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Use of bio activators in agriculture

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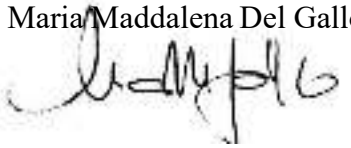

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Summary

Abstract

Biostimulants are defined as "containing substances and/or micro-organisms to stimulate natural processes in order to improve nutrient absorption and efficiency, tolerance to abiotic stress, and crop quality" according to the European Biostimulants Industry Council (EBIC, 2012), which creates a legal framework for marketing and the regulation of these products.

In the last two decades, the possible use of biostimulants to improve crop production though sustainable agriculture have attracted the interest, not only of the farmers, but also of the scientists as it could represent a tool to combat damaged farmland and the uncertainties of climate change. The objective of the research project I pursued during my PhD training was to select the plant growth-promoting bacteria, a specific category of biostimulants, among a collection of environmental isolates.

Chapter 1

1.1 Introduction

Pollution of the air, water, and soil causes approximately 40% of all fatalities worldwide. This is due to the ever-increasing impact humanity has on Earth's atmospheric, terrestrial, and marine ecosystems and their inability to degrade and tolerate the rising amount of garbage produced by human society as a result of population increase and industrialization (Glick, 2015). With the world's population expected to reach 9.5 billion by 2050, increasing crop yield has become a major concern in satisfying global food demands (Godfray et al., 2014; FAO 2017).

Population insufficient food consumption has reduced from over 60% in 1960 to 15% in 2010, indicating that food production has kept pace with population growth (Fig1). Simply expressed, the question is whether we can feed an additional 2 billion people by 2050 (Glick and Gamalero, 2021). Given the difficulties stated above, the world must act on numerous fronts, one of which is the need to drastically enhance agricultural productivity in the coming decades. In this context, it is important to first reduce global population growth, which is a highly contentious issue. Then, it is important to be able to move food more effectively from where it is produced to where it is consumed, thereby lowering much of the current food deterioration and food waste.

Agriculture must be efficient and productive on what is now considered marginal land. In the short run, it will be tempting to use even more agricultural chemicals than is already used to improve yields. This approach, however, will almost certainly be unproductive in the long run (Glick, 2015). Boosting agricultural production can be accomplished by expanding the area under cultivation (agricultural expansion) and increasing yields (agricultural intensification). In addition, conversion of natural ecosystems to agriculture or deforestation can result in considerable greenhouse gas emissions, biodiversity losses, and a deterioration in soil's ability to hold water and avoid flooding (Godfray and Garnett 2014). Moreover, climate change is causing a growing number of countries to experience worrying levels of water scarcity, which are predicted to worsen in many locations in the future years.

The twentieth-century Green Revolution permitted significant increases in global food production. The Green Revolution was an innovative approach to agricultural production issues that enabled significant increases in agricultural production in much of the world between the 1940s and 1970s

through the use of genetically selected plant varieties, fertilizers, pesticides, water, and other capital investments in the form of new technical and mechanical inputs. The Green Revolution could be substantially divided into two parts: chemical inputs (pesticides, herbicides, and chemical fertilizers) and biological inputs (Backer et al., 2018).

Chemical fertilizer application remains the simplest approach to qualitatively and quantitatively boost crop yields. As a result, demand for fertilizers and pesticides has risen considerably and is predicted to rise more in the coming years (Atieno et al., 2020). However, the uncontrolled use of these compounds, particularly pesticides, has resulted in hazardous residues accumulating in food, soil, air, and water, as well as the development of insect resistance (Campos et al., 2019). Excessive application of chemical fertilizers has exerted a major threat to soil quality and the environment, resulting in biodiversity loss. Maintaining soil biodiversity can also help stop the spread of diseases and pests, protecting human health in the process. More sustainable growth is required, one that considers the issues linked with the indiscriminate use of fertilizers and pesticides.

The United Nations identified 17 major sustainable development goals (SDGs) in the 2030 Agenda for Sustainable Development (including: zero hunger: end hunger, ensure food security, improve nutrition, and promote sustainable agriculture; life on earth: protect, restore, and promote the sustainable use of terrestrial ecosystems, sustainably manage forests, combat desertification, halt land degradation, and halt biodiversity loss); soil protection is mentioned in five of these SDGs.

Today's horticultural industry faces an increasing number of difficulties in balancing the need for environmentally friendly crop management techniques with the desire for high production. Because of the limited use of chemical fertilizers and pesticides in organic farming, some plant supplements suitable for this production are needed.

It is difficult to boost productivity while minimizing negative environmental implications. Sustainable environmental practices, low production costs, increased plant resistance to various biotic and abiotic challenges, and high seed value are among the main goals of contemporary agriculture.

Numerous studies have been conducted to find useful amendments that can be used in crop production to improve the growth, productivity and quality of plants and to help them overcome various environmental challenges. Conservation Agriculture (CA), a set of farming practices for sustainable production (such as no or minimal mechanical soil disturbance, maintenance of soil

mulch cover, and diversified cropping), has the potential to mitigate climate change by: 1) more efficient use of fertilizers, agrochemicals, and water; and (2) greater resilience to abiotic and biotic stresses. (Kassam et al., 2022).

It has been known that plants and bacteria can interact in ways that are useful, harmful, or neutral to the plant, and that the impact of a specific bacterium can occasionally alter as soil conditions change. Numerous soil and foliar diseases now threaten commercially important agricultural, horticultural, and decorative plants, causing billions of dollars of crop losses. Chemical fungicides are currently the most commonly utilized in disease management, and their use leads to fungicide resistance (Glick and Gamalero 2021).

In order to preserve productivity, the agricultural and agri-food sectors must have transition towards environmentally sustainable development. One approach to these biology-based strategies is the use of natural and environmentally safe products, such as PGPRs (Plant Growth Promoting Rhizobacteria).

Alternatively, emerging agricultural techniques known as Precision Agriculture aim to improve production while minimizing pollution through site-specific resource management based on field variability (Kassam et al., 2014). With site specific management, this agricultural approach allows the administration of nutrients, water, and pest control measures to fulfill the specific requirements at each site within a field, enhancing fertilizers use efficiency and lowering nitrate leaching. Biotechnologies have also been advocated as smart crop management solutions, boosting nutrient uptake efficiency, managing biotic adversity, and reducing fertilizer consumption (Glick , 2012 ; Souza et al., 2015).

Among these, the use of microbial inoculants, known as bio-fertilizers, is a potential technology for sustainable farming systems that aims to reduce the usage of conventional inorganic fertilizers, primarily N and P fertilizers. Both Arbuscular Mycorrhizal Fungi (AMF) and Plant Growth Promoting Bacteria (PGPB) are recognized to be bio-fertilizers because they can fix N, aid access nutrients such as P and N from organic fertilizers and soil stocks, improve drought tolerance and plant health, and boost salt tolerance.

Fig1 https://ourworldindata.org/world-population-cartogram

1.2 Soil

Soil is made up of five components: minerals, organic soil materials, living organisms, gases, and water. Thus, soil is a complex system composed of a wide range of microhabitats with vastly varying chemical and physical gradients and discontinuous environmental factors that can change dramatically over time (Sequi et al., 2017). Water availability, farming techniques, and soil type are some of the environmental and anthropogenic elements that can modify a specific ecosystem on a daily basis (Kumawat et al., 2021).

In the context of this thesis, it is not possible to delve into every component of it; however, it is necessary to briefly introduce how it is structured (Fig. 2) in order to focus attention in greater detail on that part of the soil classified as the Rhizosphere, which represent the interface between the biotic and abiotic worlds.

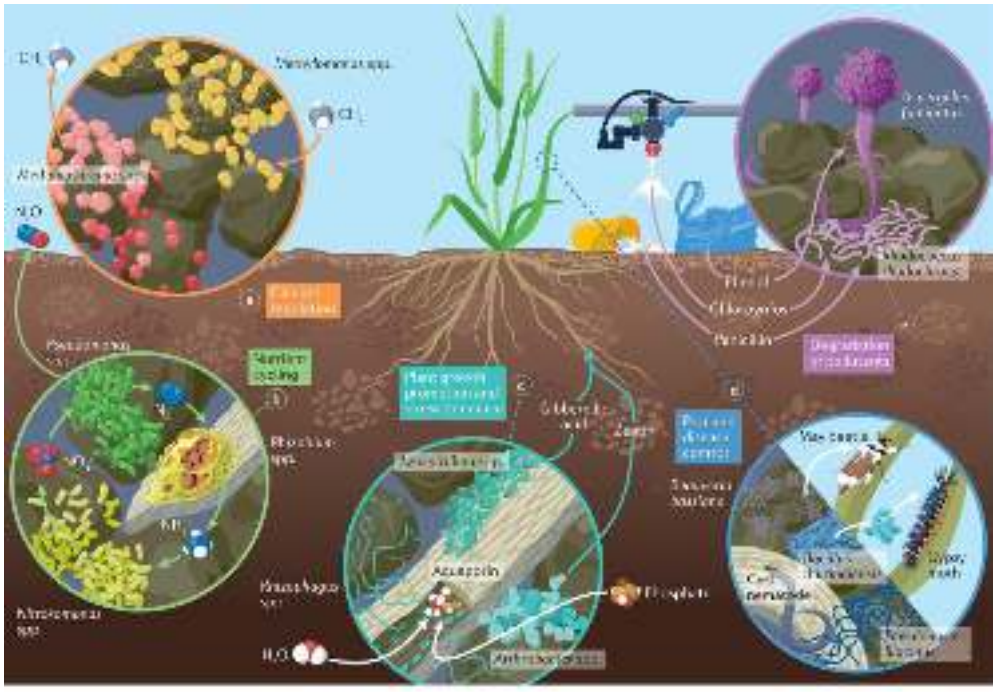

Fig. 2 Soil structure and microbiome functions in agroecosystems (Hartmann and Six, 2023)

1.2.1 The structure of Soil

As mentioned in the brief introduction, among the various soil components there is a wide variety of minerals. The chemical nature varies and, in turn, also the soil fertility because the mineral surfaces serve as possible nutrient storage sites. (Arias et al., 2005)

Weathering affects soil and minerals primarily in two ways: physical erosion and chemical erosion; the two processes produce smaller and finer particles (Fig 3). Thus, soils include particles of various sizes, ranging from enormous boulders to microscopic particles, and the fraction of these particles defines its texture (Sequi et al., 2017).

Chemical erosion, in particular, acts on the parent rock after it has been broken down into microscopic particles (physical erosion). Depending on whether, the chemical erosion is caused by water or oxygen, thus this process can occur via hydrolysis or oxidation. (Bünemann et al., 2018; Sequi, 2017). Chemical erosion, in either instance, results in the transition of primary minerals into secondary minerals, which represent the majority of the microscopic particles in the soil. Chemical erosion is critical for nutrient management because the resultant soil particles correctly store and

supply nutrients. When soils are overused, however, much of the nutrients are lost due to excessive leaching. Soils classed as highly degraded are quite infertile, whilst those classified as moderately degraded are more fertile (Bünemann et al., 2018).

Clay, silt, and sand are the three basic size classes of minerals that make up the great range of soils. They can originate from the process of aggregation. For example, smectite, a type of clay mineral, can shrink and inflate so much when wet and dry that buildings collapse, and quartz, the most prevalent mineral in soils, can produce beautiful crystals while remaining relatively inactive (Seaton et al., 2020).

The constituent minerals of a specific soil can provide a wealth of information, not only about their texture and consistency, but also about their matter content and biological activity.

A variety of indicators influences soil quality. Among these indicators the amount of organic matter (decomposed plant, animal, and microbiological remains at various states of decomposition), which contains significant amounts of phosphorus (between 20 and 80 %) and sulfur (more than 90 %) in noncalcareous soils and serves as an energy source for autotrophic microorganisms and free nitrogen-fixing bacteria, is an important criterion to evaluate soil quality (Bünemann et al., 2018; Sequi et al., 2017).

Soil organic matter is regarded as one of the most important soil fertility components and one of the best predictors of agricultural productivity, with benefits ranging from crop production to land defense against degradation and erosion; this is especially true in arid and semi-arid regions (Turrión et al., 2012).

In this context, microbial diversity and soil community structure also depend on soil aggregation and size distribution. Aggregates, in fact, offer the physical environment for microorganisms and play an important role in determining the overall number of microorganisms present. It has been estimated that one gram of soil could contain around 94 million species, most of which are bacteria (the major groups include Cyanobacteria, Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria) (Tahat et al., 2020).

The results of several analyses of the spatial distribution of bacteria/rhizobacteria at the microhabitat level in soils subjected to various fertilization treatments revealed that 80 percent of the rhizobacteria were localized in the micropores of microaggregates. These microaggregates create the optimal environment for microbial development (silt and clay) in particular contained a

diverse range of rhizobacteria from the *Holophaga/Acidobacterium* and *Prosthecobacter* divisions. Only a few members of the *Holophaga/Acidobacterium* division were discovered in large particles. Alphaproteobacteria and Rhizobacteria prevail in sand aggregates (Khan et al., 2023; Verma et al., 2017). As a result, soil microorganisms connect roots to soil, recycle nutrients, decompose organic debris, and respond quickly to changes in the soil ecosystem, acting as precise indicators of specific activities in the soil environment. Microorganisms found in soil have the potential to increase nutrient use efficiency (NUE), water usage efficiency (WUE), and plant productivity. (Meena and Meena, 2017; Salim and Raza, 2019)

The vertical series of layers known as horizons formed by the combined action of percolating water and living organisms differentiates soils from simple terrestrial materials and serves as the foundation for classification.

The USDA "Soil Taxonomy" is the soil classification system developed by the U.S. Department of Agriculture that defines the various horizons in order to classify a given soil. They include:

- **Horizon O**: surface organic horizon that has undecomposed, partially decomposed and fully decomposed organic matter through the activity of microorganisms.
- **Horizon A**: surface horizon with abundant mineral fraction and fair presence of organic matter. The color, with an increase in organic matter, takes on a darker hue.
- **Horizon B**: mineral horizon with little organic matter, red yellow in color due to the accumulation of clay, carbonates and iron. They are also called "endopedons," or depth horizons.
- **Horizon C**: mineral horizon with complete absence of organic matter consisting mainly of semi-coherent sedimentary rock.
- **Horizon R**: parent rock from which the process of pedogenesis starts.

Because soil is recognized as a natural, nonrenewable, and therefore exhaustible resource, it is critical to understand all of the processes that have a direct impact on soil quality in order to safeguard it (Sequi et al., 2017).

Soil quality could be divided into two categories: intrinsic quality and dynamic quality. Intrinsic soil quality is mainly concerned with the quality composition of the soil as a result of pedogenetic

elements, such as climate and weather, whereas dynamic soil quality is mainly concerned with the interactions between soil quality and the ecosystem's artificial components.

The globe's area of tree crops has expanded in many places around the world in recent decades, spurred by the possibility of higher economic incomes. According to FAO statistics (http://www.fao.org/faostat/), the world area used for growing woody crops was about 130 million hectares (2017). Between 2010 and 2018, the world area planted with fruits, nuts, citrus and olive trees increased. Agricultural methods have a great impact on soil structure as they can alter some of the essential functions of soil, which include nutrient cycling, plant development, gas exchange, carbon storage and waste disposal.

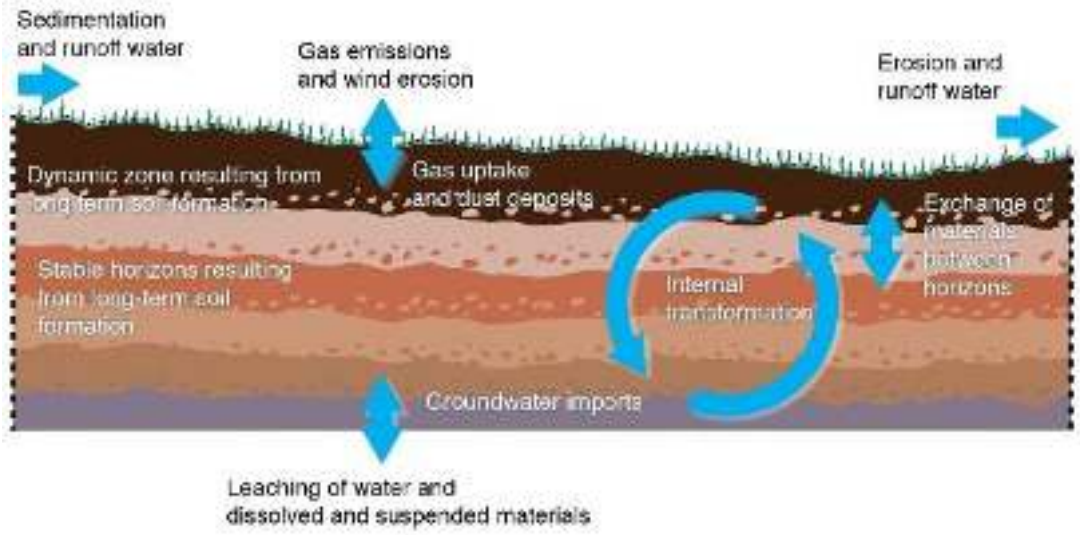

Fig.3 Soil dynamics. Needelman, B. A. (2013)

1.2.2 The Rhizosphere

The rhizosphere (from Greek rhzo = root; sphàira = sphere) is a complex and dynamic habitat described in 1904 by German Lorenz Hiltner. The rhizosphere concentrates many biological and chemical properties of soil that influence plant root secretions and is the hotspot of intensive soil-microflora interactions (Kumar et al., 2015). Thus, the rhizosphere is the small area of soil that immediately surrounds the root system and is made up of three zones (Fig4): the soil, the rhizoplane

(root surface), and the root itself (Munees and Kibret, 2013). The root zone is further divided into three sections, as indicated below, from the innermost to the outermost.

- **Endorhizosphere:** corresponds to the cortical layer of roots potentially colonized by microorganisms occupying the "free space" between cells;
- **Rhizoplane:** medial zone consisting of the root surface, closely adhering soil particles and root mucilage;
- **Ectorizosphere:** the soil layers in direct contact with the roots that border the bulk soil.

Therefore, the rhizosphere surrounds the plant root zone, which hosts a very high number of microorganisms and invertebrates and is considered, for that, one of the most dynamic interfaces on Earth. From a biological point of view, the soil, and in particular the rhizosphere, represents unique environments with the optimal conditions for the growth of diverse microbial populations. It contains at least 2 mm of the rhizoplane (the innermost part), but its effect extends to 10 mm. Plant-microbe interactions are concentrated at the interface between the root and the rhizosphere, and they assist plants in many ways during their growth.

In theory, bacteria known as endophytes in the rhizosphere can improve plant nutrition by controlling the intake of water and/or mineral nutrients, consequently altering root growth and/or, in some situations, even immune responses. Over time, scientific attention has increasingly shifted at characterizing the rhizosphere microbiome and studying its potential effects on plant health and growth (Berendsen et al., 2012). Since the rhizosphere microbiota not only influences the composition and biomass of plant communities in natural ecosystems, but also can directly and/or indirectly affect the composition and biomass of plants in agroecosystems. Disciplines such as agricultural microbiome engineering have invested their interest in understanding the key mechanisms involved (Castellano-Hinojosa and Strauss, 2021).

Not surprisingly, much of the current knowledge about Rhizosphere interactions and processes has emerged precisely from studies of agricultural or horticultural plants and model species such as *Arabidopsis thaliana* and *Medicago trancatula*.

This interface has been identified as an important solution for hyper optimizing the functions of the microbiome in agroecosystems and increasing nutrient uptake by plants, making it an important part of crop production to invest in, reducing reliance on chemical fertilizers to achieve high production yields.

Rhizospheric soil characteristics are modified by the release of rhizodepositions, which consist of whole cells or portions thereof, mucilage, and root exudates. Root exudates selectively stimulate the rhizosphere and rhizoplane regions, and we can distinguish them into:

- **Low-molecular-weight exudates**: composed of amino acids, vitamins, sugars, phenols, and organic acids, which promote the acquisition of nutrients and communication between root cells and rhizosphere microorganisms.

- **High-molecular-weight exudates**: formed of mucilage composed of polysaccharides derived from cell walls; they are responsible for facilitating the mobilization, and thus the absorption, of poorly mobile elements such as phosphorus and iron. Root exudates may also contain toxic substances, such as glycosides and hydrogen cyanide, which go to inhibit the growth of pathogens or may alter the pH of the rhizosphere and consequently cause changes in microbial growth (Ma et al., 2022).

Quantitative and qualitative variations in the root exudates of various plant genotypes exert a strong influence on the development of the rhizosphere community. Rhizodepositions are able to condition the response to stress situations, mediate interactions with other microorganisms, and are an important source of energy for microorganisms in the rhizosphere (Ma et al., 2022).

Studies have also shown that mucilage plays a key role in regulating and promoting associations with PGPRs. (Sequi et al., 2017). Exudates then carry out a process called phytostabilization where, in the outermost portion of the rhizosphere (ectorizosphere), they block contaminants by reducing their mobility (Sequi et al., 2017). The pH of the rhizosphere determines the mobility of nutrients such as nitrogen (N), phosphorus (P), potassium (K^+), sulfur (S), calcium (Ca^{2+}) and magnesium (Mg^{2+}).

Exudation from root tips are substantially influenced by diffusion rates, resulting in concentration gradients between rhizodermal cells and the soil environment. It is widely assumed that the majority of the mechanisms by which plant roots secrete/exude chemicals are passive, following the soil solution concentration gradient from high cytoplasmic to low external concentrations (since

diffusion does not need plants to expend energy). Nevertheless, many molecules are also ejected in the opposite direction of the concentration gradient via active transport (i.e., citrate and secondary compounds).

In addition to growth-promoting bacteria, in general, and rhizobacteria, in particular, other beneficial organisms, such as mycorrhizae, also occur in the rhizosphere. As with rhizobacteria, both bionts (plants and mycorrhizae) benefit but, differently, in mycorrhizae the binding is much less selective. Symbioses generally occur in the portion of the root most efficient for uptake (i.e., the secondary roots and root apices) that will be covered by the mycelium (Reed and Glick, 2023).

There are several symbiotic relationships between microbes and plants in the rhizosphere. Mycorrhizal symbiosis is the most well-known relationship between a mycelium fungus and a higher plant's root. Mycorrhizal symbiosis occurs in more than 80% of all terrestrial plants. These relationships vary greatly in structure and function, but the most frequent are the arbuscular mycorrhizal associations (AM). Arbuscular mycorrhizal fungi (AMF) belong to phylum *Glomeromycota* fungus (Reed and Glick, 2023).

Mycorrhizae can be classified according to the position of the fungus in relation to the host plant cells: in ectomycorrhizae the fungus remains external, while in endomycorrhizae there is a process of colonization of root cells by fungal hyphae (Santoyo et al., 2021).

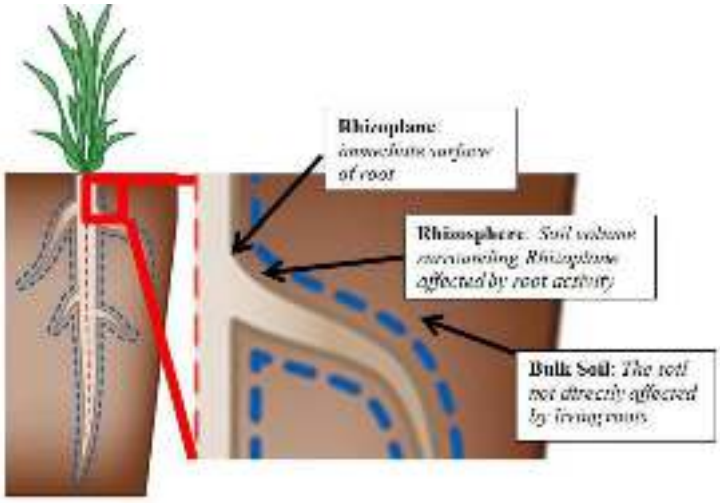

Fig4 The structure of the Rhizhospere.

1.3 Definition, classification, and regulation of plant biostimulants

Modern conventional agriculture production practices based on the abuse of pesticides and chemical fertilizers cause a slew of environmental and human health issues. Furthermore, the emergence of new pathogens related to climate change, the re-emergence of old ones, and the persistence of endemic pathogens continues to challenge our ability to safeguard plant growth and health worldwide (Pathak et al., 2022). For all these reasons, there is a growing demand for innovative and environmentally friendly strategies in agriculture. Plant biotechnology, also, has contributed to the development of new crop varieties with greater resistance to disease, drought, salt stresses, and greater nutritional value. Furthermore, plant biostimulants derived from natural environments have attracted considerable interest from both the scientific community and commercial enterprises, especially in the last two and a half decades. A number of microbial inocula are commercialized worldwide and in recent years their popularity has increased dramatically as scientific research has improved their efficacy and consistency (Fusco et al., 2022) Biostimulants have emerged as a potentially novel strategy to regulate and modify physiological processes in plants, in particular to accelerate growth, reduce stress-induced limits, and increase yield (Kunicki et al., 2010). Prof. V.P. Filatov launched the debate on the "biogenic stimulant" idea in the Soviet Union in 1933. (Yakhin OI et al., 2017). Bioformulates, and in particular biopesticides, are considered key tool for Integrated Pest Management (IPM), which the European Union, through Directive 2009/128/EC, imposes on Member States and which in Italy is implemented in the Legislative Decree of August 14, 2012, no. 150.

According to the European Regulation, the biostimulant is: "A community fertilizer product with the function of stimulating plant nutritional processes, regardless of the nutrient content of the product, with the sole purpose of improving one or more of the following characteristics of plants or their rhizosphere: a) nutrient use efficiency; b) abiotic stress tolerance; c) qualitative characteristics; and d) availability of nutrients contained in the soil or rhizosphere (Reg. (EU) 2019/1009)".

In the Agriculture Improvement Act of 2018 (U.S.A.), the biostimulant is described as that substance or microorganism which is applied to plants, seeds, or their rhizosphere to stimulate natural

processes and improve nutrient uptake and efficiency, improving crop quality and enabling plants to be more tolerant to abiotic stresses. (Bhupenchandra et al., 2022). There are no obvious differences between the two definitions (European and U.S.).

However, currently the term "biostimulant" remain poorly defined. A wide range of products, such as metabolic enhancers, plant boosters, positive plant growth regulators, elicitors, allelopathic preparations, plant conditioners, phytostimulators, biofertilizers or biofertilizers/biostimulants (Yakhin et al., 2017), have been variously labeled as biogenic stimulants.

The main categories of plant biostimulants are listed below:

- **Microorganisms**, such as beneficial bacteria, yeasts or fungi, are commonly used in the creation of biostimulants. These preparations may contain living and nonliving microorganisms and their metabolites. Preparations based on microorganisms as biostimulants are extensively described by Xavier and Boyetchko (2002). Plant diseases are responsible for 20-40% of global crop losses. Climate change has a complex impact on plant-pathogen interactions because environmental circumstances affect the entire disease triangle*:* they alter plant susceptibility as well as parasite and pathogen biological cycles. (Coakley et al., 1999). Bacteria interact with plants in a variety of ways. Functional, and ecological variety, agricultural applications of biostimulants should be explored, in particular: mutualistic endosymbionts of the type of Rhizobium and mutualistic, rhizospheric PGPRs (‘plant growth- promoting rhizobacteria) (Du Jardin, 2015). The biology and agricultural uses of the Rhizobium-based symbioses have been extensively reviewed by the scientific literature (Du Jardin, 2015; Fadiji et al., 2022). PGPRs are multifunctional and have an impact on all plant life, including nutrition and growth, as well as morphogenesis and development. Nutrition and growth, morphogenesis and development, response to biotic and abiotic challenges, and interactions with other living being in agroecosystems are all aspects of plant life (Du Jardin, 2015; Fadiji et al., 2022). The intricacy of PGPRs, as well as the varying responses of plant cultivars and receiving conditions, limit their agricultural use. Microbial inoculants can be single strains (for example, Bacillus subtilis) or combinations of microorganisms with additive or synergistic effects (Du Jardin, 2015). Among the bacteria, *Rhizobium*, *Azospirillum*, and *Azotobacter* genera have been approved by Regulation (EU) 2019/1009. *Azospirillum* and

Azotobacter are nitrogen-fixing bacteria that are free-living and are widely utilized in inoculant products. *Azospirillum,* one of the most studied PGPB genera in the world, has been used as a substrate for over 100 biostimulant products in South America alone (especially *Azospirillum. brasilense*). As stated in the introductory part of this section, both types of products must meet the allowable limit about the presence of bacteria and other pathogenic microorganisms in addition to having to pass experimental tests to ensure their effectiveness.

Fungi interact with plant roots in a variety of ways, including mutualistic symbiosis (where both organisms live in direct contact with each other and form mutually beneficial interactions) and parasitism (Behie and Bidochka, 2014). Mycorrhizal fungi are a diverse collection of taxa that form symbioses with more than 90% of all plant species. There is growing interest in using mycorrhiza to enhance sustainable agriculture, owing to the well acknowledged benefits of the symbioses to nutrition efficiency (for both macronutrients, particularly P, and micronutrients), water balance, and plant biotic and abiotic stress protection Thus, fungal-based biostimulant compounds applied to plants improve nutrition efficiency, stress tolerance, crop output, and product quality. Nevertheless, several technical difficulties mainly related to the large-scale propagating AMF, due to their biotrophic nature (Dalpé and Monreal, 2004), and, more fundamentally, the lack of understanding of host specificity determinants and population dynamics of the mycorrhizal communities in agroecosystems, represent the main limitations to their use. (Dalpé and Monreal, 2004; Du Jardin, 2015). *Trichoderma* spp. (Ascomycota) and *Sebacinales* (Basidiomycota, with *Piriformospora indica* as model organism) are gaining popularity as plant inoculants and model organisms for studying the mechanisms of nutrition transfer between fungal endosymbionts and their hosts. Some of these fungi, primarily *Trichoderma* spp., have been widely studied and used for their biopesticidal (myco-parasitic) and biocontrol (inducer of disease resistance) qualities, as well as enzyme supplies by biotechnological enterprises (Mukherjee et al., 2012; Nicolás., 2014).

- **Algae** and **plant extracts**. Several species of algae, especially marine algae, are also commonly used to produce biostimulants (Sharma et al., 2013b; Ali et al., 2021) and experimental articles by Billard et al., (2013). Aremu et al., (2015) report algae preparations

as biostimulants. Fresh seaweeds have long been used in agriculture as a source of organic matter and fertilizer, but biostimulant properties have just lately been discovered. This encourages the commercial use of seaweed extracts and purified substances such as the polysaccharides laminarin, alginates, and carrageenans, as well as their breakdown products. Other elements that promote plant growth include micro- and macronutrients, sterols, N-containing compounds such as betaines, and hormones (Craigie, 2011; Du Jardin, 2015). The majority of the algal species are members of the phylum of brown algae, which usually can be applied on soils, in hydroponic solutions or as foliar treatments (Du Jardin, 2015).

Moreover, with the term "Botanicals" can be defined compounds taken from plants that are used in medications and food products, but there appears to be the possibility of employing them as biostimulants as well, however much more research needs to be done (Du Jardin, 2015, Ertani et al., 2013; Ziosi et al., 2012). Both the upper (seeds and leaves) and the lower (roots) parts of plants, as well as the exudates of plant families belonging to *Amaryllidaceae*, *Brassicaceae*, *Ericaceae*, *Fabaceae*, *Fagaceae*, *Moringaceae*, *Plantaginaceae*, *Poaceae*, *Rosaceae*, *Solanaceae*, *Theaceae*, and *Vitaceae*, are often used as raw materials for biostimulants (Ertani et al., 2011).

- **Humic substances**, Ertani et al., (2011), and Jannin et al., (2012), Yakhin et al., (2017) have evaluated humate-based raw materials (such as humic and fulvic acids) for biostimulants. Humic substances (HS) are naturally occurring elements of soil organic matter that result from the decomposition of plant, animal, and microbial wastes, as well as the metabolic activity of soil bacteria utilising these substrates. HS are groups of heterogeneous substances that were previously classified into humins, humic acids, and fulvic acids based on their molecular weights and solubility. Humic compounds have long been recognized as important contributions to soil fertility, operating on the soil's physical, physicochemical, chemical, and biological qualities. The majority of HS biostimulant effects are related to improved root nourishment via several pathways. One of them improves macro- and micronutrient absorption due to the increased cation exchange capacity of the soil containing the polyanionic HS, as well as increased phosphorus availability due to HS interfering with calcium phosphate precipitation (du Jardin, 2015). HS seem to enhance respiration and invertase activities providing C substrates. Stress prevention is also mentioned in the suggested biostimulation action of HS. Phenylpropanoid metabolism is essential for the synthesis of phenolic chemicals, as

well as secondary metabolism and a variety of stress responses. (Canellas et al., 2015; Du Jardin, 2015).

- **Nitrogen compounds**, including hydrolyzed proteins, amino acids, peptide combinations and other nitrogen-containing compounds generated from animals, including waste (e.g. collagen, epithelial tissues) and agro industrial by-products (crop residues) (Sharp, 2013; Du Jardin, 2015). Single or mixed molecules can also be synthesized using chemical synthesis. These chemicals have been found to serve a variety of roles as plant growth biostimulants (Du Jardin, 2015). Direct impacts on plants include manipulation of N absorption and assimilation via regulation of enzymes involved in N assimilation and their structural genes, as well as acting on the N acquisition signaling pathway in roots. Increased microbial biomass and activity, soil respiration, and total soil fertility are all key indirect effects on plant nutrition and growth. (Du Jardin, 2012).

Organic compounds, including chitosan and other polymers derivatives obtained from insects (Sharp, 2013). Chitosan is a deacetylated version of the biopolymer chitin that is produced both naturally and artificially (Du Jardin, 2015; Sharp, 2013). Polymers and oligomers of varied, regulated sizes are employed in the culinary, cosmetic, medical, and agricultural industries. The physiological effects of chitosan oligomers in plants are due to their ability to bind a wide range of cellular components, including DNA, plasma membrane, and cell wall constituents, as well as specific receptors involved in defense gene activation, in a manner similar to plant defense elicitors (Hadwiger, 2013; Hidangmayum et al., 2019). Over the years, agricultural applications of chitosan have been developed, with a focus on plant protection against fungal pathogens, but broader agricultural applications bear on tolerance to abiotic stress (drought, salinity, cold stress) and quality traits related to primary and secondary metabolisms. Several poly- and oligomers of biological origin or (hemi-) synthetic versions, notably seaweed polysaccharides, are increasingly exploited in agriculture as plant defense elicitor (Vera et al., 2011).

- **Inorganic compounds.** Beneficial elements are defined, not only by their chemical properties, but also by the specific settings in which favorable impacts on plant growth and stress response can be detected (Du Jardin, 2015). Beneficial elements are chemical components that improve plant growth, plant product quality, and tolerance to abiotic stress

and for this may be vital to some species but are not required by all plants (Pilon-Smits et al., 2009). The five most important helpful elements are Al, Co, Na, Se, and Si, which are found in soils and plants as various inorganic salts and as insoluble forms such as amorphous silica (SiO_2,) in graminaceous species. These positive effects can be constitutive, such as silica deposits strengthening cell walls, or expressed in specific environmental situations, such as pathogen attack for selenium and osmotic stress for sodium. (Du Jardin, 2015). The scientific literature reports numerous beneficial element effects. The following are several of significant examples: cell wall rigidification; osmoregulation; reduced transpiration by crystal deposits; thermal regulation via radiation reflection; enzyme activity by co-factors, plant nutrition via interactions with other elements during uptake and mobility; antioxidant protection; interactions with symbionts; pathogen and herbivore response; heavy metal toxicity protection; plant hormone synthesis and signaling (Pilon-Smits et al., 2009, Du Jardin, 2015). Finally, inorganic salts of beneficial and important elements such as chlorides, phosphates, phosphites, silicates, and carbonates have been utilized as fungicides (Deliopoulos et al., 2010, Du Jardin, 2015).

Therefore, biostimulants can be defined as any material, substance or product that, applied to plants or soil, stimulates or enhances existing biological processes in plants and related microbes. It helps improve plant growth, increases crop yield and quality, and improves nutrient uptake and tolerance to abiotic stress. Thus, over the past two decades, an extensive literature has developed regarding the results brought by the use of biostimulants on different plant species. Many trials have been funded in order to find biostimulants as a solution to water scarcity in diverse crop- growing areas. The performance of biostimulants can be evaluated by various means such as measuring root mass, photosynthesis activity, and quantity harvested. In the case of grapevine, for example, it was discovered that the concentration of metabolites involved in the flavonoid pathway decreased while the concentration of metabolites associated with uptake and vigor of vegetative growth increased. There was also an increase in the expression of enzymes related to the photosynthesis process, which resulted in a reduction in berry sugar concentration and increased acidity. The latter two effects appear to be due to the higher photosynthetic efficiency of treated plants compared to control conditions (Bavaresco et al., 2020).

The mechanism of action of biostimulants can be summarized in 5 steps:

- the recognition of the active molecules by specific receptors, which allows the penetration of the active molecules of the product into the cells and tissues of the plant;
- the translocation and transformation of these active molecules;
- the manifestation of defense genes, signals and regulation of hormonal status that allow induced local resistance;
- the activation of metabolic processes;
- the transmission of signals and the transfer of induced resistance to the whole plant.(URL: https://www.unmaco.it/2021/07/biostimolanti-e-nutrizione-del-suolo-e-delle-piante/ (19/7/2022)

Since the 1980s, a number of researchers have recognized the potential of PGPBs in promoting plant growth and increasing crop yield in different soils and environments. In particular, *Azospirillum*, *Azotobacter*, *Bacillus*, *Klebsiella*, *Enterobacter*, *Arthrobacter*, *Burkholderia*, *Pseudomonas*, *Serratia*, etc. as reported by different authors have been studied and applied in different crop systems as biofertilizers (Pellegrini et al., 2020).

Biofertilizers are materials that contain efficient microorganisms (live or dormant) that enrich the soil with nutrients and stimulate plant growth by improving nutrient uptake, nutrient efficiency, abiotic stress tolerance, and crop productivity and quality.

Nitrogen fixation, phosphate solubilization, siderophores production, and hydrolytic enzyme synthesis are some of the methods used by PGPBs to fertilize soil and boost plant productivity (Kour et al., 2019; Rana et al., 2020; Yadav, 2020). Crop productivity has been challenged in recent decades by risks from both plant diseases and massive inputs of man-made pesticides to combat disease. Plant diseases globally reduce yields by 21-30% in all crops (Savary et al., 2019). Simultaneously, several plant diseases have gained resistance to long-used chemical management methods (Lucas, 2011). As a result, some economically important plant diseases have grown more difficult to control, owing mostly to a shortage of effective chemicals (Bailey, 2010); intensive agricultural production techniques and food market globalization have clearly aggravated this situation. (Fones et al., 2020; Jiao et al., 2021).

Biological control, as defined by the IOBC (International Organization for Biological Control), is the employment of living organisms to prevent or mitigate pest harm. The organisms mentioned are known as "biological control agents."

Numerous research on the use of these microorganisms as pesticide alternatives have demonstrated that these biocontrol agents can play an essential role in improving agricultural and horticultural performance (Niranjan et al., 2003). Several bacteria and fungi, particularly species of the genera *Bacillus*, *Pseudomonas*, and *Burkholderia*, have been identified as hostile microorganisms (Lee et al., 2001). Due to environmental concerns, the hunt for innovative biological control measures to prevent the proliferation of phytopathogenic microbes has become popular. Several mechanisms have been proposed to explain the inhibition of phytopathogenic fungi by bacteria, including (Bensidhoum et al., 2016; Ajijah et al., 2023; Patkowska, 2021):

- antibiotic production. Antibiosis is the inhibition of pathogens through the production of substances with antifungal and/or antibiotic properties. Several metabolic products with bioactive activities have been produced by PGPR, lytic enzymes (chitinases, proteases, glucanases, etc.), antimicrobial proteins or peptides, polyketides, phenolic compounds, and bio-surfactants. Fengycin A and B, iturin A mycosubtilin, bacillomycin D, and pyochelin, are categories of antibiotics produced by *Bacillus* and *Pseudomonas* to control aflatoxigenic fungi (Andrić et al., 2020). Another type of antibiotic are volatile compounds, and several authors have reported the ability of PGPR to produce volatile compounds such as ammonia, hydrogen cyanide, acetoin, and 2,3-butanediol.

- **The secretion of hydrolytic enzymes.** Chitinase is an enzyme that hydrolyzes insoluble linear polymers of ß(1,4) N-acetylglucosamine, which are major components of the cell wall of several fungi, insect exoskeletons, and crustacean shells. Microorganisms that produce this enzyme are also classified as biological control agents (Hamid et al., 2021). Several researchers have shown that chitinases are involved in antifungal activity and can enhance the insecticidal activity of *Bacillus* sp. (Wang et al., 2018). According to Quecine et al., (2008), most *Bacillus* sp. show high chitinase activity. Some studies have established the relationship between the chitinase of *Bacillus* sp. and *Pseudomonas* sp. and their ability to inhibit the mycelial growth of *Fusarium oxysporum* and *Fusarium solani*.

- **Induction of plant resistance**. The PGPRs-plant interaction involved in pathogen control consists of the stimulation of plant defense mechanisms. This phenomenon has been termed induced systemic resistance or ISR (Van Loon et al., 1998), making the host much more resistant to future pathogen attacks competition for nutrients and space or a combination of these mechanisms. Competition consists of consuming or controlling access to nutrients (particularly competition for carbon, one of the mechanisms responsible for the fungistatic effect that is characterized by inhibition of spore germination in the soil to space or any other factor whose availability is limited (Alabouvette et al., 2006). Backer et al., (2018) defined biocontrol competition as the ability of PGPRs to compete with pathogenic organisms by sequestering most nutrients and colonizing appropriate niches so that they constitute a significant percentage of the rhizosphere-rhizoplane population. Antagonistic PGPRs can suppress the growth of certain phytopathogens by competition for nutrients such as nitrogen, carbon, or macro- or micronutrients (Elad and Stewart, 2007; El-Saadony et al., 2022). A special case of competition for nutrients is based on competition for iron. As previously explained, to survive, microorganisms secrete siderophores by depriving phytopathogens of one of their growth factors. Competition for iron by siderophores and competition for substrate have been proposed as mechanisms of plant suppression by biocontrol agents (Pandey and Maheshwari, 2013; Burbank et al., 2015).
- **Bacterial siderophores can influence plant nutrition**. Actually, they are known for their ability to sequester iron from the rhizosphere, making it unavailable to pathogenic fungi, thus limiting their growth (Burbank et al., 2015)

  The role of pseudobactin and pyoverdin siderophores produced by *Pseudomonas fluorescens* has been clearly shown to be involved in the control of *Fusarium* species (Trapet et al., 2016). Siderophores produced by *Pseudomonas* spp. are involved in the biocontrol of plant pathogens such as *Aspergillus niger* (Sindhu et al., 2016). These compounds play an important role in stimulating plant growth, and some plants assimilate iron directly from Pseudomonas siderophore (Sah et al., 2017). Inoculation of seeds by siderophore PGPRs improves plant growth and increases chlorophyll content (Sah et al., 2017). The exploitation of siderophore-producing PGPRs in agriculture, as biocontrol agents, and plant growth-promoting bacteria is a research avenue to be explored.

- It is acknowledged that one of the strategies utilized by several biocontrol agents to indirectly control plant infections is **changing environmental factors** (such as pH, plant area, etc.) (Pandit et al., 2022).

To lessen the hazards connected with pesticide use, biofertilization and biological control have frequently been embraced as alternatives to chemical imputs in agriculture. It is a land policy that substitutes natural remedies for chemical plant protection products. The agricultural inputs market has recently seen the development of a number of products with the goal of enhancing plant-soil interactions or the functionality of soil and plants. These items, referred to as biostimulants, frequently offer ground-breaking approaches to crop protection and fertilization.

1.4PGPM

Various evidence have pointed out that microbial communities, as they interact with their host, behave in a synchronized manner (Zilber-Rosenberg, 2008; Sessitsch et al.,2019), giving rise to the concept of the holobiont. According to this viewpoint, the plant is not a separate entity closed in on itself, but rather a complex biological unit in which the plant-associated microbial community is crucial for performing many functions, such as encouraging plant development and boosting stress resilience. Plant-associated microbial communities can be termed microbiomes, microbiota, or metagenomes, depending on the entities, environments, and activities considered (Berg et al., 2021). The microbiota is defined as the set of living microorganisms present in a defined environment (Marchesi and Ravel, 2015), thus bacteria, fungi, actinomycetes, and protists (excluding viruses, plasmids, prions, viroids, and free DNA). On the other hand, when the set of microorganisms living, thriving and interacting with a particular plant organ, they are able to perform different activities, and thus can be defined as microbiome.

Plant growth-promoting microorganisms (PGPM, an acronym for 'plant growth promoting microorganisms) are microorganisms that are typically found in the rhizosphere and are capable of boosting plant growth and development. Actually, these PGPMs can belong to different categories of microorganisms, in particular they can be protozoa, bacteria and fungi that are mainly involved in solubilizing soil nutrients, producing growth regulators, counteracting phytopathogenic

organisms and reducing the magnitude of abiotic stresses. In addition, they can play a role in improving soil structure and can be used in bioremediation of polluted soils, due to their ability to sequester heavy metals and degrade xenobiotic compounds (Tarkka et al., 2008; Prasad et al., 2019). At the same time, interacting with plant roots, their growth and proliferation is supported by the low molecular weight carbon compounds that are released by the plants themselves (Prasad et al.,2019).

Bacteria are the most prevalent microorganisms in the rhizosphere, although only a small percentage of them, perhaps 2 to 5%, support plant growth (Fig. 5). In the latter situation, they are known as PGPR. Kloepper and Schroth coined the name PGPR in 1978 to describe non-pathogenic, helpful bacteria that colonize plant seeds and roots to promote plant growth.

They can exist in their natural state or as symbionts. In the first case, they generally establish themselves on the rhizoplane or in the spaces between root cortex cells and belong mainly to the genera *Agrobacterium, Azotobacter, Azospirillum, Azomonas, Bacillus, Burkholderia, Herbaspirillum, Enterobacter, Erwinia, Klebsiella, Pseudomonas,* and *Serratia*.

In the second case, however, the bacteria live in structures within root cells or in structures formed by the co-participation of bacterial and root activity and belong mainly to the genera *Rhizobium, Bradyrhizobium, Mesorhizobium,* and *Sinorhizobium,* which establish symbiosis with leguminous plants, and the genus *Frankia*, which instead establishes symbiosis with woody species (both tree and shrub) belonging to families other than *Leguminoseae*.

There is also a plurality of species belonging to the fungi kingdom that are beneficial to plants. Among the fungi that have shown positive effects on plant-growth, we distinguish mycorrhizal fungi and non-mycorrhizal fungi. Mycorrhizal symbiosis is a mutualistic association between plants and soil fungi established at the level of the root system, colonized by the structures of the fungus: the fungus receives from the plant carbohydrates derived directly from photosynthesis in exchange contributes for mineral elements and water absorbed from the surrounding soil through its extensive hyphal system. Mycorrhizal symbiosis is widespread in nature, affecting between 70 and 80 % of plant species: It is estimated that there are around 50 000 species of mycorrhizal fungi that form symbiotic associations with around 280 000 plant species (van der Heijden et al., 2015).

Mycorrhizae are important in both natural and agro-ecosystems because they regulate carbon and nitrogen cycles and influence soil fertility. We differentiate ectomycorrhizal fungi (EMF), arbuscular

mycorrhizal fungi (AMF), ericoid mycorrhizae, and orchid mycorrhizae based on morphological-structural, physiological, and functional characteristics; around 74% of plants establish AM-type relationships, whereas just 2% are EM (van der Heijden et al., 2015). This type of symbiosis can only be formed by *Brassicaceae, Juncaceae, Caryophyllaceae, Cyperaceae, and Chenopodiaceae*.

The diversity of species and functional groups is strongly associated with soil health and ecosystem multifunctionality (Wagg et al., 2014), and the ability of the soil to respond to disturbance is influenced by the resistance and resilience of the soil microbial community (Griffiths and Philippot, 2013). Interactions between microorganisms mediate the transfer of nutrients and energy to higher trophic levels (microbial loop); in addition, many other ecological services rely on this interaction and thus on the preservation of microbial biodiversity, such as disease control (suppression of unwanted organisms), detoxification of harmful chemicals, control of local microclimate, and regulation of local hydrological processes (Altieri, 1999).

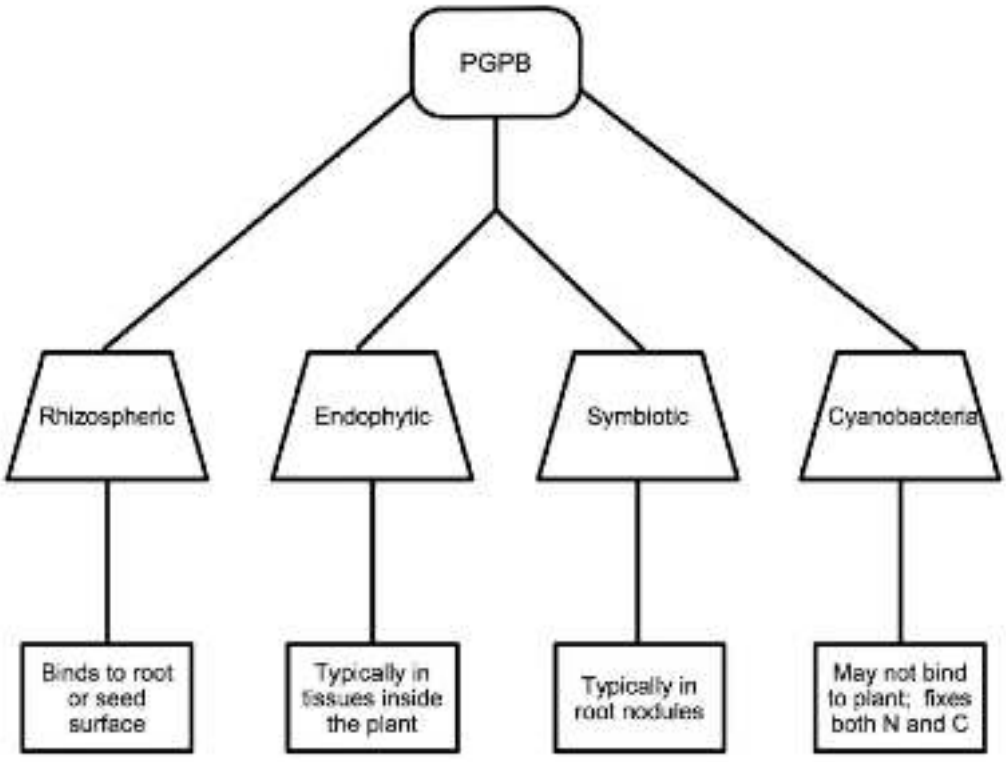

Fig. 5 Different kind of PGPR and their interaction with the host plant

1.4.1 PGPR's Mechanisms of action

As described in the section above, the most investigated rhizobacteria functions are those that affect plants and soil properties, particularly those related to nutrient cycle, symbiosis, pathogens, and plant growth promotion.

The key processes through which PGPRs enhance plant growth and development are listed below. They are many and frequently engaged at the same time, making them inextricably linked (Ahmed et al., 2019; Olanrewaju et al., 2017):

- The contribution in nitrogen fixation.
- The increased solubilization of nutrients, resulting in increased bioavailability of minerals such as phosphorus and other trace elements.
- The production of hormones such as auxins, cytokinines and gibberellins.
- The reduction of ethylene production, which allows plants to develop longer roots and anchor themselves better during the early stages of growth.
- The production of siderophores.
- The synthesis of antibiotics and other substances that can reduce pathogen activity.
- The promotion of the functioning of mycorrhizal symbiosis (Mycorrhizal helper bacteria, MHB).

In detail, some processes are direct, while some others are indirect (Fig. 6). Direct stimulation includes nitrogen fixation, phytohormone production, and mineral solubilization; whereas indirect stimulation includes antibiotic production, iron chelating molecules, and the synthesis of extracellular enzymes to hydrolyze fungal cell wall and other organisms that may pose a threat (Van Loon, 2007). Indirect effects are thus more closely tied to the biocontrol activities of pathogenic bacteria and, for this reason, will be better described in the related section.

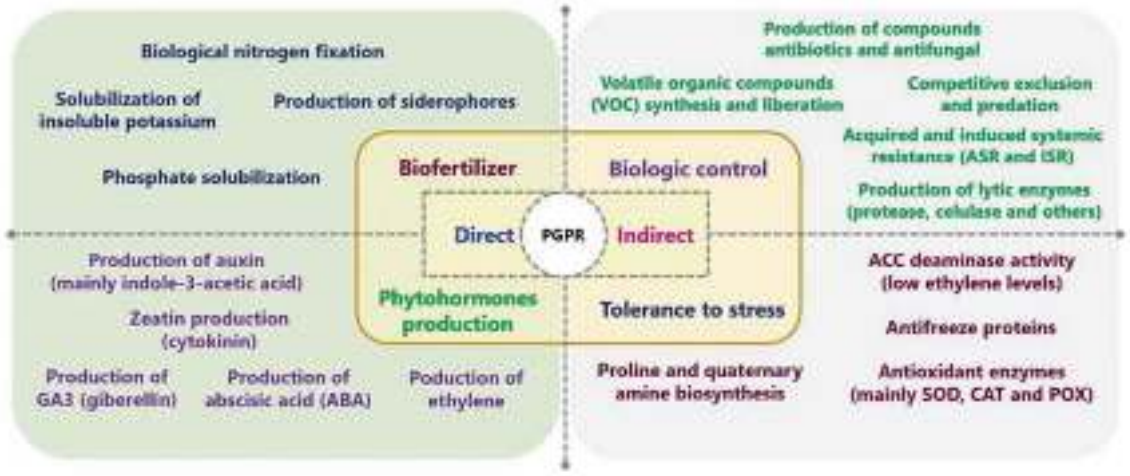

Fig. 6 Mechanisms used by plant growth promoting rhizobacteria.

1.4.2 Direct Mechanisms

Nitrogen fixation

One of the major nutrients necessary for the growth of all living organisms including plants and bacteria is nitrogen. Despite abundance of nitrogen in the earth's atmosphere, ~80 %, nitrogen must first be reduced to ammonia, before it can be metabolized by plants to become an integral component of proteins, nucleic acids and other biological molecules. Sadly, no plant species exists that can effectively manage the conversion of climatic nitrogen into ammonia and provide immediately for its growth. Ammonium (NH_4^+) and nitrate (NO_3^-) are the two forms of nitrogen that plants can use (Lugtenberg et al., 2013). Biological nitrogen fixation, also known as diazotrophy, is the process of converting atmospheric nitrogen into forms that can be assimilated (Lugtenberg et al., 2013).

This conversion requires high energy input because the N_2 triple bond is extremely stable (Fig 7).

As a result, contemporary agriculture, especially in more developed countries, relies heavily on the use of nitrogen fertilizers derived at the expense of petroleum. Production of chemical nitrogen fertilizers depletes nonrenewable resources and poses human and environmental risks (Glick, 2012). More in detail, the primary mechanism by which air nitrogen can be converted into ammonia using an unexpected catalyst called nitrogenase, is known as biological nitrogen fixation (BNF) (Gaby and Buckley, 2012). To date, all nitrogenases found have two oxygen-sensitive components. Component I is a complex made up of two identical protein subunits (approximately 50,000 Daltons each), two

identical protein subunits (about 60,000 Daltons each), 24 molecules of iron, 2 molecules of molybdenum, and FeMoCo, an iron-molybdenum cofactor. Component II contains two protein subunits (about 32,000 Daltons each, that diverge from the protein subunits found in component I) as well as a number of related iron compounds. The catalysis of nitrogen to ammonium ion requires both components I and II, a compound of magnesium, ATP, and a source of reducing equivalents. . Bacterioid respiration requires oxygen (Glick et al.,2012), nitrogenase can convert the gas acetylene to ethylene in addition to fixing nitrogen.

Both components (I and II) are extremely sensitive to oxygen. (Gupta et al., 2012). Thus, the concentration of oxygen is a significant component in regulating how much nitrogen a rhizobial strain fixes. On the other hand, oxygen is also a nitrogenase inhibitor and a negative regulator of *nif* gene expression (genes involved in the transcription of proteins that are directly involved in the nitrogen-fixing process).

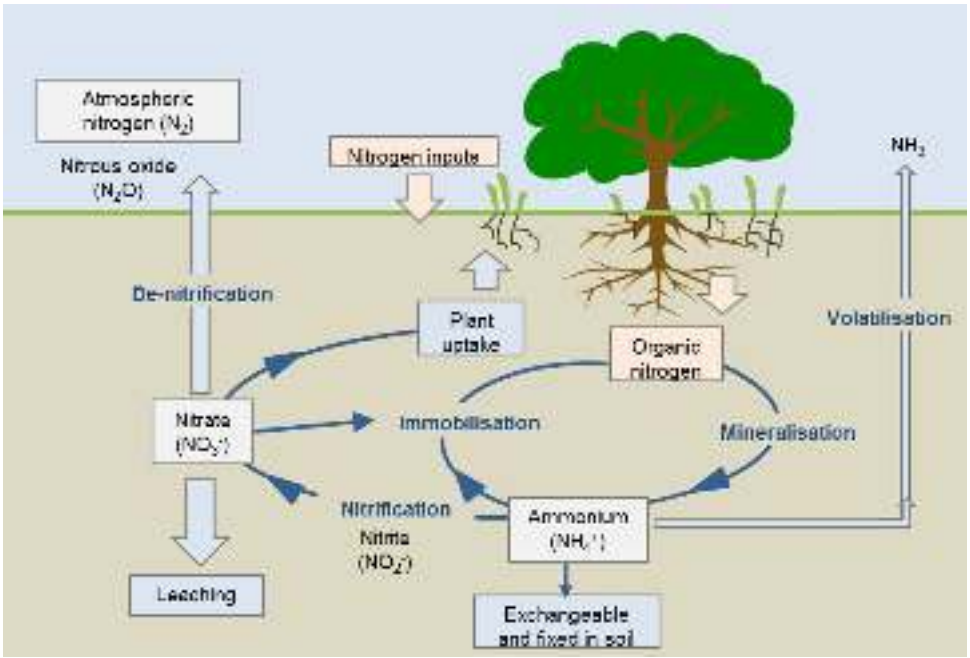

Fig. 7 The nitrogen cycle's stages. The quantity of nitrogen available for plants to absorb is determined by the nitrogen cycle, which describes how nitrogen moves through different forms in soil. Agric.wa.gov.au/soil-carbon/immobilisation-soil-nitrogen-heavy-stubble-loads

Among the great variety of bacteria that can fix nitrogen, the following are the bacteria considered to have potential to substitute agricultural fertilizers:

Cyanobacteria are all able to do photosynthesis (fixing the atmospheric CO_2), but only a subset of are able to do nitrogen fixation. Several research have been published on the use of dried cyanobacteria as a fertilizer to inoculate soils and improve fertility. A great number of studies have been conducted in which cyanobacteria have been introduced into rice fields, with the conclusion that the nitrogen that they fix and release may be taken up and used by the rice plants. (Mishra et al., 2019).

Some cyanobacteria, such as *Nostoc* and *Anabaena*, are made up of lengthy chains of two types of cells: vegetative cells and heterocysts. The vegetative cells, which account for roughly 90-95% of the total number of cells, are photosynthetic, whereas the bigger and thick-walled heterocysts fix nitrogen. Nitrogen is often carried as glutamine, but carbon may be carried as sucrose.

Fixed nitrogen is frequently regarded as the most important growth constraint for rice. In addition to rice, other crops such as vegetables, wheat, sorghum, corn, cotton, and sugarcane are cultivated in many tropical and subtropical regions employing cyanobacteria as a biofertilizers. In addition to fixed nitrogen, cyanobacteria may assist crop plants by producing a variety of growth-promoting substances such as gibberellins, auxins, vitamins, free amino acids, and different carbohydrates and sugars. Furthermore, several cyanobacteria can solubilize inorganic phosphate and make it available for crop growth. (Mishra et al., 2019).

Given their ability to fix both carbon and nitrogen, there has recently been a lot of interest in using cyanobacteria as microbial cell factories as a bioenergy production alternative.

The following are some of the benefits of employing cyanobacteria to synthesis various biofuels:

1) they grow relatively quickly,

2) they can be grown throughout the year under favorable weather conditions,

3) they can be grown in brackish water and on non-arable land,

4) they can sometimes be grown on wastewater,

5) they do not require fertilizers, pesticides, or herbicides, and,

6) after the biofuel has been extracted, the residual biomass may be useful as cattle feed (Zhara etal.,2020).

Cyanobacteria development on a big scale is frequently carried out in open tanks that can be contaminated. While no commercial products have yet been produced in the wild or with genetically modified cyanobacteria, several products, including ethanol, isobutyraldehyde, isobutanol, 1-butanol, isoprene, hydrogen, fatty acids, and fatty alcohols, have been produced on a small scale under laboratory conditions (Glick, 2012).

Rhizospheric genera, can be divided in **symbionts**, such as *Rhizobium, Bradyrhizobium, Sinorhizobium*, and *Mesorhizobium* with leguminous plants, and *Frankia* with nonleguminous trees and bushes; and **no symbiotic**, such as *Azoarcus, Azotobacter, Acetobacter, Azospirillum, Burkholderia, Diazotrophicus, Enterobacter, Gluconacetobacter, Pseudomonas* (Kundan et al., 2015; Gupta et al., 2012).

It has been observed that the symbiosis between *Rhizobium* and legumes start from radical exudates that secrete flavonoid or isoflavonoid components, which then activate specifically *nod* genes, another type of gene that are involved, as *nif* genes, in the process of fixation and that, in bacteria, are able to induce the nitrogen-fixing symbiosis (Van Loon, 2007). Once the bacteria emit nod factors, the nodules can be formed, the plants can sense them and respond to. In particular, when bacteria enter symbiosis, a plasma membrane of plant starting to originate coats to them, the bacteria enter symbiosis, allowing them to establish a strong bond with the cells of the host plant. In this moment, the volume of bacteria can rise by up to 30 times, as a result (Saeed et al., 2021).

Phosphate Solubilization

Plant growth needs large amounts of phosphorus (P), which is essential for cell membranes (lipids, and a few polysaccharides) and nucleic acids. Fortunately, phosphorus is present in most soils (400-1,200 mg/kg of soil) (Glick, 2012), even if in many soils the amount of soluble phosphorus is extremely low (around 1 mg/kg soil)(Goldstein, 1994). This is because the large amount of insoluble phosphorus in the soil cannot be absorbed directly by plants, thus limiting their growth. Insoluble phosphorus can be found in both inorganic and organic forms. Microorganisms are essential to the

biogeochemical cycle of phosphorus (Fig 8) and, as such, play an important role in mediating phosphorus availability to plants (Walia et al., 2017). Except for *Aspergillus* and *Penicillium* species and all mycorrhizal fungi, the vast majority of fungus are non-phosphate solubilizers. Phosphate-solubilizing bacteria obtained from various soils include *Rhizobium, Klebsiella, Mesorhizobium, Acinetobacter, Erwinia, Achromobacter, Enterobacter, Micrococcus, Pseudomonas,* and *Bacillus*. In particular, among these*, Mesorhizobium mediterraneum* and *Mesorhizobium ciceri* are two species of nodulating chickpea that are noted for their strong phosphate-solubilizing effectiveness (Walia et al., 2017).

The majority of the organic phosphorus in soil (usually 30-50% of total) occurs in the form of inositol hexaphosphate (phytate). Endophytic P-solubilizing bacterial populations in plant tissue have been observed to range between 10^2 and 10^4 viable bacteria per gram (Walia et al., 2017). Plant roots produce very low amount of phytases, the enzymes that break down phytate, hence is not accessible to plants. However, phytate is easily degraded by a variety of microorganisms (both bacteria and fungi). Other types of organic phosphate present in soil, in addition to phytate, include phosphomonoesters and phosphotriesters, which can be broken down by different phosphatases. Rocks are the main sources of phosphorus. The inorganic phosphorus is present in minerals such as apatite. Apatite is a phosphate mineral group that contains hydroxyapatite, fluorapatite, and chloroapatite. Apatite is frequently processed into a powder and used as a phosphorus source in fertilizers. Inorganic phosphorus is typically dissolved by plants and bacteria via the synthesis and secretion of low molecular weight organic acids such as gluconic, citric, lactic, 2-ketoglucic, oxalic, tartaric, and acetic acids. These organic acids operate as chelating agents, solubilizing phosphorus- containing minerals, while also sometimes solubilizing other nutrients.

A significant portion of the soluble inorganic phosphorus employed as a chemical fertilizer by farmers, is immobilized immediately after application and contributes to eutrophication of waters (the outflow of nutrients from fields into water bodies where these nutrients can promote algal growth). Due to the fixation of P by free metal ions in the soil, leaching, and runoff, all of which result in P loss, chemical fertilizers have a use efficiency of less than 30%. (Glick, 2012).

The effectiveness of plant and microbial phosphatases in depleting organic P in the rhizosphere and increasing P uptake by plants has been well proven (Rodriguez et al., 2006).

P is taken up by cells in a variety of ways, although the majority is as HPO_4^{2-} or $H_2PO_4^-$ (Beever and Burns, 1981). PGBPs convert phosphorus into a soluble form that can be used by plants. Most of the phosphate taken by a cell is, in fact, in the form of HPO_4^{2-} or PO_4^-. Phosphate solubilizing bacteria such as *Azospirillum, Bacillus, Burkholderia, Erwinia, Pseudomonas, Rhizobium* and *Serratia* convert insoluble phosphates into soluble form through acidification, chelation, exchange reactions and production of gluconic acid (Sharma et al., 2013; Li et al., 2020). Phosphatase mineralizes the majority of phosphorus-containing organic molecules (Rodrguez and Fraga, 1999; Alori et al., 2017). The primary microbiological source of phosphatase activity in soil is thought to be there (Liang, JL et al., 2020). Hydroxyl ions, organic acids, protons, siderophores, and carbon dioxide are released by phosphate soluble microorganisms. By chelating cations or lowering pH, organic acids with their carboxyl and hydroxyl ions liberate phosphorus (Yuquan et al., 2018). The same bacterial strain can occasionally naturally solubilize both organic and inorganic forms of phosphorus (Chaiharn and Lumyong, 2011).

Thus, P-solubilizing microorganisms (PSM) is regarded as an additional strategy for lowering chemical use in agriculture/cultivation. (Musarrat and Khan, 2014; Walia et al., 2017). It has been shown that many strains are crucial for appropriate mineral phosphate solubilization and for adequate circulating phosphatase boosted the yield of tomatoes, cauliflower, capsicum, apples, apricots, and so on in field studies (Walia et al., 2017). Another method for using PSMs as microbial inoculants is to use mixed or co-inoculation with other microorganisms. The co-inoculation of phosphate-solubilizing *Pseudomonas striata* and *Bacillus polymyxa* strains with an *Azospirillum brasilense* strain resulted in a significant shift in grain and dry matter yields, as well as an increase in N and P uptake (Alagawadi and Gaur, 1992). Similarly, in pot and field studies, phosphate-solubilizing *Agrobacterium radiobacter* coinoculated with nitrogen fixer *Azospirillum lipoferum* yielded higher grain production than single inoculations (Belimov et al., 1995). Similarly, Pandey and Maheshwari, (2006) investigated the interaction for stimulating plant growth of two species, *Burkholderia* MSSP and *Sinorhizobium meliloti* PP3, both of which may create IAA and solubilize inorganic phosphate.

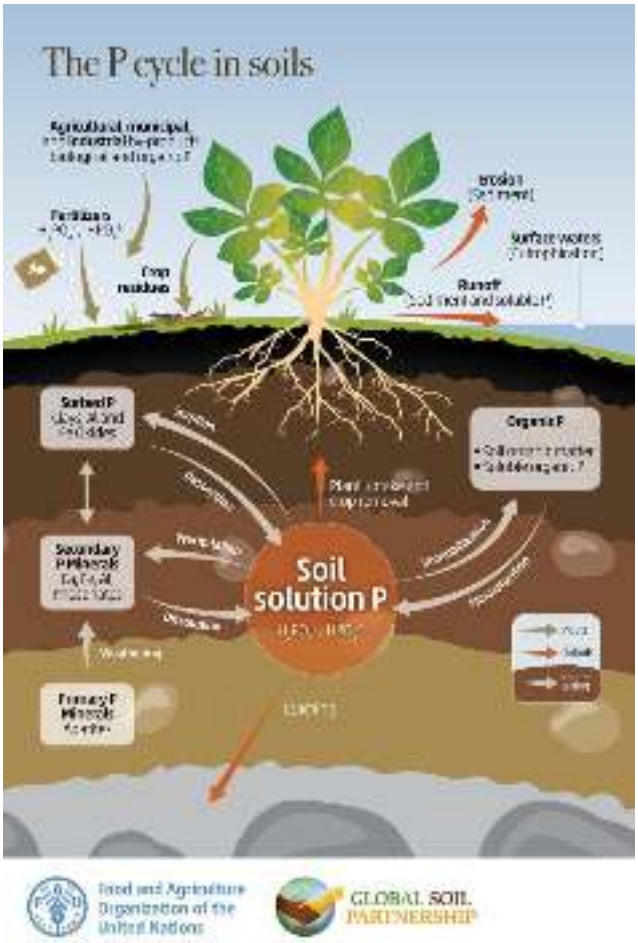

Fig. 8 Movement of phosphorous in soil FAO. 2022. *Soils for nutrition: state of the art.* Rome.

Phytohormone Production

A plant's physiological activity is controlled by one or more plant hormones (phytohormones), small molecules which include auxin, cytokinin, gibberellin, abscisic acid, ethylene, salicylic acid, jasmonic acid, and brassinosteroids (Maheshwari et al., 2015). Phytohormones help maximize plant growth rates and development by influencing numerous aspects of a plant's environmental and developmental responses.

Many soil bacteria, in addition to plants, are capable of generating and/or modifying the levels of certain of these hormones. (Glick, 2012) The study of this beneficial interaction has led to the identification of different compounds of microbial origin that mimic the effect of endogenous plant hormones. (Keswani et al., 2020).

The potential of rhizosphere bacteria to impact plant hormonal status via bacterial hormone synthesis or metabolism is regarded as an essential strategy for promoting plant growth and productivity. However, inoculating these bacteria into the plant rhizosphere can have either positive or negative consequences depending on the bacterial effects on hormone composition and amount in planta as well as the environmental conditions under which the plants grow(Kudoyarova et al., 2019). PGPB are known to produce indole-3 acetic acid (IAA,), cytokinins, gibberellins, and ethylene stress-mediating enzymes, like ACCdeaminase.

Auxin. The phytohormone that has gotten the most attention is auxin. Auxins are involved in the response of root and shoot growth to light and gravity, differentiation of vascular tissue, apical dominance, initiation of lateral and adventitious roots, stimulation of cell division, and elongation of stems and roots (Glick, 2012). Auxin levels are influenced not only by plant, but also, by bacterial synthesis and breakdown, as well as conjugate formation. It is assumed that auxin conjugates play crucial roles as storage forms for the active plant hormone IAA (Keswani et al., 2020).

IAA was found at the end of the nineteenth century, it is only recently that the multiple routes of IAA biosynthesis have been elucidated, thanks to the complete sequencing of a significant number of bacterial genomes (Glick, 2012). In fact, hormones released by plant cells or by rhizobacteria ensure that practically all of the communication in plant cells (Maheshwari et al., 2015).

There are several naturally occurring auxins, but IAA is by far the most abundant and it is produced and released by around 80% of rhizospheric bacteria (Patten and Glick, 1996; Spaepen et al., 2007). This is most likely an indicator that IAA biosynthesis is an essential (or at least extremely important) component of these bacteria's functioning and metabolism, possibly for purposes other than plant growth promotion (Glick, 2012). Some IAA precursors, such as indole-3-acetonitrile, may also have auxin action. Indole-3-butyric acid, which is produced from IAA, is another example of endogenous auxin (Glick, 2012). IAA in its free form accounts for only up to 25% of the total quantity of IAA in a plant, but the optimal levels can vary depending on the tissue and plant species analyzed (Glick 2012; Spaepen et al., 2007).

There is emerging evidence that a number of soil bacteria, not only PGPB but alsophytopathogens, include multiple IAA biosynthetic pathways. In particular, it has been reported that PGPB belonging to *Azospirillum, Aeromonas, Azotobacter, Bacillus, Paenibacillus, Burkholderia, Enterobacter, Pantoea, Pseudomonas* and *Rhizobium* genera produce IAA.

The generation of phytohormones like auxins is one of PGPB's advantageous effects on plant physiology, particularly when the batters colonize the plant's neurons during the interaction between the plant and the microbiome. (Olanrewaju et al., 2017; Maheshwari et al., 2015).

Some of the numerous IAA production pathways are found in both PGPB and pathogenic bacteria. For instance, the indole-3-acetamide (IAM) pathway is used to produce IAA, and pathogens have focused the most research on this pathway. For example, the genes in *A. tumefaciens*, which infects host plants and causes crown gall formation, are not functional inside the bacteria, but are carried into the plant cell and integrated into the plant chromosome, resulting in extremely high levels of IAA synthesis and uncontrollable tumor growth (Glick, 2012).

Briefly, IAA is synthesized in two steps from a tryptophan precursor: tryptophan is converted into indole-3-acetamide intermediate, thanks to the activity of an enzyme known as tryptophan 2-monooxygenase; secondary, the indole-3-acetamide is hydrolyzed to the final IAA product following the reaction catalyzed by an IAM specific hydrolase/amidase.

The second step, and this route is shared by many strains of PGPB, involve an aminotransferase that first deaminates L-tryptophan to indole-3-pyruvic acid (IPA). Subsequently, the indole-3-pyruvic acid is converted by a decarboxylase into indole-3-acetylaldehyde (IAAld), which is then oxidized to IAA by aldehyde dehydrogenase, mutase, or oxidase enzymes.

There is an alternative pathway in which tryptophan is directly converted into indole-3-acetylaldehyde by a reaction know like tryptophan side chain oxidase (TSO). *Azospirillum brasilense* is a nitrogen-fixing gram-negative bacteria. It is a rhizosphere resident that connects with grass and cereal roots. It promotes plant growth through a variety of methods,

including increasing the number of root hairs and lateral roots as a result of IAA production. This bacterium, for example, primarily generates IAA via the IPA pathway.

The indole-3-acetonitrile (IAN)/indole-3-acetaldoxime (IAOx) pathway can also be used to produce IAA. Tryptophan is converted into indole-3-acetaldoxime via oxidoreductase. An indoleacetaldoxime dehydratase converts the intermediate indole-3-acetaldoxime into indole-3-acetonitrile. The intermediate indole-3-acetonitrile is then transformed to IAA by a nitrilase enzyme in a single step, or by a nitrile hydratase and an amidase in a two-step process (Glick, 2012)

Cytokinins are a class of chemicals with adenine-like structures that promote cell division and other comparable actions. The ability of PGPR to synthesize cytokinins (CK) has received far less attention than auxin production. While cytokinins clearly have direct effects on different plant processes (for example, encouraging cell division), the balance of auxin and cytokinin levels is frequently regarded as a major regulator of plant organogenesis and root architecture (Kudoyarova et al., 2019). Because some PGPR can produce both of these hormones (Vacheron et al., 2013), the tissue auxin to cytokinin ratio may be crucial in predicting plant response to rhizobacterial inoculation.

Kinetin was the first cytokinin discovered in 1955 and was named for its capacity to stimulate cytokinesis or cell division. Plants' responses to extrinsic stimuli, such as light and nutrition availability, as well as to biotic and abiotic challenges are influenced by cytokinins, which regulate plant cell differentiation (Maheshwari et al., 2015; De Rybel et al., 2016). Additionally, they also control apical dominance, root elongation, seed germination, nutrient signaling, and the growth of flowers and fruits.

Based on limited data, it is assumed that PGPB produce lower cytokinin levels than phytopathogens, so that the PGPB's influence on plant growth is considered as normally stimulatory, whereas the pathogens' effect is "overstimulatory" (i.e., tumor-inducing) (Glick, 2012). According to some reports, introducing cytokinins to some plants can boost resistance to the phytopathogen *P. syringae pv. tabaci*. (Glick, 2012).

Some PGPB bacteria, including *Azotobacter* spp., *Rhizobium* spp., *P. agglomerans*, *Rhodospirillum rubrum*, *P. fluorescens*, *Bacillus subtilis*, and *Paenibacillus polymyxa*, have

been found to produce cytokinins in cell-free medium. Furthermore, cytokinin biosynthetic genes have been found in *A. tumefaciens, P. savastanoi, E. herbicola pv. gypsophilae*, and *Streptomyces turgidiscabies* (Glick, 2012).

Cytokinin-producing bacteria may restrict root development and hence salt uptake, but the significance of bacterial cytokinins in salt stress resistance is still mainly unknown, due to a lack of research. The influence of rhizobacterial cytokinin production on plant drought response was also investigated, because increased leaf area and larger stomatal opening mediated by cytokinins could hasten soil moisture depletion (Kudoyarova et al., 2019).

When cultured under osmotic stress conditions, a quarter of the pseudomonads isolated from the rhizospheres of diverse crops (*Pennisetum glaucum, Helianthus annuus, Zea mays*) planted in 25 arid and semi-arid locations in India were able to produce cytokinins. In Patel and Saraf, (2017), cytokinins were generated by *Pseudomonas stutzeri*, *Stenotrophomonas maltophilia*, and *Pseudomonas putida*. The participation of cytokinins in the plant growth-promoting impact of *Bacillus megaterium* was revealed by Ortiz-Castro et al., 2009. (Kudoyarova et al., 2019). Under stress conditions, particularly water stress at the grain-filling stage, it was observed that stay-green genotypes have the potential to exhibit increased tolerance, which was ascribed to an increased concentration of cytokinin in the xylem sap (Borrell et al., 2000). Zhang et al., (2010) demonstrated that cytokinin-over-expressing transgenic cassava plants were more drought tolerant than wild-type plants.

The invention of a simple and reproducible method for testing the ability of bacterial strains to manufacture cytokinins is an important first step in this direction. Of course, cytokinin concentrations can be easily measured using HPLC. Despite the fact that this technology is available in many labs, it is not always available in developing nations and is not easily adaptable to assaying huge numbers of biological samples. It should also be possible to detect naturally occurring bacterial cytokinin overproducers using HPLC. This is significant because the benefits of high levels of exogenous cytokinin in treated plants can only be obtained if the cytokinin is introduced as a foreign chemical or as part of a naturally occurring PGPB (Kudoyarova et al., 2019). As an alternative, some researchers have created a simple biological assay that could be useful in this attempt. Despite its simplicity, this non-quantitative technique can detect cytokinin quantities (Glick, 2012). Researchers should be

able to utilize this assay in the future to screen vast numbers of bacteria for the existence of cytokinin minus mutants and then supplement those mutants with clones that encode the manufacture of active cytokinin. (Kudoyarova et al., 2019).

Gibberelin. Another important plant growth regulator is gibberellin, closely related to *ent*-kaurene-derived diterpenoid phytohormones. Gibberellin production is common among soil bacteria, especially PGPB (Keswani et al., 2022) .

For example, gibberellin-like compounds have been detected in the cell-free medium of the heterotrophic bacteria *Azotobacter chroococcum* (Lenin and Jayanthi, 2012), *Pseudomonas fluorescens* (Lenin and Jayanthi, 2012), *Pseudomonas aeruginosa* (Katznelson and Cole, 1965), *Azotobacter chroococcum* (Brown and Burlingham, 1968), *Azotobacter vinelandii* (Lee et al., 1970), *Azotobacter paspali* (Barea and Brown, 1974), *Azospirillum brasilense* (Janzen et al., 1992); *Azospirillum lipoferum* (Bottini et al., 1989; Lenin and Jayanthi, 2012), *Thiobacillus novellus* growing autotrophically on thiosulfate (Gairola et al., 1972), *Pseudomonas monteilii* (Pandya and Desai, 2014); and the symbiotic bacteria *Rhizobium meliloti*, *Rhizobium trifolii*, and *Rhizobiumleguminusarum* (Katznelson and Cole, 1965). Gibberellic acid enhanced antioxidant enzyme activity by lowering the levels of reactive oxygen species (ROS) that contribute to better growth under stress (Manjili et al., 2012)

The first report on the characterization of gibberellins in bacteria was made by Atzorn et al., (1988), who demonstrated the existence of several gibberellins (GA1, GA4, GA9, and GA20) in gnotobiotic cultures of *Rhizobium meliloti* using only physicochemical approaches such as gas chromatography-mass spectrometry (GC-MS). In the following years, gibberellin production has been identified in various bacterial species, including *Azospirillum* spp., *Bradyrhizobium diazoefficiens*, *Acetobacter diazotrophicus*, *Herbaspirillum seropedicae*, *Bacillus* spp (Keswani et al., 2022). Members of the last group, for example, can create a wide range of physiologically active compounds, some of which are possibly inhibitory of fungal development. These bacteria's released chemicals serve a vital role in plant growth promotion. Gibberellins are released by many *Bacillus* species and have roles in plant cell growth, division, and enlargement in both symbiotic and non-symbiotic roots (Keswani et al., 2022). Experimental evidences outline that microbial gibberellins produce maize longitudinal

leaf growth, enhanced rooting of maize seedlings, dwarf maize, dwarf pea, and lettuce hypocotyl growth, lettuce hypocotyl extension and a rise in root and shoot length in wheat (Triticum aestivum L.) and chickpea (Keswani et al., 2022).

At the present time there are 136 fully characterized GAs, designated gibberellin A_1 (GA_1) through GA_{136}, that have been identified from 128 different species of vascular plants, and also from seven bacteria and seven fungi (Sponsel and Hedden, 2010).

The gibberellin biosynthesis pathways evolved independently in plants, fungi, and bacteria, with distinctions at the enzymatic and genetic levels, implying that horizontal gene transfer events of plant biosynthetic genes to microorganisms did not take place (Keswani et al., 2022; Salazar-Cerezo et al., 2018). The gibberellin biosynthesis pathway has been widely investigated in plants, and studies in fungal strains have provided new insights.

Gibberellin biosynthesis has been widely studied in the plant pathogenic fungus *Fusarium fujikuroi* (telemorph, *Gibberella fujikuroi*) as well as other fungi such as *Phaeosphaeria* sp. strain L487 (Salazar-Cerezo et al., 2018). The metabolic pathway for GA synthesis begins with geranyl-geranyl diphosphate (GGPP) and proceeds through isopentenyl diphosphate (IPP), the 5-carbon building block for all terpenoid/isoprenoid molecules (Salazar-Cerezo et al., 2018). The basic isoprenoid unit IPP is produced in most plant's green tissue via two pathways: the mevalonic acid (MVA) pathway in the cytoplasm; and the methyl erythritol phosphate (MEP) pathway in the plastids (Keswani et al., 2022; Salazar-Cerezo et al., 2018). The MVA pathway, which provides IPP for the synthesis of all terpenoids, including GAs, is involved in the biosynthetic route in fungi. In higher plants, GAs are typically produced via the methylerythritol phosphate (MEP) pathway. Trans-geranylgeranyl diphosphate is converted into bioactive GA in this mechanism (GGDP). Terpene synthases (TPSs), cytochrome P450 monooxygenases (P450s), and 2-oxoglutarate-dependent dioxygenases (DODs) are three groups of enzymes employed in the MEP route to produce GA from GGDP (2ODDs). The MEP route consists of eight phases.

The plant Arabidopsis and the fungus Gibberella fujikuroi have separate GA routes and enzymes. P450s in fungi serve tasks similar to those of KAOs in plants. In fungi, the function of CPS and KS in plants is fulfilled by a single enzyme, CPS/KS. The GA biosynthesis genes are

present on one chromosome in fungus, but on many chromosomes in plants (Tudzynski et al., 1998).

While only recently, the route in bacteria is beginning to be understood (Salazar-Cerezo et al., 2018)

Ethylene is a gaseous hormone that is active at very low concentrations (0.05 mL L 1) and it is defined as "stress hormone," increases in its concentration can be observedduring various abiotic and biotic stresses. The production of ethylene is regulated by a large number of factors including temperature, light, gravity, nutrition, and other plant hormones (Glick et al., 2005). Ethanol accumulation in response to stress may increase plant tolerance or worsen stress response symptoms and senescence. PGPR function has been examined under both stress and nonstress situations, and it frequently gives greater growth stimulation under adverse conditions, such as drought stress (Rubin et al., 2017).

The 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase generated by PGPB degrades ACC, an immediate ethylene precursor in higher plants, into -ketobutyrate and ammonium. Some PGPRs use ethylene to improve plant tolerance to stress: the PGPRs produce ACC deaminase, which lowers ethylene synthesis in plants. (Backer et al., 2018). Several forms of stress are relieved by ACC deaminase producers, such as effects of phytopathogenic microorganisms (viruses, bacteria, and fungi etc.) and resistance to stress from polyaromatic hydrocarbons, heavy metals, radiation, wounding, insect predation, high salt concentration, draft, extremes of temperature, high light intensity and flooding (Glick, 2012). Bacterial strains with ACC deaminase activity have been found in a variety of taxa, including Acinetobacter, Achromobacter, Agrobacterium, Alcaligenes, Azospirillum, Bacillus, Burkholderia, Enterobacter, Pseudomonas, Ralstonia, Serratia, and Rhizobium, among others (Nadeem et al., 2007; Zahir et al., 2009; Zahir et al., 2010; Kang et al., 2010)

1.4.3 Indirect Mechanisms

Competition

An ecological strategy is using microorganisms to control plant diseases (Glick, 2012). Many plant pathogens, including bacteria, fungus, nematodes, viruses, and insects, are antagonistically affected

by PGPBs (Saharan and Nehra, 2011). The ability to successfully compete with pathogens for nutrients or specific root niches, the production of siderophores, the synthesis of antimycotic and antimicrobial metabolites like antibiotics, hydrolytic enzymes or volatile production like hydrocyanic acid (HCN), and the capacity to induce systemic resistance (ISR) in plants are just a few of the mechanisms used by PGPB to suppress pathogens (Yang et al., 2015), Bhattacharyya and Jha (2012). Plant disease incidence and severity may be reduced through competition between the PGPB and pathogens (Glick, 2012). In addition to establishing systemic resistance in the plant against root and leaf diseases, PGPB can lower disease by causing antagonism between bacteria and pathogens present in the soil, as for pathogens on leaves and roots. Introduced resistance is an induced resistance, in other words, is a rise in the basal level of resistance to multiple diseases simultaneously, which is beneficial in environments where multiple infections are present naturally. Multiple pathogens can exist in natural settings (Van Loon and Glick 2004). Plants are equipped with a variety of active defense mechanisms that can be actively expressed in response to biotic stress caused by pathogens and parasites of all sizes, ranging from tiny viruses to (from microscopic viruses to phytophagous insects).

Iron Acquisition

Iron is required by all living species, including animals, plants, and microbes, as a component of proteins involved in vital functions such as respiration, photosynthesis, and nitrogen fixation. Despite the abundance of iron on the earth's surface, as for the other elements, soil organisms such as plants and microbes cannot readily assimilate enough iron to support their growth because iron in soil is mostly present as insoluble ferric (Fe^{+3}) hydroxides that are only sparingly soluble and cannot be readily transported into cells (Lugtenberg et al., 2013). To address this issue, bacteria, fungus, and some plants produce iron-binding molecules termed siderophores into the soil to scavenge iron. (Glick, 2015; Sayyed et al., 2013).

Siderophores are low-molecular-weight compounds (400-1,000 Da) with three functional, or iron-binding, groups joined by a flexible backbone. Each functional group contains two oxygen or, less typically, nitrogen atoms that bond to iron. Trivalent ferric iron may accommodate three of these bidentate functional groups to generate a six-coordinate complex.

Microbial siderophores often have hydroxamates or catecholates as functional groups; however, additional functional groups such as carboxylate moieties such as citrate and ethylenediamine are also commonly utilized (Khan et al., 2018). A single siderophore molecule may have several combinations of these functional groups. *Azotobacter, Azospirillum, Bacillus, Dickeya, Enterobacter, Klebsiella, Kosakonia, Methylobacterium, Nocardia, Pantoea, Paenibacillus, Pseudomonas, Rhodococcus, Serratia, Streptomyce*s, and others are among the siderophores producers described (Timofeeva et al., 2022).

In general, fungi prefer hydroxamate-type siderophores, whereas bacteria prefer catecholates, which bind iron more securely than hydroxamates (Timofeeva et al., 2022). Moreover, microorganisms that produce siderophores limit the amount of iron available to pathogens, reducing in turn the spread of those organisms (Zhang et al., 2023). Even among several siderophores generated by a single organism, there is tremendous structural variability among the hundreds of known siderophores. The simplest way to describe it is that there are two types of pigments in the world.

Pyoverdin are salicylic acid and cysteine-derived phenolated siderophores. Pyoverdin are water-soluble pigments that turn yellow-green when exposed to ultraviolet light and have a substantially higher affinity for iron (Timofeeva et al., 2022). To become available for metabolism, the bound iron must also be absorbed and released into bacterial or plant cells.

Siderophores rely on proteins since they are hydrophilic and are transported across cell membranes by membrane-bound proteins. Iron is released from the siderophore-iron complex once within the cell and is available for usage in metabolic processes. The siderophore is cleaved enzymatically or ferric iron is converted to ferrous state for this purpose. Researchers have identified, both certain esterases that can cleave siderophores (and liberate iron as a result of reduced affinity) and specific reductases that bind iron for this purpose (Glick, 2012). Siderophore-producing bacteria promote plant growth by either directly delivering iron for plant usage or by eliminating iron from the surroundings of phytopathogens, limiting their competitiveness. (Lugtenberg et al., 2013; Zhang et al., 2023). Some pyoverdin siderophores are synthesized by as many as fifteen enzymes in pseudomonads. Some pyoverdin siderophores, as well as a variety of other proteins, are essential for ferric-siderophore complex transport and the control of siderophore and receptor production. The most basic argument for why microorganisms have expended so much effort to synthesis and

utilize siderophores suggests that these resources are required because iron is required for survival. (Timofeeva et al., 2022).

The ability to collect iron efficiently in an iron-restricted environment and at the expense of other bacteria provides a bacterium with a mechanism to compete for the limited resources of the rhizosphere. The creation of siderophores allows microorganisms to recover ambient iron, although various conditions influence their ability to bind iron (Glick, 2012). The quantity of siderophores produced is an essential consideration. Because the siderophore-iron binding relationship is stoichiometric, the more siderophore molecules there are, the more iron may be bound. The stronger the siderophore's affinity for iron and the faster the rate of connection between iron and siderophore, the more successful the bacterium's iron acquisition. Moreover, the formation of a stable compound with iron is influenced by the pH of the rhizosphere (Glick 2012). The ability to manufacture huge amounts of high-affinity siderophores may not be as crucial for bacterial competitiveness and root colonization in the rhizosphere as the ability to employ a number of different ferric siderophores (Glick 2012).

It may be possible to boost the capacity of these bacteria to assist plant growth by using genetic engineering to broaden the spectrum of siderophores that a PGPB can recognize (Glick 2012).

1.4.4 Actinobacteria.

According to Boukhatem et al., (2022), among the 18 major lineages now recognized under the domain Bacteria (including 5 subclasses, 6 orders, and 14 suborders), the phylum Actinobacteria constitutes one of the greatest taxonomic groups in terms of the quantity and variety of identified species. Its biodiversity, which could have significant biotechnological uses, is reflected in its genetic diversity (Ventura et al., 2007). Physiologically and ecologically, they are aerobic Gram-positive bacteria, but there are also some obligate or facultative anaerobic actinomycetes, as *Bifidobacterium*, *Propionibacterium*, and *Actinomyces* (Sousa and Olivares, 2016*).*

These microorganisms were once considered as intermediate forms between bacteria and fungus. (Lechevalier and Lechevalier, 1967). In fact, a large number of *Actinomycetes* produce pseudomycelium with filaments, or pseudohyphae, that resemble those of fungi but showing a smaller diameter, between 0.5 and 2.0 µ (Silva et al., 2022; Barka et al., 2016; Bhatti et al., 2017). They can have a coccoid shape, similar to *Micrococcus*, a rod-coccoid structure, similar to *Arthrobacter*,

or a fragmenting hyphal form, similar to *Nocardia* spp. While certain actinomycetes, such as *Rhodococcus* and *Streptomyces* species, grow by extending filaments on the surface of the medium but do not generate true pseudomycelia, other actinomycetes, such as *Frankia* and *Streptomyces* species, do not produce any pseudomycelia. Most *Rhodococcus and Mycobacterium* species lack aerial pseudomycelia in general (Zahr et al., 2022). A noticeable number of them also reproduce by sporulation (Fig. 9). Typically, the genomes of the Actinomyces have a high concentration of guanines and cytosines (G+C), especially those that belong to the order *Actinomycetales*, which are distinguished by substrate and aerial pseudomyceliar growth. (Lechevalier e Lechevalier, 1967; Bhatti et al., 2017). Distinct actinomycetes species can be categorized using morphological characteristics of spores. Spores can have a wide range of surface properties and morphologies. Spore shapes include ovoid, reniform, globose, allantoid, and rod-shaped forms. Additionally, the surface ornamentation of spores might be parallel or uneven rugose, smooth, verrucose, hairy, warty, or spiky (Zahr et al., 2022). The process of spore formation in actinomycetes is similar to the process in other Gram-positive bacteria (Kalakoutskii and Agre, 1976). They follow essentially two different modes and, in turn, can be divided in two groups: endogenous and exogenous.

For the spores formed exogenously, frequently found among the actinomycetes, the initial steps include the division of the parental hyphal wall into two layers (Kalakoutskii and Agre, 1976). The spores, in sporangia, young colony a network of pseudomycelia of uniform appearance, which includes the substrate pseudomycelium, are released immediately after the break of the sporangial wall (bearing a sheath surface characteristic of the aerial pseudomycelium of actinomycetes). Aerial hyphae develop directly from the upper substrate pseudomycelium, and sporulation begins shortly after the appearance of the first aerial hyphae (Fig 10).

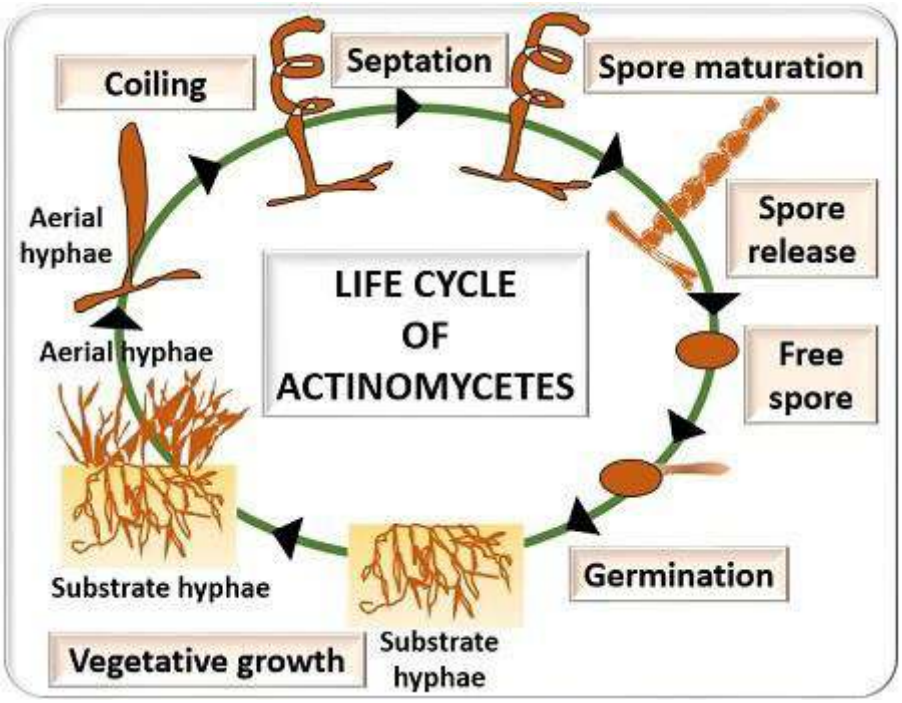

Fig. 9 Schematic representation of the life cycle of sporulating actinomycetes.

The spores formed endogenously, named endospores, are produced mainly by thermophilic actinomycetes and are similar morphologically and chemically to those of *Bacillaceae*. In particular, they contain a thick multilayered wall that surrounds the cortex, cytoplasmic membrane, cytoplasm, and ribosomes. They also contain dipicolinic acid, a chemical compound that plays an important role in the heat resistance. These kinds of spores are mainly found in the genus *Thermoactinomyces* (Kitouni, 2007).

Moreover, there are also some actinomycetes that form structures, such as sclerotids, sporangia, and synemes that do not correspond to either pseudomycelium or spores, whose function is still unknown, (Kitouni 2007; Djaballah, 2010). Sporangia vary greatly in both size and shape. They originate from substrate pseudohyphae or aerial pseudohyphae. Sporangia are a sac-like structure in which spores develop and are held together until released, usually leaving an empty sporangial envelope. Actinosynnema differentiate into substrate mycelium with long, branched hyphae that penetrate the agar forming synnemata. The synnemata, or cornemia, correspond to clusters of hyphae that sometimes coalesce and bear lateral or apical conidia (Kitouni, 2007; Djaballah, 2010; Li et al., 2015).

However, the two fundamental reasons why the Actinomycetes should be regarded as bacteria are (1) the absence of mitochondria and (2) the absence of nuclear membrane. Like bacteria, they reproduce also by division and are susceptible to penicillins and tetracyclines. Moreover, muramic acid (an amino sugar acid, in terms of chemical composition, it is the ether of lactic acid and glucosamine) is a component of their cell wall (Pepper et al., 2015)

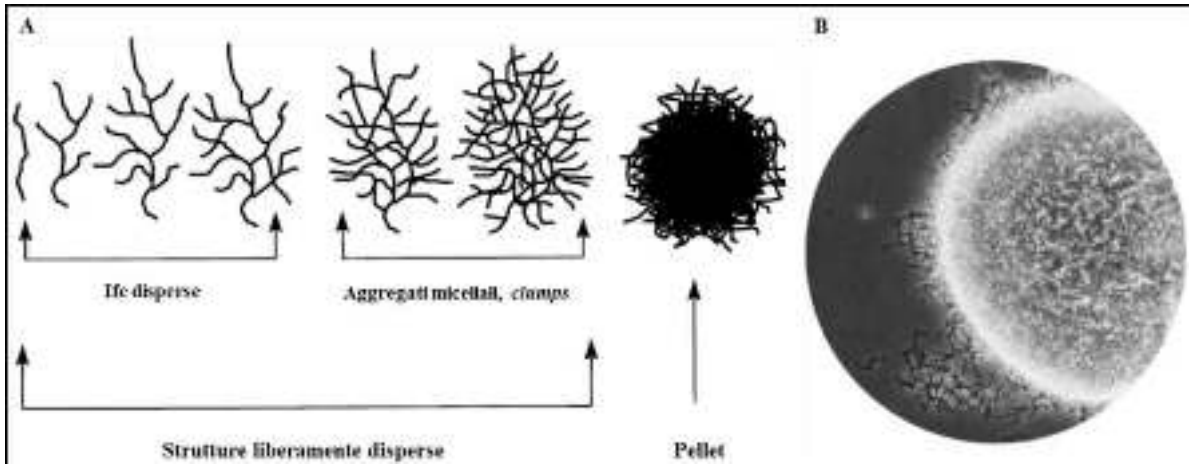

Fig 10. Differentiation of Actinomycetes

Most Actinomycetes are chemoheterotrophs and, therefore, capable of utilizing a considerable amount of nutrients, including various complex polysaccharides. Actinobacteria have been observed to release a large variety of hydrolytic enzymes under natural circumstances, making them a dominating element of the saprophytic community (Jog et al., 2016). According to Ian Pepper et al., (2015), Actinobacteria are the main organisms that break down dead organic materials, particularly lignocellulosic biomass. They show a remarkable ability to produce cellulase, xylanase, lignin peroxidase, and chitinase enzyme cocktail in addition to protease, lipase, pectinase, keratinase, amylase, invertase, and phytase that can trigger as a first step plant biomass degradation, thus processing it into simpler form for a second decomposition step initiated by secondary decomposers (Jog et al., 2016). For this reason, they have the ability to decompose organic compounds of low biodegradability such as hydrocarbons, lignin, humus and various phytochemicals. These properties allow the phylum adaptation to an extensive variety of ecological environments (Stevens et al., 2007; Pepper et al., 2015).

Compared to other microorganisms, Actinomycetes are the most abundant in soil, where their density is generally on the order of 10^6 to 10^9 cells per gram of soil, among which the genus Streptomyces is the most dominant (more than 95 percent of *Actinomycetales* strains isolated from soil) (Williams and Vickers 1988). Both the soil's surface and depths (even more than two meters) can contain them. Although they are often immobile due to being entrenched in the ground, some of them can move because of the presence of flagella (Prudence et al., 2020).

Among the factors that influence their growth are temperature, pH and soil moisture (Flowers and Williams, 1977; Pathom-Aree et al., 2006).

Mesophilic in nature, the optimal temperatures for the growth of actinobacteria are between 25 and 30 °C. However, according to Barka et al., (2016), thermophilic actinomycetes may develop at temperatures between 50° and 60°. There is evidence of the presence of thermophilic Actinomycetes everywhere, from the Mongolian steppes' desert to Argentina's subtropics (Prudence et al., 2020).

Different topographic zones, such as those with marine sediments, wetlands, hyperarid desert soils, and underground forests of cave systems, can be colonized thanks to the diverse metabolic physiology. (Benhadj et al., 2019; Kalyani et al., 2019; Long et al., 2019; Millán-Aguiñaga et al., 2019; Srivastava et al., 2019).

In fact, some of them are obligatory chemoheterotrophs, meaning they require decaying organic matter (dead plants and animals) to survive. Other thermophilic Actinomycetes, like *Acidithiomicrobium sp*. and *Streptomyces thermoautotrophicus*, are obligate chemoautotrophs, but they can only grow on CO_2+H_2 and sulfur, respectively (Prudence et al., 2020). In addition, different nutritive modes such as facultative chemoautotrophy (*Strepyomyces* G26) and facultative methyltrophy (*Amycolatopsis methanolica*) have been observed among thermophilic Actinomycetes.

Soil moisture is another crucial factor in bacterial growth. For example, when moisture is very high, the growth of *Actinomycetes*, especially vegetative growth is favored, particularly when spores are immersed in water (Zviagintsev et al., 2007).

On the contrary, in dry soils where moisture tension is higher, growth is very limited and may even be arrested. In addition, almost all the Actinomycetes grow in neutral pH soils (the range of 6 to 9

ensures good growth) although, a small amount of Streptomyces strains prefer acidic soils (pH 3.5) (Zviagintsev et al., 2007).

Actinomycetes, and *Streptomyces* in particular, constitute one of the most influential groups in the soil microbial population. However, it is now well established that they can live in all types of environments and habitats worldwide (Javed et al., 2021).

In fact, they can also be found in aquatic environments (*Streptomyces, Micromonospora, Rhodococcus,* and *Salinispora*); can live in symbiosis with plants (Frankia spp.); endophytic and actinorrhizal relationships with plants and mycorrhizae (Javed et al., 2021), protecting them from pathogenic fungi (*Streptomyces globisporus*). In some case, they can be plant or animal pathogens (*Corynebacterium, Mycobacterium, Nocardia*) such as marine sponges, tunicates (Hentschel et al., 2002; Lee et al., 2001), ants and termites (Barka et al., 2016). Or in other cases, they can be gastrointestinal commensals and probiotics (*Bifidobacterium spp*) (Barka et al., 2016).

The stimulation of plant growth by endophytic actinobacteria is of two types, direct and indirect. In the case of direct mechanism, they produce phytohormones such as IAA, cytokinins and solubilization of minerals such as iron and phosphorus through the production of siderophores. Concerning siderophores production, Actinobacteria is one the most important producer (Franco-Correa and Chavarro-Anzola, 2016). These molecules are produced to enhance plant nutrition (. PGP bacteria generate 1-aminocyclopropane-1-carboxylate (ACC) deaminase in response to the stress condition "stress ethylene," which slows down the growth of the plant (Glick, 2005). Some non-symbiotic species of *Agromyces, Arthrobacter, Corynebacterium, Micromonospora, Mycobacterium, Streptomyces, and Propionibacteria* have the ability to fix nitrogen, according to investigations on the nitrogen-fixing abilities of the Gram-positive Actinobacteria (Jain et al., 2022)

Endophytic actinobacteria indirectly assist plants by acting as a biocontrol agent. They can block dangerous phytopathogens by boosting the plant's defense mechanism. They are also capable of producing extracellular enzymes that can damage the cell walls of harmful fungi.

Endophytes associated with medicinal plants have created a number of distinctive secondary metabolites, and these metabolites have applications in the pharmaceutical, agricultural, and other industries.

It is possible to isolate endophytic actinobacteria from a number of plants. The ability to isolate these bacteria depends on a number of variables, including the type and age of the host plant, the

sample method, the season of the sampling, the growth environment, the surface sterilization technique, and the selective media employed (Gaiero et al., 2013; Kaewkla and Franco 2013).

The endophyte isolation method used must allow for the isolation of as many endophytes as possible while avoiding the proliferation of epiphytic bacteria present on plant surfaces in order to maximize the range of endophytes recovered (Hallmann et al., 2006).

The surface sterilization of plant tissues is a crucial step in the isolation of endophytic actinobacteria. Sterilizing substances, the most popular of which are sodium hypochlorite (3–10%), ethanol (70–95%), and hydrogen peroxide, can be used. The growth of endophytic fungi can be inhibited by the use of less common sterilizing treatments such sodium chlorate (5%), sodium thiosulfate (2.5%), and sodium bicarbonate (10%) (Dochhil et al., 2013).

The following step is the classification. Microscopic morphology and chemotaxonomy are the primary characteristics utilized to define the taxonomy of *Actinomycetes* at the genus and species levels. The latter is related to cell wall composition (presence/absence of specific optical isomers of the chiral amino acid 2,6-diaminopimelic acid (DAP); peptidoglycan may include LL-DAP or DL-DAP, depending on the genus) and by the distribution of sugars throughout the cell (arabinose, galactose, xylose), although the arrangement of phospholipids and the type of menaquinone (vitamin K) may also be taken into account for fine-tuning purposes. There are many different morphologies that actinomycetes can take, the main differences being the presence or absence of aerial mycelium or a mycelial substrate, the color of the mycelium, the synthesis of diffusible melanoid pigments, and the shape and appearance of the spores.

1.4.5 Streptomyces

Streptomycetes are abundant in soils, especially dry, non-acidic, organic matter-rich soils, and they frequently outnumber the total number of other bacteria (Chen et al., 2016). They degrade animal and vegetable biomass: it is in fact reported in the literature that the increase of necrotic biomass in the soil considerably increases the activity and population of streptomycetes. Streptomycetes can form either spores or vegetative pseudomycelia (Waksman and Lechevalier, 1953). Streptomycetes, like other actinobacteria, lack an exterior membrane and have a cell wall made entirely of peptidoglycan, also known as murein. The presence of LL-diaminopimelic acid (LL-DAP) in the cell wall confers a chemotaxonomic characteristic to all members of the genus *Streptomyces*. Teichoic

acids (anionic glycopolymers) constitute another important component of the cell wall that imparts a negative charge to the cell surface and contributes to physiological function and cell co-aggregation (Olanrewaju et al., 2019)

The life cycle starts when spore germination is stimulated by a suitable environment and nutrients availability. Aerial pseudohyphae are produced at the center of the colony by cell differentiation and substrate myceli''s planned cell death in response to food scarcity or other stressors (Sousa, et al., 2015) .

These aerial pseudohyphae may be easily distinguished from nurse pseudohyphae because they have a fibrous hydrophobic layer covering them, possibly to help aerial pseudohyphae in breaking the surface tension of air pockets in the soil, whereas nurse pseudohyphae have a smooth hydrophilic surface. Subapical branching and hyphal tip extension are both involved in Streptomyces growth (Sousa et al., 2015).

Streptomyces growth takes place through the development of hyphae from the cell pole, in contrast to the process that takes place in rod-shaped bacteria, in which cytokinesis is based on the construction of a cross wall by depositing murein in the side walls. The apical cells of the aerial hyphae differentiate into a spore chain during the last stage of the Streptomyces life cycle. By tip extension, a differentiated apical compartment expands and starts multiple, synchronous cell divisions that result in a developmentally regulated form (Vurukonda et al. 2018, Olanrewaju et al., 2018, Ferrer et al., 2018).

Streptomyces is a large genus with many different species, and up to 75% of its genome is made up of guanine and cytosine. This genus produces a number of physiologically active substances associated with plant growth. During the programmed cell death of substrate mycelia, antibiotics are simultaneously produced, probably to protect food sources from competing microorganisms (Sousa et al., 2016).

Two-thirds of the 23,000 bioactive secondary metabolites produced by microorganisms are produced by actinobacteria, and more than 70% of them are produced by *Streptomyces* spp. The growth of aerial hyphae in response to nutritional intake is thought to be the cause of this generation of secondary metabolites (Sousa et al., 2016; Ferrer et al., 2018).

These substance's biological activity includes metal transport, metazoan toxicity, microbial hormonal activity, and inhibitory or microbiocidal activity against microbes (i.e., antibiotics) (Vurukonda et al., 2018).

Streptomycetes produce secondary metabolites that have been shown to enhance adaptation to biological, physical, and chemical stresses; this has led to their designation as "stress metabolites." Volatile organic compounds (VOCs) are a different group of bioactive compounds that various rhizobacteria, including strains of *Streptomyces*, make and of which some are known to have antifungal activity. In their study, Viaene et al., (2016) found that rice was resistant to *Rhizoctonia solani, Brassica napus* was resistant to *Sclerotinia sclerotiorum,* and *Fragaria ananassa* was resistant to *Botrytis cinerea* due to volatiles compounds produced by *Streptomyces platensis* strain F-1 (Viaene et al., 2016). Although the structure of the volatile chemicals has yet to be clarified, 10 out of the 12 *Streptomyces* strains isolated from a disease-suppressive soil encouraged plant development through the generation of VOCs (Cordovez et al., 2015).

The overall eco-physiological traits of the genus *Streptomyces* support the concept of cosmopolitan biogeographical behavior (Vurukonda et al. 2018).

A large pH range that is permissive to growth, among various Streptomyces species, is necessary for the growth under unfavorable abiotic conditions. In fact, different *Streptomyces* species require a variety of pH values to thrive well, for example, the acidophilic *S. yeochonensis* requires a pH 4.3 (Vardharajula et al., 2016), the neutrophilic *S. roseus* requires a pH 7.0 (Benson and Silvester 1993.), and for the alkalophilic *S. alkaliphilic* alkalithermotolerans requires a pH 10 (Vurukonda et al. 2018). S*treptomycetes* are typically chemoorganotrophs with great versatility in metabolizing a wide range of carbon sources including mono- and disaccharides, polyols, organic acids (glucose, dextrose, fructose, lactose, maltose, mannitol, rhamnose, sucrose, glycerol, and glycolic acid), polysaccharides (including cellulose and starch), and more complex and recalcitrant C sources, such as humic and fulvic acids (Vurukonda et al., 2018).

Researchers from all around the world have become interested in streptomycetes since it has been seen that they bring benefits to the plant by stimulating its growth and protecting it from pathogens. Most of the fundamental and applied studies on beneficial interactions between plants and microbes concern Gram-negative bacteria. Less often studied bacteria, many representative groups of Gram-positive bacteria, particularly those belonging to the genus *Streptomyces* exhibit a number

of characteristics that may enhance plant growth using different mechanisms (Vurukonda et al., · 2018) .

It is important to clarify the biochemical processes that result in harmonious interactions in order to better comprehend and control the interactions between Plant Growth-Promoting *Streptomyces* (PGPS) and their hosts.

The majority of these Streptomycetes, as seen above, are soil-dwelling bacteria with a free-living cycle (i.e., saprophytic competence) and the capacity to effectively colonize the rhizosphere and rhizoplane compartments. Eventually, some PGPS can become endophytes and colonize the host plant's internal tissues and conduct their life cycle partially or completely within them. However, numerous studies have also demonstrated that they may be found both on land and in water (Olanrewaju and Babalola 2019). The easy-to-spread spore production of the streptomyces, could account for their prevalence of in a variety of habitats.

The three categories of biofertilization, biostimulation, and bioprotection can be used to categorize the growth-promoting effects of *Streptomyces*-plant interactions.

By solubilizing and releasing nutrients into solution, organic acids (including gluconic acid, citric acid, succinic acid, and oxalic acid) generated by diverse microorganisms can liberate nutrients that are trapped in the crystal lattice of the soil mineral component. The release of free phosphate by acidification as a result of the release of malic acid and gluconic acid by *Streptomyces* mhcr0816 and Streptomyces mhce0811, respectively, was documented by Olanrewaju and Babalola, 2019. Numerous papers have reported that *Streptomyces* spp. produces indole-3-acetic acid (IAA). In addition to producing a variety of phytohormones, including gibberellic acid, *Streptomyces* species isolated from a marine environment have improved the agronomic performance of eggplant (*Solanum melongena*) by influencing its growth parameters, such as root length and fresh and dry weight of roots (Olanrewaju and Babalola 2019; Vurukonda et al., 2018). The ability of the genus *Streptomyces* to synthesize a number of bioactive compounds that inhibit phytopathogens and give an advantage in rhizosphere or endophyte colonization is well known. It has been noted that 213 *Streptomyces* strains obtained from various habitats produced antifungal compounds that have antagonistic action in vitro against *Rhizoctonia solani* (Olanrewaju and Babalola, 2019; Vurukonda ·et al., 2018).

Abiotic stresses including heat, cold, drought, soil salinization, and nutrient depletion can all be mitigated by plant-associated streptomyces, which reduces their negative effects and consequently promotes host plant development.

Several reports have demonstrated improved symbiosis with legumes and mycorrhizal symbiosis in double inocula with different PGPRs (Barea et al., 2005); however, there is less information on Actinobacteria.

There is a growing belief that helper bacteria can promote these symbioses. *Rhodococcus, Streptomyces,* and *Arthrobacter* are considered mycorrhizal helpers (Frey-Klett et al., 2007). In addition, Schrey and Tarkka, (2008) showed that the genus *Streptomyces* promotes the formation of symbioses between plant roots and microbes, and this is partly due to their direct positive influence on the symbiotic partner, expressed as, for example, promoting hyphal elongation of symbiont fungi. Furthermore, Franco-Correa et al., (2010) showed that co-inoculation of *Streptomyces spp*. MCR9 and MCR24 and *Glomus mosseae* produced synergistic benefits on plant growth and phosphate acquisition. Arbuscular mycorrhizae (AM) were better able to form in clover plants, thanks to specific actinobacterial strains. The saprophytic strains *Streptomyces* MM40, *Actinoplanes* ME3, and *Micromonospora* MM18 were shown to function as helper bacteria in the actinorhizal symbiosis (Solans et al., 2011).

These actinobacterial strains clearly produced phytohormones (Solans et al., 2011) and possessed enzymatic activities for cellulose, hemicellulose, pectin, and lignocellulose (Solans and Vobis, 2003), but the true metabolites responsible are still unknown.

Although streptomycetes are undoubtedly a crucial component of the soil and/or root microbiome and actively aid in the defense against plant soil pathogens, there are relatively few commercial products that use streptomycetes strains or their bioactive compounds. *Streptomyces griseoviridis* K61 (MycostopR) and *Streptomyces lydicus* WYEC 108 (ActinovateR) are included in two products that are marketed as biofungicides for soil-borne and foliar and soil-borne illnesses, respectively (Viaene et al., 2016).

1.4.6 PGPB used in experiments.

Azospirillum

Azospirillum is a bacterial genus that appears to be exceptionally adaptable, able to thrive in a variety of soil and wet settings, particularly in plant rhizospheres. Although its distribution appears to be understated in comparison to the actual range, it is obvious that they are widespread in soil. *Azospirillum* has indeed been isolated from the rhizosphere of several wild and cultivated plants. In addition, it has also been reported in forest soil (Zhou et al., 2009). Some strains have even been isolated from contaminated tars (Lin et al., 2009) or oil (Young et al., 2008) or even from different extreme environments such as sulfide sources (Lavrimenko et al., 2010) or acidic environments (Magalhaes et al., 1983).

Azospirilla are gram-negative, non-nodule-forming aerobic nitrogen-fixing plants and belong to the family *Azospirillaceae* (Mehnaz, 2015). Although there are many species under this genus such as *Azospirillum amazonense, Azospirillum halopraeferans*, and *Azospirillum brasilense*, the main beneficial species include *Azospirillum lipoferum* and *Azospirillum brasilense* (Cassán et al., 2020). *Azospirillum* forms associative symbiosis with many plants, particularly those that have a C4 dicarboxylic pathway (Hatch-Slack pathway) of photosynthesis, as they grow and fix nitrogen on organic salts of malic and aspartic acid (Cassàn et al., 2020).

Azospirillum is mostly recommended for the growth of corn, sugarcane, sorghum, pearl millet, and other crops (Fig 11). They produce growth hormones (IAAs, gibberellins, and cytokinins) and enhance root development and nutrient uptake in plants (N, P and K). *Azospirillum* inoculum has a significant impact on root development and exudation (Trabelsi and Mhamdi, 2013).

When *A. brasiliense* is used as an agro-inoculant in the wheat crop, the detrimental impact of drought circumstances appears to be reduced. Indeed, wheat seedlings inoculated with Azospirillum strains benefit from enhanced water status during salt and osmotic stressors due to *A. brasiliense*-induced xylem channel expansion. Wider xylem channels caused by bacteria may also improve the water conductivity of the coleoptile, which is the pointed protective sheath that covers the shoot (Pereyra et al., 2006). Similar aspects have also been found in other plant species, for example in tomato plants. Again, in this case it has been reported that inoculation of *Azospirillum brasilense* is able to increase the area of xylem vessels and the hydraulic conductivity of the phloem thus mitigating the water stress imposed by a pathological condition affecting the vascular system (Romero et al., 2014). it has been observed that when *A. brasilense sp*. 245 was inoculated into maize, it was observed that the production of several phytohormones by the

organism increases considerably and leads to substantial improvement in maize growth (Steenhoudt and Vanderleyden, 2000; Perrig et al., 2007). As a result of the increased production of various phytohormones, the physiology and root architecture of maize are altered and lead to increased maize growth, particularly by facilitating the molecular mechanism of N_2 fixation (Steenhoudt and Vanderleyden, 2000; Perrig et al., 2007).

Nitrogenase, an enzyme complex composed of a Fe-protein (dinitrogenase reductase) and a MoFe-protein (nitrogenase), is responsible for N_2 fixation. Dinitrogenase reductase supplies electrons, and dinitrogenase converts N_2 to NH_3 using these electrons. Because the enzyme can bind to O2, inactivating it, oxygen is a potent inhibitor of the enzyme complex. Yet, because bacterial hemoglobin has a higher affinity for oxygen, free O_2 binds to it more strongly and effectively. As a result, the presence of hemoglobin keeps the nitrogenase enzyme complex active by shielding it from plant oxygen consumption (Steenhoudt and Vanderleyden, 2000).

According to Naiman et al., (2009), inoculating *Azospirillum* and *Pseudomonas* changes the culturable bacterial community in the wheat rhizosphere. It has also been shown that *Azospirillum* and *Pseudomonas* inoculation can alter the profiles of carbon source use by soil microorganisms during the tillering and grain filling stages (Naiman et al., 2009).

In addition to this, it has also been described in the literature that inoculation with two strains of *A. brasilense* (40 and 42 M) isolated from maize roots is capable of altering the community-level physiological profiles (CLPPs) of culturable microbial communities associated with rice (De Salamone et al., 2010; Trabelsi and Mhamdi 2013).

Several plant hormones can be produced by *Azospirillum*, and many plant metabolic pathways involved in phytohormone synthesis can be altered. The main phytohormones mediated by *Azospirillum* include indole-3-acetic acid (IAA), as an auxin hormone (Fendrihan et al., 2018), gibberellic acid (GA3), as a gibberellin hormone, and zeatin (Z), as a cytokinin hormone (Perrig et al., 2007), all of which are implicated in plant growth stimulation and development. This bacterium has also been reported to interfere with the synthesis of ethylene (ETH) and abscisic acid (ABA) (Cohen et al., 2008).

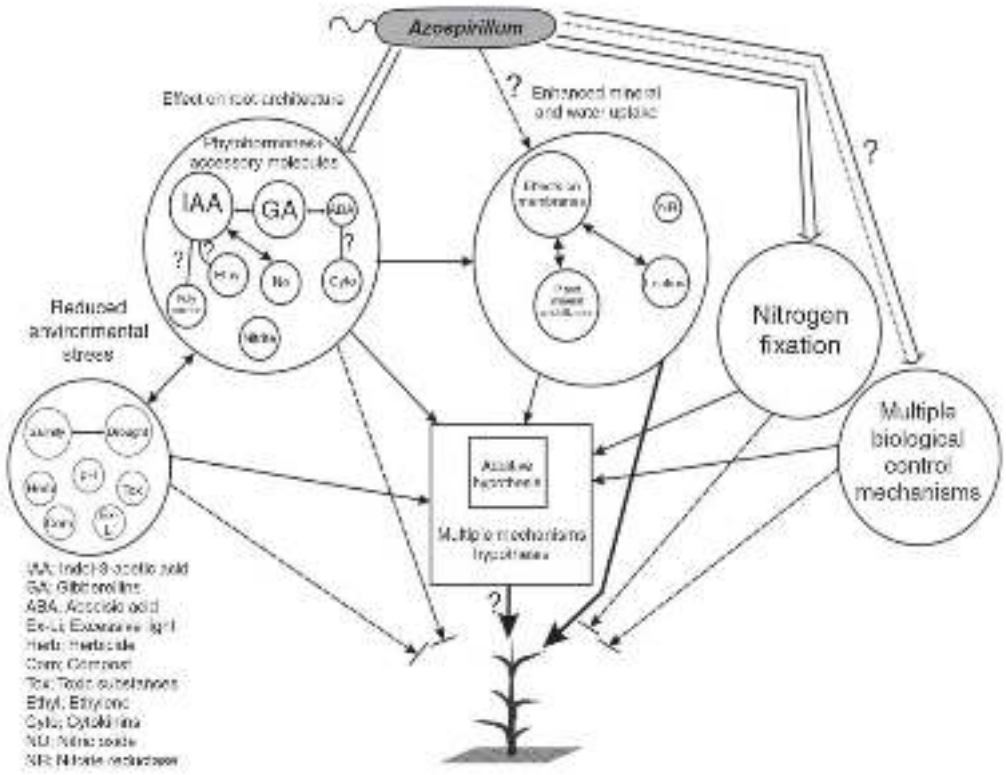

Fig. 11 Mechanisms by which Azospirillum spp. may enhance plant growth and their possible interactions grouped as biological processes. Circles represent processes containing experimental data. Squares represent theories. Size of a circle represents its relative importance according to current data (Bashan et al., 2010).

Herbaspirillum

Herbaspirillum is a genus of Betaproteobacteria with 14 species, according to the most recent update. Although most *Herbaspirillum* spp. (9 species) have not been observed in connection with plants, they may exist in aquatic habitats as well as polluted and unpolluted soils.

Herbaspirillum spp were first isolated from washed and surface-sterilized rice (*Oryza sativa*), maize (*Zea mays*), and sorghum (*Sorghum bicolor*) roots, as well as sugarcane (Saccharum hybrid) leaves (Olivares et al., 1996). Bacteria of the genus *Herbaspirillum* have been found in conjunction with a wide range of plants, particularly *Poaceae* (Monteiro et al., 2012). In addition to *Poaceae,* they have been found endophytically associated with dicotyledonous plants such as nodules of *Phaseolus vulgaris* (Valverde et al., 2003) and surface-sterilized roots of soybean (Kuklinsky-Sobral et al., 2005), though it should be noted that *Herbaspirillum* are not capable of actually nodulating these plants.

Herbaspirillum seropedicae has also been discovered in washed portions of banana (*Musa* spp.) and pineapple (*Ananas comosus*) plants (Monteiro et al., 2012).

Herbaspirillum seropedicae's interaction with plants most likely begins with attraction to roots, which supply carbon sources for the bacteria, followed by adhesion to root surfaces and subsequent colonization of lateral root emerging sites. *H. seropedicae* strain SmR1 attached to maize roots 30 minutes after inoculation, and epiphytic colonization of roots was still present 5 and 8 days later, often with bacterial cells surrounded by a halo and fibrils (Balsanelli et al., 2010).

Monteiro et al., (2012) Gyaneshwar et al., (2002) observed *H. seropedicae* within the roots of rice plants grown under nonsterile conditions, concentrated mainly in wounds or fractures of the epidermis, suggesting that most likely one of the main pathways of entry to the plant's internal tissues is through epidermis discontinuities. Under field conditions, these root epidermis discontinuities are probably more common, as the natural soil contains heterogeneous particles that can cause wounds due to friction with the root surface with the root surface. From a technology standpoint, this is a really intriguing function. The ability of these bacteria to spread into plant tissues, forming epiphytic and endophytic populations, may allow them to act as genetically modified vectors for delivering biocontrol molecules into plants, or for direct biocontrol of insect larvae and nematodes that feed on plant tissues containing.

Moreover, given the ability of *Herbaspirillum* to colonize different plant crops, it would allow the application of these biocontrol strains in a wide range of crops. The potential of *Herbaspirillum* spp. as inoculants has been demonstrated by several studies (Dall'Asta et al., 2017; Pellegrini et al., 2021)

Plant-associated *Herbaspirillum spp.* can recognize plant signals that modulate the expression of factors that facilitate plant colonization (e.g., LPS, EPS, and adhesins). In particular, lipopolysaccharides play an important role in the plant-bacterial communication process by participating in the formation of the interface between the bacterial cell and the environment. LPS appears to be a key molecule on the bacterial surface required for the connection and colonization of internal plant tissues by a wide range of endophytes. For example, Balsanelli et al., (2010) showed that *Herbaspirillum seropedicae* LPS is required for its colonization of maize; in contrast*, H. seropedicae* strains altered in LPS biosynthesis showed a severe reduction in attachment to the maize root surface, resulting in more than 90 percent fewer attached bacteria than wildtype. *H. seropedicae* LPS in plant

is thus important for colonization, the latter being enhanced by the fact that its biosynthesis is regulated by plant-derived signals. (Balsanelli et al., 2010)

Although scanning electron microscopy has shown that *Herbaspirillum seropedicae* produces a mucilaginous and fibrillar material during colonization of maize and sorghum root surfaces that could be traced back to EPS, there is still no functional evidence about the role of EPS in plant colonization by *H. seropedicae* (Balsanelli et al., 2014).

Plant-associated *Herbaspirillum spp*. may recognize elements that promote plant growth (e.g., nitrogenase activity, phytohormones, and siderophores). Parallel to this, the plant response comprises identifying the invading bacteria as nonpathogenic and then negatively modulating the expression of defense-related genes (Leandro et al., 2019).

Studies evaluating inoculation with *Herbaspirillum seropedicae* have shown that there is a significant increase in the total biomass of rice and sugarcane plants, and certain bacterial traits such as BNF, phytohormones, ACC deaminase, and siderophore production have been suggested to be responsible for the increase in plant growth. *Herbaspirillum seropedicae* produces gibberellins and indole acetic acid (Cortés-Patiño S et al., 2021).

Although there is no described role for ACC deaminase in bacterial metabolism, its effect on plant growth has been demonstrated using *Pseudomonas putida* and *A. brasilense* strains genetically manipulated to carry the gene for ACC deaminase (Bashan and De-Bashan 2005). The acdS gene, which encodes for ACC deaminase, was identified in the *H. seropedicae* SmR1 genome. The coordinated production of IAA and ACC deaminase by *H. seropedicae* is a likely mechanism for plant growth promotion by this microorganism (Pedrosa et al., 2011).

Siderophores are small iron-binding molecules used by many microorganisms to sequester iron present in the soil. Bacterial iron-binding siderophores recognized through receptors (Bashan and De-Bashan, 2005). Eighteen genes encoding for siderophore receptors have been identified in the *H. seropedicae* SmR1 genome, but only one gene among them appears to be responsible for siderophore biosynthesis (Pedrosa et al., 2011).

Canuto et al., (2003) proved that the presence of *H. seropedicae* strains can supply at least 15% of the nitrogen necessary by sugarcane. Similarly, inoculating rice with H. seropedicae strains increased total nitrogen in grain by 15% (Guimares et al., 2003; Alves et al., 2021).

Gluconacetobacter

They are bacteria belonging to the family *Acetobacteraceae* (Mitesh Dwivedi et al., 2020), are classified as rod (coccus or ellipsoidal), Gram-negative, motile and aerobic, although nitrogen is fixed in the microaerobic layers, Nitrogen fixation can occur in the presence of a nitrate concentration that can exceed 10 mM, this also reduces the deleterious effect of oxygen concentration on nitrogenase activity by using oxidative metabolism at the membrane level of the periplasmic space. These are aerobes that create organic acids as an end product of their metabolism through incomplete oxidation of sugars and alcohols. They do not grow with tricarboxylic acids and are acclimated to high osmolarity and sucrose content (10-30%). They have the ability to grow in very aggressive environments; in fact, they show a high tolerance to acidity; they are able to grow in environments presenting a pH close to 3.0-3.5 (Stephan et al., 1991).

Initially, the *acetobacterace* family was associated with representatives of the general family of *Acetobactera* and *Glucono-bacter*. This classification was based on morphological, physiological, and biochemical criteria. Later, Yamada and Kondo, (1984) proposed a new subgenus (genus) named *Gluconoacetobacter*, which was later elevated to the category of a true genus following sequence analyses of the 16S rRNA gene (Yamada et al., 1997).

Among the different species, *Gluconacetobacter diazotrophicus* (formerly *Acetobacter diazotrophicus*) was among the first to be isolated, initially from sugarcane roots, stems, and leaves in Brazil, Argentina, Uruguay, Mexico, Cuba, the United States, India, Canada, and Egypt, to name a few. Later, it was also isolated from agricultural crops such as sugar beet, rice, pineapple, coffee, carrot, and many others (Reis and Teixeira, 2015). For this reason, it was initially considered as an endophyte with a low survival rate in soil, associated only with sugarcane or, at most, with other plants that tend to accumulate sucrose (Reis and Teixeira, 2015; Estrada-De Los Santos et al., 2001). During "in vitro" inoculation studies under controlled conditions, they have shown that *Gluconacetobacter diazotrophicus* enters in sugar-textured plants are able to colonize tissue intercellular spaces (apoplasts) by crossing secondary root tissue. However, there are other routes of entry, and in particular, other possible points of infection are wounds and stomata of plants, such as in sugarcane plants. It also manages to colonize the root tips and root hairs of other plants such as wheat, sorghum, and rice, as shown using reporter genes (James and Olivares, 1998).

Subsequent observations have revealed a new mechanism for this bacterium to penetrate plants: phloem sap sucking by insects (mealybugs) carrying this species in their lymph and living within the sheath pocket of sugarcane leaves (Ashbolt and Inkerman, 1990).

De la Cruz et al., (2012) in the Philippines studied the use of *Gluconacetobacter diazotrophicus* in the germination of sugarcane stem parts. These researchers experimented with different cell densities (10^8, 10^{10} and 10^{12} cells ml-1) and application methods (spray, soaking for 2 hours and soaking for 2 minutes). Compared with the control, inoculation resulted in an increase in percentage, plant survival height and shoot/root biomass 45 days after planting. When microbial inoculation was introduced into 10^{12} cells of ml-1 making use of the dipping method, taller plants with larger biomass and root were formed compared to other treatments and the un-inoculated control. *Gluconacetobacter diazotroficus* synthetize gluconic acid, this mild non-corrosive acid not only lowers pH but also promotes chelation and exchange reactions (solubilization) of phosphate and zinc (Reis and Teixeira, 2015).

Another promising consequence of *Gluconacetobacter diazotrophicus* inoculation is the biological control of other microorganisms including *Xanthomon asalbilineans*, *Colletotrichum falcatu*, *Helminthosporium spp*, and *Fusarium spp* (Reis and Teixeira, 2015). Plant disease resistance has been shown to be effectively induced by diazotrophs. Sugarcane plants inoculated with *Gluconacetobacter diazotrophicus* are resistant to infection by *Xanthomonas albilineans* (Monteiro, et al., 2012).

Burkholderia

Burkholderia is a rapidly expanding genus of Gram-negative non-fermenting bacteria that can be found in nearly every environment on the world. Some species live in simple soil or as planktonic organisms in fresh water, but the vast majority coexist with a growing number of hosts, including people, animals (both vertebrate and invertebrate), plants, and fungi (Depoorter, all 2016).

The genus *Burkholderia* comprises 19 species (Estrada-De Los Santos et al., 2015). Lately, *Burkholderia* has been divided into seven separate groups, which include *Paraburkholderia, Robbsia, Pararobbsia, Mycetohabitans, Trinickia, Caballeronia,* and *Burkholderia sensu stricto*.

Burkholderia spp. are adaptable bacteria that create a plethora of compounds that boost their rhizosphere competence, particularly in the presence of limited nutrition availability (Bach et al., 2017). Its adaptability may be due in part to their enormous and complicated DNA (Bach et al., 2022). There has been growing interest in the genus *Burkholderia* in recent years because of its great potential value in plant growth promotion, biocontrol of plant pathogens, and phytoremediation (Bach et al., 2022). For example, An and his colleagues (An et al., 2022) discovered an endophytic bacterium XN08 with antagonistic activity against *Rhizoctonia cerealis* (a wheat spike fungus pathogen), which was then isolated from healthy wheat plants and identified as *Burkholderia ambifaria* through genetic analysis of 16S rRNA sequences.

Burkholderia ambifaria is a typical rhizosphere species that plays a crucial role in plant defense against pathogenic fungus, and it is one of the species found in persons with cystic fibrosis (a rare condition in and of itself), as well as as an occasional, never harmful, guest (Botta et al., 2013).

Burkholderia ambifaria is known for its ability to promote maize growth, increase crop yields, reduce various soilborne plant diseases, and breakdown a variety of pesticides. (Estrada-De Los Santos et al., 2001).

Bacterial endophytes from genera like *Bacillus* and *Burkholderia* have been shown to be the most successful isolates in vitro at controlling bacterial and fungal diseases. Indeed, the genus *Burkholderia* is rich in antibiotic synthesis genes (Kim et al., 2021) and has been reported to produce a large number of antifungal substances (such as pyrrolnitrin), siderophores, and phenazines (Mullins et al., 2019), all of which play an important role in the control of fungal diseases in plants (An et al., 2022). In their study, Bach and his colleagues, for example, evaluate the functionality of around twenty-one antimicrobial secondary metabolites produced exclusively by *Burkholderia* species, whereas the remaining, just seven NPs, are produced by at least five additional bacterial genera (Bach et al., 2022).

For the characterization of secondary metabolites, different methodological approaches are used, the results of which tend to return more detailed and complete information, usually the evaluation of the amplification of antibiotic synthesis genes is complemented by liquid chromatography quadrupole mass spectrometry (UPLC-QTOF-MS) analyses. Through these analyses, for example, An and his collaborators were able to show that the strain is capable of producing a potent antifungal compound known as pyrrolnitrin (An et al., 2022). Furthermore, it could be observed that

Burkholderia ambifaria XN08 has the ability to solubilize phosphates, produce indole-3-acetic acid (IAA), proteases and siderophores in vitro (An et al., 2022).

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Outline of the Project

The objective of this thesis project was focused on the study of PGPB and PGPR widely documented but not completely understood.

This Ph.D. thesis comes out in the form of collection of papers. They are presented progressively, each one corresponding to a single chapter (those from 2 to 4).

Altogether, this is the scientific production, strictly related to my Ph.D. research program, I contributed to during the "Health and Environmental Sciences" Ph.D. three years course:

The studies were conducted in the Laboratory of Microbiology at the Section of Environmental Science at the University of L'Aquila, under the supervision of my tutor Prof. Maria Maddalena Del Gallo.

During these 3 years, Prof. Maria Maddalena Del Gallo has been active within different research lines in the field of Environmental Microbiology. Among the most important ones, to highlight the study of the impact PGPB and PGPR in agriculture.

Before starting my Ph.D. studies, it had been already described that some PGPB and PGPB studied and isolated and characterized by the group of Prof. Maria Maddalena Del Gallo had important results in Laboratory and field scale on various plants of agricultural interest.

My role in this field consisted in evaluating: the activity of bacterial isolates in promoting plant growth and in biocontrol in saffron, hemp, tomato, and potato.

I also tested these selected bacteria on biocontrol of pathogenic fungi. This was possible by the use of PGPB inocula, both *in vitro* (SEM observations) and *in planta* (*Cannabis sativa* plant monitoring in greenhouse).

The research carried out during the first year of the doctorate led to the publication of the following scientific article:

> **"*Fusarium oxysporum* f. sp*. cannabis* isolated from *Cannabis sativa* L.: *in vitro* and *in planta* biocontrol through a plant growth promoting rhizobacteria consortium"** in the international journal PLANTS. Pellegrini, M.; Ercole, C.; Gianchino, C.; Bernardi, M.; Pace, L.; Del Gallo, M. Fusarium Oxysporum f. sp. Cannabis Isolated from Cannabis Sativa L.: In Vitro and In Planta Biocontrol by a Plant Growth Promoting-Bacteria Consortium. Plants 2021,10, 2436. https://doi.org/10.3390/plants10112436, (quoted in full in Chapter 2).

In view of a safe and effective use of these tools in agriculture, the next step was evaluated in bibliography the use of some metabolites derived from bacteria, in particular cell free supernatant of plant grow promoting bacteria. A cell-free supernatant (CFS) is a liquid containing the metabolites resulting from microbial growth and the residual nutrients of the medium used. CFSs can be obtained through two main unit operations, centrifugation, and filtration (i.e., microfiltration, ultrafiltration, nanofiltration, inverse osmosis). These techniques can be applied individually or in combination with other technologies according to the desired final product by summarizing studies concerning PGPB CFSs and their metabolites as biostimulant and biocontrol agents. Several databases have been used to create a collection of articles. 109 valid published works has been selected. Data organization allowed the discussion of CFSs' and their metabolites' biostimulant and soil-borne pathogen control applications (i.e., of bacteria, fungi, oomycetes). These results were published in 2020 in the journal as:

> **"Cell-free supernatants of plant-grow promoting bacteria: a review of their use as biostimulant and biocontrol agents in sustainable agriculture".** Sustainability 2020, 12(23), 9917 https://doi.org/10.3390/su12239917 . (quoted in full in Chapter 3).

In particular, I contributed at the review by performing **"**CFSs as Biocontrol Agents".
Participation in the activities related to the biocontrol of *Fusarium oxysporum* ff.spp. from fields of saffron subject to fusariosis. Purification, isolation, and characterization of relevant isolates, including fungal pathogens belonging to the genus *Fusarium oxysporum* ff.spp.

The research activities carried out also led to the publication of the following article:

> **"Bacterial Microbiota and Soil Fertility of *Crocus sativus* L. Rhizosphere in the Presence and Absence of *Fusarium* spp."** Land 2022, 11, 2048. Farda, B.; Djebaili, R.; Bernardi, M.; Pace, L.; Del Gallo, M.; Pellegrini, M. https:///doi.org/10.3390/land112048. (quoted in full in Chapter 4).

Another article that saw my contribution was the use of some actynobacteria in agricolture.

The aim of the study was to evaluate the ability of some actinomycete isolates to colonize roots and stimulate plant growth and development of (*Solanum lycopersicum* L.). 60 actynomicetes strains were isolated from two saline soils of northeast region of Algeria- Ezzemoul sebkha and Djendli sebkha. Strains were first characterized in vitro for their capability to solubilize phosphate, produce indole acetic acid, hydrocyanic acid, and ammonia, and for the presence of different enzymatic activities. Then, strains that obtained best in vitro results were investigated for their root colonization ability by scanning electron microscopy and utilized in a greenhouse experiment to assess inoculation biostimulant effects on tomato plants. Among sixty isolates, fourteen PGPR were selected based on their plantgrowth promoting traits. These strains, belonging to Streptomyces sp. And Nocardiopsis sp. genera, showed good association capability with tomato plants in vitro. Greenhouse experiment results showed that tomato plants were positively influenced by actinomycete inoculation. Inoculated plants showed better growth and morphophysiological characteristics with respect to the control.

Conference Paper: **"Actinomycete strains isolation and selection from Algerian saline soils as environment- friendly tool for *Solanum lycopersicum* fertilization"**. R. Djebaili, M. Pellegrini, M. Bernardi, M. Smati, M. del Gallo, M. Kitouni. *1st International Electronic Conference On Plant Science*.

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Chapter 2

Fusarium Oxysporum f. sp. Cannabis Isolated from Cannabis Sativa L.: In Vitro and In Planta Biocontrol by a Plant Growth Promoting-Bacteria Consortium

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Abstract

Industrial hemp (*Cannabis sativa* L.) is a multipurpose plant used in several fields. Several phytopathogens attack hemp crops. *Fusarium oxysporum* is a common fungal pathogen that causes wilt disease in nurseries and in field cultivation and causes high losses. In the present study, a pathogenic strain belonging to *F. oxysporum* f. sp. *cannabis* was isolated from a plant showing Fusarium wilt. After isolation, identification was conducted based on morphological and molecular characterizations and pathogenicity tests. Selected plant growth-promoting bacteria with interesting biocontrol properties— *Azospirillum brasilense, Gluconacetobacter diazotrophicus, Herbaspirillum seropedicae* and *Burkholderia ambifaria*—were tested against this pathogen. In vitro antagonistic activity was determined by the dual culture method. Effective strains (in vitro inhibition > of 50%) *G. diazotrophicus, H. seropedicae* and *B. ambifaria* were combined in a consortium and screened for in planta antagonistic activity in pre-emergence (before germination) and post-emergence (after germination). The consortium counteracted *Fusarium* infection both in pre-

emergence and post-emergence. Our preliminary results show that the selected consortium could be further investigated as an effective biocontrol agent for the management of this pathogen.

2.1. Introduction

Hemp (*Cannabis sativa* L.) is a crop with a rich and ancient history and is grown all over the world. Its widespread cultivation is because of the versatility of this plant in a variety of fields. Hemp can be used in the textile and manufacturing industries and for the production of biobased materials [1]. The metabolites of hemp (e.g., cannabinoids, phenolic compounds, vitamins and proteins) can be used in pharmaceutical, nutraceutical and food industries [2–4]. Hemp crops are threatened by attacks from viruses, bacteria and fungi that penetrate through the surfaces of leaves, stems and roots; spread within the tissues; and colonize the entire plant [5]. Some of these plant pathogens can cause significant damages to hemp plants by blocking plant development and causing metabolic disorders, leaves shriveling or roots destruction [6]. Previously reported pathogens that can cause wilting and collapse of *C. sativa* plants include *Fusarium oxysporum* f. sp. *cannabis* (FOC) and *F. oxysporum* f. sp. *vasinfectum* (FOV) [7]. FOV *forma specialis* affects a wide range of hosts, while FOC is specific to hemp and can result in complete crop loss. Symptoms of FOC pathogenesis begin with dark spots on lower leaflets, rapid wilting of leaves, covering of stem cortex with mycelium and death of the plant [8]. It is possible to use naturally occurring plant-microbe interactions to counteract the attacks of phytopathogens. Through various direct and indirect mechanisms, Plant Growth-Promoting Bacteria (PGPB) can be used as sustainable biocontrol agents against many phytopathogens [9,10]. In the literature, biocontrol of hemp *Fusarium* has been described for *Burkholderia cepacia, Pseudomonas fluorescens* and *Streptomyces griseoviridis* and the beneficial fungi *Trichoderma lignorum* and *Glomus intraradices* [7]. This scarcity of biocontrol agents requires the search for new effective biocontrol agents. Our study aims to investigate the efficacy of a bacterial consortium for the control of hemp *Fusarium*. Among the PGPB belonging to our Environmental Microbiology laboratory collection, we selected *Azospirillum brasilense, Gluconacetobacter diazotrophicus, Burkholderia ambifaria* and *Herbaspirillum seropedicae,* provided by several colleagues (Y. Okon, J. Döbereiner and T. Heulin). Since these bacteria have shown in planta biocontrol against other *F. oxysporum* f. sp. *radicis-lycopersici* [11] and good biostimulatory abilities on C. sativa 'Finola' [12], we hypothesized that they could be an effective

biocontrol agent for hemp against fusariosis. These bacterial species live in association with many crops, are associated with plant roots and promote plant growth through various direct (e.g., hormone production) and direct mechanisms (e.g., production of biocontrol molecules) [13–16]. We isolated a FOC *forma specialis* from a plant with specific symptoms (i.e., wilted leaves with yellow-tan colour and cortex covered by fungal mycelium). This FOC strain was characterized by internal transcribed spacer (ITS) sequencing and by pathogenesis assay. The antagonistic activities of the individual strains and the effective strain's PGPB consortium (*B. ambifaria, G. diazotrophicus* and *H. seropedicae*) against the FOC pathogen were first evaluated in vitro, examining the inhibitory ability by dual culture method and the morphological changes of the mycelium in the presence of PGPB by scanning electron microscopy (SEM). The PGPB consortium of effective strains was tested in planta in order to verify the induced protection under pre-emergence (infection before germination) and post-emergence (infection after germination) conditions.

2.2 Materials and Methods

2.2.1 Fungal Strain Isolation and Growth Conditions

The stem of a hemp plant with classic symptoms of *Fusarium* wilt was sampled from M.A.D. Biofarm SS field (42.0302, 13.4421, Avezzano, Italy) in August 2018. Several pieces of the stem cortex (~3 × 3 cm) with a clear cover of mycelium (15–20 pieces) were sampled with sterile blades, placed in sterile plastic bags and transferred to the laboratory. Small pieces of cortex tissue (0.5–1 cm) were treated with a 0.5% sodium hypochlorite solution for 30 s, 70% ethanol solution for 20 s and rinsed five times in sterile distilled water. Pieces were left to dry under hood flow and plated on SFA, supplemented after autoclaving with 20 mL L−1 of 5% streptomycin stock solution (SigmaAldrich, St. Louis, MO, USA), 12 mL L−1 of 1% neomycin stock solution (Sigma-Aldrich, St. Louis, MO, USA) and 13 mL L−1 of 0.5% 2,6-dichloro-4-nitroanaline ethanol stock solution (Sigma-Aldrich, St. Louis, MO, USA) [17]. SFA plates were incubated at 25 ◦C for 5–10 days. By using a stereomicroscope placed under Gelaire TC48 laminar flow hood (class 2 cabinet (Gelaire, Sydney, Australia)) and sterile needles, single spore isolation was carried out. Emerging colonies were transferred to fresh medium and permitted to grow. The isolates were selected based on macroscopic and microscopic observations (mycelium colour and growth rate on PDA and microconidia, macroconidia on SA [17]). FOC liquid cultures were grown in 250 mL Erlenmeyer flasks containing 150 mL of Potato Dextrose

Broth (PDB) at 25 ◦C under constant shaking (150 rpm) for 7 days (mycelial mat growth). Spore solutions were prepared from 7 days PDB cultures by filtering the broth through 4 layers of muslin cloth, centrifuging at 6000× g for 10 min and adjusting the density to 106 by a Burker chamber [38].

2.2.2. Fungal Strain Molecular Identification

The putative *Fusarium oxysporum* isolate was identified at the species level by ITS rDNA sequencing. The primers of ITS1F-ITS4 (ITS1-F 50 -CTTGGTCATTTAGAGGAAGTAA3 0 and ITS4 50 -TCCTCCGCTTATTGATATGC-30) [39,40] were used in the following reaction mixture:~150 mg of fresh mycelium; 2 µL of 20 mg µL −1 bovine sieroalbumin solution; 1.5 µL of 5U µL −1 Taq polymerase solution; 5 µL Buffer 10×; 1 µL of 10 mM dNTP; 4 µL of 50 Mm MgCl2 solution; 2 µL ITS1F Primer forward; 2 µL ITS4 primer reverse; and sterile distilled water up to 50 µL. Negative (water) and positive (known strain) controls were included. PCR reactions were carried out in a thermal cycler (SimpliAmp™ Thermal Cycler—Applied Biosystems) with the following program: 1 cycle of 8 min at 95 ◦C and 30 s at 94 ◦C; 30 cycles lasting 30 s at 55 ◦C and 45 s at 72.8 ◦C; and 1 cycle from 7 min to 72 ◦C and re-establishment and final maintenance at a temperature of 4 ◦C. Sequencing was carried out by the Microsynth AG company (Balgach, Switzerland), starting from the solution of amplicons obtained by PCR checked on 1.5% agarose gel. The ITS sequences were compared with those available in the NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/; accessed on 2 August 2021) genetic database by using the Basic Local Alignment Search Tool (BLAST) algorithm and using only sequence identity values above 99%.

2.2.3. Phylogenetic Analysis

The phylogeny was inferred using Bayesian and Maximum Likelihood methods. *Ilyonectria radicicola* (Gerlach & Nilsson) Chaverri & Salgado (AF220969) was used as the outgroup. Bayesian search and model selection were carried out in a JModel Test [41]. We selected the best model of nucleotide substitution under the corrected Akaike's Information Criterion. The optimal model for the rDNA region was GTR + G using MrBayes 3.2.7 [42]. Maximum Likelihood bootstrap analyses were assessed with RAxML [43] by bootstrap replicating the data matrix 1000 times in order to assess clade support. The obtained phylogenetic trees were visualised and edited by using FigTree v.1.3.1 (available at http: //tree.bio.ed.ac.uk/software/figtree/; accessed on 28 October 2021). The

congruence between phylogenies resulting from these two methods was determined based on sharing highly supported nodes (>70%—maximum likelihood; >95%—posterior probability).

2.2.4. Fungal Strain Formae Specialis Identification

Once the species was assigned to the isolate, the *forma specialis* was identified by utilizing a pathogenicity test. The *formae speciales* that attacked hemp included *F. oxysporum* f. sp *cannabis* (pathogen exclusive to hemp) and *F. oxysporum* f. sp*. vasinfectum* (pathogen of many plants) [18]. The pathogenicity test was carried out on *Cannabis sativa* 'Finola,' *Capsicum annuum* and *Medicago sativa* by using a 10^6 CFU mL^{-1} spore solution (see Section 2.2.1) at sowing as a dipping solution for 20 min and by observing the development of the pathogenesis for 20 days. Seed germination rates, plant development (presence of leaf wilting) and morphology (presence of black spots) were monitored as disease symptoms.

2.2.5 Bacterial Strains and Growth Conditions

Bacterial strains *A. brasilense* ATCC 29710, *B. ambifaria* PHP7, *G. diazotrophicus* ATCC 49037 and *H. seropedicae* ATCC 35892 were cultivated in 1 L Erlenmeyer flasks containing 500 mL of T4 medium (KH2PO4 10.99 g L^{-1} ; K2HPO4 3.34 g L^{-1} ; Oxoid™ Yeast Extract Powder 0.05 g L^{-1} ; fructose 10.99 g L^{-1} ; 100 mL of 10× salt solution (MgSO4 * 7H2O 2 g L^{-1} ; NaCl 1 g L−1 ; CaCl2 * 2H2O 0.26 g L^{-1} ; Na2MoO4 * 2H2O 0.01 g L^{-1} ; MnSO4 * H2O 0.02; NH4Cl 10 g L−1 ; 2 mL of Fe-EDTA solution in 1.4% KOH; pH 6.4)) [44]. Broth cultures were grown at 30◦C under constant shaking (150 rpm) for 24 h (except for *G. diazotrophicus*, cultured for 48 h).

2.2.6. In Vitro Biocontrol Activity

In vitro antagonistic activity was assessed by co-cultivation of bacterial single strains/ consortium with FOC. An amount of 10 µL of 106 CFU mL^{-1} of bacterial broth cultures at the log phase (determined spectrophotometrically by comparing obtained 600 nm optical densities with growth curves) was plated with a loop forming two vertical lines at the edges of the plate and 2.5 cm away from the centre on PDA dishes Ø 90 mm dishes with 22 mL of medium; 2 lines per plate). After incubation at 28 ◦C (48 h for *G. diazotrophicus* and 24 h for the other bacteria), a plug (Ø 5 mm) of young FOC mycelium facing the agar (5 days old) was transferred to the centre of the dish. PDA

dishes were incubated at 28 °C until the control fungal mycelium (without bacterial presence) completely covered the dish (10 days). Each trial was repeated 3 times (three independent experiments). In the presence of bacterial inhibition, we obtained no circular growth. For this reason, the growth of the fungus was measured from the centre toward both sides of bacterial streaks. The inhibition percentages were calculated as follows.

$$I\,\% = \frac{(\text{mm growth control} - \text{mm growth dual culture})}{\text{mm growth control}} \times 100$$

After the determination of the inhibition percentages of the individual bacteria, the consortium, formed by equal amounts of the most active bacteria (*B. ambifaria, G. diazotrophicus* and *H. seropedicae*) broth cultures at the log phase, was plated after vortexing, and inhibition percentages were evaluated as described above.

2.2.7. Bacterial Consortium–Pathogen Interaction

The interaction between the bacterial consortium and the FOC in in vitro biocontrol dishes was investigated by scanning electron microscopy. The part of the mycelium that develops towards the bacterial streak was sampled with the head of 1000 µL sterile pipette tips with the aid of a Greenough stereo microscope, Leica S8 APO with 8:1 apochromatic zoom. The samples were fixed overnight with a 2.5% glutaraldehyde solution in 0.05 M phosphate buffer (pH 7.3), washed with distilled water and dehydrated with a few drops of hexamethyldisilazane (HMDS—Sigma-Aldrich, St. Louis, MO, USA). The dried samples were fixed with carbon tape (Agar Scientific, Stansted, UK) on stubs and coated with chromium for SEM observations (Gemini SEM 500 SEM—Zeiss, Oberkochen, Germany). Acquisitions were performed with an acceleration voltage of 5 kV and type II secondary electrons (SE2 signal).

2.2.8. In Planta Biocontrol Activity

The in planta biocontrol activity of the consortium against FOC was assessed both during pre-emergence and post-emergence (before and after germination, respectively). The experiments were carried out by utilizing certified *Cannabis sativa* 'Finola' seeds (Hemp Farm Italia, Tortoreto, Italy). In the pre-emergence experiment, the bacterial inoculation was obtained by soaking the seeds for 20 min under constant stirring in the consortium solution (1010 CFU mL^{-1}, determined

spectrophotometrically by comparing obtained 600 nm optical densities with growth curves) and prepared with equal amounts of *B. ambifaria, G. diazotrophicus* and *H. seropedicae* broth cultures. After drying overnight, the bacterial density of the seeds (106 CFU g−*1*) was estimated by plating serial dilutions. One gram of seed was homogenized in sterile saline with 0.1% of Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) with a lab blender Stomacher® 80 (Seward, Worthing, UK) for 1 h, and 100 µL of serial dilutions 10−3 to 10−7 was plated on T4 agar plates (Ø 90 mm). Colonies developed on plates were counted after 48 h of incubation at 28 ◦C, and CFU g^{-1} was calculated by considering serial dilutions used (the trial was repeated three times in three independent experiments). In post-emergence experiments, seedlings with the first leaves unfolded were inoculated after transplanting with a consortium solution of 106 CFU mL^{-1} (adjusted spectrophotometrically by absorbance measurements at 600 nm). An amount of 10 mL of consortium solution was directly deposited to the base of each seedling. In both experiments, infections were induced with FOC 106 mL^{-1} spore suspensions (see Section 4.1). The experimental conditions investigated were (i) FOC, no bacterial inoculation/with fungal infection; (ii) Consortium + FOC, with bacterial inoculation/with fungal infection; (iii) Consortium, with bacterial inoculation/no fungal infection; and (iv) Control, no bacterial inoculation/no fungal infection. Each experimental unit consisted of 8 pots with 5 seeds/plants per pot (filled with 3 L of commercial common soil) left to grow in a greenhouse under a natural spring photoperiod (25–27 ◦C). Plants were checked daily and watered with 10 mL per plant every 2 days. The growth was stopped when infected plants showed evident disease symptoms 20 days after sowing for the pre-emergence trial and 30 days after sowing for the post- emergence trial. Once growth was stopped, plants from both experiments were analyzed for the following parameters: germination/survival (%), plant height (cm), root length (cm), number of true leaves, total chlorophyll content (mg g FW−1) [45] and chlorophylls a/b ratio. The degree of damage was estimated as follows: 0 = no damages; 1 = 0.1–3 mm; 2 = 3–6 mm; 3 = 6–9 mm; 4 = 9–12 mm; 5 = > 12 mm/plant death.

2.2.9. Statistical Analysis

Mean values differences among experimental conditions were estimated by two-way analysis of variance (ANOVA). Comparison and separation of the means were performed by Fisher's LSD post hoc test at a 5% level of significance ($p < 0.05$) using XLSTAT 2016 software (Addinsoft, Paris, France).

2.3 Results

2.3.1. Fungal Isolate Morphological and Molecular Identification

Fusarium isolates obtained on Selective *Fusarium* Agar (SFA) [17] were screened based on macroscopic and microscopic observations. Based on the colour of mycelium and growth rate on Potato Dextrose Agar (PDA, Oxoid, United Kingdom) and microconidia and macroconidia on Soil Agar (SA) [17], a putative *Fusarium oxysporum* isolate was selected. As shown in Figure 1, the 8 cm mycelium that developed from the isolate after 7 days has a pale purple/deep pink colour (Figure 1A). Oval-shaped microconidia (Figure 1B) are formed in false heads on monophialides (Figure 1C); the macroconidium has five septa (Figure 1D), and single and terminal chlamydospores are present (Figure 1E).

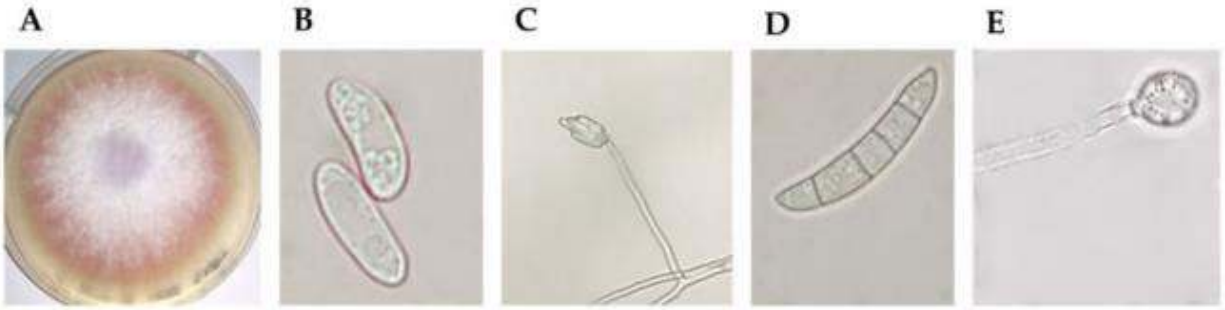

Figure 1. Putative *Fusarium oxysporum* morphological characteristics. In the figure: **(A)** pale violet colour of mycelia; **(B)** oval-shaped microconidia; **(C)** false heads of microconidia on a monophialide; **(D)** sickle-shaped macroconidia; **(E)** terminal chlamydospore.

The isolate was then characterized by ITS sequencing and identified with 100% identity as *Fusarium oxysporum* (Figure 2). Phylogenetic analyses grouped the FOC isolate with a high degree of sequence identity (99–100%) within the *Fusarium oxysporum* complex. Figure 2 shows the phylogenetic tree inferred from maximum likelihood and Bayesian analyses from ITS regions of 24 representative species of *Fusarium*, the isolate of this study and the Ilyonectria radicicola outgroup. The formae speciales that caused pathogenicity on hemp include *F. oxysporum* f. sp. *vasinfectum*, which attacks other plants such as *Capsicum annuum* and *Medicago sativa,* and *F. oxysporum* f. sp. cannabis, which occurs only on hemp [18]. We infected the seeds of *C. sativa, M. sativa* and *C. annuum* at sowing with a spore solution of 106 CFU mL^{-1} and observed the development of pathogenesis for 20 days.

The seeds of *C. sativa* that germinate developed rachitic plants with dark spots on the leaves and wilting of the leaves. In *M. sativa* and *C. annuum,* there were no changes in seed germination, plant development (no wilting leaves) and morphology (no black spots). Therefore, based on the ability to induce pathogenesis on *C. sativa* and not on *Medicago sativa* and *C. annuum*, the isolate was classified as *F. oxysporum* f. sp. cannabis.

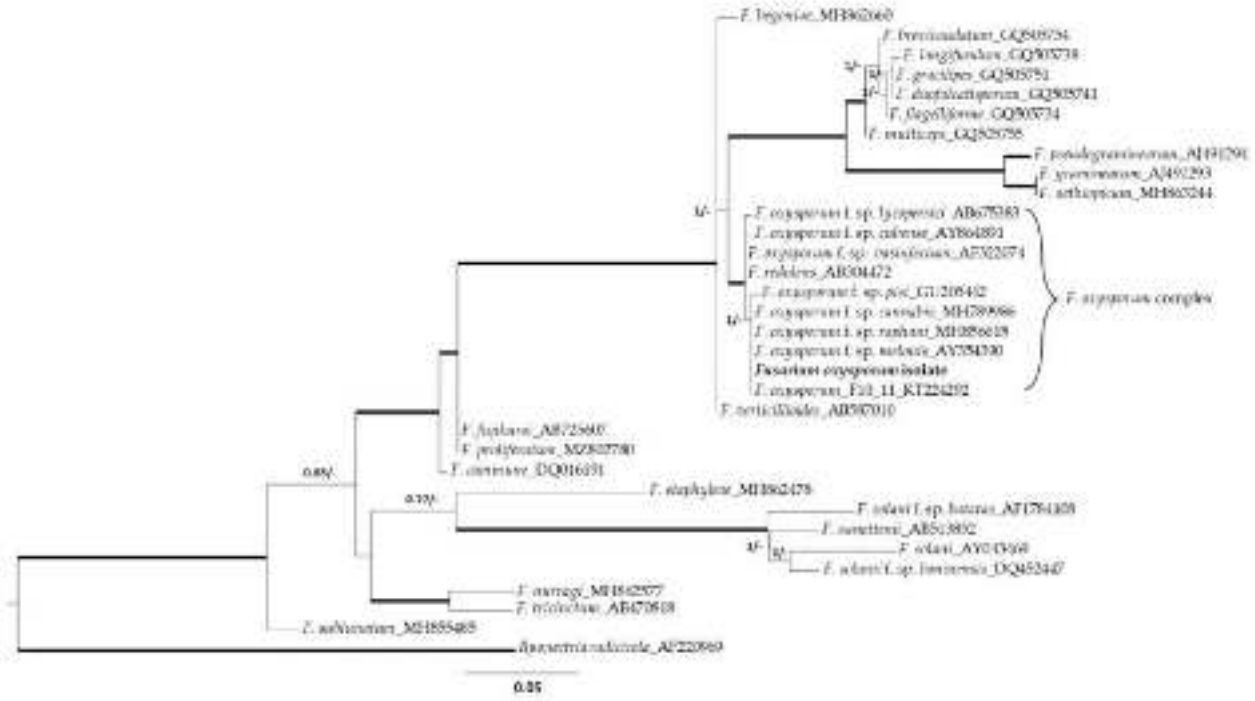

Figure 2. Phylogenetic tree inferred from maximum likelihood and Bayesian analyses from internal transcribed spacer (ITS) regions of 24 representative species of *Fusarium*, the isolate of this study and the Ilyonectria radicicola outgroup. Thickened branches indicate those that are supported both by likelihood bootstrap values of >70% and by Bayesian posterior probabilities of >95%. The definition of MrBayes and RAxML percentages bootstraps are defined next to the branches at each node (probabilities/bootstrap). Scale bar represents the number of substitutions per nucleotide site for a unit of branch length.

2.3.2 In Vitro Antagonistic Activity

PGPB antagonistic activity against FOC was tested in vitro by dual culture (cultivation of single bacteria/consortium and FOC on PDA medium). Effective growth inhibition was assumed when the percentage of inhibition was higher than 20%. Based on the distribution of mycelium in the centre and bacterial streaks at the edges of the plate, values below 20% were associated with the growth

of mycelium on and across bacterial streaks. The percentages of inhibition obtained after 7 days of culture are presented in Table 1. The in vitro antagonistic activity of *B. ambifaria, G. diazotrophicus* and *H. seropedicae* was statistically similar ($p > 0.05$), with an average inhibition of 68%. For these bacteria, mycelial growth ceased before the bacterial streaks (Figure 3A). For *A. brasilense*, no effective inhibition was observed, the inhibition was less than 20% and mycelium grew across the bacteria streaks (Figure 3B). The latter was excluded from the consortium, which comprised equal amounts of *B. ambifaria, G. diazotrophicus* and *H. seropedicae* broth cultures. The combination of strains in the consortium did not alter antagonistic activity (no statistically significant differences from the values of the individual strains, $p > 0.05$), with an inhibition rate of 71%.

Table 1. In vitro antagonistic activity of single bacterial strains and the bacterial consortium formed by *Burkholderia ambifaria, Gluconacetobacter diazotrophicus* and *Herbaspirillum seropedicae* against *Fusarium oxysporum* f. sp. cannabis.

Strains	Inhibition (%)
Azospirillum brasilense	<20%
Burkholderia ambifaria	65.0 a
Gluconacetobacter diazotrophicus	64.1 a
Herbaspirillum seropedicae	66.9 a
Consortium	70.6 a
LSD	6.7

The results are the mean of three replicates (three independent experiments). Results followed by the same case letter are not significantly different according to Fisher's least significant difference (LSD) post hoc test ($p < 0.05$)

2.3.3. Bacterial Effects on Fungal Mycelium

Scanning electron microscopy (SEM) observations of the inhibition zones of the consortium–FOC dual cultures showed the effects of the bacterial consortium on the fungal mycelium. Figures 3 and 4 present the micrographs obtained by SEM. In the absence of PGPB during growth (Figure 3A), the mycelium exhibits normal growth with continuous overlapping and abundant hyphae (green circles). In the presence of PGPB (Figure 3B), the mycelium is discontinuous, with sparse and deformed hyphae (swelling and vacuolation are shown by blue and red arrows, respectively). The 5000× micrograph details in Figure 4 show the bacterial effects on the hyphal structures. Figure 4A shows the disaggregation of fungal branches (arrows) and lytic fragments (circles), while Figure 4B shows the thinning of hyphal branches.

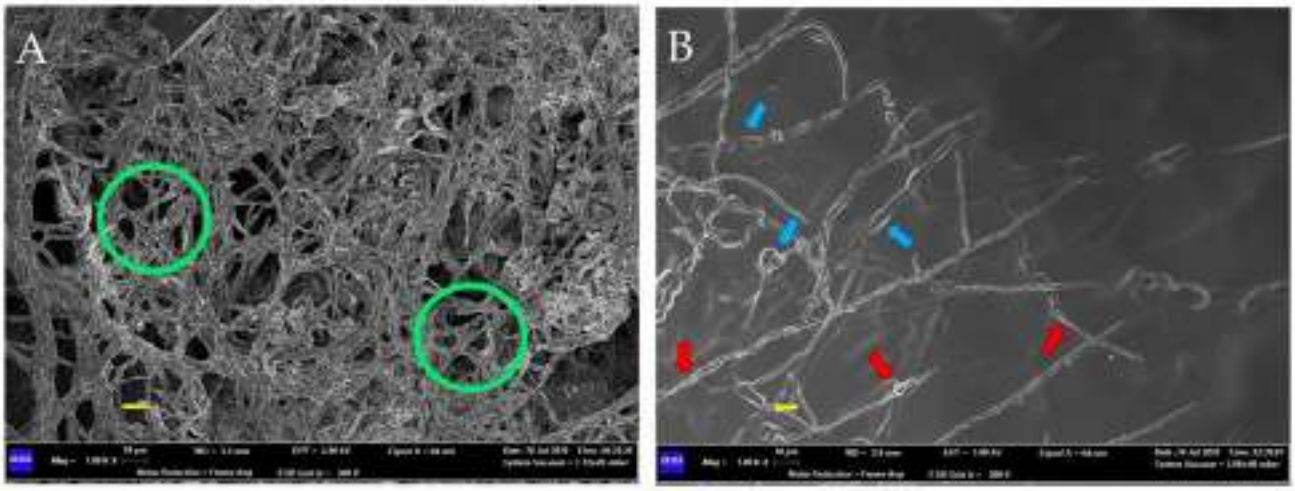

Figure 3. Scanning Electron Microscope (SEM) micrographs at 1000X showing differences in mycelium development of *Fusarium oxysporum* f. sp. *cannabis*. (A) Control mycelium with continuous and normal hyphae and branching; (B) mycelium with swelling and vacuolation of the hyphae present in an interaction zone between *F. oxysporum* f. sp. cannabis and the bacterial consortium formed by *Gluconacetobacter diazotrophicus, Herbaspirillum seropedicae* and *Burkholderia ambifaria*. Scale bars (in yellow) 10 µm.

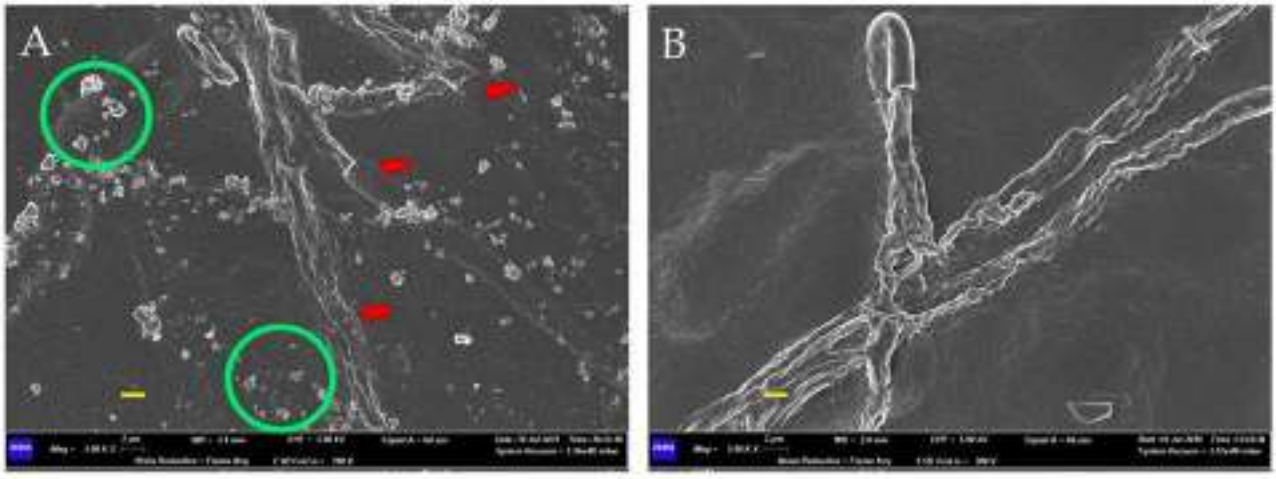

Figure 4. Scanning Electron Microscope (SEM) micrographs at 5000 X that show the abnormalities of the *Fusarium oxysporum* f. sp. cannabis mycelium. In the presence of the bacterial consortium formed by *Gluconacetobacter diazotrophicus, Herbaspirillum seropedicae* and *Burkholderia ambifaria,* the mycelium presented irregular and desegregated hyphae (A), with a distorted development (B). Scale bars (in yellow) 2 µm.

2.3.4. In Planta Biocontrol

The ability of the consortium, formed by *G. diazotrophicus, H. seropedicae* and *B. ambifaria*, to induce protection against FOC in C. sativa was investigated in pre-emergence and post-emergence pot experiments. Figure 5 shows the comparisons of the four experimental units for pre-emergence (Figure 5A) and post-emergence (Figure 5B) trials. In both pre-emergence and post-

emergence trials, treatment of the plant with the bacterial consortium alone (Consortium) promoted good plant growth. Similar plant development was observed in Consortium + FOC (presence of the bacterial consortium and the fungal pathogen). The plants under these two experimental conditions were longer than those of the control. Under the experimental condition Consortium + FOC, the plants were healthier than those of FOC (not treated with bacteria and infected with the fungal pathogen). The results of the pre-emergence and post-emergence in planta trials are shown in Table 2.

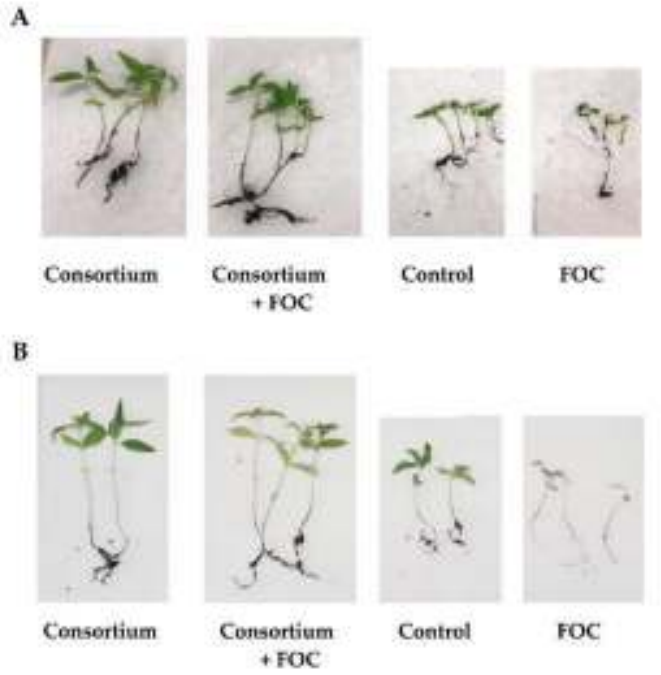

Figure 5. Comparison of experimental units obtained for pre-emergence (A) and postemergence (B) experiments. In the figure: FOC*, Fusarium oxysporum* f. sp. *cannabis*

Table 2. In planta pre-emergence and post-emergence antagonistic activity of bacterial consortium formed by Gluconacetobacter diazotrophicus, Herbaspirillum seropedicae and Burkholderia ambifaria against Fusarium oxysporum f. sp. cannabis.

		Germination	C g	Damages	C g	Roots	C g	Shoots	C g	Leaves	C g	Chl tot	C g	Chl a/b Ratio	C g
Consortium	Pre	100^{a}	A	-	-	3.3^{c}	A	8.7^{a}	A	4.3^{c}	A	2.23^{a}	A	4.99^{d}	AB
	Post	100^{a}		-		6.0^{a}		6.5^{c}		9.5^{a}		0.49^{d}		5.43^{c}	
Control	Pre	100^{a}	A	-	-	2.1^{e}	C	4.0^{e}	C	3.5^{cd}	B	1.11^{c}	C	3.26^{f}	B
	Post	100^{a}		-		3.6^{c}		4.2^{e}		5.5^{b}		0.20^{f}		6.70^{a}	
Consortium + FOC	Pre	89^{b}	B	2^{c}	B	2.6^{d}	B	7.6^{b}	B	4.0^{c}	B	1.78^{b}	B	4.61^{de}	A
	Post	85^{c}		2^{b}		5.2^{b}		5.5^{d}		6.0^{b}		0.21^{f}		6.12^{b}	
FOC	Pre	55^{d}	C	5^{a}	A	1.4^{f}	D	2.2^{g}	D	1.5^{e}	C	0.43^{e}	D	1.02^{g}	C
	Post	42^{e}		5^{a}		2.8^{d}		3.0^{f}		2.8^{d}		0.02^{g}		4.36^{e}	
LSD Condition		1.4		0.3		0.2		0.4		0.6		0.04		0.31	
LSD Trial		0.9 *		0.2 ns		0.2 *		0.3 *		0.4 *		0.03 *		0.22 *	
LSD Condition x Trial		1.9		0.4		0.3		0.5		0.9		0.06		0.44	

In the Table: FOC*, Fusarium oxysporum* f. sp. *cannabis*; Pre, pre-emergence trial; Post, post-emergence trial; LSD, least significant difference; C g , Fisher's LSD grouping based on Condition; Chl, chlorophylls; *, pre-emergence and post-emergence trials are significantly different based on Fisher's LSD post hoc test; ns, pre-emergence and post-emergence trials are not significantly different based on Fisher's LSD post hoc test. For the same column, results followed by the same case letter are not significantly different according to Fisher's LSD post hoc test.

Two-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) post hoc test showed that the two variables, Condition and Trial, and their interaction (Condition × Trial) had a significant effect. The best plant growth parameters were obtained under the Consortium experimental condition followed by Consortium + FOC and Control. The lowest results were registered under FOC. Except for damages, plant growth parameters of pre-emergence and post-emergence trials differed significantly. The summary of multiple pairwise comparisons for Condition x Trial (Fisher (LSD) interaction is presented in Table S1 in Supplementary Materials. In the pre-emergence trial, FOC infection significantly reduced germination, with a decrease of −45% compared to the control. Plants that germinated and grew despite the fungal infection (FOC) exhibited damages and recorded a decrease in all growth parameters. Plant height and root length decreased significantly ($p < 0.05$) compared to control (−33% and −44%, respectively). The number of true leaves, chlorophylls content and chlorophyll a/b ratio ($p < 0.05$) was also lower than the control ($p < 0.05$). In the absence of fungal infection (Consortium), the bacteria promoted good plant growth and development, with the highest values for all parameters ($p < 0.05$). Plant growth and development promoted by the bacterial consortium was flawed in the presence of fungal infection (Consortium + FOC). However, the severity of infection was less and resulted in a lower decrease in germination (−11% than control). For the plants under Consortium + FOC, fewer damages and better growth parameters were recorded compared to those under FOC. The number of true leaves and the chlorophyll a/b ratio was statistically comparable to the control ($p > 0.05$), while plant height, root length and chlorophylls contents had higher values than the

control ($p < 0.05$). In the post-emergence experiment, the FOC infection (FOC) induced a massive loss of plants (plant survival −58%). Plants that survived fungal infection exhibited extended damages and recorded the lowest growth parameters ($p < 0.05$). In the presence of the bacterial consortium, there was a substantial reduction in fungal infection symptoms (Consortium + FOC parameters lower than Consortium, $p < 0.05$). The improvement in all parameters investigated highlighted the effective antagonistic activity of bacterial consortium against FOC (Consortium + FOC parameters higher than FOC, $p < 0.05$). The number of true leaves and chlorophylls content was similar to the control ($p > 0.05$), while plant height and root length were higher than the control ($p < 0.05$).

2.4 Discussion

In this study, the application of a consortium of three beneficial bacteria significantly reduced FOC disease in both pre-emergence and post-emergence trials. The biocontrol agents available for preventing and countering FOC are limited, and the literature lacks scientific studies on biocontrol agents against *forma specialis*. However, our findings are consistent with previous studies on microbial consortia as biocontrol agents against plant fungal diseases [19]. The biocontrol potential of the bacterial strains that form our consortium against Fusarium spp. has been described in various studies. Simonetti et al., demonstrated that *B. ambifaria* has strong activities against *Fusarium* spp. (i.e., *F. graminearum, F. oxysporum* and *F. solani*) when using fusaric acid (responsible for the disease) as an energy source [20]. *B. ambifaria* is a valid biocontrol strain thanks to a set of numerous diffusible and volatile antifungal molecules. Among the diffusible molecules, we can find the powerful antifungals burkholdines, occidiofungin, pyrrolnitrin and 4-hydroxy-2- alkylquinoline [21–23]. *B. ambifaria* volatile antifungal compounds include dimethyl disulfide, dimethyl trisulfide, 4-octanone, S-methyl methanethiosulphonate, 1-phenylpropan1-one and 2-undecanone [13]. Mehnaz and Lazarovits showed in vitro inhibitory activity of *G. diazotrophicus* against *Fusarium* spp. [24]. The same results were reported by Logeshwarn against *F. oxysporum* of sweet potato, ascribing the inhibition capabilities to 2,4-diacetylphloroglucinol, pyrrolnitrin and pyoluteorin [14]. Weber et al., described effective control of *F. oxysporum* f. sp. *cubense* in banana seedlings in the presence of the co-inoculation of *H. seropedicae* and *Burkholderia cepacia* [25]. *H. seropedicae* intervenes in the modulation of the host plant's defence responses [15,26] and produces siderophores (serobactins) that contribute to competition within the

plants [27,28]. The production of metabolites by beneficial bacteria is essential to help the plant fight fungal diseases by interfering with the growth and activities of pathogens. In addition to diffusible (e.g., organic acids, lipopeptides and pyrroles) and volatiles (e.g., hydrocyanide, ammonia and sulphides), other metabolites can counteract fungal infection. Lytic enzymes, for example, can directly break down constitutive polymeric compounds (i.e., chitin, proteins, and DNA) [29]. Another effect exerted by beneficial bacteria is competition for nutrient sources particularly against soil-borne pathogens, such as Fusarium [29]. Trophic competition can involve carbon, nitrogen and iron and can be an effective biocontrol mechanism against phytopathogenic fungi [30]. Biocontrol inoculants based on microbial consortia are an effective strategy for crop protection against phytopathogens [31]. Bacterial inoculation induces the activation of the defence response of host plants and increases nutrient uptake and root structure by reducing the propagation of pathogens [32]. The presence of more strains broadens the antagonistic spectrum and improves performance [33]. Other direct plant growth-promoting traits also counteracted fungal pathogens. Our findings demonstrated that the bacterial consortium enhanced the growth of plants both in pre-emergence and post-emergence trials. This positive effect on plant growth is related to the ability of *B. ambifaria, G. diazotrophicus* and *H. seropedicae* in producing phytohormones, solubilizing nutrients and fixing atmospheric nitrogen [34]. Fungal diseases are a major concern in agriculture given the huge losses induced annually. The control of fungal diseases in crops is achieved by using agrochemicals. These substances, extensively applied in prevention campaigns, have resulted in severe consequences for the environment and human health. Pollution of soil, groundwater and surface water by agrochemicals is toxic to both humans and animals and induces the growth of algae, which unbalances the life cycle of aquatic animals [35]. This situation drives the scientific community and agriculture to search for valid alternative techniques for the control of fungal infections. In this study, we focused our attention on hemp. Many fungal diseases threaten the crops of this multipurpose plant every year. FOC is a devastating fungal disease of hemp [7]. The severity of its pathogenesis is so strong that this fungus is used as a bioherbicide to destroy the illegal fields of *C. sativa* subsp. *indica* [36]. To the best of our knowledge, this study is the first report on the biocontrol ability of a bacterial consortium against FOC. Further studies should be directed toward the evaluation of this consortium in greenhouse (repeated experiments with different light and soil characteristics and a major number of plants) and open field experiments (different pedoclimatic conditions). In order to clarify the mechanism's underlying the biocontrol activity, the characterization of the bioactive molecules

produced by the bacteria against FOC should also be carried out, as well as the response of the plant to fungal infection in the presence of bacteria. The preliminary results obtained so far suggest that this consortium may have activity against *F. oxysporum* ff. spp. and other fungal pathogens [11]. Future research should investigate the biocontrol ability of the consortium against *F. oxysporum* f. sp. *vasinfectum* and other fungal pathogens in hemp and other crop plants. More detailed studies of the translation elongation factor alpha genetic region of the pathogenic fungus could also provide additional information on the phylogeny of the isolate [37].

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Chapter 3

Cell-Free Supernatants of Plant Growth-Promoting Bacteria: A Review of Their Use as Biostimulant and Microbial Biocontrol Agents in Sustainable Agriculture

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Abstract

Plant growth-promoting bacteria (PGPB) afford plants several advantages (i.e., improvement of nutrient acquisition, growth, and development; induction of abiotic and biotic stress tolerance). Numerous PGPB strains have been isolated and studied over the years. However, only a few of them are available on the market, mainly due to the failed bacterial survival within the formulations and after application inside agroecosystems. PGPB strains with these challenging limitations can be used for the formulation of cell-free supernatants (CFSs), broth cultures processed through several mechanical and physical processes for cell removal. In the scientific literature there are diverse reviews and updates on PGPB in agriculture. However, no review deals with CFSs and the CFS metabolites obtainable by PGPB. The main objective of this review is to provide useful information for future research on CFSs as biostimulant and biocontrol agents in sustainable agriculture. Studies

on CFS agricultural applications, both for biostimulant and biocontrol applications, have been reviewed, presenting limitations and advantages. Among the 109 articles selected and examined, the Bacillus genus seems to be the most promising due to the numerous articles that support its biostimulant and biocontrol potentialities. The present review underlines that research about this topic needs to be encouraged; evidence so far obtained has demonstrated that PGPB could be a valid source of secondary metabolites useful in sustainable agriculture.

3.1 Introduction

Plant growth-promoting bacteria (PGPB) are a widespread group of bacteria generally living in association with plants, having several beneficial effects related to (i) improvement of plant nutrient acquisition [1], (ii) promotion of plant growth and development [2], and (iii) induction of tolerance towards abiotic and biotic stress [3]. Although the mechanisms behind these effects are complex and not fully known, most of the effects can be ascribed to the bacterial ability to produce metabolites with stimulant and/or protective effects. Among stimulant molecules, a meaningful role is played by phytohormones (i.e., abscisic acid, auxins, cytokinins, ethylene, and gibberellins). These substances regulate plant growth at all stages of development, by stimulating growth, coordination between cells, tissues and organs, and by preserving certain functions [4]. Stimulant effects are also ascribed to organic acids, which induce the release of nutrients from insoluble complexes by lowering soil pH, chelation, and mineralization [5,6]. The promotion of plant growth and development are also induced by several other secondary metabolites, volatile compounds, and exopolysaccharides [2,7]. Phytohormones, organic acids, secondary metabolites, volatile organic compounds, and exopolysaccharides also provide protection/tolerance against several stresses, both abiotic (e.g., salt and drought) and biotic (e.g., bacterial and fungal pathogens). Due to the above characteristics and their sustainability, PGPB have received increasing attention in recent decades and their use is highly regulated by the European Parliament and by the European Council by the Regulation (EU) 2019/1009. However, formulation and effectiveness of PGPB cells present challenges. The main limit for bacterial cell suspension without an adequate carrier or formulation is that, after inoculation in the soil, there is a decrease in bacterial population for most of the PGPB species. This low persistence, combined with low production of bacterial biomass, makes it difficult to support the activity in the rhizosphere. The non-optimal bacterial physiological status at the time

of application can prevent the accumulation of a sufficiently large PGPB population in the rhizosphere. Besides, these bacteria must compete with the adapted native microbial community and resist predation by soil microfauna [8]. In the scientific literature, many potential PGPB strains are described; however, only a few are on the market. This situation is mainly due to low bacterial survival during product shelf life and, once applied, inside the agroecosystems. PGPB strains with these challenging limitations can be used for the formulation of a cell-free supernatant (CFS). CFSs, are mixtures derived from broth cultures by several mechanical and physical processes that allow the removal of cells. CFSs can be obtained through two main unit operations, centrifugation, and filtration (i.e., microfiltration, ultrafiltration, nanofiltration, inverse osmosis). These techniques can be applied individually or in combination with other technologies according to the desired final product. Several other downstream processes can be applied to isolate and purify target metabolites, also from the inside of cells [9]. Many studies of CFSs deal with metabolites utilized in medical and food sectors; studies on the biostimulant and biocontrol properties of these formulations in plants are limited to in vitro tests, controlled conditions experiments, and/or addressed to the characterization of target metabolites. Numerous reviews and updates concerning PGPB in agriculture, from their isolation to their formulation, can be found in the literature. However, as far as we know, there are no reviews dealing with applications of CFSs obtained by PGPB. The present review aimed at summarizing studies concerning PGPB CFSs and their metabolites as biostimulant and biocontrol agents. Several databases have been used to create a collection of articles. After article screening, a total of 109 valid published works has been selected. Data organization allowed the discussion of CFSs' and their metabolites' biostimulant and soil-borne pathogen control applications (i.e., of bacteria, fungi, oomycetes). This review provides useful information for future research on CFSs as biostimulant and biocontrol agents in sustainable agriculture.

3.2 Methods

To find relevant publications on CFSs and their metabolites an online literature search was conducted. The following databases were employed in the search:

- CAB Direct (cabdirect.org)
- Google scholar (scholar.google.com)

• Science Direct (sciencedirect.com)
• Scopus (scopus.com)
• Springer Link (springerlink.com)
• Taylor and Francis (tandfonline.com)
• Web of Science (webofknowledge.com)
• Wiley Online Library (onlinelibrary.wiley.com)

Several combinations of search terms were attempted in each database. The terms “cell-free supernatant”, “spent supernatant”, “bacterial broth”, “bacterial culture”, and “bacterial metabolites” were combined with “biostimulant”, “biocontrol”, “phytopathogens”, “fungi”, “bacteria” “oomycetes”, and “sustainable agriculture”. The search was extended to all manuscript sections. The online literature search produced a large collection of articles that have been screened according to Title and Abstract contents (Initial check). Then, articles were read completely and related papers were included in the collection if they were not already present (Related paper check). The reading and screening allowed us to discard irrelevant papers from the collection and to find a total of 109 relevant articles. The complete reading of the articles also allowed the organization of the collection based on two main categories: “biostimulant” and “biocontrol”. The Biostimulant category was organized based on details about PGPB strain, compound, production technique utilized to obtain CFS/metabolites (C, centrifugation; F, Filtration; E, solvent extraction; DP, several downstream processes), crop, and experiment (P, in vitro growth; G, greenhouse growth; O, open field growth). The Biocontrol category was organized depending on the type of phytopathogen (i.e., bacteria, fungi and oomycetes) and based on details about PGPB strain, pathogen, compound, production technique utilized to obtain CFS/metabolites (C, centrifugation; F, Filtration; E, solvent extraction; DP, several downstream processes), and experiment (V, in vitro antagonism; X, ex vivo antagonism; P, in vitro growth; G, greenhouse/pot growth). For each category, tables were prepared to provide these details per reference.

3.3 CFSs as Biostimulant Agents

Over the years, the application of synthetic fertilizers in agriculture has increased to the maximum requested by global demand–crop yield [10]. Continuous fertilization campaigns repeated over the years involve considerable production costs, environmental pollution and soil degradation [11,12].

The use of PGPB-CFSs and isolated metabolites can represent an alternative sustainable technique to synthetic fertilizers. Table 1 summarizes details of the studies concerning the application of CFSs and their metabolites as biostimulant agents. These studies reported interesting biostimulant properties of CFSs in vitro and in planta (both in greenhouse and in open field experiments). The capability of CFSs to stimulate in vitro growth of seedlings has been reported for *Medicago polymorpha* [13], *Oryza sativa* [14], *Glycine max* [15,16], *Zea mays* [17], *Lemna minor* [18], *Solanum lycopersicum* [19], *Glycine max*, and *Triticum aestivum* [20]. The CFS obtained from *A. brasilense* Cd strain has been reported to be able to promote growth in an *M. polymorpha* seedling inoculated with *Rhizobium meliloti* RT1 early nodulation and changes in root morphology and function by ethylene production [13]. An 8% (v/v) CFS-based formulation obtained from *A. brasilense* Cd strain showed a good capability to increase in vitro *O. sativa* growth. In particular, the presence of CFS in the culture medium promoted better elongation, root surface area, root dry matter, and development of lateral roots of *O. sativa* seedlings than those grown on culture media without CFSs addition [14]. Idris et al., also described concentration-related positive effects of *Bacillus* spp. CFSs in *Z. mays* L. in a coleoptiles cylinder test [17] and in L. minor in 48-well microtiter plates growth [18]. *Bacillus amyloliquefaciens* KPS46 CFS metabolites positively affected growth and development of *G. max* under gnotobiotic condition [15]. The CFSs obtained from *Burkholderia seminalis* (an isolated strain selected for high levels of Indole-3-Acetic Acid (IAA) production) showed a positive impact on in vitro germination of tomato seeds [19]. Ethyl acetate extract of *Methylobacterium* spp. CFSs, composed mostly of cytokinins, demonstrated positive effects on *Triticum aestivum* L. seed germination and seedling growth [20]. To assess the actual capability of a certain compound to stimulate plant growth, in vitro experiments should be followed by in planta ones. However, among the above-mentioned reports, only a few studies [13,15] confirmed in planta effectiveness in greenhouse experiments. Effectiveness of CFSs' biostimulant properties in greenhouse experiments was also reported for *Manihot esculenta* [21], *Musa* spp. [22], *Vigna unguiculata* [23], *Pisum sativum* [24], *Vicia villosa* [24], *G. max* [16,25,26], and *M. sativa* [27]. *Bacillus* sp. CaSUT007 CFS solvent extracts containing lipo-chittinligosaccharides (LCOs), phytohormone and extracellular proteins promoted the growth of *M. esculenta* Crantz [21]. Posada et al., [22] reported that CFSs of *Bacillus subtilis* EA-CB0575, either from vegetative cells or from spores, significantly increased shoot length and total dry weight of *Musa* plants compared with control. CFSs of *Streptomyces acidiscabies*,

containing siderophores and auxins, were able to promote growth and alleviate metal toxicity in *Vigna unguiculata* L. [23]. *Rhizobium leguminosarum* bv. *viciae* CFSs rich in LCOs were able to ameliorate *Pisum sativum* and *Vicia villosa* growth [24]. *G. max* was positively affected by treatment with *A. brasilense* Sp7 CFSs, inducing better root growth than experimental condition treated with the bacterial inoculum [25]. For this plant, the enhancement of biostimulant effectiveness has been reported when a combination of different treatments was tested. The application of CFSs of *A. brasilense* strains Ab-V5 (CNPSo 2083) and Ab-V6 (CNPSo 2084) via seeds improved root morphology and nodulation in *G. max* inoculated with *Bradyrhizobium* spp. [16]. However, the efficacy was lower than co-inoculation with *Bradyrhizobium* spp. single strains. Positive effects on *G. max* were reported by Moretti et al., [26]. In their work the best results were obtained with a combination of (i) *Bradyrhizobium diazoefficiens* (USDA 110) and *Rhizobium tropici* (CIAT 889) metabolites enriched in LCO seed treatment, (ii) *Bradyrhizobium japonicum* (SEMIA 5079) and *B. diazoefficiens* (SEMIA 5080) inoculation; and (iii) *A. brasilense* (Ab-V5 and Ab-V6) foliar application. Efficient combination was also reported by Morel et al., [27]. These authors indicated that hydroponic solution added with bacterial and root-secreted molecules (i.e., flavonoids, phytohormones, and lipophilic chitin oligosaccharides obtained during a co-inoculation of *Medicago sativa* L. with *Sinorhizobium* and Delftia strains) increased growth of *M. sativa*. Overall, this combination was the most effective in terms of root development, activity (i.e., greater exploitation of the soil), nodulation, and crop grain yield (+10%) compared with plants inoculated only with *Bradyrhizobium* strains and other formulations.

The final confirmation of the effectiveness of a formulation can be reached in open-field experiments, where the environmental conditions are extremely variable. Open-field studies of CFS biostimulant activity are few. Marks et al., [28] reported the enhancement of grain yields of *Glycine max* L. and *Zea mays* L. when rhizobial metabolites (exopolysaccharides, phytohormones, and LCOs) were co-inoculated with both *Bradyrhizobium* spp. and *Azospirillum* spp. Similar trends were also obtained by adding *Bacillus subtilis* QST 713 to this combination within the foliar application. The recent article by Tewari et al., [29] indicated that a combined formulation of *Bradyrhizobium* sp. IC-4059, its CFSs, and exopolysaccharides (EPS) increased the productivity and nodulation of *Cajanus cajan* in the field, compared to both bacterial inoculum and CFS applied alone.

From all these reports it is evident that further processing of CFSs provides several metabolites with interesting stimulant properties. Among these metabolites, LCOs are the most tested. Lesueur et al., [30] summarize the effective applications of different LCOs on legume–rhizobia symbiosis, with positive outcomes on plant growth. Positive LCO application effects have also been recorded for non-leguminous plants, e.g., *Zea mais, Solanum lycopersicum, Picea abies, Daucus carota*, *Arabidopsis thaliana* [31]. Biostimulant PGPB metabolites can also be obtained from lactic acid bacteria (LABs). In addition to their probiotic properties, metabolites of these strains showed interesting biostimulant and biocontrol potential in agriculture [32]. Rodríguez-Morgado et al., [33] reported that L-lactic acid obtained from *Lactobacillus rhamnosus* whey-waste stimulated soil microbial activity and release of soluble phosphates. PGPB inoculation enriched with lactic acid was also involved in shaping the composition of soil bacterial communities. In a second study, the same research team published similar results on metabolites isolated by *L. rhamnosus* whey fermentation and separated by physicochemical processes [34]. The protein hydrolysates and the lactic acid-induces soil microbial activity. Lactic acid also positively influenced microbial biodiversity, favoring some plant growth promoter families (i.e., Bacilliaceae and Veillonellaceae family). Several PGPB strains can also be exploited to produce biosurfactants (BFs) and bacteriocins. Positive outcomes on soil quality and plant growth promotion have been extensively reviewed both for BFs [35–37] and for bacteriocins [38,39].

3.4 CFSs as Biocontrol Agents

Beyond biostimulant activity, CFSs and metabolites of PGPB can be used for the inhibition of microbial soil-borne pathogens. The strategies behind this antagonistic activity are mainly related to antibiosis and induction of plant defense response (i.e., induced systemic resistance - ISR) mechanisms [40]. The use of bioformulations in agriculture can be interesting, as it offers a valid tool for phytopathogen control whilst safeguarding ecosystems [40]. Pathogen control is a major concern in agriculture. Nowadays, the most effective strategy against plant pathogens is the use of resistant cultivars. However, due to its high costs, the application of agrochemicals remains one of the most utilized techniques [41]. Agrochemicals cause environmental pollution, with serious consequences for human health. These issues force agriculture towards effective and sustainable techniques to manage bacterial, fungal, and oomycete pathogens.

Table 1. Studies of stimulant properties of plant growth-promoting bacteria (PGPB) cell-free supernatants (CFSs) and CFS metabolites.

PGPB Strain	Compound	PT	Crop/Experiment	Ref.
Azospirillum brasilense Cd	IAA	C	*Medicago polymorpha* – P+G	[13]
Azospirillum brasilense Cd	IAA	C+F	*Oryza sativa* – P	[14]
Bacillus amyloliquefaciens KPS46	EP; LP; indoles	C+F	*Glycine max* – P+G	[15]
Bacillus amyloliquefaciens FZB24, FZB42, FZB45	IAA	F	*Zea mays* – P	[17]
Bacillus subtilis FZB37	IAA	F	*Zea mays* – P	[17]
Bacillus amyloliquefaciens FZB42	IAA	F	*Lemna minor* – P	[18]
Burkholderia seminalis	IAA	C	*Solanum lycopersicum* – P	[19]
Methylobacterium spp.	LCO	C+E	*Triticum aestivum*– P	[20]
Azospirillum brasilense Sp7	IAA, ILA and GA	C+F	*Glycine max* – G	[25]
Azospirillum brasilense Ab–V5, Ab–V6	Indolic compounds	C+F	*Glycine max* – G	[16]
Bacillus sp. CaSLT0017	EP and indoles	C+E	*Manihot esculenta* – G	[21]
Bacillus subtilis EA–CB0575	IAA, Siderophores	C+F	*Musa* spp. – G	[22]
Streptomyces acidiscabies E13	Siderophores	DP	*Vigna unguiculata* – G	[23]
Rhizobium leguminosarum bv. *viciae* GR09	LCO	E	*Pisum sativum*, *Vicia villosa* – G	[24]
Sinorhizobium meliloti U143	Flav, IAA, Trp	C+F	*Medicago sativa*– G	[27]
Delftia sp. JD2	Flav, IAA, Trp	C+F	*Medicago sativa*– G	[27]
Bradyrhizobium diazoefficiens USDA100 + *R. tropici* CIAT899	LCO	C+F	*Glycine max* – G	[26]
Rhizobium tropici CIAT 899	Flav	C+F+E	*Zea mays* – O	[28]
Bradyrhizobium diazoefficiens USDA 110	Flav	C+F+E	*Zea mays*, *Glycine max* – O	[28]
Bradyrhizobium sp. IC–4059	EPS	C+DP	*Cajanus cajan* – O	[29]
Lactobacillus rhamnosus	LLA	F+E	soil properties	[33]
Lactobacillus rhamnosus	LLA, peptides, AA	F+E	microbial growth	[34]

IAA, 3-indoleacetic acid; ILA, indole-3-lactic acid; GA, Gibberellins; LP, lipopeptides; EP, extracellular proteins; LCO, lipo-chitin oligosaccharide; LLA, L-lactic acid; AA, amino acids; Trp, tryptophan; Flav, flavonoids; EPS, exopolysaccharides; PT, production technique utilized to obtain CFS/metabolites; C, centrifugation; F, Filtration; E, solvent extraction; DP, several downstream processes; P, in vitro growth; G, greenhouse growth; O, open field growth.

3.4.1. Bacterial Pathogen Control

Among soil-borne pathogens, phytopathogenic bacteria are one of the major threats for agriculture, due to the deficiency of effective agrochemicals, the absence of host plants' resistance or immunity, and the accidental and undetected spread or latency [42]. Plant bacterial diseases cause devastating damage to cultivation with huge economic losses [43]. Sustainability 2020, 12, 9917 6 of 22 Studies of CFSs and PGPB useful to counteract this risk are limited. In Table 2 details of the studies concerning the application of CFSs or their metabolites against bacterial phytopathogens are summarized. Literature on bacterial biocontrol by CFSs/metabolites is mainly on tests carried out in

vitro against pathogens belonging to *Bacillus, Clavibacter, Ralstonia, Erwinia, Micrococcus, Agrobacterium, Pectobacterium* and *Xanthomonas* genera. Several CFS/metabolites obtained from *Bacillus* spp. demonstrated activity against these pathogens. In particular, the *B. amyloliquefaciens* species is one of the most promising. The antagonistic capability of *B. amyloliquefaciens* CFSs was first reported by Yoshida et al., [45], who described good inhibition of *Agrobacterium tumefaciens* and *Xanthomonas campestris* pv. Campestris in ex situ *Morus alba* leaves. *B. amyloliquefaciens* Bk7, together with *Bacillus laterosporus* spp. (B4, S5), and *Alcaligenes faecalis* spp. (Bk1, P1), showed good in vitro biocontrol capabilities against *Xanthomonas oryzae* pv. oryzae [49]. Interesting results in planta biocontrol of *X. oryzae* pv. *oryzae* were reported by Kong et al., for CFS extracts (i.e., surfactin, iturin, and acid precipitate with a concentration of 500 μg mL^{-1}) obtained from *Bacillus licheniformis* N1 [50]. Among several PGPB strains isolated from the rhizosphere of three horticultural and tree crops (i.e., apple, apricot, and strawberry), biocontrol capabilities were showed by B. amyloliquefaciens KM658175 CFSs against *Clavibacter michiganensis* ssp. *michiganensis* [46]; best in vitro inhibition was achieved utilizing 1% (v/v) concentration of the CFS of this strain. Extracts of *B. subtilis* ATCC 6633 and BBG100 CFSs inhibited in vitro growth of *Erwinia chrysanthemi*, *Pseudomonas aeruginosa*, and *Micrococcus luteus* due to the presence of mycosubtilin, surfactin, subtilin, subtilosin, and rhizocticins [55]. CFS of B. *subtilis* 14B was able to reduce the *Agrobacterium tumefaciens* infection both in vitro and in planta in *Solanum lycopersicum* [54]. The main active compounds identified in *Bacillus* CFSs are iturins. Iturins extracted from Bacillus sp. SS12.9 CFSs showed effective antagonism against *X. oryzae* pv. *oryzae* in in vitro experiment [51]. Iturins were also found in CFSs successfully applied in *Beta vulgaris, Oryza sativa,* and *Cucumis sativus*, in which they were able to inhibit several bacterial phytopathogens. CFSs of *B. amyloliquefaciens* and *Bacillus pumilus* inhibited *Pseudomonas syringae* pv. apta pathogenic activity in *B. vulgaris* in vitro cultivation [48]. CFS of B. subtilis NB22 and UB24 counteracted infections of *X. oryzae* and *Pseudomonas lachrymans* in *O. sativa* and *C. sativus*, respectively, during ex vivo and in planta experiments [53]. Other studies demonstrated the capability of different compounds to counteract several bacterial diseases. The ability of B. amyloliquefaciens CFSs to decrease Glycine max pustule disease severity caused by *Xanthomonas axonopodis* pv. *glycines* in a greenhouse experiment to surfactin has been ascribed [47]. Inhibition capabilities of *Bacillus brevis, B. subtilis, Paenibacillus granivorans*, and *M. luteus* strains to amylocyclicin isolated by *B. amyloliquefaciens*

FZB42 has been recognized [44]. The ability of a lipopeptide mixture from *Bacillus* sp. EA-CB0959 to decrease the incidence of *R. solanacearum* disease in *Musa* plants to fengycin, and in a lesser extent to surfactin and iturin, has been ascribed [52]. In vitro antibacterial properties against A. tumefaciens to the bacteriocin BAC IH7, isolated from *B. subtilis* IH7, have been recognized [56]. In addition to the Bacillus genus, several CFSs obtained by LABs, showed significant in vitro inhibition against *P. syringae* pv. a*ctinidiae*, *Xanthomonas arboricola* pv. pruni and *Xanthomonas. fragariae* [57], thanks to the presence of organic acids. Antibacterial effects have been inactivated by pH neutralization of CFS. CFSs containing siderophores produced by *P. aeruginosa* RZS3 and *Alcaligenes* sp. STC1 strains efficiently inhibited in vitro growth of *Pseudomonas solanacerum* [58]. Metabolites present in the culture supernatant of *Ochrobactrum lupini* KUDC1013 were able to elicit ISR against *Pectobacterium carotovorum* ssp. *carotovorum* in *Nicotiana leaves* [59]. Several CFSs of bacterial strains isolated from suppressive soils showed in vitro antagonistic activity against *X. campestris*. Among them, CFSs from *Peanibacillus polymyxa* also revealed a strong in vivo inhibition activity against this black rot causal agent [60]. Interesting results were also reported for the purified CFS of *Paenibacillus* sp. strain B2; superdex-purified CFS, constituted mainly by polymyxin B, inhibited in vitro growth of *Pseudomonas viridiflava* and *Erwinia carotovora* pathogens with minimal inhibitory concentrations (MICs) of 0.6 and 6.7 µg mL^{-1}, respectively [61].

3.4.2. Fungal Pathogens Control

In addition to bacteria, phytopathogenic fungi are one of the other major microbial soil-borne pathogens that threaten productive landscapes. Fungal plant pathogens cause enormous losses in yield and quality of plants [62]. A broad-spectrum antifungal activity has been observed for diverse CFSs against the genera *Fusarium, Rhizoctonia, Botrytis, Sclerotinia, Colletrotrichum,* and *Ralstonia*. However, the majority of the studies report results on in vitro assays. Most of the studies are on *Bacillus*. Table 3 summarizes studies on CFS and extracted metabolites from this genus.

B. amyloliquefaciens and *B. subtilis* are the most studied species. *B. amyloliquefaciens* strains were utilized to produce CFSs [66,67] and CFS metabolites [45,63–66,68,69,96] valid to inhibit in vitro growth of several fungal pathogens of both Ascomycota (e.g., *Fusarium* spp., *Colletotrichum* spp.) and Basidiomycota (e.g., *Rhizoctonia* spp.) phyla. The inhibition capacities of these CFSs and their

metabolites were correlated with the presence of lipopeptides (e.g., iturins, fengycins, surfactins, and sphingofungins); however, no records about the in planta control are available in the literature. *B. subtilis* CFSs and metabolites obtained by *B. subtilis* strains have been assayed against several fungal pathogenic strains, in vitro, ex vivo, and in planta [53,55,56,80–90,92–97,100].

Table 2. Studies of biocontrol properties of cell-free supernatants (CFS) and CFSs metabolites of plant growth-promoting bacteria (PGPB) against bacterial phytopathogens.

PGPB Strain	Pathogen	Compound	PI – Experiment	Ref
Bacillus amyloliquefaciens strain FZB42	*Bacillus brevis*; *Bacillus subtilis*; *Paenibacillus graminis*; *Micrococcus luteus*	Amylocyclicin	C+E+DP – V	[44]
Bacillus amyloliquefaciens strain BC-2	*Agrobacterium tumefaciens*; *Xanthomonas campestris* pv. *campestris*	Iturin	C+F – X (*Morus alba*)	[45]
Bacillus amyloliquefaciens strain KM658175	*Clavibacter michiganensis* ssp. *michiganensis*	-	C – V	[46]
Bacillus amyloliquefaciens strain KP546	*Xanthomonas axonopodis* pv. *glycines*	Surfactin	C+F – G (*Glycine max*)	[47]
Bacillus amyloliquefaciens (SS-12.6, SS-38.4); *Bacillus pumilus* SS-10.7	*Pseudomonas syringae* pv. *aptata*	Iturin	C+E – V+P (*Beta vulgaris*)	[48]
Bacillus amyloliquefaciens Bk7; *Brevibacillus laterosporus* spp. (B4, S5); *Alcaligenes faecalis* spp. (Bk1, P1)	*Xanthomonas oryzae* pv. *oryzae*	-	C+F – V	[49]
Bacillus licheniformis N1	*Xanthomonas oryzae* pv. *oryzae*	Iturin A, Surfactin	C+DP – V+G (*Oryza sativa*)	[50]
Bacillus sp. SS12.9	*Xanthomonas oryzae*	Iturins	C+E – V	[51]
Bacillus sp. EA-CB0959	*Ralstonia solanacearum*	Fengycin, Iturin, surfactin,	C/E/DP- V + G (*Musa*)	[52]
Bacillus subtilis NB22, UB24	*Xanthomonas oryzae*; *Pseudomonas fuchrysea*	Iturin	C+F+E – V+X+G (*Oryza sativa*; *Cucumis sativus*)	[53]
Bacillus subtilis 14B	*Agrobacterium tumefaciens*	-	C+DP – V+G (*Solanum lycopersicum*)	[54]
Bacillus subtilis (ATCC 6633; BBG100)	*Erwinia chrysanthemi*; *Pseudomonas aeruginosa*; *Micrococcus luteus*	Mycosubtilin, surfactin, subtilin, subtilosin, rhizocticins	C+E – V	[55]
Bacillus subtilis B47	*Agrobacterium tumefaciens*	Bac B47	C+E+DP – V	[56]
Lactic acid bacteria	*Pseudomonas syringae* pv. *actinidiae*; *Xanthomonas arboricola* pv. *pruni*; *Xanthomonas fragariae*	D- and L-lactic acid	C+E – V	[57]
Pseudomonas aeruginosa RZ53; *Alcaligenes* sp. STC1	*Pseudomonas solanacearum*	Siderophores	C – V	[58]
Ochrobactrum lupini KUDC1013	*Pectobacterium carotovorum*	PAA, H, LA,LPs/Flagella	C+EC+DP – P (*Nicotiana tabacum*)	[59]
Paenibacillus polymyxa	*Xanthomonas campestris* pv. *campestris*	-	C – V C+F – G (*Brassica oleracea* var. *acephala*)	[60]
Paenibacillus sp. B2	*Pseudomonas viridiflava*; *Erwinia carotovora*	Polymyxin B	C+DP – V	[61]

PAA, Phenylacetic acid; H, 1-hexadecene; LA, Linoleic acid; LPs, lipopolysaccharides; PT, production technique utilized to obtain CFS/metabolites; C, centrifugation; F, Filtration; E, solvent extraction; DP, several downstream processes; V, in vitro antagonism; X, ex vivo antagonism; P, in vitro growth; G, greenhouse/pot growth.

Table 3. Studies of biocontrol properties of cell-free supernatants (CFSs) and CFSs metabolites of Bacillus spp. against fungal phytopathogens.

Bacillus Strain	Pathogen	Compound	PI – Experiment	Ref
Bacillus amyloliquefaciens B4	*Rhizoctonia solani*	Iturin A2	C+F+E – V	[64]
Bacillus amyloliquefaciens BNM 122	*Rhizoctonia solani*; *Sclerotinia sclerotiorum*	Surfactin, Iturin	C+E – V	[64]
Bacillus amyloliquefaciens BS-2	*Botrytis cinerea*; *Fusarium culmorum*; *Botryosphaeria dothidea*; *Magnaporthe grisea*; *Rhizoctonia solani*; *Phytophthora capsici*; *Colletotrichum musae*; *Exserohilum turcicum*; *Valsa mali*	Fengycin, surfactin	C+F+E – V	[65]
Bacillus amyloliquefaciens LSM 5006	*Aspergillus* spp.; *Fusarium* spp.; *Aphanomyces* sp.; *Bipolaris sorokiniana*; *Cercospora sojina*; *Diplodia* spp.; *Drechslera* spp.; *Rhizoctonia* spp.; *Verticillium albo-atrum*	Iturin, fengycin	C+F – V	[66]
Bacillus amyloliquefaciens LZN01	*Fusarium oxysporum f. sp. niveum*	Mycocidin, sphingofungin C, sphingofungin F, 3-methyl-2-oxovaleric acid, gaberpentin, sphingofungin C	C+F – V	[67]
Bacillus amyloliquefaciens PG12	*Botryosphaeria dothidea*	Iturin A	C+F+E – V	[68]
Bacillus amyloliquefaciens RC-2	*Colletotrichum dematium*	Iturin A2	C+F+DF – V	[69]
Bacillus amyloliquefaciens Sfw-3	*Fusarium graminearum*	Iturin A, plipastatin A	C+F+E – V	[64]
Bacillus amyloliquefaciens (KT379983); *Bacillus cereus* (KT599643)	*Fusarium solani*	Surfactin, fengycin	C+F – V	[70]
Bacillus licheniformis BC98	*Magnaporthe grisea*	-	C+E – V	[71]
Bacillus licheniformis N3	*Rhizoctonia solani*; *Botrytis cinerea*; *Colletotrichum* spp.; *Rhizoctonia graminis*	Iturin A, Surfactin	C – V+G.Gsfusarium graminearum, *Fusarium oxysporum*, *C. acutatum*, *Rhizoctonia solani*	[59]
Bacillus megaterium, *B. subtilis*, *B. subtilis* ssp. *Subtilis*	*Aspergillus niger*; *Aspergillus flavus*	-	C+F+DF – V	[72]
Bacillus pumilus	*Aspergillus*; *Penicillium*; *Fusarium*	Iturin A	C+F+E+DF – V	[73]
Bacillus pumilus SH8/A4	*Rhizoctonia solani*; *Fusarium oxysporum*	Surfactin	C+F – V	[74]
Bacillus spp.	*Sclerotinia sclerotiorum*	-	C+F+E – V	[75]
Bacillus sp. 1-CPI (KP795799)	*Rhizoctonia* sp. *Sclerotium* sp.	Surfactin, Iturin, fengycin	C – V	[76]
Bacillus sp. SJ-5	*Rhizoctonia solani*; *Fusarium oxysporum*	Linoleic Acid	F+DF – V	[77]
Bacillus spp.	*Fusarium oxysporum f. sp. lycopersici*	Iturin A	F+E – V	[78]
Bacillus spp. (gyrB1-4, Cipro500, Cipro22, Cipro20)	*Colletotrichum acutatum*	Iturin, surfactin, fengycin	F – V	[79]
Bacillus subtilis AF1	*Fusarium moniliforme*; *Aspergillus niger*	β-(1,4)-N-acetylglucosaminidase (NAGase)	C+F – V	[80]
Bacillus subtilis (ATCC 6633, BBG100)	*Botrytis cinerea*; *Fusarium oxysporum*	Mycosubtilin, surfactin, subtilin, subtilosin, rhizocticins	C+E – V	[81]
Bacillus subtilis AU195 *Bacillus subtilis* B47	*Aspergillus flavus* *Rhizoctonia solani*	Iturin Iturin A2	C+F – V C+E+DF – V	[82] [83]
Bacillus subtilis B-916	*Rhizoctonia solani*; *Magnaporthe grisea*; *Sclerotinia sclerotiorum*; *Alternaria oleracea*; *Alternaria brassicae*; *Botrytis cinerea*	Bacisubin	C – V	[84]
Bacillus subtilis B-FS01	*Fusarium moniliforme*	Fengycin A, fengycin B	C+F+E – V	[85]
Bacillus subtilis CL27; *Bacillus pumilus* CL45	*Alternaria brassicola*; *Botrytis cinerea*	-	C+E – V+P (Antifly)	[86]
Bacillus subtilis EA-CB0015	*Botrytis cinerea*; *Colletotrichum acutatum*	Iturin A, fengycin C	C+E+LIV – V	[87]
Bacillus subtilis ES-1	*Penicillium digitatum*; *Botrytis cinerea*	Iturin A	F+DF – V+E (*Citrus limon*, *Eriobotrya japonica*)	[88]
Bacillus subtilis B9844-11	*Ophiostoma ulmi*; *Verticillium dahliae*; *Ceratocystis fagacearum*; *Cryphonectria parasitica*	Iturin	F+E – V	[89]

Table 3. *Cont.*

Bacillus Strain	Pathogen	Compound	Pt – Experiment	Ref
Bacillus subtilis GA1	*Botrytis cinerea*	Fengycins, iturins, surfactins	C+DP – V	[89]
Bacillus subtilis HC8	*Fusarium oxisporum f. sp. radicis-lycopersici*	Iturins, fengycins, surfactin	F+E – V	[90]
Bacillus subtilis HussainT-AMU	*Rhizoctonia solani*	Surfactin	C+F+E – V+G+O (*Solanum tuberosum*)	[91]
Bacillus subtilis IH7	*Alternaria solani*	Bac IH7	C+E+DP – V	[56]
Bacillus subtilis KS03	*Gloeosporium gloeosporioides*	Iturin A	C+F+E – V	[92]
Bacillus subtilis NB22, UB24	*Alternaria mali; Cercospora kikuchii; Botrytis cinerea; Puccinia coronata; Rhizoctonia solani; Pyricularia oryzae; Cochliobolus miyabeanus*	Iturin	C+F+E – V+X+G (*Malus domestica; Cucumis sativus; Glycine max; Avena sativa*)	[53]
Bacillus subtilis SCB-1	*Saccharicola bicolor; Neodeightonia subglobosa; Cochliobolus hawaiiensis; Curvularia senegalensis; Curvularia lunata; Alternaria alternata;; Fusarium oxysporum; Fusarium verticillioides; Fusarium* sp.; *Phomopsis* sp.	Surfactin	C+F+E – V	[93]
Bacillus subtilis ssp.	*Fusarium oxysporum f. sp. radicis-lycopersici; Rosellinia necatrix*	Surfactin, fengycin, iturin A	C+E – V	[94]
Bacillus subtilis ssp. *subtilis*	*Setophoma terrestris*	-	C+F – V	[95]
Bacillus subtilis ssp. *subtilis* PGPMori7; *Bacillus amyloliquefaciens* PGPBacCA1	*Macrophomina phaseolina*	Iturin, surfactin, fengycin	C+F+E+DP – V	[96]
Bacillus subtilis UMAF6614, UMAF6619, UMAF6639, UMAF8561	*Podosphaera fusca*	Iturin, fengycin	C+F – V+X (*Cucumis melo*)	[97]
Bacillus vallismortis ZZ185	*Fusarium graminearum; Alternaria alternata; Rhizoctonia solani; Cryphonectria parasitica*	Bacillomycin D (n-C14, iso-C15)	C+F+E – V	[98]
Bacillus velezensis Y6, F7	*Ralstonia solanacearum; Fusarium oxysporum*	Surfactin, iturin, fengycin	C+F – V	[99]
Bacillus subtilis; Pseudomonas fluorescens	*Macrophomina phaseolina*	-	C+F – V	[100]
Bacillus mycoides (+ *Pichia guilermondii*)	*Botrytis cinerea*	-	C – V+R (*Fragaria* × *ananassa*)	[101]

PT, production technique utilized to obtain CFS/metabolites; C, centrifugation; F, Filtration; E, solvent extraction; DP, several downstream processes; V, in vitro antagonism; X, ex vivo antagonism; R, antagonism on fruit; P, in vitro growth; G, greenhouse/pot growth; O, open field growth.

Noteworthy is the recent work of Hussain et al., in which the potentialities of metabolites of CFSs produced by *B. subtilis* HussainT-AMU were assessed in vitro and in planta, both in greenhouse and open field experiments [91]. Thanks to the presence of surfactin, the CFS of this strain was able to decrease *Rhizoctonia solani* infections by up to 71% and 50% under greenhouse and open field conditions, respectively. CFSs [70,74,76,79,99] and CFS extracted metabolites [71–73,75,77,78,98] from other *Bacillus* species were reported to inhibit the in vitro growth of several fungal phytopathogens belonging mainly to *Aspergillus, Fusarium, Sclerotinia,* and *Rhizoctonia* genera. Interesting are the results obtained by Guetsky et al., who reported effective *B. cynerea* biocontrol on ex vivo strawberries by CFSs obtained from *Bacillus mycoides* and *Pichia guilermondii* [101]. Moreover, Kong et al., reported effective fungal inhibition by *B. licheniformis* N1 CFS and purified

metabolites. In their work surfactin and iturin A formulates at a concentration of 500 µg mL^{-1} were shown to control in planta disease caused by *R. solani, Botrytis cinerea, Colletotrichum* spp., and Blumeria graminis under greenhouse experiments [50]. In addition to *Bacillus* genus, other genera can be valid sources of CFSs and metabolites for the biocontrol of fungal phytopathogens. In Table 4 the details of studies of species belonging to these other genera are shown.

One of the first studies available in the literature reports the *Erwinia herbicola* CFS in planta biocontrol capability against *Puccinia recondita* f. sp. tritici in a *Triticum aestivum* greenhouse experiment, thanks to the presence of herbicolin A [104]. However, no other reports can be found on this species. In the recent literature, there are many studies of the in vitro biocontrol potential of *Pseudomonas* spp. CFSs [58,107] and CFS metabolites [72,100], thanks to the presence of siderophores, phenazines, and 2-hexyl 5-propyl resorcinol N-Butylbenzenesulphonamide [108–110]. The in vitro inhibition of fungal pathogens has also been demonstrated for the CFSs and metabolites of other species of *Alcaligenes* [58,102], *Chryseobacterium* [103], and *Paenibacillus* [61] genera. Actinomycetes are also a source of formulates for the management of fungal plant diseases However, only a few studies have evaluated CFSs or metabolites obtainable by these microorganisms [124] and dealing exclusively with the *Streptomyces* genus [112–116,119]. Noteworthy are the studies of Kaur et al., and Jacob et al., who reported good in planta biocontrol capabilities of CFS on *Fusarium moniliforme* on *S. lycopersicum* [117] and *Sclerotium rolfsii* on *Arachis hypogaea* [118], respectively.

Table 4. Studies of biocontrol properties of cell-free supernatants (CFSs) and CFS metabolites of plant growth-promoting bacteria (PGPB) strains other than Bacillus spp. against fungal phytopathogens.

PGPB Strain	Pathogen	Compound	Pt – Experiment	Ref
Alcaligenes faecalis BCCM ID 2374	*Fusarium oxysporum; Alteraria alternata*	Siderophores	C/C+DP – V	[102]
Chryseobacterium aquaticum	*Pestalotia theae; Rhizoctonia solani; Curvularia lunata*	-	C – V	[103]
Erwinia herbicola	*Puccinia recondita f. sp. Tritici*	Herbicolin A	C+F – V+G (*Triticum aestivum*)	[104]
Lactobacillus coryniformis ssp. *coryniformis*	*Mucor hiemalis; Fusarium poae; Fusarium graminearum; Fusarium culmorum; Fusarium sporotrichioides*	-	C+F – V	[105]
Lactobacillus plantarum	*Colletotrichum capsici*	-	C+F – V+P (*Capsicum annuum*)	[106]
Paenibacillus sp. B2	*Fusarium solani; Fusarium acuminatum*	Polymyxin B	C+DP –V	[61]
Pseudomonas aeruginosa RZS3; *Alcaligenes* sp. STC1	*F. oxysporum; Alternaria alternata; Cercospora arachichola;*	Siderophores	C – V	[58]
Pseudomonas batumici EB132; *Pseudomonas trivialis* EB133; *Pseudomonas grimontii* EB150; *Burkholderia stabilis* (EB159, EB193)	*Alternaria panax; Botrytis cinerea; Cylindrocarpon destructans; Rhizoctonia solani*	-	C+F – V	[107]
Pseudomonas chlororaphis PCL1391	*Fusarium oxysporum f. sp. radicis-lycopersici*	Phenazines	C+E – V	[108]
Pseudomonas fluorescens	*Macrophomina phaseolina*	-	C+F – V	[100]
Pseudomonas fluorescens PCL1606	*F. oxysporum f. sp. radicis-lycopersici*	2-hexyl 5-propyl resorcinol	C+E+DP – V	[109]
Pseudomonas sp. AB2	*Rhizoctonia solani, Botrytis cinerea, Fusarium oxysporum*	N-Butylbenzenesulphonamide	C+E+DP – V	[110]
Pseudomonas spp.	*Aspergillus niger; Aspergillus flavus*	-	C+F+DP – V	[72]
Serratia sp. ZoB14	*Sclerotium rolfsii; Colletotricum acutatum; Fusarium oxysporum; Rhizoctonia solani*	-	C+DP – V	[111]
Streptomyces goshikiensis	*F. oxysporum sp. niveum*	-	C+E – V	[112]
Streptomyces pactum Act12; *Streptomyces rochei* D74	*Sclerotium rolfsii; Fusarium oxysporum*	-	F – V	[113]
Streptomyces roseoflavus US80	*Fusarium sp.; Verticillium dahliae*	irumamycin; X-14952B, 17-hydroxy-venturicidin A	C+E+DP – V	[114,115]
Streptomyces sp. 3–10	*Amphobotrys ricini; Alternaria alternata; Aspergillus flavus; Aspergillus niger; Aspergillus parasiticus; Bipolaris maydis; Botrytis cinerea; Colletotrichum siamense; Curvularia lunata; Drechslera graminea; Fusarium oxysporum; Monilia fructigena; Pestalotia theae; Sclerotinia minor; Sclerotinia sclerotiorum; Rhizoctonia solani; Sclerotium rolfsii*	Reveromycin A, B	C+E – V+X (*Fragaria* × *ananassa*)	[116]

Table 4. *Cont.*

PGPB Strain	Pathogen	Compound	Pt – Experiment	Ref
Streptomyces sp. MR14	*Fusarium moniliforme*	-	C/E – V+G (*Solanum lycopersicum*)	[117]
Streptomyces sp. RP1A-12	*Sclerotium rolfsii*	-	C+E – V+G (*Arachis hypogaea*)	[118]
Streptomyces spp.	*Botrytis cinerea; F. oxysporum f. sp. ciceri; Fusarium andiyazi; Fusarium proliferatum; Macrophomina phaseolina; Rhizoctonia bataticola;*	-	C+F – V	[119]
Xenorhabdus nematophila mutant	*Botrytis cinerea; Rhizoctonia solani; Exserohilum turcicum; Physalospora piricola; Curvularia lunata; Gaeumannomyces graminis; Fusarium graminearum*	-	F – V	[120]
Xenorhabdus nematophila TB	*Botrytis cinerea*; Alternaria solani; Bipolaria maydis; Bipolaris sorokiniana; Dothiorella gregaria; Exserohilum turcicum; Physalospora piricola; Rhizoctonia cerealis; Sclerotinia sclerotiorum*	-	C+F – V *C+F+E – P (*Solanum lycopersicum*)	[121]
Xenorhabdus nematophila YL001	*Alternaria brassicae; Alternaria solani; Botrys cinerea; Clomerela cingulate; Curvularia lunata; Exserohilum turcicum; Magnaporthe grisea; Physalospora piricola; Sclerotinia sclerotiorum; Verticillium dahliae*	Xenocoumacin 1, 2	C+F – V	[122]
Xenorhabdus spp.C19A1:D25	*Fusicladium carpophilum; Fusicladium effusum; Monilinia fructicola; Glomerella cingulata; Armillaria tabescens*	-	C+F – V	[123]

PT, production technique utilized to obtain CFS/metabolites; C, centrifugation; F, Filtration; E, solvent extraction; DP, several downstream processes; V, in vitro antagonism; X, ex vivo antagonism; P, in vitro growth; G, greenhouse/pot growth.

LABs are capable of producing several bioactive metabolites that effectively counteracted several plant diseases [32,105]. El-Mabrok et al., for example, reported L. plantarum CFS' effective inhibition of *Colletotrichum capsici,* both in vitro and during a *Capsicum annum* seed germination experiment under sterile conditions [106]. Several works report the capability of CFSs of *Xenorhabdus* spp. to inhibit some fungal phytopathogens in vitro [120,122,123]. For this genus, relevant is the study of Fang et al., who reported that the extracted metabolites from *X. nematophila* TB CFS can inhibit B. cinerea under in vitro *S. lycopersicum* cultivation [121].

3.4.3. Oomycete Phytopathogens

Oomycetes are endemic phytopathogens responsible for destructive outcomes in several crop plants. There are only a few anti-oomycete compounds for the control of their diseases. These pathogens are spreading severely and developing resistant strains [125]. In Table 5, details of the studies concerning the application of CFSs and their metabolites against oomycetes phytopathogens are summarized.

Only a limited number of works are present in the literature, mostly addressing the biocontrol of *Phytophthora* spp. and *Pythium* spp. Members of these fungal-like genera have been widely studied throughout the world due to the serious losses they cause [137]. *Phytophtora* spp. effective biocontrol has been obtained on: (i) *S. lycopersicum* by CFS metabolites of *B. subtilis* NB22 and UB24 [53], *B. licheniformis* N1 CFS [50], and *Pseudomonas fluorescens* SS101 CFS metabolites [131]; (ii) *Carica papaya* by CFS of *Photorhabdus* spp. [130]; (iii)*C. annuum* by *X. nematophila* TB CFS metabolites [121]; (iv) *Solanum tuberosum* by *X. nematophilus* var. *pekingensis* CFS metabolites [136]. *Pythium* spp. biocontrol has been obtained on: (i) *Phaseolus vulgaris* by B. subtilis M4 CFS metabolites [126]; (ii) *S. tuberosum* by *Streptomyces* sp. TN258 CFS [134]; (iii) *Fragaria × ananassa* by *Streptomyces* sp. 3–10 CFS metabolites [116]. Beyond *Phytophthora* spp. and *Pythium* spp., the control of *Plasmopara viticola* infection on ex vivo *Vitis vinifera* leaves has been obtained by B. subtilis CFS application [127]. Biocontrol of bacterial, oomycetes, and fungal pathogens can also be achieved by bacterial BFs, bacteriocins, and hydrolytic enzymes. Several formulations of these molecules have great potential for use in agriculture. Mode of action and inhibition effectiveness have been extensively reviewed for BFs [35–37], bacteriocins [38,39], and hydrolytic enzymes [138].

Table 5. Studies of biocontrol properties of cell-free supernatants (CFSs) and CFSs metabolites of plant growth-promoting bacteria (PGPB) against oomycetes phytopathogens.

PGPB Strain	Pathogen	Compound	Pt – Experiment	Ref
Bacillus subtilis NB22, UB24	*Phytophtora infestans*	Iturin	C+F+E – V+X+G (*Solanum lycopersicum*)	[53]
Bacillus subtilis M4	*Phytium ultimum*	Fengycin, iturin, surfactin	C+E+DP – G (*Phaseolus vulgaris*)	[126]
Bacillus subtilis	*Plasmopara viticola*	Fengycin, Surfactin	C+F – X (*Vitis vinifera*)	[127]
Bacillus subtilis CU12	*Pythium sulcatum*	Fengycin	C+DP – V	[128]
Bacillus subtilis mutant	*Phytium aphanidermatum*	Mycosubtilin	C+F – V	[55]
Bacillus sp. LCF1 (KP257289)	*Phytophthora sp.*	Surfactin, iturin, fengycin	C – V	[76]
Bacillus licheniformis N1	*Phytophtora infestans*	Iturin A, Surfactin	C – V+G (*Solanum lycopersicum*)	[50]
Bacillus toyonensis EB70; *Paenibacillus terrae* EB72	*Pythium* sp.; *Phytophthora cactorum*	-	C+F – V	[107]
Bacillus vallismortis ZZ185	*Phytophthora capsici*	Bacillomycin D	F+E – V	[98]
Lactobacillus plantarum IMAU10014	*Phytophthora drechsleri*	3-phenyllactic acid; Benzeneacetic acid, 2-propenyl ester	C+F+DP – V	[129]
Photorhabdus spp.	*Phytophthora* sp.	-	C – V+G (*Carica papaya*)	[130]
Pseudomonas fluorescens SS101	*Phytophtora infestans*	Massetolide A	C+DP – G (*Solanum lycopersicum*)	[131]
Pseudomonas aeruginosa	*Pythium myriotylum*	phenazine 1-carboxylic acid	C+DP – V	[132]
Pseudomonas sp. AB2	*Pythium ultimum*, *Phytophthora capsici*	N-Butylbenzenesulphonamide	C+E+DP – V	[110]
Serratia sp. ZoB14	*Pythium myriotylum*; *Phytophthora infestan*	-	C+DP – V	[111]
Streptomyces similaensis	*Phytophthora* sp. D4	β-glucanase extracts	C+DP – V	[133]
Streptomyces sp.TN258	*Pythium ultimum*	-	C+F – V+G (*Solanum tuberosum*)	[134]
Streptomyces sp. 3–10	*Pythium aphanidermatum*; *Pythium ultimum*	Reveromycin A, B	C+E – V+G (*Fragaria* × *ananassa*)	[116]
Xenorhabdus nematophila	*Phytophthora infestans*	SID	C+E – V	[135]
Xenorhabdus nematophila TB	*Phytophthora capsici*	-	C+F+E – V+P (*Capsicum annuum*)	[121]
Xenorhabdus nematophila	*Phytophthora capsici*	Xenocoumacin 1, 2	C+F – V	[122]
Xenorhabdus nematophilus var. *pekingensis*	*Phytophthora infestans*	Xenocoumacin 1	C+DP – V+X+G (*Solanum tuberosum*)	[136]
Xenorhabdus nematophila mutant	*Phytophthora capsici*	-	F – V	[120]

PT, production technique utilized to obtain CFS/metabolites; C, centrifugation; F, Filtration; E, solvent extraction; DP, several downstream processes; V, in vitro antagonism; X, ex vivo antagonism; P, in vitro growth; G, greenhouse/pot growth. SID, racemic 3-indoleethyl(3′-methyl-2′-oxo)pentanamide.

3.5 CFSs and Metabolites - Limitations and Advantages

Data on the use of CFS in agriculture are extremely limited and their application in agriculture has been completely ignored in recent decades. No published studies have investigated formulation and shelf life of CFSs; thus, the limitations are mainly related to the downstream processes for their production. According to Doran et al., [9] downstream processes can often be technically challenging due to:

• Metabolites' lability: these compounds are sensitive to temperature, high salt concentrations, and addition of chemicals (i.e., solvents, strong acids and bases).

• the complexity of the broth mixture.

• contamination susceptibility.

These factors limit the operation units that can be applied, lowering the purity and stability of final products. Concerning the use of CFSs as fertilizers, other possible limitations are similar to those found for other biofertilizers, namely [139]:

• lower nutrient content that may be inadequate for maximum crop growth.

• slower nutrient release rate.

• highly variable nutrient composition.

On the other hand, CFSs have more advantages than synthetic fertilizers that can overcome these negative aspects:

• a more balanced nutrient supply.

• soil biological and fertility status enhancement.

• soil structure improvement.

These advantages sustain crop production whilst safeguarding agroecosystem health. Concerning bacteriocins, purified metabolites, hydrolytic enzymes, and BFs, currently large-scale application and production are limited mostly due to the high cost of production [31,140,141].

3.6 Perspectives

Our literature survey underlined that studies of CFSs and their metabolites should be encouraged. This resource from bacteria is in our opinion very interesting both from the scientific and commercial point of view. The metabolites present in CFS-based formulations have demonstrated effectiveness against a certain number of species. The biocontrol potential against fungi, bacteria and actinomycetes has also been demonstrated. The biostimulant market is in constant increase, with an annual growth rate of 10.4% in 2016–2021. Thus, the formulation of new products by biostimulant producers could be a valid financial investment in such a lucrative market. However, the formulation of new products ready to be commercialized would require new scientific and industrial scale-up studies. This request would challenge the scientific world as a not yet fully explored field. New studies should deal with the: (i) identification of PGPB species with interesting

metabolite profile; (ii) selection of procedures to obtain cost-effective formulation; (iii) chemical characterization of formulates; (iv) modes of action; (v) effectiveness studies under different environmental conditions; (vi) studies on formulation stabilities (vii) product registration and commercialization. Even if this process is long and challenging, we think that these formulations could be one of the new tools useful for sustainable agriculture, equal to the biostimulants present on the market. Our literature survey shows that *Bacillus* is the most promising genus for the isolation of CFSs and/or their metabolites. Moreover, several *Bacillus* strains are already commercialized in biostimulant/biocontrol products. Thus, the scale-up procedures for reaching the formulation stage should also be less challenging. The collaboration of different field specialists (i.e., academics, industrial and commercial fields, farmers) should be activated to explore the CFS field and obtain new biostimulant products. We believe that the formulation of natural products for agriculture is not only important at the scientific and economic level but also for our planet. To cope with an increasingly global food demand, agriculture is maximizing production by excessive use of chemicals. The development of new fields of study and the publication of scientific reports can lead to the awareness of farmers and companies engaged in food production.

3.7. Conclusions

From the data reported, it is evident that the literature contains only a few reports useful for the creation of valid scientific evidence to support the development of CFS formulations. The majority of the reports deal with environmental controlled biostimulant and in vitro microbial biocontrol experiments. Among the 109 articles selected and examined, the *Bacillus* genus seems to be the most promising due to the numerous articles that support its biostimulant and biocontrol potentialities. Several CFSs and CFS metabolites of *Bacillus* strains demonstrated activity against a broad spectrum of bacterial, fungal, and oomycete pathogens, under different cultivation conditions. The present review underlined that research on this topic needs to be encouraged; evidence so far obtained has demonstrated that PGPB could be a valid source of secondary metabolites useful in sustainable agriculture. For the production of CFS-based formulations useful for agriculture, new PGPB strains/metabolites should be studied and obtained. Moreover, through advanced biotechnologies, standardized formulations and shelf life investigations should be carried out. To introduce these formulations in agriculture, future studies of CFSs should include

effectiveness tests with trials in greenhouse and field experiments. The present review creates the first literature summary of CFSs and their metabolites as plant growth-promoting bacteria. Data organization provided details of their use as biostimulant and microbial biocontrol agents in agriculture. This review can also be used as a starting point for drawing up new reviews regarding the use of CFSs and their metabolites. These formulations can be exploited for other purposes in agriculture (e.g., biocontrol of nematodes, insects, protozoa).

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Chapter 4

Bacterial Microbiota and Soil Fertility of *Crocus sativus* L. Rhizosphere in the Presence and Absence of *Fusarium* spp.

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Abstract

Intensive agricultural practices have led to intense soil degradation and soil fertility losses. Many soil-borne diseases affect these intensive agricultural soils, worsening the physicalchemical and fertility imbalances. Among the numerous pathogens, the genus *Fusarium* includes members that destroy many crops, including *Crocus sativus* L., which also impairs the composition and functions of the microbial communities. This work aimed to investigate, for the first time, the bacterial communities of the rhizosphere of saffron in the presence and absence of fusariosis. The rhizosphere of the saffron fields in the territory of L'Aquila (Italy) with and without fusariosis was sampled and subjected to a microbiological analysis. Culture-dependent methods characterized the fusariosis. The dehydrogenase activity assay was estimated. The metabarcoding of the 16S rRNA gene, a metagenome functioning prediction, and a network analysis were also carried out. The results showed that fusariosis, when it is linked to intensive agricultural practices, causes alterations in the microbial communities of the rhizosphere. The culture-dependent and independent approaches have shown changes in the bacterial community in the presence of fusariosis, with functional and enzymatic imbalances. The samples showed a prevalence of uncultured and unknown taxa. Most of the known Amplicon Sequence Variants (ASVs) were associated with the Pseudomonadoa (syn. Proteobacteria) lineage. The composition and richness of this phylum were significantly altered by the presence of *Fusarium*. Moreover, pathogenesis appeared to improve the ASVs interconnections. The metagenome functions were also modified in the presence of fusariosis.

4.1 Introduction

The growing demand for healthy food from a growing human population requires intensive and efficient land management practices and crop control to reduce the disease losses [1]. However, intensive farming practices are leading to the degradation of agricultural soils and a gradual loss of their fertility [2]. Soil degradation leads, in turn, to the loss of its functions. Climate change also increases the uncertain and complex management of agricultural soil, jeopardizing its long-term viability, its biodiversity, and consequently, its functions. The use of chemical fertilizers is considered to be the fastest way to increase agricultural production. However, their cost and other constraints are increasingly discouraging farmers from using them [3]. These products also cause environmental pollution with negative consequences for human health [4]. A lack of knowledge about soil biodiversity has been identified as the main limitation to its management. The diversity of soil microbial communities can be critical for soil resilience to various abiotic and biotic stressors [5]. Microorganisms in agricultural soils have a significant impact on soil fertility, on the availability of nutrients for the plant and on the suppression of soil-borne plant diseases [6]. The conservation and sustainable use of soil microbial diversity are crucial for increasing agricultural productivity [7]. The loss of biodiversity has a detrimental impact of productivity, stability, and services [4]. According to a recent meta-analysis, fields that undergo organic management practices had between 32% and 84% higher soil microbial biomasses (carbon, nitrogen, total phospholipid fatty acids) and enzymatic activities (dehydrogenase, urease, protease) than the conventional systems do. Crop rotation, legume intercropping, and organic inputs have all been linked to an increased microbial richness in agricultural soils [8,9]. The loss of soil biodiversity is also linked to the increase in soil-borne diseases, especially in agricultural ecosystems, resulting in higher production costs [6]. Among the numerous pathogens, the genus *Fusarium* includes members that cause diseases in many plants. *Fusarium* diseases are mainly associated with vascular wilt, but several species can cause the seedling wilt, crown, lower stem, root and seed rot, and head and seed plague [10]. *Fusarium* spp. live saprophytically on the roots, stems, leaves, flowers, and seeds of diseased and dead plants [11]. The fungus can survive on seeds (internal and external) or as spores or mycelium in the dead or infected tissues [12]. Within the *Fusarium* genus, *Fusarium oxysporum* is responsible for wilting of plants in nurseries and field crops, causing significant losses [11].

Saffron (Crocus sativus L.) is one of the valuable crops that is affected by F. oxysporum. Several fungal species belonging to *Fusarium, Rhizoctonia, Penicillium, Aspergillus, Sclerotium, Phoma, Stromatinia, Cochliobolus,* and *Rhizopus* genera affect saffron [13]. *Fusarium* corm rot, which is caused by *F. oxysporum,* is the most destructive disease [14]. Infected plants die early, thus reducing the corm yield, quality, and flower and stigma production [15]. *F. oxysporum* causes vascular wilt, as shown by yellowing of the leaf, the loss of turgidity, necrosis, wilting, and the plant's death.

A *Fusarium* infection occurs when the mycelium or germinating spores penetrate the plant's roots, enter the xylem, and produce microconidia. Vascular vessels become clogged by the accumulation of mycelium, spores, and the oxidation of the degradation products of enzymatic lysis. Toxins can cause vein clearing (the loss of chlorophyll production along the veins), a reduction in the photosynthesis rate, and tissue damage that leads to excessive water loss through transpiration [16]. Fusariosis also harms microbial communities' composition and functions. The recent study by Wang and collaborators highlighted the increase in the carbon cycle, the Calvin cycle, and the expression of hemicellulose and chitin degradation genes in watermelon soil in the presence of *Fusarium* [17]. The literature lacks studies which investigate the effect of *Fusarium* on the quality of the saffron rhizosphere. We hypothesized that *Fusarium* is closely associated with microbial biodiversity loss and a loss of the soil enzymatic activity. This work is aimed at investigating the bacterial communities of the saffron rhizosphere in the presence and absence of fusariosis. The rhizospheres of four saffron fields in the L'Aquila area (Italy) with different extensions of fusariosis were sampled. We performed the metabarcoding of 16s rRNA and the dehydrogenase activity assay. The same analyses were also carried out on the rhizosphere of six saffron fields without fusariosis.

4.2. Materials and Methods

4.2.1. Soil Sampling

Ten saffron fields in the L'Aquila territory (Abruzzo region) were subjected to rhizosphere sampling at 20 cm depth in March 2021. Four fields showed evident fusariosis (ZF1, ZF2, ZF3, and ZF4) and six fields showed no evident pathogenesis (ZB1, ZB2, ZB3, ZB5, ZB6, and ZB7). Field ZF3 presented a less evident presence of the pathogen. Figure 1 shows an example of an evident fusariosis. Five soil sub-samples were collected per field following a non-systematic pattern. The soil samples were sieved (<2 mm) to remove large particles and plant debris. Fresh homogeneous aliquots of each soil sample

were immediately processed for culturable approaches and enzymatic activity estimations. Ten aliquots of each soil sample were stored at −80° until they were processed for DNA extraction.

Figure 1. Geolocalization map of the sampling area and examples of a field and a corm with an evident Fusarium pathogenesis.

4.2.2. Fusariosis Pathogenesis Confirmation

The *Fusarium* pathogenesis was confirmed by the corms inspection and microbial culturable approaches. Three aliquots of each rhizosphere were processed in saline with 1% of Tween 20 (1:10 ratio) in a bag mixer for 30 min. After centrifugation at 4000 for 10 min, the supernatants were subjected to serial dilutions up to 1×10^{-7}. One hundred µL of each serial dilution were plated on Selective *Fusarium* Agar (SFA) [18] and incubated at 25 ◦C for five days. We confirmed the presence of Fusarium by macro- and microscopic observations of hyphae and spores and by spores sub-culturing on Potato Dextrose Agar PDA (Sigma-Aldrich, St. Louis, USA).

4.2.3. DNA Extraction and 16S rRNA Metabarcoding

The genomic DNA was extracted using 500 mg of homogenous samples according to the manufacturer's protocol (NucleoSpin®Soil, Macherey Nagel, Germany). The DNA content and purity were verified using a Nanodrop spectrophotometer (Thermo ScientificTM, Waltham, MA, USA) and

a Qubit fluorometer (Thermo ScientificTM, Waltham, MA, USA). For each sample, the individual replicates were combined in an equimolar ratio. We performed paired-end 16S rRNA community sequencing on the Mi-Seq Illumina technology (Bio-Fab Investigation, Rome, Italy), focusing on the V3 and V4 regions of the 16S rRNA gene [19]. The filtering was performed, and the readings were evaluated for reliability, and they were counted. Using QIIME2 (qiime2-2020.2 version), the DADA2 plugin was used to build ASV (Amplicon Sequence Variant) [20]. The V3–V4 specific area was taken from the 16S file that was obtained from the SILVA database (https://www.arb-silva.de/ accessed on 14 October 2021) and used to train the classifier using the fit-classifier-naive-Bayes plugin.

4.2.4. Prediction of Metagenomic Functions

PICRUSt 2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) was used to predict the functional abundances based on 16S rRNA gene sequencing data [21]. Pathways (PWYs), Enzyme on (EC) numbers and KEGG Orthologs (KOs) were predicted based on the Amplicon Sequence Variants (ASVs) sequence profiles/abundances (BIOM file format obtained from qiime2). PICRUSt 2 was run as a plugin of qiime2 with default parameters. We used the ALDEx2 (ANOVA-like differential expression) to perform the differential abundance testing between the two conditions with 1000 Monte Carlo samples and a One-way ANOVA test. An effect size that is greater than 1 was used as a significance cutoff with or without the BH correction of the raw *p* values.

4.2.5. Network Analysis

The network analyses were performed following Barberán et al., [22]. Briefly, the network was inferred by all of the possible Spearman rank correlation comparisons between the ASVs with more than 5 sequences (Spearman's correlation coefficient > 0.6 and statistically significant p value < 0.01). The networks were reconstructed with 90% identity ASVs as nodes and strong and significant correlations between the nodes as edges. The network topology was estimated by a metrics calculation (i.e., average node connectivity and path length, diameter, cumulative degree distribution, clustering coefficient, and modularity) [23]. All of the statistical analyses were performed in the R program using the Igraph [24] package. The networks were investigated and visualized using the interactive platform Cytoscape v 3.9.1 [25] and the Network analyzer v 4.4.8 tool [26].

4.2.6. Dehydrogenase Activity of Soil Samples

The soil dehydrogenase activity (DHA) was estimated using fresh soil samples [27]: Three aliquots of each soil sample (6 g) were placed in test tubes and mixed with 4 mL of distilled water. Each mixture was supplemented with 120 mg of CaCO3 and 1 mL of 2,3,5-triphenyltetrazolium chloride (TTC 3% v/w) and incubated at 30 ◦C for 20 h. The samples were filtered, and triphenylformazane (TPF) was extracted using ethanol. The samples were then mixed and placed in the dark for 1 h. After incubation, the supernatant was recovered by centrifugation and analyzed at λ = 485 nm (Multiskan GO™—Thermo Scientific, Waltham, MA, USA). The results are expressed as µg TPF g™1 min™1 using a calibration curve (y = 0.0132x + 0.0083, R^2 = 0.999) [28].

4.2.7. Statistical Analysis

The data were analyzed by One-way Analysis of Variance (ANOVA) using the XLSTAT 2016 software (Addinsoft, Paris, France). Significant differences were calculated with Tukey's post hoc test at $p < 0.05$. The Primer 7 and PAST 4.03 software allowed the realization of the taxonomic bar plots of ASVs at the phylum (1%) and genus (1.5%) level and the calculation of alpha-diversity metrics (i.e., Simpson, Shannon, and Chao1 indices) of the different samples.

4.3. Results

4.3.1. Fusariosis Pathogenesis Confirmation

The presence of *Fusarium* spp. was confirmed by the microbiological approaches in all of the field where the pathogenesis was evident (ZF1-ZF4). Culturable fungal microflora that were developed on SFA showed a huge presence of *Fusarium.* Based on the morphology of the colonies that were observed, many species of *Fusarium* were present. Some of the isolates were allegedly identified as *Fusarium oxysporum* based on the shapes and sizes of the macro- and microconidia, the presence or absence of chlamydospores, the colony pigments, and the growth rates on PDA. No *Fusarium* isolates were observed from the fields where a pathogenesis was not evident.

4.3.2. DNA Extraction and 16S rRNA Metabarcoding

The 16S rRNA gene metabarcoding results were used to investigate the diversity of the samples. As shown in Table 1, a high diversity was present both in the presence and absence of *Fusarium* (Shannon H values higher than 3.5). Sample ZB1 showed more taxa numbers (1454), individuals (36,299), and a high diversity index (Chao-1) when it was compared to the other field with fusariosis. Sample ZF3 presented the highest taxa values, individuals, and diversity indices.

Table 1. Diversity indices calculated on 16S rRNA metabarcoding results using PAST 4.03 software. Soil samples were labelled as follows: ZB1–ZB7 labels refer to saffron soil samples without evident Fusarium pathogenesis; ZF1–ZF4 labels refer to saffron soil samples with *Fusarium* pathogenesis.

	ZB1	ZB2	ZB3	ZB5	ZB6	ZB7	ZF1	ZF2	ZF3	ZF4
Taxa_S	1454	1283	1141	1309	958	1345	1155	1270	2078	1440
Individuals (Richness ASVs level)	36,299	32,009	27,465	28,502	19,823	27,429	25,296	25,719	54,646	33,625
Shannon_H	6.657	6.538	6.41	6.594	6.281	6.657	6.501	6.587	6.994	6.761
Evenness_e^H/S	0.5354	0.5385	0.5329	0.5583	0.5577	0.5788	0.5765	0.5711	0.5248	0.5996
Chao-1	1455	1286	1142	1310	958.7	1347	1156	1271	2080	1444

In the Table: ASVs, Amplicon Sequence Variants.

The 16S rRNA metabarcoding results were also investigated for their structure and abundance. Figure 2 depicts the ASVs composition and abundances at the phylum level. Most of the ASVs were associated with Pseudomonadota (syn. Proteobacteria), which was followed by Actinobateriota. Latescibaterota and Entotheonellaeota were only present in ZB1 and ZB3, respectively. Firmicutes was only present in ZB2, ZB3, ZB6, and ZF2. Except for ZF1, Nitrospirota was absent in all of the ZF samples. Patescibacteria was not found in ZB3 and all of the ZF samples (except for ZF3). Except for ZB2, Planctomycetota was always present. The other phyla were shared by all of the samples.

Given the relevance of the Pseudomonadota phylum within the bacterial communities in all of the fields, we carried out a comparison of the abundances and the composition of the ASVs based on the *Fusarium* presence/absence variable. Figure S1 shows the stacked boxplot of the comparison. In the presence of *Fusarium*, the abundances of the ASVs were lower than those that were observed in the absence of pathogenesis. This finding suggested a strong impact of the pathogenesis on richness of the ASVs associated with this phylum. At the genus level, the common ASVs were those that were associated with uncultured and unknown taxa, which was followed by *Sphingomonas.* (Figure 3). *Vicinamibacteraceae*, *WD2101_soil_group*, RB41, and *Rubrobacter* were also present in almost all of the samples. However, the occurrence of some genera was absent in the presence of

Fusarium pathogenesis, i.e., *Streptomyces, Bacillus, Pseudomonas*, 67-14*, Nitrospira, Nocardioides, Adhaeribacter, Flavisolibacter, Flavobacterium, Gaiella,* KD4-96, MB-A2-108*, Stenotrophomonas, Terrimonas,* and UTCFX1. *Ellin6067* and *Massilia* were only present in the samples under the *Fusarium* pathogenesis condition.

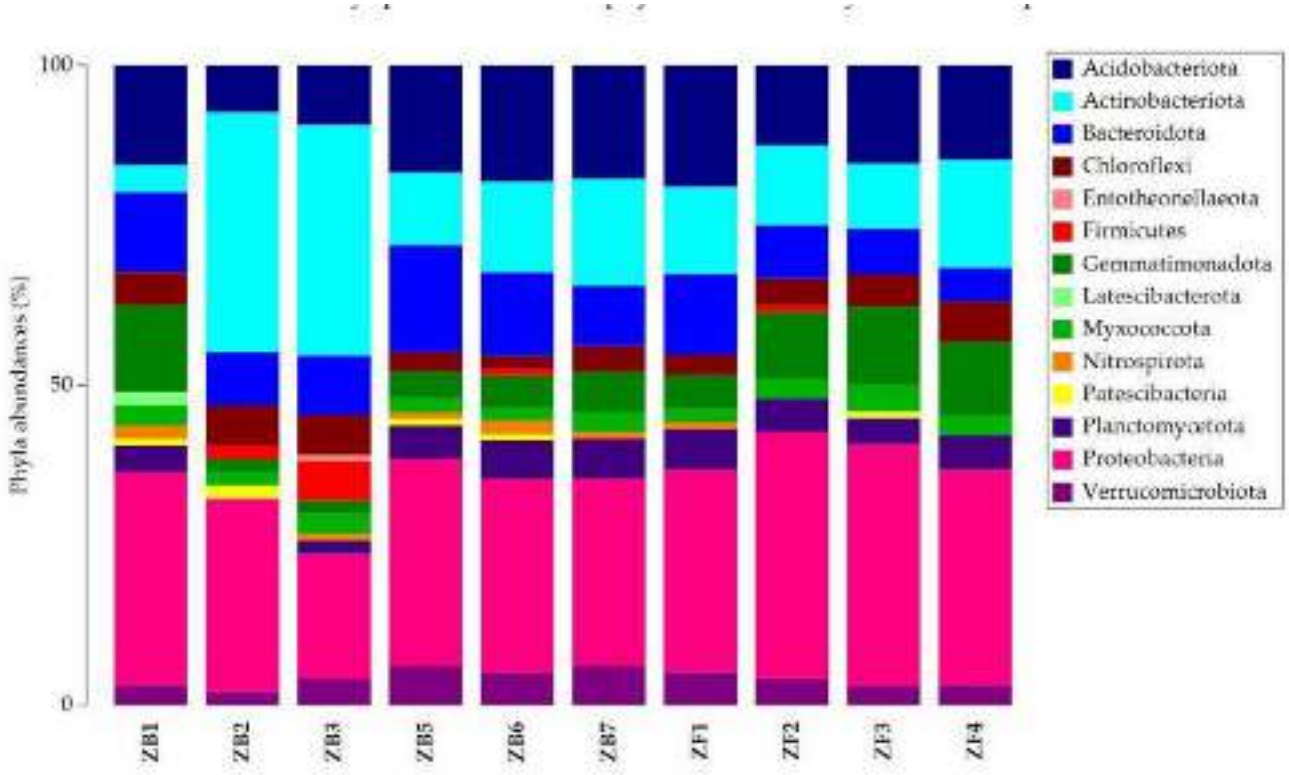

Figure 2. Taxonomic bar plot of the relative abundances of bacterial phyla associated with individual soil samples.

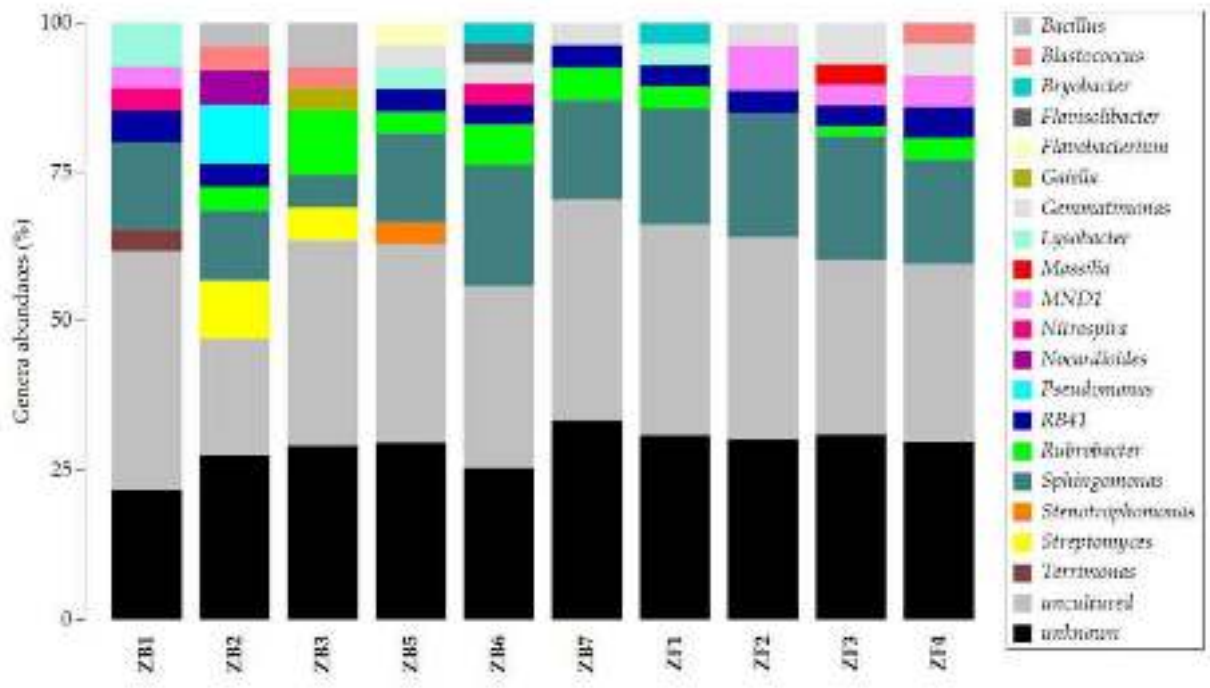

Figure 3. Taxonomic bar plot of the relative abundances of bacterial genera associated with individual soil samples.

4.3.3. Prediction of Metagenomic Functions

Some of the metabolic predictions showed differential abundances in the presence of fusariosis. Figures 4–6 show the Bland–Altman and Effect plots that shows the relationship between the effect size and the BH-adjusted p values (0.05 and 0.01) in the tests that were carried out for the ECs, KOs, and PWYs. Among the ECs (Figure S2), the most significant differences were observed for feature 1 (EC:1.1.1.21—aldose reductase) and 10 (EC 1.12.2.1—cytochrome-c3 hydrogenase), which were higher in the presence of *Fusarium*, and 11 (EC:1.3.1.87—3-(cis-5,6-dihydroxycyclohexa-1,3-dien-1-yl)propanoate dehydrogenase) and 61 (EC:4.3.1.29—D-glucosaminate-6-phosphate ammonia-lyase), which were higher in the absence of fusariosis.

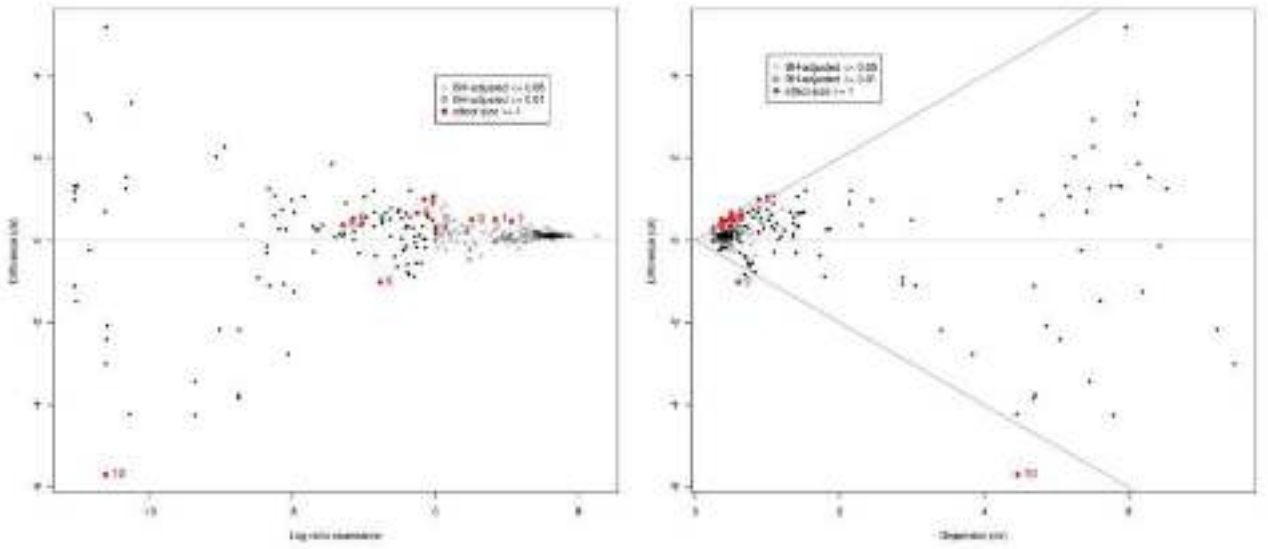

Figure 4. The panel on the left displays the Bland–Altman plot that shows the relationship between Abundance and Difference of the predicted pathways (PWYs) in the presence (lower part) and absence (upper part) of fusariosis. The panel on the right displays the Effect plot that shows the relationship between Difference and Dispersion of the PWYs between *Fusarium* and not *Fusarium* groups. In both of the plots, the 'not significant' features are shown in grey and black. Features that are statistically significant are in red.

Among the KOs (Figure S3), in the presence of Fusarium, higher counts were recorded for features 2 (K00011—aldehyde reductase), 24 (K02205—arginine/ornithine permease), 63 (K11601—manganese transport system substrate-binding protein), and 65 (K11638—twocomponent system, CitB family, response regulator CitT). In the absence of pathogenesis, the higher counts were recorded for the features 28 (K02791—maltose/glucose PTS system EIICB component), 32 (K03078—3-dehydro-L-gulonate-6-phosphate decarboxylase), and 35 (K03290—MFS transporter, SHS family, sialic acid transporter).

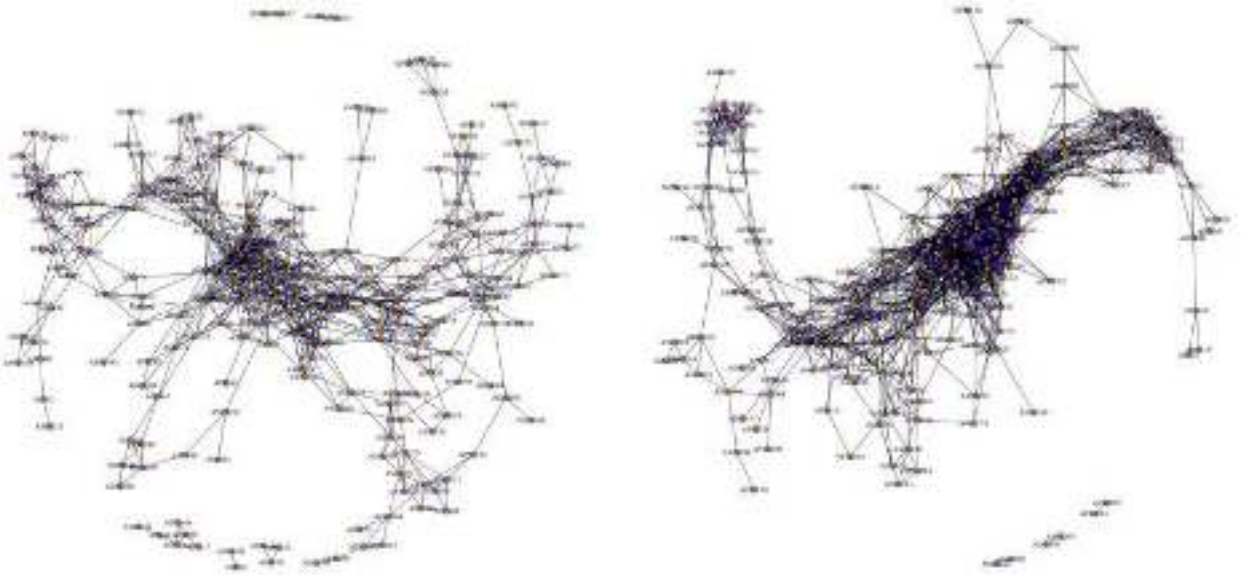

Figure 5. Network analyses carried out on saffron rhizosphere samples in the absence (on the left) and presence (on the right) of fusariosis. Jaccard similarity coefficient: 4.

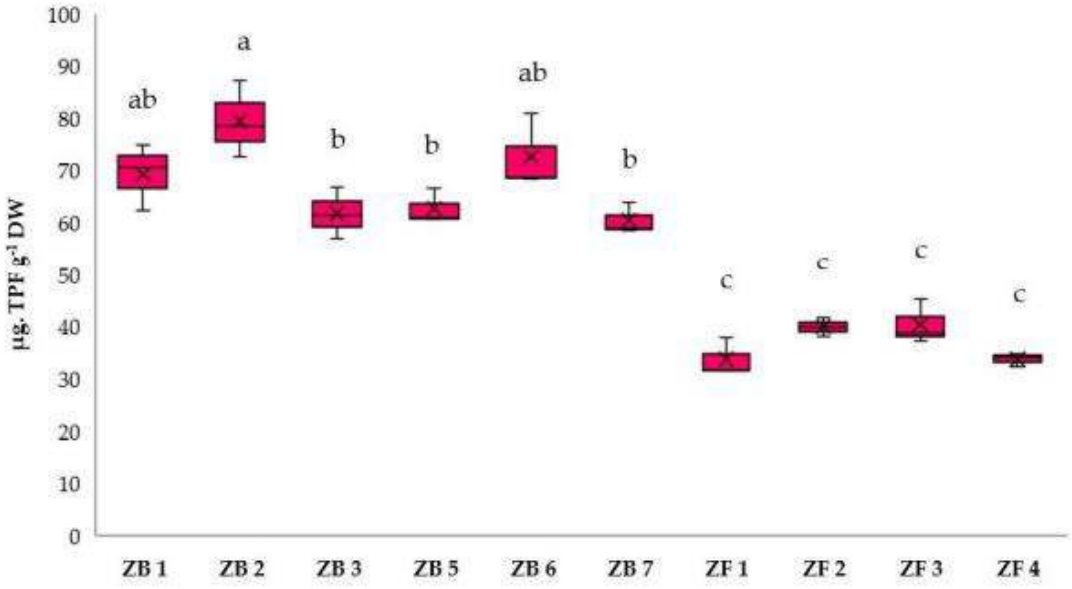

Figure 6. Dehydrogenase activity expressed as µg TPF g−1 DW. Results followed by the same case letter (a-c) are not significantly different according to Tukey's HSD post hoc test ($p > 0.05$).

Among the PWYs (Figure 4), the features 9 (PWY-922—mevalonate pathway I) and 10 (THREOCAT-PWY—L-threonine metabolism) showed higher values in the presence of fusariosis. The pathogenesis altered the other PWYs, with low counts for features 1 (P124- PWY—fructose 6-phosphate pathway), 2 (P125-PWY—superpathway of (R,R)-butanediol biosynthesis), 3 (P161-PWY—acetylene degradation—anaerobic), 4 (PWY-5415—catechol degradation I), 5 (PWY-5529—

superpathway of bacteriochlorophyll a biosynthesis), 6 (PWY5531—3,8-divinyl-chlorophyllide a biosynthesis II—anaerobic), 7 (PWY-7254—TCA cycle VII—acetic acid-producers), and 8 (PWY-7315—dTDP-N-acetylthomosamine biosynthesis).

4.3.4. Network Analysis

The DNA sequencing results were also processed through a network analysis. Figure 5 shows the networks that were obtained for the soil samples with the presence and absence of Fusariosis. At a Jaccard similarity coefficient of four, the samples without *Fusarium* had a total number of 270 nodes and 989 edges, with an average number of neighbors of 7647. In the presence of pathogenesis, higher counts of all of the features were observed (295 nodes; 2750 edges; 19,010 average number of neighbors). A complete dataset of both groups was also processed, creating a network with the sample distribution base on the ASVs features. Figure S4 shows the interconnections among all of the samples based on shared ASVs occurrences, highlighting a close relationship among all of the samples.

4.3.5. Dehydrogenase (DHA) Activity

The results of the dehydrogenase activity analysis are presented in Figure 6. The samples without fusariosis showed the highest values of DHA ($p < 0.05$), with results of up to 79.43 µg TPF g^{-1} DW. Conversely, the samples with Fusarium pathogenesis recorded the lowest values ($p < 0.05$). No significant values among the fields with Fusarium pathogenesis were recorded ($p > 0.05$), with an average value of 36.84 µg TPF g^{-1} DW. Moreover, these samples presented the lowest values when they were compared to those from the field without the presence of *Fusarium*.

4.4 Discussion

The microbial diversity of the rhizosphere of numerous plants, including saffron, has been thoroughly studied using culture-dependent and -independent methodologies [29–32]. In this study, we investigated the changes that occur in the saffron rhizosphere in the presence of the *Fusarium* pathogenesis. The L'Aquila territory (Abruzzo, Italy) and the *"Zafferano dell'Aquila"* (a fine saffron variety with a protected designation of origin) were taken as a case study. Overall, the results suggest that pathogenesis does not affect the rhizosphere microbiota diversity and richness.

However, the microbial communities' composition, structure, and functions were altered in the presence of the *Fusarium* pathogenesis. A presence of uncultured and unknown taxa were found by the 16S rRNA gene metabarcoding. Uncultured microorganisms are widespread in many environments. They play a crucial role in the biodegradation of various pollutants [33]. They constitute a buried group with a genetic resource encoding for unique valuable functions [34]. The uncultured microorganisms are detected in numerous degradation processes, allowing for efficient bioremediation by targeting specific eco-physiological niches [33]. The metagenomic analysis of chronically polluted coastal sediments revealed the presence of aromatic-ringhydroxylating oxygenase, which is related to the biodegradation of polycyclic aromatic hydrocarbon as reported by Loviso et al., [35]. Likewise, the genus *Sphingomonas* is a part of the rhizospheric population, and it is linked with several biogeochemical cycles in soil and different metabolic processes [36].

In addition to the uncultured and unknown taxa, most of the ASVs were associated with Proteobacteria. In the presence of fusariosis, the abundances and taxa associated with this phylum were lower than they were in the healthy soils. Proteobacteria is one of the major phyla in soil ecosystems [37–40], with them having crucial roles in fixing the atmospheric nitrogen and mineralizing numerous soil nutrients [36]. This decrease in Proteobacteria is in line with the findings of Zhou et al., who described the same behavior for the banana rhizobacteria microbiota that were infected by *Fusarium* [41]. Proteobacteria have been closely associated with fungal pathogenesis in other plant species. Shen et al., for example, found that the prevalence of Proteobacteria is linked to the epidemic stage of wheat take-all disease [42]. In our case, this phylum is the most prevalent in the saffron rhizosphere, with it comprising up to 54% of the population [43].

At the genus level, the exclusive presence of *Bacillus, Nitrospira, Pseudomonas*, and *Streptomyces* in the healthy rhizospheres may indicate the presence of beneficial bacteria. These genera are usually associated with plant growth-promoting rhizobacteria (PGPR), with important biostimulant and biocontrol abilities [44–47]. Conversely, the exclusive presence of *Massilia* in the rhizospheres of samples with the pathogenesis indicates an unhealthy status. This lineage exploits the succession of communities within niches [48] and colonizes fungal hyphae with biocontrol effects [49]. A similar situation has been described by Bejarano-Bolívar et al., who described the presence of genera with biocontrol abilities (e.g., *Myxococcus* or *Lysobacter*) in the rhizosphere of an avocado that was affected by *Fusarium oxysporum* [50].

Metabolic predictions have highlighted interesting differences between the two groups. Among the most relevant, the increase in the mevalonate pathway I shows the increase in isoprenoids production. These compounds induce plant growth and development and improve the plant's response to environmental stresses [51]. The increase in the metabolic pathway of L-threonine indicates a high functionality of the community in the degradation of this amino acid [52]. These aspects suggest an attempt to counteract the pathogenesis by the microbial community of the rhizosphere.

Conversely, low counts of the other pathways related to the degradation of sugars, aromatic compounds, and hydrocarbons, the production of acetic acid and chlorophylls, and the production of sucrose metabolites were found. In line with previous reports, these decreases show less functionality in the presence of pathogenesis. The study by Wu et al., for example, described a higher carbohydrate and energy biosynthesis and secondary metabolites in the *Panax notoginseng* rhizosphere in the presence of root-rot fungal pathogens [53]. The network analyses also confirmed the attempt to counter the pathogenesis by the rhizosphere microbial community. Pathogenesis appeared to improve the ASVs interconnections. As reported by the recent review by Siles et al., [54]. Conversely, in the presence of pathogenesis, the organic matter increases due to the plant's degradation. This organic supply can increase the saprotrophic and symbiotrophic interactions, producing a more interconnected network [54]. Estimating the soil enzymatic activity is another approach to studying soil microbial community alterations [55–58]. Among the soil enzymes, dehydrogenase converts hydrogen from an organic material to inorganic acceptors, oxidizing the soil organic substances [59,60]. Soil DHA is an early indicator of alterations in the biological activities of the soil [55]. In the presence of Fusariosis, we found a significant decrease in DHA, which is in line with the results of the literature. Low DHA values have been described for the tomato rhizosphere in the presence of fusariosis by Dukare et al., [61]. A negative correlation between the DHA and pathogenesis was also found in the tomato rhizosphere in the presence of *Ralstonia solanacearum* pathogenesis [62]. This finding confirms the lower metabolic functions of the saffron rhizosphere in the presence of fusariosis which is underlined by the prediction of metagenome functions.

4.5 Conclusions

In this study, we investigated changes in the saffron rhizosphere in the presence of *Fusarium* pathogenesis. The territory of L'Aquila (Abruzzo, Italy) and *Zafferano dell'Aquila* were taken as a case study. We found alterations in the microbial communities' composition, structure, and functions in the presence of the *Fusarium* pathogenesis. Conversely, the diversity and richness of the rhizosphere microbiota were not affected. A predominance of uncultured and unknown taxa was reported using 16S rRNA gene metabarcoding, and most of the ASVs were attributed to Proteobacteria. Additionally, the taxa that are associated with this phylum were less abundant in the presence of fusariosis when they were compared to those in the healthy soil. A noteworthy presence of beneficial bacteria in the healthy rhizospheres and genera with biocontrol activity in the samples with the pathogen was signaled. The microbial taxa interconnections have also improved to face the pathogen attack. To our knowledge, this is the first study on the saffron rhizosphere. Therefore, our findings help to enrich the knowledge on the subject. These results can be used as a starting point for future investigation on the microbial taxa of the rhizosphere that are involved in the suppression of *Fusarium* wilt disease to be used as sustainable disease control agents. Intensive agricultural practices are the most common reasons for fusariosis. Intensive managements, that are associated with agrochemical use and mechanizations, unbalance the soil microbiota and lead to outbreaks of fungal pathogenesis. For this reason, future studies should also investigate the agricultural practices that are used in fields to highlight the possible variables that induce Fusariosis and to develop strategies to avoid or control *Fusarium* outbreaks early.

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CONCLUSIONS

The data presented in this last section, although partial, have led us to demonstrate that these species of bacteria and fungi are indeed useful tools in which we can invest for an optimization of plant crops.

In today's civilization, environmental microbiology and biotechnology are essential. They have an impact on environmental conditions, human health, and the production of new bioproducts and energy. The activities carried out in this PhD project allowed the identification of several plant growth-promoting bacteria useful in sustainable agriculture. The findings obtained and supported by scientific productions underlined the possibilities for these isolates to be used both for biostimulant and biocontrol agents in hemp, tomato, and potato. Literature research carried out with this PhD activities allowed to publish the first review on the possible use of CFS as biostimulant and biocontrol agents in sustainable agriculture. This research product has an important relevance in the field as gives a good summary of the scientific knowledge on the production and application of these products. Being the first review on a subject not well investigated it might pave the road for research in the field. The results are of particular interest for Gram-negative, potential pathogenic, and metabolites-producing strains that present challenging formulations, scale up, and stabilization at industrial levels. The use of the CFS of these strains, in fact, might allow the exploitation of the metabolites produced by these strains without industrial and commercial constraints. The laboratory and on field activities allowed to identify several fungal strains causal agent of hemp, saffron and Solanaceae diseases and responsible for huge crop losses. Within the research activities carried out with saffron the effects of pathogenesis on soil bacterial microbiota were also assessed. Overall, findings obtained with these studies contribute significantly to the field. The description of the type of pathogens, their effects on plants and bacterial microbiota and the selection of possible biocontrol agents contribute to enrich the scientific knowledge, prospecting a substitution of pesticides with bioformulations. The different activities on *A. brasilense, B. ambifaria, G. gluconacetobacter,* and *H. seropedicae* allowed to collect scientific evidence of the efficacy of these strains as biostimulant and biocontrol agents. The participation to the research carried out on actinomycetes allowed to shad the light on these important bacteria not fully explored for agriculture. Even if further research is required, the results obtained during these three years are a good starting point for the development of microbial inoculants that are useful for

overcoming abiotic and biotic stresses and in substitution of agrochemicals. The evidence created can be used by biostimulant industries to formulate and produce biostimulant and biocontrol agents. Future research should be directed towards the application of the strains singularly and in consortia on different plants and in the presence of diverse pedoclimatic conditions. Furthermore, the biotechnological potentialities and industrial level production should be explored for commercialization perspective.

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