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Development of innovative microbial-based biostimulants from agri-food waste for sustainable agricultural productions

Ida Romano

Thesis Committe

Thesis supervisor

Prof. ssa Olimpia Pepe

Professor at the Dept. of Agricultural Sciences,

Division of Microbiology

University of Naples Federico II, Naples, Italy

Thesis co- supervisors

Prof. Albino Maggio

Professor at the Dept. of Agricultural Sciences,

Division of Microbiology

University of Naples Federico II, Naples, Italy

Dr.ssa Valeria Ventorino

Researcher at the Dept. of Agricultural Sciences,

Division of Microbiology

University of Naples Federico II, Naples, Italy

PhD program coordinator

Prof. Albino Maggio

Professor at the Dept. of Agricultural Sciences,

Division of Microbiology

University of Naples Federico II, Naples, Italy

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1 Biostimulant for agricultural applications

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1.1 Background

The Green Revolution of the 20th century allowed the development of the global food production (Backer et al., 2018). It was characterized by two main advances: chemical inputs (such as pesticides, herbicides, and chemical fertilizers) and improving crop plants through targeted breeding and genetic manipulations (Backer et al., 2018). However, advantages achieved through chemical fertilization had high environmental costs. In the last years there was an increasing demand to reduce the use of chemical products and to develop more sustainable agri-food systems both for environmental and human health. This "Fresh Green Revolution" is based on intensive inputs with reduced environmental impact, like utilization of microbial based inoculants and manipulations of the microbiome community structure (Backer et al., 2018). This new trend increased after the adaptation of agricultural legislation in several countries (Romano et al., 2020). Soil microorganisms, which only comprise less than 1% of the total mass of soil, play an important role in agriculture. Some of them being essential for decomposing organic matter and recycling of nutrients, others form relationships with plant roots and provide important nutrients (Desbrosses and Stougaard, 2011); their potential was recognized, which led to their commercialization (Backer et al., 2018). Intensive farming reduce the abundance and activity of soil microbes, thus the application of microbial based inocula might help to restore microbial populations (Alori and Babalola, 2018).

The use of microbial inoculants has a long history, it began with broad-scale rhizobial inoculation of legumes in the early 20th century (Trabelsi and Mhamdi, 2013). Recently, strains of *Bacillus*, *Pseudomonas*, *Glomus*, *Azotobacter*, *Trichoderma*,and others have been commercialized due to their abilities to enhance plant productions, that have been extensively studied and described (Alori and Babalola, 2018; Trabelsi and Mhamdi, 2013).

1.2 Definition of Biostimulants and Biofertilizers

Microbial inoculants known as "soil inoculants" or "bioinoculants" are agricultural amendments containing beneficial rhizospheric or endophytic microbes that promote plant health. Considering their function, two kinds of microbial inoculants are defined: biostimulants and biofertilizers.

Plant biostimulants are used to improve crop production and the nutritional quality of agri-food products. They are included in agricultural management practices to reduce chemical inputs, to increase the productivity and to restore the natural equilibrium in agro-ecosystems. The widely accepted definition of plant biostimulants by du Jardin, (2015) is: substance (s) and/or micro-organisms whose function, when applied to plants or the soil rhizosphere, stimulates the natural processes to enhance/benefit nutrient uptake and efficiency, improve abiotic stress tolerance and crop quality (Woo and Pepe, 2018; De Pascale et al., 2017). Biofertilizers are defined as natural fertilizers that contain bacteria, algae, fungi alone or in combination. The microbial components are able to colonize the rhizosphere or the interior of the plants and promote their growth, by

improving, in particular, the acquisition of primary nutrients to target crops when applied to soils, seeds or plant surfaces (Sheraz Mahdi et al., 2010).

1.3 Mode of action

Biostimulant are products that contain living or latent cells of efficient bacterial or fungal strains able to increase the number of microorganisms in soils and to accelerate processes which augment the availability of nutrients assimilated by plants (Sheraz Mahdi et al., 2010).

Biofertilizers can promote plant growth, increase crop yields and quality by several mechanisms like: nitrogen (N) fixation, phosphorus (P) and potassium solubilization, plant growth promotion, solubilization of micronutrients, preventing the depletion of the soil organic matter and maintaining the natural habitat of the soil (Brenner et al., 2008; Chakdar et al., 2018). Several microorganisms and their association with crop plants are being exploited in the production of biostimulant and biofertilizers and can be grouped in different ways based on their nature and/or functions.

1.3.1 Nitrogen fixers

Some bacterial strains and algae are able to fix atmospheric nitrogen (N_2) into plant available forms like ammonia and nitrate, this process is known as Biological Nitrogen Fixation (BNF; Gothwal et al., 2008). This mechanism allowed the utilization of some microorganisms as biofertilizers, which may act as a substitute for mineral N fertilizers and might help to maintain soil N reserves (Peoples and Craswell, 1992).They can be divided into three groups: free-living bacteria like *Azotobacter*, associative like *Azospirillium*, and symbiotic bacteria like *Rhizobium*, *Frankia* and *Azolla* (Kumar et al., 2018). It's important to highlight that symbiotic N fixers are the main contributors for BNF in nature (Kumar et al., 2018).

Azotobacter: it is a free–living N fixing diazotroph. Strains belonging to this genus have several beneficial effects on crop growth, yield and quality, as well as increase BNF (Jnawali et al., 2015). *Azotobacter* strains promote plant growth by regulating the level of other substances like auxins, cytokinins and giberellic acid (Jnawali et al., 2015). In addition, *Azotobacter* cells can stimulate rhizospheric microbes, have antimicrobial activity and improve plant nutrient uptake (Jnawali et al., 2015; Viscardi et al., 2016).

Azospirillum: among the associative N fixing bacteria they are one of the earliest discovered and well characterized (Van Dommelen and Vanderleyden, 2007). *Azospirillum* have a positive influence on plant growth, crop yields and N content of plants. The plant growth promoting effects exerted by *Azospirillum* has been attributed to several mechanisms like production of Indole Acetic Acid (IAA), disease resistance and drought tolerance, but especially to BNF (Sheraz Mahdi et al., 2010; Van Dommelen and Vanderleyden, 2007). *Azospirillum* strains are able to develop associative symbiotic relationship with *graminaceous* plants (Sheraz Mahdi et al., 2010).

Rhizobium: rhizobia are symbiontic N fixing bacteria. They induce the formation of nodules with their legume hosts in which they differentiate into bacteroids (Poole et al., 2018). This symbiosis contributes the major share of N in the biosphere (Poole et al., 2018). This interaction is interesting if considering that, legumes are among the world's most important crops and fodder plants. But the introduction of industrial N fertilizers reduced the attention paid to this group not without consequences for the global N cycle (Poole et al., 2018).

Other three important groups of N fixers are *Frankia*, *Azolla* and *Cyanobacteria. Frankia* is a soil *actinomycetes,* well described for its ability to form N fixing root nodule symbioses with actinorhizal plants (Chaia et al., 2010). *Azolla* is usually called mosquito fern, duckweed fern, fairy moss or water fern, and is a small freely floating aquatic fern (Roy et al., 2016). *Azolla*, used ad biofertilizers in paddy field, significantly increase N levels in paddy soils (Roy et al., 2016). Finally, *Cyanobacteria* are both free living or symbionts with lichens, ferns and cycads. Their contribution in total BFN is high, but they are capable to fix atmospheric N only under N limited conditions (Kaushik, 2014).

1.3.2 Phosphorus solubilizers

Phosphorus in soil is an essential macronutrient, necessary for growth and development of plants (Chakdar et al., 2018). It is involved in various fundamental biological functions, but its availability is limited (Chakdar et al., 2018). Thus P fertilizers become the second most applied agrochemical in world after N fertilizers (Chakdar et al., 2018). Of the total P-solubilizing microbial population in soil, P solubilizing bacteria (PSB) account from 1 to 50%, while fungi (PSF) have a P-solubility potential of only 0.1 to 0.5% (Chen et al., 2006). It is assumed that 20–25% of plants' requirement is fulfilled by PSB and PSF (Chen et al., 2006). Well studied PSB in soil are *Pseudomonas putida* and *Bacillus megaterium* while most know fungal genera are *Aspergillus* and *Penicillium* (Kumar et al., 2018). Some actinomycetes are also known for P-solubilization activity, and they are gaining popularity due to their capability of surviving in extreme environments (Kumar et al., 2018). Approximately 20% of actinomycetes are able to solubilize P, including those of common genera like *Streptomyces* (Hamdali et al., 2008).

1.3.3 Potassium and Zinc solubilizers

Potassium (K) is an essential macronutrient for plant development. Naturally, soils contain large amounts of K but only 1 to 2% of it is available for plants uptake (Kumar et al., 2018). Bacteria, fungi and actinomycetes are able to solubilize K in soil achieved through different chemical reactions (Kumar et al., 2018; Archana et al., 2013). *Bacillus licheniformis*, *Pseudomonas azotoformans* and *Enterobacter hormoechei* are among the most effective K-solubilizers, as inoculation studies on rice (Meena et al., 2015) and cucumber (Saha et al., 2016) have shown (Kumar and Verma, 2018). Zinc (Zn) can be solubilized by different microbial species like *Bacillus subtilis*, *Thiobacillus thioxidans* and *Saccharomyces* sp. (Prajapati and Modi, 2016). These microorganisms can be applied as biofertilizers to increase Zn availability for plants (Sheraz Mahdi

et al., 2010). Interesting results were obtained after inoculation of a Zn solubilizer *Bacillus* strain in soils with high levels of insoluble zinc, where it became more available for plants (Sheraz Mahdi et al., 2010).

1.3.4 Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal fungi (AMF) are obligate symbionts, belonging to the phylum *Glomeromycota* that form symbiotic association with about 80% of all land plants, including several agricultural crops (Brenner et al., 2008; Kumar et al., 2018). They represent a fundamental link between plants and soil mineral nutrients, as they were shown to increase the uptake of several macro- and micro-elements such as P, N, K, Zn, magnesium, calcium and sulfur (Berruti et al., 2016; Bolan, 1991). In addition, AMF provide other kind of benefits to plants, such as an improvement of drought and salinity tolerance and disease resistance (Berruti et al., 2016). Thus, in last year they are receiving growing interest to be employed as biofertilizers in agriculture, horticulture, afforestation and reclamation of deserts (Bolan, 1991).

1.3.5 Other mycorrhizae

In general, mycorrhiza is the symbiotic association between a fungus with a plant root. Many tree species in worldwide forests lean on ectomycorrhizal (ECM) fungi to comply their nutrient requirements. The fungi that form ECM associations taxonomically belong to *basidiomycetes* and, to a lesser extent, *ascomycetes* (Anderson and Cairney, 2007). These fungi improve the nutrition of trees by mobilizing nutrients from organic compounds. At the same time they also contribute to the carbon supply of soils and are thus responsible for carbon flows within forests (Anderson and Cairney, 2007). Ericoid mycorrhiza is an association among plants of the order *Ericales* and soil fungi (Perotto et al., 2002), while orchid mycorrhizae are formed between plants of the family *Orchidaceae* and soil fungi (Sathiyadash et al., 2012). The latter being fundamental during germination for the delivery of carbon to the seedling (McCormick et al., 2012).

1.3.6 Plant growth promoting rhizobacteria (PGPR)

Beneficial free-living soil bacteria are usually referred to as plant growth promoting rhizobacteria or PGPR (Glick, 1995). They can affect plant growth in direct or indirect ways. The direct promotion of plant growth by PGPR is due to their production of beneficial compounds, or by facilitating the uptake of certain nutrients from the environment, as above mentioned (Glick, 1995). The indirect promotion of plant growth occurs when PGPR alleviates the deleterious effects of plant pathogens (Glick, 1995). The mechanisms of PGPR-mediated enhancement of plant growth and yield of many crops are not yet fully understood (Dey et al., 2004). However, additional explanations also include skills like:

1) the ability to produce a vital enzyme such as 1-aminocyclopropane-1-carboxylate (ACC) deaminase to reduce the level of ethylene in the root of developing plants thereby increasing the root length and growth (Penrose and Glick, 2003);

2) the ability to produce important phyto-hormones like auxin, i.e indole acetic acid (IAA), abscisic acid (ABA), gibberellic acid (GA) and cytokinins (Hayat et al., 2010);

3) antagonism against phyto-phatogenic bacterial or fungal strains by producing different compounds like siderophores, ß-1, 3-glucanase, chitinases, antibiotic, fluorescent pigment and cyanide (Glick and Patten, 2017);

4) enhancement of resistance to drought, salinity, water logging andoxidative stresses (Hayat et al., 2010) for instance, production of exopolysaccharides by PGPR helps in maintaining a good hydration level of roots and/or help re-establishing water potential gradients when water limitations occur (Van Oosten et al., 2017);

5) production of water-soluble B group vitamins niacin, pantothenic acid, thiamine, riboflavine and biotin (Hayat et al., 2010).

The application of PGPR has also been extended to remediate contaminated soils in association with plants (Ventorino et al., 2014).

1.3.7 Microbial Consortia

Combinations of microbial strains such as rhizobacteria and fungi present a good strategy to develop biofertilizer products for sustainable agriculture (Woo and Pepe, 2018). Many recent studies demonstrate the potential as plant biostimulants of consortia constituted of both rhizobacteria, and rhizofungi (Woo and Pepe 2018). It has been suggested that, products containing consortia might better survive in various environments than single strain inocula due to the communication and differentiation of microbial cells (Brenner et al., 2008). It was also observed their efficient enhancement of plant growth and performance under abiotic stresses (extreme temperature, pH, salinity, drought, plus heavy metal, and pesticide pollution) (Woo and Pepe, 2018).

The identification and culturing of interesting PGPMs, with a complete analysis and selection of the various components, and the evaluation of the synergy between new strains, can finally, allow the development of adequate formulation recipes for the distribution of new technologies and technical support to end-users (Woo and Pepe 2018).

1.4 Roadmap to formulations of Biostimulants for Sustainable Agriculture

Laboratory isolation and screening based on plant growth promoting traits of new microbial strains are the first fundamental steps to develop a new microbial inoculants (Backer et al., 2018). These phases are followed by *in vitro*, greenhouse and/or field experiments including a range of crops to evaluate the microbial effectiveness and persistence in the soil (Romano et al., 2020). Even if

interesting laboratory evidence are obtained, they not always result in plant growth promotion under field conditions.

Finally, the microbes must be multiplied and formulated in a product that meets various requirements: high concentration of vital microbial cells and a *shelf-life* of at least six months. For this, the choice of an appropriate formulation that best preserve the vitality of the microbes from their production until their application, is of major importance (Backer et al., 2018).

1.4.1 Solid formulations

The carriers used in solid (or carrier based) formulations can consist of organic, inorganic, or synthetic, low-cost materials easy to process and sterilize. They should provide a short-time protective niche for the microbes in the soil, either by physical protection or providing specific nutrients (Arora et al., 2010; Bashan et al., 2014). There are two kinds of solid formulations, peat, and granules-based formulations. Peat is an inhomogeneous and complex material which inconsistently affects microbial cell growth and survival during multiplication (Malusá et al., 2012). In addition, toxic compounds might be released during sterilization processes resulting in a reduction of microbial growth and survival, which might further hamper microbial efficiency (Bashan et al., 2014; Mahanty et al., 2017). Granule based formulations are made of peat prill, small marble, calcite, vermiculite, or silica grains coated or impregnated with the selected microbial strains (Backer et al., 2018). The application procedures for solid biofertilizers can be easily controlled, in fact they can be placed near to the seeds to facilitate the microbial interaction with the rhizosphere (Bashan et al., 2014). However, there are some general disadvantages in their use, for instance their voluminous size results in high costs of transport and storage. The microbial concentration quickly decade in solid formulation due to the absence of nutrient or protectors for microbial cells; as consequence, the rate of application has to be increased to achieve desired results (Backer et al., 2018) (Table 1.1). A special type of solid formulation are *freeze-dried powders*, obtained by direct freeze-drying of target cells in presence of a cryo-protector such as pure glucose, milk powder (Morgan et al., 2006).

1.4.2 Liquid formulations

Besides the microbial cells, liquid formulations can also contain nutrients, special cell protectants or chemical substances that promote the formation of resting spores or cysts to increase the products' *shelf-life* and the microbes' stress tolerance (Sheraz Mahdi et al., 2010). Liquid formulations are the solution to many challenges associated with solid formulations (Sheraz Mahdi et al., 2010). They have a higher *shelf-life* of up to two years instead of six months like solid formulations and they are more tolerant to high temperatures up to 55 °C (Table 1.1). They have higher population densities of up to 10^9 colony forming units (cfu) ml^{-1} at starting *shelf-life* time instead of only 10^8 cfu g^{-1} found for solid media (Sheraz Mahdi et al., 2010). In addition, they can be easily applied using e.g. hand sprayers, power sprayers or by fertigation (Sheraz Mahdi et al., 2010).

Table 1.1 Inoculants formulations – overview on advantages and disadvantages of solid and liquid formulations

		Advantages	Disadvantages
Solid formulation	**Carrier based biofertilizers**	Cheap Easy to produce Less investiment	Low shelf-life Temperature sensitive Contamination prone Low cell counts
	Freeze-dried powders	Longer shelf life High cell counts Contamination-free Product can be 100% sterile	Very high cost Higher investment for production unit
Liquid formulation		Longer shelf life Temperature tolerant High cell counts Contamination-free More effective Product can be 100% sterile	High cost Higher investment for production unit

1.5 Areas of application

Biostimulants are mainly applied in horticulture to increase yield and product quality in a sustainable way (Colla and Rouphael, 2019; Ortas, 2008; Pathak et al., 2017). The use in this field is justified by the following reasons: the high yield of crops, the controlled environmental conditions (unlike the open field), and easiness of application (Colla and Rouphael, 2019). Moreover, the high specialization of crops and the use of intensive cultivation practices in horticulture cause losses of soil fertility. In these contexts, biostimulants may contribute to plant growth and replenish microbial populations in soil (Colla and Rouphael, 2019). Microbial inoculants used alone or in combination with other inputs are usually applied in different horticulture field including vegetable production, floriculture, arboriculture, and hobby gardening (Ortas, 2008; Pathak et al., 2017). The application on banana or apple plants of biofertilizers, containing *Azotobacter* cells alone or in combination with *Glomus* strains, allowed to obtain good results about plant growth and fruit quality (Sharma et al., 2011; Pathak et al., 2017). Furthermore, pepper plants inoculated with a mycorrhizal strain in the seedling stage, had a good response to the different stress factors applied during the trial (Pivonia et al., 2008). Considering the importance of floriculture, the application of sustainable practices in this field are needed to reduce its environmental impact (Wani et al., 2018). Integrated Nutrient Management (INM) has proved particularly effective in floriculture. It consists of integration of natural and chemical inputs to

increase crop productivity and product quality, maintaining soil fertility and its physical-chemical-biological health (Wani et al., 2018). Combined application farmyard manure and PSB significantly improved both yield and N P K content in marigold leaf (Mukesh et al., 2006). Another investigation revealed that the inoculation of *Azotobacter* positively affects growth of tulips (Khan et al., 2009).

The applications in Netherlands of commercial biofertilizers containing mycorrhiza gave positive results in different real cases like sustainable management of rose gardens and golf greens, or for revegetation and urban landscaping (Weissenhorn and Külling, 2008).

The response of the inoculums, under field conditions, is influenced by different factors like soil status, methods of application, environmental factors, and other ecological aspects. Although these relevant are issues, microbial inoculants application in open field gave significant positive results.

Inoculation of *Azospirillum* cells in combination with other microbial strains gave positive results on different crops: with *Pseudomonas* positively affected grain yield of maize and cotton plants (Mohammadi and Sohrabi, 2012); with *Azotobacter* increased yields of pearl millet, sorghum wheat and rice (Wani, 1990), with *Arthrobacter* and a PSB strains significantly affect grain yield of barley (Belimov et al., 1995).

Moreover, research is needed for application of biofertilizers in several crops to make productions completely organic.

1.6 Effectiveness of biostimulant

Scientific communities across the globe extensively studied the effectivity of biofertilizers on many different crops in all kinds of ecosystems resulting in a large number of publications summarized the benefits of different biofertilizer types such as AMF, P solubilizers and N fixers (Berruti et al., 2016; McGonigle, 1988). In contrast, results obtained by farmers applying bio-inoculant were often insubstantial. The reasons for this lack of growth improvement are manifold and mostly result from an incompatible combination of environmental factors especially soil conditions, inoculant type and crop/genotype (Schütz et al., 2018). So far, specific recommendations for the use of certain products can hardly be made with few exceptions concerning brady-/rhizobia products for cultivation of non-regional legumes.

However, a recent global analysis revealed some overall patterns to predict biofertiliser effectiveness in relationship to soil and climatic conditions as well as crop and biofertiliser type (Schütz et al., 2018). The study highlighted that the biofertiliser effectiveness strongly depends on the soil conditions and that the conditions triggering the best performance differ depending on the type of biofertiliser applied. In particular, AMF exhibit the best performance under low levels of organic carbon and plant-available soil P (10–25 kg P/ha); P solubilisers also reveal the best

effectiveness under low organic carbon contents but slightly higher plant- available soil P levels (25–35 kg P ha^{-1}); whereas N fixers show the best success under increasing soil organic carbon contents and plant-available P soil levels higher than 45 kg P ha^{-1}.

Besides soil conditions, crop type also affects biofertiliser effectiveness. In fact, legumes and vegetables are more responsive to inoculation than root crops and cereals, which might result from their increased needs for nutrients (Schütz et al., 2018).

However, in controlled conditions or in greenhouse the effect of competition with native microorganisms living in the soil is excluded or minimized (Berg et al., 2020). Product efficacy can be strongly compromised by the inability of microbial inoculants to persist in soil due to adverse abiotic conditions, unsuccessful colonization of host roots and competition with native soil microorganisms (Berg et al., 2020).

Microbes inoculated may compete for niches by several mechanisms: being efficient root colonisers (e.g., biofilm formers), producing antibiotic compounds that affect the growth of other microbes, or by depleting resources that are essential for other microbes, and thereby indirectly reducing the presence of native bacteria and fungi (Berg et al., 2020). Many microbial biostimulant products include only one or very few taxa, especially in regulated markets such as EU countries. Other products, have a mixed culture. These products aim to deliver a rich mixture of effective microorganisms, to better compete, colonize and persist in the soil (Berg et al., 2020).

Another factor driving biofertiliser effectiveness is the climate. Biofertilisers were shown to be more effective in dry regions, followed by tropical and continental climates. The main reason is the lower soil fertility with low soil organic matter, N and P contents typically observed in dry regions. Low soil fertility also means a lower abundance and activity of native soil microbes, which consequently makes the application of microbial inoculants more effective. In addition, crops growing in dry climates are more tolerant to stress including heat, drought, and salinity. Microbes can produce several molecules such as plant hormones, enzymes and secondary compounds, which help to reduce stress in plants, thus stabilizing their yields.

1.7 Risks and constraints in biostimulant production and use

Previous sections described limitations regarding biostimulants' effectiveness, which depends on environmental factors, antagonism/competition with soil microorganism and inappropriate handling, transport and storage of products. Further problems related to the production and use of these kinds of inoculants and potential solutions are summarized in table 1.2.

Table 1.2 Problems related to the production and use of biofertilizers and potential solutions

Problem	Solution
Introduction of invasive microbes (Corkidi et al., 2004)	Selection of suitable and competitive strains for specific climatic regions, crops and soils (Sheraz Mahdi et al., 2010)
Inefficient products (Sheraz Mahdi et al., 2010)	
Low quality products (lack of vital propagules) (Corkidi et al., 2004)	Major quality assurance and research by producers (Sheraz Mahdi et al., 2010)
Mutations of microbial cells during fermentation (Sheraz Mahdi et al., 2010)	
High investment costs (Xu and Danny, 2018)	Research in the field of alternative growth media such as industrial by-products (Xu and Danny, 2018)
Poor understanding of the importance of microbes for below-ground processes	Raising farmers' awareness of the benefits of biofertilisers by increased communication through specialized journals (Sheraz Mahdi et al., 2010)

1.8 Alternatives: management of native soil microbes

An effective long-term alternative to the use of bio-inoculants is the propagation of the native microbial populations inhabiting the soil in order to improve soil processes and consequently promote plant growth. This can be achieved by the implementation of a range of management practices typically found in organic agriculture such as crop rotation, integration of legumes and cover crops in the rotation and the application of organic amendments such as compost. Some of these practices can be easily integrated into existing farming systems and help to increase the size and activity of the microbial communities (Lori et al., 2017).

1.9 Conclusions

Several studies successfully showed the potential of microbial inoculants in increasing yield and quality of various crops. Considering that biostimulants' effectiveness depends both on plant and environmental factors, products should be carefully selected, and applications should accurately follow the producers' instructions. Especially in dry regions, biostimulants represent a valuable tool for sustainable farming where crops are challenged by abiotic stresses and low soil fertility. Considering that in future global dryland areas are expected to increase, bio inoculants will become increasingly important. Moreover, biostimulants can (partially) replace the use of chemical fertilizers, thus reducing the risks associated with soil pollution and human health.

The approach of “rhizosphere engineering” is becoming increasingly important in agriculture because the sector is recognizing the importance of microbes for resilient farming systems. It proposes the addition of efficient microbial inoculants, selected farming practices and crop genotypes that effectively manipulate the rhizosphere by stimulating functional, beneficial microbial groups positively linked to soil fertility (Woo and Pepe, 2018; Brenner et al., 2008).

2 How to assess root colonization and persistence of a microbial inoculant

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2.1 Introduction

The increasing demand to reduce the use of chemical fertilizers and pesticides for the development of an agri-food system sustainable for environmental and human health, as well as the current shifting in the agricultural legislation of several countries, have led to an expanded use of bioinoculants. Chemical inputs usually alter the natural physico-chemical and biological equilibrium of soil, and microbial consortia used in agricultural management practices could return soil to its natural status (Lucy et al., 2004; Woo and Pepe, 2018). Although the manipulation of soil microbiomes to optimize crop productivity is an ancient practice, it is still little explored, especially regarding mechanistic studies of plant-microbe interactions and microbial persistence in heterogeneous communities in diverse locations, soils and hosts (Finkel et al., 2017). Among the numerous bacterial or fungal strains used as bioinoculants, plant growth-promoting microbes (PGPM) are the most commonly applied. PGPM may affect plant performance through multiple mechanisms of action, operating directly by the production of specific substances that are able to promote plant growth and increase the availability and uptake of nutrients in soil (i.e., phosphate solubilization, siderophore and indole-3-acetic acid production, nitrogen fixation) or indirectly through the suppression of plant pathogens (Ribeiro and Cardoso, 2012). Several plant growth-promoting rhizobacteria (PGPR) have also been demonstrated to exert a beneficial effect on plant growth under nutritional and abiotic stress (Sharma et al., 2014; Singh and Sharma, 2016; Van Oosten et al., 2018) or during the restoration of polluted soils (Ventorino et al., 2014). Moreover, plants could also establish symbiosis with arbuscular mycorrhizal fungi (AMF), which increase the root surface area for nutrient acquisition (Wu et al., 2005). A successful microbial inoculant has to colonize the external and/or internal part of plant tissues and establish a compatible interaction with the host as well as to persist in the soil against autochthonous microorganisms living in environment through its rhizocompetence traits (Finkel et al., 2017). In general, rhizosphere colonization occurs through several different mechanisms, such as bacterial movement, survival in the rhizosphere by competition against other microbes, adherence to and colonization of root surfaces, for instance by biofilm formation, and the creation of synergistic interactions with the host plant (Bhattacharya et al., 2017). Moreover, even if PGP inoculants colonize the plant initially, their persistence over time is not guaranteed. Measuring the persistence of microbial inoculants in soil poses technical difficulties, as the inoculant needs to be identified from within a complex community. The tracking and monitoring of the persistence of PGPM released in the environment have been widely studied (Brandt and Kluepfel, 1991; Kloepper and Beauchamp, 1992; Stahl and Kane, 1992; Gamalero et al., 2003; Podile and Kishore, 2006; Ahmad et al., 2011; Glick, 2015; Rilling et al., 2019) to understand their behavior in soil and which factors influence their survival under various conditions. Several sets of techniques are currently used to detect root colonization and persistence in the soils: microbial enumerations by culture-based methods, microscopy-based techniques, and DNA-based methods. The results may depend on the choice of technique since each has advantages and limitations, and each technique may have bias in favor of specific microbial taxa. This review examines and presents an overview of the current methodological approaches that could be used to assess and detect plant colonization and soil persistence of microbial bioinoculants in the

rhizosphere environment and considers multidisciplinary approaches to track and monitor inoculated microorganisms.

2.2 Good practices for rhizosphere sampling and soil preparation

In natural ecosystems such as soils, several variables or factors can influence the results due to the highly heterogeneous distribution of microbial cells in the environment. Therefore, a well-organized experimental plan to investigate microbial populations from plant roots and soil is necessary. Usually, in field experiments, the simplest approach used to overcome spatial variables is a completely randomized design with replicates since the treatments are assigned completely at random, creating homogeneous treatment groups (Fiorentino et al., 2018; Lusiba et al., 2018). To ensure good results in microbiological analysis, the first fundamental prerequisite is the correct soil sampling, both in laboratory and in greenhouse trials and in field experiments, to obtain representative samples for each treatment to be analyzed (Pennock et al., 2008). Temporal and spatial aspects could be considered during rhizosphere (soil area influenced by plant roots and their exudates; Barillot et al., 2013) or bulk soil (soil not adhering to roots and not influenced by exudates; Barillot et al., 2013) sampling since changes in microbial diversity over time are usually related to environmental changes. Therefore, soil or rhizosphere microbial diversity studies are usually carried out over years or seasons (Lombard et al., 2011). Moreover, it is known that other factors, such as plant age and developmental stage, could also influence plant microbial community structure (Compant et al., 2019); therefore, these variables could also be considered for soil sampling. Soil and rhizosphere samples can be collected by different sampling approaches, as extensively detailed by Wollum (1994): i) simple random, which ensures that each sample has the same opportunity to be selected, usually by using a grid; ii) stratified random, similar to simple random, except the area to be sampled is broken into smaller subareas; or iii) systematic, which ensures that the entire area is sampled and represented by individual samples that are obtained by establishing predetermined points. The number of soil samples to take depends on the microbial population distribution and can be calculated using the formula suggested by Wollum (1994), which considers a pre-study sampling, the sample variance and the sample mean. However, it is recommended to brush away stone, rubbish, trash, or grass from the soil surface before taking samples. Then, using a sanitized shovel, it is possible to take the samples from topsoil to an adequate depth (for instance, 0-20 cm) or to collect plant roots by excavating or uprooting plants to study microbial diversity in bulk soil and/or rhizosphere. For rhizosphere studies, after plant sampling, roots should be shaken vigorously by hand to remove bulk soil and to collect soil adhering to roots (Ventorino et al., 2012; Barillot et al., 2013). Moreover, during the sampling, it is necessary to avoid root damage. Manual excavation using spades and hand tools and working progressively in layers or sectors could minimize the corruption of soil architecture and ensure the safety of the roots. It is also fundamental to take enough replications for data analysis (Neumann et al., 2009). Following this, the samples must be recovered in sterile polyethylene bags or vessels and stored at 4°C to avoid desiccation during transport to the laboratory. To evaluate external and

internal root colonization, which generally occur in the rhizoplane and endosphere, respectively, several steps for sample preparation are necessary (Figure 2.1). In particular, plant roots should be washed by agitation in sterile water or buffer (e.g., phosphate buffered saline-PBS or physiological buffers) without tearing or cutting plant tissues to facilitate the separation between soil/root particles and microorganisms (Kloepper and Beauchamp, 1992). For instance, a good practice to detach the bacteria from the soil particles is shaking for 30 min at 120-130 rpm in an adequate volume of isotonic solution containing tetrasodium pyrophosphate (16%w/v) (Ventorino et al., 2014). Barillot et al. (2013) reported that after vigorously hand-shaking roots to separate bulk soil from rhizospheric soil, shaking the roots a second time in a sterile 0.9% NaCl solution allowed rhizosphere collection, and shaking the roots a third time in the same sterile solution containing Tween 80 (0.01% v/v) allowed the rhizoplane fraction (thin layer of soil strongly adhering to the roots; Barillot et al., 2013) to be collected (Figure 2.1). Indeed, to study microbial endophytes, it is necessary to surface sterilize the roots prior to grinding, chopping or blending them (McInroy and Kloepper, 1991). Several works describe a prior wash with 1%chloramine and cycles of washing/agitation treatments using ethanol and phosphate-buffered saline (PBS) (Ladha et al., 1997; Dennis et al., 2008; Richter-Heitmann et al., 2016). Cleaned roots to be analyzed by culture-independent methods can be stored in a solution of PBS buffer and 70%ethanol (2:3 v/v) for a long time at -20 °C (Dennis et al., 2008; Richter-Heitmann et al., 2016). However, fresh root samples used to evaluate the density of the cultivable microorganisms by plating on growth media should be analyzed within a short time (24-48 h).

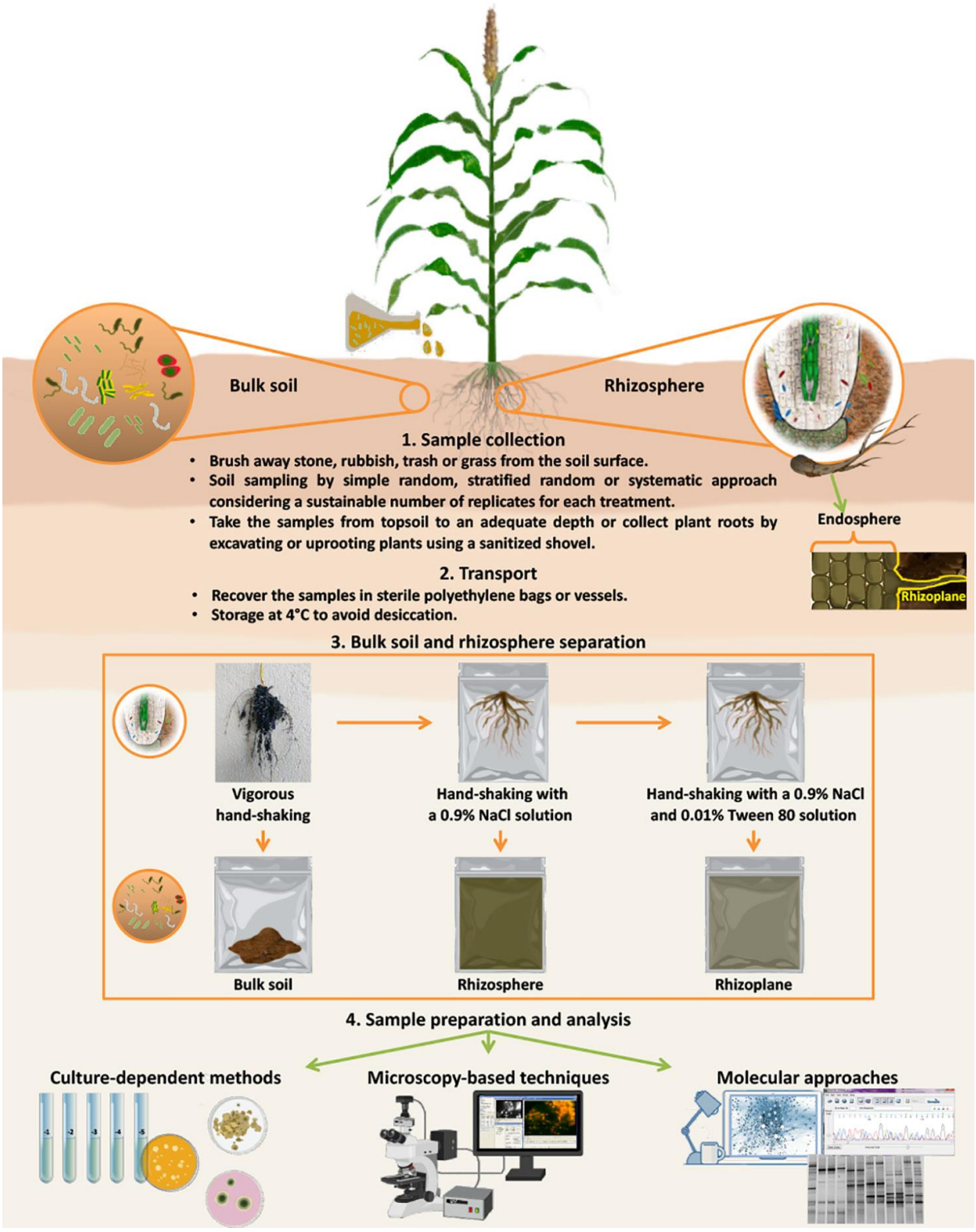


Figure 2.2 Schematic description of sampling collection, separation of different soil fractions, and methods (culture-dependent methods, microscopy-based techniques and molecular approaches) for the detection of microbial inoculants. After plant sampling, roots should be shaken vigorously by hand to collect bulk soil (soil not adhering to roots and not influenced by exudates). Shaking the roots, a second time in a sterile 0.9% NaCl solution allowed rhizosphere (soil area influenced by plant roots and their

exudates) collection and shaking the roots a third time in the same sterile solution containing Tween 80 (0.01% v/v) allowed the rhizoplane (thin layer of soil strongly adhering to the roots) fraction to be collected. To study microbial endophytes, it is necessary to add a step of sterilization of the root surfaces prior to grinding, chopping or blending them. Root samples should be analyzed in a short time (24-48 h) to evaluate the density of the cultivable microorganisms by plating on growth media or they can be stored in a solution PBS buffer and 70% ethanol at -20 °C for later analysis by culture-independent methods (microscopic and molecular methods).

2.3 Microbial enumerations by culture-dependent methods

Mainly because of their ease of use, culture-dependent methods are commonly used to estimate the persistence of inoculated microorganisms in soil and/or rhizosphere. However, these methods are limited since it is difficult to represent the high diversity of bacteria on culture media because only 0.1% to 1.0% of soil bacteria are cultivable (Daniel, 2005), and at the same time, it is difficult to differentiate inoculated organisms from native populations based on morphological characteristics (Lima et al., 2003).

To increase the likelihood of cultivating a high number of microbial strains, enrichment, selective and differential media are usually used as well as synthetic media mimicking the soil environment, typically containing soil extracts, are also developed. This approach has been successful, and it allowed the detection of a higher diversity of cultivable populations compared with other methods (Andreote et al., 2009). Although culture-dependent methods have been used to detect bioinoculants in different experimental conditions (growth chamber, greenhouse, open field), they are especially useful when the experiment is carried out in sterile conditions and interference by soil autochthonous microbial populations can be avoided. Therefore, advantages and limitations of culture-dependent approaches will be discussed on the basis of experimental conditions (i.e., growth chamber, greenhouse, field).

2.3.1 Growth chamber

Experiments conducted in growth chambers are usually performed using sterile synthetic substrates or hydroponic conditions for plant growth, allowing the control of all environmental parameters, such as temperature, relative humidity, light/dark cycle, and light intensity. Therefore, this approach is particularly suitable for the detection of inoculated strains in plant tissues by enumeration on culture media. Castanheira et al. (2017) used viable counts to assess the colonizing abilities of a bacterial consortium composed of *Pseudomonas* sp. G1Dc10, *Paenibacillus* sp. G3Ac9 and *Sphingomona s* (*S.*) *azotifigens* DSMZ 18530 on the rhizoplane and surface-disinfected roots, stems and leaves of annual ryegrass plants grown under gnotobiotic conditions (Table 2.1). Sterile experimental conditions allow the use of a unique generic growth substrate to perform total bacterial counts and can allow three different bacterial strains to be distinguished on the basis of colony morphology. Indirect viable counts on solid medium also allowed the assessment of the survival of endophytic trans-conjugant *Pseudomonas* sp. strains tagged with

green fluorescent protein (GFP) in different tissues of poplar trees for 10 weeks (Germaine et al., 2004; Table 2.1). Since the plants were grown in a sterilized substrate but were not maintained under sterile conditions throughout the experiment, a number of indigenous endophytic strains were also isolated on growth medium. Therefore, to exclusively count the inoculated strains, only the colonies expressing GFP were enumerated by examining the plates under an epifluorescence microscope (Germaine et al., 2004). Similarly, Kandel et al. (2015) used trans-conjugant GFP-tagged strains of *Burkholderia* sp., *Rhizobium tropici* PTD1 and *Rahnella* sp. WP5 to evaluate their colonization abilities in rice plants (Table 2.1). At 20 days after inoculation, the use of a selective growth medium allowed them to enumerate the total number of inoculated endophytes in the plant tissues. However, the use of axenic experimental conditions ensures ease of study and that only inoculated strains will be recovered.

2.3.2 Greenhouse

Greenhouse experimental conditions could be considered a variation of farming in a controlled environment, which provides favorable growing conditions and protects crops from unfavorable weather and various pests. Therefore, this approach could be suitable for evaluating the viability of inoculated microorganisms by culture-dependent methods. In pot greenhouse conditions, Wu et al. (2005) counted viable bacteria to demonstrate the successful colonization and the synergistic effect of beneficial rhizobacteria such as *Azotobacter* (*A.*) *chroococcum* and *Bacillus* (*B.*) (*B. megaterium and B. mucilaginous*) combined with mycorrhizal fungi belonging to the genus *Glomus* (*G.*) (*G. mosseae* or *G.intraradices*) in the rhizosphere of *Zea mays* plants (Table 2.1). The use of differential culture media allowed the detection and enumeration of groups of bacteria similar to the inoculants on the basis of their specific plant growth promoting activities, such as nitrogen fixation, phosphate and potassium solubilization.

Similarly, culture-dependent methods, based on the use of differentiation media for plant growth-promoting properties, were also useful to assess the persistence of bacterial (*A. chroococcum, B.megaterium* and *B. mucilaginous*) and fungal (*G. mosseae* or *G. fasciculatum*) consortia (Khalid etal., 2017; Table 2.1). The use of this approach demonstrated that the microbial concentration and root colonization of *Spinacia oleracea* L. was improved by the application of a consortium of microorganisms, suggesting the synergistic behavior of the strains. The plate count method was also used to analyze the survival of five *Azotobacter* strains (ST3, ST6, ST9, ST17 and ST24) at different stages of wheat (*Triticum aestivum* L.) plant growth. These strains were inoculated in earthen pots containing saline soil under greenhouse conditions. The results of rhizosphere soil monitoring showed that the concentration of the inoculated strains increased up to 60 days of sampling (Chaudhary et al., 2013; Table 2.1). However, this approach did not allow the identification of microorganisms present in the culture at genus and species level in non steril condition. In fact, it is difficult to distinguish bioinoculants from indigenous microbial populations living in soils based on morphological characteristics. Van Oosten et al. (2018) used viable microbial counts to assess the persistence of the inoculated *A. chroococcum* 76A in the rhizosphere of tomato plants cultivated under abiotic stress conditions (Table 2.1). A

differentiating culture nitrogen-free medium for N fixers allowed them to demonstrate that the strain *A. chroococcum* 76A, inoculated at a concentration of approximately 10^6 CFU/g, was able to grow in all experimental conditions, increasing by approximately one order of magnitude at the end of the experiment. Interestingly, Solanki and Garg (2014) described a novel technique to enumerate viable cells of *A. chroococcum* in the unsterilized rhizoplane of *Brassica campestris* using a trans-conjugant strain of *A. chroococcum* Mac 27 containing a lacZ fusion (*A. chroococcum* Mac 27 L; Table 2.1). Using this approach, it was possible to monitor the growth and survival of the LacZ-tagged bacteria that formed blue-colored colonies on Burks medium containing X-gal.

2.3.3 Field

Although the field represents the natural and real condition for assessing the effectiveness of a microbial consortium or biofertilizer in soil, it is difficult to differentially enumerate inoculated microorganisms in this experimental state by culture-dependent methods. However, some works have reported general results on the variation of microbial concentration in the rhizosphere of plants grown in agricultural fields. Sharma et al. (2011) used a culture-dependent approach to assess microbial changes due to the application of a consortium formed by *A. chroococcum* AZ1 and AZ2 in association with *G. fasciculatum* and *G. mosseae* on apple plants grown in rainfed fields. As a general result, an increase in the concentration of bacteria and/or fungal strains in the inoculated tests was observed, although the results were more or less significant depending on the inoculant (used alone or in combination) and experimental conditions (Table 2.1). A field experiment was also conducted to evaluate the inoculation effect of *Azotobacter*, *Azospirillum* (*Az.*), and arbuscular mycorrhizal fungi, either alone or in combination, on seedlings of apple cultivars. The viable counts of *A. chroococcum* and *Az. brasilense* in the rhizosphere were significantly higher in all the treatments than in the controls. In fact, the microbial concentration in the treatment with multi-inoculation of all the strains was significantly higher than those in all the other biological treatments but lower than that of the chemical fertilizer treatment (Singh et al., 2013; Table 2.1). Culture-dependent methods have several advantages such as they are practical and useful techniques to quantify bioinoculants especially in sterile experimental conditions, and they allow to detect only viable cells and therefore bacterial inoculants that are competitive and able to persist overtime. Moreover, as reported in several works (Al-Awadhi et al., 2013; Ngom and Liu, 2014; Pitkäranta et al., 2007), it is difficult to detect the inoculated strain in unsterilized conditions. Culture-dependent methods cannot provide a comprehensive analysis of the endophytic ability of selected strains in unsterilized conditions since a portion of epiphytes that are resistant to sterilizing agents could determine an overestimation of their counts (Kandel et al., 2017). To explain the behavior of the bioinoculants in the natural soil ecosystem, culture-based methods should always be complemented with culture-independent approaches to examine the variations in the microbial community after inoculation treatment and to track the inoculated microbial strains.

Table 2.1 Culture-dependent approach used to monitor plant growth–promoting bacteria and root interaction.

Strains	Experimental conditions	Microbial media	Plants ubstrate	Results	References
Pseudomonas sp. G1Dc10 *Paenibacillus* sp. G3Ac9 *Sphingomonas azotifigens*DSMZ18530	Gnotobiotic conditions in controlled-environment chamber (16-h light/8-h dark, 18-23°C)	TY agar	Modified Evans medium supplemented with 8% agar	Colonization density in the rhizoplane and in the leaves was about 9 and 4 $\log_{10}$ CFU/g, respectively. Colonization was more abundant in the rhizoplane than in plant tissues.	Castanheira et al., 2017
Pseudomonas sp.VM1449 *Pseudomonas* sp.VM1450 *Pseudomona s*sp. VM1453	Pots (16-h light/8-h dark, 20-25 °C)	PCA containing 100 µg/mL kanamycin	Sterilized compost/vermiculite (3:1 ratio)	The three bacterial strains showed different colonization behavior (CFU/g) for rhizosphere, interior root tissues stems or leaves	Germaine et al., 2004
Burkholderia sp. WPB *Rhizobium tropici* PTD1 *Rahnella* sp. WP5	Axenic conditions in growth chamber	MG/L with 100 µg/mL of gentamycin and carbenicillin	N-free MS agar	Higher endophyte populations (CFU/g) were observed in the roots when compared with the stem and leaves	Kandel et al., 2015
Azotobacter chroococcum HKN-5 *Bacillus megaterium* HKP-2 *Bacillus mucilaginous* HKK-2 *Glomus mosseae* *Glomus intraradice*	Pots in greenhouse (20± 4°C; 87 days)	Specific media for N-fixing bacteria, P solubilizer and K solubilizer	Soil (pH 5.46, organic matter 1.08%, total N 0.062%, total K 7408 mg/kg, total P 1090 mg/kg)	The population size of the inoculated rhizobacteria varied in accordance with the levels of fertilization and AMF colonization in the rhizosphere	Wuet al., 2005
Azotobacte rchroococcum *Bacillus megaterium* *Bacillus mucilaginous* *Glomus fasciculatum* *Glomu smosseae*	Greenhouse (21± 5°C; 45days)	Differentiating media for N-fixing bacteria, P solubilizer and K solubilizer	Sterilized soil (pH 7.32, EC 0.14 dS/m, total C 1.92%, total N, 0.19%, total K 2063 ppm)	Root colonization by AMF was increased in the presence of bacterial consortium application in comparison to individual inoculation treatments	Khalidet al., 2017
Azotobacter strainST3 *Azotobacter* strainST6 *Azotobacter* strainST9 *Azotobacter* strainST17 *Azotobacter* strainST24	Pot house; sampling at 30, 60 and 90 days	Nutrient agar	Four different unsterilized saline soil	Survival of inoculated strains increased up to 60 days of sampling	Chaudharyet al., 2013
Azotobacter chroococcum 76A	Greenhouse (10 cm plasticpots)	LG agar	Pure peat moss under salt stress	The bacterial strain was able to grow in the rhizosphere of tomato plants under abiotic stress conditions increasing of 1 Log	Van Oosten et al., 2018

Table 2.1 *continuous.*

Strains	Experimentalconditions	Microbial media	Plantsubstrate	Results	References
Azotobacter chroococcum Mac 27L	Pots; sampling after 30 and 60 days of growth	Burks medium plates with and without X-gal	Unsterilizedsoil	The bacterial strain was able to survive in the rhizoplane of *Brassica campestris* up to 30 days after sowing	Solanki and Garg, 2014
Azotobacter chroococcum AZ1 *Azotobacter chroococcum* AZ2 *Glomus mosseae* *Glomus fasciculatum*	Plots, temperate rain-fed conditions	Nutrient agar medium, coal-vitamin medium, potato-dextrose supplemented with Rose-Bengal and streptomycin (30g/mL)	Solarized, disinfected and natural soil plots (21% sand, 35.7% silt 43.3% clay, pH 7.4)	An increase of concentration of bacteria and/or fungal strains in the inoculated tests has been registered	Sharma et al., 2011
Azotobacter chroococcum *Azospirillum brasilense* *Glomus fasciculatum*	Open field	Jensen's medium and N-free maltase medium	Soil (pH 7.12, organic carbon 9.6 g/kg)	Viable counts of microbial population in the rhizosphere increased significantly in all the treatments over control but decreased under chemical fertilizers treatment	Singh et al., 2013

2.4 Microscopy-based techniques

Today, a wide range of microscopy-based techniques are available and have been used to detect microorganisms inoculated on plant tissues and to evaluate the colonization patterns of bacterial endophytes through molecular interactions and dynamics within living cells in specific vegetative tissues (Kandel et al., 2017).

Root colonization by bacteria and AMF has been studied by several types of microscopy, which can be divided into three major groups: light microscopy, electron microscopy and fluorescence microscopy.

2.4.1 Optical microscopy

Light microscopy is the most common microscopic technique for assessing microorganisms in root systems due to its low costs of purchasing, maintaining, and servicing (Hulse, 2018). Bright-field light microscopy was employed by White et al. (2014), who developed a combination of stains to evaluate the bacterial colonization of seedling root tissues. This approach was based on the use of 3,3'-diaminobenzidine tetrachloride (DAB) to stain hydrogen peroxide associated with bacterial invasion of eukaryotic cells followed by counterstaining with aniline blue/lactophenol to stain protein in bacterial cells. This elementary technique allowed the visualization of bacteria and their eventual lysis in seedling roots, providing information on the defensive response of host cells and the bacterial degradation process (White et al., 2014). Microscopy techniques that use different dyes are also usually used to assess mycorrhizal relationships with host plants. A wide number of staining procedures, which each have advantages and disadvantages, have been developed for studying AMF colonization, as extensively reported by Hulse (2018). Among these is a very simple, nontoxic, reliable and inexpensive staining technique for AMF colonization in root tissues; this technique is based on the use of an ink-vinegar solution after adequate clearing with KOH (Vierheilig et al., 1998). This solution stains all fungal structures, rendering them clearly visible by bright-field light microscopy. The level of root colonization by mycorrhizal strains is usually evaluated using the microscopic procedure described by Phillips and Hayman (1970) and by Giovannetti and Mosse (Newman's intersection method, 1980). This method requires a stereomicroscope for observation; randomly dispersed roots are stained, placed on a grid in a 9-cm Petri plate and quantified by counting the number of intersections between grid lines and colonized roots. Although this method is strongly influenced by operator skill, it could provide sufficient information to evaluate the mycorrhizal colonization level. In fact, the gridline intersect method has been extensively used in many works to assess and quantify root colonization of mycorrhizal fungi (Sharma et al., 2009; Sharma et al., 2011; Sharma et al., 2012; Singh et al., 2013).

2.4.2 Electron microscopy

Electron microscopy was further developed into scanning electron microscopy (SEM), which can be used to examine plant surfaces and microorganisms at high resolution, highlighting the adhesion

of microbial cells to plant tissues. SEM was used to observe chickpea root colonization by *A. chroococcum* and *Trichoderma viride* (Velmourougane et al.,2017; Table 2.2). The plants were cultivated in sterile media composed of sand and vermiculite (1:1), and samples were taken at 40 days post inoculation. SEM microphotographs revealed the proliferation of *Azotobacter* cells, both individually and attached to the fungal mycelia. SEM observations have also highlighted the production of exopolysaccharides by *A. chroococcum.* These polymers improve the survival of EPS-producing microbial cells in natural ecosystems, exhibit beneficial effects in plant growth promotion and abiotic stress (Gauri et al., 2012; Van Oosten et al., 2017) and could be interesting for biopolymer production (Ventorino et al., 2019). Although SEM produces 3D images, it provides information only on surface morphology and colonization and is not as powerful as transmission electron microscopy (TEM). Although TEM is not considered a user-friendly technique since sample preparation is complex and time consuming, it is the most powerful microscopy technique, with a maximum potential magnification of 1 nanometer. TEM allows 2D ultrahigh resolution images to be obtained, providing information about the internal structure of a root sample; therefore, it is useful to establish endophytic interaction as reported by Singh and Sharma (2016). Hairy roots of *Arnebia hispidissima* were inoculated *in vitro* with five different *A. chroococcum* strains (Table 2.2). After 10 days of incubation, TEM showed that *A. chroococcum* strains were only inside hairy roots of inoculated plants, revealing the endophytic ability of *A. chroococcum* strains. However, since TEM allows only a small area of a sample to be explored, which provides information about the inner part of a sample, and SEM can explore a larger external area, these two techniques could be used in combination to obtain better detailed results about the rhizosphere environment and inoculant colonization (Thokchom et al., 2017).

Environmental scanning electron microscopy (ESEM) is another powerful method to evaluate the survival of a bacterial inoculant and its ability to colonize plant tissues. It provides new possibilities compared to conventional SEM and enables the investigation of nonconductive and hydrated samples without complex histological preparation steps (i.e., air drying, chemical fixation, dehydration, and coating), which are critical in conventional SEM (Stabentheiner et al., 2010). This approach was recently used by Dal Cortivo et al. (2017) to evaluate the colonization level of a commercial biofertilizer containing a bacterial consortium on wheat in sterile conditions (Table 2.2). ESEM imaging revealed good survival rates as well as external and internal colonization of leaf and root tissues by a bacterial consortium.

Although electron microscopy allows clear visualization of cells outside and inside plant tissues at a very high resolution, this technique can be used only in limited sterile conditions since it is unable to distinguish bioinoculants from indigenous microbial populations living in soils.

2.4.3 Fluorescence microscopy

Fluorescence microscopy has become an essential technique in biology for the study of living tissues or cells. Although this method requires more complex and expensive instrumentation than conventional transmitted-light microscopy, it is widely used for the detection of bacteria inside

plant tissues. This is possible because fluorescence microscopy reveals the position of fluorescent substances that were previously introduced into living cells. Several fluorescent dyes and protein tags and other methods to fluorescently label cells can be employed, providing a range of tools to track a microbial inoculant.

Narula and coworkers (2007) proposed the use of serological methods such as double-antibody sandwich enzyme-linked immuno sorbent assay (DAS-ELISA) and immuno-fluorescence as potential techniques for investigating the colonization behavior of bioinoculants. They revealed the presence of *A. chroococcum* Mac 27 L in root fragments of hydroponically grown wheat plants using immunofluorescence (Table 2.2). However, one of the most commonly used methods for tracking endophytic inoculated bacteria within plant tissues is the use of green fluorescent protein (GFP), which emits fluorescent green light when irradiated with blue light or near-ultraviolet (UV) light (Wang et al.,2015). The detection and quantification of GFP-tagged strains is possible using epifluorescence microscopy (Leff and Leff, 1996), confocal laser-scanning microscopy (CLSM) (Götz et al., 2006; Fan et al., 2011; Krzyzanowska et al., 2012), flow cytometry (Elvang et al., 2001), and UV exposure for solid agar plates (Errampalli et al., 1999). The use of GFP allowed the evaluation the colonization abilities of tagged *Burkholderia* sp., *Rhizobium tropici* PTD1 and *Rahnella* sp. WP5 in rice plants grown in N-free MS agar for twenty days in a growth chamber (Kandel et al., 2015; Table 2.2). The presence of three inoculated GFP-tagged endophytic *Pseudomonas* sp. strains in different poplar tree tissues (leaf, stem and root) was verified by Germaine et al. (2004) using an epifluorescence microscope (Table 2.2). An innovative transparent soil made of a polymer with a low refractive index was used by Downie et al. (2012) to evaluate the abundance of GFP-tagged *P. fluorescens* SBW25 on *Lactuca sativa* roots (Table 2.2). The transparency of the substrate allowed them to capture images using confocal microscopy, which showed a high bacterial abundance on the root tips and at root branching zones. Although the use of GFP-tagged microbial strains has various advantages, such as no influence of autochthonous bacteria and the possibility of *in situ* detection, it can be used only in laboratory/greenhouse experiments since this method requires that the microbe be transformed before any application (Compant and Mathieu, 2013). In addition, the visualization of GFP expression is sometimes difficult due to the auto-fluorescence of the plant cell walls (Germaine et al., 2004), and it is difficult to detect inoculated microbes *in situ* because of interference by soil particles (Quadt-Hallmann and Kloepper, 1996). Finally, the procedure for the transformation of the GFP-plasmid involves exposure to $CaCl_2$, which promotes cyst formation in some endophytic strains, such as *A. chroococcum*; therefore, the procedure is unsuccessful in certain organisms. This is the main reason for developing an alternative procedure based on fluorescence resonance energy transfer (FRET) to visualize endophytes inside plant tissues when the use of GFP is restricted. This technique is based on the use of a novel specific rhodamine-pyrene conjugate as an Al^{3+} selective colorimetric and fluorescence sensor to visualize the endophytes with minimum interference of background autofluorescence, unlike GFP tagging. The FRET-based technique was used by Banik et al. (2016) to track the *A. chroococcum* Avi2 strain after inoculation on sterile rice seedlings (Table 2.2). The results showed intracellular root colonization by the *A. chroococcum* Avi2 strain since a clear and

stable green fluorescence was emitted by bacterial cells and detected by fluorescence microscopy, whereas a blue fluorescence was emitted by root tissues, proving the feasibility of this approach. In fact, the authors demonstrated that the rhodamine–pyrene conjugate was an excellent fluorescence ligand that was green-shifted only by the Al^{3+}-treated bacterial cells since it was able to detect only intercellular Al^{3+} (Banik et al., 2016).

The fluorescent Al^{3+}-siderophore complex produced by *A. chroococcum* strains was used by Viscardi et al. (2016) in combination with CLSM to assess the rhizocompetence of inoculated bacteria on tomato plants under sterile conditions *in vitro*, demonstrating the ability of the two selected bacteria to colonize plant roots (Table 2.2).

To determine the colonization ability of microbes on and inside plants, other methods, such as fluorescence *in situ* hybridization (FISH), have been employed. FISH is a molecular method based on the use of fluorescently tagged oligonucleotide probes, which are able to bind ribosomal RNA sequences to target metabolically active and intact cells (Moter and Göbel, 2000), combined with microscopy techniques such as epifluorescence microscopy (Compant and Mathieu, 2013) or CLSM (Rothballer et al., 2003; Wu et al., 2008). The range of available and developed probes for the detection of microbial cells using universal probes or strain-specific probes limits this technique. In addition, the long and complex sample preparation protocol (Moter and Göbel, 2000) could represent a disadvantage of this approach. Recently, the colonization ability of a multi-strain inoculant composed of *Pseudomonas* sp. G1Dc10, *Paenibacillus* sp. G3Ac9 and *S.azotifigens* DSMZ 18530 on annual ryegrass plants was analyzed using FISH combined with CLSM (Castanheira et al.,2017; Table 2.2). However, in plant tissues, FISH showed several limitations due to weak and/or unsuccessful hybridization signals of the probe. In fact, it was reported that in the FISH method, a low signal intensity of some of the detected microbes can occur due to a low cellular concentration of the target molecules or due to the low *in situ* accessibility of rRNA regions for singly labeled probes, thus preventing their successful visualization in plants (Wagner et al., 2003; Compant and Mathieu, 2013). Therefore, to overcome this problem, a combination of FISH, GFP-labeling methods and CLSM was employed. In detail, the use of FISH to detect a GFP-labeled *S. azotifigens* strain increased the signal, improving the visualization of bacterial cells and enabling the visualization and localization of inoculated strains in different parts of plants (Castanheira et al., 2017).

Although bioinoculants inside plant tissues can be clearly visualized by microscopy-based techniques, these techniques can suffer from several limitations (Pantanella et al., 2013; Emerson et al., 2017). For example, it is not always possible to distinguish living cells from dead cells by direct observation, and the autofluorescence of the plant cells sometimes makes it difficult to visualize microbial cells inside different plant tissues. Moreover, tagged microbial cells should be used only in limited and controlled experimental conditions (growth chamber and greenhouse) since it is not always permitted the dispersion of modified microorganisms in the environment, preventing the evaluation of survival and colonization ability of the bioinoculant in natural real ecosystems.

Table 2.2 Microscopy-based techniques used to monitor plant growth–promoting bacteria and root interaction.

Strains	Experimentalconditions	Methods	Plants ubstrate	Results	Reference
Burkholderia gladioli	Laboratory experimet on *Panicum virgatum*	Bright field microscopy	Water agar plates	Bacterial cells adhered to surfaces of root hairs and root epidermal parenchyma	White et al., 2014
Azotobacter chroococcum W5 *Trichoderma viride* ITCC 2211	Pot (day/night temperature 22–24/18 °C, humidity 60%)	SEM	Sterile sand and vermiculite (1:1)	Presence of *Azotobacter* cells, both individually both attached to the fungal mycelia, on root tissues	Velmouroug ane et al., 2017
Azotobacter chroococcum ATCC9043 *Azotobacter chroococcum* BCRC10599 *Azotobacter chroococcum* CCRC10599 *Azotobacter chroococcum* DSM2286 *Azotobacter chroococcum* IAM12666	*In vitro* assay on *Arnebia hispidissima* (25±1°C, 60% relative humidity, 10days)	TEM	MS culture medium	Endophytic interaction between bacterial strains and hairy roots	Singh and Sharma, 2016
Azospirillum spp. *Azoarcus* spp. *Azorhizobium* spp**.**	Controlled conditions (22°C; 16-h/8-h light/dark; relative humidity 75%)	ESEM	MS agar medium	Colonization of root cavities, bacterial biofilm formation, colonization of inner root tissues	Dal Cortivo et al., 2017
Azotobacter chroococcum Mac 27L	Phytotron chamber (12h light, ca. 30000 lux, 15-17°C/8-10°C day/night temperature, 28 days)	Immuno-fluorescence microscopy	Semi solid nutrient media	Bacteria were clearly detectable after 7 days of inoculation	Narula et al., 2007
Burkholderia sp. WPB *Rhizobium tropici* PTD1 *Rahnella* sp. WP5	Axenic conditions in growth chamber	GFP	N-free MS agar	Bacterial cells reside outside plant tissues in the apoplastic spaces and xylem tissue of rice plants	Kandel et al., 2015
Pseudomonas sp.VM1449 *Pseudomonas* sp.VM1450 *Pseudomonas* sp. VM1453	Pots (20–25 °C, 16-h light/8-h dark)	GFP	Sterile compost/vermiculite substrate (3:1 ratio)	GFP-tagged cells were clearly visible in the rhizosphere and on different root tissues	Germaine et al., 2004
Pseudomonas fluorescens SBW25	Laboratory experiment on 5 days growth lettuce	GFP	Transparent soil of particles of Nafion (polymer with a low refractive index)	Colonization of root surfaces, rhizoplane, and surfaces of Nafion particles	Downie et al., 2012
Azotobacter chroococcum Avi2	*In vitro* assay on sterile rice seedlings (14-h light cycle, 30±2°C, 7 days)	FRET-based technique	MS agar medium	Intracellular roots colonization (green fluorescence emitted by bacterial cells and blue fluorescence emitted by root tissues)	Banik et al., 2016

Table2.2 *Continuous.*

Strains	Experimentalconditions	Methods	Plant substrate	Results	Reference
Azotobacter chroococcum 67B *Azotobacter chroococcum* 76A	*In vitro* assay (sterile conditions)	Fluorescent Al^{3+}-siderophore complex combined with CLSM	Pots containing a growth medium added of 2 mM of Al^{3+}	Ability of the two bacterial strains to colonize tomato roots	Viscardi et al., 2016
Sphingomonas azotifigens DSMZ18530	Gnotobiotic conditions in controlled-environment chamber (16-h light/8-h dark, 18-23°C)	GFP	Modified Evans medium supplemented with 8% agar	Visualization and localization of bacterial strain in different parts of annual ryegrass plants (preferentially localized along root hairs and in stem epidermis)	Castanheira et al., 2017
Pseudomonas sp. G1Dc10 *Paenibacillus* sp. G3Ac9	Gnotobiotic conditions in controlled-environment chamber (16-h light/8-h dark, 18-23°C)	FISH/Confocal laser-scanning microscopy	Modified Evans medium supplemented with 8% agar	Visualization and localization of bacterial strains in different parts of annual ryegrass plants (preferentially localized along root hairs and in stem epidermis)	Castanheira et al., 2017

2.5 Molecular approaches

Methods based on the analysis of nucleic acids extracted directly from soil/rhizosphere samples have been developed to overcome cultivation limitations. In fact, the development of molecular tools allows new species of un-culturable microorganisms associated with the root system to be discovered or helps to understand the ecological function of several microbial species (Lebeis et al., 2012; Bulgarelli et al., 2013). The total genetic material recovered directly from soil samples represents the soil metagenome (Daniel et al., 2005), and metagenomics is the field of molecular genetics and ecology that studies this "collective" genome to determine the phylogenetic and functional gene complements of a sample (Pershina et al., 2013; Jansson, 2015).The development of metagenomic techniques, including the use of DNA probes (Bouvier and del Giorgio, 2003), polymerase chain reaction (PCR)-based techniques (Ruppel et al., 2006) and next-generation sequencing (NGS, Mardis, 2008), has greatly increased the ability to track microorganisms in natural environments (Ahmad et al., 2011). However, considering the high microbial diversity and the complex environmental matrix, DNA extraction is a fundamental step that could affect the detection and quantification of microbial taxa inferred from metagenomic sequences in all molecular methods; therefore, specific microbial groups can be underrepresented (Morgan et al., 2010; Montella et al., 2017). Currently, two main approaches are used for microbial DNA extraction from soil (Lombard et al., 2011): i) direct extraction, based on the direct lysis of microbial cells inside the soil matrix followed by DNA extraction and purification; and ii) indirect extraction, based on the initial recovery of microbial cells from the soil samples followed by lysis and DNA extraction and purification. Although both DNA extraction approaches are suitable for metagenomic analysis, they have different advantages and drawbacks in terms of DNA quantity and quality, even when starting from the same matrix (Ventorino et al., 2015; Montella et al., 2017), as extensively reported by Lombard et al. (2011), depending on the soil type. Therefore, when beginning a metagenomic analysis of soil, it is critical to define which DNA extraction method will be optimal by considering the subsequent genomic analysis (Lombard et al., 2011). For a more detailed discussion on this topic see Lombard et al. (2011).

2.5.1 PCR-based methods

In recent decades, several molecular approaches, such as quantitative real-time PCR (qPCR), denaturing gradient gel electrophoresis (DGGE), automatic ribosomal interspace spacer analysis (ARISA), amplified ribosomal DNA restriction analysis (ARDRA) and next-generation sequencing (NGS), have been used to investigate the presence of microbial inoculant in the soil system and to determine its impact on the rhizosphere community (Ciccillo et al., 2002; Steddom et al., 2002; Gamalero et al., 2003).These approaches allow the detection of specific microorganisms and/or the abundance of different microbial populations or species on the basis of the amplification of specific genes. Among these techniques, qPCR is a sensitive and suitable approach for determining the abundance of functional genes from soil-derived DNA and RNA (Fiorentino et al., 2016), and it has therefore been extensively used to track and quantify inoculated strains in soil systems (Providenti

et al., 2009; Timmusk et al., 2009). For instance, Sorte et al. (2014) used this method to design specific PCR primers targeting a 16S rRNA variable region to specifically measure the abundance of *Gluconacetobacter diazotrophicus* following co-inoculation with other diazotrophic strains in sugarcane plants grown under field conditions (Table 2.3). The validation of employed species-specific primers allow the use of this method to evaluate the occurrence of endophytic diazotrophic *Gluconacetobacter diazotrophicus* species in any soil type and plant tissue. A qPCR protocol was also developed by Couillerot et al. (2010) for the strain-specific quantification of *Az. brasilense* UAP-154 and CFN-535 in the maize rhizosphere using BOX-based sequence characterized amplified region (SCAR) markers, although the detection limit ranged from 10^4 to 10^8 CFU g^{-1} (Table 2.3). The success of this approach has led other authors to use it. In fact, strain-specific primers recovered from draft genome sequence analysis were employed for qPCR to quantify *Az. brasilense* FP2 in wheat roots as well as to assess its competitiveness following co-inoculation with other PGPR (Stets et al., 2015; Table 2.3). All of these works demonstrate the high effectiveness and specificity of this culture-independent approach based on the use of strain-specific primers, allowing rapid and inexpensive detection of bioinoculants in the plant rhizosphere for monitoring and quantification purposes, which is also useful in non-sterile and uncontrolled conditions.

The addition of bioinoculants in a soil could determine variations in the native microbial community structure, as recently reported by Fiorentino et al. (2018). PCR-DGGE followed by sequence analysis of bands is a metagenomic approach able to describe changes in soil microbial communities after inoculation of bacterial or fungal strains as well as to test the persistence of microbial inoculant in the soil. By DGGE and gene sequence analyses, Chen et al. (2013) detected heavy metal-resistant *Burkholderia* sp. J62 and *P. thivervalensis* Y-1-3-9 in both root interiors and rhizosphere soil of *Brassicanapus* L., demonstrating their influence on the rape-associated bacterial community structures in artificially Cd-contaminated soil (Table 2.3). The presence of *Az. brasilense* Cd (DSM 1843) in the rhizosphere of sorghum plants was monitored by Lopez et al. (2013) by gene sequencing of DGGE bands for three crop cycles (Table 2.3), highlighting its rhizocompetence against indigenous populations. However, since DGGE allows us to distinguish microbial populations at the species level, when the experiments are carried out in non-sterile soil, it is difficult to ensure that a sequence of bands originated from inoculated microbial strains or from other autochthonous strains belonging to the same species. Therefore, DGGE analysis is usually performed in combination with other techniques, such as FISH (Lopez et al., 2013), GFP (Piromyou et al., 2013), SEM and TEM (Thokchom et al., 2017). In some cases, the combination of DGGE and qPCR is a suitable approach to investigate the abundance of specific microbial groups and the survival of bioinoculants in the soil, as recently reported by Kumar et al. (2018) in a pot trial-based study (Table 2.3). In this case, DGGE was a useful approach to check bioinoculants because no band corresponding to inoculated *Dyadobacter* sp. was recovered in the control soil.

2.5.2 Next-generation sequencing (NGS)

In recent decades, the development of massive DNA sequencing technology, known as NGS, and bioinformatic tools has provided a powerful alternative to other molecular studies of microbial

ecology in natural environments, enabling the study of taxonomic diversity at a high resolution (Ventorino et al., 2018). Indeed, analyzing the rhizosphere microbiome with the high-throughput sequencing (HTS) approach has different prospective results that could allow understanding the community structure of root-associated bacteria and, as a consequence, novel bacteria with plant growth promoting traits to be discovered. This approach could also help to understand changes in the microbial community dynamics and structure after inoculation treatments. NGS could be performed following two different approaches: i) amplicon sequencing based on the amplification of phylogenetic marker genes, usually hypervariable regions from small-subunit ribosomal RNA genes (i.e., 16S rRNA), followed by bioinformatic analysis; ii) shotgun sequencing based on random sequencing across entire genomes followed by genome assembly and bioinformatics analysis. The construction of environment-based libraries was a major advance in soil metagenomics, and these libraries could be screened by functional and sequence-based approaches to clarify several functions of organisms in soil communities and to simplify genomic analyses of uncultured soil microorganisms (Garza and Dutilh, 2015). Recently, NGS of 16S rRNA genes was used to evaluate the behavior of the strain *Streptomyces* sp. AH-B after it was inoculated in quinclorac-contaminated soil, as well as its influence on soil microbial communities (Lang et al., 2018). After alignment, sequences were clustered into operational taxonomic units (OTUs) at 97% identity, which revealed that *Streptomyces* sp. AH-B became the dominant species following inoculation and that the bacterial and fungal diversity in treated soil was higher than that in the control, probably due to the degradation activity of inoculant that could reduce quinclorac toxicity to microorganisms. However, due to the high and complex biodiversity of soil microbial communities and the presence of various PCR and library preparation inhibitors, such as humic substances, full coverage of the soil metagenome is a difficult task. Moreover, the identification of OTUs at 97% identity thresholds allow to discriminate microbial populations at the species level but not at the strain level, so different strains with different plant growth promoting activities could be pooled together. In addition, identical OTUs do not necessarily mean the same species, since there are several databases for microbial identification, and it could be difficult to compare different studies, since the determination of sequences depends on sequences entered into DNA collections. Finally, high-quality DNA extraction for NGS is challenging for soil studies and is dependent on the extraction method and soil characteristics (Daniel, 2005).

2.5.3 Whole-genome sequencing and pangenome

The determination of the entire genomic DNA sequence at a single time sequence (whole-genome sequencing -WGS) of a microbial strain could be a powerful approach to investigate the potential PGP activities of a strain as well as its plant colonization and survival efficiency in the rhizosphere, leading to the identification of specific genes related and involved in plant-microbe interactions. In recent years, this approach was used to characterize new PGPR strains. Functional annotation of WGS of the strain *B. aryabhattai* AB211 revealed the presence of common genes involved in PGP activities and in abiotic/biotic stress tolerance as well as genes conferring resistance to oxidative stresses in plants demonstrating its high potential as a PGPM (Bhattacharyya et al., 2017). However, the presence of PGP-related genes is essential but not sufficient for a bacterium to exert

beneficial effects on plant growth in a real environment. In fact, although the presence of key attributes essential for possible colonization and interaction with the host plant were recovered in two *Rhodopseudomonas palustris* strains (PS3 and YSC3), these strains exhibited different expression patterns of genes related to PGP activities, probably due to the different physiological responses of these strains to specific compounds in the root exudates that act as signal molecules (Lo et al., 2018). Therefore, the effectiveness of PGP activities of a specific strain could also be affected by the different exudates released into the soil by different plants.

WGS could also be used in combination with metagenomic studies to identify microbial strains in the soil metagenome. Using this approach, the presence of the plant-associated strain *B. amyloliquefaciens* FZB42 on lettuce was assessed by Kröber et al. (2014; Table 2.3). Fragment recruitments of metagenome sequence reads on the referenced genome sequence of *B. amyloliquefaciens* FZB42 following shotgun sequencing of whole rhizosphere microbial communities of inoculated plants evidenced that the strain was present for over 5 weeks. Therefore, the combination of WGS and shotgun sequencing could be a suitable approach to identify the persistence of a microbial inoculant in the rhizosphere of plants grown in a natural environment.

Another method for the detection and identification of key genes responsible for the adaptation and evolution of a microbe as an endophyte is the pangenome. The pangenome can be defined as the entire genetic repertoire of a species; it comprises a core genome, which is composed of the genes present in all strains of the species, and an accessory genome, comprising the genes that are unique to specific strains (Mira et al., 2010; De Maayer et al., 2014). By analyzing the pangenome of eight sequenced *Pantoea ananatis* strains isolated from different sources, De Maayer and coworkers (2014) identified proteins with a potential role in plant-microbe interactions. Despite the large amount of information that could be retrieved from the pangenome, this method is still rarely used for studying the genetic traits of endophytes since it is based on the cultivation of microbial strains; therefore, non-culturable endophytes remain unexplored (Kaul et al., 2016).

Recently, Albanese and Donati (2017) proposed a novel method (StrainEst) based on the use of single-nucleotide variant (SNV) profiles of the referenced available genomes of selected species to identify and quantify the strains of interest present in metagenomic samples. This novel approach could be useful to highlight differences at the strain level that could allow us to track a microbial inoculant in the rhizosphere.

The increasing database of sequenced microbial genomes also allows genome-wide computational searches for clustered, regularly interspaced short palindromic repeats (CRISPRs) in microbial species (Sorek et al., 2008). These repetitive sequences have been detected in a wide number of bacterial and archaeal genomes (Horvath and Barrangou, 2010), including PGPM. CRISPRs are usually used as molecular markers for the detection of pathogenic microbes or for the evaluation of phage-resistance mechanisms in bacteria (Sorek et al., 2008). Although the CRISPR approach has been applied to plant-soil environments only to detect plant pathogenic strains such as *Erwinia*

amylovora (McGhee and Sundin, 2012), it could be exploited in the future for developing molecular markers to monitor PGPR for plant-microbe interactions (Rilling et al., 2019).

The development of molecular techniques based on the analysis of nucleic acids provides an approach useful to understand plant-soil-microbe interactions. These methods have greatly increased the ability to track microorganisms in natural environments and some of them allow a rapid and inexpensive detection of bioinoculants in the plant rhizosphere for monitoring and quantification purposes overcoming cultivation limitations. The use of one or a combination of these methods allow the investigation of the abundance of specific microbial groups and the survival of bioinoculants in the soil as well as variations in the native microbial community dynamics and structure (Kumar et al., 2018). Although, DNA-based approaches have improved our knowledge of microbial ecology, they are not able to differentiate between live and dead cells. Therefore, it is recommended to use them in combination with conventional methods, such as culture enumerations, for investigating bacterial ecology in natural habitats. Finally, molecular methods are highly influenced by DNA quality and quantity that is dependent on the extraction method and soil characteristics (Daniel, 2005; Lombard et al., 2011).

Table 2.3 Molecular approaches used to monitor plant growth–promoting bacteria and root interaction

Strains	Experimental conditions	Method	Plant substrate	Results	Reference
Gluconacetobacter diazotrophicus	Field experiment on sugarcane	qPCR	Soil (pH 5.3, P 6.1, 6.8 mg/dm^3, K 44 mg/dm^3, organic matter 1.3%)	Quantification of bacterial cells in plant tissues using species-specific primers	Sorte et al., 2014
Azospirillum brasilense UAP-154 *Azospirillum brasilense* CFN-535	Pots in greenhouse on maize (18-h/6-h light/dark, 18-22°C, 10 days)	qPCR	Sieved non sterile soil from La Côte St André adjusted to 20% (w/w) water content	Quantification of bacterial cells in the rhizosphereusing primers designed on strain-specific SCAR markers	Couillerot et al., 2010
Azospirillum brasilense FP2	Wheat plants germinated under sterile conditions, incubated in a greenhouse (14-h light/10-h dark, 23°C, humidity above 50%)	qPCR	Hoagland solution and quartz beads in glass tubes	Quantification of *A. brasilense* FP2 in the rhizosphere under sterile conditions	Stets et al., 2015
Azospirillum brasilense FP2 alone or co-inoculated with *Azospirillum brasilense* NH, *Herbaspirillum seropedicae* Z67, *Gluconacetobacter diazotrophicus* DSM 5601, *Azospirillum lipoferum* DSM 1691	Wheat plants germinated under non sterile conditions, incubated in a greenhouse (14-h light/10-h dark, 23°C, humidity above 50%)	qPCR	Quartz beads in glass tubes	Quantification of *A. brasilense* FP2 in the rhizosphere even under non sterile conditions and when co-inoculated with other rhizobacteria using strain-specific primers	Stets et al., 2015
Burkholderia sp. J62 *Pseudomonas thivervalensis* Y-1-3-9	Pot with rape plants (30.4±4.6°C/ 18.3±3.2°C day/night, relative humidity 67.5±12.9%)	PCR-DGGE	Contaminated soils (0.50 mg/kg of Cd and 100 mg/kg of $CdSO_4$)	Inoculated bacteria were detected in the root interiors and rhizosphere soils	Chen et al., 2013
Azospirillum brasilense Cd	Shade house with sorghum (temperature ~29 °C, light intensity of ~1,000 µmol photons m^2/s, 20 days; three crop cycles)	PCR-DGGE	Highly degraded alluvial desert soil	Persistence of the inoculant within the bacterial community of the rhizosphere of sorghum plants by purification and sequencing od DGGE bands	Lopez et al., 2013
Dyadobacter sp.	Pot trial in a net house (sampling at 30, 45, 60, and 90 days)	PCR-DGGE - qPCR	Soil (pH 7.5, oxidazable organic carbon 0.3-0.5%; phosphorus pentoxide< 22 kg/ha, ammonia 15 kg/ha, nitrate 4 kg/ha)	Quantification of diazotrophic abundance by qPCR and persistence of inoculantin the soil by detection of a specific DGGE band.	Kumar et al., 2018

Table 2.3 Continous

Streptomyces sp. AH-B	Containers with dry natural soil sprayed with quinclorac solution	NGS	-	*Streptomyces* sp. AH-B became the dominant species following inoculation in quinclorac-contaminated soil	**Lang et al., 2018**
Bacillu samyloliquefaciens FZB42	Field trial on lettuce rhizosphere	WGS-Metagenomic study	Soil (alluvial loam, total N 112 mg/100 g, P 32.3 mg/100 g, K 17.4 mg/100 g, Mg 9.1 mg/100 g, pH 6.5	Presence of the strain in the rhizosphere over 5 weeks in field. Marginal changes in the bacterial community after inoculant application.	Kröber et al., 2014

2.6 Conclusion

Assessing the root colonization of inoculants with beneficial effects on plant growth as well as their persistence over time in a soil is a critical issue in sustainable agriculture. Currently, several approaches that use culture-dependent, microscopic and molecular methods have been developed to follow bioinoculants in the soil and on the plant surface. However, to ensure good results in microbiological analysis, the first fundamental prerequisite is the correct soil sampling and sample preparation for the different methodological approaches that will be assayed.

Although plant colonization of bacterial endophytes can be assessed by microscopy-based techniques through molecular interactions and dynamics within living cells in a specific vegetable tissue, the measurement of the persistence of inoculants in soil poses technical difficulties, as the inoculant needs to be identified from a complex community. Methods to detect persistence include cultural enumeration or molecular approaches using PCR-based methods and next-generation sequencing. Culture-dependent methods are commonly used to estimate the persistence of inoculated bacteria in soil and/or rhizosphere, mainly for their ease of use, but this analysis is limited since it is difficult to represent the high diversity of bacteria on culture media and, at the same time, it is difficult to differentiate inoculated organisms from native populations based on morphological characteristics. Therefore, culture-dependent methods are especially useful when the experiment is carried out in sterile conditions to avoid interference by native microbial populations living in the soil. Molecular analysis allows the detection of bioinoculants or their activity in soil and contemporaneous evaluation of the effect of rhizosphere engineering on native microbial communities. However, most of the molecular techniques are based on the preliminary genomic characterization of the microbial strain used as inoculant and the specific molecular markers of the strain for its detection in the soil metagenome. Molecular approaches help to improve our knowledge of microbial ecology, but they cannot be considered as a substitute for more conventional methods, such as culture enumerations. In fact, if DNA is analyzed, there is the disadvantage of the inability to differentiate between live and dead cells; therefore, these methods should be considered complementary for investigating bacterial ecology in natural habitats. Future perspectives in the assessment of colonization and soil persistence should have a polyphasic approach combining several molecular and microbiological techniques to allow the tracking of inoculated strains or microbial consortia.

Moreover, a microscopy-based approach allows us to obtain a picture of bacterial colonization outside and inside plant tissues, but it is not possible to always distinguish living cells from dead cells by direct observation. The autofluorescence of the plant cells and interference by soil particles make it difficult to visualize microbial cells inside different plant tissues. Tagged microbial cells should be used only in limited and controlled experimental conditions (growth chamber and greenhouse), and the evaluation of the survival and colonization ability of an inoculant in a natural real ecosystem can not be performed because the strains could be released into the environment.

All the described methods have advantages and disadvantages and provide only partial results, and most of them are time-consuming, expensive and unable to detect specific inoculated microbial strains. Therefore, to better explain the behavior of bioinoculants in the natural soil ecosystems, culture-dependent and culture-independent (molecular and microscopic approaches) methods should be used in combination to examine the variations in microbial communities after inoculation treatment and to track the inoculated microbial strains in different systems.

The main challenge for the application of PGPM as bioinoculants in unsterilized greenhouse or field conditions is the establishment of effective methods for the assessment of plant colonization and soil persistence. Moreover, modern soil microbiology lacks efficient methods for the detection and estimation of the effective PGP activities that inoculated strains have on the soil. This is another main bottleneck in the use of microbial inocula for rhizosphere engineering. Therefore, the development of specific and easy methodologies for the evaluation of PGP activities could help to understand what actually occurs in a natural soil system during plant-soil-microbe interactions.

3 Development of innovative microbial-based biostimulants from agri-food waste for sustainable agricultural productions

This chapter reports the contents of original paper:

Romano I., Ventorino V., Ambrosino P., Testa A., Chouyia F. E., Pepe O. "*Development and application of low-cost and eco-sustainable bio-stimulant containing a new plant growth-promoting strain Kosakonia pseudosacchari TL13*". Frontiers in Microbiology 11:2044. doi: 10.3389/fmicb.2020.02044

This chapter also reports the contents of the original paper:

Chouyia F. E., Romano I., Fechtali T., Fagnano M., Fiorentino N., Visconti D., Idbella M., Valeria V., and Pepe O. "*P-solubilizing Streptomyces roseocinereus MS1B15 with multiple plant growth-promoting traits, enhance barley development and regulate rhizosphere microbial population*". Frontiers in Plant Science. 11:1137. doi: 10.3389/fpls.2020.01137

Moreover, this chapter also reports part of experimental activities performed at Research and Developments laboratory of Agriges s.r.l. (San Salvatore Telesino, Italy).
- Supervisors: Patrizia Ambrosino ph.D. and Marisa di Santo ph.D.

3.1 Introduction

According to the Food and Agriculture Organization (FAO), the estimated world population for 2025 will be nearly 8.5×10^9 inhabitants (Timmusk et al., 2017). Such an increase in agricultural production of 60% within the next years could be required to satisfy global food demand (Berger et al., 2018). Actually, in order to maintain a high quality of agricultural productions and eliminate or minimize yield loss, chemicals (fertilizers, pesticides, herbicides, etc.), hormones and antibiotics are commonly used for crops. The use of agrochemicals at industrial level allows to produce a large number of agricultural products at low costs with high profits for farmers. However, serious concerns regarding human and environmental health resulting from chemical residues in soil, water and food as well as farm workers' exposure have posed great attention (Alori and Babalola, 2018). Indeed, in the last two decades, the demand for organically grown agricultural products increased as consequence to the request for healthy and safe products (Dorais and Alsanius, 2016). Therefore, new eco-compatible strategies to improve agricultural systems and crop production are needed. The use of plant beneficial microorganisms as bio-inoculants offers an attractive eco-friendly alternative strategy to chemical inputs to ensure crop yield and nutritional quality (Fiorentino et al., 2018) acting as agricultural probiotics. Probiotics are live microorganisms that offer benefits to the host providing nutritional inputs and protecting it from pathogens (Hossain et al., 2017). Among the beneficial microbes employed in agriculture, plant growth-promoting rhizobacteria (PGPR) are the most commonly used. These microbes are able by a wide range of mechanisms to improve nutrient availability in soil, plant nutrient uptake and assimilation (i.e., nitrogen fixation, phosphate solubilization, siderophore, indole-3-acetic acid and ammonia production) and/or providing protection against plant pathogens (Backer et al., 2018; Woo and Pepe, 2018). Indeed, these microbes could also act as bio-stimulants ameliorating plant growth and crop production in response to abiotic stress in hostile environments (Van Oosten et al., 2017; Viscardi et al., 2016). Important examples of PGPR include *Pseudomonas*, *Bacillus*, *Azotobacter*, *Azospirillum*, *Burkholderia*, and *Sphingomonas* (Castanheira et al., 2017; Dal Cortivo et al., 2017; Kandel et al., 2017; Khalid et al., 2017; Sharma et al., 2011; Singh et al., 2013).

Kosakonia is a genus within the *Enterobacteria* complex. The order *Enterobacteriales* is a large and diverse group conformed by rod-shaped, non-spore forming, Gram-negative, facultatively anaerobic bacteria. The members of this group inhabit several different ecologic niches such as soil, water, and living in association with plants, insects, animals, or humans (Brenner and Farmer, 2005).
The participation of *Kosakonia* spp. in promoting plant growth has been poorly studied. But in the last few years, the interest around this genus is rising for its potential PGP activities.
The isolation or detection of different species of *Kosakonia* from rhizospheric habitat of different crops (such as corn or wheat) is the evidence of the close relationship between *Kosakonia* and plants (Jan-Roblero et al., 2020). Recently, several members of *Kosakonia* genus have been recognized as endophyte of different agricultural plants and their growth-promoting effects and crop yield improvement were demonstrated (Berger et al., 2017; Kämpfer et al., 2016). Several species of *Kosakonia* present some of the PGP traits above mentioned (Berger et al., 2017; Jan-Roblero et

al., 2020), and they are commonly described as N_2-fixing bacteria (Chen et al., 2014; Chin et al., 2017; Sun et al., 2018), thus, they can be classified as a PGPB.
However, not all *Kosakonia* species have the same functions. Indeed, *Kosakonia* (*K.*) *sacchari* is commonly considered phytopathogenic (Jan-Roblero et al., 2020). Although *Kosakonia* has been associated with plant growth, it has been demonstrated that it does not exert the same effect on all plants. The inoculation of sorghum with *Kosakonia* cells did not present any beneficial effect on plant growth (Schlemper et al., 2018).
However, this genus being relatively young, is less investigated and many of its features remain still unexplored.

Phosphorous (P) is the second most important plant growth-limiting nutrient after nitrogen, it is indispensable in many physiological and biochemical processes. Phosphorus deficiency is a common phenomenon in worldwide agricultural soils, thus most of the farmers regularly use chemical P fertilizers which get incorporated into the soil to avoid P limiting conditions in cropping systems. The applied P usually precipitate after the application by the formation of non-bioavailable complexes, whether in acid or alkaline soils (Urrutia et al., 2014). This mechanism generally causes a slow release of P, generating great challenges for remediation of these soils, with high accumulation of P not available to crops (Roy, 2017). Major attentions have to be given to phosphate solubilizing bacteria (PSB) considering that this nutrient will be less available in soil in the next future (Granada et al., 2018).
The use of PSB as microbial inoculants in soils is an attractive, eco-compatible and low-cost alternative strategy to exploit soil native P, limiting the application of chemical fertilizers with both environmental and economic benefits (Zaidi et al., 2009).
Among known beneficial soil microbes, Actinobacteria is one of the dominant prokaryotic taxa living in the soil. These microorganisms belong to an extensive and diverse group of Gram-positive, aerobic and filamentous prokaryotes. Actinobacteria can solubilize phosphate and promote plant growth besides several mechanisms such as siderophore and phytohormone productions (Jog et al., 2012). The most described genus belonging to this taxon is *Streptomyces* which is gaining popularity thanks to their ability to survive under stress conditions and is attracting special interest due to PGP activities (Jog et al., 2012; 2014), and for its beneficial effects on several crop plants (Gopalakrishnan et al., 2014). In fact, some *Streptomyces* species have been reported as PGPR in some crops such as tomato (El-Tarabily, 2008), wheat (Sadeghi et al., 2012) and chili (Passari et al., 2015).

The establishment of a low-cost and eco-sustainable process, as well as an effective and stable formulation, are among the main biotechnological challenges for the development of microbial inoculants. The use of agro-industrial organic waste and by-products as carbon source for the growth and production of microbial biomass is an attractive strategy to reduce the production costs, to valorize organic waste and by-products and to develop a sustainable and environmentally friendly process for bioinoculant production at industrial level. Moreover, it is also very important the form (solid or liquid) of microbial inoculant as well as its shelf-life. In fact, the form of the inoculant could influence its cost production, affect its efficiency and determine the method of

application in agriculture on large scale (Alori and Babaloa, 2018). The bioinoculant must be easy to handle in the field but it should maintain its features during the process and an adequate viability and shelf-life since it is required that it should be stable for at least six months (Berger et al., 2018).

Both papers presented in this chapter were aimed at isolation, selection, and characterization of rhizobacteria with multiple PGP traits and antimicrobial activity. Considering that two of the three bacterial strains selected in these studies were poorly investigated, they were deeply characterized. Two selected PGPB strains were also tested for their ability to tolerate abiotic stress and to be able to efficiently colonize plant roots in *in vitro* experiments.
Additionally, the selected strains were tested to develop a new bioinoculant using agro-industrial by-products as sole carbon source for microbial growth. Finally, new low-cost and eco-sustainable bio-formulates were obtained and tested in two forms (solid or liquid) in pot experiments to improve growth performance of maize plant.

3.2 Materials and Methods

3.2.1 Soil sampling and microbial isolation from different Ecosystems

Samples collected from the Rhizosphere of wheat plants

Rhizosphere samples were collected according to Romano et al. (2020) from wheat plants grown, under drought stress and nitrogen deficiency, in a greenhouse at the experimental station of the University of Naples Federico II (Bellizzi, Italy; 43°31'N, 14°58′E, 60 m a.s.l.). Ten grams of samples were shaken for 30 min in 90 mL of quarter strength Ringer's solution (Oxoid, Milan, Italy) containing tetrasodium pyrophosphate (16% w/v) as previously described (Ventorino et al., 2012a). After shaking, tenfold serial dilutions (1:10) were performed and used to inoculate liquid Augier medium (Pepe et al., 2013) for the detection of free-living (N_2)-fixing aerobic bacteria. After incubation for 14 days at 28 °C, the brown rings formed by microorganisms grew in the liquid medium were used to inoculate LG agar medium (Aquilanti et al., 2004). The plates were incubated for 7 days at 28 °C. Isolated colonies were picked from plates, purified by streaking on the same isolation medium, characterized by different morphologies examined by microscopy, gram staining and catalase reaction and stored at 4 °C as slant cultures until their characterization.

Samples collected in Morocco

Rhizospheric samples were collected from two different site in northwest of Morocco (33° 32′ 00″N, 7° 35′ 00″W) in November 2018. In each field, five different oat plants were randomly selected for sampling, collected and stored at 4 °C before analysis (Romano et al., 2020). Bacterial isolation, was performed as described above. Dilutions were performed from each sample followed by streaking in modified Pikovskaya's (MPVK) without yeast extract (Nautiyal, 1999) and containing $CaHPO_4$ as the only inorganic phosphate source. After incubation for 7 days at 30 °C, colonies distinguished based on phenotypic features such as morphology and biochemical characteristics (Gram reaction and catalase activity) were picked from plates and purified by

repetitive streaking on plate count agar (PCA, Oxoid). The isolates obtained were stored at 4 °C as slant cultures for further analysis.

3.2.2 Preliminary screening for plant growth promoting traits

Isolates from Wheat plants

Thirteen bacterial isolates from wheat plants were screened on the basis of their potential plant growth promotion activities. Detection and quantification of indol-3-acetic acid (IAA) production was determined by the Salkowski colorimetric assay using Nutrient Broth (Oxoid) with and without l-tryptophan (2 mg L^{-1}; Sigma-Aldrich, Milan, Italy) as previously described (Ventorino et al., 2014).

Semi-quantitative agar spot method was used to determine the ability of bacterial isolates to produce siderophores by Chrome-azurol S (CAS) assay as described by Silva-Stenico et al. (2005). After 14-21 days of incubation at 28 °C, the formation of an orange or yellow halo around the colony indicated the production of siderophores by the microorganism.

Determination of ACC deaminase activity of isolates was performed by assessing the growth on nitrogen-free minimal medium (MM) agar supplemented with 3 mM ACC (Sigma-Aldrich) after incubation at 28 °C in the dark for 7 days as described by Jaemsaeng et al. (2018). MM agar supplemented with 2 g L^{-1} $(NH_4)_2SO_4$ was used as control.

Isolates from Moroccan soil

The screening procedure comprised two-fold steps. For the first screening, sixteen isolates representative of different bacterial groups with similar morphological and biochemical characteristics, were selected and tested in vitro for P-solubilizing activity on MPVK agar by semi-quantitative spot method. Inoculated plates were incubated at 30 °C for 14 days, and the phosphate solubilization index (PSI) was calculated according to Gupta et al. (2012) using the formula reported by Qureshi et al. (2012). The second step was the quantitative estimation of solubilized P on MPVK liquid medium. During 15 days of incubation at 30 °C in agitation (150 rpm), 1 mL of the culture was sampled every 72 h, centrifuged at 18,620 x g for 5 min and the supernatant was collected to measure the pH of the medium as well as to estimate released soluble P by the molybdenum blue assay (Murphy and Riley, 1962). The concentration of P solubilized was quantified by spectroscopic absorbance measurements at 430mμ according to the standard curve. Un-inoculated samples were used as negative control. All experiments were performed in triplicate.

3.2.3 Identification of selected strains

The bacterial isolates showing the highest plant growth promoting activities or the best P-solubilizing activity were selected for further investigations and identified by the sequencing of the 16S rRNA gene. In detail, total genomic DNA of selected strains was extracted by boiling for 10 min and then used as template for the PCR assay. The PCR mixture was employed according to

Alfonzo et al., (2012) using the primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-AAGGAGGTGATCCAGCC-3′). The PCR conditions were as described by Ventorino et al., (2017). PCR products were purified using the QIAquick PCR Purification Kit (Quiagen, Milan, Italy) according to the supplier's recommendations and sequenced as previously reported (Ventorino et al., 2016). The DNA sequences were compared to the GenBank nucleotide data library using the BLAST software at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/Blast.cgi).

The nearly full-length 16S rRNA sequences of the selected bacterial strain with multiple PGP traits and 30 type strains belonging to different genera related to *K. pseudosacchari* species as described by Kämpfer et al. (2016) were used to perform multiple nucleotide alignments using the ClustalW program (Thompson et al., 1994) from MEGA version 4.0 (Tamura et al., 2007). The nucleotide sequences of the type strains were retrieved from the Ribosomal Database Project (RDP, http://rdp.cme.msu.edu/). The phylogenetic tree was inferred using the Neighbor-Joining me thod with the Maximum Composite Likelihood model in the MEGA4 program, with bootstrap values based on 1,000 replications.

The 16S rRNA gene sequences of *Kosakonia* were deposited in the GenBank nucleotide database under accession numbers MN607213, MN607214 (http://www.ncbi.nlm.nih.gov) the ones of *Streptomyces* under accession numbers MN607213 and MN607214.

3.2.4 In vitro plant growth promotion and antimicrobial activities

Phosphate solubilization ability of *Kosakonia* strains was quantified by molybdenum blue quantitative assay in PKV liquid medium. Briefly, 10 mL of PKV medium was inoculated with 0.1 mL of bacterial cultures (approximately 1.5×10^8 CFU mL^{-1}) and incubated for 15 days at 30 °C. After incubation, cultures were centrifuged (5 min at 18,620 ×g) and supernatant was collected to estimate released soluble phosphorus as described by Murphy and Riley (1962). The concentration of P solubilized was determined by spectroscopic absorbance measurements at 430 mμ according to the standard curve (Murphy and Riley, 1962).

Ammonia production of selected *Kosakonia* and *Streptomyces* strains was estimated by inoculating the microorganisms in 5 mL of peptone water according to Cappuccino and Sherman (1987) and incubating under shaking (100 rpm) at 30 °C for 7 days. The presence of ammonia was detected by the development of a brown to yellow color after adding 0.5 mL of Nessler's reagent (Sigma-Aldrich) to the culture and then quantified by spectroscopic absorbance measurements at 420 nm according to the standard curve (Passari et al., 2017).

The presence of the target gene *nif*H, encoding nitrogenase reductase enzyme, was assessed by PCR assay using the synthetic oligonucleotide primers *nif*H-F (5′-AAAGGYGGWATCGGYAARTCCACCAC-3′; Rösch et al., 2002) and *nif*H-R, (5′-TTGTTSGCSGCRTACATSGCCATCAT-3′; Rösch et al., 2002) using conditions reported by

Fiorentino et al. (2016). The presence of the target gene was assessed by visualization of a 475 bp band by agarose (1.5% w/v) gel electrophoresis (100 V for about 1h).

ACC deaminase activity was quantified according to Penrose and Glick (2003) by measuring the amount of α-ketobutyrate (Sigma-Aldrich) produced when the enzyme ACC deaminase cleaves ACC. In detail, bacterial strains were inoculated in 5 mL of DF salt medium containing $(NH_4)_2SO_4$ as sole nitrogen source (Penrose and Glick 2003). After incubation at 30 °C for 48 h, the cultures were used to inoculate 5 mL of DF salt medium containing 3 mM ACC (Oxoid) as nitrogen source. The amount of α-ketobutyrate (µmol) produced was estimated by measuring the absorbance at 540 nm according to the standard curve (α-ketobutyrate concentration ranged from 0.1 to 100 µmol).

Quantitative estimation of siderophores was performed according to Arora and Verma (2017) using CAS reagent and expresses as percent siderophore unit (psu) using the following formula (Payne, 1993):

$$\text{psu} = [\,(\text{Ar} - \text{As})\ \text{x}\ 100\,] \,/\, \text{Ar}$$

where Ar is the absorbance of reference (CAS solution and uninoculated medium), and As is the absorbance of sample (CAS solution and cell-free sample supernatant).

Antimicrobial antagonism was evaluated using the dual culture method described by Hammami et al. (2013) against eight pathogenic eukaryotic strains belonging to the microbial collection of Division of Biology and Protection of Agricultural and Forest Systems (Department of Agricultural Sciences, University of Naples Federico II): *Botrytis cinerea* B11, *Botrytis cinerea* B12, *Fusarium oxysporum* F3, *Fusarium oxysporum* F5, *Aspergillus niger* A31, *Phytophthora infestans* ph1, *Phytophthora cactorum* ph3, and *Phytophthora cryptogea* ph4. Fungi were grown on Potato Dextrose Agar (PDA, Oxoid) at 28 °C for 7 days, while Oomycetes were grown on V8 agar (200 mL of V8 juice, 2.5 g $CaCO_3$, 800 mL of distilled water and 17 g of bacteriological agar) at 28 °C for 21 days. Conidia were harvested from the surface of plates by flooding the cultures with 9 mL of sterilized distilled water and gently scraping with a sterilized glass rod. The conidial concentration was determined using the counting chamber Thoma (Hawksley, UK). An over-layer agar (agar 0.7%) containing a concentration of 10^5 conidia mL^{-1} of each plant pathogen was poured on BHI agar plates previously spotted with the bacterial strains. After incubation for 7 or 21 days at 28 °C, the antimicrobial activity of the bacterial strains was highlighted by the presence of a halo around the colony without fungal growth.

Tolerance to abiotic stress of Kosakonia strains

The two selected *Kosakonia* strains were tested for their salt tolerance in liquid medium as previously described by Ventorino et al. (2012b). Briefly, 5 mL Brain Heart Infusion (BHI) medium supplemented with NaCl up to 15% (w/v) was inoculated with each bacterial strain. The standard BHI medium with 0.5% (w/v) NaCl was used as control. Bacterial growth was determined by observing the development of turbidity of cultures at 24 h and 48 h and comparing them with McFarland Turbidity Standard.

Similarly, temperature tolerance was investigated comparing bacterial growth in BHI liquid medium with McFarland Turbidity Standard after 24 h and 48 h of incubation at 28, 30, 37 and 42 °C.

Finally, pH tolerance was determined by evaluating the growth of bacterial strains in BHI liquid medium in which pH was adjusted at pH 5, 6, 7 and 8 by the addition of HCl or NaOH. After incubation, bacterial growth was estimated at 24 h and 48 h comparing their turbidity to McFarland Turbidity Standard.

Rhizosphere competence of Kosakonia strains

Tomato seeds (*Solanum lycopersicum* var. *cerasiforme*) were carefully de-husked without damaging the embryo and surface sterilized as described by Banik et al. (2016). Briefly, seeds were treated with 2% sodium hypochlorite (5 min) followed by washing with sterile distilled water, then seeds were treated with 75% ethanol (5 min), washed again with sterile water and treated with 30% hydrogen peroxide (2 min) as suggested by Amarasinghe et al. (2018); finally, they were carefully rinsed ten times with sterile distilled water. Seeds sterility was checked by plating on Plate Count Agar (PCA; Oxoid). Seeds germination took place in darkness at 30 ± 2°C.

Microbial cells were grown in BHI medium (30 ± 2°C, 24 h). Cells were harvested by centrifugation (2000 ×g for 5 min) at the end of their exponential phase of growth, washed twice in HEPES buffer (0.1 M) and then suspended in quarter strength Ringer's solution (Oxoid) until achieving microbial concentration of approximately 5×10^8 CFU mL^{-1} (counting chamber Thoma 0.02 depth, Hawksley UK). Finally, tomato seedlings were treated with bacterial suspension for 48 h at 30 ± 2°C and then rinsed five times with sterile HEPES buffer (0.1 M) to remove the loosely associated bacteria from the radicle surface. Tomato seedlings treated with sterile water were used as control. Bacteria-infected radicles and controls were treated with LIVE/DEAD® BacLight™ bacterial viability kit (Thermo Fisher Scientific) following the manufacturer's instructions. Treated radicles were observed by fluorescence microscope (Axiovert 200M, Zeiss, Göttingen, Germany) under UV light (50-W mercury lamp) and using a Green Fluorescent Protein Filter (38 HE-GFP; excitation wavelength of 450-490 nm) and Rhodamine Filter (Rh-20; excitation wavelength of 540-552 nm).

3.2.5 Studies for production of a low-cost bacterial inoculants

Bacterial growth of Kosakonia pseudosacchari TL13

The strain TL13 was inoculated in 200 µL of BHI using 96-well flat-bottom microplate in a Microplate Reader (BioTek Elx808) and incubated at 30 °C for 24 h with moderate shaking every 30 min. $O.D._{600nm}$ measurements were performed every 30 min to define the growth curve.

Preliminary batch growth tests were performed to assess the best growth conditions for the strain TL13. In details, 500 mL flasks filled with BHI medium were inoculated with 2% bacterial cells suspension (8.45 ± 0.20 CFU mL^{-1}) and incubated at 30 °C for 24 h using three different growth conditions: 1) Batch 1, shaking at 130 rpm (Grant-bio, Orbital Shaker-Incubator ES80); 2) Batch 2, shaking at 130 rpm and sterile air sparging at 0.5 vvm; control, no shaking and no air sparging. Samples were withdrawn every 2 h and cell growth was determined by viable counting on BHI medium.

A scale-up batch experiment was performed in a 10 L fermentor (New Brunswick BioFlo®/CelliGen® 115, Eppendorf) to evaluate the microbial growth using the best conditions assessed in the preliminary batch experiments. The experiment was performed in a working volume of 4 L of BHI medium inoculated with 2% bacterial cells suspension (8.67 ± 0.40 CFU mL^{-1}), using the following parameters: 30 °C, pH 7.00, agitation of 130 rpm, air sparging at 0.5 vvm, 40 mL of a solution 3% of Antifoam 204 (Sigma-Aldrich) added at the beginning of the process. Samples were withdrawn every 2 h and cell growth was determined by viable counting on BHI medium. After 24 h, the culture was centrifuged (45 minutes at 3428 ×g) and recovered cells were suspended in a 5% sucrose solution at the ratio 1:5 (w:v). The strain was freeze-dried, and cell viability was determined by counting on BHI medium immediately after freeze-drying and after 3 and 6 months of storage at room temperature.

Microbial growth in liquid media containing food by-products of Kosakonia pseudosacchari TL13

The strain TL13 was inoculated in several liquid media containing agro-food industrial by-products to find a low-cost carbon source useful for its growth. To this end, the strain was inoculated into 10 mL of liquid substrates containing 1%, 5% or 10% of whey, protein hydrolysate, exhausted yeasts, molasse or vinasse, kindly provided by Agriges S.r.l. (San Salvatore Telesino, Benevento, Italy). The strain TL13 grown in BHI was used as control. Samples were withdrawn after 48 h of incubation at 30 °C, to determine bacterial growth.

Production of Kosakonia pseudosacchari TL13 inoculants on nutrient-supplemented vermiculite

Solid State Fermentation (SSF) was performed in gas permeable polypropylene bags (SacO2, Belgium). Growth on inert support was carried out by adopting the procedures described by Graham-Weiss et al. (1987). Sterile vermiculite, moistened with BHI broth or with a solution of exhausted yeasts and vinasse, was inoculated with the selected strain TL13 (10^6 bacterial cells per g

of vermiculite). After incubation (15 days at 30 ± 1 °C), an aliquot was used to develop liquid bio-inoculants recovering the bacterial cells and added them in a raw castor oil/alginate based emulsion following the protocol described by Fravel et al. (1985) with some modifications. Another amount of inoculated vermiculite was dried for 15 days at 30 ± 2 °C to achieve a microbial-based solid formulation. Samples were withdrawn immediately after incubation and after the development of formulations to determine bacterial growth by viable counting on BHI medium.

Production of Streptomyces roseocinereus MS1B15 by Solid State Fermentation

Different SSF tests were performed as above described to define the best conditions to apply this technology for *Streptomyces* (*S.*) *roseocinereus* MS1B15 Sterile vermiculite, moistened with SC broth, was inoculated with the selected strain MS1B15 (10^6 bacterial cells per g of vermiculite). After incubation (15 days at 30 ± 1 °C), an aliquot was used to assess the growth of the microbial strain. Another test was performed using rice, alone or combined with grain, moistened with the International media for *Streptomyces* n. 2 (IM n.2) or with Whey as alternative low-cost carbon source in the ratio (5:1 or 8:4:3). Also, in this case after the incubation (15 days at 30 ± 1 °C), an aliquot was used to assess the growth of the microbial strain.

3.2.6 Pot trials

The ability of the selected strain TL13 to promote plant growth was evaluated in growth chamber pot trials. The experimental set up was performed according to standard procedure (DM 27/01/2014) with some modifications. Maize (*Zea mays*, Class FAO 400/gg 120) seeds were surface sterilized by 5 min washing in NaClO 5% solution and germinated on damp tissue paper for 48 h. Seeds were planted in 10 cm Ø plastic pots filled with 0.5 kg of unsterilized soil. At planting, soil was inoculated with the strain TL13 at a concentration of approximately 1×10^6 cells g^{-1}.

The strain TL13 was inoculated in three different formulates: raw castor oil/alginate-based emulsion (E-TL13), dried vermiculite (V-TL13) and recovered cells (R-TL13) diluted in sterile Ringer's solution (Oxoid). Un-inoculated soil (C) was used as control. All tests were performed in triplicate and three seeds were planted for each pot.

Plants were grown under controlled conditions with a constant temperature of 28 ± 0.5 °C, a 16 h light / 8 h dark photoperiod, relative moisture 70% and daily watered for 15 days.

After 15 days, the plants were sampled and were measured vegetative parameters as total plant length, root and shoot length, root and shoot fresh weight, root and shoot dry weight percentage.

3.2.7 Statistical analyses

Data were analyzed by one-way ANOVA followed by Duncan's HSD post hoc for pairwise comparison of means (at $P< 0.05$) using SPSS 19.0 statistical software package (SPSS Inc., Cary, NC, USA).

3.3 Results

3.3.1 Plant growth promoting activities of bacterial isolates from wheat rhizosphere

A total of 13 bacterial isolates (from TL1 to TL13) were obtained from the rhizosphere of wheat plants using Augier liquid medium followed by streaking on LG agar medium. Isolates were preliminarily screened for their potential plant growth promoting activities, as IAA and siderophores production and ACC-deaminase activity (Table 3.1).

The results indicated that about 85% of isolates were able to synthetize IAA, although most of them at low amounts (ranging from 1.32 to 5.98 mg L^{-1}). The two isolates, TL8 and TL13, showed the highest IAA production up to 13.20 ± 1.80 or 22.16 ± 2.67 and 12.91 ± 0.64 or 33.26 ± 1.67 mg L^{-1}, respectively, in the absence and in the presence of L-tryptophan (Table 3.1).

Ten isolates produced siderophores showing orange haloes around the colony in CAS agar ranging from 10 to 35 mm (Table 3.1). Among these, the isolates TL3, TL4 and TL13 exhibited the highest siderophores production (halo dimension 30-33 mm); while the isolates TL1, TL3, TL7, TL8 and TL12 produced haloes ranging approximately from 20 to 23 mm.

Moreover, seven isolates (TL1, TL2, TL4, TL6, TL7, TL8 and TL13), corresponding to about 54%, revealed ACC-deaminase activity because they were able to grow on MM medium supplemented with ACC (Table 3.1).

Table 3.1 Preliminary screening for the assessing the plant growth-promoting activities of bacterial isolates obtained from wheat rhizosphere.

Isolate	IAA† in NB (mg L^{-1})	IAA§ in NB+TRP (mg L^{-1})	Siderophores# (mm)	ACC-deaminase activity*
TL1	2.59 ± 0.06^{g-m}	0.00 ± 0.00n	20.0 ± 0.00^{cd}	++
TL2	3.16 ± 0.50^{g-i}	1.82 ± 0.31^{i-m}	0.00 ± 0.00^{f}	+
TL3	1.56 ± 0.07^{l-n}	1.32 ± 0.02^{mn}	30.0 ± 0.00^{ab}	-
TL4	3.40 ± 0.02^{f-h}	4.90 ± 0.63^{de}	33.33 ± 5.77^{a}	+
TL5	1.62 ± 0.02^{i-m}	1.42 ± 0.00^{mn}	23.33 ± 5.77^{bc}	-
TL6	5.98 ± 1.03^{d}	3.95 ± 0.33^{e-g}	10.0 ± 0.00^{e}	++
TL7	5.89 ± 0.60^{d}	5.15 ± 0.51^{de}	23.33 ± 5.77^{bc}	++
TL8	13.20 ± 1.80^{c}	12.91 ± 0.64^{c}	23.33 ± 11.55^{bc}	++
TL9	0.00 ± 0.00^{n}	0.00 ± 0.00^{n}	0.00 ± 0.00^{f}	-
TL10	1.68 ± 0.67^{i-m}	4.69 ± 1.32^{d-f}	0.00 ± 0.00^{f}	-
TL11	2.23 ± 0.02^{h-m}	0.00 ± 0.00^{n}	13.33 ± 5.77^{e}	-
TL12	1.43 ± 0.02^{mn}	3.05 ± 0.07^{g-l}	20.0 ± 0.00^{cd}	-
TL13	22.16 ± 2.67^{b}	33.26 ± 1.67^{a}	30.0 ± 0.00^{ab}	++

†IAA production in Nutrient Broth without L-tryptophan, values represent the means ± SD of three replicates.
§IAA production in Nutrient Broth supplemented with L-tryptophan, values represent the means ± SD of three replicates.
#Halo size (mm) = diameter of clearing or halo zone/colony diameter, values represent the means ± SD of three replicates
*- no growth; + middle growth; ++ high growth

Identification and phylogenetic analysis of selected strains

The preliminary screening for the assessment of plant growth promotion activities allowed for the selection of the TL8 and TL13 isolates. The nearly full-length sequence of 16S rRNA gene (about 1,450 bp) of the strains TL8 and TL13 revealed an identity of 99% with *K. sacchari*, *K. pseudosacchari*, *K. oryzae* and *K. radicincitans* species using Blast software. To establish the identification of the two selected strains, a consensus tree, generated from the distance data using the Neighbor-Joining method with the Maximum Composite Likelihood model in the MEGA4 Program was constructed including the 16 S rRNA sequences of type strains related to *Kosakonia* genus (Figure 3.1). High bootstrap values, ranging from 51% to 99%, were observed and indicated significant branching points in the phylogenetic tree. The phylogenetic tree indicated that the closest relative species of the two selected strains was *K. pseudosacchari* (cluster with bootstrap value of 97%), demonstrating that the strains TL8 and TL13 can be classified as belonging to this species (Figure 3.1).

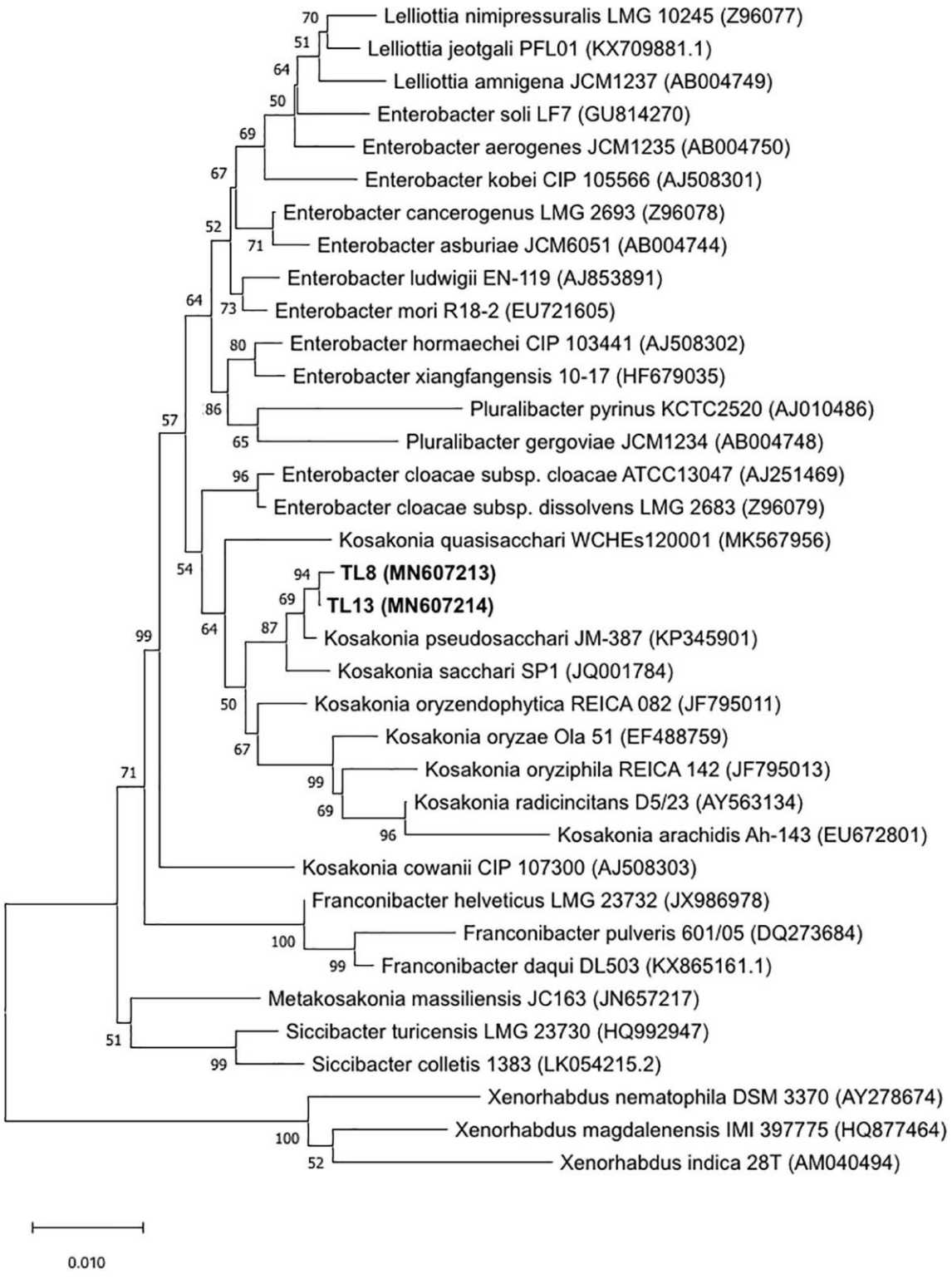


Figure 3.1 Neighbor-Joining tree based on the comparison of 16S rRNA gene sequences of bacterial strains TL8 and TL13 and 30 type strains related to genus *Kosakonia* sequences from RDP. Bootstrap values (expressed as percentages of 1,000 replications) are given at the nodes. The sequence accession numbers used for the phylogenetic analysis are shown in parentheses following the species name. *Xenorhabdus* type strain sequences were used as out group. The scale bar estimates the number of substitutions per site.

3.3.2 Phosphate solubilizing activities of bacteria isolates from Moroccan soil

A total of sixteen isolates were evaluated for *invitro* P solubilizing activity using MPVK agar containing $CaHPO_4$ as sole P source. Out of 16 isolates, five strains (31.3%) were able to solubilize the P showing a clear halo around the colony with a PSI value ranging from 1.17 to 1.75. The highest PSI was exhibited by the isolates MS1B15 (PSI = 1.75) followed by MS1B13 (PSI =1.63).

On the basis of this preliminary screening the isolates MS1B15 and MS1B13 were selected for further investigations and identified by 16S rRNA gene sequencing. Using the BLAST software, the nearly full-length gene sequence of the bacterial strains MS1B15 and MS1B13 showed 98.69% identity to *Streptomyces roseocinereus* and 99.59% identity to *Streptomyces natalensis*, respectively.

Quantitative assay in liquid medium confirmed that *S. roseocinereus* MS1B15 and *S. natalensis* MS1B13 had high P-solubilizing efficiency. The soluble P concentration was slow during the first three days, after that it gradually increased reaching a value of 245.6 ± 11.8 mg/L and 207.9 ±3 .3 mg/L for MS1B15 and MS1B13, respectively (Figure 3.2). Maximum P solubilization was observed by *S. roseocinereus* MS1B15 which is consistent with the highest PSI. It has been also found that the soluble-P concentration increased as the pH decreased in liquid medium from an initial pH of 7.00 to 5.55 ± 0.11 and 6.13 ± 0.06 by MS1B15 and MS1B13, respectively. Neither soluble P (Figure 3.2) nor a decrease in pH (7.00) were detected in the control treatment.

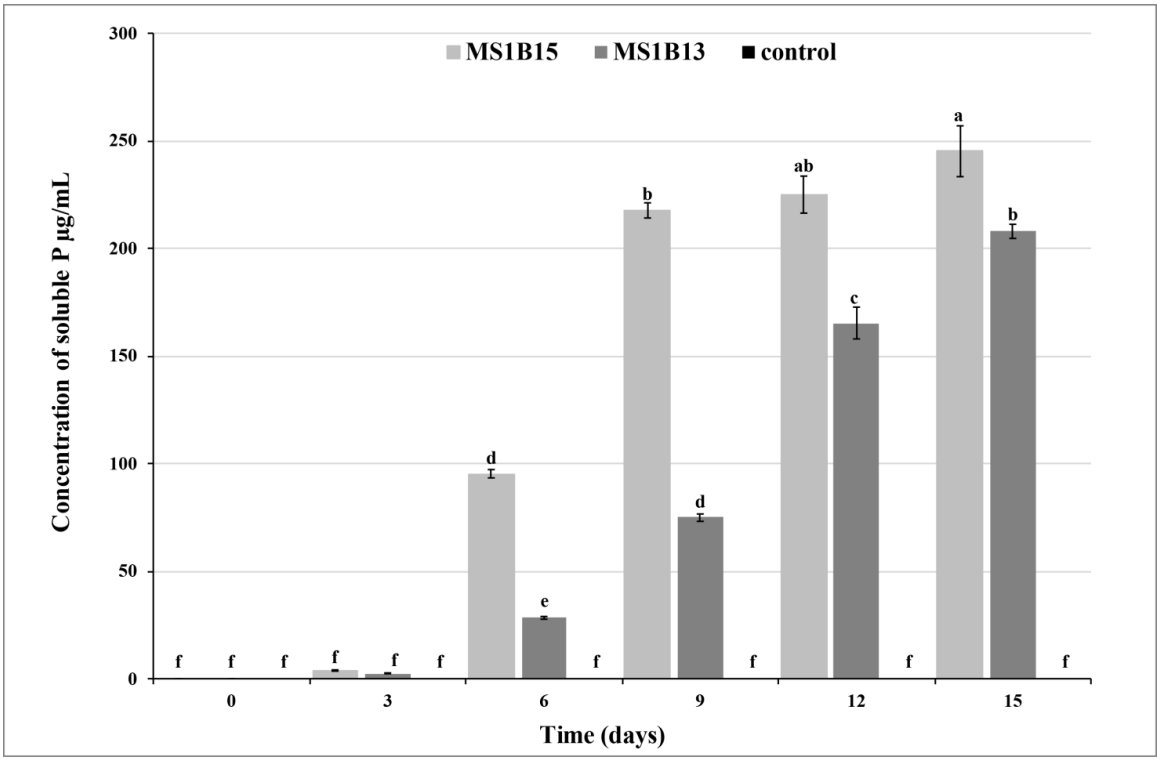


Figure 3.2 Phosphate solubilizing activity of MS1B15 and MS1B13 isolates during 15 days of incubation in MPVK liquid medium.

3.3.3 PGP traits, phenotypic characteristics and rhizosphere competence of *Kosakonia pseudosacchari* strains

The selected strains *K. pseudosacchari* TL8 and TL13 were further characterized to evaluate other plant growth promotion activities as well as antagonistic behaviors. Quantitative estimation of phosphate solubilization by molybdenum blue assay in PKV liquid medium indicated that the strains TL8 and TL13 were able to solubilize up to 348.05 ± 12.77 and 346.05 ± 25.62 mg L^{-1} of phosphate starting from dicalcium phosphate (Table 3.2). Measurement of ammonia in peptone water liquid medium by quantitative Nessler's reagent test highlighted that both bacterial strains TL8 and TL13 were capable to produce ammonia (2.24 ± 0.03 mg L^{-1} and 2.37 ± 0.03 mg L^{-1}, respectively; Table 3.2) in medium without nitrogen source. Moreover, *K. pseudosacchari* TL8 and *K. pseudosacchari* TL13 were potentially able to fix atmospheric nitrogen (N_2) due to the presence of the nifH gene detected by specific PCR amplification as well as exhibited ACC deaminase activity producing up to 3.04 ± 0.10 μM and 3.31 ± 0.11 μM of α-ketobutyrate protein mg^{-1} in 30 min (Table 3.2). As reported in Table 3.2, quantitative assay showed a siderophore concentration produced by the strains *K. pseudosacchari* TL8 and TL13 equal to 32.00 ± 0.92 and 29.77 ± 1.8 psu, respectively. Indeed, both strains exerted antimicrobial activity against soil-borne plant pathogens (Table 3.2) revealed by a considerable reduction of mycelium growth of *Botrytis cinerea* B12, *Phytophthora infestans* ph1, *Phytophthora cactorum* ph3, and *Phytophthora cryptogea* ph4, in respect to the control plates.

K. pseudosacchari TL8 and TL13 were found to be salt-tolerant because they were able to grow in the liquid culture medium containing up to 13% w/v of NaCl (Table 2). In detail, no differences were found in the bacterial growth up to 8.0% w/v of NaCl reaching a concentration of about 1×10^8 CFU mL^{-1} after 24 h of incubation (Table 3.2). At higher NaCl concentration (from 9.0 to 13 % w/v) the two strains grew slowly reaching a bacterial growth of two orders of magnitude lower (about 1×10^6CFU mL^{-1}) after 48 h of incubation (Table 3.2). The two strains *K. pseudosacchari* TL8 and TL13 grew also up to about 1×108 CFU mL^{-1} after 24 h of incubation at different temperatures (28, 30, 37, and 42 °C). Finally, both strains tolerated a pH range between 4.0 and 8.0 reaching a final concentration of about 1×10^8 CFU mL^{-1} after 24 h (Table 3.2).

In order to test the ability of the two selected strains *K. pseudosacchari* strains TL8 and TL13 to colonize the root surface, sterile tomato radicles were inoculated and observed by fluorescence microscope after staining with the LIVE/DEAD® BacLight™ kit reagents. As shown in Figure 3.3, bacterial cells were clearly visualized on plant tissues highlighting that both *K. pseudosacchari* TL8 and *K. pseudosacchari* TL13 successfully colonized tomato's radicle. In particular, bacterial cells of the strains TL8 resulted congregated on root surfaces (Figure 3.3a), whereas cells of the strain TL13 appeared scattered (Figure 3.3b).

Table 3.2 Differential phenotypic characteristics and plant growth-promoting traits of bacterial strains *Kosakonia pseudosacchari* TL8 and TL13.

Characteristic/Activity	*Kosakonia pseudosacchari* TL8	*Kosakonia pseudosacchari* TL13
IAA in NB[†] (mg L^{-1})	13.20 ± 1.80	22.16 ± 2.67
IAA in NB+T[§] (mg L^{-1})	12.91 ± 0.64	33.26 ± 1.67
Siderophores production (psu)	32.00 ± 0.92	29.77 ± 1.80
ACC-deaminase activity (μM of α-ketobutyrate protein mg^{-1} in 30 min)	3.04 ± 0.10	3.31 ± 0.11
Ca_2HPO_4 solubilization (mg L^{-1})	348.0 ± 12.77	346.05 ± 25.62
*Nif*H gene	+	+
Ammonia accumulation (mg L^{-1})	2.24 ± 0.03	2.37 ± 0.03
NaCl tolerance range (w/v, 0.5-8%) 24 h	1×10^8 CFU mL^{-1}	1×10^8 CFU mL^{-1}
NaCl tolerance range (w/v, 9-13%) 48h	1×10^6 CFU mL^{-1}	1×10^6 CFU mL^{-1}
pH range at 24 h	5-8	5-8
Temperature range (°C) at 24 h	28-42	28-42
Antagonistic activity	+ against *Botrytis cinerea* B12, *Phytophthora infestans* ph1, *Phytophthora cactorum* ph3, *Phytophthora cryptogea* ph4	+ against *Botrytis cinerea* B12, *Phytophthora infestans* ph1, *Phytophthora cactorum* ph3, *Phytophthora cryptogea* ph4

[†]NB = Nutrient Broth.
[§] NB+T = Nutrient Broth supplemented with L-tryptophan.

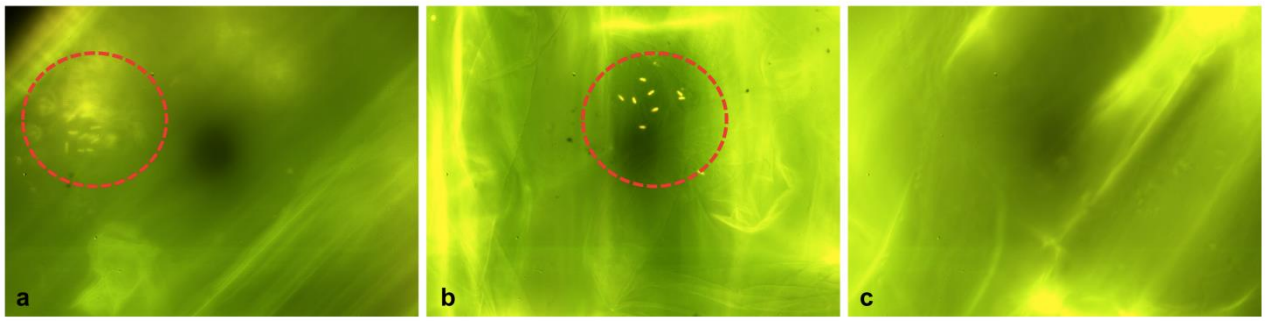


Figure 3.3 Colonization of tomato's radicles by *Kosakonia pseudosacchari* TL8 (a), *Kosakonia pseudosacchari* TL13 (b) and uninoculated control (c) detected byLIVE/DEAD BacLigh bacterial viability kit and observed under fluorescence microscope

3.3.4 Characterization of plant growth promotion and antimicrobial activities of *Streptomyces roseocinereus* MS1B15

Based on the results obtained by quantitative assay in liquid medium, the strain *S. roseocinereus* MS1B15 was selected for further characterization as other plant growth promotion activities and antimicrobial ability.

Quantitative analysis revealed that the strain *S. roseocinereus* MS1B15 was able to produce siderophores up to 14.09 ± 1.10 psu as well as to synthesize IAA in liquid medium with and without tryptophan (1.43 ± 0.02 and 6.34 ± 0.33 mg/L, respectively). The strain *S. roseocinereus* MS1B15 was also found positive to the ACC deaminase test, by growing on DF agar medium amended with ACC as the sole nitrogen source; whereas it resulted negative to *nif*H gene amplifications indicating that it was unable to fix nitrogen.

Interestingly, *S. roseocinereus* MS1B15 exerted antimicrobial activity against several tested soil-borne pathogens as *Fusarium oxysporum* F3, *Botrytis cinerea* B12, *Phytophthora cactorum* ph3, and *Phytophthora cryptogea* ph4.

3.3.5 Investigation and optimization of *K. pseudosacchari* TL13 growth conditions

On the basis of PGP traits, the strain *K. pseudosacchari* TL13 was selected for further investigations in order to produce an innovative bacterial inoculant.

The first step was to explore and define the best growth conditions of the strain *K. pseudosacchari* TL13. To this end, a kinetic growth curve of the strain TL13 was obtained by Microplate Reader test. This preliminary investigation showed that the exponential phase started after 4h of incubation and continued until 10 h, when begun the stationary phases (data not shown).

Batch experiments were then performed to investigate the effect of agitation and air sparging on the bacterial growth. The highest bacterial concentration in the shorter time was recorded in the batch 2 reaching a value of 8.87 ± 0.02 log CFU mL^{-1} after 8 h of incubation (Figure 3.4), after that, a significant decrease in its concentration was observed. Similarly, in the batch 1 was detected an increase of three orders of magnitude at 10 and 12 h (8.88 ± 0.00 log CFU mL^{-1} and 8.89 ± 0.00 log CFU mL^{-1}) in respect to the beginning of the experiment (0 h; 5.90 ± 0.04 log CFU mL^{-1}), decreasing up to 8.26 ± 0.15 log CFU mL^{-1} at 24 h (Figure 3.4). However, in both conditions, the *K. pseudosacchari* TL13 load was approximately one order of magnitude greater than that recovered in the control at the same sampling time (ranging from 5.89 ± 0.07 to 8.35 ± 0.03 log CFU mL^{-1}; Figure 3.4).

On the basis of these results, growth conditions of batch 2 (shaking at 130 rpm and air sparging at 0.5 vvm) were chosen to perform the scale-up of the experiment in a 10 L fermentor. In this condition, although at 8 h was detected a bacterial concentration (8.66 ± 0.02 log CFU mL^{-1}) similar to that recorded in the previous batch experiment, the exponential phase persisted up to 24 h reaching a bacterial load of 9.33 ± 0.18 log CFU mL^{-1} (Figure 3.5). Moreover, to explore the tolerance of the strain *K. pseudosacchari* TL13 to desiccation and to test its shelf-life, the viability

of freeze-dried bacterial cells obtained by fermentor experiment was estimated over time. Immediately after freeze-drying, a bacterial concentration of 10.43 ± 0.10 log CFU g^{-1} was determined. This value remained constant after 3 months of storage (10.40 ± 0.06 log CFU g^{-1}) and decrease of about 1 log after 6 months reaching a concentration of 9.57 ± 0.14 log CFU g^{-1}.

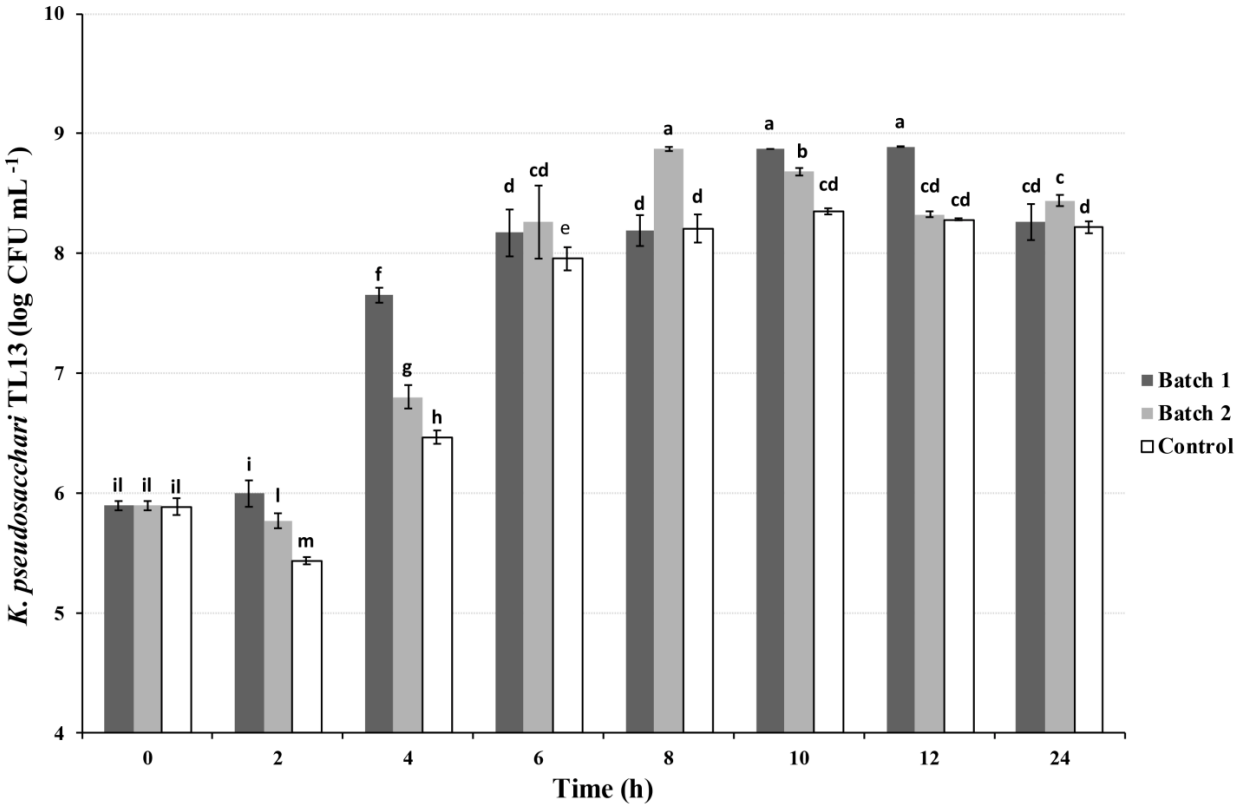


Figure 3.4 Viable count of *Kosakonia pseudosacchari* TL13 during its growth in batch experiments using BHI medium (30 °C and pH 7.00). Batch 1: shaking at 130 rpm; Batch 2: shaking at 130 rpm and air sparging at 0.5 vvm; Control: no shaking and no air sparging. The error bars represent the means ± SD of three replicates. Different letters indicate significant differences ($P < 0.05$).

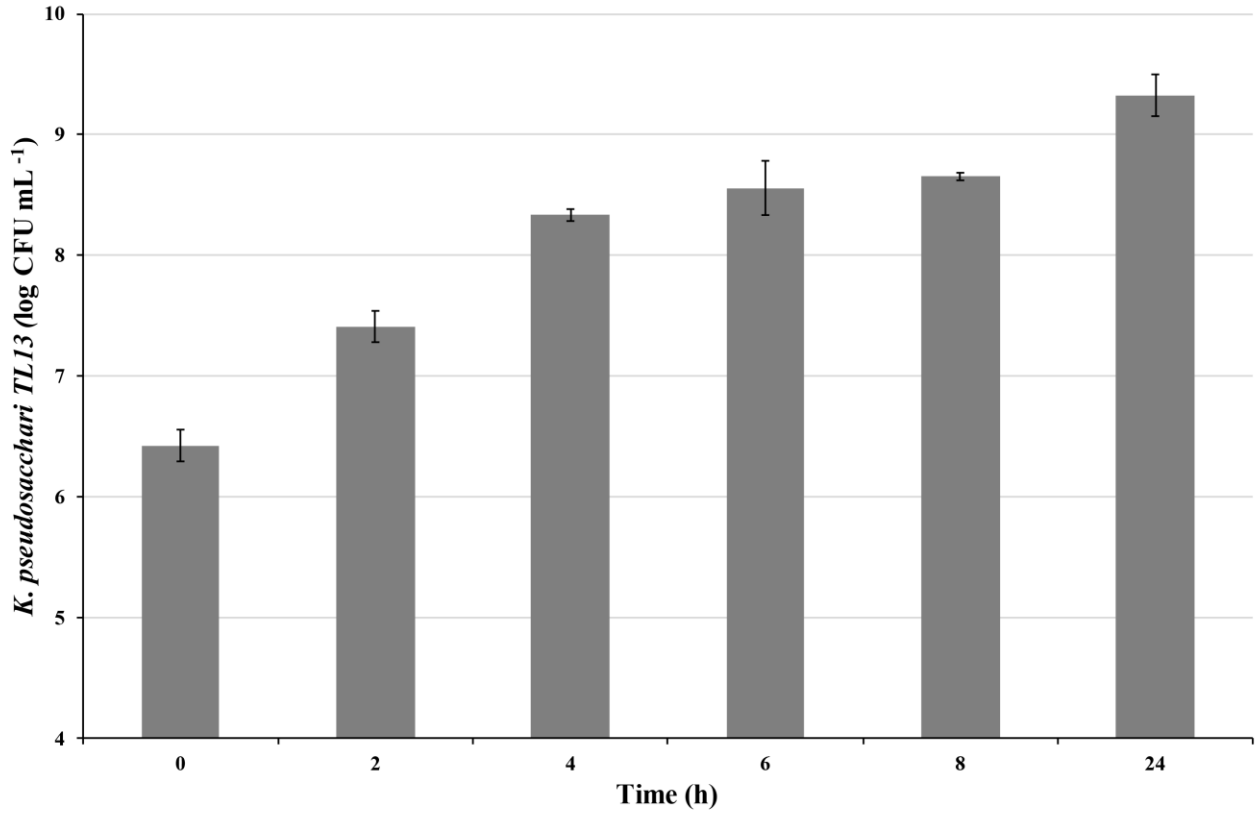


Figure 3.5 Viable count of *Kosakonia pseudosacchari* TL13 during its growth in 10 L fermentor at 30 °C, pH 7.00, shaking at 130 rpm and air sparging at 0.5 vvm. The error bars represent the means ± SD of two replicates.

3.3.6 Production of eco-friendly and low-cost bacterial inoculants with *Kosakonia* strain

Different agro-food industrial by-products were used to obtain an eco-sustainable and cheap carbon source for the growth at industrial level of *K. pseudosacchari* TL13 and its use as bioinoculant. The strain TL13 resulted able to grow in presence of several carbon sources (whey, protein hydrolysate, exhausted yeasts, or vinasse) at different concentrations (1, 5 and 10 %) reaching a bacterial load of about 8-9 log CFU mL^{-1} (Table 3.3). The only exception was the liquid medium containing molasse that determined the lowest bacterial growth at 5% (7.08 ± 0.18 log CFU mL^{-1}) and no growth at 1% and 10% (Table 3.3). The highest bacterial growth was detected in the medium containing 10% exhausted yeasts (8.86 ± 0.21 log CFU mL^{-1}), that was comparable to the optimal synthetic medium used as control (8.93 ± 0.01 log CFU mL^{-1}), followed by the liquid medium containing 5% vinasse (8.81 ± 0.07 log CFU mL^{-1}; Table 3.3). Therefore, SSF on nutrient-supplemented vermiculite of the strain *K. pseudosacchari* TL13 for the production of inoculant was performed using a solution of exhausted yeasts and vinasse. Microbial concentration increased after 15 days of incubation of about three orders of magnitude from 6.81±0.05 to 9.34±0.11 log CFU g^{-1}. No significant differences (P> 0.05) were detected between *K. pseudosacchari* TL13 grown on vermiculite moistened with exhausted yeasts and vinasse and the vermiculite moistened with BHI used as control (9.20±0.65 log CFU g^{-1}).

SSF products were used to develop solid and liquid inoculants containing a microbial load of about 6.7-6.9 log CFU g^{-1} or mL^{-1}, which remained constant up to 28 days.

Table 3.3 Viable counts of *Kosakonia pseudosacchari* TL13 (log CFU mL^{-1}) after 48 h of growth at 30 °C in several liquid media containg different agro-industrial by-products as carbon source at three percentage (1%, 5% and 10%).

Agro-industrial by-products	Percentage of by-products in the liquid medium		
	1%	5%	10%
Whey	8.18 ± 0.02^{e}	7.98 ± 0.07^{fg}	7.95 ± 0.03^{fg}
Protein hydrolysate	8.05 ± 0.01^{f}	8.30 ± 0.03^{d}	8.18 ± 0.07^{e}
Exhausted Yeast	8.30 ± 0.09^{d}	8.48 ± 0.08^{c}	8.86 ± 0.21^{ab}
Molasse	0.00 ± 0.00^{i}	7.08 ± 0.18^{h}	0.00 ± 0.00^{i}
Vinasse	7.94 ± 0.02^{g}	8.81 ± 0.07^{b}	7.98 ± 0.02^{fg}
BHI (control)	---	8.93 ± 0.01^{a}	---

Values represent the means ± SD of three replicates.
Different letters indicate significant differences ($P< 0.05$).

3.3.7 Response of *S. roseocinereus* MS1B15 to Solid State Fermentation process

Several conditions were tested to find an eco-sustainable and cheap strategy for industrial-scale production of *S. roseocinereus* MS1B15. The strain MS1B15 was not able to grow on vermiculite moistened with the SC broth, or on rice moistened with the IM for *Streptomyces* n.2 (Table 3.4). Bacterial persistance was observed in the tests rice and grain impregnated with the IM n.2 (3.34 ± 0.37 log CFU g^{-1} after 15 days at 30°C**;** table 3.4), and in the tests made using rice alone or in combination with grain moistened with whey as alternative and low-cost carbon source 3.64 ± 0.18 and 3.75 ± 0.64 log CFU g^{-1} respectively (table 3.4).

Table 3.4 Viable counts of *S. roseocinereus* MS1B15 (log CFU g^{-1}) after 15 days of growth at 30°C in several solid media moistened with different liquid media as carbon source.

Solid support	Impregnating agent	Viable Count 1 days	Viable Count 15 days
Vermiculite	SC broth	5.56±0.03	n.d.
Rice	IM n.2	5.56±0.03	n.d
Rice and grain	IM n.2	5.56±0.03	3.34±0.37
Rice	Whey	5.56±0.03	3.64±0.18
Rice and grain	Whey	5.56±0.03	3.75±0.64

n.d.: not detected

3.3.8 In vivo pot experiments

Maize plants were positively affected by inoculation with the strain *K. pseudosacchari* TL13. Indeed, several plant growth parameters significantly increased in the soils treated with solid or liquid bioinoculants as shown in Table 3.5. In particular, E-TL13 treatment (raw castor oil/alginate-based emulsion containing *K. pseudosacchari* TL13 cells) showed the best results, in which a significant increase ($P< 0.05$) of total plant length (63.83 ± 4.51 cm), root length (23.67 ± 2.57 cm) and root fresh weight (1.28 ± 0.11 g) was recorded in E-TL13 treated plants in respect to the un-

inoculated control (49.17 ± 3.40 cm, 11.06 ± 0.90 cm and 0.80 ± 0.13 g, respectively; Table 3.5). Similarly, a significant increase in the root length was also observed in the V-TL13 (dried vermiculite containing *K. pseudosacchari* TL13 cells) and R-TL13 (*K. pseudosacchari* TL13 cells diluted in sterile Ringer's solution) treatments reaching values of 18.50 ± 2.26 cm and 23.13 ± 1.99 cm, respectively (Table 3.5). Interestingly, V-TL13 treatment induced a significant increase of shoot dry weight percentage (9.62 ± 0.29%) compared to un-inoculated control (6.84 ± 0.40%; Table 3.5). However, also E-TL13 and R-TL13 treatments showed a similar trend of this plant parameter although no significant differences were detected (P>0.05; Table 3.5).

Table 3.5 Effect of different inoculant formulations on total plant length (cm), root length (cm), shoot length (cm), root fresh weight (g), shoot fresh weight (g), root dry weight (%), shoot dry weight (%) of maize plants.

Plant parameters	Soil treatment			
	V-TL13	E-TL13	R-TL13	C
Total plant length (cm)	51.50 ± 4.63^{ab}	63.83 ± 4.5^{a}	58.56 ± 4.78^{ab}	49.17 ± 3.40^{b}
Root length (cm)	18.50 ± 2.26^{ab}	23.67 ± 2.57^{a}	23.13 ± 1.99^{a}	11.06 ± 0.90^{b}
Shoot length (cm)	33.00 ± 2.87^{a}	40.17 ± 2.55^{a}	35.44 ± 3.68^{a}	38.11 ± 2.77^{a}
Root fresh weight (g)	1.01 ± 0.15^{ab}	1.28 ± 0.11^{a}	0.81 ± 0.15^{b}	0.80 ± 0.13^{b}
Shoot fresh weight (g)	1.19 ± 0.21^{a}	1.58 ± 0.14^{a}	1.54 ± 0.30^{a}	1.30 ± 0.27^{a}
Root dry weight (%)	15.30 ± 1.64^{a}	15.03 ± 1.52^{a}	19.73 ± 1.08^{a}	17.25 ± 2.42^{a}
Shoot dry weight (%)	9.62 ± 0.29^{a}	7.75 ± 0.64^{ab}	8.53 ± 0.83^{ab}	6.84 ± 0.40^{b}

V-TL13, soil inoculated with dried vermiculite containing *K. pseudosacchari* TL13;
E-TL13, soil inoculated with raw castor oil/alginate-based emulsion containing *K. pseudosacchari* TL13;
R-TL13, soil inoculated with *K. pseudosacchari* TL13 cells diluted in sterile Ringer's solution;
C, un-inoculated soil.
Values represent the means ± SD of three replicates.Different letters indicate significant differences (P< 0.05).

3.4 Discussions

3.4.1 PGP traits, phenotypic characteristics and rhizosphere competence of *Kosakonia* strains

In the last decades, the development and the use of microbial inoculants have elicited great interest as an ecofriendly alternative strategy to the application of synthetic fertilizers for plant growth promotion and pest management. This new approach improves the sustainability of agricultural systems by reducing environmental and human health risks due to the application of chemical fertilizers and pesticides in crop production (Rahman et al., 2018). In this context, it is necessary to find new microorganisms that can exert multiple plant beneficial activities to develop a low-cost bioinoculant. The ecological approach developed in this study enabled the isolation of new plant growth-promoting strains *K. pseudosacchari* TL8 and *K. pseudosacchari* TL13. This species belongs to the phylum *Proteobacteria*, and in particular to the γ-*proteobacteria* class. This bacterial class, that commonly colonize the rhizosphere of crop plants (Sheridan et al., 2017) or is associated

to plant biomass (Montella et al., 2017), is ubiquitous in the soil environment (Ventorino et al., 2019). Indeed, it includes different species that were known to synthesize substances which promote plant growth (i.e., hormones such as indole acetic acids, ethylene, and gibberellins), to increase nutrient availability (i.e., N, P, Fe) and their uptake in soil (Kim et al., 2011) and they act as plant disease-suppressive bacteria (Haas and Défago, 2005; Kobayashi et al., 2002). Therefore, the presence of these populations in the soil highlight its high biological fertility potential because they could improve the growth, fitness and health of agricultural plants playing an important role in the bionetwork function of soils (Ventorino et al., 2018). Although many members belonging to the genus *Kosakonia*, as *K. radicincitans*, are known to interact and exert beneficial effects on plant growth (Kämpfer et al., 2016; Bergottini et al., 2015; Berger et al., 2017;Brock et al., 2018), PGP properties in *K. pseudosacchari* species are poorly investigated. Indeed, it was recognized as a novel endophyte species only recently (Kämpfer et al., 2016) and siderophore production was the sole PGP activity previously documented (Arora and Verma 2017). The main PGP activity by the new PGPR strain *K. pseudosacchari* TL13 was the production of IAA. About 80% of rhizospheric microorganisms are able to produce and release auxins as a secondary metabolite, among these IAA is the most common that can contribute to plant-microbe interaction (Olanrewaju et al., 2017). It is an important growth enhancer because it plays a central role in cell division, elongation, fruit development and senescence, and it has a significant effect on plant root system development (Duca et al., 2014). The concentration of IAA produced by the strain TL13 is similar or higher to that recovered in *K. radicincitans* YD4 strain (about 24 μg mL^{-1}) by Bergottini et al. (2015). Interestingly, an increase of 50% of this phytohormone synthesis was observed in the strain grown in the presence of L-tryptophan suggesting a tryptophan-dependent IAA biosynthesis pathway. The synthesis and secretion of IAA could also be linked to the synthesis of ACC synthase in the plant to catalyze the formation of ACC (Glick, 2014). Synthesis of ACC deaminase is also one of the crucial bacterial traits that can facilitate plant growth in the presence of several abiotic or biotic stress (Ali et al., 2014; Glick, 2014). Indeed, *K. pseudosacchari* strains isolated in this work were able to produce ACC deaminase. As for IAA, this is the first work reporting ACC deaminase activity in *K. pseudosacchari* species.

Another interesting PGP activity is the production of siderophores. These are iron-chelating agents with low molecular masses (200–2000 Da), which are produced by microorganisms especially when the bioavailability of Fe is low (Ahmed and Holmström, 2014). Siderophore producing bacteria can improve plant growth by reducing the Fe availability for the phytopathogens and increasing nutrient availability to the plant (Ahmed and Holmström, 2014). As expected, the two *K. pseudosacchari* strains TL8 and TL13 were able to produce iron chelating siderophores, a trait commonly present in *Kosakonia* genus as largely reported by the literature (Arora and Verma, 2017; Chimwamurombe et al., 2016; Lambrese et al., 2018). Siderophore production could be involved also in disease suppression. Indeed, PGPR could act also as biocontrol agents against soil-borne plant pathogens by different ways like competing for nutrients or space, limiting available Fe supply through producing siderophores or by the production of lytic enzymes and antibiosis (Bhattacharyya and Jha, 2012). *K. pseudosacchari* TL8 and TL13 exerted antagonistic activity

against *Botrytis* and *Phytophthora* species. To the best of our knowledge, this is the first work reporting suppressive effect against plant pathogens in *K. pseudosacchari* species highlighting that these strains could use also for pest control in agricultural plants.

In addition, *K. pseudosacchari* TL8 and TL13 were also able to solubilize phosphate. Phosphorus is one of the major growth-limiting nutrients required by plants due to its limited availability. There is a great interest in searching phosphate solubilizing bacteria that are able to increase phosphate content and bioavailability in the soil and therefore they are considered promising bio-fertilizers for agriculture enhancement (Kalayu, 2019). Within genus *Kosakonia* this ability was previously reported only in the strain *Kosakonia* sp. A37 (Chakdaret al., 2018).

It is known that some PGPB can fix atmospheric nitrogen into ammonium, and consequently increase the availability of this nutrient in the rhizosphere. The use of these microorganisms in agriculture could decrease the use of chemical N-based fertilizers and therefore their negative impact on the environment as soil quality depletion, pollution and human health (Noar and Bruno-Bárcena, 2018). According to previous works in which several *Kosakonia* species were described as N_2-fixing bacteria (Chen et al., 2014; Chin et al., 2017; Sun et al., 2018), the new strains *K. pseudosacchari* TL8 and TL13 were able to produce ammonia and potentially able to fix atmospheric nitrogen due to the presence of the *nif*H gene encoding nitrogenase reductase enzyme.

K. pseudosacchari TL8 and *K. pseudosacchari* TL13 showed also interesting abiotic stress tolerance because they were able to grow in a wide range of temperature, pH and salt. These phenotypic properties could help the tolerance of crops cultivated in stress conditions. In particular, salinity is one of the most common abiotic stress in modern agriculture because the irrigation of summer crops with saline water, especially in the coastal regions, lead to an increase of soil salinization in many areas of the world causing major problems for the productivity of agricultural crops and reducing the soil microbial activity (Kumar and Verma, 2018).

Finally, according to Kämpfer et al. (2016) which describe this species as an endophyte of maize, *K. pseudosacchari* TL8 and TL13were able to colonize tomato radicles as observed *in vitro* assay under fluorescence microscope after treatment with BacLight bacterial viability kit. This ability was also described for other *Kosakonia* species as *K. radicincitans*, able to colonize the root surface of winter wheat (Witzel et al., 2017), or of cucumbers (Sun et al., 2018).

3.4.2 PGP traits of *Streptomyces* strains

The ecological approach developed in this study enabled the isolation of new PSB with multiple PGP activities. Out of 16 isolates from Moroccan oat rhizosphere, two strains belonging to the genus *Streptomyces* showed the best P-solubilizing activity on the solid assay as well as in liquid assay. P-solubilizing microorganisms are active in the conversion of insoluble P to soluble forms making it accessible to plants (Rajput et al., 2013). In this study, the maximum concentration of solubilized P ranged from 207.9 ± 3.3 mg/L to 245.6 ± 11.8 mg/L and therefore the presence of the *Streptomyces* strains MS1B15 and MS1B13 in the rhizosphere could improve plant P assimilation.

Although these values are lower than those reported by Jog et al. (2014) for *Streptomyces* sp. isolated from wheat plants (950–1916 mg/L), they exceed the level of the other bacterial genera commonly used as biostimulants, including *Bacillus* and *Pseudomonas* which solubilized 128.10 mg/L and 166.53 mg/L, respectively (Habil-Addas et al., 2017; Tiwari et al., 2018).

During the growth of the two PSB strains *S. roseocinereus* MS1B15 and *S. natalensis* MS1B13, the pH of the medium decreased as soluble P increased. This result confirm the observations of Jog et al. (2014) who reported a strong pH decreasing and soluble P increasing during the growth of the two strains *S. cellulosae* and *S. tricolor*. Wei et al. (2018) observed that PBS inoculation affected pH, total acidity and the production of several acids during composting of organic wastes supposing that the lower pH in PBS enriched compost than un-inoculated compost might be attributed to organic acids produced by microbial inoculum accompanied with the degradation of organic matter. Furthermore, Marra et al. (2011) established that there was a significantly negative linear correlation ($P < 0.05$) between culture pH and solubilized inorganic P. By observing the negative correlation between pH and soluble P ($r = -0.940$; $P < 0.05$), it could be inferred that the acidification of the medium could facilitate phosphate solubilization.

S. roseocinereus MS1B15 showed other interesting PGP activities as siderophore and IAA productions as well as ACC deaminase and antimicrobial activities. Siderophore compounds are potential plant growth promoters and disease suppressers against phyto-phatogenic bacterial or fungal strains. Khamna et al. (2009) suggested that *Streptomyces* sp. can produce hydroxymate-type siderophores, which inhibit the growth of phytopathogens by limiting iron in the rhizosphere. The strain *S. roseocinereus* MS1B15 exerted also antimicrobial activity against plant pathogenic fungi such as Fusarium, *Botrytis*, and *Phytophthora*. These results concur with other studies which have shown that several *Streptomyces* strains play a key role in protecting plants against several soil borne plant pathogens reporting them as biocontrol agents (Errakhi et al., 2007; Joo, 2005).

Previous research has documented that *Streptomyces* genus is also able to synthesize IAA. It is an important phyto-hormones responsible for improving plant growth by helping it to uptake a large volume of nutrients, absorption of water, increasing seed germination, and root elongation (El-Tarabily, 2008). According to Abd-Alla et al. (2013) that reported the ability of *Streptomyces* sp. isolated from wheat, corn and faba bean to produce IAA in a range from 3.55 µg/mL to 22.56 µg/mL, the strain *S. roseocinereus* MS1B15 selected in this work exhibited a IAA production of 6.34±0.33 µg/mL. As other bacterial genera living in soil including *Pseudomonas* (Hall et al., 1996), *Enterobacter* (Li et al., 2000), and *Bacillus* (Ghosh et al., 2003) able to produce ACC, also the strain *S. roseocinereus* MS1B15 was able to grow on DF agar medium amended with ACC as the sole nitrogen source. This is an interesting ability because ACC deaminase-producing bacteria have been known to promote plant growth by reducing the level of ethylene in the root of developing plants thereby increasing the root length and growth (Husen et al., 2011).

Although some *Streptomyces* isolates are able to fix atmospheric nitrogen (Sellstedt and Richau, 2013) having nitrogen-fixing genes (Dahal et al., 2017), the strain *S. roseocinereus* MS1B15 didn’t

show positive amplification of *nif*H genes. This could be attributed to a difference in the genes sequences which didn't allow the primers annealing (Gaby and Buckley, 2012).

3.4.3 Production of a low-cost and eco-sustainable bacterial inoculants and their effectiveness in inoculated plants

Based on PGP traits, the strain *K. pseudosacchari* TL13 was selected for the production of a new low-cost and eco-sustainable bacterial inoculant. In order to develop new bacterial inoculants and to ensure the application of a suitable number of viable and active microbial cells, high biomass production, formulation and shelf life are crucial steps (Bashan et al., 2014). Preliminary investigations in synthetic medium allowed us to assess the growth curve and the best growth condition and parameters to increase bacterial biomass and to obtain a suitable microbial concentration of the strain *K. pseudosacchari* TL13. Besides, microbial cells of TL13 were also subjected to freeze-drying, a common method for preserving bacteria, in order to evaluate their shelf life over time. Although freeze-dried *K. pseudosacchari* TL13 remained viable up to six months, this approach could not be suitable at industrial level for its higher production costs than others as foam drying (Morgan et al., 2006). Indeed, production costs of a bio-formulate, which include raw material, equipment and staff, must be competitive in relation to that for the production of chemical fertilizers (Lobo et al., 2019). In general, the use of a low-cost culture medium for the growth and production of microbial biomass is an important issue (Liu et al., 2014; Xu et al., 2015). In this work, to reduce the costs and to develop an eco-sustainable bioinoculant, the use of several agro-industrial by-products as carbon source was evaluated for the production of *K. pseudosacchari* TL13 by SSF on vermiculite. Indeed, valorization of organic waste biomass and by-products derived from agriculture and food processing factories by a sustainable and harmless disposal have generated interest in microbial biotechnologies (Pagliano et al., 2019). This new approach to by-products management is eco-friendly, easy to be conducted and economically advantageous. Interestingly, *K. pseudosacchari* TL13 was able to use different organic by-products as carbon source although the highest bacterial growth was observed in liquid media containing exhausted yeasts or vinasse. This approach allowed to obtain a suitable bacterial concentration (10^6 CFU mL^{-1}or g^{-1}) in the two final, solid (vermiculite-based) or liquid (raw castor oil/alginate-based emulsion), bio-formulations. The development of two kinds of formulations was important to evaluate their different advantages. Indeed, liquid emulsion formulation allowed to protect the bio-inoculant from desiccation as well as from osmotic and oxidative stress (John et al., 2010); whereas, solid vermiculite-based inoculants were very stable, require no special storage and has good seed-sticking properties (Graham-Weiss et al., 1987). Although, both *K. pseudosacchari* TL13 formulations exerted positive effects on maize plants cultivated in unsterilized soil, the liquid raw castor oil/alginate-based emulsion showed the best results increasing several plant parameters. Liquid formulations are often preferred by users because the product is ready to use. However, the stable and low-cost solid vermiculite-based formulation could be used in agricultural crops for increasing dry matter. These results are in accord with previous works in which inoculum of *Kosakonia* sp. strains were able to exert positive effects in various crops as radish (Berger et al., 2015), yerba mate (Bergottini et al., 2015), tomato (Berger et al., 2017) and maize (Berger et al., 2018).

Preliminary investigations on response of *S. roseocinereus* MS1B15 to SSF allowed us to establish a starting point to define conditions for a low-cost and eco-sustainable production process. It is known that a successful microbial-based formulation must respect various requirements: a high concentration of vital cells and a long shelf-life (at least six months). For these reasons, research for appropriate growth conditions and formulations are of major importance (Backer et al., 2018). Growth condition and parameters for *S. roseocinereus* MS1B15 must be deepen investigated to increase bacterial biomass and to obtain a suitable microbial concentration to develop a marketable product.

Further investigations are necessary to evaluate the impact of these innovative bio-inoculants on the rhizosphere microbial community. In fact, the use of these inoculums may correspond to the introduction in a natural system of new microbes, which may alter the community structure and ecosystem functioning of different habitats (Litchman, 2010). On the other hand, it is necessary to study the effect of microbial inoculation to evaluate if the selected strains can efficiently colonize and persist in soil habitats or if they succeed in competing with native microorganisms. As previously suggested (Romano et al., 2020), the application and combination of multiple approaches, e.g., fluorescence microscopy or amplicon/shotgun sequencing, could allow the respond to these questions.

3.5 Conclusions

The *K. pseudosacchari* strains isolated in this study showed multiple PGP traits as well as antimicrobial activity against several soilborne plant pathogens. In particular, the new selected strain *K. pseudosacchari* TL13 was able to colonize plant roots and improve plant growth. To our knowledge, this is the first work reporting effective multiple PGP abilities and antimicrobial activity in *K. pseudosacchari* species. Moreover, the ability of *K. pseudosacchari* TL13 to efficiently use agro-industrial organic by-products as carbon source for its metabolism makes this strain a promising candidate for the development of new biofertilizers for sustainable agriculture.

The ecological approach used in this study allowed to isolate and select the new phosphate-solubilizing strain *S. roseocinereus* MS1B15.To the best of our knowledge this is the first study reporting the ability of the *S. roseocinereus* species to solubilize phosphate. Moreover, the new selected strain *S. roseocinereus* MS1B15 showed multiple plant growth promoting activities and antimicrobial activity against several soilborne plant pathogens as well as was able to improve plant growth. Therefore, further investigations are needed to develop an effective technology for industrial massive production of this strain.

4 Effect of pre-crops on potatoes associated microbiome cultivated under varying water and nitrogen availability

This chapter reports the experimental activities performed at FiBL - Research Institute of Organic Agriculture (Switzerland).

- Supervisors: *Sarah Symanczik* ph.D. and *Natacha Bodenhausen* ph.D.

4.1 Introduction

The future of agriculture production is dominated by the idea that food productions have to be doubled by 2050. It is also expected that human population reach 8 billion in 2025. To avoid or minimize food shortage, soils have to be managed to increase agricultural production of approximately 25%–70% above current production levels to meet worldwide demands for food in 2050 (Hunter et al., 2017). However, arable land will only increase by five percent by 2050, and today 25 % of arable land is already significantly degraded (Shiferaw et al., 2011). Both increasing food demands and diminishing arable land call for strategies to intensify agricultural systems without harming the environment (Ladeiro, 2012; Berger et al., 2018).

4.1.1 Soil management

Conventional agriculture embraces the philosophy of industrial production, emphasizing efficient productions with low financial budgets, dependence on external inputs for fertility and pest management, simplified monocultures, and economies of scale and specialization evident on large farms (Shennan et al., 2017). Intensive soil management causes the loss of biodiversity in European agricultural land due to the reduction of some important key soil functions (Tsiafouli et al., 2015). This problem stressed the need to develop knowledge about innovative practices that enhance soil biodiversity and function.

As reaction to the environmental damage caused using chemical pesticides and synthetic fertilizers in conventional agriculture, modern organic farming has been developed. Organic farming is characterized by ecologically based pest controls and biological fertilizers, it emphasizes techniques such as crop rotation and companion planting (Shennan et al., 2017). This approach has several ecological advantages: by adopting sustainable practices, farmers will reduce chemical use and save scarce resources.

Bakker et al. (2012) suggested two interesting strategies for sustainable agriculture based on promotion of plant-microbe interactions i) the direct manipulation of microbial communities by applying microbial inoculants, or ii) their indirect manipulation via agro-ecosystem management practices and/or selection/combination with plant genotypes (Bakker et al., 2012).

Sustainable management practices are promising for the development of low input agro-ecosystems, through the improvement of naturally occurring biotic interactions both among and within species, and providing essential nutrients (Bilsborrow et al., 2013). These agro-ecological innovations may be used to promote biological processes within agro-ecosystems and maximize the delivery of key ecosystem services (Doré et al., 2011; Duru et al., 2015), including nutrient cycling (Wagg et al., 2014) and disease suppression (Garbeva et al., 2004).

4.1.2 Overview of issues related to water stress and reduced nutrient

Abiotic stresses are among the most important constraints for global agricultural productions, losses depending from these conditions are estimated at 70% worldwide (Etesami and Beattie, 2017). The

pressure to increase agricultural productions expand cultivation on marginal lands and accelerated the rate of soil degradation. Irrigation, for example, led to salinity across large areas of agricultural land.

Drought is one of the most significant environmental stress. Moreover, it strongly impacts global agricultural production (Kijne, 2006; Cattivelli et al., 2008; Berger et al., 2015). Considering that approximately, 60% of all crops produced in developing countries are grown without irrigation (FAO 2009b), it is clear that the majority of crops are vulnerable to drought. Crop productions accounts for approximately 70% of global water use, and irrigation contribute up to 90% of total water withdrawals in arid countries (Council 2008; FAO 2009a). An increase of 14% of water use requirement for irrigation is expected in developing countries by 2030, further increase of 10% for every 1 °C additional in temperature in arid and semiarid regions (Grover et al., 2011); these data show how strategies to decrease water requirement for agricultural practices are important.

Furthermore, the intense use of synthetic fertilizers incurred environmental costs in the form of nitrate contamination of groundwater, greenhouse gas production associated with industrial nitrogen fixation (Etesami and Beattie, 2017). Both nitrogen and phosphorus losses in soil surface run off from fertilized land depend upon the rate of transporting water, and the time and quantity of fertilizer applied. In fact, fertilizer applications before a wet season or snowmelt, or on frozen soil, increase losses if compared with fertilization made in the springtime. Uncontrolled application of fertilizers and bad management practices increase nutrient loss from the soil. Lesser amounts of nitrogen and phosphorus are lost to the surface water if soils are shrewdly fertilized and well-managed (Khan and Mohammad, 2014).

4.1.3 Features of crop rotation

Annual crop sequences can produce progressive loss of soil fertility due to reduction in soil organic matter (soil fertility and organic matter content are strongly correlated) both by leaving scanty residues on (litter) and beneath (roots) the soil surface and by requiring tillage for seed bed preparation (Caporali and Onnis, 1992). One possible solution is the crop rotation or sequential cropping compasses growing two or more crop species on the same land in sequence but not concomitantly (Liebman and Dyck, 1993). Crop rotation can reduce agriculture's dependence on external inputs through internal nutrient recycling, maintenance of the long-term productivity of the land, and breaking weed and disease cycles (Gebremedhin and Schwab, 1998). The key element for a successful crop rotation is the alternance of a polyannual forage legume with annual cash crops (winter and summer cereals, sunflower, sugar beet, etc.), in fact, the legumes can restore high fertility levels, after depletion induced by other crops. Crop rotation systems positively impact on soil quality and fertility, environmental quality, and farm profitability. Wheat and potato grown in rotation with legumes tends to outperform continuous wheat/potato both in terms of yield, profitability and income risk (Gebremedhin and Schwab, 1998). By contrast, comparing profitability performance of continuous corn vs. corn grown in rotation, results did not show significative differences (Gebremedhin and Schwab, 1998). Clearly, applying crop rotations is

necessary to also consider the environmental benefits/costs both on and off the farm site that accrue to society.

4.1.4 Fungal communities in agro-ecosystems

Soil microorganisms constitute less than 0.5% (w/w) of the soil mass, but play a key role in its properties and processes (Yan et al., 2015). Soil biota, in particular fungi and bacteria, play a major role in soil quality and functioning, largely determining its structure and nutrient cycling, as well as pest and disease regulation (Barrios, 2007; Lori et al., 2017), ultimately impacting plant performance through nutrient mobilization, root growth, and plant health, and enhancing crop yields under stressful conditions (Naveed et al., 2014). Improvement of these functions are fundamental to achieve food security in the coming decades. Fungi, in particular, are the dominant eukaryotic lineage in terms of biomass in soil, where they play key roles as decomposers, pathogens, and mycorrhizal mutualists (Orgiazzi et al., 2012). One of the most widely known fungal role in soil is the decomposition and mineralization of complex compounds of plant and animal origin, such as cellulose, hemicellulose, lignin, and chitin. Other important functions of soil fungi include their participation in beneficial symbioses with plant roots. Mutually beneficial mycorrhizae allow plants to resist against several stress factors like nutrient and water limitations. In addition, fungi play a key role in controlling soil structure and water content and regulating above-ground biodiversity (Orgiazzi et al., 2012).

4.1.5 Soil habitats

There are specific terms to define the different areas from the inside of the roots to the outside, the “endosphere” indicates the internal root area, the “rhizosphere” is the external soil area influenced by plant roots and their exudates, while the “bulk soil” is the soil area not adhering to roots and not influenced by exudates (Barillot et al., 2013).

The term “habitat” indicates a specific space inhabited by a community of organisms for growth and reproduction. Thus, plant organs or root surrounding areas colonized by microbial communities with a distinctive phylogenetic structure represent different habitats (Bulgarelli et al., 2013).

Cultivation-independent surveys of the bacterial rhizosphere and endosphere communities carried on different plant species grown in different soils, indicates that these two close ecological habitats are formed by a soil biome community shifts that give rise to a distinctive phylogenetic structure with a few dominating phyla (Bulgarelli et al., 2013).

The understanding of the complex and dynamic root/soil/microbial interactions has increased significantly over the past few decades (Hinsinger et al., 2011; White et al., 2014), notably through molecular ecology approaches, which have considerably expanded the scientific knowledge of soil microbial communities (Philippot et al., 2013). Harnessing the rhizosphere microbiome through plant breeding (Peiffer et al., 2013) and agroecosystem management is in its infancy, in spite of being highly promising for the sustainable intensification of agricultural systems (Bakker et al., 2012), and the development of a biodiversity-based agriculture (Duru et al., 2015).

The rhizodeposition is a potential molecular mechanism explaining the formation of a distinctive rhizosphere microbiota from soil biomes. This process is related with intertwined of plant developmental and secretory activities in the root system. Rhizodermis cells secrete a wide range of compounds (organic acid ions, inorganic ions, sugars, vitamins, amino acids, purines, and nucleosides) and the root cap produces polysaccharide mucilage (Bulgarelli et al., 2013).

In addition, composition, and functions of the rhizosphere microbiome, and of endophytic microbiome, can vary with genotypic differences in plant traits. Specific plant genotypes were reported to promote beneficial microbiomes, which supports the hypothesis that there is a degree in specification in the interaction between crop species and microbial community. Therefore, when selecting a specific plant varieties, it is important to consider the association with beneficial microorganisms (Hardoim et al., 2015; Peiffer et al., 2013; Philippot et al., 2013; Quambusch et al., 2014).

4.1.6 Methodology to evaluate microbial activities and compositions

Enzymatic activity represents the most used early-response factor to evaluate soil quality. Protease and phosphatase potential microbial activities are often evaluated by enzymatic essays. They are also indirect indices of soil status, and several studies have demonstrated a strong seasonal and crop-dependence variability (Panettieri et al., 2014).

To quantify DNA, the real-time polymerase chain reaction (qPCR) is a well-established approach. The method – like the basic PCR – is based on the amplification of nucleic acids but fluorescent labeling allows the quantification of the amplified DNA. Thus, the amount of amplified nucleic acids can be quantified after every amplification cycle in real-time. The typical target regions to quantify bacteria and fungi abundance are the 16S and 18S genes, (two highly conserved genes) in ribosomal DNA (rDNA) Similarly, the abundance of functional genes can be quantified to make assumptions about the potential activity of certain enzymes. Proteolysis, for instance, is a process that strongly affect the supply of plant-available N for crop growth and thus indigenous extracellular protease encoding microbial communities play an important role in regulating proteolysis and subsequent N transformations. Figure 4.1 shows enzymes and functional genes involved in the nitrogen (N) mineralization process. Organic nitrogen (N_{org}) is mineralized via different steps into mineral nitrogen (N_{min}). Functional genes involved in this process are alkaline metallopeptidase (*apr*), neutral metallopeptidase (*npr*), urease (*ure*C), bacterial ammonia-oxidase (*amo*AOB), and archaeal ammonia-oxidase (*amo*AOA). Quantification of these genes and interpretation of the results could allow to make hypotheses about nitrogen mineralization.

In recent decades, the development of massive DNA sequencing technology, known as next generation sequencing (NGS), and bioinformatic tools provided a powerful alternative to other molecular studies of microbial ecology in natural environments, enabling the study of taxonomic diversity at a high resolution. Microbiome analysis can be roughly divided into two approaches as showed: targeted amplicon-based approaches analyze the same gene of different organism with the goal to investigate microbial diversity and composition, whereas shotgun approaches analyze

different genes from different organisms to analyze for example diversity and metabolic potential (Zhou et al., 2015). For the targeted approach it is important to identify an optimal gene region. For analyzing fungal communities the internal transcribed spacer (ITS) is a commonly used region, which is situated between the ribosomal small and large subunit genes (O'Donnell, 1992). Therefore, the ITS region is well suited to study fungal communities.

Two approaches are suitable for analyzing diversity in community data the Alpha and Beta diversity. Alpha diversity analyses the diversity within one sample. It can be expressed in richness or number of species, which counts all species present in one sample. However, this value is depending on the size of the sample. Another index describing Alpha diversity is Evenness. A sample with a few species very abundant and all other species rare would be called uneven, another sample where all species have the same abundance would be called even. But this index is dependent on richness, which is why it is biased. An often used index for alpha diversity is the Shannon diversity, which takes into account both evenness of distribution of species and total amount of different species (Borcard et al., 2018). Beta diversity expresses the difference between samples. The most popular index used in ecology is the Bray-Curtis dissimilarity, which quantifies the compositional dissimilarity between two different sites, based on counts at each site.

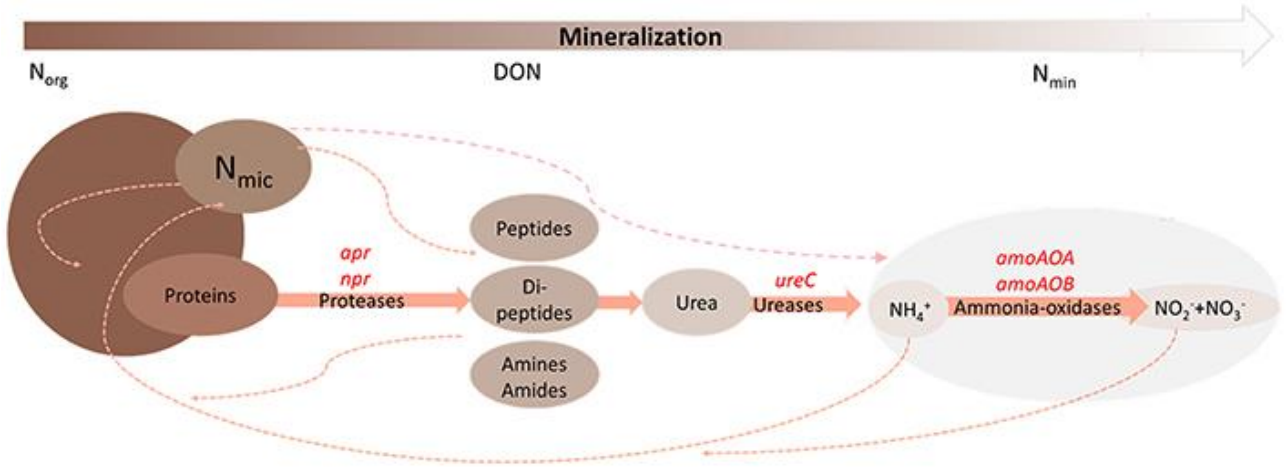


Figure 4.1 Simplified scheme of soil microbial mediated nitrogen mineralization from Lori et al., (2018). Organic nitrogen (N_{org}) is mineralized into mineral nitrogen (N_{min}) via different steps and enzymes. Functional genes encoding for the respective enzymes are highlighted in red and abbreviated as alkaline metallopeptidase (*apr*), neutral metallopeptidase (*npr*), urease (*ure*C), bacterial ammonia-oxidase (*amo*AOB), archaeal ammonia-oxidase (*amo*AOA). Nitrogen losses like leaching or N_2O production are not considered in this scheme. Microbial bound nitrogen (Nmic) dissolved organic nitrogen (DON) and mineral nitrogen (Nmin = NO−2NO^{2-} + NO−3NO^{3-} + NH+4NH^{4+}) represent the labile N pool (N_{labile}).

4.1.7 Aims of the study

This study aims to increase our knowledge of how root and rhizosphere microbiome functioning may enhance potato growth. Potato (*Solanum tuberosum* L.) is one of the most widely grown vegetables in the world, ranking as the third most important food crop (International Potato Centre-

2017), it can be cultivated in many ways (e.g., systems based on high versus low inputs, in the context of conventional farming).

Combining different agro-ecological approaches it is possible to promote biological processes within agro-ecosystems and maximize the delivery of key ecosystem services (Doré et al., 2011; Duru et al., 2015), including nutrient cycling (Wagg et al., 2014) and disease suppression (Garbeva et al., 2004).

In this study, we assessed differences in microbial activity, fungal community diversity and composition of the bulk soil, rhizosphere and roots of potato grown under different fertilization treatments. The crops were grown in rotation with rye and soja under adequate and reduced irrigation and nitrogen fertilization.

Crop physiology and genetics, combined with abiotic stresses, may improve the water and nutrient use efficiency by microbial community. Genotypic differences may vary the rhizospheric and of endophytic microbiome, thus impacting plant performance through nutrient mobilization, root growth, plant health, and possibly increasing crop yield under stress conditions.

Crop rotations with grain legumes will be tested regarding their potential to improve resilience against water and nitrogen limitations and variable conditions regarding this deficit. This strategy was chosen considering that legume crop rotation can reduce the use of chemical fertilizers through internal nutrient recycling, and it can restore high fertility levels, after depletion due by other cultivations.

4.2 Material and methods

4.2.1 Site description and experimental set up

In 2019, a two-year Crop Rotation Experiment was established in Conthey (VS), a representative European pedoclimatic region in the southern part of Switzerland (46° 13′ N 7°18′ E, 504 m above sea level). The average annual precipitation is 753 mm and the mean annual temperature is 9.8°C (climate-data.org).

The previous crop rye and soya were cultivated, and after their harvest, potatoes were planted on all the plots to evaluate rotation effect and its potential to improve resilience against combined water (W) and nitrogen (N) limitations. Irrigation was applied to compare the performances of potatoes growth in optimal conditions (adequate irrigation, and 120 kg N ha^{-1}) with that exposed to combined water and nitrogen limitations (reduced irrigation, and. 0 kg N ha^{-1}).

The trial was performed with a Nested randomized design, four different potato genotypes were tested “Cara, Pentland Dell, Agria and Charlotte”, schematic representations of the field design are shown in figure 4.2 a - b. Each experimental block had parallel plots of 6 m^2 with 30 plants per row

(192 plots in total). Seed tuber pieces were planted at beginning of April, at 10 cm of depth. Two border rows were used to surround the trials.

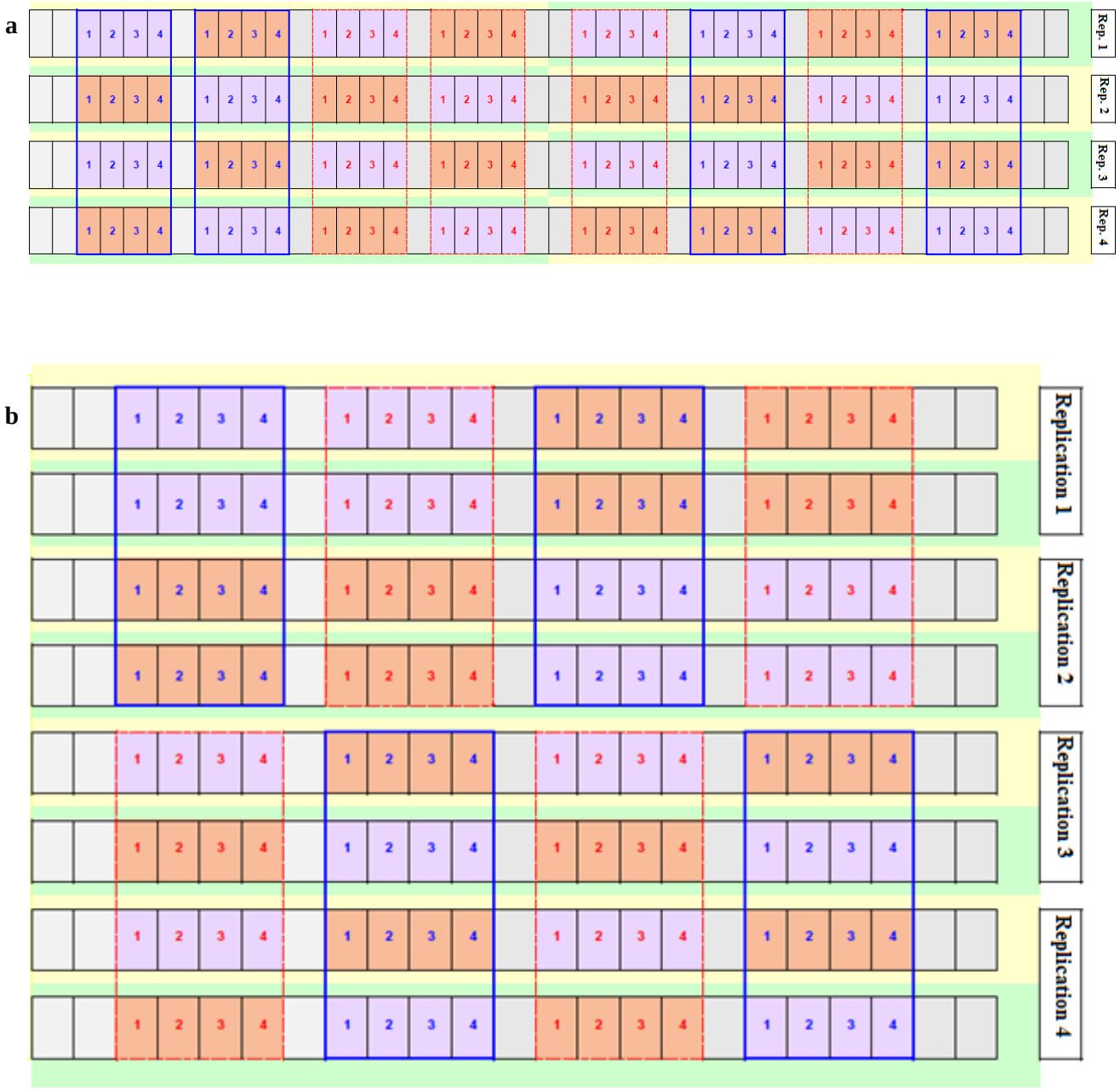


Figure 4.2 Field plan of the long-term trial in Conthey (VS), Switzerland a) in 2019 and b) in 2020. The numbers from 1 to 4 indicate the different genotypes (1: Cara, 2: Pentland Dell, 3: Agria, 4: Charlotte); red border indicates the reduced water regimes, blue border indicate the optimal water regime; the light-yellow background indicates rye as pre crop, whereas the light green background indicates soybean. The blue colored boxes represent the plots cultivated without nitrogen limitations, while the light red colored boxes represent the areas under nitrogen limitations.

4.2.2 Sampling

In both 2019-2020 years, the vegetative stage chosen for the sampling of potatoes was "tuber initiation" (diameter of mini-tubers 0,5 – 2,0 cm). From each plot, one plant was harvested yielding a total of four replicates per treatment to provide high representatively of each plot and to avoid edge effect, plants from the upper and lower end of the rows were not sampled. Potato roots and rhizosphere were sampled from 1 to 30 cm of depth. Each root with attached soil as well as bulk soil was collected into a 50 ml Falcon tube, additional rhizospheric soil was collected in a sterile polyethylene plastic core bag. These samples were then stored in a cooling box until the end of each sampling day (Romano et al., 2020). Bulk soil was sampled as far as possible from genotype affected sites to avoid the influence of root exudates, from each plot 4 – 5 samples were taken by using a soil borer/auger, then they were mixed to get a composite sample. A sub-sample was transferred in a sterile 50 ml Falcon tube for later analysis. The whole plant was removed including the root system by using a potato fork. The root system was placed into a sterile plastic bag and gently shaken to collect approximately 100 g of attached rhizospheric soil. Only horizontal roots (Figure 4.3 a - c) were sampled and placed into a sterile plastic bag. All the samples were frozen before further investigations.

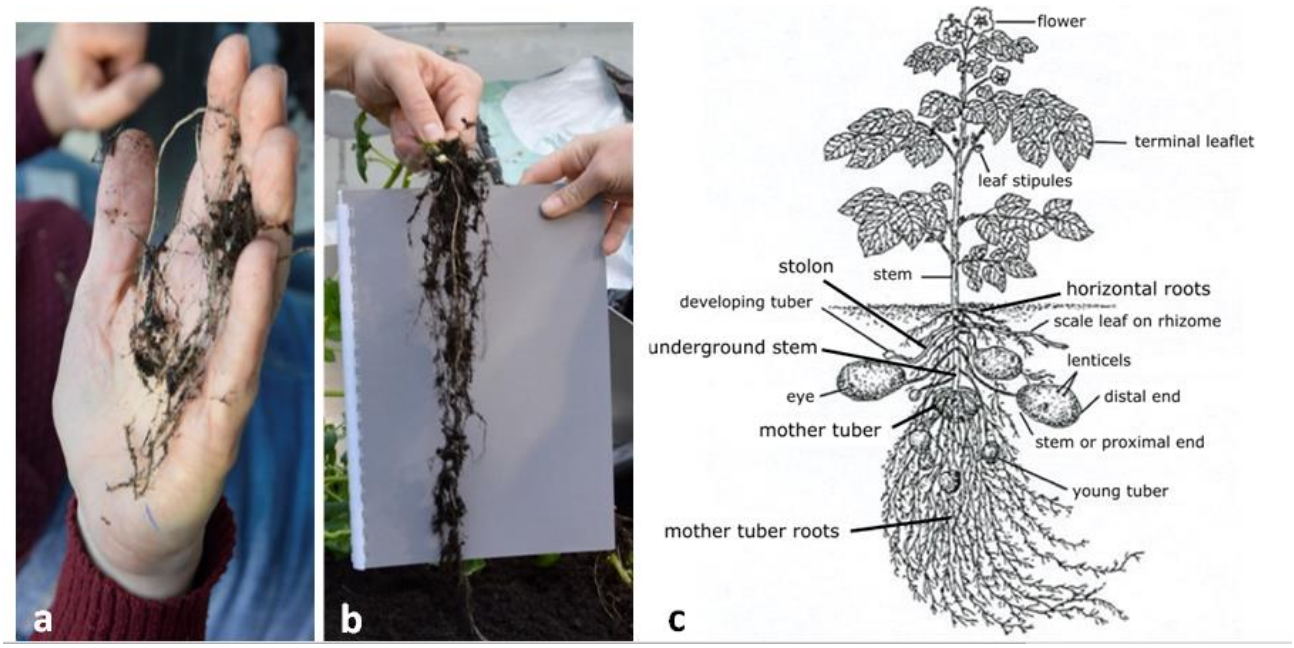


Figure 4.3 Pictures of a) one potato root, b) the root system of a young pot grown potato plant, c) the anatomy of a potato plant useful for sampling.

4.2.3 Enzymatic essay

4.2.3.1 Protease activity

The casein-protease (PRO) activity was estimated applying the protocols described by Schinner (1991), and Ladd and Butler (1972) adapted for 96 well approach. Briefly, 5 g of fresh frozen soil

was added with 10 ml of Tris-Buffer and shacked by vortex. Then 350 µl of the soil slurry were transferred in four technical replicates (three samples and one control) into a 96 deep well plate, and the three samples were added with 250 µl of the casein substrate. Plates were incubated at 50 °C (130 rpm, 180 minutes) before centrifugation at 5000 x g (5 min, 10°C). For each soil sample, triplicate standard curves were prepared by mixing adequate volumes of Tris-buffer with different concentrations of Tyrosine stock solution (from 0 to 300 µl), with 500 µl of Casein stock solution and 500 µl of TCA (Trichloroacetic acid solution 0.92 M) in 96 microplates. After the incubation, the deep-well plate was taken out of the incubator and put immediately on ice to stop enzyme activity. After centrifugation (5000 x g, 5 min, 10°C), 250 µl of supernatant was transferred to a clean deep well plate and of 250 µl TCA was added, at this point the controls were supplemented with250 µl of the casein substrate before centrifugation (5000 x g, 5 min, 10 °C). Avoiding touching the pellet 50 µl of the supernatant was transferred into a 96-well microplate then added with 79 µl of Alkali reagent and 50 µl of the Folin reagent. After 10 minutes of incubation at room temperature, tyrosine concentration was measured photometrically at 680nm in a plate reader.

The measure of protease activity was calculated in amount of Tyrosine equivalents, which are released from Sodium-Casein during the incubation time (here 180 min) in the samples. The measurement was calculated according to the following formula:

$$[(\text{Mean TE in sample} - \text{Mean TE in control}) * 100] / \% \text{ TS} = \text{TE} / (\text{g TS} * 3\text{h})$$

Where TE is Tyrosine equivalents in µg, and TS is the amount of Tyrosine in micromoles in the standard series.

4.2.3.2 Phosphatase activity

Acid phosphomonoesterase activity was determined applying the protocols described by Margesin (1993) and Tabatabai and Bremner (1969) adapted to 96 deep-wells microplates. Briefly, 5 g of soil was mixed with10 ml of Modified universal buffer (MUB - pH 6.5 or 11). The soil slurry (200 µL) was added four times appropriate wells of a 96-deep-well plate (3 full samples and one blank), then 50 µl of p-Nitrophenyl Phosphate (pNPP) solution (6.5 pH) was add to the 3 full samples. Plates were incubated into the water bath at 37°C for 60 minutes. After incubation, 50 µl of $CaCL_2$ (0.5 M), 200 µl of NaOH (0.5 M) and 500 µl of demineralized water were directly added to all wells. Then 50 µl of pNPP (pH 6.5) was added to all blank samples

before centrifugation at 5000 x g (5 min). The supernatant was transferred and diluted (10 µl supernatant + 190 µl demineralized water) into microplates, and finally the absorbance was measured at 405 nm.

The standard curve was prepared in triplicate by mixing different volumes of nitrophenol standard solution (0 – 22 % of nitrophenol solution), with 10 µl of $CaCl_2$ and 40 µl of NaOH adjusted with H_2O up to a final volume of 1000 µl.

Final p-Nitrophenol (µg/g/h) concentration was calculated according to the following formula:

$$(C \times v)/ (EW \times t) – (C\ Bl \times v) / (EWBl \times t) = \text{p-Nitrophenol (µg/g/h)}$$

Where: C represent µg/ml nitrophenol in filtrate, C Bl is µg/ml nitrophenol in the blank sample, V is the Volume of the suspension, EW is the initial weight (related to dry matter), EWBl is the Weight of the blank sample (related to dry matter), and t represent the incubation time (1h).

4.2.4 DNA Extraction

DNA from bulk soil, rhizosphere and roots were extracted from 250 mg of bulk soil and rhizosphere, respectively and 50 mg of roots with the DNeasy PowerSoil HTP 96 Kit (Qiagen, Hilden, Germany) following supplier's instructions. The extracted DNA was used for both qPCRs and Illumina sequencing.

4.2.5 Q PCR

DNA concentrations were measured using a Qubit Fluorometer (Thermo Fisher Scientific, Waltham, USA).

Abundance of 16S ribosomal RNA, 18S ribosomal RNA, and of selected microbial genes linked with N mineralization activity was assessed by quantitative polymerase chain reaction (qPCR) using degenerated oligonucleotides (Lori et al., 2018). The selected genes were alkaline metallopeptidase (*apr*), urease subunit alpha (*ure*C), ammonia monooxygenase of Archea (*amo*AOA) and of Bacteria (*amo*AOB) genes. The 16S primers (Bact-0341_F, Bact-0515_R) were designed by Muyzer et al., (1993). The 18S primers (FF390, FR1) were designed by Vainio and Hantula (1999). For *apr*, FR aprI and RP aprtII primers were used, which were described by Bach et al., (2001). *amo*AOA was analyzed with amo19F and crenamo primer, which were described by Leininger et al., (2006) and Schauss et al., (2009), respectively. Primers quantifying *amo*AOB (amoA1F, amoA2R) were described by Rotthauwe et al., (1997). For *ure*C L2F and L2R primers were used Gresham et al., (2007). Gene sequences are indicated in the table 4.1.

Prior to qPCR, cycling conditions of oligonucleotides were optimized using different DNA dilutions and annealing temperatures to reach standard curves with an $R2 > 0.999$ and amplification efficiencies between 0.8 and 1. qPCR reactions were performed using a SYBR green approach (Kapa SYBR Fast qPCR Kit Master Mix (2×) Universal; Kapa Biosystems, Wilmington, MA, USA) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Switzerland). All cycling

profiles and master mix compositions can be found in the supplementary tables (table S 4.1 a - f). The quantifications were performed using three technical replicates. In each quantification run internal control samples, negative controls and a dilution series of the plasmid standards used for the calibration curve were integrated. The qPCR reaction was performed in a CFX96 Touch 15 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, USA) and analyzed by the related software (Bio-Rad CFX Manager 3.1, Bio-Rad Laboratories, Hercules, USA).

Table 4.1 list of primers used for qPCR quantification.

Target gene	Name	Sequence	Reference
16S	Bact-0341_F	CCTACGGGNGGCWGCAG	Muyzer et al., (1993)
	Bact-0515_R	GGACTACHVGGGTMTCTAATC	
18S	FF390	CGATAACGAACGAGACCT	Vainio and Hantula (1999)
	FR1	AICCATTCAATCGGTAIT	
apr	FP aprI	TAYGGBTTCAAYTCCAAYAC	Bach et al., (2001)
	RP aprII	VGCGATSGAMACRTTRCC	
*ure*C	L2F	ATH GGY AAR GCN GGN AAY CC	Gresham et al., (2007)
	L2R	GTB SHN CCCC ART CYT CRT G	
*amo*AOA	amo19F	ATGGTCTGGCTWAGACG	Leininger et al., (2006)
	crenamo	GCCATCCABCKRTANGTCCA	Schauss et al., (2009)
*amo*AOB	amoA1F	GGGGTTTCTACTGGTGGT	Rotthauwe et al., (1997)
	amoA2R	CCCCTCKGSAAAGCCTTCTTC	

4.2.6 Composition and diversity of fungal communities

Fungal community structure was analyzed using amplicon sequencing of the ribosomal internal transcribed spacer (ITS 1) region. The instructions for amplicons preparation of the, which were developed in cooperation with the Genetic Diversity Centre in Zurich (GDC). The library preparation was performed at FiBL. The sequencing on the MiSeq was conducted at the GDC according to the work flow described by Hartman and his team (2017). Before starting the amplicon PCR, the annealing temperature, and the functionality of the different nextera adapters were tested with a qPCR. The first PCR was performed with primers with overhang adapters. The PCR was performed in 20 µL using Kapa SYBR green (SYBR® Fast qPCR Kit Master Mix (2x) Universal; Kapa Biosystems, Wilmington, MA, USA). The PCR was repeated three times for each sample with different DNA template concentrations (1:5, 1:10, 1:15 dilutions). After the PCR, the three reaction replicates were 16 pooled and purified with self-made Siri beads following the protocol described by Jean-Claude Walser (GDC Zurich) and his team. To estimate the DNA concentration, the amplicons were visualized by gel electrophoresis and selected samples differing in band brightness were quantified with a NanoDrop to estimate how the samples need to be diluted for the next step. In a second PCR, indices and Illumina sequencing adapters were attached to the amplicons so that each sample has a specific index combination. This PCR was performed in 10 µL using Kapa SYBR green (SYBR® Fast qPCR Kit Master Mix (2x) Universal; Kapa Biosystems, Wilmington, MA, USA). Cycling conditions and master mix concentrations can be found in the supplementary material (table S 4.2). The PCR was followed by a second purification with self-

made Siri beads. The remaining steps were performed at the GDC in Zurich. The samples were normalized and pooled in two libraries. The amplicons were sequenced on the MiSeq (Illumina® MiSeq™). The bioinformatic analyses were performed by Jean-Claude Walser (GDC Zurich) using UNITE ITS Referenz v8.2 (2020) for the annotation and ITSx to discover and correctly categorize the ITS. The results were arranged in an operational taxonomic units (OTU) table excluding reads of vascular plants and rhizobia.

4.2.7 Statistical analysis

Linear mixed models with fixed effects ($p \leq 0.05$) were used to assess the difference in microbial activities and abundances. Variety, pre crop, and stress were used as fixed factors. Block was added as random factors and comparing 2019 with 2020 the factor "year" was added as covariate. Statistical analyses were performed using the SPSS 21.0 statistical software package (SPSS, Inc., Cary, NC, United States). GGplot R packages was used for graphical design (Wickham et al., 2016).

Fungal community data was organized and analyzed with R package phyloseq (McMurdie and Holmes, 2013) and vegan 2.5-6 (Oksanen et al., 2013). The quality of sequencing was controlled with rarefaction analysis using the rarecurve function from vegan package. Alpha diversity was assessed with Shannon diversity (Bodenhausen et al., 2013). Beta diversity was examined by permutational multivariate analysis of variance (PERMANOVA) using the adonis function from vegan. To visualize the differences between samples principal coordinate analysis (PCoA) on Bray-Curtis dissimilarities was used.

4.3 Results

In this study crop rotations of potatoes with rye and soja was tested regarding its potential to improve resilience against water and nitrogen limitations (adequate irrigation, and 120 kg N /ha vs reduced irrigation, and. 0 kg N/ha). Thus, 4 different potatoes genotypes Charlotte, Pentland Dell, Agria and Cara were tested in a two-year field trial (2019 - 2020). The idea behind this study was that crop physiology and genetics, combined with abiotic stresses and sequential cropping, may improve the water and nutrient use efficiency by microbial community. The final goals were the reduction of the use of chemical fertilizers through alternative sustainable strategies, and the restoring of soil fertility.

Summarizing, the experimental variables were: 2 water treatments, 2 N treatments, 2 previous crops, 4 cultivars and 4 replications for each condition for a total of 128 plots.

4.3.1 Enzyme activities

In first analysis, we measured protease and phosphatase activity as indirect indices of the soil status and of potential microbial activities.

4.3.1.1 Protease activity

Looking at whole results of the two years trial it has been possible to detect difference of performances due to water and nitrogen limitations. In detail, these stresses significantly affect the trial ($p \leq 0.05$, Supplementary material table S 4.3 a). The no stress rhizosphere had a higher activity ([redacted] µg of Tyrosine equivalents) compared to stressed ones ([redacted] µg of Tyrosine equivalents). The effects of the different factors on protease activity in both years are shown in Figure 4.4 a. Analyzing the years separately, it was found that in 2019, differences of protease activity were depending on potato genotypes ($p \leq 0.05$, Supplementary material table S 4.3 a), the highest activity resulted for rhizosphere from Charlotte variety, followed by Pentland Dell, while the lowest results were recorded in Agria and Cara genotypes. In general, in the first year of trial values ranged from [redacted] µg of Tyrosine equivalents. The genotype effect was also highlighted in 2020 ($p \leq 0.05$, Supplementary material table S 4.3 a), but in this case, the best performance resulted in Cara, while the lowest was Pentland Dell. Meanwhile, values ranged from [redacted] µg of Tyrosine equivalents.

4.3.1.2 Phosphatase activity

Water and nitrogen limitations resulted the main discriminating element also for phosphatase activity comparing whole results ($p \leq 0.05$, Supplementary material table S 4.3 b). A lower phosphatase activity was detected in stressed rhizosphere ([redacted] µg/g/h of p-Nitrophenol) compared to optimal ones ([redacted] µg/g/h of p-Nitrophenol). In 2019, statistically significant effect due to stress factors were noted ($p \leq 0.05$, Supplementary material table S 4.3 b), the phosphatase activity measured in samples subjected to optimal conditions ([redacted] µg/g/h of p-Nitrophenol) was higher than in limited ones (591.74 ± 34.18 µg/g/h of p-Nitrophenol). In the first-year, values ranged from [redacted] µg/g/h of p-Nitrophenol. Lower results were detected in the second experimental year (minimal value: 101.76 ± 0.21; maximal value: [redacted] µg/g/h of p-Nitrophenol, Figure 4.4 b) and no statistical relevant effects were observed.

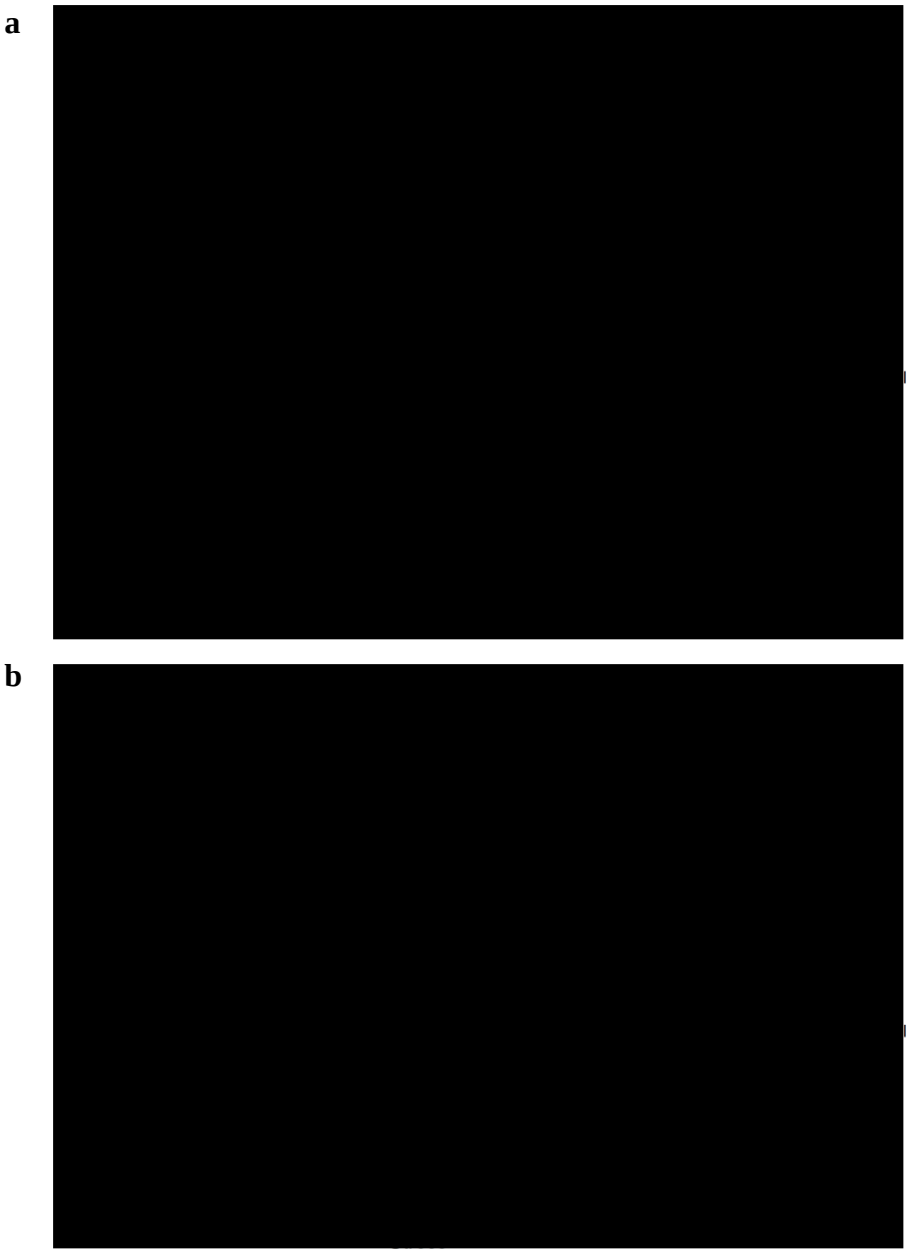


Figure 4.4 Effects of the different factors on a) protease activity, and b) phosphatase activity. The error bars represent the means ± standard error (SE) of three replicates. With NO are indicated potatoes cultivated under adequate irrigation and with120 kg /ha while with YES the ones with reduced irrigation and 0 kg ha^{-1}.

4.3.2 Abundances Microbial community and of functional genes involved in N cycling

Bacterial and fungal abundances were quantified by qPCR to evaluate potential changes due to experimental plan. 18 S and 16 S abundances of were not significantly affected by trial conditions ($p > 0.05$, Supplementary material table S 4.4 a - b, Figures 4.5 a - b). Further analyses were performed to quantify functional genes involved in the nitrogen mineralization. The genes targeted

in the study were *apr*, *ure*C, *amo*AOB, and *amo*AOA. Analyzing both years it was possible to evaluate that, the genotypes significantly affected abundance of *apr* gene ($p \leq 0.05$, Supplementary material table S 4.4 c), Pentland Dell resulted as the highest one while Cara the lowest. Overall, *apr* encoding microbes were more abundant in 2020 than 2019 ($p \leq 0.05$, Supplementary material table S 4.4 c), [redacted] (q gene copies/ g dw of roots) respectively (Figure 4.5 c).

Abundances of the functional gene *ure*C, were most affected by pre crop factor, in 2019 ($p \leq 0.05$, Supplementary material table S 4.4 d) the gene was more abundant with soja (6.37 ±0.31 $\log_{10}$ (q gene copies/ g dw of roots)) as pre crop then rye ([redacted] (q gene copies/ g dw of roots)); while no effects were observed in 2020 (Figure 4.5 d). The pre crop factor is statistically relevant also comparing both years ($p \leq 0.05$, table 3 d). Abundances of *amo*AOA and *amo*AOB genes (Figures 4.5 e - f) significantly vary comparing the two years of trial ($p \leq 0.05$, Supplementary material table S 4.4 e - f), the first one resulted effected also by the pre crop ($p \leq 0.05$, Supplementary material table S 4.4 e). Copies of the archeal gene in 2019 were [redacted] (q gene copies/ g dw of roots) and in 2020 [redacted] (q gene copies/ g dw of roots), in contrary for the second gene we observed [redacted] (q gene copies/ g dw of roots) in the first and the second year, respectively.

a

[redacted]

b

c

d

e

f

Figure 4.5 Abundance of a) 16S, b) 18S, c) *apr*, d) *ure*C, e) *amo*AOA, and of *amo*AOB genes. The error bars represent the means ± SE of three replicates. With NO are indicated potatoes cultivated under adequate irrigation and with 120 kg ha^{-1} while with YES the ones with reduced irrigation and 0 kg ha^{-1}.

4.3.3 Fungal Community in Potatoes roots, rhizosphere and bulk soil

To study the dynamics of root, rhizosphere and bulk-soil microbial was applied ITS amplicon sequencing.

The number of sequences per sample in 2019 was quite low, after applying the threshold, so the library should be re-sequenced. In general, ITS amplicon sequencing revealed a total of 2473 taxa for 298 samples. The rarefaction analysis showed similar pattern in 2019 (Supplementary material Figure S 4.1 a) and 2020 (Supplementary material Figure S 4.1 b), samples reached an asymptote, maximizing the number of distinguishable operational taxonomic units (OTUs). This analysis also showed a higher eukaryotic diversity in bulk soil and rhizosphere soil compared to root samples (Supplementary material Figures S 4.1 a - b). Alpha diversity analyzed by Shannon's diversity index gave different evidence. In both years, Shannon diversity indices was not significantly affected by any factor considering in the complete trial (Supplementary material Figures S 4.2 a - b), but looking separately at each habitat the stress effect resulted significant for roots in 2019 and for rhizosphere in 2020 (Figures 4.6 a – b, Supplementary material Table S 4.5 a - b). In the first year, the interaction of stress with pre crop or with variety factor was significant (Figure 4.5 a, Supplementary Material Table S 4.5 a).

Principal coordinate analysis (PCoA) on Bray-Curtis dissimilarities complemented with PERMANOVA test allow to evaluate the relationship between fungal community and factors applied in the trial. PCoA results of all the samples of 2019 (Figure 4.7 a) indicated that the

different habitats have characteristically different fungal communities, whereas the pre-crop factor and the experimental block also affected this result as well as the interactions among stress and pre crop (Supplementary material Table S 4.6 a). Analyzing separately the different habitats, it was possible to show that the rhizospheric fungal community was affected by the pre crop and stress, while in the bulk soil the factor pre crop influenced the beta diversity (Supplementary material Figures S 4.3 a -c - e, and Table S 4.6 a). In 2020 looking at all data it has been possible to detect characteristically different fungal communities, due to material, block factors as well their interaction (Figure 4.7 b and Supplementary material Table S 4.6 b). Observing a strong "Block" effect, it has been applied a conditioned ordination to reduce its influence. Thus, the beta diversity of whole fungal community resulted influenced by water and nitrogen limitations while rhizospheric community by pre crop and stress factors as resulted in 2019. (Supplementary material Figures S 4.3 d, Table S 4.6 b).

Eukaryotic communities have similar patterns in bulk soil and rhizosphere (Supplementary material Figure 4.4 a - d) in both year of trial. There were high proportions of *Ascomycota* and *Mortierellomycota* (Figures 4.8 a – b and Supplementary material Figure 4.4 a - d), there was also a small representation of *Basidiomycota*, *Chytridiomycota* and *Olpidiomycota*. Even though, it is important to highlight that majority of OTUs result unclassified (indicated as NA).

Although it was possible to find the same phyla in the roots, it was evident that the behavior of the relative abundances was very variable. The figures 4.8 a - b show different abundances of the most representative phyla at the root level for each factor applied in the test.

a

b

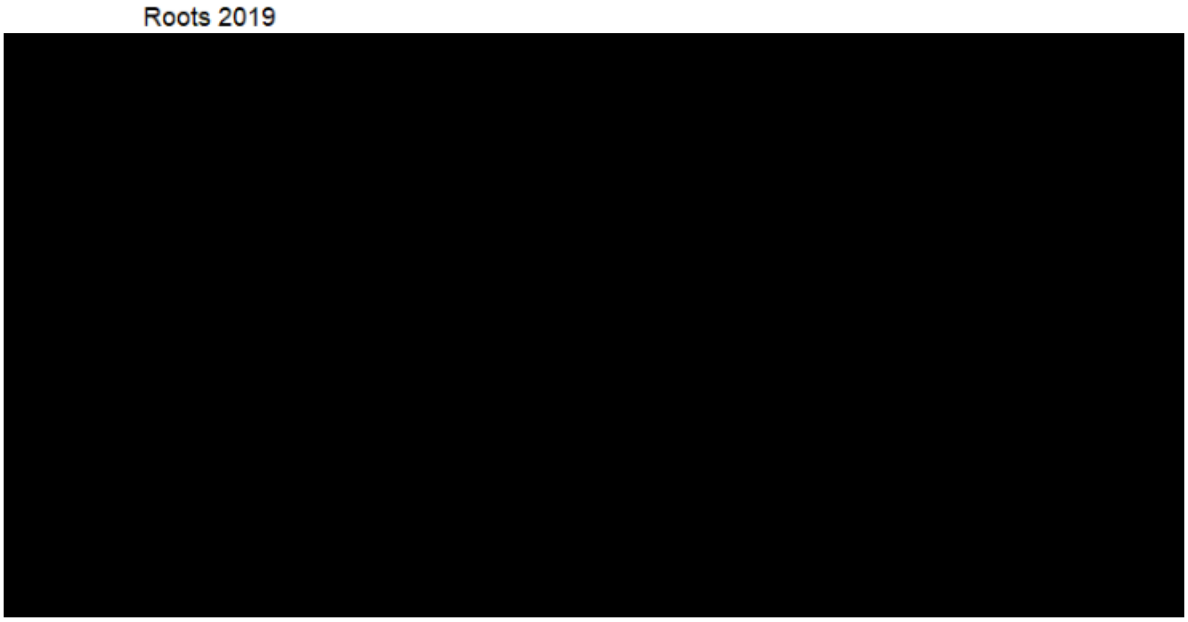
Figure 4.6 Boxplot of Alpha-diversity with Shannon index. Shannon indices reflect the diversity of OTU in samples of a) roots in 2019, and b) rhizosphere in 2020. Boxes represent the interquartile range (IQR) between the first and third quartiles (25th and 75th percentiles, respectively), and the horizontal line inside the box defines the median. Whiskers represent the lowest and highest values within 1.5 times the IQR from the first and third quartiles, respectively.

a

b

Figure 4.7 Effects of habitats on community composition. Ordinations with PCoA using Bray-Curtis dissimilarities were performed on the a) roots in 2019, and b) rhizosphere in 2020, fungal communities associated with potatoes crop.

a



b

Figure 4.8 Changes in the abundance of soil fungi at the phylum level in roots a) in 2019 and b) in 2020. Average of relative abundance of fungal phyla.

4.4 Discussions

In this two-year field trial, we studied the effect of two different water and nitrogen (W: N) treatments (adequate and reduced), four potatoes genotypes and two previous crops on microbial abundances and activities, as well as on the patterns of root, rhizosphere and soil associated fungal communities.

4.4.1 Stress effect on microbial activities and fungal community

Abiotic stresses combined with other factors such as crop physiology, may affect water and nutrient use efficiency by microbial community. Protease and phosphatase activities show higher values in cultivations under optimal conditions. Previous studies clearly demonstrated that lack of water reduces microbial activities and growth (Bottner, 1985; Kieft, 1987), may affect several process such as N mineralization (Pulleman and Tietema, 1999; Sleutel et al., 2008) and may cause shifts of microbial community structure (Hueso et al., 2012; Sorensen et al., 2013; Yan et al., 2015). Protease activity was significantly influenced by farming practices as reported by Sawicka et al., (2020). In their trial, it was observed that the enzymatic activity was significantly higher in soils fertilized with adequate amounts of nitrogen (N), than in the control soil (without nitrogen fertilization), confirming our observations.

In our study, fungal alpha diversity was strongly affected by stress factors (W: N limitations) in both years. In 2019 this effect was observed in roots while in 2020 in the rhizospheric soil. In 2019 and 2020, stress and the pre crop factors (as well as their interactions in the first year) explained more fungal community variance than other factors. In detail, the diversity of rhizospheric fungal community resulted influenced by water and nitrogen limitations in both years of trial.

Previous researches showed that the type and quantity of nitrogen fertilizer affect physical, chemical and biochemical properties of soil, as well as microbial communities in the rhizosphere (Paungfoo-Lonhienne et al., 2015). In general, increasing the dose of N fertilizer has been associated with an increased abundance of fungi in crop soils (Paungfoo-Lonhienne et al., 2015). Furthermore, it is known that “drought” influence microbial respiration, diversity, and community composition. Kundel et al., (2020) in their study did not observe relationship among water limitations and fungal community. Based on this, we can assume that nitrogen limitations affect the fungal community more than water deficiency in this trial.

4.4.2 Precrop effect on microbial activities and fungal community

In this work, crop rotations were tested regarding the possibility to ameliorate response to water and nitrogen limitations. We hypothesize that crop rotation of soja with potatoes could restore high fertility levels, having a better impact on soil quality and fertility than rotation with rye.

The abundance of the genes *amo*AoA and *ure*C was significantly affected by crop rotation, but not by water and nitrogen limitations. Generally, the highest total was observed in soils after soja rotations. This result implies a close relationship between soil N availability and its abundance; Xue

et al., (2013) in their study demonstrated that *ure*C abundance, was significantly correlated with soil NH_4^+ content rather than NO_3^-.

In 2019 and 2020, the pre-crop factor, as well as the interactions with W : N limitations in the first year, explained more the beta diversity in rhizospheric soil than other factors.

Sequential cropping is expected to stabilize soil structure and fertility, and to affect pathogen and weed control. Furthermore, it can exert selective power on soil mycobiome structures. Many potential plant beneficial fungi responded positively to pre-crop practices (Sommermann et al., 2018).

4.4.3 Genotype effects

Plant genotypes may alter the rhizospheric and of endophytic microbiome, thus impacting crop performances. Several studies demonstrated that plants microbiome and microbial activities may be influenced by several factors such as genotype, root system, developmental stage and the ecosystem they colonize (Grayston et al., 1998; Adair and Douglas, 2017; Soonvald et al., 2020). Different genotypes of the same plant species may have significant influence on selecting "rhizospheric partners" through production of diverse root exudates (Aira et al., 2010; Patel et al., 2015). In particular, the release of these compounds affects microbial transformations in soil by modifying their abundance and activities (Rocha et al., 2020). and the use of nutrients appropriate for the species, cultivars and genotypes.

4.4.4 Influence of the soil habitats

We found that fungal richness is significantly different in root, rhizosphere, and bulk soil habitats, confirming previous observations (Urbina et al., 2018).

Our results revealed that bulk soil, rhizosphere and root fungal community were mostly dominated by *Ascomycota* and *Mortierellomycota*, while the relative abundance of *Basidiomycota*, *Chytridiomycota* and *Olpidiomycota* were quite low, confirming the observations of Kundel et al., (2020) made on winter wheat with soybeans as pre-crop.

Roots and rhizospheric fungi are closely related to plant status, due to their roles against plant pathogens, to decompose plant residues, and to provide nutrients (Ehrmann and Ritz, 2014). Variation in the fungal community of these habitats is suggested to be plant-dependent because of peculiarity of roots of the release several organic compounds that contribute to a unique rhizospheric nutrient pool, which is accessible to soil microorganisms (Klaubauf et al., 2010; Han et al., 2017). Indeed, differences in root traits and exudates affect also fungal community composition (Broeckling et al., 2008; Hu et al., 2018).

In general, there is no single biotic or abiotic factor that can be considered the most important in influencing the composition and the activity of the soil microbiome (Fierer, 2017).

Furthermore, the availability of carbon and nitrogen in the different soil compartments may affect a part of fungal community composition; additionally it can be influenced by oxygen concentrations in soil which vary from 20% to <1% from the outside to the inside of single soil aggregates of only a few millimeters in size (Sexstone et al., 1985). Indeed, the microbial communities found in proximity to a plant root can differ substantially from those found in 'bulk' soil environments even if a few centimeters away (Philippot et al., 2013).

4.4.5 Influence of spatial and temporal factors

Abundances of *apr*, *amo*AOA and *amo*AOB functional genes result affected by year variations but not by genotype and farming practices. The *apr* gene varies significantly over two years and in different genotypes.

As general grounds to all results, part of the variation in the composition of the microbiome may be due to spatial and temporal variability, and to specific characteristics of the field. The shifts of climate factors, such as temperature and precipitation, during seasons and over years are often the strongest factors influencing microbial composition and dynamics (Cruz-Martínez et al., 2009; Xue et al., 2011). The relative abundances of microbial communities in the soil microbiome can vary considerably also depending on the soil characteristics. This is true even also when soil samples are collected from sampling areas that are a few centimeters apart (O'Brien et al., 2016). In open field trial biotic and abiotic factors, including the presence of microbial predators (like protists or nematodes) and the amount of available carbon, can affect the whole soil microbial community at any time (Fierer, 2017). Thus, it is quite easy to assume the extent of variability this kind of experiments.

Whole bacterial and fungal abundances were not significantly affected by any trial conditions; different explanations can justify this result. Previous work demonstrated that after a cell dies, amplifiable extracellular DNA can persist in soils for weeks to years (Carini et al., 2016). Extracellular DNA is not quantifiable in all kid of soils; it is more represented in soils with low exchangeable base cation concentrations. These imply that this 'relic DNA' remaining in soil after cell death can alter treatment effects, spatiotemporal patterns and relationships between microbial taxa and environmental conditions (Carini et al., 2016). Furthermore, microbes can form spores or resting structures to resist to moderate or short-term drought, without suffering severe declines in biomass (Kundel et al., 2020).

4.5 Conclusions

It is known that soil type, year and vegetative stage are main factors influencing microbiota of plants. Thanks to this study, we could also provide evidence for the influence of cultivars, combined stresses and pre crop on microbial activity and fungal community composition. The goal of this complex experimental design was to select one or more potato genotypes that combined with the

right sequential cropping can stimulate microbial activities to address more effectively water and nitrogen limitations. This study demonstrated that the experimental variables affect differently the response variables. Comparison with other field experiments performed by project partners with same design but in other countries (Hungary) as well as further investigations are necessary to deepen evaluate the effects on plant and on microbial community in detail.

Acknowledgments

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Supplementary material

Tables

Table S 4.1 PCR set up and cycling conditions of a)18 S b) 16 S, c) *apr*, d) *ure*C, e) *amo*AOA, and f) *amo*AOB,

a) PCR set up and cycling conditions of 18 S

PCR set up			
SYBR		MM	
		1x	66.0
ddH2O		4.5	297
SYBR	1x	7.5	495
F-primer (10µM)		0.75	49.5
R-primer (10µM)		0.75	49.5
Master Mix volume (distributed in every sample tube)		13.5	891.0
DNA template (individually distributed)		1.5	
final volume:		15	

PCR cycling conditions			
Block:	preheated at 95°		
Step	temp °	time	
1	95	3	
2	95	15	
3	50	15	35x
4	72	30	
5	Melt curve		
6	10°	break	

b) PCR set up and cycling conditions of 16S

PCR set up			
SYBR		MM	
		1x	66.0
ddH2O		2.4	158.4
SYBR	1x	7.5	495
F-primer (10µM)		1.8	118.8
R-primer (10µM)		1.8	118.8
Master Mix volume (distributed in every sample tube)		13.5	891
DNA template (individually distributed)		1.5	
final volume:		15	

PCR cycling conditions			
Block:	preheated at 95°		
Step	temp °	time	
1	95	3	
2	95	15	
3	62	15	39x
4	72	30	
5	Melt curve		
6	10°	break	

c) PCR set up and cycling conditions of alkaline metallopeptidase (*apr*)

PCR set up			
		MM	
SYBR		1x	27.0
ddH2O		3	81
SYBR	1x	7.5	202.5
F-primer (10µM)		1.5	40.5
R-primer (10µM)		1.5	40.5
Master Mix volume (distributed in every sample tube)		13.5	364.5
DNA template (individually distributed)		1.5	
final volume:		15	

PCR cycling conditions			
Block:	preheated at 95°		
Step	temp °	time	
1	95	3	
2	95	15	
3	55	15	34x
4	72	20	
5	Melt curve		65-95
6	10°	break	

d) PCR cycling conditions of urease (*ure*C)

PCR set up			
		MM	
SYBR		1x	27.0
ddH2O		3	81
SYBR	1x	7.5	202.5
F-primer (10µM)		1	27
R-primer (10µM)		1	27
Master Mix volume (distributed in every sample tube)		13.5	337.5
DNA template (individually distributed)		1.5	
final volume:		15	

PCR cycling conditions			
Block:	preheated at 95°		
Step	temp °	time	
1	95	3	
2	95	10	
3	57	15	39x
4	72	30	
5	Melt curve		55-95
6	10°	break	

e) PCR set up and cycling conditions of ammonia monooxygenase Archaea (*amo*AOA)

PCR set up			
		MM	
SYBR		1x	288.0
ddH2O		5.0	1440
SYBR	1x	7.5	2160
F-primer (10µM)		1.5	0.5
R-primer (10µM)		1.5	0.5
Master Mix volume (distributed in every sample tube)		13.5	13.5
DNA template (individually distributed)		1.5	1.5
final volume:		15	

PCR cycling conditions			
Block:	preheated at 95°		
1	95	3	
2	95	15	
3	55	15	39x
4	72	30	
5	Melt curve		55-95
5	6	10°	break
6	1	95	3

f) PCR cycling conditions of ammonia monooxygenase Bacteria (*amo*AOB)

PCR set up			
		MM	
SYBR		1x	70.0
ddH2O		5.2	364
SYBR	1x	7.5	525
F-primer (10µM)		1	0.40
R-primer (10µM)		1	0.4
Master Mix volume (distributed in every sample tube)		13.5	13.5
DNA template (individually distributed)		1.5	1.5
final volume:		15	

PCR cycling conditions			
Block:	preheated at 95°		
1	95	3	
2	95	15	
3	59.5	15	39x
4	72	30	
5	Melt curve		55-95
5	6	10°	break
6	1	95	3

* MM : Master Mix

Table S 4.2 ITS Amplicon PCR a) primers, b) PCR set up and cycling conditions

a) **Primers:**

ABC-F_nex0	5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGACTTGGTCATTTAGAGGAAGTAA -3'
ABC-F_nex1	5 '- CGTCGGCAGCGTCAGATGTGTATAAGAGACAGNGACTTGGTCATTTAGGAGAAGTAA -3'
ABC-F_nex2	5 '- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNGACTTGGTCATTTAGAGGAAGTAA -3'
ABC-F_nex3	5 '- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNGACTTGGTCATATTGAGGAAGTAA -3'
ABC-R_nex0	5 '- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCAGCTGCGTTCTTCCATGATGC -3'
ABC-R_nex1	5 '- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNCAGCTGCGTTCTTCATCGATGC -3'
ABC-R_nex2	5 '- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNCAGCTGCGTTCTTCATCGATGC -3'
ABC-R_nex3	5 '- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNCAGCTGCGTTCTTCATCGATGC -3'

b) PCR set up and cycling conditions

Items	**Volume (µl)**
Kapa SYBR	10
ddH2O	7,8
F-primer (10µM)	0.6
R-primer (10µM)	0.6
Dna Template	1

PCR cycling conditions			
Step	**temp °**	**time**	
1	95	3	
2	95	15	
3	60	20	30-35x
4	72	20	
5	72	10	
6	10		

Tables S 4.3 Output of the linear mixed models with fixed effects analysis of a) protease and b) phosphatase activities, and bold test indicates whether there is a statistically significant difference between our group means.

a	***stress***	***precrop***	***Variety***	***stress * precrop***	***stress * Variety***	***precrop * Variety***	***stress * precrop * Variety***	**year**
				Protease activity in 2019				
F	2.70	0.57	3.43	1.36	0.44	0.39	1.16	-
P-value.	0.11	0.45	**0.02**	0.25	0.73	0.76	0.33	-
				Protease activity in 2020				
F	3.07	0.00	2.80	0.11	0.65	0.11	0.52	-
P-value.	0.09	0.95	**0.05**	0.74	0.58	0.96	0.67	-
				Protease activity in 2019 and in 2020				
F	5.65	0.32	2.58	0.33	0.52	0.34	0.64	64.51
P-value.	**0.02**	0.57	0.06	0.56	0.67	0.80	0.59	**0.00**

b	***stress***	***precrop***	***Variety***	***stress * precrop***	***stress * Variety***	***precrop * Variety***	***stress * precrop * Variety***	**year**
				Phosphatase in 2019				
F	7.05	0.05	0.99	1.90	0.43	0.35	2.54	-
P-value.	**0.01**	0.83	0.41	0.17	0.73	0.79	0.07	-
				Phosphatase in 2020				
F	3.51	0.15	0.11	0.22	0.77	0.65	2.49	-
P-value.	0.07	0.70	0.95	0.64	0.52	0.59	0.07	-
				Phosphatase in 2019 and 2020				
F	6.93	0.05	0.94	1.82	0.43	0.34	2.24	488.23
P-value.	**0.01**	0.83	0.42	0.18	0.73	0.80	0.09	**0.00**

Tables S 4.4 Output of the linear mixed models with fixed effects analysis on abundances of six microbial genes a) 16 S, b) 18 S, c) *apr*, d) *ure*C, e) *amo*AOA, and f) *amo*AOB, and bold test indicates whether there is a statistically significant difference between our group means.

a	***stress***	***precrop***	***Variety***	***stress * precrop***	***stress * Variety***	***precrop * Variety***	***stress * precrop * Variety***	**year**
				log q16S copies/g dw root in 2019				
F	3.34	3.57	0.88	0.10	0.78	0.18	0.56	-
P-value.	0.07	0.07	0.46	0.75	0.51	0.91	0.64	-
				log q16S copies/g dw root in 2020				
F	0.91	0.11	2.19	0.71	0.22	0.01	0.89	-
P-value.	0.35	0.74	0.10	0.40	0.89	1.00	0.45	-
				log q16S copies/g dw root in 2019 and 2020				
F	0.42	1.24	1.96	0.71	0.57	0.08	0.38	2.93
P-value.	0.52	0.27	0.13	0.40	0.64	0.97	0.77	0.09

b	***stress***	***precrop***	***Variety***	***stress * precrop***	***stress * Variety***	***precrop * Variety***	***stress * precrop * Variety***	**year**
				log q18S copies/g dw root in 2019				
F	0.15	0.00	0.21	0.77	0.84	0.71	0.16	-
P-value.	0.70	0.95	0.89	0.38	0.48	0.55	0.92	-
				log q18S copies/g dw root in 2020				
F	1.90	0.00	1.26	1.60	0.44	0.08	0.35	-
P-value.	0.17	0.96	0.30	0.21	0.72	0.97	0.79	-
				log q18S copies/g dw root in 2019 and 2020				
F	0.27	0.00	0.71	0.00	1.21	0.67	0.05	0.07
P-value.	0.60	0.98	0.55	0.97	0.31	0.57	0.99	0.79

c	***stress***	***precrop***	***Variety***	***stress * precrop***	***stress * Variety***	***precrop * Variety***	***stress * precrop * Variety***	**year**
				log q apr copies/g dw root in 2019				
F	0.08	0.08	3.58	8.15	1.50	0.68	0.41	-
P-value.	0.82	0.83	0.35	0.06	0.35	0.62	0.74	-
				log q apr copies/g dw root in 2020				
F	0.16	0.06	1.11	**0.04**	0.45	0.46	0.30	-
P-value.	0.69	0.81	0.36	0.85	0.72	0.71	0.83	-
				log q apr copies/g dw root in 2019 and 2020				
F	0.08	0.14	2.87	2.74	0.95	0.28	0.21	7.77
P-value.	0.78	0.71	**0.04**	0.10	0.42	0.84	0.89	**0.01**

d	*stress*	*precrop*	*Variety*	*stress * precrop*	*stress * Variety*	*precrop * Variety*	*stress * precrop * Variety*	year
				log q ureC copies/g dw root in 2019				
F	**0.02**	5.27	0.10	**0.03**	0.14	0.09	0.16	-
P-value.	0.89	**0.03**	0.96	0.87	0.93	0.97	0.92	-
				log q ureC copies/g dw root in 2020				
F	0.22	0.28	0.64	0.06	0.42	0.42	0.89	-
P-value.	0.64	0.60	0.59	0.81	0.74	0.74	0.46	-
				log q ureC copies/g dw root in 2019 and 2020				
F	**0.01**	4.73	0.09	**0.00**	0.10	0.05	0.10	35.80
P-value.	0.91	**0.03**	0.97	0.95	0.96	0.99	0.96	**0.00**

e	*stress*	*precrop*	*Variety*	*stress * precrop*	*stress * Variety*	*precrop * Variety*	*stress * precrop * Variety*	year
				log amoAOA copies/g dw root in 2019				
F	0.34	0.55	0.06	0.18	0.35	**0.00**	**0.03**	-
P-value.	0.56	0.46	0.98	0.68	0.79	1.00	0.99	-
				log amoAOA copies/g dw root in 2020				
F	0.34	110.46	1.74	0.30	0.70	0.78	0.76	-
P-value.	0.56	0.95	0.70	0.62	0.61	0.58	0.52	-
				log amoAOA copies/g dw root in 2019 and in 2020				
F	**0.00**	5.16	0.17	0.00	0.52	0.25	0.47	32.40
P-value.	0.96	**0.03**	0.92	0.99	0.67	0.86	0.71	**0.00**

f	*stress*	*precrop*	*Variety*	*stress * precrop*	*stress * Variety*	*precrop * Variety*	*stress * precrop * Variety*	year
				log q amoAOB copies/g dw root in 2019				
F	0.15	0.04	0.48	0.21	0.30	0.35	0.41	-
P-value.	0.70	0.84	0.70	0.65	0.82	0.79	0.75	-
				log q amoAOB copies/g dw root in 2020				
F	0.96	0.15	0.42	0.17	0.11	0.07	**0.02**	-
P-value.	0.33	0.93	0.52	0.92	0.74	0.98	1.00	-
				log q amoAOB copies/g dw root in 2019 and 2020				
F	1.25	0.54	0.52	0.00	0.07	0.14	0.10	40.58
P-value.	0.27	0.46	0.67	0.96	0.97	0.93	0.96	**0.00**

Table S 4.5 Shows the output of analysis of Variance of Alpha diversity analyzed by Shannon's diversity, in a) 2019 and in b) 2020.

Analysis of Variance Table Shannon all samples 2019

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)	sign.
Precrop	1	139.5	139.54	0.5825	0.44707	
Variety	4	1949.3	487.32	2.0344	0.09503	.
Stress	1	69	69.04	0.2882	0.59252	
Precrop:Variety	4	366.9	91.72	0.3829	0.82042	
Precrop:Stress	1	36.7	36.72	0.1533	0.69622	
Variety:Stress	4	124.7	31.17	0.1301	0.97111	
Precrop:Variety:Stress	4	223.7	55.92	0.2335	0.9	
Residuals	103	24672.3	239.54			

Analysis of Variance Table Shannon Rhizosphere 2019

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	sign.
Precrop	1	0.4	0.417	0.0049	0.9445	
Variety	3	108.5	36.176	0.4256	0.7357	
Stress	1	121.5	121.46	1.4291	0.2393	
Precrop:Variety	3	407.8	135.928	1.5993	0.2056	
Precrop:Stress	1	13.6	13.568	0.1596	0.6917	
Variety:Stress	3	54.3	18.093	0.2129	0.8869	
Precrop:Variety:Stress	3	154.4	51.483	0.6057	0.6153	
Residuals	38	3229.7	84.992			

Analysis of Variance Table Shannon Roots 2019

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	sign.
Precrop	1	1.969	1.969	0.114	0.74715	
Variety	3	43.146	14.382	0.8325	0.52294	
Stress	1	173.811	173.811	10.061	0.01927	*
Precrop:Variety	3	45.026	15.009	0.8688	0.50718	
Precrop:Stress	1	171.888	171.888	9.9501	0.01971	*
Variety:Stress	3	288.671	96.224	5.5701	0.0361	*
Precrop:Variety:Stress	2	61.732	30.866	1.7867	0.24618	
Residuals	6	103.65	17.275			

Analysis of Variance Table Shannon Bulk soil 2019

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	sign.
Precrop	1	34.826	34.826	1.6726	0.225	
Stress	1	45.117	45.117	2.1668	0.1718	
Precrop:Stress	1	30.396	30.396	1.4598	0.2548	
Residuals	10	208.222	20.822			

Signif. Codes: 0 ’***’ 0.001 ’**’ 0.01 ’*’ 0.05 ’.’

Analysis of Variance Table Shannon all samples 2020

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)	sign.
Precrop	1	15	14.86	0.0421	0.83769	
Variety	4	2816	703.91	1.9968	0.09944	.
Stress	1	439	438.53	1.244	0.26697	
Precrop:Variety	4	135	33.78	0.0958	0.98361	
Precrop:Stress	1	170	169.98	0.4822	0.4888	
Variety:Stress	4	249	62.28	0.1767	0.95003	
Precrop:Variety:Stress	4	186	46.47	0.1318	0.97046	
Residuals	118	41597	352.52			

Analysis of Variance Table Shannon Rhizosphere 2020

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)	sign.
Precrop	1	199	199.03	1.7344	0.19482	
Variety	3	218.1	72.71	0.6337	0.59736	
Stress	1	546.8	546.83	4.7653	0.03454	*
Precrop:Variety	3	134.8	44.94	0.3916	0.75965	
Precrop:Stress	1	29.5	29.51	0.2572	0.61466	
Variety:Stress	3	124.9	41.62	0.3627	0.78023	
Precrop:Variety:Stress	3	44.7	14.91	0.13	0.94176	
Residuals	43	4934.3	114.75			

Analysis of Variance Table Shannon Roots 2020

	Df	Sum Sq	Mean Sq	F Value	Pr(>F)	sign.
Precrop	1	4.7	4.738	0.0429	0.8371	
Variety	3	425.3	141.757	1.2843	0.296	
Stress	1	1.2	1.166	0.0106	0.9187	
Precrop:Variety	3	171.3	57.108	0.5174	0.6732	
Precrop:Stress	1	171.3	171.325	1.5522	0.2216	
Variety:Stress	3	294.8	98.279	0.8904	0.4563	
Precrop:Variety:Stress	3	118.5	39.507	0.3579	0.7837	
Residuals	33	3642.5	110.379			

Analysis of Variance Table Shannon Bulk soil 2020

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	sign.
Precrop	1	1.99	1.989	0.0291	0.8677	
Stress	1	62.53	62.526	0.9136	0.3597	
Precrop:Stress	1	90.83	90.832	1.3272	0.2737	
Residuals	11	752.81	68.438			

Signif. Codes: 0 ’***’ 0.001 ’**’ 0.01 ’*’ 0.05 ’.’

Table S 4.6 Output of PERMANOVA analysis used to complement the Principal coordinate analysis (PCoA) on Bray-Curtis dissimilarities in a) 2019 and b) 2020.

a PERMANOVA analysis all samples 2019

Number of permutation:	999.00					
	Df	**Sums Of Sqs**	**Mean Sqs**	**F. Model**	**R2**	**Pr(>F)**	**Sign.**
Block	1	0.923	0.9226	38.909	0.02140	0.001	***
Material	2	13.237	66.184	279.111	0.30700	0.001	***
Stress	1	0.416	0.4160	17.544	0.00965	0.077	.
Precrop	1	0.513	0.5126	21.619	0.01189	0.033	*
Variety	3	0.760	0.2535	10.690	0.01764	0.318	
Material:Stress	2	0.348	0.1739	0.7333	0.00807	0.781	
Material:Precrop	2	0.593	0.2964	12.498	0.01375	0.184	
Stress:Precrop	1	0.620	0.6201	26.153	0.01438	0.016	*
Material:Variety	3	0.694	0.2314	0.9760	0.01610	0.455	
Stress:Variety	3	0.591	0.1970	0.8308	0.01371	0.688	
Precrop:Variety	3	0.673	0.2243	0.9460	0.01561	0.493	
Material:Stress:Precrop	2	0.681	0.3404	14.356	0.01579	0.097	.
Material:Stress:Variety	3	0.557	0.1858	0.7834	0.01293	0.746	
Material:Precrop:Variety	3	0.659	0.2197	0.9267	0.01529	0.535	
Stress:Precrop:Variety	3	0.743	0.2476	10.441	0.01723	0.351	
Material:Stress:Precrop: Variety	3	0.716	0.2386	10.061	0.01660	0.430	
Residuals	86	20.393	0.2371	0.47297			
Total	122	43.116	100.000				

PERMANOVA analysis Rhizosphere 2019							
Number of permutation:	999.00						
	Df	**Sums Of Sqs**	**Mean Sqs**	**F. Model**	**R2**	**Pr(>F)**	**Sign.**
Block	3	0.5746	0.191541	191.649	0.09827	0.014	*
Precrop	1	0.2103	0.210275	210.394	0.03596	0.036	*
Variety	3	0.2806	0.093528	0.93581	0.04799	0.519	
Stress	1	0.2656	0.265643	265.792	0.04543	0.014	*
Precrop:Variety	3	0.2455	0.081820	0.81867	0.04198	0.721	
Precrop:Stress	1	0.1247	0.124711	124.781	0.02133	0.227	
Variety:Stress	3	0.2045	0.068178	0.68217	0.03498	0.938	
Precrop:Variety:Stress	3	0.4434	0.147803	147.886	0.07583	0.066	.
Residuals	35	34.980	0.099944	0.59823			
Total	53	58.473	100.000				

PERMANOVA Roots 2019							
Number of permutation:	999.00						
	Df	**Sums Of Sqs**	**Mean Sqs**	**F. Model**	**R2**	**Pr(>F)**	**Sign.**
Block	2	0.9753	0.48763	116.131	0.11640	0.139	
Precrop	1	0.5185	0.51853	123.491	0.06189	0.124	
Variety	3	12.027	0.40089	0.95474	0.14354	0.625	
Stress	1	0.4326	0.43265	103.038	0.05164	0.382	
Precrop:Variety	3	11.482	0.38274	0.91153	0.13705	0.766	
Precrop:Stress	1	0.3731	0.37313	0.88862	0.04453	0.718	
Variety:Stress	3	11.515	0.38384	0.91413	0.13744	0.775	
Precrop:Variety:Stress	2	0.8969	0.44843	106.797	0.10705	0.287	
Residuals	4	16.796	0.41989	0.20046			
Total	20	83.784	100.000				
Block	2	0.9753	0.48763	116.131	0.11640	0.139	

PERMANOVA analysis Bulk soil 2019							
Number of permutation:	999.00						
	Df	**Sums Of Sqs**	**Mean Sqs**	**F. Model**	**R2**	**Pr(>F)**	**Sign.**
Block	3	0.18917	0.063057	120.209	0.24426	0.163	
Precrop	1	0.09393	0.093928	179.060	0.12128	0.022	*
Stress	1	0.07714	0.077138	147.053	0.09960	0.087	.
Precrop:Stress	1	0.04702	0.047022	0.89639	0.06072	0.592	
Residuals	7	0.36719	0.052456	0.47413			
Total	13	0.77445	10.000				

Signif. Codes:	0.00	'***'	0.00	'**'	0.01	'*'	0.05

b PERMANOVA analysis all samples 2020

Number of permutation:	999.00						
	Df	**Sums Of Sqs**	**Mean Sqs**	**F. Model**	**R2**	**Pr(>F)**	**Sign.**
Block	1	0.961	0.9611	44.665	0.02599	0.001	***
Material	2	6.797	33.986	157.936	0.18382	0.001	***
Stress	1	0.346	0.3459	16.074	0.00935	0.044	*
Precrop	1	0.303	0.3029	14.074	0.00819	0.109	
Variety	3	0.801	0.2671	12.413	0.02167	0.122	
Material:Stress	2	0.422	0.2110	0.9807	0.01141	0.459	
Material:Precrop	2	0.394	0.1968	0.9145	0.01064	0.550	
Stress:Precrop	1	0.196	0.1960	0.9111	0.00530	0.514	
Material:Variety	3	0.581	0.1936	0.8999	0.01571	0.608	
Stress:Variety	3	0.619	0.2064	0.9593	0.01675	0.508	
Precrop:Variety	3	0.667	0.2223	10.333	0.01804	0.366	
Material:Stress:Precrop	2	0.400	0.2001	0.9298	0.01082	0.530	
Material:Stress:Variety	3	0.725	0.2418	11.236	0.01962	0.209	
Material:Precrop:Variety	3	0.585	0.1950	0.9061	0.01582	0.617	
Stress:Precrop:Variety	3	0.756	0.2521	11.716	0.02046	0.163	
Material:Stress:Precrop:Variety	3	0.689	0.2296	10.670	0.01863	0.332	
Residuals	101	21.734	0.2152	0.58777			
Total	137	36.976	100.000				

PERMANOVA analysis Rhizosphere 2020							
Number of permutation:	999.00						
	Df	**Sums Of Sqs**	**Mean Sqs**	**F. Model**	**R2**	**Pr(>F)**	**Sign.**
Block	3	20.108	0.67028	104.873	0.35182	0.001	***
Precrop	1	0.1965	0.19646	30.738	0.03437	0.005	**
Variety	3	0.2642	0.08805	13.777	0.04622	0.097	.
Stress	1	0.1770	0.17701	27.696	0.03097	0.010	**
Precrop:Variety	3	0.1569	0.05229	0.8182	0.02745	0.716	
Precrop:Stress	1	0.0513	0.05126	0.8021	0.00897	0.628	
Variety:Stress	3	0.1424	0.04746	0.7426	0.02491	0.848	
Precrop:Variety:Stress	3	0.1601	0.05336	0.8349	0.02801	0.716	
Residuals	40	25.565	0.06391	0.44729			
Total	58	57.156	100.000				

PERMANOVA Roots 2020							
Number of permutation:	999.00						
	Df	**Sums Of Sqs**	**Mean Sqs**	**F. Model**	**R2**	**Pr(>F)**	**Sign.**
Block	3	12.900	0.43002	136.953	0.08311	0.018	*
Precrop	1	0.3137	0.31366	0.99896	0.02021	0.461	
Variety	3	0.8646	0.28822	0.91792	0.05571	0.658	
Stress	1	0.3616	0.36160	115.164	0.02330	0.253	
Precrop:Variety	3	0.8039	0.26797	0.85344	0.05179	0.824	
Precrop:Stress	1	0.3871	0.38711	123.288	0.02494	0.178	
Variety:Stress	3	10.554	0.35180	112.043	0.06799	0.201	
Precrop:Variety:Stress	3	10.258	0.34194	108.903	0.06609	0.244	
Residuals	30	94.196	0.31399	0.60686			
Total	48	155.219	100.000				

PERMANOVA analysis Bulk soil 2020							
Number of permutation:	999.00						
	Df	**Sums Of Sqs**	**Mean Sqs**	**F. Model**	**R2**	**Pr(>F)**	**Sign.**
Block	3	0.44496	0.148319	181.869	0.32989	0.007	**
Precrop	1	0.06465	0.064654	0.79279	0.04793	0.674	
Stress	1	0.13264	0.132640	162.643	0.09834	0.082	.
Precrop:Stress	1	0.05415	0.054147	0.66395	0.04014	0.869	
Residuals	8	0.65242	0.081553	0.48370			
Total	14	134.882	100.000				

Signif. Codes:	0.00	'***'	0.00	'**'	0.01	'*'	0.05

Figures

a

b

Figure S 4.1 Rarefaction of fungal community OTUs in rhizosphere (red), roots (green) and bulk soil (black) of a) 2019, b) and 2020. Threshold was set at 5000 sequences in 2019 and 8000 in 2020.

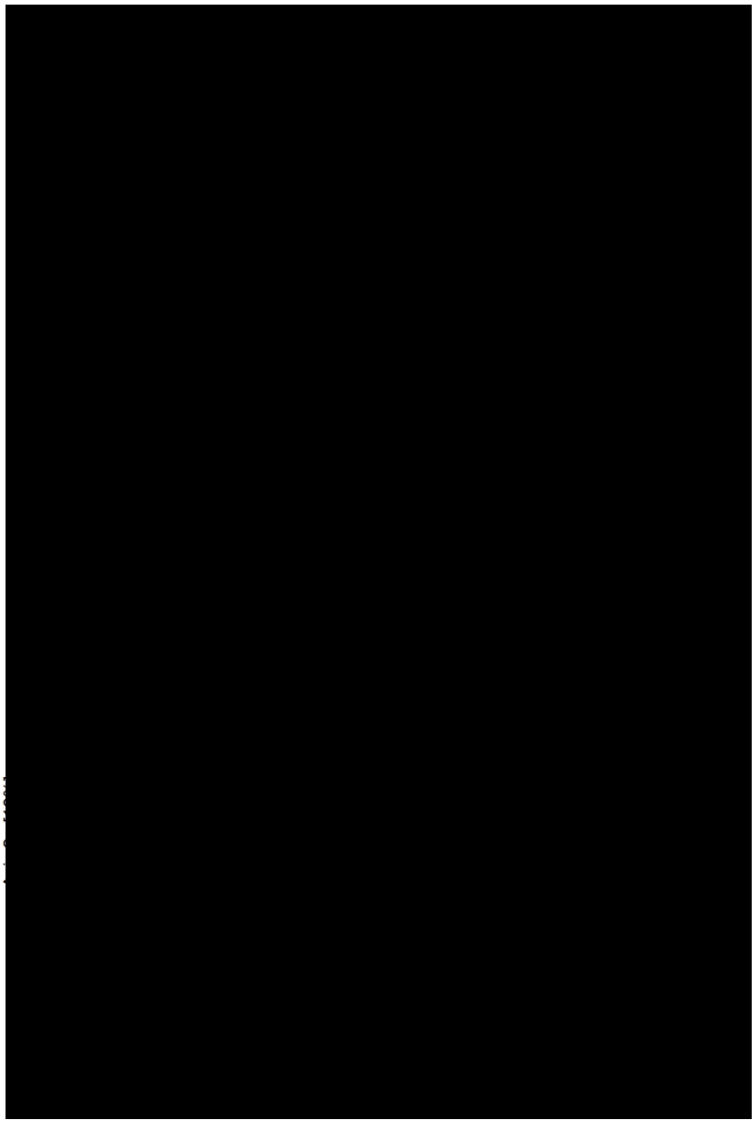
a

b

Figure S 4.2 Boxplot of Alpha-diversity with Shannon index. Shannon indices reflect the diversity of OTU in samples of a) 2019, and b) 2020. Boxes represent the interquartile range (IQR) between the first and third quartiles (25th and 75th percentiles, respectively), and the horizontal line inside the box defines the median. Whiskers represent the lowest and highest values within 1.5 times the IQR from the first and third quartiles, respectively.

a

b



c

d

e

f

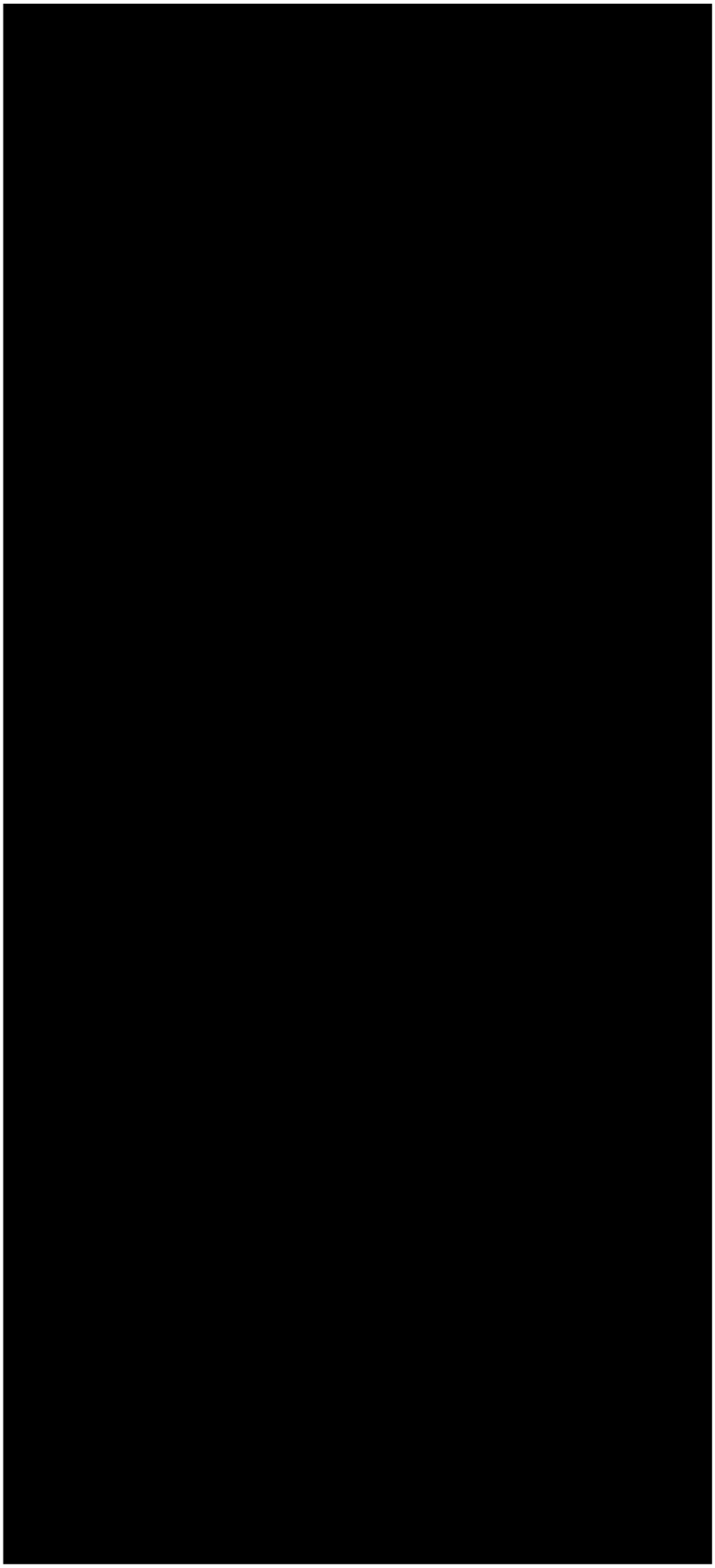
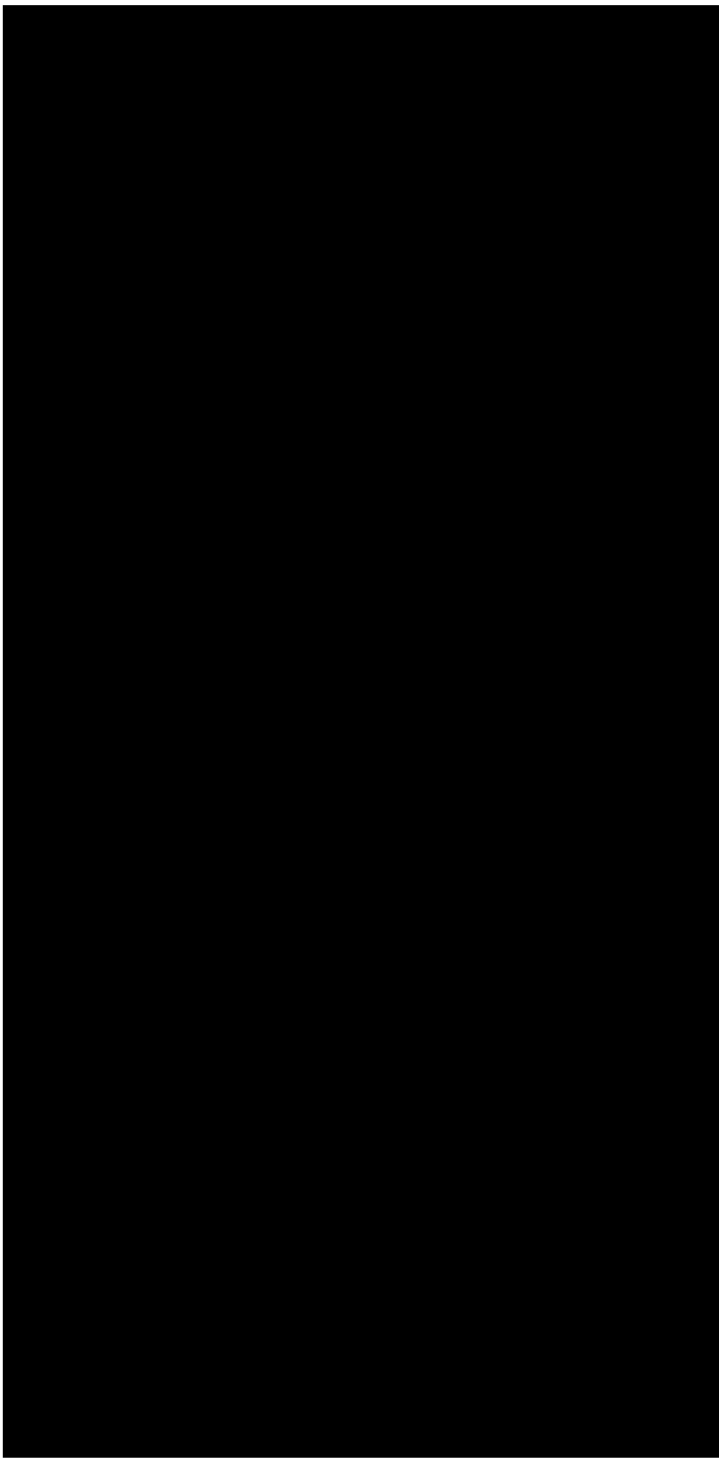


Figure S 4.3 Effects of habitats on community composition. Ordinations with PCoA using Bray-Curtis dissimilarities were performed on root samples in a) 2019, and in b) 2020, rhizosphere samples in c) 2019, and in d) 2020, bulk soil samples in e) 2019 and in f) 2020.

a

b

c



d

Figure S 4.4 Changes in the abundance of soil fungi at the phylum level in a) rhizosphere in 2019 and b) in 2020 and c) in bulk soil in 2019 and in d) 2020. Average of relative abundance of fungal phyla.

Conclusions

Although positive effects of microbial-based biostimulants have been widely reported in literature, they are rarely introduced into standard cultivation technologies. This relates to the insufficient knowledge of farmers on functions and employment, high production costs of biostimulants what results in a fear of an increase in the cultivation expenses and reduced plant quality and quantity, which would affect crop profitability. Despite this, especially in dry regions, biostimulants are a valuable tool for sustainable crop productions, and they can also limit the use of mineral fertilizers introduced into the environment, thus reducing the risks associate with pollution of soils, water, and air and human health. The various challenges in the use of microbial inocula include the establishment of effective methods for the assessment of plant colonization and unsterilized-soil persistence in greenhouse and field trials. *Chapter* 2 deals with several critical issues in sustainable agriculture related to the assessment of root colonization as well as their persistence over time. Modern soil microbiology lacks efficient and standardized methods for the tracking of the effective PGP activities of inoculated strains. Therefore, in *Chapter* 2 it is proposed multi-technique approaches to explain the behaviour of bio-inoculants in the natural soil ecosystems, the combination of cultural-dependent and independent methods should be the main solution to examine the variations in microbial communities after inoculation treatment and to track the inoculated microbial strains in different systems.

Another issue is the multitude of preparations and the need to select a proper biostimulant for a specific crop to ameliorate quality yields. The market has many requirements, the formulates need to have a broad spectrum of functionality, to be easy to apply, and combined with other agents. The new technologies of bio-preparations give a significant contribution to environmental protection, but mainly they are linked with sustainable agricultural and horticultural production to obtain cheap, easily available, and high-quality food.

Chapter 3 proposed new microbial strains *K. pseudosacchari* TL13 and *S. Roseocinereus* MS1B15 with multiple PGP traits both innovative low-cost methodologies to produce innovative microbial-based bio-stimulant. In particular, *K. pseudosacchari* TL13 can efficiently use several agro-industrial organic by-products as carbon sources for its metabolism, thus it is a promising candidate for the development of new and cheap biofertilizers for sustainable agriculture. Therefore, inoculation with beneficial bacteria such as *S. roseocinereus* MS1B15 and *K. pseudosacchari* could be a suitable option for low-input systems, where environmental constraints and limited chemical fertilization may affect the potential yield.

Although the microbial-based biostimulant prototypes proposed in this work are a valuable tool for sustainable farming further investigations in field are necessary before commercialization, such as use of different crops, geographic locations, planting dates, soil types, different soil management practices (e.g., crop rotation), effect on soil microbiota, use in combinations with other PGP strains, refinement of the product, and finally experiments to evaluate the absence of eco-toxicological effects. Another important issue to be considered is also their ability to compete with native soil

microorganisms and to persist in the soil. To this purpose, the use of a multi-disciplinary approach proposed in *Chapter* 2 could be a possible strategy.

Finally, *Chapter* 4 proposed an effective and long-term sustainable alternative to the use of bio-inoculants. It aims to deal with the native microbial populations inhabiting the soil to enhance microbial activities in soil and consequently promote plant growth. This can be reached by implementing a range of management practices typical of organic agriculture such as sequential cropping, integration of legumes and cover crops in rotation with high values productions, and the application of organic amendments such as compost. In this two-year field experiment, four potato genotypes combined with rye and soja sequential cropping should stimulate microbial activities and fungal community to address more effectively water and nitrogen limitations.

The studies presented in this work, regarding both the production of an innovative microbial-based biostimulant and the assessment of alternative management practices, support the principles of the "rhizosphere engineering", which represent the natural follow up of the "fresh green revolution". Agriculture sector begins to recognize the importance of microbes for resilient farming systems. Indeed, the rhizosphere engineering approach proposes the combination of efficient microbial inoculants, selected farming practices, and crop genotypes to effectively stimulate functional and beneficial microbial groups in the rhizosphere, which are positively linked to soil fertility (Woo and Pepe, 2018). Natural rhizospheres are characterized by highly structured and interactive microbiomes and food net, due to plants' co-evolution with their microbiomes, indeed they support them through several mechanisms such as the production of root exudates (Wallenstein, 2017). The final aim is to learn how to engineer the interactive rhizosphere, in order to enhance the efficiency and sustainability of crop production by emulating the symbiotic interactions between plants, soils, and microbes that evolved over millions of years in nature (Wallenstein, 2017).

Rhizosphere engineering is fundamental and in the coming years the research that will focus on the combination of multiple practices, such as microbial inoculation, soil management practices and addition of amendments, will help choose the most effective farming management. The work presented could be help the future research in this regard since it proposed an effective method to select, characterize, and study the growth condition of microbial strains potentially able to promote plant growth (*Chapter* 3) and it also suggested several methods to evaluate the effectiveness of microbial inoculum in a complex system (*Chapter* 2). Moreover, this work gave insights on how to evaluate the effect of biostimulants prototypes combined with different management practices on microbial community (*Chapter* 4).

"*The nation that destroys its soil destroys itself*." – F. D. Roosevelt

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Scientific curriculum

Personal information	
First name / Surname	***Ida Romano***
E-mail	idaromano91@gmail.com ida.romano@unina.it
Nationality	Italy
Date of birth	12.09.1991
Bibliometric indexes (scopus)	
Documents	9
Citations	128
h-index	5
Work experiences	
Current position	Current position PhD candidate at Department of Agricultural Sciences of University of Naples Federico II
March 2020- December 2020	**Ph.D. fellowship**: SolACE project- Solutions for improving Agroecosystem and Crop Efficiency for water and nutrient use. Research Institute of Organic Agriculture (FiBL) -. Ackerstrasse 113, Postfach 219, CH-5070 Frick (Switzerland) Telephone +41 62 865 72 72.
November 2020	**Tutoring grant** at the Agricultural Department of the University of Naples Federico II
June 2019-December 2019	**Ph.D. fellowship**: Development of microbial-based solid and liquid formulations using food wastes. AGRIGES SRL - Nutrizione Speciale per L'Agricoltura Biologica e Integrata, Contrada Selva di Sotto Zona Industriale, 82035 San Salvatore Telesino BN, Telefono: 0824 947065.
January 2018- May 2021	**Ph.D**.: in Sustainable Agriculture and Forestry Systems and Food Security PON R& I 2014/2020. **Project:** Developing innovative microbial-based bio-stimulants fromagri-food wastes for sustainable agricultural productions. University of Naples Federico II, Agricultural Department via Università 100, 80055 (Na).
March 2017-June 2017	**Scholarship**: "Isolation and selection of bacteria from microbial community of contaminated soil on the basis of their biodegradative potential and ability to produce structured biofilms with the aim to prepare formulates for *in situ* application and evaluation of the microbial community structure of contaminated soil by PCR-DGGE". University of Naples Federico II, Agricultural Department via Università 100, 80055 (Na).

May 2016-December 2016	**Scholarship**: "Optimization of the process parameters for the bioconversion of lignocellulosic biomass into biochemicals through the use of selected bacteria". University of Naples Federico II, Agricultural Department via Università 100, 80055 (Na).
November 2013 - June 2014	**Part-time contract** at the Agricultural Department of the University ofNaples Federico II

Project's participation

BIOMAT MISE Bando Horizon 2020 PON I&C 2014-2020.

BENEVEGEFIT MISE-Agrifood PON I&C 2014-2020.

SolACE - Solutions for improving Agroecosystem and Crop Efficiency for water and nutrient use Horizon2020 (EU Research & Innovation programme).

Ph.D. project in Science, Technical, Engineering, and Sustainable Development: "Identification andevaluation of Phosphate Solubilizing Bacteria to improve the agronomic efficiency of phosphate fertilizers". Università di Hassan II (Casablanca, Marocco)

Ph.D. project in Scienze e Tecnologie Agrarie, Forestali e degli Alimenti: "Mixed starter yeasts as biotechnological tool to produce wine with low alcoholic content". Università degli studi della Basilicata.

Education and training

2-6 September 2019,	Summer school titled "High-Throughput Phenomics"Organized by SIMTREA. University Florence.
4-7 June 2019	Summer school titled: "Biodiversity and bioindicators in monitoring and management of contaminated soils". Organized by Italian Society of Soil Science. Department of Agricultural Sciences University of Naples Federico II.
13-15 December 2016	Winter school titled: "Innovative methods for the analysis and management of degraded and/or contaminated sites". Department of Agricultural Sciences, University of Naples Federico II.
October 2013 - December 2015	Master's degree in Food Science and Technologies (106/110) at the Department of Agricultural Sciences, University of Naples Federico II.
September 2010 - October 2013	Bachelor's degree in food technologies (103/110) at The Department of Agricultural Sciences, University of Naples Federico II.

Publications

• Di Mola I., Ventorino V., Cozzolino E., Ottaiano L., **Romano I**., Duri L. G., Pepe O., and Mori M., (2021) "Biodegradable mulching vs traditional polyethylene film for sustainable solarization: chemical properties and microbial community response to soil management". Applied Soil Ecology. 163: 103921. DOI: 10.1016/j.apsoil.2021.103921.

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• Chouyia F. E., **Romano I**., Fechtali T., Fagnano M., Fiorentino N., Visconti D., Idbella M., Valeria V. and Pepe O., (2020) "P-solubilizing *Streptomyces roseocinereus* MS1B15 with multiple plant growth-promoting traits, enhance barley development and regulate rhizosphere microbial population". Frontiers in Plant Science. 11:1137. DOI: 10.3389/fpls.2020.01137

• **Romano I.**, Ventorino V., and Pepe O. (2020). "Effectiveness of plant beneficial microbes: overview on the methodological approaches for the assessment of root colonization and persistence". Frontiers in plant science. Frontiers in Plant Science, 6, 2020. DOI: 10.3389/fpls.2020.00006

• Pagliano, G., Ventorino, V., Panico, A., **Romano, I**., Pirozzi, F., and Pepe, O. (2018). "Anaerobic process for bioenergy recovery from dairy waste: meta-analysis and enumeration of microbial community related to intermediates production". Frontiers in microbiology, 9: 3229. DOI: 10.3389/fmicb.2018.03229

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• Pagliano G., Ventorino V., Panico A., **Romano I**., Robertiello A., Pirozzi F. and Pepe O. (2018). "The effect of bacterial and archaeal populations on anaerobic process fed with mozzarella cheese whey and buttermilk". Journal of Environmental Management, 217:110-122. DOI: 10.1016/j.jenvman.2018.03.085

• Ventorino V., **Romano I**., Pagliano G., Robertiello A., and Pepe O. (2018). "Pre-treatment and inoculum affect the microbial community structure and enhance the biogas reactor performance in a pilot-scale biodigestion of municipal solid waste". Waste Management, 73: 69-77. DOI: 10.1016/j.wasman.2017.12.005

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