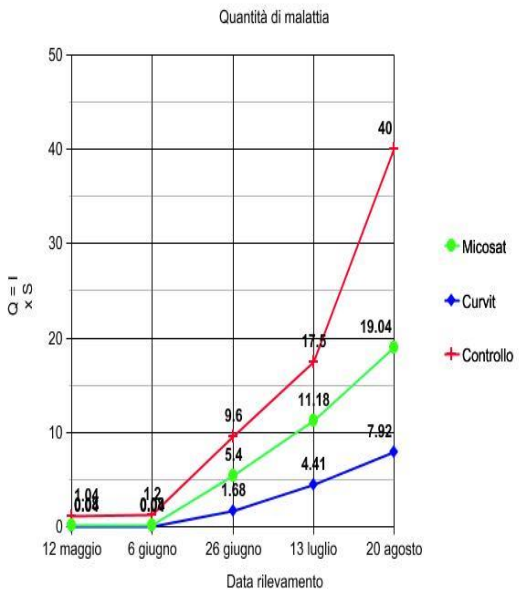


Figure 5.1 a, b, c: Increase of Esca disease quantity in vineyard plots after treatments with Micosat F microbial consortium and Curvit, an innovative copper compounds amended with plant nutrients, as compared with untreated plots.

Disease quantity started to be visible at the end of June, increasing until harvest (September). Disease quantity increase in Micosat treated plots was significantly lower in all seasons, when compared with other treatments. Curvit did not perform well during the first and second season, but the last season, when disease quantity reached an index of 7.92, compared to the control (Q = 40), had the best performance. If all results (three years) are summarised, then Micosat treated plots showed the slowest disease progression.

Parameters of grape juice before fermentation showed a better biochemical profile, when compared to the control: total flavonoids and co-pigments increased significantly after treatments with Micosat F1. Colour intensity also increased significantly **(Table 5.2)**.

	CONTROL	Micosat F	Fvalue
620 nm	0.013 ± 0.000	0.012 ± 0.002	n.s
520 nm	0.103 ± 0.000	0.083 ± 0.019	n.s
420 nm	0.085 ± 0.000	0.074 ± 0.013	n.s
580 nm	0.030 ± 0.000	0.025 ± 0.005	n.s
Total flavonoids mg/L	**752 ± 2.4**	**571 ± 68**	**5 57***
IC	0.201 ± 0.000	0.169 ± 0.033	n.s
Colour intensity	**0.817 ± 0.002**	**0.902 ± 0.047**	**6.56****
Copigments	**0.287 ± 0.000**	**0.306 ± 0.013**	**4.78***
Yellow	**0.420 ± 0.001**	**0.439 ± 0.010**	**7.21****
Red	**0.515 ± 0.000**	**0.487 ± 0.014**	**7.19****
Blue	0.065 ± 0.000	0.074 ± 0.005	n.s
Red colour purity	0.472 ± 0.001	0.527 ± 0.031	n.s
Total anthocyans mg/L	98.0 ± 0.02	78.4 ± 17.64	n.s
Productivity %	54.5 ± 7.01	55.3 ± 7.11	n.s

Table 5.2: Biochemical parameters values in grape juice prior to fermentation into wine in plots treated with microbial consortia. Numerical values are mean ± SD (n=20).

5.2. Expression profile of genes up- and down-regulated in response to microbial treatment

To study the relative gene expression, mRNA was extracted and converted to cDNA from leaves of grapevine plants after exposure to microbial consortium at 0, 2, 4, 10 and 24 hpi. This allowed the analysis of gene expression in a synchronous time

course experiment. Grapevine α-tubulin and elongation factor genes were selected as an internal control to analyse quality of RNA and to normalise the different samples for differences in the amount of plant RNA. No amplification was obtained when RT-PCR was performed for no template control indicating that all primers were specific for grapevine. Different genes like plant growth promotion and pathogenicity related (PR) genes along with two reference genes (Tubulin alpha and Elongation factor) **(Table 5.1)** were selected to understand the differentiation of gene pattern upon microbial inoculation compared with uninoculated control plants. Major classes of genes involved in a broad spectrum of biological pathways were chosen to study (**Table 5.1**).

Currently, data normalization using a set of reference genes is considered to be the gold standard method for accurate measurement of qPCR expression levels of target genes. RNA quality is one of the crucial parameters that must be addressed in a gene expression profiling experiment. In the present study, all samples were analysed spectrophotometrically and in agarose gels showing absorbance ratios at 260/280 and 260/230 nm above 1.8, well-defined bands corresponding to the rRNA and absence of nucleic acid degradation. To confirm the absence of contaminating gDNA, positive and no RT controls were used for each candidate gene amplification. DNase treatment (DNase I Digestion, Sigma-Aldrich) was followed by a careful check for the absence of gDNA through qPCR analysis of a target on the crude RNA.

In qPCR, when using a SYBR Green approach, amplification specificity of several genes should be supported by both melting curves and gel electrophoresis. In our samples, single PCR amplification products with the expected size for each gene were found. Melting curves of the genes tested were analysed to detect the absence/presence of primer dimer or non-specific PCR products. For all genes, no-template controls (NTCs) had no Cq values or the Cq values ranged between 29 and 34 Cq. Since no amplicon peak was obtained from melting curve analysis, the Cq

values observed on NTCs were attributed to primer dimer formation/hairpins, and thus disregarded.

PCR efficiency of each primer pair was calculated through the standard curve method using the pool of all cDNA samples in a ten-fold serial dilution. The amplification efficiency (E) of the reactions ranged from 1.907 (90%) to 1.992 (99%), with correlation coefficients R^2 varying from 0.993 to 0.998. To account that any variation between biological replicates was not due to the treatments but intrinsic to the gene itself, data from the biological replicates were analysed separately by statistical algorithms.

5.2. Expression of *PR* genes

Comparative analysis of differentially expressed genes in inoculated plant leaves compared to control plant leaves showed a distinct pattern of gene regulation. *PR* protein family genes, namely *PR1, PR3, PR5, PR11* and *PR12* that play significant role in defence mechanisms were expressed. At T_0 time point, all these genes were downregulated with statistical significance of $p < 0.05$ and biological fold change difference is observed. After two hours *i.e.,* at T_2 hpi, microbial inoculation started to induce these genes actively. *PR12* was significantly downregulated ($p < 0.05$) without any biological fold change, whereas *PR11* was downregulated by 5.2-fold; no expression was observed in other genes **(Figure 5.2)**. Differential expression of gene pattern was observed from 4 hpi, at T_4 *PR5* was upregulated by 3.7-fold and *PR3* (5.3-fold) & *PR11* (6.3-fold) were significantly downregulated with a $p < 0.05$. Interestingly at T_{10} (10 hpi), *PR11* was upregulated by 6.7-fold and *PR3* downregulated by 4.5-fold difference with comparison to *PR1, PR5 & PR12* these genes downregulated but without any biological significant expression. After 24 hpi (T_{24}), all the PR proteins were upregulated, but only *PR3* by 3.7-fold, *PR5* by 4.2-fold and *PR11* by 4.1-fold were upregulated with highly statistical significance of $p< 0.01$. *PR1* and *PR12* genes were

also upregulated, but there is no statistical and biological significance observed **(Figure 5.2).**

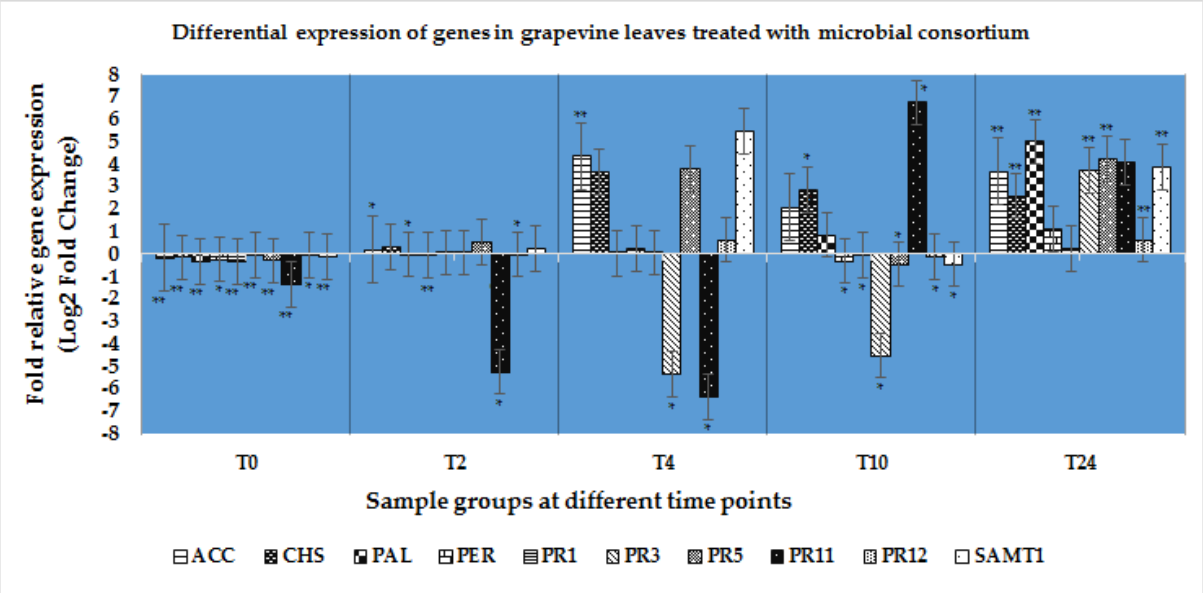


Figure 5.2: Relative gene expression in treated leaf samples compared with control leaf samples at different time points after inoculation with microbial consortium. Data were normalized on the most stable housekeeping genes Elongation factor (*EF1*) and α-Tubulin (*TA*). Fold change and statistical analysis according to REST. Data are the means ± SEM from three independent assays. One star (*): significance difference at $p < 0.05$; two stars (**): highly significance difference at $p< 0.01$; no star: no significance difference observed. Three independent biological replicates were used for each sample for the analysis. The threshold fold change difference considered as 1 for Log2 scale.

5.3. Expression of secondary metabolites, cell wall reinforcement and signalling gens

Results also showed the differential expression of genes involved in the pathways of secondary metabolites (*PAL* and *CHS*), growth promoting signalling pathways (*ACC* and *SAMT1*) and cell wall integrity (*PER*) involved genes **(Table 4.1 and Figure 5.2)**. Soon after microbial consortium treatment *i.e.*, at T_0 all these genes were downregulated without any biological significance, but statistically they are

highly significant ($p < 0.01$). After two hours (T_2), microbial consortium slowly started to induce some of the specific genes, at this time point only *PAL* and *PER* were downregulated without any fold change difference. From T_4 (4 hpi), it was clearly evident that microbes present in the consortium started to induce genes effectively. At this time point *ACC, CHS* and *SAMT1* genes were significantly upregulated by 4.3-fold ($p < 0.01$), 3.6-fold and 5.4-fold change respectively. Similarly, at T_{10} (10 hpi), both *ACC* and *CHS* were upregulated by biological fold change of 2, whereas *PER* and *SAMT1* were downregulated without any biological significance, but these genes show statistical significance of $p < 0.05$. From Figure 1, it was evident that at T_{24} (24 hpi), all the genes including *ACC* (3.6-fold, $p < 0.01$), *CHS* (2.6-fold, $p < 0.01$), *PAL* (5.0-fold. $p < 0.01$) and *SAMT1* (3.8-fold, $p < 0.01$) were significantly upregulated, whereas *PER* was upregulated without any biological and statistical significance.

6. DISCUSSION

Biologicals are an alternative method for combating plant pathogens **(Harman, 2000)**, and there are commercially available examples **(Velivelli et al., 2014)**. Beneficial rhizobacteria may secrete antibiotics and other compounds antagonistic to plant pathogens. Production of antibiotics is one of the more common biocontrol mechanisms **(Fravel, 1988; Doumbou et al., 2001)**. There are commercially available examples of biocontrol agents **(Velivelli et al., 2014)**. Flavescence Dorée and Esca are two grapevine diseases, whose control is particularly cumbersome: indeed, both diseases have an epidemic aspect, slowly or fast progressing according season, plant genotype, pathogen(s) virulence, agricultural practices. In case of Flavescence Dorée, results were not satisfactory and no differences in disease progression and quantity were observed between treated and untreated plots. This can be explained with the total lack of vector control: the microbial consortium might have a positive effect on the plant physiology, but no chance to control *S. titanus*, the insect vector on the phytoplasma. Therefore, lacking an efficient control of the vector, beneficial microbes have no chance to control the disease. In case of Esca, experiments showed a remarkable effect of sprays with the microbial consortium in slowering disease progression in commercial vineyards. Since grapevine is a multiannual crop – a vineyard may last over 30 years – a continuous disease slowering may have a positive impact in grapevine longevity and productivity. In our case, the vineyard was managed mechanically using mechanical pruning and mechanical harvesting. Mechanical operations may cause a large quantity of wounds on plants and, additionally, blades can serve for inoculum dispersal. Since Esca is caused by phytopathogenic fungi, spores and other vegetative parts are spread by machines that, in the same time, produce wounds. In our case, mechanisation reduced the beneficial effects of Micosat F. Among bacterial biocontrol agents, *Bacillus subtilis* was the most tested toward Esca disease. Its ascertained *in vitro* efficiency against Esca pathogens was confirmed as wound protectant, with different biocontrol degrees according to both Esca and selected pathogens **(Halleen et al., 2010; Kotze et al., 2011; Schmidt et al., 2001)**. In nurseries, *B. subtilis* reduced the incidence of the vascular pathogen

associated to Esca complex, but the severity of internal symptoms increased **(Fourie and Halleen, 2004)**.

The nitrogen, phenolic and volatile compositions of grape berries have important roles in the final quality of wines. Must nitrogen composition affect the growth and development of the yeast during alcoholic fermentation, defines the fermentation kinetics, and contributes to the formation of fermentative compounds, mainly as certain higher alcohols and esters that constitute the 'fermentation bouquet' of wines **(Bisson and Butzke, 2000; Bell and Henschke, 2008)**. Beneficial microbes may have a positive impact on quality of fruits and vegetables, increasing their nutritional value. Grape juice, prior to fermentation into wines, showed a better quality, especially in term of pigmentation and stability towards oxidation. This is one the basics for a production of high-quality wines. In some crops, volatile compounds are important for the flavor of the final products, as in the case of grapevine **(Vitis vinifera** L.). In fact, in this plant, compounds such as 1-propanol and 2-butanone have a strong impact on the aroma of the produced wines and give them their characteristic flavor. Verginer et al. **(2010)** reported the isolation and identification of different rhizospheric microorganisms (bacteria and fungi) that influenced the production of volatile compounds in fruits.

Normalization is one of the key factors affecting the accuracy and reliability of quantitative gene expression analysis. Here, we randomly selected ten genes with two reference genes for their use as internal controls in gene expression studies for the interaction between grapevine and microbial consortium. The dataset was used representing two different samples: the dataset compares each control and treated (inoculated with microbial consortium) grapevine leaf samples analysed at different time course like 0, 2, 4, 10 and 24 hours post inoculation (hpi).

Plants have evolved complicated signaling and defense pathways in response to microbes over time. Identification and understanding of such processes will be a platform to develop genomic based breeding for economically important crops.

Grapes have become one of the main fruits and beverage crops worldwide over the last few decades. In this study, a total of ten (*ACC, CHS, PAL, PER, PR1, PR3, PR5, PR11, PR12* and *SAMT1*) **(Table 1)** differentially expressed genes (DEGs) were selected for the analysis of grapevine leaf RNA sample, and upon blast search on NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) database showed that these genes related to the pathways associated with secondary metabolites, pathogenicity related (PR), cell wall reinforcement and also signalling pathways. Therefore, these results indicated that microbial consortium induced a wide range of responses in the host.

PR proteins were discovered for the first time in tobacco leaves, indicating the plant's hypersensitive reaction to tobacco mosaic virus (TMV) **(Van Loon et al., 1970)**. These proteins are found in many plant species **(Van Loon, 1985)**, including grape. PR proteins are proteins encoded but not expressed in host plant in the absence of interaction with a pathogen. They are also defined as proteins generally induced in an infection **(Antoniw and White, 1980)**. PR proteins are also induced under conditions of non-pathogenic origin, such as stress. Examples include cytoplasm separation **(Wagih and Coutts, 1981)** and high concentrations of plant hormones production **(Antoniw et al., 1981)**. In the last several decades, researchers have conferred systemic resistance against pathogens to decrease disease severity. The studies have shown that plants can obtain systemic resistance by exposure to certain strains of PGPR, which is termed ISR (Induced Systemic Resistance) **(Raupach et al., 1996; Ryu et al., 2004; Whipps, 2001)**.

Accumulation of pathogenesis-related (PR) proteins is known to be associated with systemic acquired resistance (SAR) in plants **(Ryals et al., 1996)**. Studies have shown that PR-proteins are also induced in plants upon treatment with *P. fluorescens* **(M'Piga et al., 1997)**. PR protein like chitinases (*PR3* and *PR11*) has the potential to hydrolyse chitin, which is major component of fungal cell wall. Moreover, the chitinase releases elicitors from the walls of fungi which, in turn, stimulate various defense responses in plants **(Ren and West, 1992)**. Microbial consortium treated plants

in the present study revealed that microbes present in the consortium significantly induced the all the PR proteins studied **(Figure 1)** like PR1 (Defence related), PR3 (Chitinase group), PR5 (Thaumatin), PR11 (Chitinase like protein) and PR12 (Defensins). In general, these genes expressed upon pathogen recognition, but salicylic acid (SA) mediated pathway also induce these types of genes in plants. In this study, microbial treatment induced salicylic acid expression gradually as time course increased. In our study, all these PR genes were significantly downregulated at T_0 and at T_2 both *PR12* and *PR11* (5.2-fold) were significantly downregulated. Microbial inoculation induced upregulation of PR3 (5.3-fold), PR5 (3.7-fold) and PR11 (6.3-fold) genes with a statistical significance of $P < 0.05$. at 10 hpi only PR11 (6.7-fold) gene was upregulated whereas, at 24 hpi, all the genes were significantly upregulated.

Peroxidase (PER) represents a component of an early response in plants to pathogen attack and plays a key role in the biosynthesis of lignin which limits the extent of pathogen spread **(Bruce and West, 1989)**. The products of this enzyme in the presence of hydrogen donor and hydrogen peroxide have antimicrobial activity and even antiviral activity **(Van Loon and Callow, 1983)**. Increased peroxidase (PO) has been observed in a number of resistant interactions involving plant pathogenic fungi, bacteria and virus **(Chen et al., 2009)**. In the present study, the activity of peroxidase was one-fold greater than the uninoculated control in microbial consortium treated plants. The expression was differentially changed from 0 hpi to 24 hpi. Initially, after treatment with microbes, *PER* gene was significantly downregulated but after 24 hours from inoculation it was upregulated with biological significance. Similarly, increased activity of cell wall bound peroxidase has been reported in different plants such as cucumber **(Chen et al., 2009)**, rice **(Reimers et al., 1992)** and tomato **(Mohan et al., 1993)**. Increased activities of PO were also observed in *P. chlororaphis* isolate (BCA) and *B. subtilis* isolate (CBE4) treated hot pepper seedlings after challenge inoculation with the pathogen *P. aphanidermatum* **(Nakkeeran et al., 2006)**. However, cell suspension or its cell-free culture filtrate of *B. amyloliquefaciens* B014 induced an

increased activities of defense-related enzymes phenylalanine ammonia lyase, peroxidase and polyphenol oxidase, when compared to control in *Anthurium* plants challenged with the blight pathogen *Xanthomonas axonopodis* pv. *dieffenbachiae* **(Li et al., 2012)**.

Phenylalanine ammonia-lyase (*PAL*) plays an important role in the biosynthesis of various defense chemicals in phenylpropanoid metabolism. Activity of PAL could be induced in plant-pathogen interactions and fungal elicitor treatment. De Meyer et al. **(1999)** reported that rhizosphere colonization by *P. aeruginosa* 7NSK2 activated PAL in bean roots and increased the salicylic acid levels in leaves. In the present study, increased activity of PAL was recorded in bioformulation treated plants. The time required to activate the defense mechanisms is important for the suppression of the invading pathogen. Earlier and higher levels of expression of defense enzymes and accumulation of inhibitory compounds at the infection site certainly prevent the fungal mycelial colonization. In the treated grapevine, the activity of *PAL* gradually increased its level after treatment while the enzyme activity in control plants remained constant. The activity of PAL was at T_0 and T_2 downregulated, without any fold change and remained without change in expression at T_4 and T10. Whereas at T_{24}, was upregulated with a fold change of 5.0. Several studies have shown that PAL activity is induced in plants upon treatment with *P. fluorescens* **(Chen et al., 2000; Sundaravadana, 2002; Saravanakumar et al., 2003)**.

The *CHS* (Chalcone synthase) gene family plays important roles in the growth and development of plants. Most plant genomes contain smaller *CHS* gene families. For example, in *Petunia hybrida*, eight complete *CHS* genes and four partial genes have been cloned and sequenced **(Koes et al., 1989)**. Six *CHS* genes were identified in *Ipomoea* **(Durbin et al., 2000)**. At least eight *CHS* members were identified in pea (*Pisum sativum*) **(Ito et al., 1997)**. Flavonoids are synthesized by condensation of a phenylpropanoid compound with three molecules of malonyl coenzyme A. This reaction is catalyzed by the enzyme chalcone synthase that leads to the formation

of chalcones. The chalcones are subsequently cyclized under acidic conditions to form flavonoids **(Shahidi and Naczk, 2004)**. In the present study there is no expression at initial time points T_0 and T_2, but from T_4 *CHS* significantly upregulated.

Salicylic acid (SA) is an important signal molecule of defense responses directed by resistance genes and a methylated form of SA has been implicated in the establishment of SAR **(Park et al., 2007; Singh et al., 2004; Tsuda et al., 2008; Vlot et al., 2009)**. Methyl salicylate (MeSA) is a volatile plant secondary metabolite that is an important contributor to taste and scent of many fruits and flowers. It is synthesized from salicylic acid (SA), a phytohormone that contributes to plant pathogen defense. MeSA is synthesized by members of a family of O-methyltransferases **(Tieman et al., 2010)**. Volatile compounds are important to many aspects of plant growth and development as well as inter-kingdom interactions. They act as attractants and repellents of insects **(James, 2003; Zhu and Park, 2005)**, promote defense against microbial pathogens **(Durrant and Dong, 2004; Loake and Grant, 2007)** and act as attractants for seed dispersing organisms. MeSA is one of several phenylpropanoids that significantly contribute to the unique flavor of grapes. Salicylate O-methyltransferase gene (*SAMT1*) is differentially expressed in treated leaf samples. At 0 hpi, this gene was significantly downregulated, and no expression was observed at 2 hpi. At 4 hpi significant fold change upregulation was evidenced. Again, down regulated at 10 hpi, until 24 hpi all the SA was pooled up and MeSA gene was significantly upregulated by 3.8-fold. This putative gene encodes an enzyme catalysing the synthesis of MeSA from salicylic acid, a well-known plant defense messenger. This result suggests that MeSA might play a role SAR **(Tieman et al., 2010)**.

1-Aminocyclopropane-1-carboxylic acid (ACC) is best known as the direct precursor of ethylene in the ethylene biosynthesis pathway. ACC has been identified as a potential signaling molecule, independent of ethylene. This property of ACC is perhaps the most exciting, opening new avenues in ACC research, with potentially profound effects on plant physiology. The molecular mechanism by which ACC is

signaling and the identity of other putative signaling components in such an 'ACC pathway' remain to be discovered **(Amrhein et al., 1981; Van de Poel and Van Der Straeten, 2014)**. During berry development, veraison is a key developmental stage in grapes as most of the compositional changes that determine quality are triggered in this stage. Recently, it was demonstrated that ethylene biosynthesis was up-regulated at veraison. In this work, we evaluated the expression of 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase genes that catalyze the last step in ethylene biosynthesis during berry development. At initial time points like 0 & 2 hpi there is no significant expression of *ACC* gene but, from 4 to 24 hpi there is a significant upregulation in the expression. Our results similar to the findings of **Defilippi et al. (2013).**

7. CONCLUSION:

Our data confirm that plant responses to microbes are dynamic processes that induce deep changes in the kind, quantity and timing of the genes involved. This establishes novel homeostasis between plants and their environment that can enhance plant defense mechanisms against pathogens. The crucial impact of microbial consortium on the grapevine leaves generally begins with down-regulation, followed by over-expression of fundamental genes such as PR proteins, secondary metabolites and phytohormones. This helps to maintain the imbalance/balance of SAR signaling, and attributes a key role to the plants as the sensors of environmental changes, which allows them to protect the from stresses. The typical SA signaling during plant immunity was found to be associated with plant-microbe interactions. However, the involvement of PR proteins with microbes, and in particular of PR1, which is one of the SAR response markers, suggests SAR induction also for microbial inoculation. We have highlighted here that the resistance inducers that are effective in the control of Esca disease of grapevine but not the FD. The genes identified in the present study can represent markers to better elucidate plant/pathogen/resistance inducer interactions and to design novel sustainable disease management strategies.

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