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CICLO XXXV

Plant Growth Promoting Rhizobacteria characterization. Dissecting the role of *Beijerinckia fluminensis* in reducing salt stress in *A. thaliana*

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INTRODUCTION

1. Challenges to agriculture

The 21st century is probably the most complex century to face for the agriculture, presenting a series of complex challenges. It must: i) produce more food to feed a growing population, with a reduction in the labour force, ii) adopt a more sustainable and efficient production and adapt to climate change, iii) produce more feedstocks for a potentially huge bioenergy market, and iv) sustain the development of the many agriculture-dependent developing countries (FAO, 2009).

The United Nations projects that the world's population will be 9.7 billion by 2050, 10.8 billion by 2080, and 11.2 billion by 2100. Compared to approximately 7.3 billion people in 2015, the population will increase by around 32, 47, and 53 % in these three future periods, respectively (UN, 2015). Almost all of this growth is expected to take place in developing countries where the population of sub-Saharan Africa would grow the fastest (plus 114 %). Urbanization will also continue to grow at the same rate: by 2050, 70 % of the world's population will reside in urban areas (up from 49 % today), while the rural population, after peaking in the next decade, will decline.

The demand for food will continue to grow. For example, it is predicted that by 2050 the demand for cereals, both for human and animal food, will reach 3 billion tons, up from the current 2.1 billion tons (FAO, 2009). The advent of biofuels also has the potential to change some of the predicted trends and increase global demand; indeed, greater competition between food and non-food uses of biomass has intensified the interdependence between food, feed, and energy markets (FAO, 2017).

Food production is projected to need to increase by about 70 % by 2050 to feed a world population of 9.1 billion people, with a doubling of production of various staple foods in developing countries (FAO, 2009). Although agricultural production globally has become more efficient, competition for natural resources has intensified in recent decades due to population growth, changing dietary patterns, industrial development, urbanization, and climate change. The most visible manifestations of

this unsustainable competition are land degradation, deforestation, water scarcity, fertilizer and pesticide pollution, and reduced biodiversity (FAO, 2018).

Globally, there are few opportunities left to further expand agricultural areas. Much of the exploitable land is concentrated in a few countries in Latin America and sub-Saharan Africa, but in many countries much of the land is suitable for growing only a limited number of crops, which are not necessarily those most in demand. In addition, much of the unused land is subject to chemical, physical, endemic disease etc., limitations that cannot be easily overcome. For these reasons, 90 % of the growth in agricultural output globally (80 % in developing countries) is expected to come from higher yields and increased cropping intensity, with the rest coming from land expansion (FAO, 2009). Unfortunately, since the 1990s average annual increases in maize, rice, and wheat yields at the global level have reached just over 1% while those of soybeans and sugarcane were below 1 % (FAO, 2017).

Climate change will be negatively deterministic on the future state of natural resources, as well as the future conditions and limitations of agricultural production, thus affecting food availability and food supply stability (FAO, 2018).

2. Salt stress affects plants growth

2.1 Soil salinization

Soil salinity is a major abiotic stress in agricultural crop productivity worldwide. The causes of soil salinization can be grouped into two: i) primary, due to natural sources and ii) secondary, largely anthropogenic and exacerbating the problem of salinization (Kumar and Sharma, 2020; Omuto et al., 2020).

A primary source is saline parent material that releases soil mineral constituents during chemical erosion of rock or sediment minerals, which react with air and water to produce soluble salts that are then transported away from their source of origin

through watercourses. Another factor influencing the development of saline soils is salty groundwater that rises through the soil profile by capillarity and releases salts, which as a result of water evaporation remain in the soil. Coastal areas are particularly prone to salinization. Winds and sea spray along the coasts transport salts from the sea inland in sufficient quantities to cause salinization of these areas. Sea spray can have an impact up to 80 km inland or even beyond (Kumar and Sharma, 2020). Coastal regions are also at risk of progressive salinization due to storms, cyclones, floods, etc. Climate is another factor in the formation of saline soils, especially in arid and semi-arid regions, where evaporation results in the release of salt crystals on the surface of the soil. Furthermore, low precipitation does not facilitate salt leaching (Omuto et al., 2020).

The main secondary cause is irrigation. Irrigation can cause salinity when salty water is used or when it causes inadequate leaching of salts into the soil. Irrigation water can also recharge underground wells and cause them to rise, gradually introducing salts contained in groundwater into the soil. The use of fertilized water can also contribute to the introduction of salts, which accumulate over time and increase with repeated application. In addition to irrigation, waste and wastewater are also secondary contributors to soil salinity. Variation in land use or land cover is an important secondary factor; in fact, alteration of vegetation type can change the water use and evapotranspiration characteristics of plants, with consequences such as soil drying and salt accumulation. Soil salinization has been exacerbated by excessive use of chemical fertilizers, improper irrigation practices and industrial pollution (Omuto et al., 2020). Global warming is exacerbating the accumulation of salts in soils due to expanded drylands, water scarcity, and rising sea levels.

According to the GSASmap (FAO, 2021), more than 1.257 million hectares of soil are affected by salt and recent studies reported and estimated 20% of cultivated lands and 33% of irrigated lands are subjected to high salinity, with an expected increase of 10% annually (Abdelraheem et al., 2019; Farhangi-Abriz and Ghassemi-

Golezani, 2016). Soil salinity will continue to be an environmental problem in the future, with an estimated 50% of salinized arable land by 2050.

Based on their adaptative evolution, plants can be classified into two main categories: halophytes capable of co-existence with salinity and glycophytes incapable of co-existence with salinity. All the most important crops belong to the second category of plants, and for this reason, salt stress is considered the most lethal of the abiotic stresses and likely to have the greatest effect on crop yields worldwide**.**

The agricultural sector has estimated an annual loss of 27.3 billion US dollars due to agricultural damages caused by saline soils (Kumar and Sharma, 2020). Crops growing in salinity-affected soils exhibit a spectrum of complex responses and interactions of physiological, morphological and biochemical processes, which ultimately lead to very low crop production and quality (Liu et al., 2017). Most domesticated crops are salt sensitive and cannot be cultivated in saline soil (Yang and Guo, 2018).

Generally, soil is considered saline when the electrical conductivity (EC) of the saturation extract in the root zone exceeds 40 mM at 25 °C, with 15% of unbound Na^+ ions (Shrivastava and Kumar, 2015). Among the different ions that can cause salinization, sodium is the most soluble and widespread and it is considered the most deleterious. In fact, unlike other ions, such as Ca^{2+}, Mg^{2+} or K^+, which plants can exploit for normal physiological and biochemical processes, Na^+ turns out to be useless and extremely toxic to glycophytes (Maathuis, 2014).

Salt-affected soils have serious impacts on some of the ecosystem services that soils typically provide, which are critical to supporting human life and biodiversity, leading to an array of consequences including: i) decreased agricultural productivity, water quality, soil biodiversity, and increased soil erosion; ii) decreased ability to act as a buffer and filter against contaminants; iii) degraded soil structure; iv) decreased functions of ecological systems such as the hydrological and nutrient cycles; v) increased concentration of ions that are toxic to plants; vi) reduced ability of crops to take up water; vii) reduced soil fertility and availability of micronutrients (FAO, 2021).

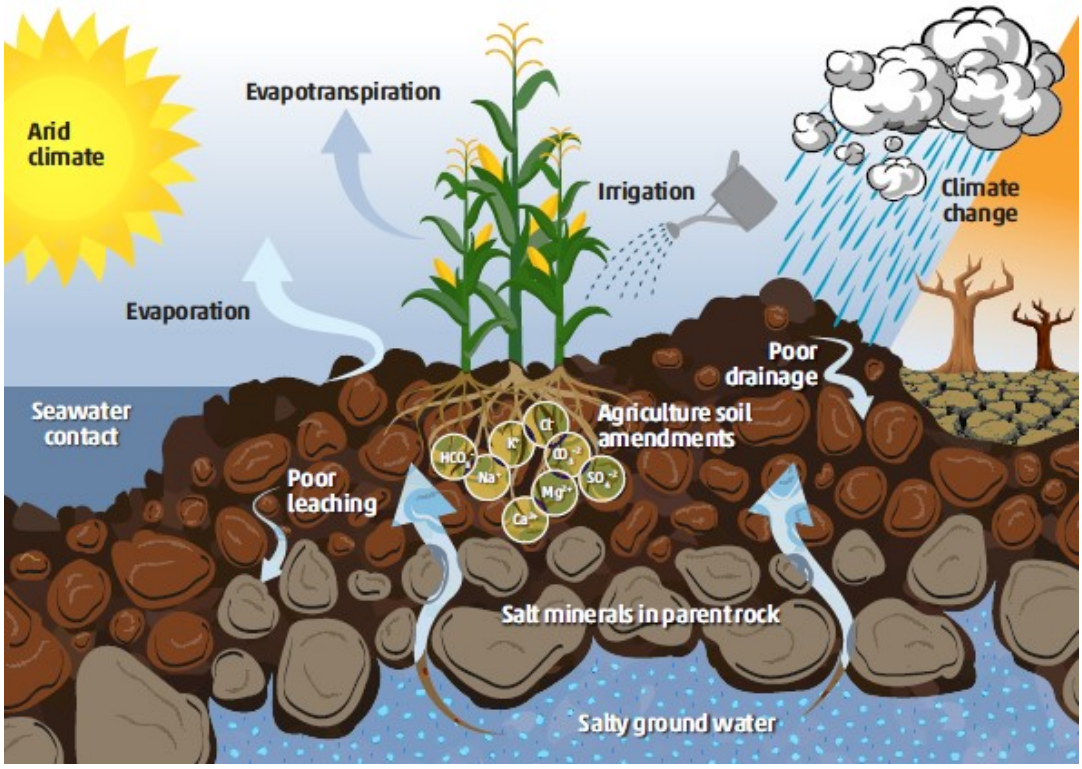

Figure 1. Causes of soil salinization *(FAO, 2021).*

2.2 Salt stress and plant tolerance mechanisms

Salinity stress involves changes in various plant physiological and metabolic processes, depending on the severity and duration of the stress, and ultimately inhibits plant growth. High salinity affects plants in several ways: water stress, ion toxicity, nutritional disorders, oxidative stress, alteration of metabolic processes, membrane disorganization, reduction of cell division and expansion, genotoxicity (Carillo et al., 2011).

NaCl is the most soluble and widespread salt; therefore, it is not surprising that plants have evolved mechanisms to regulate its accumulation. By analysing the different growth responses under salinity conditions, plants differ considerably in their tolerance (Munns and Taster, 2008). Among cereals, barley is the most tolerant crop, while rice is the most sensitive. Similarly, soft wheat (*Triticum aestivum*) is moderately tolerant, and durum wheat is less so (*Triticum turgidum* ssp. *durum*). Tall

grass (*Thinopyrum ponticum*, syn. *Agropyron elongatum*) is a halophytic relative of wheat and is one of the most tolerant plants of monocotyledons. Among dicotyledons, the variation in salinity tolerance is even greater. For example, some legumes are very sensitive, while saltbush (*Atriplex* spp.) can grow at salinity concentrations higher than those in the sea. *Arabidopsis*, when compared with other species under similar conditions, is a salt-sensitive species; indeed, continuous exposure of *Arabidopsis* to 100 mM NaCl does not allow it to complete its life cycle (Munns and Taster, 2008).

Elevated concentration of salt reduces shoot growth and affects plants in two main ways: it makes complicated for roots to uptake water reducing the osmotic potential (osmotic effect), and inside the plants results in ions toxicity (ionic effect). Indeed, a two-phases model has been proposed to describe the effects of salt stress. The osmotic phase begins immediately after exposure of the roots to a threshold level of salinity, which is usually 40 mM NaCl or less for such plants as *Arabidopsis*. During this phase, the immediate response is stomatal closure, which also helps to reduce ion flux to the shoot; the rate of shoot growth falls and new leaves emerge more slowly (Carillo et al., 2011; Munns and Taster, 2008). However, because of the difference in water potential between the atmosphere and the leaf cells but especially the need to fix carbon, this is an unsustainable solution and therefore is bound to be discontinued in a short time. An interesting phenomenon that has not yet been mechanistically explained is that shoot growth is more affected than root growth. The teleological explanation is that a reduction in leaf area development in relation to root growth would decrease the use of water by the plant, thus allowing it to conserve soil moisture and prevent an increase in salt concentration in the soil. The second phase is determined by the ion toxicity. At this stage, the plant's response to salinity begins when salt accumulates to toxic concentrations in older leaves, which are no longer expanding and thus no longer dilute the incoming salt as younger growing leaves do, leading them to death. If the leaves die at a faster rate than they are produced, the plant's photosynthetic capacity is compromised, and it will no longer be able to supply

the carbohydrates needed by the young leaves, further reducing their growth rate (Munns and Tester, 2008).

Usually, osmotic stress has an immediate and greater effect on the growth rate than ionic stress, except for a high salinity concentration or in sensitive species that do not have a strong ability to control Na^+ transport. For most plant species, Na^+ toxicity is greater than Cl^- toxicity, for this reason, almost all studies have focused on the mechanisms involved in Na^+ control rather than Cl^- (Munns and Tester, 2008).

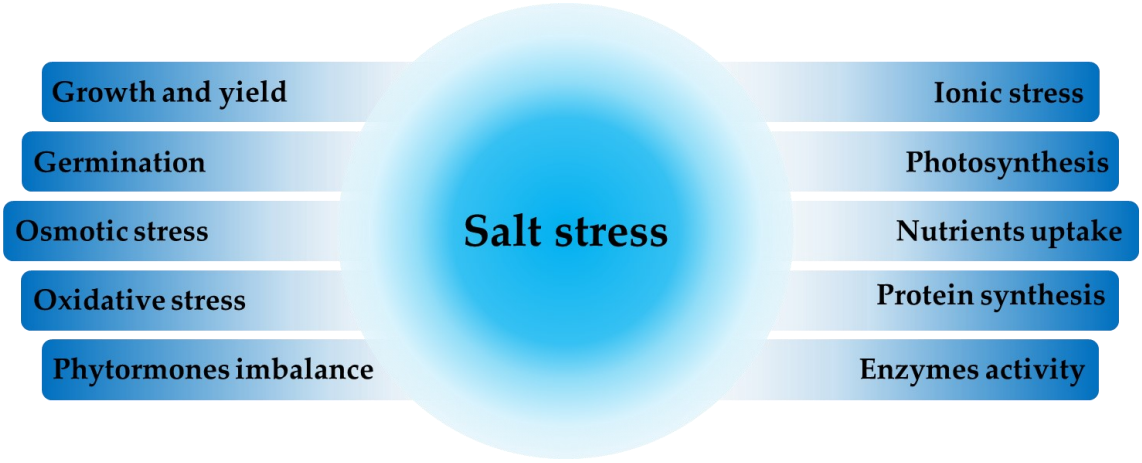

Figure 2. Overview of the salt stress impact on plants.

For their survival against the unfavourable conditions, plants have evolved a well-coordinated defence system to sense and respond to stress factors. Mechanisms of plant salinity tolerance can be classified into three main categories (Munns and Tester, 2008):

- **Tolerance to osmotic stress.** Reduced response to osmotic stress would increase leaf growth and stomatal conductance, but the resulting increased leaf area would benefit only plants with sufficient soil water.
- **Na^+ exclusion.** The exclusion of Na+ by roots ensures that Na^+ does not accumulate at toxic concentrations within the leaves.

- **Na^+ tissue tolerance.** Tolerance requires the compartmentation of Na^+ at the cellular and intracellular level to avoid toxic concentrations within the cytoplasm.

The main physiological, molecular, and biochemical mechanisms, for survival under high salt concentration, include (Gupta and Huang, 2014) i) ion homeostasis and compartmentalization, ii) ion transport and uptake, iii) biosynthesis of osmoprotectants and compatible solutes, iv) activation of the antioxidant enzyme and synthesis of antioxidant compounds, v) modulation of hormones.

At the cell and molecular level, plant defence is characterised by highly regulated and multi-layered complex mechanisms that cover a number of events regulating stress tolerance. These mechanisms include the perception and transduction of stress signals and the reprogramming of genetic, transcriptomic, and metabolic machineries, which are translated into biochemical and physiological combined stress-responsive phenomenology to achieve a substantial increase in salt tolerance (Chele et al., 2021; Zhang and Shi, 2013).

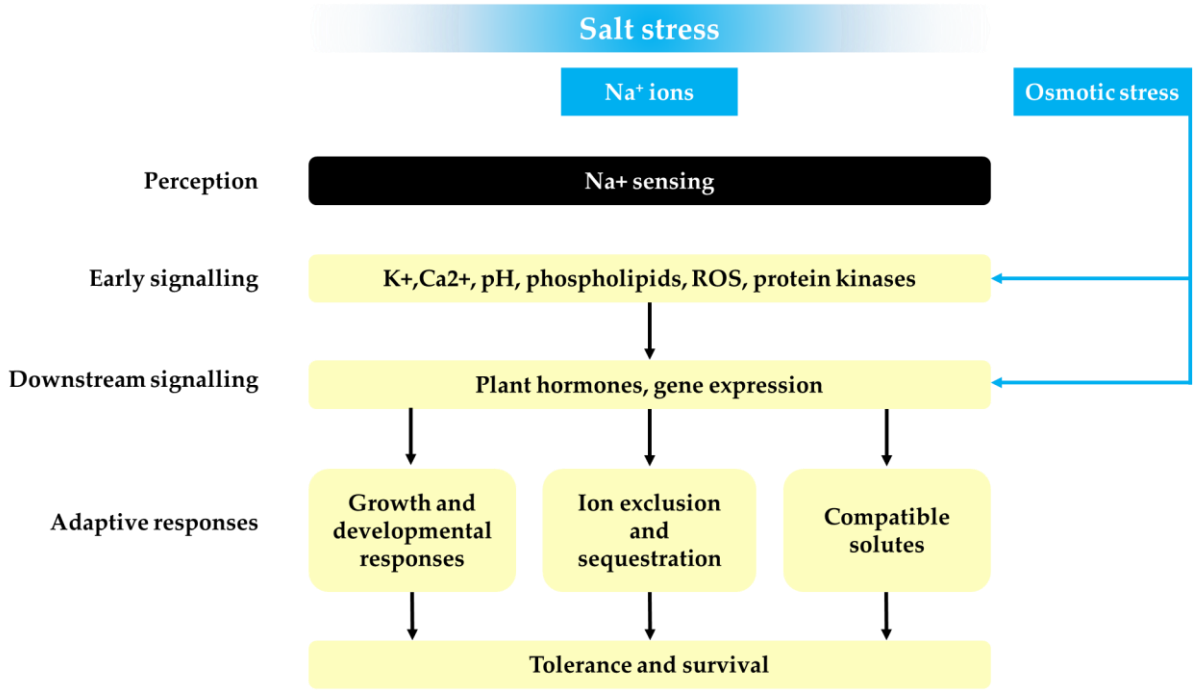

***Figure 3. The salt-induced signaling cascade from sensing to adaptive responses**. Part of the early signaling and downstream signaling (edited from van Zelm et al., 2020).*

Table 1 shows examples of processes and proteins involved in the salt stress response and their possible roles in determining tolerance.

***Table 1**. Main categories of proteins that appeared related to salt-stress response (Sahi et al., 2006).*

Function(s)	Examples	Possibile role in salt stress response
Signalling molecules	Receptor like protein kinase, MAP kinase, histidine kinases, protein phosphatase 2C, G-protein coupled receptor protein, AAA-type ATPase, calmodulin, calcineurin, EF-hand containing proteins, serine-threonine protein kinase, ADPRFs	Stress signal transduction and gene expression
Transcriptional and post transcriptional machinery	DREB, EREBP, MYB, MYC and Zn-finger transcription factors, RING finger proteins, MADS box proteins, homeodomain leucine zipper, CBF, TATA-binding protein, General Control Non-repressed (GCN)-like proteins, glycine-rich and zinc finger RNA-binding proteins, RNA polymerase, splicing factors, microRNAs	Transcriptional regulation of stress gene expression, transcript stability, turnover, processing
Translational machinery	Ribosomal proteins, translation initiation and elongation factors, t-RNA synthetases	Stress-regulated protein translation, selective translation, transport, localization
Protein folding	F-box, WW, WD40, Postsynaptic density protein, Disc-large, Zo1 (PDZ), Tetratricopeptide repeat (TPR)-domain-containing proteins, HSPs, PPIases, PDIase, DnaJ, DnaK like proteins, calrecticulin	Maintenance of protein structures, protein folding, preventing protein denaturation, Protein sorting, targeting
Protein turnover	Polyubiquitins, ubiquitin conjugating enzymes and ligases, components of the proteasome pathway, proteases, protease inhibitors	Regulation of protein metabolism, targeted protein degradation in response to stress
Osmoprotectants	Proteins encoding for enzymes that govern levels of proline (pyrroline carboxylate reductase, proline oxidase), glycinebetaine (choline oxidase), trehalose (TPS), mannitol (mt1D) and sorbitol (sac B); LEA, COR, dehydrins, WSP (water stress proteins)	Osmotic adjustment, protection of cellular structures and macromolecules
Transport protein	Water, amino acid, sugar and metal transporters, aquaporins, membrane proteins, antiporters, ion channels, sulphate transporters, ABC-type transporters, amino acid permease, Naþ and Kþ transporters, plasma membrane and vacuolar ATPases, TiP	Ion homeostasis during stress, compartmentalization of solutes and amino acids
ROS scavengers, cell death, senescence, ageing	SOD, peroxidases, oxido-reductases, PAL, catalase, glutathione S-transferase, cytochrome c-oxidase, glyoxalase, cyclin H1, histones, tumour suppressors	Detoxification of free oxygen radicals, cell death, hypersensitive response

Metal-binding proteins	Metallothionin, ferritin, Cu- and Zn-binding proteins, calmodulin	Affecting cellular metabolism, metal ion homeostasis, acting as cofactors for critical reactions, signalling, metal toxicity, secondary stress responses, oxidative stress
Photosynthesis	Chlorophyll a/b-binding protein, photosystem I subunit PSI-like protein, ATP sulphurylase, rubisco activase	Regulation of photosynthesis
Defence-related proteins	WRKY family of transcription factors, chitinase, glucanases, protease inhibitors, myrosinase-binding protein, other PR proteins such as thaumatin	Protection against biotic stress including viral, bacterial and fungal infestation
Hormone-related proteins	Zeaxanthin epoxidase, gda-1 (GA-induced gene), asr-1 (abscisic acid responsive), ACC synthase, ABI-3 interacting protein, allene oxide synthases	Hormonal homeostasis and gene expression
General metabolism	Nucleosidediphosphate Kinase (NDPK), arginine decarboxylase, glucosyltransferases, mannosyltransferases, methyl and acetyl transferases, choline kinase, lipoxygenase, fatty acid desaturase, GAPDH, lipase, ferredoxin nitrite reductase, aldolase, enolase, alanine transaminases, methionine synthase, asparagine synthetase, tryptophan synthase, acetohydroxyacid synthase, NADP-ME, fructose bis-phosphatase, malate dehydrogenase, enzymes of the photorespiratory and pyruvate cycle pathways, acetyl Co-A synthetase, phenylpropanoid pathway	Overall cellular function, housekeeping metabolic pathways carbohydrate, fatty acid and protein synthesis and modifications membrane fluidity, nitrogen metabolism, carbon and nitrogen fixation
Unclassified proteins	Hypothetical and putative proteins, including genes encoding proteins with uncharacterized domains and tissue specific genes	Unknown

2.2.1 Osmotic adjustments

The decrease in osmotic pressure induces the loss of water and represents one of the problems plants face when growing in saline soils. Changes in osmotic pressure must be compensated to maintain cell volume and turgor (van Zelm et al., 2020). To maintain osmotic balance both inside and outside the cell, plants increase the synthesis of low-molecular-weight compatible solutes into the cytosol, also referred to as osmolytes. The most common osmolytes include proline, glycine betaine, and soluble sugars (Ilangumaran and Smith, 2017). They are not charged, polar, soluble, and do not interfere with cell metabolism even at high concentration (Gupta and Huang, 2014). Osmotic adjustment is an energy-intensive process; in fact, it results in a reduction in growth to direct energy to osmolyte synthesis. On the other hand, it is a

necessary measure to alleviate the effects of salt stress (Munns and Gilliham, 2015; Raven, 1985). In addition to its role in osmotic adjustment, proline acts as an antioxidant that scavenges excessive stress-induced ROS, buffers the cell redox potential, stabilizes proteins, enzymes, membrane structures and the electron transport system complex II (Asensi-Fabado et al., 2017; Cui et al., 2020; Reza Amirjani, 2011). Proline is considered as a sensitive physiological markers of salt stress.

2.1.2 Antioxidant activity

One of the major secondary stresses induces by salt stress is oxidative stress. During salt stress, the content of reactive oxygen species (ROS) increases dramatically, mainly due to the disruption of electron transport chains (ETC) during photoinhibition and/or decrease in the water potential (Acosta-Motos, et al., 2017). The ROS comprising different compounds such as $O_2^{\bullet -}$, H_2O_2, $^1O_2$, $HO_2^{\bullet -}$, $OH^{\bullet}$, ROOH, $ROO^{\bullet}$, and $RO^{\bullet}$, which react spontaneously with organic molecules and cause membrane lipid peroxidation, protein oxidation, enzyme inhibition, and DNA and RNA damage (Vinocur and Altman, 2005). To detoxify ROS plants have developed antioxidant systems including antioxidant enzymes and non-enzymatic compounds. Salinity tolerance is positively correlated with the activity of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), ascorbate peroxidase (APX) and glutathione reductase (GR) and with the accumulation of non-enzymatic antioxidant compounds (carotenoids, flavonoids and other phenolics, proline) (Gupta and Huang, 2014).

2.1.3 Roles of SOS1, NHX1, and HKT1 in ion homeostasis

Regardless of their nature, both glycophytes and halophytes cannot tolerate a high concentration of Na^+ in their cytoplasm. In fact, maintenance of ion homeostasis through Na^+ ion compartmentalization is critical for growth during salt stress (Carillo et al., 2011). The regulation of Na^+ uptake and transport in salt stressed plants has been interpreted in the context of maintaining high K^+/Na^+ ratio in the cytoplasm. A

deleterious effect of salt stress is the imbalance of nutrients, and K^+ is one of the many ions involved in this phenomenon. K^+ is an essential macronutrient that plays important functions related to enzyme activation, osmotic adjustment and turgor generation, regulation of membrane potential, and cytoplasmatic pH homeostasis (Barragan et al., 2012). Because Na^+ and K^+ possess very similar physical and chemical properties, the two ions compete for many key metabolic processes in the cytoplasm. In fact, Na^+ inhibits the enzymatic activity of many enzymes that require K^+ to function. Since more than 50 different cytoplasmic enzymes are activated by K^+, the disruption of the activity of these enzymes leads to a dramatic effect on cellular metabolism (Almeida et al., 2017). Na^+ control mechanisms involved the Na^+ exclusion from roots, Na^+ long distance transport, and Na^+ compartmentation (Munns, 2005).

Three main proton pumps have been identified related to salt stress tolerance in plants: i) vacuolar-proton phosphatase which generates a proton gradient by using energy from pyrophosphatase (PPi), ii) plasma membrane H^+/ATPase, and iii) vacuolar H^+/ATPase which couple H^+ transport and ATP hydrolysis (Singh and Roychoudhury, 2021).

Increasing evidence demonstrates the roles of a Salt Overly Sensitive (SOS) stress signalling pathway in ion homeostasis and salt tolerance, which is considered the first mechanism used by plants involved in the Na^+ exclusion. SOS signalling pathway is composed of three proteins: SOS3 (Ca^{2+}-binding protein), SOS2 (serine/threonine kinase) and SOS1 (Na^+/H^+ antiporter). High salinity triggers increase in cytosolic Ca^{2+} concentration, inducing the Ca^{2+}-mediated activation of SOS3. The C-terminal regulatory domain of the SOS2 protein contains a FISL motif (also known as NAF domain), which is about 21 amino acid long sequence, and serves as an interaction site for the Ca^{2+} binding protein SOS3. The interaction between SOS2 and the SOS3 protein results in activation of the kinase and release of the SOS3/SOS2 complex to the cytosol where it phosphorylates SOS1, activating its transport function. The SOS1 protein is characterised by a long cytosolic C-terminal tail, approximately 700 amino acids long, comprising a putative nucleotide binding motif and an

autoinhibitory domain. This autoinhibitory domain is the target site for SOS2 phosphorylation (Chele, et al., 2021; Guo et al., 2004).

SOS1 is autoinhibited under normal conditions, and SOS2 release inhibition under salt stress by phosphorylating Ser1044 in the C-terminal domain of SOS1. In particular, whereas *sos* mutants have hypersensitive phenotype under high salinity, they normally grow under osmotic stress imposed by mannitol or PEG (polyethylene glycol), indicating that the SOS signalling pathway is specifically involved in the response to ionic stress (Zhao et al., 2020). SOS1 is required to extract excess Na^+ from cells (i.e., into the rhizosphere through root epidermal cells, or into the xylem through parenchyma cells of the xylem) and thus reduce ionic stress. Upregulation of the gene encoding SOS1 confers salt tolerance in plants (Shi et al., 2000; Shi et al., 2002).

In addition to extruding Na^+ from the cell through the action of SOS1, plants possess an additional system of controlling Na^+ concentrations in the cytosol, represented by Na^+ translocation within the vacuole. This function is performed by Na^+/H^+ exchangers based on tonoplasts in the NHX family. The NHX family is composed of many isoforms, differing mainly in their localization. For instance, *Arabidopsis* NHX family members are divided into two classes: class I isoforms (AtNHX1-4) are located on the tonoplast membrane, and class II (AtNHX5 and 6) on endosomal membranes of the Golgi, trans-Golgi network and pre-vacuolar compartment. Among all isoforms, the role of NHX1 in determining salt stress tolerance is the well characterized. As is the case with SOS1, up-regulation of NHX1 results in increased tolerance to salt stress (Apse et al., 1999; Sottosanto et al., 2007). The NHX antiporter family is also involved in pH homeostasis, growth and development, stomatal functions, and protein and vesicle trafficking. Under normal conditions, the major role of NHXs is as a K^+/H^+ antiporter. Na^+ transport mediated by NHX would only occur under conditions of high Na+ concentration (Assaha et al., 2017). The mechanism underlying this change in selectivity is hypothesized for the NHX1 protein of *Arabidopsis*. The *Arabidopsis* NHX1 C-terminus interacted with a calmodulin like protein15 (AtCaM15) within the vacuolar lumen in a Ca^{2+}-dependent

and pH-dependent manner. Under normal physiological conditions, where the vacuole is acidic (pH 5.5) and Ca^{2+} activity high, AtCaM15 is bound to AtNHX1 and results in higher activity of K^+/H^+ than Na^+/H^+. At a higher pH (6.0–7.5), the AtCaM15 binding to AtNHX1 was reduced and the activity of Na^+/H^+ increased. As salinity causes alkalinization of the vacuole, the change in pH results in a change in protein selectivity from K^+ to Na^+ (Bassil and Blumwald 2014).

Other important proteins involved in salt stress tolerance in plants are the High Affinity Potassium Transporter (HKT) proteins. According to their transport characteristics, the HKT family is divided into two different classes. HKT1, characterized by a preference for Na^+ conductance over other cations, and HKT2, characterized by transport of Na^+ or K^+ depending on the external concentration of these two ions (Mäser et al., 2002a). The most characterized member of HKT class I is AtHKT1 from *Arabidopsis*, the only member of the HKT family in *Arabidopsis*. AtHKT1 is involved in Na^+ entry, and two other complementary functions have been proposed. In the phloem recirculation model, AtHKT1 loads Na^+ into the phloem cells of the shoot and transfers it to the roots through the downward stream, thus preventing Na^+ accumulation in the shoot. Another function of AtHKT1 is to unload Na^+ from the xylem transpiration stream, thus limiting the amount of Na^+ reaching the photosynthetic tissues and supporting salt stress tolerance (Almeida et al., 2017).

2.1.4 ABA dependent pathway

Among all phytohormones, abscisic acid (ABA) is the major factor in the regulation of resistance to abiotic stresses in plants, which coordinates a variety of functions. Its activity depends on its concentration in the plant. At a normal level, ABA regulates various physiological processes such as stomatal opening, embryo morphogenesis, seed development, dormancy, and synthesis of storage proteins and lipids (Finkelstein and Rock, 2002; Sreenivasulu et al., 2012), while at high concentration ABA inhibits plant growth.

Under conditions of abiotic stress, such as drought and salt stresses, ABA biosynthesis is greatly induced, leading to an increase in its content in the plant and determining stomatal closure and a change in gene expression, which may favour plant adaptation and survival (Sah et al., 2016). ABA regulates a multitude of salt-responsive genes. Comparison of *Arabidopsis* and rice transcriptomes exposed to ABA and various abiotic stresses showed that transcriptional changes affect 5-10% of the genome and more than half of them were common to salinity, drought and ABA treatments (Nakashima et al., 2009; Shinozaki et al., 2003).

The importance that ABA has in this phenomenon is represented by the fact that in *Arabidopsis* seedlings, the genes that are modulated by ABA comprise about 10 % of the genome, evenly divided between induced and repressed genes; two to six times more than those modulated by other plant hormones (Nemhauser et al., 2006). Most ABA-modulated genes encode proteins involved in stress tolerance, such as dehydrins and enzymes that detoxify ROS, those involved in osmolyte metabolism, several transporters, transcription factors, protein kinases and phosphatases, and enzymes involved in phospholipid signalling (Cutler et al., 2010). Furthermore, ABA biosynthesis is also regulated by its end products, since ABA negatively regulates its own accumulation by activating its catabolic enzymes (Cutler and Krochko, 1999).

During salt stress, many transcription factors (TFs) play a fundamental role in the response to stress. TFs usually act as key negative or positive regulators of gene expression. With two distinct domains, a DNA binding domain and a transcriptional activation/repression domain, they regulate diverse cellular processes, governing the transcriptional rates of target genes. TFs such as MYC, MYB, bZIP, MADS, and BHLB have been characterised and assigned to various families and superfamilies based on their DNA binding domains. The MYB family, found in all eukaryotes, represents one of the large and functionally diverse classes of proteins. (Roy, 2016). In general, most of the MYB proteins function as transcription factors and are characterized by the presence of variable numbers of conserved MYB repeats (R) at the N-terminus, mainly associated with DNA-binding and protein-protein interactions. The variable C-

terminal region is responsible for modulating the regulatory activity of the protein. Based on the number of MYB domains, the MYB protein family has been classified into four different groups, such as 1R-, R2R3-, 3R- and 4RMYB proteins, respectively.

In plants, most MYB TFs belong to the R2R3-MYB subfamily (in *Arabidopsis*, 126 members), involved in the regulation of specific plant processes such as i) primary and secondary metabolism, ii) cell fate and identity, iii) developmental processes and iv) responses to biotic and abiotic stresses (Dubos et al., 2010). Regulation of MYB TFs in response to salt stress involves ABA. When plants grow under high salinity conditions, the ABA content increases significantly, initiating a cascade of salt stress response signals in the ABA-dependent pathway that leads to up or down regulation of downstream response genes. The specific effects are to alleviate osmotic stress and ionic stress caused by excessive salt, maintain water balance, and maintain integrity of the cell membrane structure (Jaschke et al., 1997; Li et al., 2019).

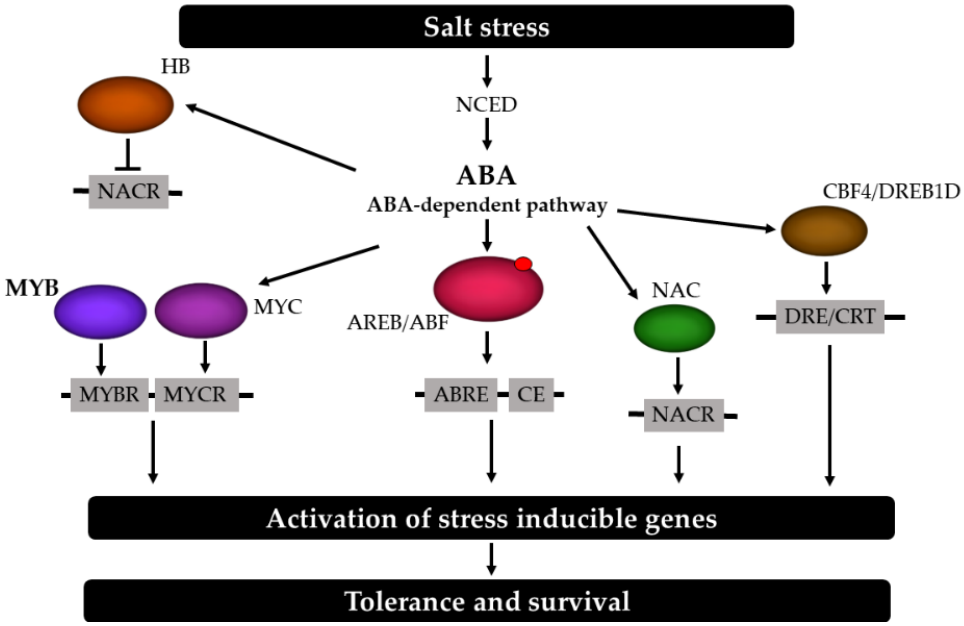

Figure 4. ABA dependent pathway. Activation of some TFs in response to stress signal*. (edited from Roychoudhury et al., 2013).*

3. Rhizosphere environment

3.1 Rhizosphere microbial community

A plant growing under field conditions is not an individual, but a complex community (Lundberg et al., 2012) with subtle and relatively constant microbial partner relationships, which can be established in all parts of the plants, from the roots to the apical region, to the inner parts of the plants, and capable of both adverse and beneficial effects (Jones et al., 2019). Since their earliest evolution, land-plants have been associated with a complex microbial community that included beneficial microorganisms. The presence of beneficial microorganisms helped early land plants respond to the environment and face challenges such as access to nutrients, new and/or stressful conditions, and pathogens (Backer et al., 2018; Smith et al., 2015).

In soil, there is a gradient of intimacy between plant roots and microorganisms, and the influence of the plant on the microbial community is greater near the root surface. A volume of soil specially influenced by plant roots or associated with the roots and plant-produced material is defined as rhizosphere (Bringhurst et al., 2001), a term originally coined by Hiltner (1904) to describe the microorganisms of soil around and inside the roots. Now, microorganisms living on the surface of roots are said to inhabit the rhizosphere and the rhizoplane, and those living inside the roots are called endophytes (Gray and Smith, 2005; Zhang et al., 2017).

Considering the different regions of the plant colonized by microbial communities (flowers, fruits, stems, leaves, and roots), the microbial community associated with the roots (rhizomicrobiome) is certainly the most populated and most sophisticated of all microbial communities associated with high plants (Backer et al., 2018). The rhizosphere effect is caused by the enormous influence exerted by the plant root exudates, a phenomenon called rhizodeposition, including a range of organic acids, amino acids, sugars, nucleosides, vitamins, and other small molecules that act as strong chemo-attractants of the soil microbiota (Marschner, 1995). The term

rhizodeposition also refers to the release into the rhizosphere of a specialized cell population called root cap boundary cells. This cell type is considered crucial for the effect of the rhizosphere, as it generally remains alive after desquamation from the root (Bulgarelli et al., 2013). Rhizodeposits account for approximately 11% of net carbon fixed by photosynthesis and 10-16% of total nitrogen in plants, but these values are very different depending on plant species and age (Jones et al., 2019). Net sequestration of organic carbon and nitrogen by roots stimulates the multiplication of soil microbials near root tissues because i) most known soil bacteria are organotrophic (they derive energy for growth from organic substrates), and ii) the accessibility and availability of organic compounds are limited in most soils.

The microbial population colonizing the rhizosphere includes bacteria, which are the most abundant, fungi, actinomycetes, protozoa, archaea and algae, and the composition is deeply different from that of its surrounding soil (Burdman et al., 2000; Kaymak, 2010). Regarding bacteria, Weller and Thomashow (1994) proved that the number of bacteria present in the rhizosphere is generally 10 to 100 times greater than in the bulk soil. Of course, all plant-microbe interactions can have mutualistic or detrimental effects on the host during their attempts to obtain nutrients and environmental protection, regardless of the physical interaction (Soto et al., 2011). For this reason, plants need a selection mechanism that allows the colonization of the rhizosphere to beneficial microorganisms and non-pathogenic.

The available literature suggests a two-step selection process that gradually distinguishes the root microbiota from the surrounding soil biomes and determines differences in the dimension and composition of the microbial community. In the first step, it is suggested that rhizodeposition and recognition of the host cell wall promote organotrophic bacteria growth, thus initiating a first community shift of the soil biome. In the second step, the microbial subpopulation goes through a second selection process based on a host genotype-dependent selection close and within the root, fine-tunes community profiles thriving on the rhizoplane and within plant roots (Bulgarelli et al., 2013; Santoyo et al., 2021). Similarly, the extent of differences between the

rhizosphere community and the soil biome may vary for soil types and soil types. A prediction of this model is the existence of a host genetic adaptations to different soil types, i.e., optimal plant growth depends on specific combinations of the host genotype–dependent and soil type–dependent bacterial start inoculum. In addition, microorganisms present mechanisms that allow them to recognize plant molecules, obtain nutrients, occupy space, and directly or indirectly inhibit other microorganisms in order to survive and colonize the rhizosphere (Glick, 2012).

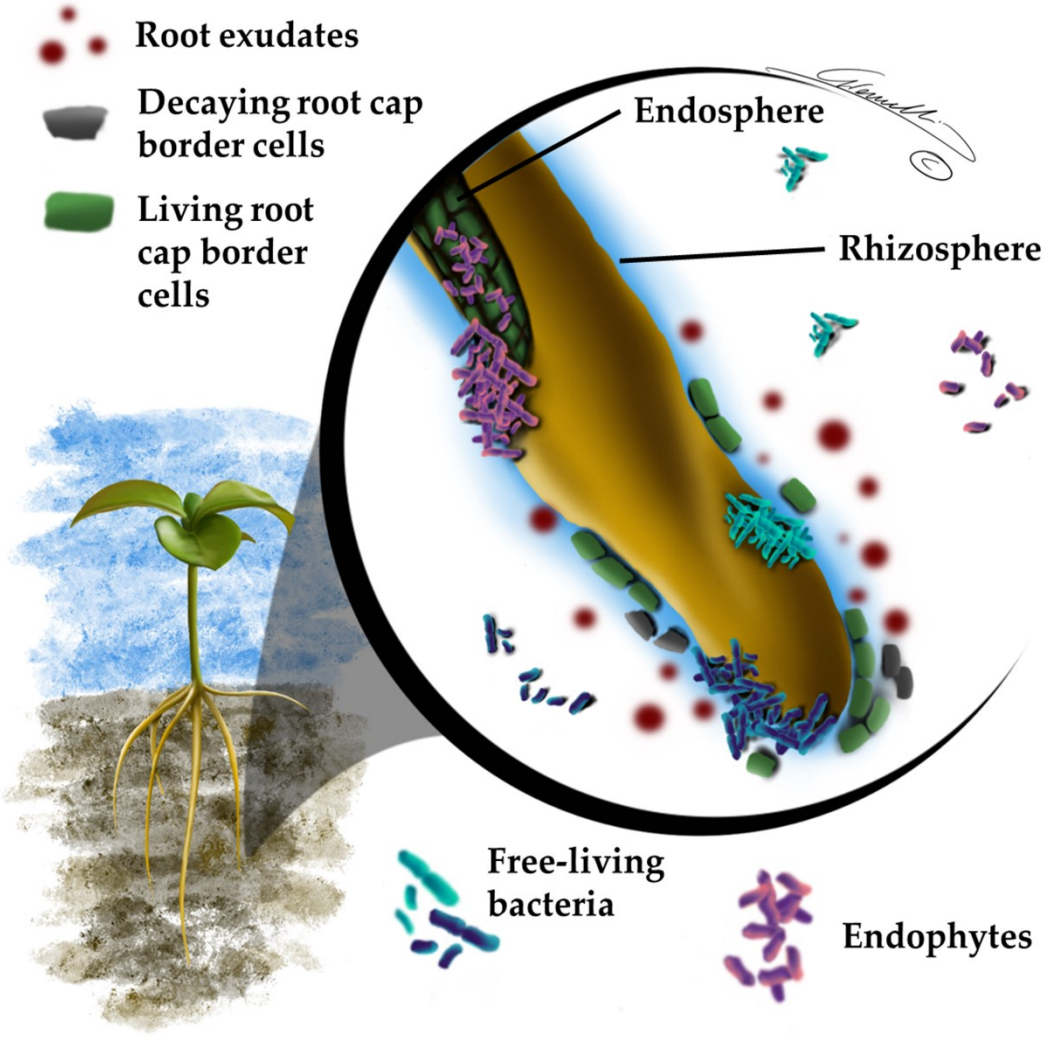

Figure 5. Representation of the main components of the rhizosphere and some processes.

3.2 Plant growth promoting rhizobacteria

In 1978 Kloepper and Schroth (1978) introduced the term "rhizobacteria" to indicate the soil bacterial community capable of competitively colonising plant roots and stimulating growth, thus reducing the incidence of plant disease. They were then renamed Plant Growth Promoting Rhizobacteria in 1981 (Kloepper and Schroth, 1981). PGPR represent about 2-5% of total rhizosphere bacteria (Antoun and Kloepper, 2001). According to Nakkeeran et al. (2005), an ideal PGPR should possess high rhizosphere competence, enhance plant growth capabilities, have a broad spectrum of action, be safe for the environment, be compatible with other rhizobacteria, and be tolerant to heat, UV radiation, and oxidizing agent.

Based on the interaction with plants and their degree of association, PGPR can be classified as intracellular PGPR (iPGPR or symbiotic bacteria), whereby they live inside the plants and exchange metabolites with them directly, and free-living rhizobacteria or extracellular PGPR (ePGPR), which live outside plant cells (Gray and Smith, 2005; Martinez-Viveros et al. 2010). iPGPR includes a wide range of soil bacterial genera that are usually located inside the specialized nodular structures of root cells such as *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium,* and *Rhizobium* of the family *Rhizobiaceae* (Wang and Martinez-Romero, 2000). The ePGPRs may exist in the rhizosphere, on the rhizoplane or in the spaces between the cells of root cortex and include genera such as *Agrobacterium*, *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Caulobacter*, *Chromobacterium*, *Erwinia*, *Flavobacterium*, *Micrococcous*, *Pseudomonas* and *Serratia* (Gray and Smith, 2005).

3.3 Traits of PGPRs involved in growth promotion

The beneficial action of PGPRs is exerted through a series of processes that result in three macro effects on the host plant: i) increased nutrient assimilation; ii) increased resistance to phytopathogen infections (viruses, fungi, bacteria, nematodes, insects, etc.) also referred to as biotic stresses; iii) increased tolerance to abiotic stresses

(heavy metal in soils, drought, nutrient deficiency, salinity, temperature). This leads to improved plant performance and, therefore, increased growth.

The mechanisms underlying these phenomena are diverse and include: i) N_2 supply for plant through biological nitrogen fixation; ii) production of siderophores; iii) solubilization and mineralization of nutrients; iv) synthesis of ACC (1-aminocyclopropane-1-carboxylate) deaminase; v) production and modulation of phytohormones (e.g. indole 3-acetic acid (IAA), cytokinins (CK), abscisic acid (ABA), gibberellic acid (GA)); vi) control of plant pathogens through various mechanisms such as secretion of enzymes able to hydrolase the fungal cell wall or competing for nutrients, induction of systemic resistance (ISR), production of siderophores and antibiotic; vii) bio-remediation of heavy metals contaminated soils; viii) improvement of abiotic stress resistance (Glick, 2004; Glick, 2010; Glick, 2014; Hayat et al., 2010; Choudhary et al., 2011; Kloepper et al., 1980).

3.1.1 Biological nitrogen fixation

Although nitrogen is the most common element in the atmosphere in nature, the most part of nitrogen is in gaseous form that is inaccessible to animals and plants, and it is abundantly present in the soil, but in insoluble forms that are not available to the plants. Among all nutrients for plant growth and development, nitrogen is the main limiting factor (Aerts and Chapin, 1999). Nucleotides, amino acids, and lipid membrane are all macromolecules constitute by nitrogen (Marschner, 1995).

Plants absorb nitrogen in the form of ammonium (NH_4^+) and nitrate (NO_3^-) and the conversion of atmospheric N_2 to ammonium is known as nitrogen fixation or diazotrophy. The ability to fix nitrogen is widespread among prokaryotes (Singh et al., 2019). According to an estimation, 83% of the biological nitrogen fixation comes from symbiotic association, while the rest part it is provided by free-living (diazotrops) or associative systems (Graham, 1988). Non-symbiotic nitrogen fixing rhizobacteria belong mainly to the *Azoarcus*, *Azotobacter*, *Acetobacter*, *Azospirillum*, *Burkholderia*, *Enterobacter*, *Gluconacetobacter*, and *Pseudomonas* genera (Bhattacharyya and Jha, 2012;

Vessey, 2003). The inoculation of biological N_2-fixing PGPR on crops and crop fields revitalizes growth promoting activity, disease management, and maintains the nitrogen level in agricultural soil (Damam et al., 2016).

3.1.2 Phosphate, potassium and zinc solubilization

After nitrogen, phosphorus is the most important element in plant nutrition. It is involved in almost all major metabolic processes, such as signal transduction, respiration, photosynthesis, macromolecular biosynthesis, and energy transfer (Khan et al., 2010). Phosphorus can be found in organic form (generally 30 to 50% of the total) mainly as phytate, and inorganic form which often forms insoluble compounds with aluminium, calcium, or manganese (Alori et al., 2017; Rodríguez and Fraga, 1999). Most agricultural soils contain a large amount of phosphorus that accumulates mainly due to fertilizations with inorganic phosphate, but unfortunately this is largely inaccessible to plants due to its rapid immobilization. In fact, the amount of soluble phosphorous in the soil is approximately 1 mg kg^{-1} of soil, insufficient to support the growth of the plant (Dey, 1988; Rodríguez and Fraga, 1999). Precipitation and fixation of phosphorus in soil is highly dependent on soil type and pH. For this reason, phosphorus is fixed by oxides and hydroxides of aluminium and iron in acid soils, while by calcium in alkaline soils (Goldstein, 1986; Goldstein, 1994).

The cell may take up several forms of P, but the largest part is absorbed in the forms of HPO_4^{2-} or $H_2PO_4^-$, or in the form of phytate after its enzymatic breakdown (Beever and Burns, 1980). From this perspective, phosphate solubilizing bacteria (rhizosphere colonizing bacteria and endophytic) play an important role in the liberation of organic phosphates or in the solubilization of insoluble inorganic phosphate (Oteino et al., 2015). There are many theories that explain the mechanisms behind this process, but the principal is the production of mineral dissolving organic acids, siderophores, protons, CO_2 and hydroxyl ions (Rodríguez and Fraga, 1999; Sharma et al., 2013). Gluconic acid is the most frequent agent of mineral phosphate

solubilization, acting by chelating the cation bound to phosphate, making it available to plants (Alori et al., 2017).

The other source of phosphorous is organic phosphorus. In addition to the soil phytate, phospholipids, nucleic acid, and other compounds are reported to be in the soil (Rodríguez and Fraga, 1999). In addition, many xenobiotic phosphonates containing phosphorus (detergents, antibiotics, and pesticides) are regularly released into the environment. Mineralisation of phosphorus refers to the solubilization of organic phosphorus and the degradation of the remaining portion of the molecule. The sink theory is an important theory proposed by Halvorson et al. (1990) for the solubilisation of organic P. This refers to the continuous removal of P, resulting in the dissolution of Ca-P compounds. Thus, the decomposition of P in organic substrates is consistent with the P content in the biomass of phosphate solubilizing microorganisms (PSM) (Dighton and Boddy, 1989). This biological process plays an important role in the phosphorus cycle. An enzyme produced by PSM in the organic P mineralization process is phytase. This enzyme releases phosphorus from the soil's organic materials that are stored in the form of phytate. Phytate degradation by phytase releases phosphorus in a form that is available to the plant (Alori et al., 2017).

The third essential and limiting plant nutrient, which plays an essential role for enzyme activity, photosynthesis, and protein synthesis, is K^+. As for phosphorous, the concentration of soluble potassium in the soil is very low, because 90% of potassium exists in the form of insoluble rock and silicate minerals (Parmar and Sindhu, 2013). It is generally believed that microorganisms contribute to the release of potassium from minerals by releasing H^+ or, as with phosphorus, by producing organic or inorganic acids and protons (acidolysis mechanism), or by chelating ions associated with potassium minerals (Etesami et al., 2017).

Zinc is one of the imperative micronutrients required relatively at a small concentration (5-100 mg kg^{-1}). Zinc is an element present in the enzyme system as a co-factor and metal activator of many enzymes (Parisi and Vallee, 1969), and its deficiency in plants determines reduced synthesis of carbohydrates, auxins, nucleotides and

affected the membrane integrity (Singh et al., 2005). The application of inorganic zinc to the soil satisfies only partially the plant need because 96-99% of applied zinc is converted into insoluble forms depending on the soil type and physiochemical reactions (Saravanan et al., 2004). Several rhizosphere bacteria exhibit potential influence on the availability of Zn for the plant. The Zn-solubilizing bacterial strains solubilize the unavailable form of zinc by producing chelating ligands, secreting organic acids, vitamins, and phytohormones and through oxido-reductive systems and proton extrusion (Saravanan et al., 2011).

3.1.3 Siderophore production

Iron is a key component of various metabolic pathways. More than 100 metabolic enzymes require iron as a cofactor and are essential for many plant processes, including photosynthesis, electron transport, oxidative phosphorylation, and hormone production (Timofeeva et al., 2022). Although it is the fourth most abundant element on earth, this huge amount of iron is not bioavailable for plants since free Fe(II) is rapidly oxidized to Fe(III), which is not assimilable due to its low solubility (Ammari and Mengel, 2006). Siderophores are low-molecular-weight compounds (500–1500 Da) that exhibit, without exception, a higher affinity for Fe (III) ($Kf > 10^{30}$) than for Fe (II). In addition, their affinity for Fe(III) is much higher than that of other bivalent or trivalent metals. The affinity of siderophores for Fe is so high that it removes Fe from the molecules of Fe-binding proteins, for example, ferritin, transferrin, and lactoferrin (Li et al., 2016; Ratledge and Dover, 2000).

Thus, the main function of siderophores is to convert Fe bound to proteins or water-soluble compounds into a form accessible to microorganisms (Dertz et al., 2006), but siderophores produced by bacteria can also be beneficial to plants. In fact, siderophores supply Fe to plants and promote their growth (Scavino and Pedraza, 2013); furthermore, bacterial siderophores are responsible for limiting the development of some phytopathogenic fungal and bacterial species (Matthijs et al., 2007). The siderophore molecule usually has its iron atom coordinating with oxygen

atoms, with the most common geometry being octahedral, allowing the six ligands to be arranged around the Fe centre with minimal ligand repulsion. The octahedral field contributes to the formation of high-spin Fe(III) particles that are thermodynamically stable. Depending on their chemical nature, siderophores are classified into catecholates and phenolates, hydroxamates, carboxylates, and mixed-type siderophores (Butler and Theisen, 2010; Hider and Kong, 2010). There are more than 500 biomolecules classified as siderophores (Dimkpa, 2016).

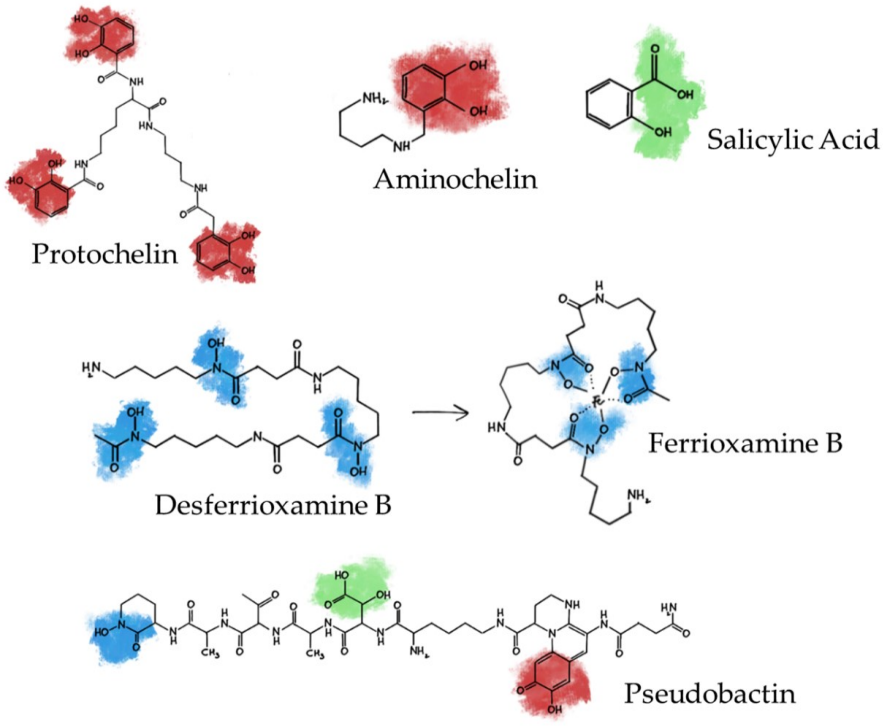

Figure 6. Example of siderophores*. Hydroxamate groups are shown in blue, catecholate groups in red, carboxylate groups in green.*

In bacteria, Fe ions are released from the Fe–siderophore complexes and become available for metabolic processes. To date, two main mechanisms for the release of Fe have been described. In the first, siderophore-bound Fe(III) is reduced to Fe(II), followed by the spontaneous release or competitive binding of reduced ions. (Hider and Kong, 2010). Two families of proteins associated with this process, the siderophore-interacting protein (SIP) and Fe–siderophore reductase (FSR), have been

identified. The second pathway of iron release uses specialized enzymes that hydrolyse and destabilize the Fe–siderophore complex (Miethke and Marahiel, 2007). Examples of such enzymes are esterases from the α/β-hydrolase family. (Larsen et al., 2006). Due to the destruction of siderophore backbone, the hydrolytic release of Fe is more expensive than the reduction of the Fe siderophore, mostly allowing the siderophore to be reused. After the release of Fe in bacterial cell, it can bind to spare proteins such as ferritins, bacterioferritins, and ferrochelatin (Miethke and Marahiel, 2007).

Siderophores are known to provide plants with iron and promote their growth. The exact mechanisms behind this process have not been established, but two theoretical mechanisms have been described, even if they have not been experimentally confirmed. In the first mechanism, bacterial siderophores (Fe(III)-siderophore complex) with a high redox potential are first transported to the plant apoplast and then reduced to give back Fe(II) to the plant transport system; in the second mechanism, the bacterial siderophores chelate Fe from the soil and perform ligand exchange with phyto-siderophores (Ahmed and Holmström, 2014).

In addition to being involved in the increase in nutrients, siderophores play an important role in the biological control of some phytopathogens. Since siderophores firmly bind iron and reduce its bioavailability to plant pathogens, they can facilitate the destruction of phytopathogens (Ahmed and Holmström, 2014; Beneduzi et al., 2012). Siderophores are also involved in the detoxification mechanisms of metals present in the soil, binding other metal species that are different from iron. The binding of metals to siderophores in the extracellular medium decreases the concentration of free metals, thus probably reducing diffusion and therefore their toxicity, since the molecular weight of the formed siderophore–metal complex is too large for its diffusion through porins.

Siderophores can modify the degree of oxidation of heavy metals such as Cd, Cu, Ni, Pb, Zn, Th, U, and Pu, making them less toxic (Schalk et al., 2011). Siderophores also bind to various toxic Cr, Cu, Pb, Cu, V, and Al (Baysse et al., 2000; Braud et al.,

2009). Thus, the ability of siderophore to detoxify and bind to toxic heavy metals plays a prominent role in plant growth in soil contaminated with heavy metals.

3.1.4 Plant growth regulators production and modulation

Plant hormones, also referred to as phytohormones, are organic substances that act as chemical messengers that affect plant growth, differentiation, development, and influencing the plant's response to the environment, prominently affect metabolic activity of plants for their normal functioning and are involved in the stimulation of defence as well as abiotic stress management. Phytohormones are effective at very low concentrations and are mainly synthesized in certain parts of the plant and transported to other locations (Waadt et al., 2015; Wani et al., 2016). In alleviation of biotic and abiotic stresses, PGPR can produce phytohormones themselves or can induce or alter their production in the plant; PGPR produce mainly indole-3-acetic acid (IAA), cytokinins (CK), gibberellins (GB) (Abd_Allah et al., 2018; Egamberdieva et al., 2017). In addition, they can produce inhibitors of ethylene production and modulate the concentration of abscisic acid (ABA). Since hormones stimulate or inhibit plant growth, they are also called plant growth regulators (Porcel et al., 2014).

Among all the different molecules that make up the auxin family, IAA is certainly the best known. Exogenous IAA is considered the most physiologically active phytohormone synthesized by PGPR, which directly and indirectly controls a wide range of processes in plant development and growth (Bhardwaj et al., 2014). Depending on the concentration, IAA acts differently. At low concentrations IAA can stimulate the elongation of the primary root, while high amounts of IAA decrease primary root length, increase root hair formation, and stimulate the formation of lateral roots. By increasing both the surface area and the length of the roots, IAA allows plants to have greater access to soil nutrients (Spaepen et al., 2007). In addition to directly regulating plant growth and development, exogenous IAA enhance the level of protection against external adverse conditions by enhancing the coordination of various cellular defence systems. Furthermore, by increasing the absorption of

nutrients and water, IAA alleviates the effects of some abiotic stresses such as salinity and drought (Etesami and Beattie, 2018).

Gibberellins (GA) promote the processes of seed germination and emergence, floral induction, development of flower and fruit, root elongation, lateral root extension and shoot elongation (Castro-Camba et al., 2022). CK stimulate plant's cell division, vascular cambium sensitivity, and vascular differentiation, induce the proliferation of root hairs, inhibit lateral root formation and primary root elongation (Aloni et al., 2006).

Ethylene is a key gaseous phytohormone with a wide range of biological activities that can affect plant growth and development. It plays an important role in root initiation, inhibits root elongation, promotes fruit ripening, promotes lower wilting, stimulates seed germination, promotes leaf abscission, and activates the synthesis of other plant hormones. It is known as a senescence hormone and growth inhibitor, but when present at low concentration, it can promote growth in several plants (Pierik et al., 2006). However, at high concentrations, it induces defoliation and other cellular processes that may lead to inhibition of root and stem growth and premature senescence, reducing crop performance (Bhattacharyya and Jha, 2012; Li et al., 2005). Under normal conditions, ethylene is produced starting from 1-aminocyclopropane-1-carboxylate (ACC) through the action of the enzyme ACC synthetase. As a response to exposure to various environmental stresses such as drought, salt stress, cold, infections, heavy metals, and flooding, plants increase the production of ACC, resulting in an increase in ethylene concentration (Glick, 2012). The production of the enzyme ACC deaminase is one of the most important physiological traits of PGPR that facilitates plant growth (Etesami et al., 2015; Glick, 2004). Indeed, under stressing conditions, when the level of ethylene in the plant might reach inhibitory levels, this enzyme supports plant growth degrading ACC (Gamalero and Glick, 2015; Glick, 2014; Glick et al., 2007).

ABA is involved in tuning responses against several abiotic stresses and have remarkable impact on the plant defence against different phytopathogens (Alazem

and Lin, 2017). Several strains of PGPR have been reported to be ABA producers (Karadeniz et al., 2006) helping plants to counteract mainly abiotic stresses, but also indirectly PGPR can modulate ABA production.

3.1.5 Biofilm formation

Biofilms are extracellular matrices composed of proteins, nucleic acids, lipids, exopolysaccharides, and microorganisms embedded in them, which allow rhizosphere bacteria to adhere to the surface of the plant roots (Danhorn and Fuqua, 2007). The biofilms produced by PGPR also protect plants from stress conditions such as drought and salinity, as their components can coordinately function as osmoprotectors (Rojas-Solis et al., 2020).

3.1.6 Modulation of plant antioxidant defence systems

In plants, reactive oxygen species are produced as bioproducts of different metabolic pathways localized in various cellular compartments such as chloroplasts, mitochondria, and peroxisomes (Foyer and Harbinson, 1994). Under physiological conditions, these molecules are scavenged by the antioxidative defence system of the plant. Many adverse environmental factors, such as biotic stress, abiotic stress, excess or deficit in physical and chemical environment, can alter the equilibrium between ROS production and scavenging activity (Apel and Hirt, 2004). In this context, intracellular ROS levels increase, a phenomenon known as "oxidative burst" (Apostol and Heinstein, 1989). Increasing evidence suggests an active role for PGPR in modulating the antioxidant defence system of plants, increasing the activity of various antioxidant enzymes under stress conditions (Damodaran et al., 2014; Etesami and Beattie, 2017; Kim et al., 2014). In other studies, plants inoculated with PGPR under stress conditions showed reduced levels of enzymatic antioxidants, suggesting that these plants have been subjected to a less stress compared to uninoculated plants (Armada et al., 2014; Kang et al., 2014; Vardharajula et al., 2011).

3.1.7 Antimicrobial compounds

The rhizosphere represents a highly competitive environment in which microorganisms are in constant competition for nutrients and spaces. For this reason, they have developed different strategies to compete, which include the synthesis of various antimicrobial compounds, allowing their establishment in a specific niche (Santoyo et al., 2012).

Based on their effect, antimicrobial compounds have been classified as bactericidal or bacteriostatic, denoting compounds with a lethal effect on cells or capable of temporarily inhibiting microorganism development, respectively (Fira et al., 2018). Indeed, many PGPR species have evolved various mechanisms to reduce the competition excreting antibiotics, lytic enzymes, siderophores or weak organic acid to their environment (Tariq et al., 2017). Some plants are known to recruit microorganisms to deal with infection when attacked by phytopathogenic fungi (Berendsen et al., 2012). The production of antibiotics is considered to be the most effective antagonistic activity for the suppression of phytopathogens. In the rhizosphere, the production of antibiotics by PGPR give them an important competitive advantage by stopping or eliminating the growth of bacterial and fungal phytopathogens. Therefore, the production of these compounds is strongly associated with the ability to colonize the rhizosphere (Romero et al., 2007; Stokes et al., 2019). Antibiotics produced by PGPR include 2, 4-diacetylphloroglucinol (2, 4-DAPG), butyrolactones, xanthobaccin phenazine-1-carboxylic acid, kanosamine, zwittermycin A, viscosinamide, oligomycin A, pyrrolnitrin (Martinez-Viveros et al., 2010). Bacteriocins are proteinaceous or peptide toxins secreted by bacteria living in a competitive environment. Bacteriocins are very effective in reducing or inhibiting the growth of phytopathogens. However, compared to antibiotics, they have a narrow killing spectrum and have a damaging effect on bacteria closely related to bacteriocin producing bacteria (Riley and Wertz, 2002). Other important antimicrobial compounds are represented by lytic enzymes. The cell wall of fungi is composed of chitin, cellulase, glucane, etc., so they can be targeted by some lytic enzymes produced

by some PGPR. Lytic enzymes include 1,3-glucanases, lipases, cellulases, protease, and chitinases (Bhagwat et al., 2019). Moreover, these enzymes also decompose non-living organic matter and plant residues to obtain carbon nutrition. Production of hydrogen cyanide (HCN) is another important PGP activity, commonly used as a biocontrol agent based on toxicity against phytopathogens, but also useful in chelating metals and indirectly involved in phosphate solubilization (Rijavec and Lapanje, 2016).

As described previously, because of their role as iron chelators, siderophores can also inhibit pathogen growth.

3.1.8 Induced systemic resistance

PGPR can induce in plants induced systemic resistance (ISR). ISR can be defined as a state of improved defensive capacity mainly involved in the response to pathogenic invasion but can helps plant also to control diverse disease (Kamal et al., 2014). It is characterized by the activation of a huge number of defence enzymes such as chitinase, lipoxygenase, SOD, CAT, APX, β-1, 3- glucanase, peroxidase. A huge of individual bacterial components induce ISR, such as lipopolysaccharides, cyclic lipopeptides, siderophores, 2, 4- diacetylphloroglucinol, homoserine lactones, and volatiles like 2, 3- butanediol and acetoin (Berendsen et al., 2015).

3.1.9 Production of VOCs

Volatile organic compounds (VOCs) are produced by a wide range of PGPR that directly or indirectly mediate resistance to disease, tolerance to abiotic stress, and plant biomass, as well as inhibit bacterial, fungal and nematodes phytopathogens and induce ISR (Raza et al., 2016a, b). VOCs include cyclohexane, 2-(benzyloxy)ethanamine, benzene, methyl, decane, 1-(N-phenylcarbamyl)-2-morpholinocyclohexene, dodecane, benzene(1-methylnonadecyl), 1-chlorooctadecane, tetradecane, 2,6,10-trimethyl, dotriacontane and 11-decyldocosane (Kanchiswamy et al., 2015).

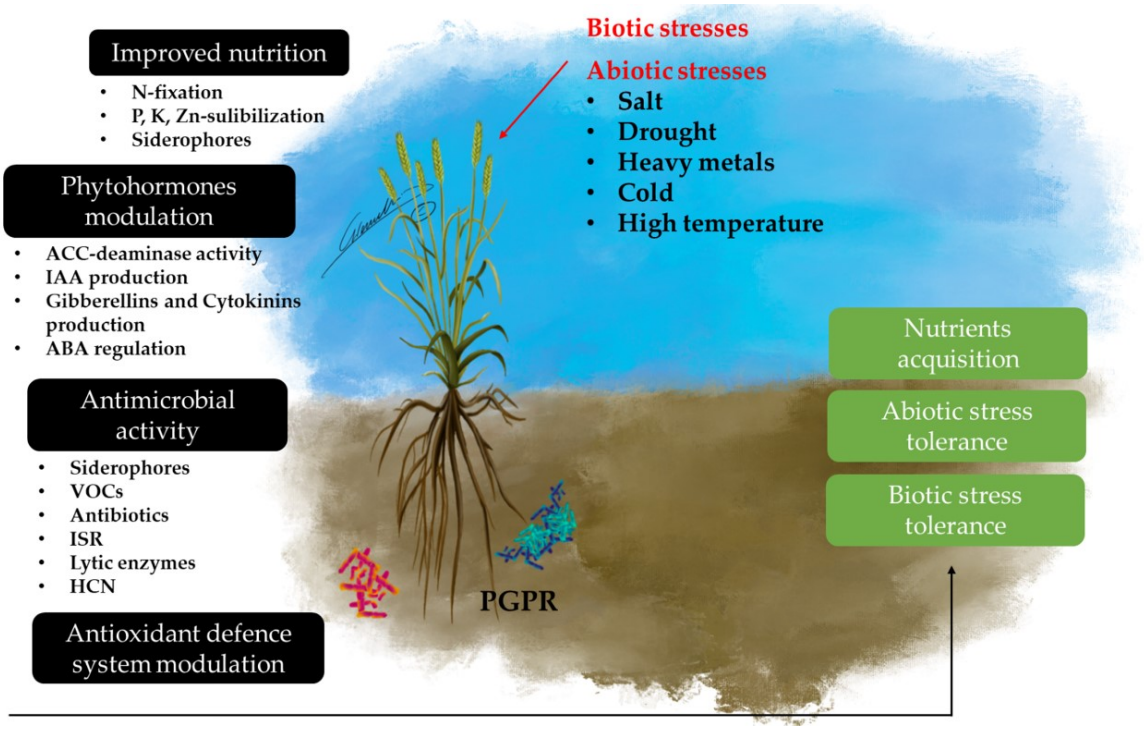

Figure 7. Overview of the main mechanisms of PGPR for reducing biotic and abiotic stress.

4. PGPR in sustainable agriculture

Green revolution has greatly improved plant productivity and yields by introducing new high yield seeds and increasing the use of chemical fertilizers, pesticides, and other agrochemicals (Basu et al., 2021; Kesavan and Swaminathan,

2018). Since then, the global agricultural situation has changed drastically due to the excessive use of synthetic compounds to improve crop yields, resulting in a progressive degradation of the biological and physiochemical health of arable land and a decline in agricultural productivity worldwide in recent decades (Pingali, 2012; Yang and Fang, 2015). For the reasons described in Chapter 1 (Section – Introduction), it is clear that soon the main goal of the world's agricultural system will be to increase agricultural production in a dimension that will prove increasingly complex. Therefore, current and future dimensions, require a rethinking of some methods to decrease the impacts of agriculture on the environment and the development of sustainable technologies (Etesami and Maheshwari, 2018). Unfortunately, there is no simple solution to the complex ecological, socio-economic and technical problems in promoting sustainable agriculture (Kesavan and Swaminathan, 2018). To achieve sustainable environmentally friendly agriculture, the use of beneficial microorganisms is a promising approach with very low environmental impacts.

But why are PGPR considered so interesting in terms of sustainable agriculture and as a response to future agricultural challenges? The answer lies in their enormous potential and wide range of applications. PGPR activities are often classified as growth promotion, biocontrol activities, and induction of tolerance to abiotic stresses. The same classification applies to products for the agricultural market. In fact, they are often referred to as biofertilizers or biopesticides. However, these classifications are very often limited in terms of PGPR capabilities, since they have more characteristics that make them able to stimulate plant growth by acting on multiple levels and not only, for example, by increasing the uptake of nutrients or fighting some plant pathogens. Under the circumstances described above, applying appropriate microbial practices, and using the positive interaction of plant roots microorganisms to improve both crop productivity and soil health could be very important (Lugtenberg et al., 2002).

The use of microorganisms can alleviate stresses in various crops, regardless of whether they are due to nutritional deficiencies, biotic or abiotic factors, and is a

promising strategy for sustainable agriculture. (Shrivastava and Kumar, 2015). For example, the most known, studied, and exploited beneficial plant-bacteria relationship is represented by the N-fixing symbiosis between rhizobia and legumes, which is also the early example of commercial microbial product in agriculture and still represents the most widely used agricultural inoculants (Bashan, 1998). This is a very strong relationship between iPGPR and plants, where legumes provide reduced C and a favourable environment for rhizobia (protected, anaerobic for nitrogenase activity), while rhizobia provide legumes with biologically available nitrogen (Backer et al., 2018; Oke and Long, 1999). With this symbiosis, both rhizobia and legumes undergo significant changes. Although these relationships are very effective, the close interaction between rhizobia and legumes, determined by strict mutual recognition, also represents a limit to their applicability. For this reason, the use of free-living bacteria (ePGPR) could represent a better solution with a view to broad applications in different environments and plant species.

Understanding the principles of communication between microorganisms and plant and microorganisms may lead to generate beneficial microbial communities in agricultural soils. It is important to consider whether such microbial communities are feasible and stable enough to function under agricultural conditions (Timmusk et al., 2017).

Table 2 shows some examples of the practical application of PGPR in different crops, under different conditions, and the most relevant effects induced on plants.

Table 2. *Overview of different PGPR tested in different crops (edited from Kumar et al., 2021; Ahemad et al., 2014)*

PGPR	Crop	Effect on crops	References
B. pumilus SE34	*S. lycopersicum*	ISR during infection	Zafar-Ul-Hye et al., 2020
J. huakuii NBRI 13E	*S. lycopersicum*, *A. esculentus*, *Zea mays*	Increased yield and ameliorating salt stress	Misra et al., 2019

B. megaterium var. *phosphaticum*	*S. oleracea*	Ensured efficient absorption of P, water, and other microelements to alleviate water stress and resist fungal disease	Khalid et al., 2017
B. cereus	*S. lycopersicum*	Biotic stress resistance against speck disease caused by *Pseudomonas syringae* pv	Niu et al., 2012
P. fluorescens	*Arachis hypogea*	Produced ACC deaminase to confer resilience to salt stress	Saravanakumar and Samiyappan 2006
A. brasiliense, *P. fluorescens*, *B. megaterium*	*Cucumis sativus*	Improved fruit quality	Salim et al., 2021
S. maltophilia, *A. fabrum*	*Momordica charantia*	Immobilized Cd in Cd-rich soil to improve growth	Zafar-Ul-Hye et al., 2020
P. putida, *Azosprillum*, *Azotobacter*	*Cynara scolymus*	Significant increase in radicle and shoot length, shoot weight, germination, decrease in time of germination	Jahanian et al., 2012
Bradyrhizobium sp. 750, *Pseudomonas* sp., *O. cytisi*	*Lupinus luteus* (FIELD CONDITION)	Increased both biomass, nitrogen content, accumulation of metals	Dary et al., 2010
P. putida strain R-168, *P.fluorescens* strain R-93, *P. fluorescens* DSM 50090, *P. putida* DSM291, *A. lipoferum* DSM 1691, *A. Brasiliense* DSM 1690	*Zea mays* L. (FIELD CONDITION)	Plant height, seed weight, number of seed per ear and leaf area, shoot dry weight increased	Gholami et al., 2009
P. aeruginosa, *P. fluorescens*, *R. metallidurans*	*Zea mays*	Promoted growth, facilitated soil metal mobilization, enhanced Cr and Pb uptake	Braud et al., 2009
Brevibacillus	*Trifolium repens*	Enhanced plant growth and nutrition and decreased zinc concentration in plant tissues	Vivas et al., 2006
P. jessenii PS06, *M. ciceri* C-2/2	*Cicer arietinum* (FIELD CONDITION)	Co-inoculation treatment increased the seed yield, and nodule fresh weight	Valverde et al., 2006
A. brasilense, *G. diazotrophicus*, *H. seropedicae*, *B. ambifaria*	*Allium cepa* L. /FIELD CONDITION)	Co-inoculation increased plant height, total chlorophylls, crop yields, bulb dry matter. Increased phenolic content and antioxidant activities. Increased organic carbon, organic matter, available phosphorous, nutrients concentrations.	Pellegrini et al., 2021

4.1 PGPR in salt stress tolerance induction

As already described in Chapter 2.2 (Section – Introduction), salt stress has a wide range of effects on plants. It could be argued that when plants grow under salinity conditions, almost all normal physiological processes are compromised. For this reason, the biological responses enacted by plants to reduce the damage caused by salt are never unique or specific, but broad and complex, involving many different pathways.

PGPR exhibit beneficial traits that allow them to mitigate the toxic effects caused by high salt concentrations. PGPR application can improve the conditions of plants grown in a salt environment in two main ways: i. PGPR can activate or modulate the response systems of plants after exposure to salt; ii. synthesize anti-stress molecules (Fouda et al., 2019). The mechanisms to improve the growth and resistance of plants exposed to salinity are different and include: i) improvement of nutrients uptake (e.g., N_2 fixation, release of bound P and K^+ from the soil, chelating iron) and maintaining water balance; ii) influence on ions homeostasis; iii) inducing the selective absorption of K^+ and excluding Na^+ to maintain a high K^+/Na^+ ratio; iv) biofilm formation to reduce toxicity of Na^+; v) changing roots architecture; vi) modulation of the antioxidant system; vii) modulation of osmotic substances; viii) modulation of plant hormonal levels; ix) modulating the expression of salt-responsive genes (Bhat et al., 2020). PGPR can positively influence the response of the plant. Although PGPRs are classified according to their mechanisms for discrete quality, in the context of salt stress, analysing how they influence the response of the plant, we realize that their promoting activity is never due to a single mechanism. In **Table 3** are reported different works demonstrating the positive roles of different PGPR species in alleviating the symptoms of salt stress in different species of plants.

***Table 3**. Overview of different PGPR activities in plants under salt stress conditions*

PGPR	Plant	Beneficial effects	References
A. johnsonii SUA-14		Increasing in activity of urease, acid and alkaline phosphatase and dehydrogenase. Enzymatic activity positive correlate with P and N_2 content in straw and grains. Reduction in catalase (CAT) and superoxide dismutase (SOD) activity and malondialdehyde (MDA) content.	Shabaan et al., 2022
Enterobacter cloacae PM23		Enhancement in plant growth, biomass, and photosynthetic pigments. Up-regulation of ascorbate peroxidase (APX) and SOD. Enhancement in radical scavenging capacity, relative water content, soluble sugars, proteins, total phenolic and flavonoid content. Elevated levels of antioxidant enzymes and osmoprotectants (free amino acids, glycine betaine, and proline). Reduction in oxidative stress markers.	Ali et al., 2022
B. safensis NBRI 12 M, *B. subtilis* NBRI 28B, *B. subtilis* NBRI 33 N	*Zea mays*	Mitigation of the adverse effects of ethylene by modulating 1-aminocyclopropane-1-carboxylic acid (ACC) accumulation, ACC-oxidase (ACO), and ACC-synthase (ACS) activities. *Bacillus* sp. inoculation induced plant response for defence enzymes, chlorophyll, proline and soluble sugar under salt stress.	Misra et al., 2020
S. liquefaciens KM4		Reduction in oxidative stress markers. Increasing in maize growth and biomass production, better leaf gas exchange, osmoregulation, antioxidant defence systems, and nutrient uptake. All these improvements were accompanied with the upregulation of stress-related genes (*APX*, *CAT*, *SOD*, Rubisco small subunit (*RBCS*), Rubisco large subunit (*RBCL*), *H+-PPase*, *HKT1*, and *NHX1*), and downregulation of the key gene in ABA biosynthesis (*NCED*).	El-Esawi et al., 2018 a
B. atrophaeus WZYH01 *P. soli* WZYH02		Improved maize growth performance, biomass yield, and antioxidant levels. Protection from salt stress by regulating plant hormones IAA and abscisic acid (ABA) levels and increasing nutrient acquisition. Increasing in the content of K^+ accompanied by an effective decrease in Na^+ in tissues. Increasing in the the transcription levels of salt tolerance genes (*ZmNHX1*, *ZmNHX2*, *ZmHKT*, *ZmWRKY58*, and *ZmDREB2A*).	Hou et al., 2022
P. oryzihabitans AXSa06	*Solanum lycopersicum*	Differences in transcript levels and metabolites following inoculation. Positive effects on plant growth and photosynthetic parameters, inducing plants to a primed state, that allow them to respond more efficiently to salt stress probably by activating antioxidant metabolism, by dampening stress signals, by detoxifying Na^+ and effectively assimilating carbon and nitrogen. The primed state was supported by the increase in leaf lipid peroxidation, ascorbate content, and enhanced activities of antioxidant enzymes before stress treatment. The identified signatory molecules of AXSa06-mediated salt tolerance included the amino acids aspartate, threonine, serine, and glutamate, key genes associated with ethylene or abscisic acid homeostasis and perception, and ion antiporters.	Mellidou et al., 2021

Enterobacter 64S1, *Pseudomonas* 42P4		Increasing in root and shoot dry weight, stem diameter, plant height, and leaf area. Reduced electrolyte leakage (improved membrane stability) and lipid peroxidation and increased chlorophyll quantum efficiency (Fv/Fm) and the performance index. Increased accumulation of proline and antioxidant non-enzymatic compounds, such as carotenes and total phenolic compounds. Reduction in the activity of CAT and POD after inoculation of *Enterobacter* 64S1.	Pérez - Rodriguez et al., 2022
Bacillus sp. wp-6	*Triticum aestivum*	Increasing in plant biomass (57%) and root length (25%). Reduction in the Na^+ content, while the K^+ content and K^+/Na^+ ratio were increased. Decreased levels of MDA by 31.94%, and increased content of proline (7.48%), soluble sugar (12.34%), and soluble protein (4.12%). Increasing in the activity of POD, CAT and SOD. Interaction analysis of differentially expressed proteins and metabolites found that energy production and transformation-related proteins and six metabolites (D-arginine, palmitoleic acid, chlorophyllide b, rutin, pheophorbide a, and vanillylamine) were mainly involved in the growth of wheat seedlings after the inoculation with wp-6.	Zhao et al., 2022
A. protophormiae, SA3, *D.natronolimnaea* STR1		Enhanced photosynthetic efficiency. increase indole-3-acetic acid (IAA) content. SA3 counteracted the increase of abscisic acid (ABA) and 1-aminocyclopropane-1-carboxylate (ACC). Enhanced levels of the *TaCTR1* gene and *TaDREB2*. Modulating expression of a regulatory component (CTR1) of ethylene signalling pathway, and DREB2 transcription factor.	Barnawal et al., 2017
A.woluwensis AK1, *M. oxydans* AK2, *A. aurescens*AK3, *B. megaterium* AK4, *B. aryabhattai* AK5	*Glycine max*	Elevated SOD and GSH level and absorption of K^+, reduced Na^+ ion concentration. A reduction in the ABA level and an increase in plant growth and chlorophyll content. The salt-tolerant gene *GmST1* was highly expressed with the highest expression of 42.85% in AK1-treated plants, whereas the lowest expression observed was 13.46% in AK5-treated plants. Similarly, the expression of the IAA regulating gene *GmLAX3* was highly depleted in salt-stressed plants by 38.92%, which was upregulated from 11.26% to 43.13% upon inoculation with the microorganism.	Khan et al., 2019
ALT29, ALT43		Increasing in the shoot length (13%), root length (21%), shoot fresh and dry weight (44 and 35%), root fresh and dry weight (9%), chlorophyll content (16–24%), Chl a (8–43%), Chl b (13–46%), and carotenoid (14–39%). Decreased endogenous ABA levels (0.77-fold) and increased endogenous SA contents (6–16%), increased total protein (10–20%) and glutathione contents, and reduced lipid peroxidation (0.8–5-fold), superoxide anion (21–68%), peroxidase (12.14–17.97%), and polyphenol oxidase (11.76–27.06%). Higher K^+ uptake (9.34–67.03%) and reduced Na^+ content (2–4.5-fold). Down-regulation of *GmFLD19* and *GmNARK* genes.	Khan et al., 2021a

Bacillus firmus SW5		Increased growth and biomass yield, synthesis of chlorophyll, nutrient uptake, gas exchange parameters, osmolytes content, total phenolic and flavonoid contents, and antioxidant enzymes activities. H_2O_2 and MDA contents were reduced in salinity-stressed soybean plants inoculated. The antioxidant enzyme-encoding genes and stress-related genes exhibited the highest expression levels in soybean plants inoculated.	El-Esawi et al., 2018 b
Pseudomonas sp., *Bacillus sp.*, *Mucilaginibacter sp.*, *Rhizobium sp.*		Increased *A. thaliana* growth under both normal and high salinity conditions. The activity of APX, CAT and POD, proline content and total antioxidative capacity also differed in the inoculated *A. thaliana* plants.	Fan et al., 2020
ZS-3	*Arabidopsis thaliana*	Soluble sugar content increased by 288%. Proline content reduced by 41.43%. Reduction in the accumulation of Na^+ and increased K+/Na+ ratio. ZS-3 isolated Na^+ in vesicles by upregulating *NHX1* and *AVP1* expression while limiting Na^+ uptake by downregulating *HKT1*. Higher levels of POD and CAT activity and reduced glutathione. In addition, it was revealed that ZS-3 activates salicylic acid (*NPR1* and *PR1*) and jasmonic acid/ethylene (*AOS*, *LOX2*, *PDF1.2*, and *ERF1*) signaling pathways to induce systemic tolerance.	Shi et al., 2022
P. knackmussii MLR6		Improved stomatal conductance, transpiration rate, total chlorophyll and carotenoid contents. Increased fresh/dry weight and height. Positive effect on cell membrane stability by reducing the electrolyte leakage and priming the ROS accumulation after the salt exposition. Additionally, the expression of *NHX1*, *HKT1*, *SOS2* and *SOS3* as well as *SAG13* and *PR1* was maintained in MLR6-bacterized plant at a similar level of controls.	Rabhi et al., 2018
B. subtilis, *P. mandelii*	*Triticum durum*	The inoculation of both bacteria resulted in a faster appearance of Casparian bands in the root endodermis and an increased growth of plants exposed to salt. *B. subtilis* prevented a decrease in both K^+ and P concentrations and increased concentration of cytokinins in salt-stressed plants. *P. mandelii* decreased the level of Na^+ accumulation and increased the concentration of auxin. Changes in the concentration of hormone in plants.	Martynenko et al., 2022
K. radicincitans KR-17	*Raphanus sativus L.*	Increasing in germination efficiency, dry biomass, and leaf pigments. Improved plant mineral nutrients (Na^+, K^+, Ca^{2+}, Mg^{2+}, Zn, Fe, Cu, P, and N). Enhanced protein, carbohydrates, root pigments, amino acids (AsA and Lys), lipids, and root alkaloids. Membrane damage, RLWC, stressor metabolites, and antioxidant defence enzymes were dramatically reduced.	Shahid et al., 2022
Pseudomonas sp. M30-35	*Chenopodium quinoa*	Increased biomass production and root activity. Higher content of chlorophyll photosynthetic pigment and b. The stability of P content was also maintained. The content of saponin, an important secondary metabolite in *C. quinoa*, was increased.	Cai et al., 2021
P. putida R4, *P. chlororaphis* R5	*Gossypium hirsutum*	Improved seed germination and seedling growth. Shoot and root growth and dry matter increased, and disease incidence was reduced by bacterial inoculants in natural saline soil.	Egamberdieva et al., 2015

B. fortis SSB21	*Capsicum annum L.*	Highest increase in shoot length, root length, and fresh and dry biomass production of capsicum plants grown under saline conditions. Increased biosynthesis of proline and up-regulation in the expression profiles of stress related genes including *CAPIP2*, *CaKR1*, *CaOSM1*, and *CAChi2*. Reduced level of ethylene, lipid peroxidation, and reactive oxygen species (ROS).	Yasin et al., 2018
Klebsiella sp. IG 3	*Avena sativa*	Higher shoot length, root length, shoot dry weight, root dry weight and relative water content (RWC). Decreased proline, electrolyte leakage and malondialdehyde (MDA content, and activities of antioxidant enzymes lesser. Positively modulated the expression profile of *RBCL* and *WRKY1* genes.	Sapre et al., 2018
Siccibacter sp. C2	*Hordeum vulgare*	Promoted growth of barley, increasing biomass, root length, and chlorophyll contents. Positive effect on the photosynthetic efficiency, concomitantly with lower intercellular CO_2 contents. Increased accumulation of proline and soluble sugars, alleviation in the oxidative stress correlated with H_2O_2 and MDA contents. Positive effect corroborates with a significant activation in the expression of a subset of barley stress responsive genes, including *HVA1*, *HvDREB1*, *HvWRKY38* and *HvP5CS*.	Sayahi et al., 2022
Enterobacter sp. S16-3, *Pseudomonas sp.* C16-2O	*Brassica napus*	Increasing in leaf area, root length, shoot length, chlorophyll fluorescence indexes and RWC. Decreasing in electrolyte leakage index, MDA and H_2O_2. Antioxidant capacity, proline, and antioxidant enzymes were increased. The amount of K^+ as an index of salinity tolerance significantly increased, and leaf Na^+ content was significantly decreased.	Neshat et al., 2022
P. putida KT2440, *Novosphingobium* sp. HR1a	*Citrus macrophylla*	Both rhizobacteria reduced salt stress-induced damage. Levels of abscisic acid (ABA) and salicylic acid (SA) were lower. Maximum efficiency of under stress conditions maximum efficiency of photosystem II (Fv/Fm) in inoculated plants decreased to a lower extent than in non-inoculated ones. *Novosphingobium* sp. HR1a also induced leaf accumulation of 3-indole acetic acid (IAA) and a delay in the decrease of quantum yield (ΦPSII). *P. putida* KT2440 inhibited root chloride and proline accumulation in response to salt stress.	Vives-Peris et al., 2018
P. endophytica SK1	*Trigonella foenum-graecum*	Increased biomass and metabolites content. The nitrogen and phosphorus content were increased under salt (100 mM NaCl) stress as compared to control plants. The production of H_2O_2 and lipid peroxidation as stress markers were high in control plants, while inoculation of SK1 a reduced these parameters. In addition, a significant effect was found on the phenolic compounds and trigonelline content in fenugreek plant inoculated with SK1 bacterium.	Sharma et al., 2022
S. maltophilia BJ01	*Arachis hypogaea*	Enhanced growth and protection of photosynthetic pigments. Lower electrolyte leakage (about 20%), lipid peroxidation (2.1 μmol g^{-1} Fw), proline (2.9 μg mg^{-1} Fw) content and H_2O_2 (55 μmol g^{-1} Fw) content were observed in plants, co-cultivated with PGPR compared to untreated plants under stress condition. The growth hormone auxin (0.4 mg g^{-1} Fw) and total amino acid content (0.3 mg g^{-1} Fw) were enhanced in plants co-cultivated with PGPR under stress conditions.	Alexander et al., 2020

In all the cases shown in **Table 3**, the bacterial inoculum results in physiological, biochemical, and molecular changes that ultimately lead to improved plant performance under salt stress.

4.2 Global market, challenges and constrain to application of PGPR-based products

The first approach to the development of commercial products based on microorganisms dates back more than 100 years ago. The first commercial biopesticide is *Bacillus thurigensis*, which was discovered as an insect pathogen in the early 1900s (Bashan, 1998). In 1938, the first commercial production of *B. thurigensis*-based biopesticides was launched under the name of Sporeine, but the industrial development of these products began in the 1960s-1970s.

The progress and positive agronomical effects of the application of microbial-based products stimulated the research on the isolation and selection of the best PGPR capabilities, and the world market was opened for new types of PGPR-based fertilizers, which have been commercially available in many countries since the 1950s. The first use of rhizobacteria in soil and plant parts to eradicate phytopathogens was in the Soviet Union in 1958 (Suslow et al., 1979). In recent decades, the ever-increasing demand for agricultural products to meet the growing needs of population has accompanied an equally growing use of microorganisms as biofertilizers; indeed, the global market for biofertilizers has grown significantly. However, the market for biofertilizers is still only a small fraction compared to that for synthetic agrochemicals (**Figure 8**) (Timmusk et al., 2017).

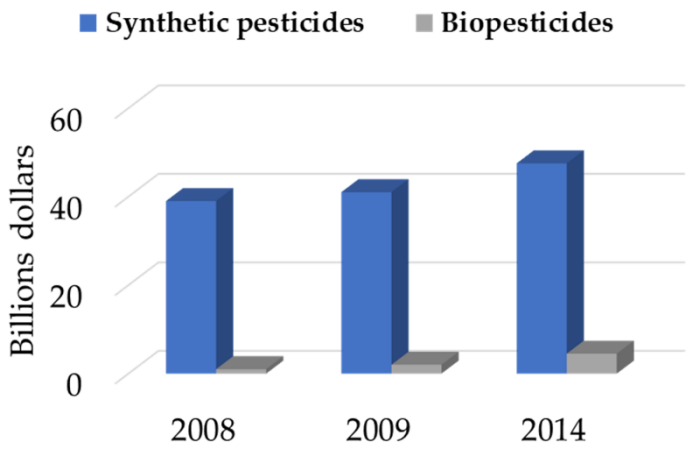

Figure 8. Comparison of the market for synthetic pesticides and biopesticides *(edited from Timmusk et al., 2017).*

Commercially, PGPR are addressed as biofertilizers and biopesticides. The global market covers almost the entire world and in terms of revenues generated from the production of biofertilizers, North America (USA, Canada and Mexico) dominates the global biofertilizer market, followed by Europe (Germany, UK, Spain, Italy, Hungary, and France) and the Asia-Pacific region (China, Japan, India, Australia, New Zealand and the rest of Asia) (**Figure 9A**).

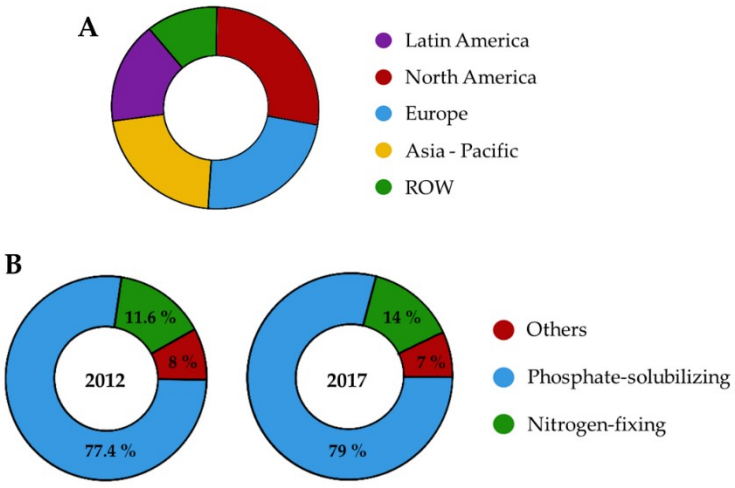

Figure 9. A. *Worldwide market for biofertilizers by 2014.* ***B.*** *Global biofertilizer market share by product typology (nitrogen-fixing and phosphatesolubilizing microbe-based biofertilizers and others). (Edited from Timmusk et al., 2017 (**A**) and Basu et al., 2021 (**B**)).*

In 2017, the biofertilizer markets were valued at USD 495 million in North America, USD 450 million in Europe, USD 284 million in Asia-Pacific, USD 240 million in South America, and USD 44 million in Africa (**Figure 10**) and it is estimated that the global biofertilizer market would reach USD 3.5 billion by 2025 (Basu et al., 2021). The market is dominated by nitrogen-fixing biofertilizers with a share of approximately 80%, followed by phosphate-solubilizing biofertilizers, with around 14% (**Figure 9B**).

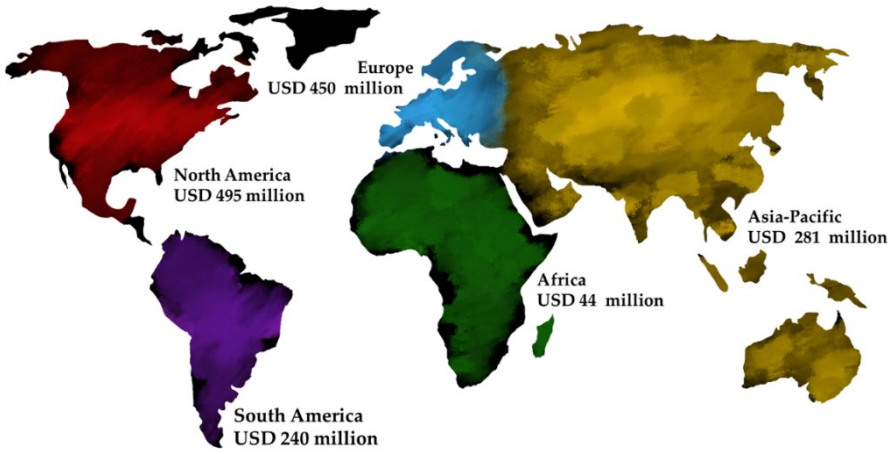

Figure 10. *Size and distribution of the global biofertilizer market in USD million per region (edited from Basu et al., 2021).*

Table 4 shows some PGPR-based products available on the market, their classification based on the functions, the country of production and the regions where they can be found on the market.

Table 4. *Overview of some globally available PGPR-based products (edited from Basu et al., 2021)*

Type of commercial product	Name	PGPR Strain(s)	Manufacturers's country	Market region	References
Nitrogen fixer	Nitragin Gold ®	Rhizobia	USA	North America	García-Fraile et al., 2017
	Bioboost	*Delftia acidovorans*, *Bradyrhizobium* sp.	Canada	North America	García-Fraile et al., 2015
	Rhizosum Aqua	*Azosprillum* sp.	Spain	Europe	García-Fraile et al., 2015
	TwinN ®	*Azorhizobium* sp. *Azoarcus* sp. *Azosprillum* sp.	Australia	Asia-Pacific	Adeleke et al., 2019
	Mamezo ®	Rhizobia	Japan	Asia-Pacific	García-Fraile et al., 2015

	BioAgro 10 ®	*Bradyrhizobium Japonicum*	Argentina, Brazil, Bolivia	South America	Uribe et al., 2010
	Azo-N Plus	*Azosprillum brasiliense, A. lipoferum, Azotobacter chroococcum*	South Africa	Africa	Adeleke et al., 2019
Phosphate solubilizer	Fosforina ®	*Pseudomonas fluorescens*	Cuba	North America	Uribe et al., 2010
	Phosphobacterin	*Bacissul megaterium* var. p*hosphaticum*	Russia	Europe	García-Fraile et al., 2017
	Rhizosum PK ®	*Bacillus megaterium, Frateuria aurantia, Rhizophagus irregularis*	Spain	Europe	García-Fraile et al., 2017
	CBF	*Bacillus mucilaginous, B. subtilis*	China	Asia-Pacific	Uribe et al., 2010
Potassium solubilizer	Rhizosum K	*Frateuria aurantia*	Spain	Europe	García-Fraile et al., 2015
	K Sol B	*Frateuria aurantia*	India	Asia-Pacific	Mehnaz et al., 2016
Zinc solubilizer	Biozink ®	PGPR consortia	India	Asia-Pacific	García-Fraile et al., 2017
	Zn Sol B	*Thiobacillus thiooxidans*	India	Asia-Pacific	Mehnaz et al., 2016
Biocontrol	Cedomon ®	*Pseudomonas chlororaphis*	Sweden	Europe	Mustafa et al., 2019
	Biotilis	*B. subtilis*	India	Asia-Pacific	Mehnaz et al., 2016
	Soilfix	*Brevibacillus laterosporus, Paenibacillus chitinolyticus*	South Africa	Africa	Aloo et al., 2022

Many publications on PGPR demonstrate that there is growing evidence supporting the use of the products as agricultural inputs. In many developing countries, bacteria are effectively used, but in developed countries, where chemical products are inexpensive, their application is growing, but it remains marginal. In all respects, the potential and benefits of microbial products are more than chemicals; they represent a renewable and eco-friendly source which, in addition to directly stimulating plant growth or inhibiting phytopathogens growth, they are also able to alleviate abiotic stresses that, taken together, are the major cause of yield loss (Bharti et al., 2016; Gao et al., 2022).

Despite this, many problems with microbial-based products need to be resolved before an extensive application that is equal to or exceeds that of chemicals. In fact,

there are many obstacles that stand between research laboratories and their effective application in agriculture. One of the main limitations is represented by the spectrum of action; in fact, conventional products have a wide spectrum of action, making them effective for many plant species and phytopathogens, and are less sensitive to environmental conditions. Microbial-based products are more selective and sensitive to environmental changes, obviously resulting in variable efficacy under highly variable field conditions. The cost of these products is also an obstacle (e.g., in Europe, the cost is about 25% higher than that of conventional products (Timmusk et al., 2017), as well as some bureaucratic processes for their approval and placement on the market.

Agricultural biofertilizer products can be developed on the basis of a single or multi-species live inoculum. A fundamental characteristic that PGPR must possess to be approved and placed on the market is their biosafety. In fact, they must not have harmful effects on humans or the environment. In the World Health Organization (WHO) guidelines on biosafety, microorganisms are classified into four groups based on the risk they pose (BSL 1-4). The selected microbial strains for agricultural use should preferably be classified as group 1 (low-risk, non-pathogenic) (Basu et al., 2021).

The first challenge is to select the PGPR strain itself. To develop a new PGPR strain(s) as a biofertilizer, the first step is to isolate it from the rhizosphere and then to analyse i*n vitro* traits. The results can then be validated in plant and field conditions. This is already crucial, because many PGPR strains will be overlooked because not all PGPR strains can be cultivable under laboratory conditions (Backer et al., 2018). Very often, the potential of PGPR is determined by using a variety of analyses to determine all the PGP traits using axenic cultures. In many cases, the result obtained under axenic conditions does not guarantee effective plant growth promotion in the crop of interest or under field conditions. At the same time, similarly, it may happen that isolates that do not show interesting PGP traits *in vitro* may be very effective on crop and in field conditions (Cardinale et al., 2015).

One of the major limitations is represented by the selectivity of the strains; in fact, the strain should not be selective and should show a broad host spectrum. The selection of a good growth promoting strain is a long process; indeed, potential isolates should be selected for their performance not only in the laboratory, but also after a subsequent extensive analysis in a wide range of cultures and different types of soil and environmental conditions (Meena et al., 2020).

Rhizobacterial strains can be considered PGPR if they show specific characteristics that promote plant growth and can improve plant growth after inoculation. A series of criteria have been established to identify ideal PGPR strains (Vejan et al., 2016): i) they should be highly rhizosphere-competent and environmentally friendly, ii) they should be able to promote plant growth, iii) they should be able to promote plant growth, iv) they should show a broad range of action, v) they should be compatible with other bacteria in the rhizosphere, vi) they should withstand physical and chemical factors such as heat, decomposition, radiation, and oxidants, vii) they should demonstrate a better competitive capability in the existing rhizobacterial community.

One of the main challenges encountered during the development of a PGPR-based product and the commercialization of an effective PGPR strain is its shelf life (Khare and Maheshwari, 2010; Zandi and Basu, 2016). PGPR-based products with a short shelf life are at risk of being destroyed if they are not used or sold before their expiry date, resulting in economic losses for the producer. Since they contain living microbial cells, their storage and transportation require extra attention and precaution. Technical constraints include the risk of product deterioration due to shorter storage life or spontaneous mutations during storage that reduce efficiency. (Mahajan and Gupta, 2009).

Another important aspect to consider is the actual effectiveness of these microorganisms when they move from the laboratory to the open field. The response of crops to applied biofertilizers is very slow because the inoculum will takes a long time to build its concentration and colonize the roots. As a result, farmers are less

willing to accept biofertilizers. The purity of the inoculants and the inoculation techniques play a vital role in field application. Furthermore, when inoculums are exposed to field conditions, many factors may have a negative impact.

The most critical step for the success of an inoculum is represented by colonization of the rhizosphere by the added microorganisms, which is a complex process that requires the ability of bacteria to compete in the rhizosphere to establish a positive plant-microbe interaction. Therefore, in this context, it is essential that inoculated microorganisms can compete with existing micro and macrobiota (Kumar et al., 2017).

In addition, positive effects can be influenced by chemical residues previously used and abiotic stresses. The soil composition is another important factor in determining the effectiveness of an inoculum, such as soil acidity, the presence of nitrates, heavy metals, or the lack of some essential nutrients. (Bhardwaj et al., 2014; Ndeddy et al., 2016; Parnell et al., 2016).

To ensure a greater probability of success, it is important to formulate specific products for the soil conditions under which they will be applied or to identify region-specific strains. In addition, little has been done to integrate microbiome-based plant breeding to achieve a heritable PGPR community (Mitter et al., 2013). Therefore, it is essential to carry out the selection and study of PGPR by implementing an approach not limited to the intrinsic potential of PGPR alone, but to use a broader and multidisciplinary approach that ultimately leads to obtaining a product that is as effective as possible (Backer et al., 2018; Basu et al., 2021).

4.1.1 Marketing regulations

Until recently, there were no rules in the European Community that determined the uniformity of the rules for the issuance of PGPR-based products on the market, so national or regional rules were applied and were an important obstacle to approval and commercialization. One of the reasons of this situation was the absence of a formal

definition of the concept by the regulatory systems. The practical results of this lack of rules in the European regulatory system have been well described by du Jardin (2015).

In Europe, there were two main routes to launch PGPR-based products on the market: one was the national fertilizers regulation, and the other was the European pesticides legislation, which combined both supranational and national provisions for the introduction of plant protection products (PPPs) on the market. The European Community (EC) Regulation No. 1107/2009 on PPPs applies to all categories of PGPR-based products, considering the very board definition of PPPs; Article 2 states: "This Regulation shall apply to products consisting of or containing active substances, safeners or synergists, and intended for one of the following uses:

(b) influencing the life processes of plants, such as substances influencing their growth, other than as a nutrient."

Because any PGPR affects plant life processes in ways other than nutrients, microorganisms and synthetic compounds are included in this regulation. However, because the procedure for placing PPPs on the European market is long and expensive, an alternative route has been chosen, namely the 'fertilisers route', in which national legislation is applied. National fertiliser laws have been used as an alternative to introduce PGPR-based products on the European market, but unfortunately there are significant differences in the data requirements for efficacy, toxicity, and ecotoxicity assessment between Member States (du Jardin, 2005; La Torre et al., 2015; Traon et al., 2014).

Why not the European law on EC fertilisers (regulation (EC) No. 2003/2003)? Because the definition of fertilizers laid down by this regulation is very restrictive and cannot include PGPR-based products. Article 2 reads:

"For the purposes of this Regulation the following definitions shall apply:

(a) 'Fertiliser' means material, the main function of which is to provide nutrients to plants. (b) The 'primary nutrient' means only the elements nitrogen, phosphorus, and potassium. (c) The 'secondary nutrient' means the elements calcium, magnesium, sodium, and sulphur. (d) 'Micronutrients' means the elements boron, cobalt, copper,

iron, manganese, molybdenum and zinc essential for plant growth in quantities small compared to those of primary and secondary nutrients'" (du Jardin, 2015).
According to these definitions, any fertilizer must provide nutrients as main function, but this is not the case of PGPR, which stimulate plant growth with a wide range of activities as well as providing nutrients.

On 5 June 2019, the European Parliament adopted Regulation (EU) 2019/1009, amending Regulation (EC) n. 1107/2009 and repealing the regulation (EC) n. 2003/2003, with the aim of regulating the use of fertilizers and harmonizing the market. This regulation has been applicable in all member states since July 2022. For the first time, microorganisms have been recognized as biostimulants in organic fertilizers. In Chapter 1, Article 2, the definition of "fertilizing product" includes "substance, mixture, microorganism, or any other material, applied or intended to be applied to plants or their rhizosphere or to mushrooms or their mycosphere, or intended to constitute the rhizosphere or mycosphere, either on its own or mixed with another material, for the purpose of providing the plants or mushrooms with nutrient or improving their nutrition efficiency". The Article 47 contains, as amended to Regulations (EC) No. 1107/2009, article 3, the definition of plant biostimulant as "a product stimulating plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere as a) nutrient use efficiency, b) tolerance to abiotic stress, c) quality traits and d) availability of confined nutrients in soil or rhizosphere" which clearly reports the definition provided by du Jardin (2015) (Barros-Rodríguez et al., 2020).

In this way, while the possibility of approving a product in a national state remains in force, it is possible to place a product with the CE mark on the market, thus facilitating its trade in the single European market.

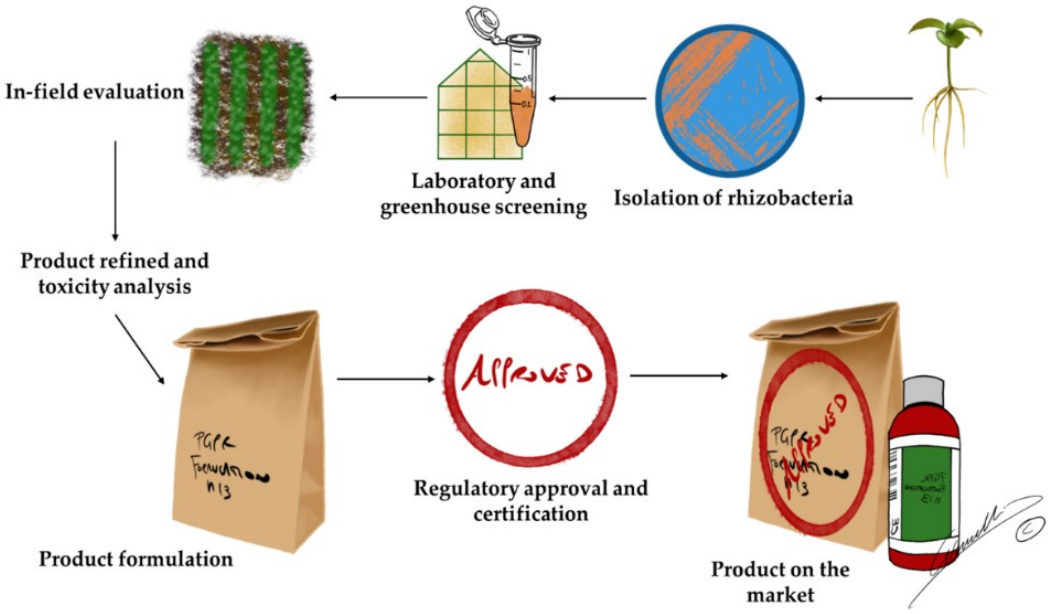

Figure 11. *Roadmap for PGPR-based products (edited from Basu et al., 2021).*

AIM OF THE WORK

The purpose of this work is to identify possible bacteria strains capable of exhibiting PGPR characteristics: and in particular their enhancement of plant growth under control and salt stress conditions and their antimicrobial activities, as first steps toward their possible field application on plants of agronomic interest. Furthermore, it is of extreme scientific interest to understand some of the phenomena underlying PGPR-induced tolerance to salt stress in plants.

At this purpose, this thesis can be divided into two parts: i) characterization of several bacteria isolated from harsh environments for their PGP traits *in vitro*; inoculation of *Arabidopsis thaliana* plants with the different strains to understand which bacteria induce growth enhancement in association with the plant; evaluation of the antimicrobial activity of the different bacterial strains in the phytopathogenic fungi *Aspergillus flavus*, *Fusarium verticillioides*, and *Fusarium proliferatum*; ii) selection of the most promising bacteria for the evaluation of its ability to reduce salt stress *in vitro* in the highly sensitive plant *A. thaliana*; application of targeted molecular and metabolic analyses to identify molecular changes induced in *Arabidopsis* by the bacterium which could be determinant for the increased tolerance; evaluation of the salt stress tolerance traits induced by the bacterium in *Arabidopsis in vitro* also in real soil conditions.

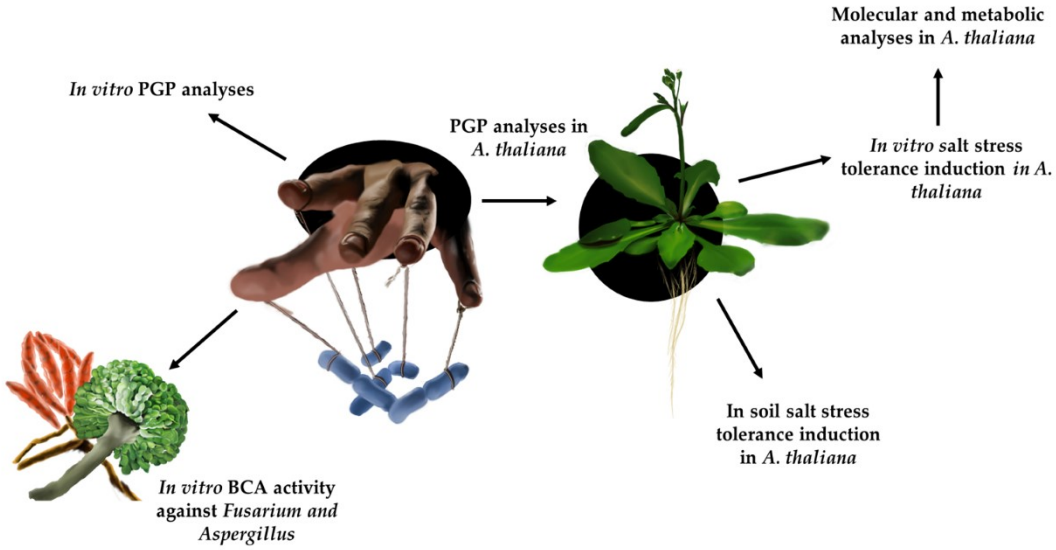

Figure 12. Graphical abstract *(edited from Giannelli et al., 2022).*

MATERIALS AND METHODS

1. Microorganisms utilized in this study and growth conditions

In the present study, six different bacterial strains isolated from different harsh environments have been selected from a collection of bacteria present in our laboratory (**Table 5**). Pvr_5 (homologous to *Paenarthrobacter ureafaciens*) and Pvr_9 (homologous to *Beijerinckia fluminensis*) were isolated from the rhizosphere of *Pteris vittata*, an As-hyperaccumulator fern, naturally grown in arsenic contaminated soils (Antenozio et al., 2021). PHA_1 (homologous to *Pseudomonas protegens*) was isolated from a hydrocarbons contaminated soil (Rizzo et al., 2020). NCr-1 (homologous to *Arthrobacter* sp.) is an endophyte bacterial strain isolated from the roots of *Noccaea caerulescens*, a nichel hyperaccumulator plant (Visioli et al., 2014). Bioch_2 (homologous to *Arthrobacter defluvii*) and Bioch_7 (homologous to *Arthrobacter nicotinovorans*) strains were isolated by a biochar derived from maize and utilized as amendment in a three-year poplar short rotation coppice plantation (Bertola et al., 2019). All the bacterial strains were maintained on plate count agar medium (PCA; Oxoid, Thermo Fisher Scientific Waltham, MA, USA).

Table 5. *Name, isolation source, homology of bacterial strains with those present in gene bank and 16S rDNA sequence accession number.*

Strain	Isolated from	Homology	GenBank Accession nr.	References
PVr_5	*P. vittata* rhizosphere	*Paenarthrobacter ureafaciens* (98.16%)	MT013510	Antenozio et al., 2021
PVr_9	*P. vittata* rhizosphere	*Beijerinckia fluminensis* (100%)	MT013514	Antenozio et al., 2021
PHA_1	Hydrocarbons contaminated water	*Pseudomonas protegens* (98%)	MT703035	Rizzo et al., 2020
NCr-1	*N. cearulescens* (Endophyte)	*Arthrobacter* sp. (99%)	KJ792857	Visioli et al., 2014
Bioch_2	Biochar amended soil	*Arthrobacter defluii* (98%)	MK143398	Bertola et al., 2019
Bioch_7	Biochar amended soil	*Arthrobacter nicotinovorans* (99%)	MK143403	Bertola et al., 2019

To assess the bacterial antifungal activity, the aflatoxigenic *Aspergillus flavus* CR10 and two strains of *Fusarium proliferatum* and *Fusarium verticillioides* were used,

kindly provided by Dr. Francesca Degola from University of Parma. The fungal strains were maintained on potato dextrose agar medium (PDA; Oxoid, Thermo Fisher Scientific Waltham, MA, USA). For conidia production, *A. flavus* was cultured at 28 °C for 14 days on PDA while *F. proliferatum* and *F. verticillioides* strains were cultured for 20 days on nutrient synthetic medium (SNA; KH_2PO_4 1.0 g L^{-1}, KNO_3 1.0 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.5 g L^{-1}, KCl 0.5 g L^{-1}, glucose 0.2 g L^{-1}, sucrose 0.2 g L^{-1}, agar 15.0 g L^{-1}).

2. *In vitro* Plant Growth Promoting activities of isolates

For PVr_5, PVr_9, NCr-1, Bioch_2 and Bioch_7 bacterial strains some PGPR activities were already evaluated (IAA production, ACC deaminase activity) (Antenozio et al., 2021; Bertola et al., 2019; Visioli et al., 2014). To gain greater insight into the PGP potential of the bacteria under investigation, additional PGP activities were evaluated.

2.1 Inorganic phosphate solubilization activity

Selected bacterial strains were tested for inorganic phosphate solubilization activity using Pikovskaya medium (PVK; dextrose 10 g L^{-1}, yeast extract 0.5 g L^{-1}, $Ca_3(PO_4)_2$ 5 g L^{-1}, $(NH_4)_2SO_4$ 0.5 g L^{-1}, KCl 0.2 g L^{-1}, $MgSO_4$ 0.1 g L^{-1}, $MnSO_4$ 0.0001 g L^{-1}, $FeSO_4$ 0.0001 g L^{-1}, agar 10 g L^{-1}) (Nautiyal , 1999). Bacterial strains were streaked on PVK agar medium and incubated at 28 °C. After 5 days of growth the phosphate solubilization activity was assessed by the visualization of a clear halo surrounding the bacterial colony.

2.2 Protease activity

Protease activity was evaluated in skim milk agar plate medium (casein hydrolysate 10 g L^{-1}, yeast extract 5 g L^{-1}, NaCl 4 g L^{-1}, skim milk powder 20 g L^{-1}, agar 10 g L^{-1}). Bacterial strains were streaked and incubated at 28 °C. After 5 days of growth protease production was determined by the presence of a clear halo surrounding the bacterial colony (Kavitha et al., 2013).

2.3 Biofilm formation

Biofilm formation was assessed following the protocol described by O'Toole, with some modifications (O'Toole, 2011). An overnight bacterial culture in liquid plate count agar medium (PCA; enzymatic digest of casein 5 g L^{-1}, yeast extract 2.5 g L^{-1}, glucose 1 g L^{-1}) medium was diluted 1:100 in fresh PCA liquid medium. 100 µL were inoculated in a well of a 96-well plate and then placed in static growth at 28 °C. After 5 days of incubation, the medium was discarded, and the plate submerged in water twice. Then, 125 µL of a 0.1% solution of crystal violet (w/v) for each well was added and the plate incubated for 15 min at room temperature. The plate was rinsed 3 times with water and after water removal, dried for 2 h. A volume of 125 µL of 30% acetic acid solution was added; after 15 min of incubation, absorbance was quantified at 595 nm wavelength.

2.4 Siderophores production

To measure siderophore production, bacteria were grown in either SM (succinic acid 4%, $(NH_4)_2SO_4$ 1%, KH_2PO_4 3%, K_2HPO_4 0.1%, $MgSO_4$ 0.2%) or SMS (sucrose 1% (w/v), $(NH_4)2SO_4$ 0.1%, K_2HPO_4 0.2%, $MgSO_4$ 0.05%, NaCl 0.01%, yeast extract 0.05%, $CaCO_3$ 0.05%, tryptophan 0.5 mg mL^{-1}) for 3 days. Bacterial cultures were then centrifuged, and the cells removed. 500 µL of supernatant were added to 500 µL of CAS assay solution (6 mL of 10 mM hexadecyltrimethylammonium bromide

(HDTMA), 1.5 mL of 1 mM $FeCl_3$, 7.5 mL of 2 mM CAS, 4.307 g of piperazine, and 6.25 mL of 12 M HCl, then diluted to 100 mL with double-distilled water according to Jeong et al. (2014) and incubated for 20 min at room temperature. The siderophore production by each strain was quantified determining the absorbance at 630 nm wavelength, and the results were expressed as siderophore unit (percentage) (Payne, 1994). Three replicates per bacterial strain were analysed. Results are expressed as mean ± S.D.

3. Identification of siderophore produced by bacterial strains

Bacterial broths obtained from a 3-days culture in SMS or SM medium were centrifuged and the supernatant was recovered, filtered, and added with methanol at a 3:1 volume ratio. Then, four volumes of ethanol were added, and the samples were left undisturbed overnight at 4 °C (Masalha et al., 2000). After a centrifugation at 4000 rpm for 20 min the supernatant was recovered and concentrated at 45 °C with a vacuum rotary evaporator and utilized for the following analyses.

3.1 Functional groups detection

For iron-chelating functional groups detection each sample was subjected to two different tests. The tetrazolium test was employed to verify the presence of hydroxamate type of siderophore (Snow, 1954). A pinch of tetrazolium salt was added in a test tube to which 1–2 drops of 2 N NaOH was added and subsequently 1 mL of test sample. Immediate development of a deep red colour was taken as a positive reaction by hydroxamate-type siderophore. Moreover, to determine functional groups belonging to the catecholate type of siderophores the Arnow's test was used (Arnow, 1937). Arnow's test is based on the reaction between catechol and nitrite–molybdate reagent (prepared by dissolving 10 g of $NaNO_2$ and 10 g of Na_2MoO_4 in 100 mL of

deionized water), in acidic conditions, producing a yellow colour. The colour changes to an intense orange-red in alkaline conditions. For this purpose, 1 mL of HCl 0.5 mol L^{-1} was combined with 1 mL of test sample. Subsequently, 1 mL of nitrite–molybdate reagent was added and then 1 mL of NaOH 1 mol L^{-1}. The mixture was then incubated for 5 min to allow the full colour development. As a blank control sample, 1 mL of deionized water was used. The presence of an orange-red colour solution detects the catecholate type siderophore. The colour intensity depends on the amount of catechol present (Arnow, 1937; Ferreira et al., 2019).

3.2 UPLC determination

The solutions containing siderophores were tested by means of ultra-performance liquid chromatography (UPLC) (Waters S.p.A. Sesto San Giovanni (MI), Italy) to better identify the siderophores.

To separate active components, each sample was injected and separated on a C18 column (Waters Acquity UPLC BEH300 C18 1.7 μm, 2.1·50 mm) using a gradient of 0.1% aqueous formic acid (A) and acetonitrile (B) as mobile phase (0–5 min 1.5–45% B, 5–16 min 45–100% B and then 16–19 min 100% B; flow rate 0.2 mL·min^{-1}; temperature 30 °C). The capillary and cone voltages in ESI mode were 3.8 kV and 25 V, respectively (Dimkpa et al., 2008; Ferreira et al., 2019). Ion transfer capillary was heated at 300 °C. Cone and desolvation gas flow was, respectively, at 100 and 480 L·h^{-1}. Positive-ion full-scan mass spectra were recorded from *m*/*z* 50 to 2000.

3.3 LC–ESI–MS/MS determination

High resolution mass spectrometry was performed also on the solutions containing siderophores by using a HPLC DIONEX Ultimate3000 interfaced with a LTQ-Orbitrap XL Thermo Fisher Scientific (Waltham, MA, USA). Samples were injected on an Aeris Peptide 3.6u XB-C18 2.1 mm × 15 cm (Phenomenex; Via M.

Serenari, 15/D, 40013 Castel Maggiore (BO), Italy). The mobile phase consisted of water with 0.1% formic acid (solvent A) and methanol with 0.1% formic acid (solvent B); gradient: 0–5 min 99% A, 5–35 min from 99% A to 5% A, 35–40 min 5% A, 40–41 min from 5% A to 99% A, 41–50 min 99% A; flow rate was 0.2 mL/min; column temperature 35 °C; injection volume 5 µL. Samples were acquired in positive and negative mode. Electrospray ionization at positive (spray voltage 3 kV; capillary voltage 13 V; source temperature 275 °C; tube lens 100 V; sheath gas flow rate 40; aux gas flow rate 10; and sweep gas flow rate 5) and negative (spray voltage 3.2 kV; capillary voltage −35 V; source temperature 275 °C; tube lens −110 V; sheath gas flow rate 40; aux gas flow rate 10; and sweep gas flow rate 5) ion modes. The mass data acquisition was performed by four scan events. Data were analyzed using a database dedicated to microbial siderophores and created by Prof. Samuel Bertrand (**http://bertrandsamuel.free.fr/siderophore_base/index.php** released on 8 June 2011, accessed on 14 December 2021); compounds were identified through the main adduct encountered using LC–ESI–MS, namely, $[M + H]^+$, $[M - 2H + Fe]^+$, and $[M - H]^-$.

4. Bacterial PGP activity in *A. thaliana*

4.1 Seed preparation and bacterial inoculation

Arabidopsis thaliana (L.) Heynh. Columbia-0 seeds were used. *A. thaliana* seeds were surface sterilized with 2% NaClO solution for 5 min and then washed 4 times with double-distilled sterile water. After washing, seeds were kept in the dark at 4 °C for 3 days to allow the synchronization of germination.

Bacterial strains were grown in 3 mL of Luria and Bertani medium (LB; tryptone 10 g L^{-1}, yeast extract 5 g L^{-1}, NaCl 5 g L^{-1}) on shaking (130 rpm) at 28 °C for 24 h.

Seed inoculation with the different strains was performed as follows: for PVr_5, PHA_1, NCr_1, Bioch_2 and Bioch_7 seeds were kept for 1.5 h in a bacterial solution

(1×10^8 cells mL^{-1}) on shaking. For control, seeds were kept in double-distilled sterile water following the same time and shaking conditions. Seeds were then recovered and plated on half strength Murashige and Skoog (Murashige and Skoog, 1962) (Duchefa Biochemie, Haarlem, Netherlands) agar medium (MS; MS 4.302 g L^{-1}, sucrose 1% (w/v), agar 10 g L^{-1}). Plates were incubated in a vertical position in an environmentally controlled chamber growth (24 °C; 16/8 h light/dark photoperiod; 120 μmol m^{-2} s^{-1} photosynthetically active radiation, 75% relative humidity (RH)) for germination and root elongation. After 14 days of growth, seedlings were collected for measurements.

For PVr_9 a different inoculation method was used. Synchronized seeds were plated and incubated without bacterial inoculum for 7 days to allow germination and root growth. After 7 days, 2 μL bacterial solution (1×10^6) were spotted on the root tips of seedlings while 2 μL of double-distilled sterile water were spotted for control seedlings. Plants were incubated in the same conditions indicated above for other 7 days. After 14 days of growth seedlings were collected for measurements.

4.2 Plants growth parameters measurements

Primary root length, number of lateral roots and projected rosette area were measured on 14-day-old seedlings inoculated or not with bacteria isolates. The number of total lateral roots was normalized for the total length of the primary root. All the measures were performed using ImageJ software (available at http://rsb.info.nih.gov/ij/ accessed on 20 September 2021; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). The results are expressed as mean ± Standard Deviation (S.D.). A total of 30 plants per treatment were analyzed.

5. Bacterial direct antifungal activity

To assess the bacterial antifungal activity of each of the bacterial strain analysed in this study, the aflatoxigenic *Aspergillus flavus* CR10 and two strains of *Fusarium proliferatum* and *Fusarium verticillioides* were used. For conidia recovery, mature cultures were treated with a solution of water and 0.1% (v/v) Tween-20 to promote conidia release. The solution was then filtered through sterile nylon filter to remove medium debris and hyphae. Conidia concentration was evaluated by detecting the absorbance at 600 nm wavelength according to a standard curve previously obtained.

To determine the antifungal activity of bacteria a 96-multiwell plate cultivation system and two different methods have been used. In the first assay bacteria were grown in PCA liquid medium at 28 °C for 3 days on shaking (130 rpm); cells were recovered, washed twice in double-distilled sterile water to remove the culture medium, and then diluted. 2.5 x 10^3 or 5 x 10^3 of bacterial cells were co-inoculated in 96-multiwell plates with 5 x 10^3 fungal conidia in a final volume of 200 µL/well of PCA liquid medium. Plates were incubated in static growth in the dark at 28 °C. In the second assay the bacterial broth was used: bacteria were grown in PCA liquid medium at 28 °C for 3 days on shaking (130 rpm); each culture was then centrifuged at 4000 rpm for 20 min and the culture broth was recovered and filtered through a 0.22 µm filter. Spores of each fungal species (5 × 10^3) were inoculated in 96-multiwell plates with 50 or 100 µL of filtered broth to a final volume of 200 µL/well of PCA liquid medium, corresponding to the 25 and 50% (*v*/*v*) of the culture, respectively. Biomass production was assessed after 10 days of incubation for *Aspergillus flavus*, while *Fusarium proliferatum* and *Fusarium verticillioides* biomass production was evaluated after 14 days. From single well mycelium was recovered, slightly dried on paper and weighted. Values were expressed as % of inhibition with respect to the control. Inocula were performed in quadruplicate, and experiments were performed in triplicate.

6. Bacterial salt tolerance induction in *A. thaliana*

6.1 Bacterial growth evaluation under saline conditions

PVr_9 bacterial strain was grown overnight in 3 mL of liquid Luria and Bertani medium on shaking (130 rpm) at 28 °C. A number of 1 x 10^6 bacterial cells were inoculated in 20 mL of SMS medium (sucrose, 0.1% $(NH_4)_2SO_4$, 0.2% K_2HPO_4, 0.05% $MgSO_4$, 0.01% NaCl, 0.05% yeast extract, 0.05% $CaCO_3$, 0.5 mg mL^{-1} tryptophan), added with 0 mM NaCl, 100 mM NaCl and 200 mM NaCl, and incubated on shaking (130 rpm) at 28 °C. The growth was followed measuring the OD_{600} at 0, 14, 19, 24, and 38 h after the inoculum.

6.2 Plant growth conditions and treatments

Arabidopsis thaliana (L.) Heynh. Columbia-0 seeds were used. *A. thaliana* seeds were surface sterilized with 2% NaClO solution for 5 min and then washed 4 times with double-distilled sterile water. After washing, seeds were kept in the dark at 4 °C for 3 days to allow the synchronization of germination. Seeds were plated on ½ MS (Murashige and Skoog, 1962) and 1% sucrose and 10 g L^{-1} plant agar (Duchefa Biochemie, Haarlem, Netherlands) and then incubated in a vertical position in an environmentally controlled chamber growth (24 °C; 16/8 h light/dark photoperiod; 120 μmol m^{-2} s^{-1} photosynthetically active radiation, 75% relative humidity) for germination and root elongation.

For root inoculation, PVr_9 was grown in 3 mL of LB medium on shaking (130 rpm) at 28 °C for 24 h. Then, bacterial cells suspension was diluted to 10^6 cells mL^{-1} with sterile double-distilled water.

After 7 days of growth, 2 μl of bacterial cell suspension was spotted on the primary root tip for the treated seedlings, while 2 μl of sterile double-distilled water

was applied to the controls. The seedlings were then incubated in the same conditions previously described for other 7 days.

6.3 Plant morphological parameters measurements

Fourteen days-old seedlings inoculated or uninoculated with PVr_9 were transferred to a fresh ½ MS, 1% sucrose and 10 g L^{-1} plant agar (Duchefa Biochemie, Haarlem, Netherlands) with 0 mM NaCl, 75 mM NaCl, 100 mM NaCl and 150 mM NaCl. After 4 days of growth, seedlings were collected and analysed.

To evaluate PVr_9 salt stress tolerance alleviation two morphological parameters were evaluated: primary root growth inhibition (percentage) and projected rosette area reduction (percentage). All measures were performed using ImageJ software (available at http://rsb.info.nih.gov/ij/ accessed on 20 September 2021; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). The results are expressed as mean ± S.D. A total of 30 plants per treatment were analysed. Since the results indicates positive effects of PVr_9 under 150 mM NaCl, all subsequent analyses were performed under this condition.

6.4 Determination of Reactive Oxygen Species (ROS) and 8-oxo-dG

The total ROS content of the samples was assessed using the 2',7'-dichlorofluorescein (H_2DCF) method (Jambunathan , 2010**)** with some modifications. Fourteen days-old seedlings, inoculated or uninoculated with PVr_9, were transferred to a fresh ½ MS, 1%sucrose and 10 g L^{-1} plant agar (Duchefa Biochemie, Haarlem, Netherlands) with 0 mM NaCl or 150 mM NaCl. After 4 days of growth seedlings were frozen in liquid nitrogen, grounded, and homogenized with 1 mL of 10 mM Tris-HCl, pH 7.2. The homogenates were centrifuged for 20 min at 4°C at 14000 rpm and then the supernatants were transferred to a fresh 1.5 mL tube. Twenty µL of supernatant was then diluted with 180 µL of 10 mM Tris-HCl, pH 7.2 in a 96 multi-well black flat

bottom plate (SARSTEDT, Numbrecht, Germany) and 2 µL of DCFDA was added (10 µM final concentration). After 10 min of incubation at room temperature in the dark, the fluorescence emission was recorded (TECAN SpectraFluor Plus microplate reader, Männedorf, Switzerland; λex = 485 nm; λem = 535 nm; optimal gain = 106 ; lag time = 0 µs; number of flashes = 3; integration time = 40 µs). The ROS values were expressed as relative fluorescence units mg^{-1} fresh weight (FW). Four samples for each treatment were analysed and the experiment was conducted in triplicate.

For 8-oxo-dG quantification, fourteen days-old seedlings, inoculated or uninoculated with PVr_9, were transferred to a fresh ½ MS, 1%sucrose and 10 g L^{-1} plant agar (Duchefa Biochemie, Haarlem, Netherlands) with 0 mM NaCl or 150 mM NaCl. 8-oxo-dG was quantified on genomic DNA extracted from 50 mg of frozen tissues, previously harvested from *Arabidopsis thaliana* plants, submitted to treatment for four days. DNA was isolated by grinding the tissue in liquid nitrogen to fine powder. Upon liquid nitrogen evaporation, isolation buffer was added (0.3 M NaCl, 50 mM Tris–Cl pH7.5, 20 mM EDTA, 0.5% SDS). After 5-min incubation at RT, an equal volume of PCI (Phenol:Chloroform:Isoamyl Alcohol, 25:24:1) was added. After centrifugation, DNA was precipitated adding 0.8 V isopropanol to the upper phase and recovered by centrifugation at 12.000 *g* for 5 min at 4 °C. The DNA pellet was washed in 75% cold ethanol and after brief air-drying, resuspended in water. Four micrograms of DNA were digested with a nuclease mix that degrades DNA to its individual nucleoside components (DNA Degradase Plus, Zymo Research, CA, USA). Base quantification was done with UHPL-MS/ MS (ExionLC-API 6500 +, Sciex). Chromatographic separation was performed in elution gradient (Phase A 10 mM HCOOH pH 3.75, Phase B MeOH) with column (ATLANTIS C18, Waters). The detection was performed in MRM mode and positive ionization.

6.5 Proline and total soluble sugars extraction and quantification

Proline was extracted and quantified according to Santangeli et al. (2019) with some modifications. Approximately 50 mg of sample was frozen in liquid nitrogen, grounded, and homogenized with ethanol 95%, then heated at 55 °C for 20 minutes. A volume of extract equal to 250 µl of the extract were added to a 1 mL of a reaction mixture composed of ninhydrin (2,2-dihydroxyindane-1,3-dione) 1% (w/v) dissolved in a mixture of 60% (v/v) acetic acid and 20% (v/v) ethanol. The samples were heated at 95 °C for 20 minutes in the dark and then centrifuged for 1 min at 7000 rpm. The proline concentration was evaluated by detecting the absorbance at 520 nm wavelength according to a standard curve made with a standard solution of L-proline ranging from 0.05 and 1 mM. Data are expressed as µmoles mg^{-1} FW.

Total soluble sugar quantification was assessed according to Khalofah et al. (2021) with some modifications. Approximately 50 mg of fresh leaves sample was washed with 2 mL 70% (v/v) ethanol and homogenized in 1.5 mL of 96% ethanol and then placed in a boiling water bath at 80 °C for 10 min. After cooling, the extract was centrifuged at 4000 rpm for 10 min, and the supernatant was kept on ice for measurement. Total soluble sugar concentrations were determined by reacting 100 µL of the ethanolic extract with 1 mL of freshly prepared anthrone reagent (150 mg anthrone plus 100 mL of 72 % sulfuric acid) and heated at 80 °C for 15 min. After cooling, the absorbance was detected at 625 nm using spectrophotometer to measure the quantity of total soluble sugars (mg mg^{-1} FW) using a glucose standard curve ranging from 0.01 mg mL^{-1} to 1 mg mL^{-1}.

6.6 Extraction and quantification of ABA from plant tissues

Fourteen days-old seedlings inoculated or uninoculated with PVr_9 were transferred to a fresh ½ MS, 1% sucrose and 10 g L^{-1} plant agar (Duchefa Biochemie, Haarlem, Netherlands) with 0 mM NaCl, 75 mM NaCl, 100 mM NaCl and 150 mM NaCl. After 4 days of growth, seedlings were collected. Endogenous ABA was

extracted by double extraction method. Briefly, 50 mg of sample was frozen in liquid nitrogen, grounded, and homogenized with methanol 20% (v/v), then centrifuged 10 min at 7500 rpm. The supernatant was collected in a new centrifuge tube. The resultant pellet was re-extracted adding 500 μL of methanol 20%, resuspended and sonicated for 3 min and centrifuged at 7500 rpm for 10 min. Total extract was dried and used for ABA quantification.

[2H6]-ABA was used as stable isotope-labelled internal standard (SIL) at fortification level of 1 ng/mL. Dried plant extracts were re-suspended in 10% MeOH. The sample was homogenized using vortexing (2000 rpm for 3 min), then centrifuged for 10 min at 8000 rpm, and supernatant purified by solid-phase extraction (SPE) using StrataX columns (1 mL/30 mg, Phenomenex). The SPE cartridge was conditioned with 1 mL MeOH and equilibrated with 1 mL MilliQ water. After sample loading, the column was washed with 1 mL 10% MeOH and then eluted with 80% MeOH acidified with 1% CH3COOH. The eluate was evaporated to dryness in vacuo and stored at -20°C until analyzed. Prior to LC-MS/MS analysis, the evaporated samples were dissolved in 140 μL 10% MeOH.

All samples were analyzed by LC-MS/MS using an UltiMate™ 3000 Basic Automated LC System (Thermo Fisher Scientific, San Jose, CA) coupled to an LTQ linear ion trap spectrometer (Thermo Fisher Scientific) equipped with an electrospray ionization source. For LC separation, a Luna Omega PS C18 (100 mm x 2.1 mm, 5 μm, 100 Å) (Phenomenex, CA, USA) was used. Purified plant extracts were analyzed using a binary solvent gradient of 0.1% (v/v) formic acid aqueous solution (solvent A) and methanol (solvent B). After column equilibration, samples were injected (20 ◎L) and eluted at a constant flow of 0.2 mL/min. The gradient started with 2% B for 1 min, then increased to 80% B in 21 min and to 90% B in 2 min, where it was maintained for 2 min. At 28 min the gradient returned to 2% B and equilibrated for 7 min before the next sample injection. The mass spectrometer was operated in negative ion mode, with the following tune parameters: electrospray voltage, -3.5 kV; sheath gas, auxiliary gas and sweep gas set at 50, 20 and 0 arbitrary units, respectively; capillary temperature, 250

°C; capillary voltage, -50V; tube lens, -120 V. MS/MS ananlysis was performed by pseudo selected reaction mode (pSRM), using the following transitions for quantitative purpose (normalized collision energy: 20): ABA, m/z = 263 ⊚ 153; [2H6]-ABA, m/z = 269 ⊚ 159. The concentration of was calculated by fitting the ratio of the analytical response of the analyte and the SIL into five-point calibration curve built in matrix. All analyses were performed in triplicate.

6.7 Ions extraction and quantification

Concentrations of Ca, Fe, K, Na, and P in leaves and roots were determined by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-OES; EPA 6010C 2007). Seedlings, leaves, and roots were mineralized in duplicate by a high-pressure microwave-assisted acid digestion as previously described (Gullì et al., 2018), weighing in quartz tubes approximately 0.02-0.05 g of oven-dried samples and adding 1 mLof high-purity deionized water (0.05 μS cm^{-1}, Purelab® Ultra ELGA, High Wycombe, UK) for rehydration and 2 mL of ultrapure HNO_3 (67–69% m v^{-1}, J. T. Baker®, Ultrex™ II, Avantor, Center Valley, PA). The digested solutions were transferred into graduated PP tubes (DigiTUBEs, SCP Science, Champlain, NY, USA), adjusting the volume to 10 ml with high-purity deionized water and filtered on 0.45 μm filters (Millex®-HA, Millipore, Merck, Darmstadt, Germany). Five-fold further dilutions were necessary for determining Ca in all samples, and fifty-fold further dilutions were used for quantifying Na and K in all samples and Fe. The analytic measurement was carried out by a ICP-OES spectrometer (Vista-MPX, Varian, Agilent Technologies, Santa Clara, CA); the instrument configuration and measurement conditions were previously described (Gullì et al., 2018). Ca (393.366 nm), Fe (259.940 nm), K (766.491 nm), Na (588.995 nm), and P (213.618 nm) were quantified through calibration lines (1.0 – 16 mgL^{-1} for Ca, Fe, K, Na and 1.0 – 50 mgL^{-1} for P), prepared from a 100 mg L^{-1} multi-element solutions of Ca, Fe, Na, and K (TraceCERT®, Sigma-

Aldrich, St. Louis, MI, USA) and a 1000 mgL^{-1} mono-element standard solution of P (TraceCERT® Fluka Analytical, Sigma-Aldrich, St. Louis, MI, USA).

6.8 Total RNA extraction from plant tissues, cDNA synthesis and qRT-PCR analyses

For the gene expression analyses of *SOS1*, *NHX1* and *HKT1*, RNA extraction was performed on plants treated with 0 mM NaCl or 150 mM NaCl for 24 h and 48 h. For the gene expression analyses of *MYB1*, *MYB52*, *MYB73* and *MYB96*, RNA extraction was performed on plants treated with 0 mM NaCl or 150 mM NaCl for 6 h. Leaves and roots were separated. Before the RNA extraction, roots treated with PVr_9 were washed three times with double-distilled sterile water to remove bacterial cells. 100 mg of tissue was used for the RNA extraction following the RNeasy Plant Mini kit (QIAGEN, Hilden, Germany) protocol. Samples quality and purity were analysed by agarose gel electrophoresis and the A_{260}/A_{280} and A_{260}/A_{230}. Reverse-transcription of 1 µg of total RNA was obtained with HiScript III RT SuperMix (Vazyme, Nanjing, PRC) following the manufacturer instructions.

The genes specific primers were identified on the primer designing tool Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and purchased from BMR Genomics (Padua, Italy). The list of primer used, and their target genes, are shown in **Table 6**. The total RNA extracted was analysed by qPCR using Universal SyBr Green kit and Quantstudio 3 (Thermo Fisher Scientific). The data were analysed with the $2^{-\Delta\Delta Ct}$ method, and the results are expressed as fold change ($2^{-\Delta\Delta Ct}$) of the means. Tubulin was used as housekeeping gene. Three biological and three technical replicates per condition were performed. Amplification was conducted as follows: 2 min 50 °C, 5 min 95 °C; 15 sec 95 °C, 1 min 60 °C (40 cycles); dissociation curves were obtained with 15 sec 95°C, 1 min 60°C, 1 sec 95 °C.

Table 6. *Genes analysed in this study and primers utilized for RT-qPCR*

Gene name	Accession number	Function	Primer sequence (5′-3′)	Amplicon length (bp)
TUB	NM_100360	Tubulin alpha-4 chain	F - TAAAGACGTGAACGCAGCTGTT R- TGAATCCAGTAGGACACCAGT	80
SOS1	NM_126259.4	Sodium proton exchanger	F- CACTTCTGGGAAATGGTTGC R- TGCCTTCAGCAATGACAACAC	76
HKT1	NM_117099.6	High-Affinity K^+ transporter 1	F- TCTTCTTGGAGTGACGGTGC R- TAGTTTCTCCGGTGTGTCGC	158
NHX1	NM_122597.3	Na^+/H^+ exchanger 1	F- GCTTCTGTGGTTGCGTTGAA R- CCAGTGCCTAGCCCAATCAA	128
MYB1	NM_111757.4	Myb domain protein 1	F- CGCTCGGAGTATTCCTGGTC R- GGAGGAGCTTCGCGATAACA	167
MYB52	NM_101658.3	Myb domain protein 52	F- TTGGCAACCACAACCGCTAT R- GTTTGGTCTATTGCTCCTTCTTGT	140
MYB73	NM_119889.2	Myb domain protein 73	F- CGGAAGTCCATCGGGATCTG R- GTTGACTCGAACCGTCTCGT	163
MYB96	NM_125641.4	Myb domain protein 96	F- TTCAAAAGGTGAAAACAGAATGGG R- TGCTACATCTTCTCAAACCTGTGT	170

7. PVr_9 salt tolerance induction in *A. thaliana* grown in-soil

To evaluate the ability of PVr_9 to induce salt stress tolerance in a less controlled system and in a long-term stress an in-soil experiment was performed. Seeds and PVr_9 culture for inoculation were prepared as indicated in the section **6.2**.

Commercial soil was sterilized trough autoclaving and 14 days-old seedlings inoculated and uninoculated were transferred from *in vitro* to individual pots with sterile soil moistened with double-distilled sterile water. After 7 days of acclimation, plants were irrigated once every two days with double-distilled sterile water or 150 mM NaCl. After one month of treatment, plants were collected. Leaves fresh weight,

leaves dry weight and primary root length were measured. The results are expressed as mean ± S.D. A total of 6 plants per treatment were analysed.

8. Statistical analysis

For statistical analyses, one-way analysis of variance (ANOVA) was used in the Past 4.06b software (Hammer et al., 2001). Results of plant growth measures and antifungal activity were analysed by Tukey's test; differences were considered significant at $p < 0.05$. When experiments considered two factor (salt and bacteria) two-ways ANOVA was used. Shapiro-Wilk test was used for normality evaluation and Tukey's test for multiple comparisons; differences were considered significant at $p < 0.05$.

RESULTS

1. Evaluation of bacterial strains properties

In this study, six different bacterial strains isolated from harsh environments were tested for some Plant Growth Promoting characteristics. The bacterial strains had already been analysed in previous works for their ability to produce the auxin hormone indole-3-acetic acid (IAA) and for 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity, as well as the production of siderophores, which was previously investigated by using a qualitative method. In this study a deeper characterization of their properties was carried out.

The six bacterial strains were tested for siderophores production using the semiquantitative assay liquid chrome azurol S (CAS) assay in two different culture media, the SMS medium containing low amounts of iron and the succinic acid medium that does not contain iron. Moreover, tests for the phosphate solubilization activity, for protease activity and for biofilm formation were also carried out. All the results are summarized in the **Table 7**.

As revealed by the CAS assay, all the bacterial strains were able to produce in both growth media siderophores involved in chelating ferric ions, as indicated by the elevated percent siderophore unit (PSU) values observed. A common but slight decrease in the PSU is evident when bacteria were grown in succinic medium, but this could be explained by the less growth of all the selected strains in this medium, which is very poor in nutrients compared to SMS medium.

Table 7. *Characteristics of PGP bacteria strains isolated from different harsh environments.*

Strain	Siderophore Production (PSU)[a]		IAA Production (mg L^{-1})	ACC Deaminase activity [b]	Phosphate solubilization ability [c]	Protease activity [d]	Biofilm formation (Abs units)
	SMS	Succinic					
PVr_5	88.64 ± 0.74	91.5 ± 1.05	62.48 ± 6.3	+	-	+	0.037 ± 0.010
PVr_9	91.90 ± 0.11	70.7 ± 2.60	82.08 ± 1.7	+	-	-	1.048 ± 0.141
PHA_1	90.38 ± 0.09	76.89 ± 4.94	n.d.	n.d.	+	-	0.134 ± 0.007
NCr-1	93.04 ± 0.08	58.78 ± 2.78	25.6 ± 1.3	+	-	+	0.059 ± 0.003
Bioch_2	91.29 ± 0.56	85.91 ± 4.70	44.02 ± 2.3	+	-	+	0.1 ± 0.007
Bioch_7	92.33 ± 0.70	89.02 ± 1.12	58.65 ± 4.2	+	-	+	0.216 ± 0.032

[a] Siderophore production on SMS and succinic media (see Material and Methods for media composition).
[b] ACC deaminase activity: (-) no bacterial growth on medium containing 1-aminocyclopropane-1-carboxylate as the only N source; (+) bacterial growth on medium containing 1-aminocyclopropane-1-carboxylate as the only N source.
[c] Phosphate solubilization: (-) absence of solubilization halo; (+) presence of solubilization halo.
[d] Protease activity. (-) absence of solubilization halo; (+) presence of solubilization halo.
Data are average of three independent experiments ± S.D.

The ability to solubilize phosphate as well as protease activity was assessed using a qualitative method by which a positive result was represented by the formation of a clear halo in the region surrounding the bacterial colony. As shown in **Figure 13A**, among the tested bacterial strains, PHA_1 is the only one capable to solubilize phosphate. On the other hand, PVr_5, NCr-1, Bioch_7 and Bioch_7 showed protease activity as reported in **Figure 13B**.

The importance of microbial peptidases is based on their crucial role in the nitrogen cycle in soils, since their activity makes nitrogen more available to plants (Bach and Munch, 2000). Finally, PVr_9 and Bioch_7 show an *in vitro* capacity to form a biofilm.

Since siderophores are considered very interesting molecules, the identification of siderophores produced by bacteria was carried out. UPLC–MS and LC–ESI–MS

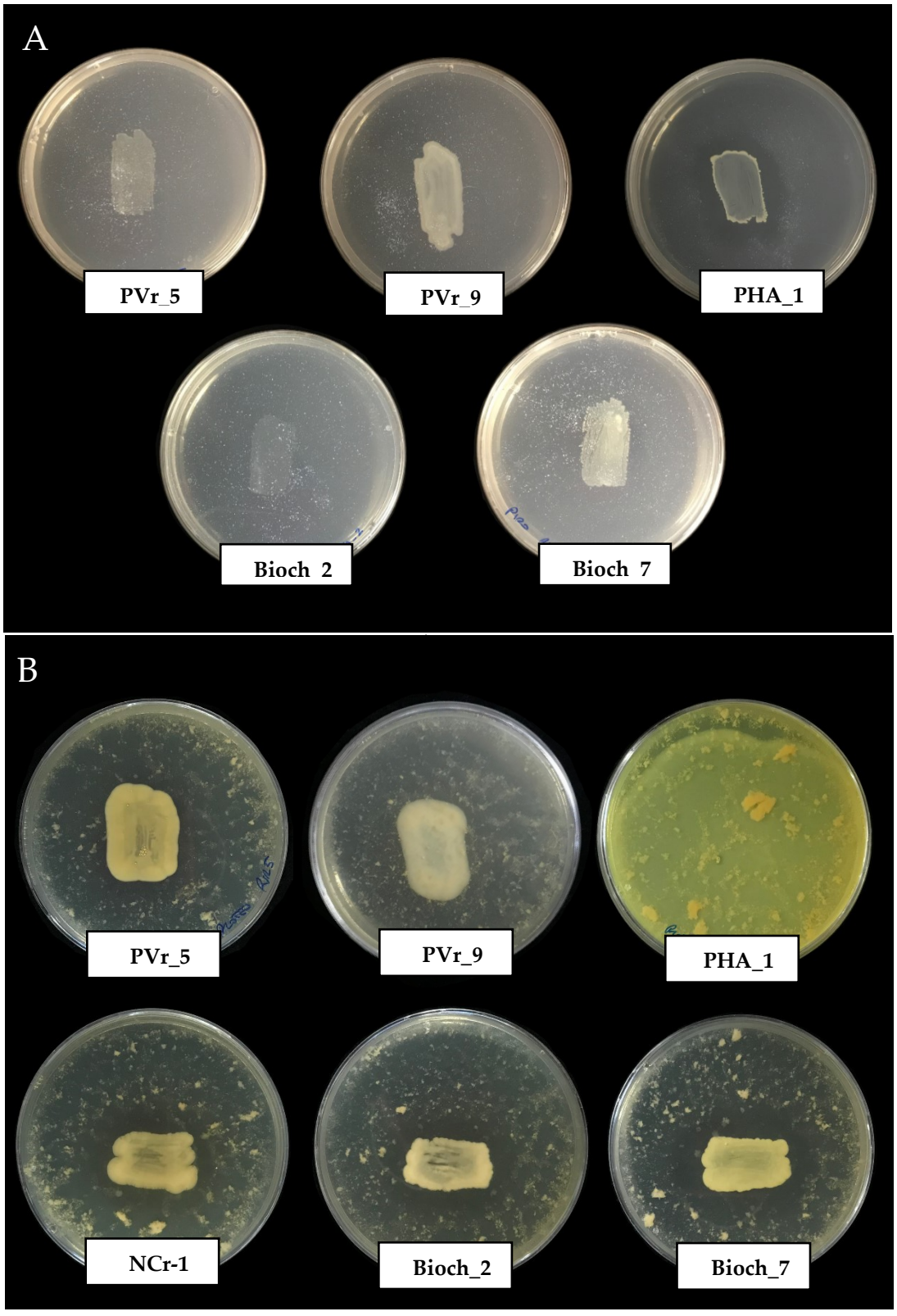

Figure 13. A. Phosphate solubilization assay*. Positive solubilization is represented by the clear halo formation around the bacterial colony.* ***B. Protease activity assay****. Positive solubilization is represented by the clear halo formation around the bacterial colony.*

analyses of SMS and SM culture broths from bacteria were performed to identify compounds. Salicylic acid was found in the culture broth of Pvr_5 and Bioch_7 grown in SMS medium, and in the culture broth of PVr_9, PHA_1, and NCr-1 grown in SMS and SM medium. Among the molecules identified, the UPLC–MS analysis revealed the presence of the hydroxamate desferrioxamine B in the SMS broth of Pvr_5, while the LC–ESI–MS/MS analysis was able to detect the presence of the catecholate aminochelin in the SM broth of PVr_9. In SM medium, NCr-1 was found to produce the hydroxamate siderophore asperchrome B, and Bioch_2 the quinolobactin, a carboxylate siderophore (**Table 8**). The discovery of salicylic acid is extremely interesting because, although its role as a siderophore is still debated, this hormone is produced by plants mainly in response to pathogens attacks.

Table 8. *Identified molecules and their relative functional groups produced by bacterial strain, along with the growth medium and the techniques used for the analysis (n.d., not detected).*

Strain	Functional group	SMS Medium	Succinic Medium
PVr_5	Carboxylate	Salicylic Acid (UPLC–MS)	n.d.
	Hydroxamate	Desferrioxamine B (UPLC–MS)	n.d.
PVr_9	Carboxylate	Salicylic Acid (UPLC–MS; LC–ESI–MS/MS)	Salicylic Acid (UPLC–MS; LC–ESI–MS/MS)
	Catecholate	n.d.	Aminochelin (LC–ESI–MS/MS)
PHA_1	Carboxylate	Salicylic Acid (UPLC–MS; LC–ESI–MS/MS)	Salicylic Acid (UPLC–MS; LC–ESI–MS/MS)
NCr-1	Carboxylate	Salicylic Acid (UPLC–MS; LC–ESI–MS/MS)	Salicylic Acid (UPLC–MS; LC–ESI–MS/MS)
	Hydroxamate	n.d.	Asperchrome B (UPLC–MS)
Bioch_2	Carboxylate	Quinolobactin (UPLC–MS)	n.d.
Bioch_7	Carboxylate	Salicylic Acid (UPLC–MS)	n.d.

2. Effect of bacterial inoculation on *A. thaliana* morphological features

The effects of bacterial inocula on plant growth characteristics were evaluated by analysing three different morphological parameters such as the primary root length, the secondary roots density, and projected leaves area on 14-day-old *A. thaliana* seedlings grown *in vitro*. As reported in **Figure 14A, B** PVr_9 was the only bacterial strain able to increase the primary root length of the seedlings with respect to the uninoculated plants. Seedlings grown in the presence of PHA_1 and Bioch_2, on the contrary, showed a slight decrease in primary root length. No significant differences were observed for roots of seedlings associated with PVr_5, NCr-1, and Bioch_7. **Figure 15A, B** shows the effect on the density of the secondary roots in seedlings inoculated with the different bacterial strains. The highest value was found for plants treated with PHA_1, with an increase in secondary roots of about three times with respect to the control condition. An increase in the density of the secondary roots was also observed for NCr-1 and Bioch_2 treated seedlings. No differences were observed with the application of the other three bacteria considered. Leaf projection area was the last parameter evaluated. Seedlings inoculated with PVr_9 and NCr-1 were able to significantly increase the rosette area with respect to the control conditions, indicating a more developed rosettes as shown in **Figure 16A, B**.

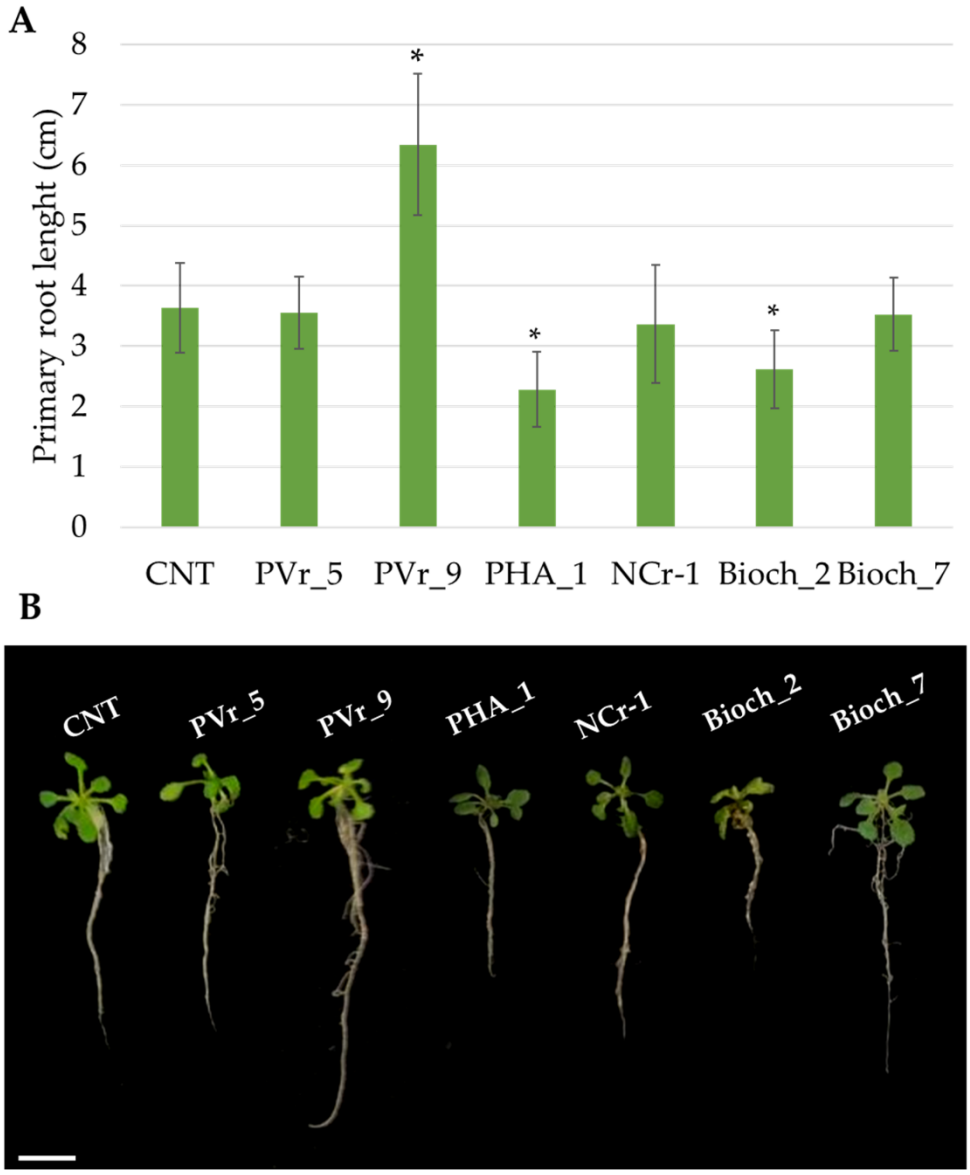

***Figure 14**. Effect of bacterial inoculation on Arabidopsis 14-day-old seedling morphological traits. (A) Primary root length, expressed in cm; **(B)** Representative photograph of difference in primary root length of uninoculated (CNT) and inoculated plants. Data presented are means of 30 biological replicates ± standard deviation (S.D.). Asterisks indicate statistically significant differences between control condition (not inoculated) and treatments (inoculated), according to ANOVA and Tukey's test ($p < 0.05$).*

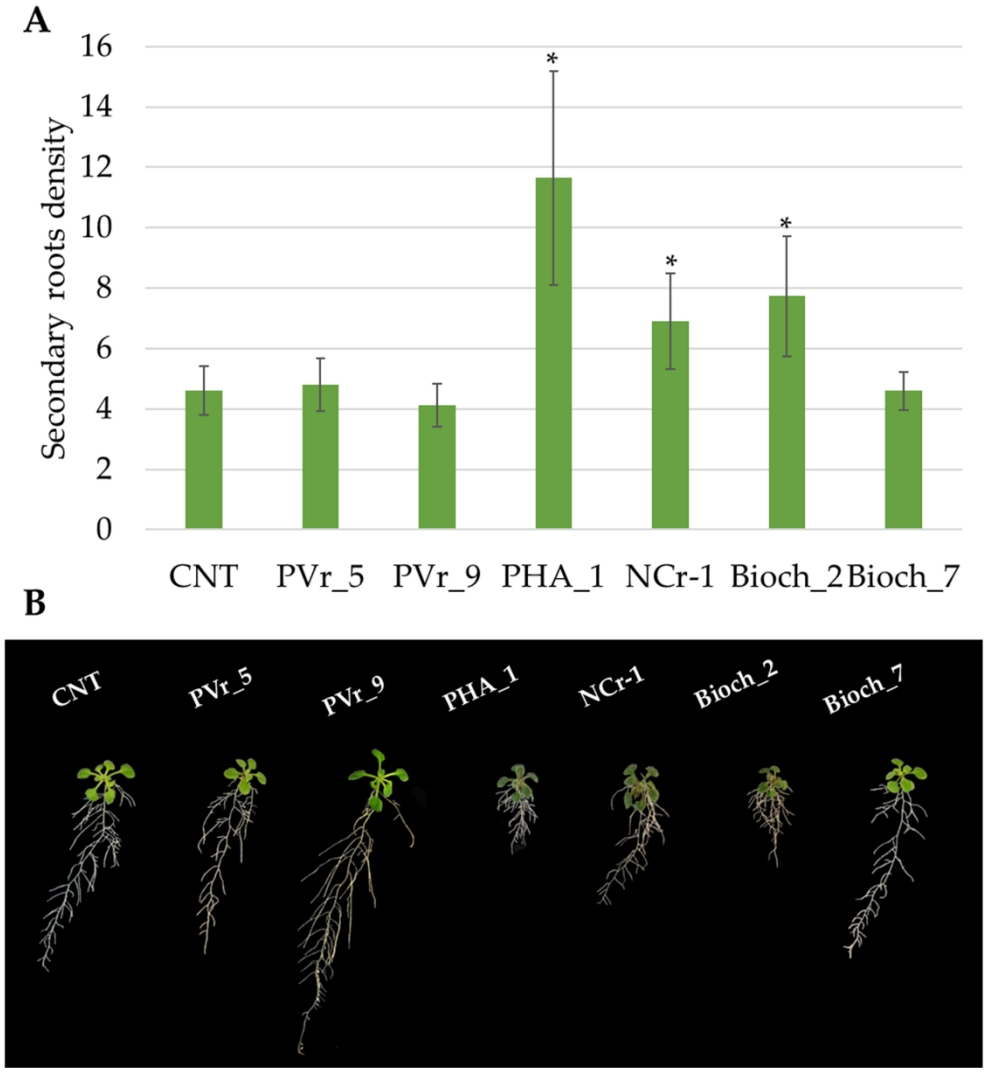

Figure 15. Effect of bacterial inoculation on Arabidopsis 14-day-old seedling morphological traits. *(**A**) secondary roots density, expressed as number of secondary roots per cm of primary root;* ***(B)*** *Representative photograph representing difference in secondary roots density of uninoculated (CNT) and inoculated plants. Data presented are means of 30 biological replicates ± standard deviation (S.D.). Asterisks indicate statistically significant differences between control condition (not inoculated) and treatments (inoculated), according to ANOVA and Tukey's test ($p < 0.05$).*

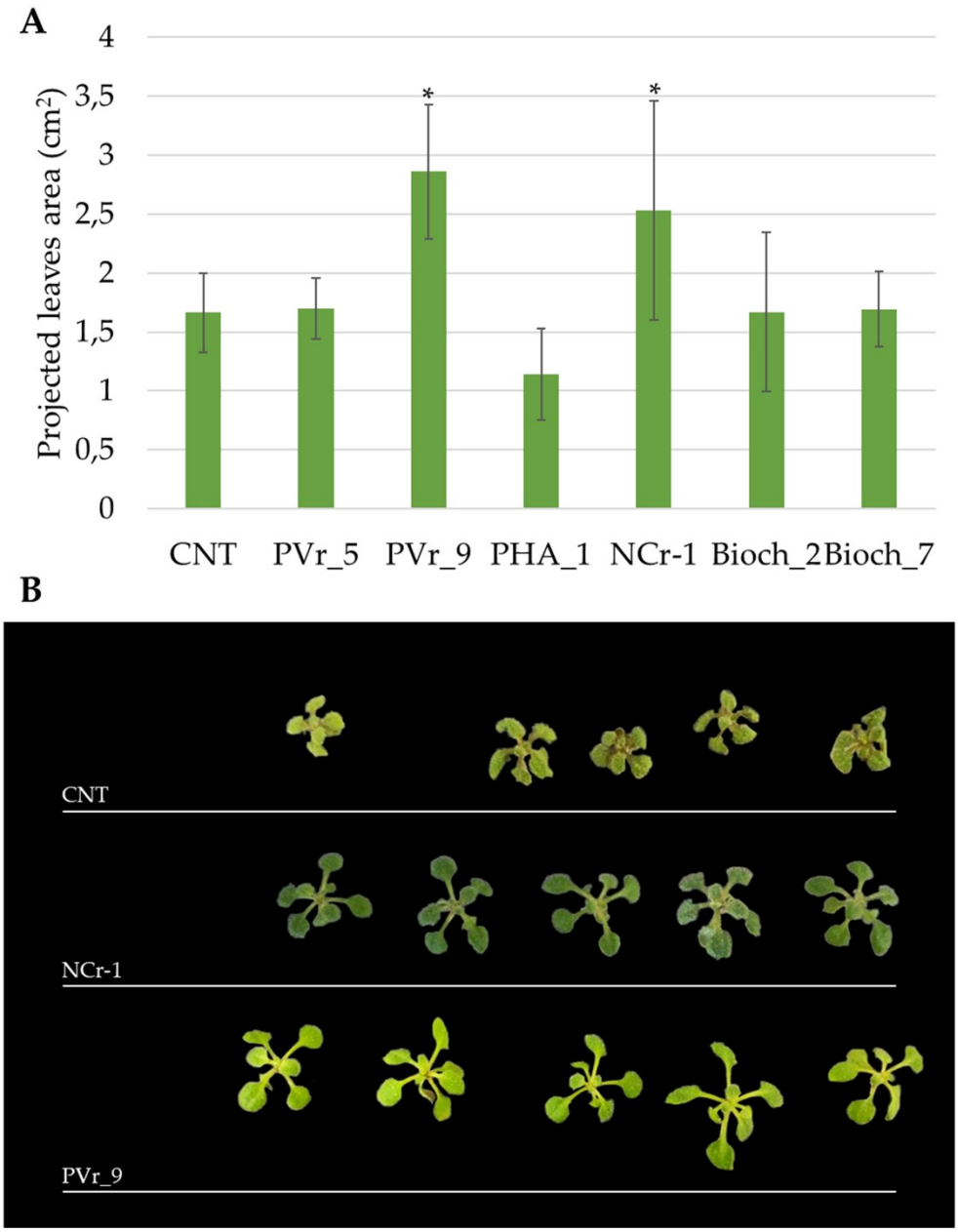

Figure 16. Effect of bacterial inoculation on Arabidopsis 14-day-old seedling morphological traits. (A) *Projected leaves area, expressed in square cm.* ***(B)*** *Example image representing difference in projected leaves area of uninoculated (CNT) and inoculated plants. Data presented are means of 30 biological replicates ± standard deviation (S.D.). Asterisks indicate statistically significant differences between control condition (not inoculated) and treatments (inoculated), according to ANOVA and Tukey's test ($p < 0.05$).*

3. Antifungal activity against selected phytopathogenic fungi

To determine direct antifungal activity, the selected bacterial strains were tested against the three different phytopathogenic fungal species *Aspergillus flavus*, *Fusarium verticillioides* and *Fusarium proliferatum.*

The antifungal potential was assayed by two methods using the bacterial cells in a co-inoculation test with the fungal spores or by cultivating the different fungal strains in a medium administrated with the filtered bacterial culture broth (**Figures 17 and 18**). Bacteria were tested at two different concentrations (2.5 or 5 x 10^3 cells/well).

PVr_9 and PHA_1 showed the highest antifungal activity at both cellular concentrations used against *A. flavus*, which reached 100% inhibition (**Figure 17A**). The same impressive antifungal activity was also observed against the other two fungal species investigated, *F. verticillioides* and *F. proliferatum* (**Figure 17B, C**). A 40% inhibition of *A. flavus* growth was observed with co-inoculation of 5 x 10^3 Bioch_7 cells (**Figure 17A**), and the same percentage of growth inhibition was observed with co-inoculation of both concentrations of Bioch_7 in *F. proliferatum* (**Figure 17C**). The highest bacterial cells concentrations of Bioch_7 was also effective in reducing the *F. verticillioides* growth of around 20% (**Figure 17B**). Co-inoculation of 5 x 10^3 cells of NCr-1 and Bioch_2 determines an inhibition of 20% and 35% in *F. verticillioides,* respectively, and an inhibition of around 50% and 40%, respectively, with both the concentrations in *F. proliferatum* (**Figure 17B, C**). When co-inoculating the cellular concentration of PVr_5, the growth of *F. proliferatum* was impaired but not the other fungal species (**Figure 17**). No antifungal activity was found for PVr_5, NCr-1 and Bioch_2 against *A. flavus* (**Figure 17**).

The antifungal effect of the bacterial broths was assayed by adding 25 and 50 % (v/v) of bacterial broth to the fungal culture medium, and 5 x 10^3 conidia/well were inoculated. Through the administration of the filtered bacterial broth, PVr_9 was

shown to be the only bacterium able to interfere with all the three fungal species growth (**Figure 18**).

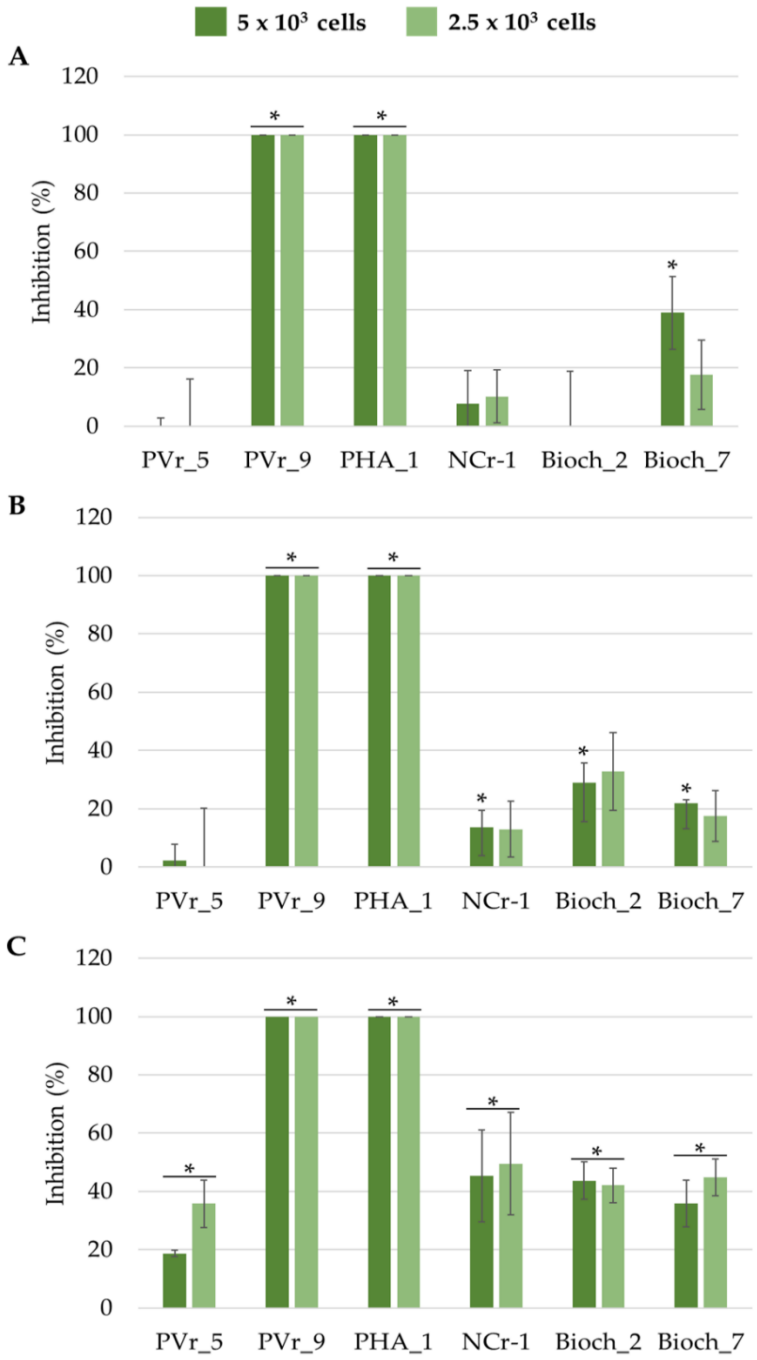

Figure 17. Antifungal activity of bacteria co-inoculum against A. flavus (A), F. verticillioides (B), and F. proliferatum (C). *Concentrations of 5 or 2.5 × 10³ bacterial cells were co-inoculated with 5 × 10³ of fungal conidia. Data are presented as percentage inhibition with respect to the control (only fungal cultures) and are the means of six biological replicates ± standard deviation (S.D.). Asterisks indicate statistically significant differences between control and co-inoculated cultures according to ANOVA and Tukey's test ($p < 0.05$).*

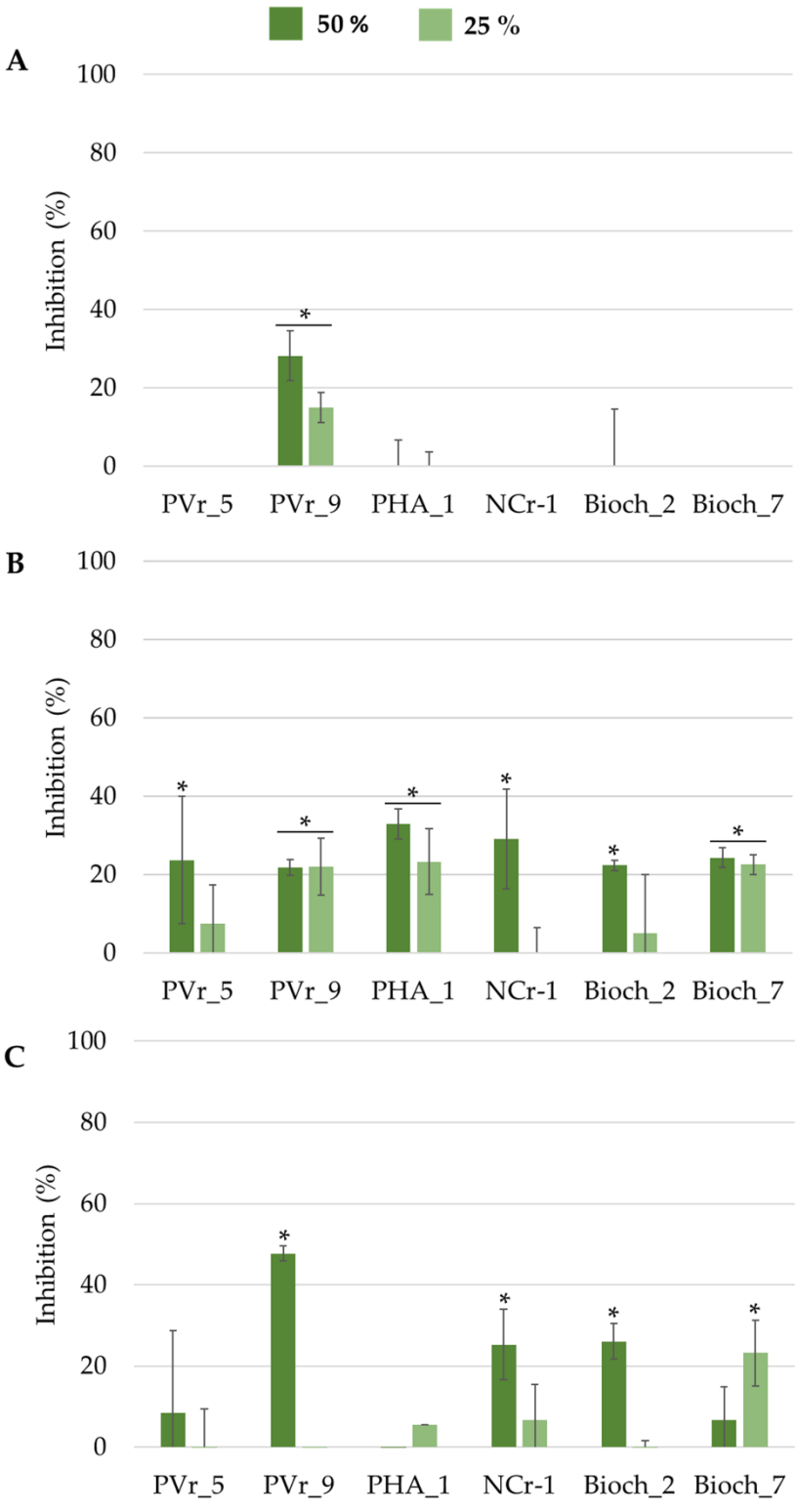

Figure 18. Antifungal activity of filtered bacterial culture broth against A. flavus (A), F. verticillioides (B), and F. proliferatum (C). *Cultures of 5 × 10³ of fungal conidia/well were amended with 25 or 50% bacterial broth. Data are presented as percentage inhibition with respect to the control (fungal cultures only) and are the means of six biological replicates ± standard deviation (S.D.). Asterisks indicate statistically significant differences between control and co-inoculated cultures according to ANOVA and Tukey's test ($p < 0.05$).*

Amending the medium with 50% or 25% of filtered culture broth, the inhibition of *A. flavus* was around 30% and 15%, respectively. At both broth concentrations used, 20% inhibition was observed against *F. verticillioides,* while around 50% inhibition of mycelium growth was observed when 50% of broth was added to *F. proliferatum* (**Figure 18B, C**). The PHA_1 broth was effective in reducing the mycelium development of *Fusarium verticillioides*: 35% and 20% inhibition was evidenced through the administration of 50% and 25% of broth (**Figure 18B**), respectively, while no effect was exerted on *F. proliferatum* (**Figure 18C**). Approximately 30 % inhibition of the growth of the two *Fusarium* fungi was achieved by amending the culture medium with 50% of NCr-1 and Bioch_2 filtrates (**Figure 18B, C**). Interestingly, 25% of Bioch_7 broth resulted in a stronger inhibition effect on *F. proliferatum* with respect to the higher concentration (50%), a peculiarity that could be attributed to a combined effect of specific and nonspecific inhibitors that differentially act on mycelium development (**Figure 18C**). Finally, a 20% inhibition of *F. verticilliodes* growth was recorded when using Bioch_7 filtered culture broth at every percentage, while the same percentage of inhibition was observed by 50% Pvr_5 filtered culture broth (**Figure 18B**).

4. PVr_9 salt tolerance induction in *A. thaliana*

From the analyses reported, PVr_9 bacterial strain was the one showing higher PGP potential than all the other bacteria tested. PVr_9 was capable of increasing the root apparatus of *A. thaliana* and the above ground biomass, as well as capable of inhibiting three phytopathogenic fungi tested. For these reasons, PVr_9 was selected for further analyses directed at evaluating its potential to alleviate salt stress, an abiotic stress of extreme relevance.

4.1 Effect of NaCl on PVr_9 growth

To understand PVr_9 salt tolerance, a growth curve of bacterial strain subjected to 0, 100 or 200 mM NaCl was carried out. The analysis was conducted measuring the OD_{600} at time 0, 14, 19, 24 and 38 hours after inoculation of the bacterial cells in 20 mL of SMS liquid medium. As shown in **Figure 19**, no difference in bacterial growth was observed between controls and both NaCl conditions after 14 hours. Starting at 19 hrs the presence of sodium chloride (both 100 and 200 mM NaCl) in the medium results in a greater bacterial growth than in controls. At 38 hrs, bacterial growth is greater at 200 mM NaCl than at 100 mM NaCl and under control conditions.

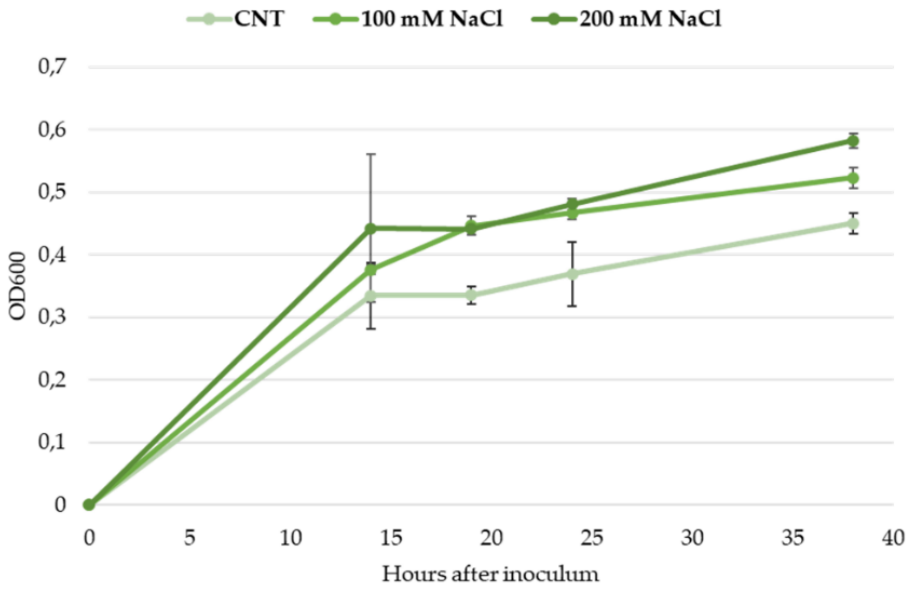

Figure 19. Growth curve of PVr_9 grown in a SMS medium of varying NaCl concentration.

4.2 The effects of PVr_9 on morphological parameters of *A. thaliana* under different NaCl conditions

To evaluate the ability of PVr_9 to reduce the deleterious effects of salt on *A. thaliana*, analyses were conducted on two different morphological parameters (primary root length inhibition and projected rosette area reduction) in seedlings grown in the presence of three different concentrations of NaCl (75, 100 and 150 mM NaCl) for four days.

At the concentrations of 75 mM and 100 mM NaCl, no significant differences were observed in the inhibition of the primary root growth between inoculated and uninoculated plants. On the contrary, at 150 mM NaCl the inhibition of primary root was around 30% in uninoculated plants, while in PVr_9 inoculated plants the inhibition was only around 5% (**Figure 20A, B**). Similarly, at the concentrations of 75 mM and 100 mM NaCl, no differences were observed in the rosettes of inoculated and uninoculated plants, while at 150 mM NaCl, uninoculated *Arabidopsis* plants showed a reduction in the projected area of around 45%, while those inoculated with PVr_9 showed a reduction of around 30% (**Figure 21A, B**). These results allow us to select the concentration of 150 mM NaCl for the following analyses. Furthermore, as shown in the example **Figure 21B (c,d)**, the rosettes of plants grown in 150 mM NaCl and inoculated generally appear healthier than plants grown in 150 mM NaCl and uninoculated. In fact, rosettes of stressed and uninoculated plants showed the first signs of chlorosis and yellowing, as well as increased stiffening of the leaves that probably results in the contraction evident as a reduction in projected area.

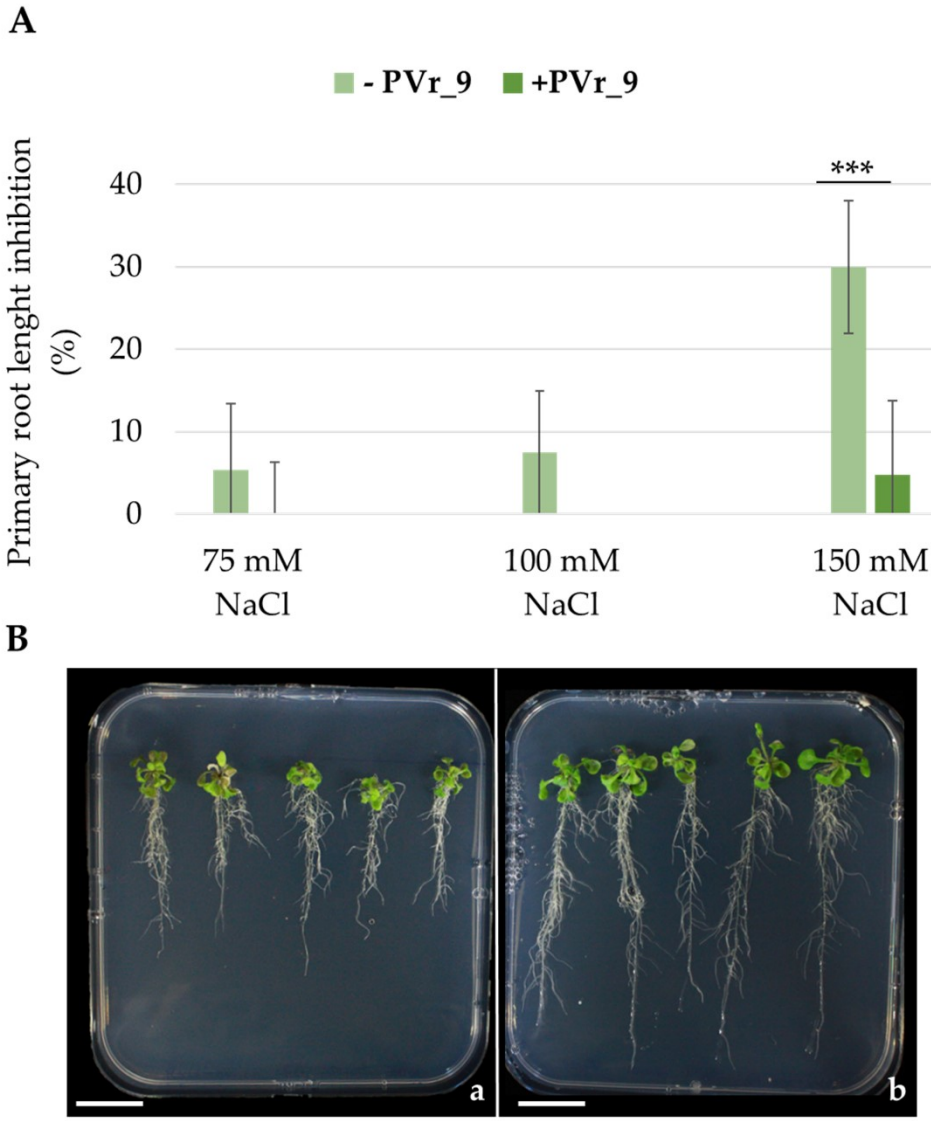

Figure 20. Effects of PVr_9 capacity in reducing Arabidopsis NaCl detrimental effects on root elongation. ***(A)*** *Ability of PVr_9 to reduce the negative effects of 75, 100 and 150 mM NaCl on the primary root growth after four days of treatment.* ***(B)*** *Representative photographs of A. thaliana plants not inoculated* ***(a)*** *or inoculated* ***(b)*** *with PVr_9 after four days of 150 mM NaCl treatment. Data were means of 30 plants per treatment ± S.D. and are calculated by the differences between the root length of stressed and not stressed plants, then converted to percentage. Asterisks indicate significant differences between plants inoculated or not with PVr_9 (One-way ANOVA and Tukey's test, *** $p < 0.001$). White bars correspond to 1 cm.*

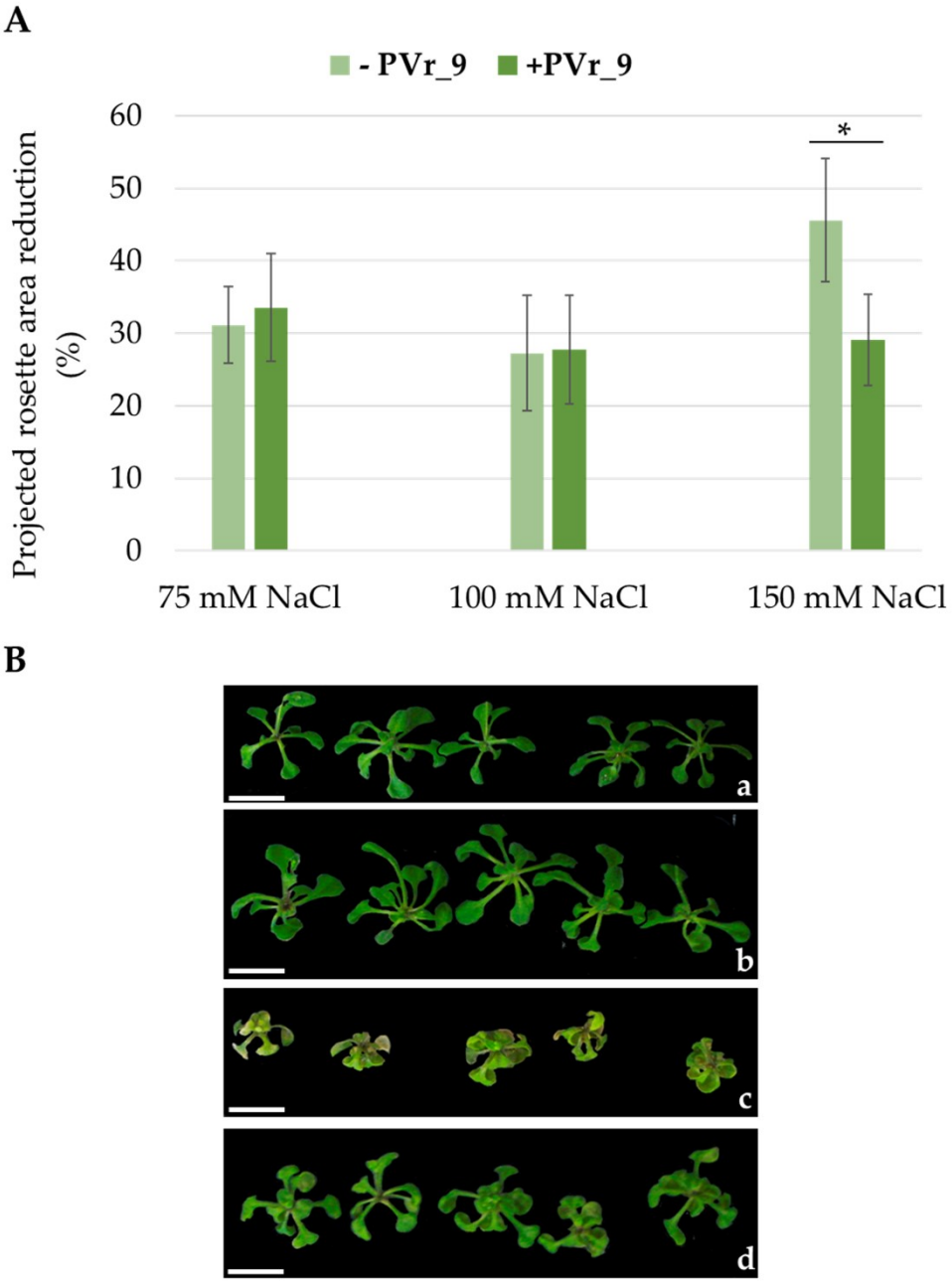

Figure 21. PVr_9 capacity in reducing NaCl detrimental effects on Arabidopsis rosette. ***(A)*** *Ability of PVr_9 to alleviate negative symptoms on rosette of Arabidopsis plants grown at 75, 100 and 150 mM NaCl for four days.* ***(B)*** *Representative photographs of rosette of A. thaliana plants inoculated or not with PVr_9;* ***(a)*** *CNT,* ***(b)*** *PVr_9,* ***(c)*** *150 mM NaCl,* ***(d)*** *150 mM NaCl + PVr_9. Data were means of 30 plants per treatment ± S.D. and are calculated by the differences between the rosette area of stressed and not stressed seedlings, then converted to percentages. Asterisks indicate significant differences between plants inoculated and not inoculated with PVr_9 grown at different NaCl concentration (One-way ANOVA and Tukey's test, * $p < 0.05$). White bars in photographs correspond to 1 cm.*

4.3 The effects of PVr_9 on ROS, 8-oxo-dG, osmolyte and ABA accumulation in *A. thaliana* under 150 mM NaCl

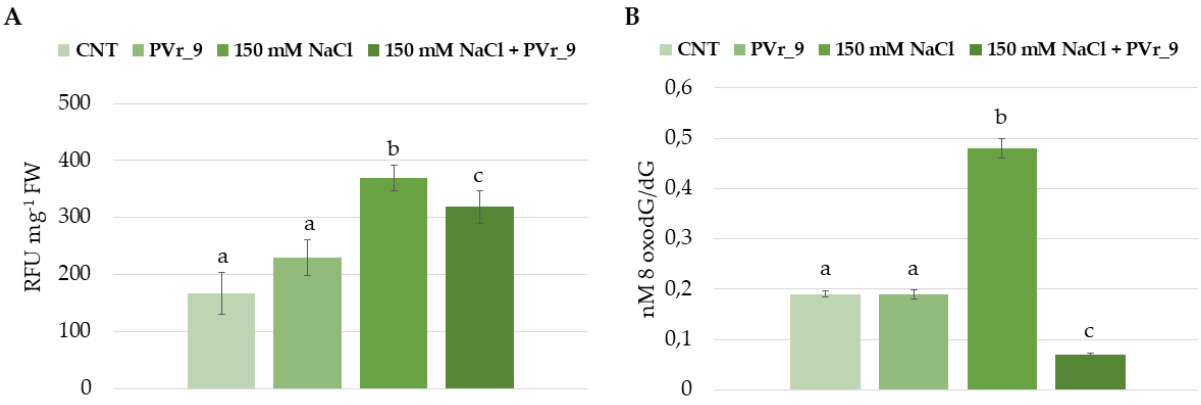

Figure 22. Oxidative stress markers accumulation. ***(A)*** *ROS content and in A. thaliana leaves exposed to 0 mM and 150 mM of NaCl, inoculated or not with PVr_9 for 4 days. Data were expressed as mean of n=6 biological replicates ± S.D. Different letters represent significant differences between treatments (Two-way ANOVA and Tukey's test, $p < 0.05$).* ***(B)*** *Quantification of 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxodG) on 4 µg of DNA digested to its individual nucleosides. Data were expressed as mean of n=6 biological replicates ± S.D. Different letters represent significant differences between treatments (Two-way ANOVA and Tukey's test, $p < 0.05$); Relative Fluorescence Units (RFU).*

No difference in ROS accumulation was observed between plants inoculated and uninoculated with PVr_9 grown under control conditions. Salt stress increased ROS content, but uninoculated plants showed a significantly higher level of ROS accumulation, than those inoculated with PVr_9 (**Figure 22A**).

As showed in **Figure 22B**, the 8-oxo-dG/dG ratio was significantly higher in the plant exposed to 150 mM NaCl, while no differences were observed between the uninoculated and inoculated plants under control conditions. Interestingly, the 8-oxo-dG/dG ratio in stressed plants inoculated with PVr_9 was significantly lower than that of uninoculated plants grown on 150 mM NaCl.

Differences in proline and soluble sugars were also determined between *A. thaliana* grown at 0 and 150 mM NaCl, inoculated or not with PVr_9, and the same

trend of ROS quantification was observed. No differences were observed in the accumulation of compatible solutes in plants grown under control conditions. In contrast, at 150 mM the accumulation of proline and soluble sugars increased in plants grown in salt conditions, but in uninoculated plants were significantly higher respect to PVr_9 inoculated ones (**Figure 23A, B**).

As shown in **Figure 24**, among plants grown under normal condition both uninoculated and inoculated with PVr_9, a low level in ABA contents was observed. As observed for the other physiological markers, salt induces an increase in ABA content in both leaves and roots, however, the inoculation with PVr_9 significantly decreased the ABA content of seedlings by around 60% in the leaves, and approximately 30% in the roots, compared to uninoculated plants. The reduction of ABA accumulation in seedlings grown under salt stress conditions in the presence of PVr_9 indicates a possible role for this strain in limiting the perception of salt stress by plants.

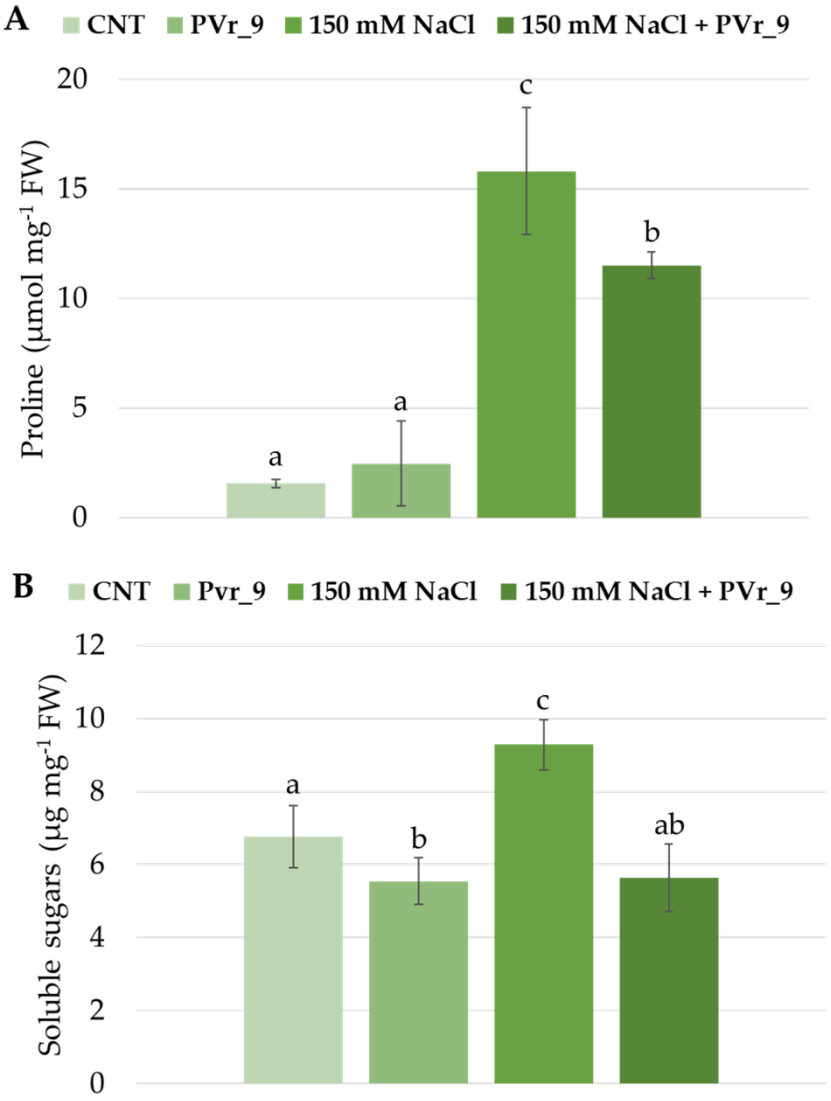

Figure 23. Content of compatible solutes. ***(A)*** *Proline content in A. thaliana leaves exposed to 0 mM and 150 mM of NaCl, inoculated or not with PVr_9 for 4 days. Data were expressed as mean of n=6 biological replicates ± S.D.* ***(B)*** *Soluble sugars in A. thaliana leaves (exposed to 0 mM and 150 mM of NaCl, inoculated or not with PVr_9 for 4 days. Data were expressed as mean of n=6 biological replicates ± S.D. Different letters represent significant differences between treatments (Two-way ANOVA and Tukey's test, $p < 0.05$).*

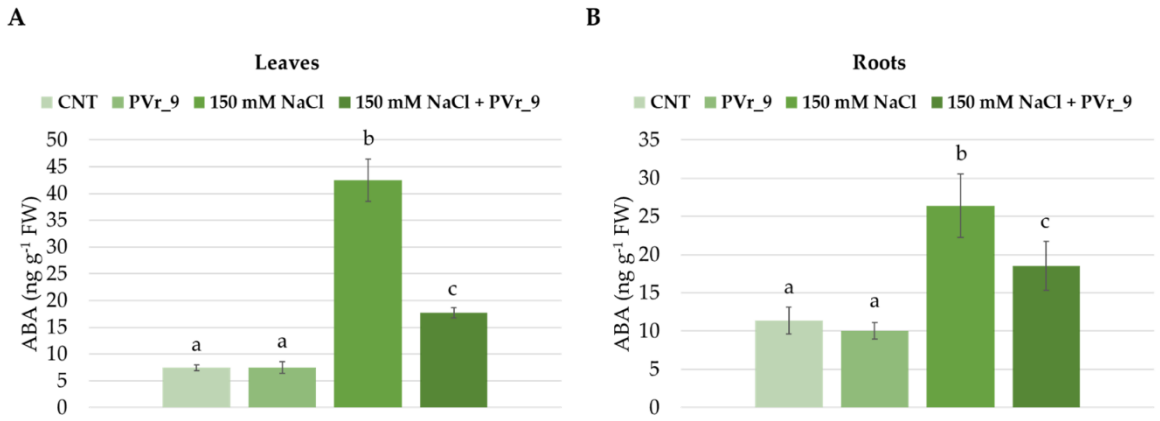

Figure 24. Hormonal content. ***(A)*** *ABA content in leaves of A. thaliana plants exposed to 0 mM and 150 mM of NaCl, inoculated or not with PVr_9 for 4 days.* ***(B)*** *ABA content in roots of A. thaliana plants exposed to 0 mM and 150 mM of NaCl, inoculated or not with PVr_9 for 4 days. Data were expressed as mean of n=3 biological replicates ± S.D. Different letters represent significant differences between treatments (Two-way ANOVA and Tukey's test, $p < 0.05$).*

4.4 The effects of PVr_9 on selected ions contents in A. *thaliana* plants

The content of Na^+, K^+, P, Fe and Ca^{2+} was evaluated in *A. thaliana* plants inoculated or not with PVr_9 under control and 150 mM NaCl conditions to determine whether the presence of the bacterium caused a change in the uptake of different ions. After 4 days of treatment, no difference in the content of selected ions in *A. thaliana* plants was appreciable, although a slight decrease in Na^+ was observed in inoculated vs. uninoculated plants at 150 mM NaCl (**Figure 25A**). However, when the Na^+ content distribution between leaves and roots was analysed, a slight but significant difference was observed. Indeed, in *Arabidopsis* salt stressed seedlings inoculated with PVr_9, increased compartmentalization of Na^+ ions in roots was observed relative to not inoculated **(Figure 25B).**

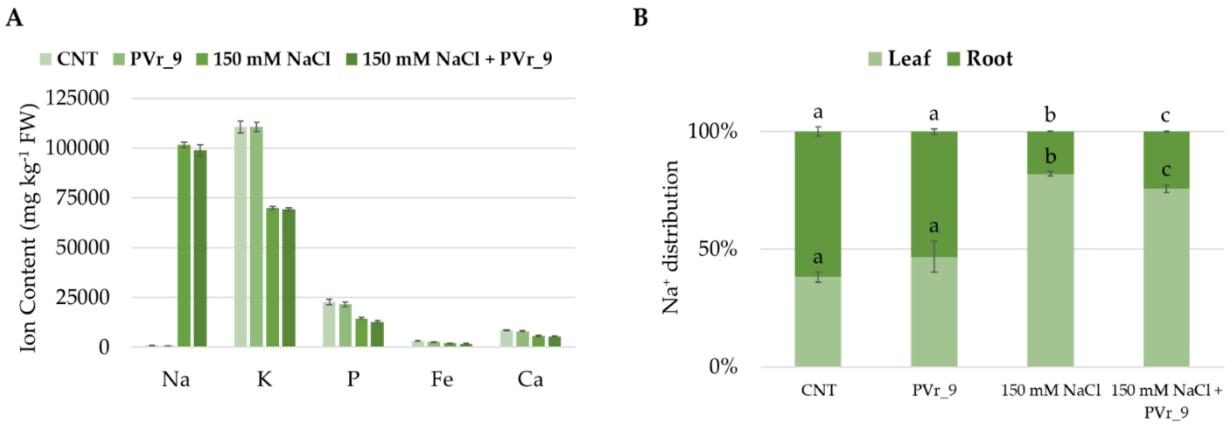

Figure 25. Ions content. ***(A)*** *Na^+, K^+, P, Fe, Ca^{2+} content in A. thaliana grown for 4 days at 0 mM NaCl and 150 mM NaCl, inoculated or not with PVr_9.* ***(B)*** *content of Na^+ in the leaves of A. thaliana grown for 4 days at 0 mM NaCl and 150 mM NaCl, inoculated and not inoculated. Data were expressed as means ± S.D. (n=6). Asterisks represent significant differences between plants grown on 0 mM and 150 mM NaCl, inoculated, and not inoculated (Two-ways ANOVA and Tukey's test, * $p < 0.05$).*

4.5 Gene expression analyses

To investigate the molecular mechanisms involved in alleviation of salt stress symptoms in *A. thaliana* by PVr_9, *SOS1*, *HKT1* and *NHX1* genes, related to salt tolerance, were chosen and analysed trough qRT-PCR both in roots and in shoots in PVr_9 inoculated and uninoculated plants, in control condition and after 24 and 48 hrs of 150 mM NaCl treatments.

In roots, at 24 hrs, *SOS1* expression was increased by 2-fold in PVr_9 inoculated plants and in uninoculated treated plants, while in the roots of PVr_9 inoculated *A. thaliana* seedlings treated with 150 mM NaCl for 24 hrs an increase of 5-fold of the *SOS1* transcript was observed. After 48 hrs, *SOS1* increase in expression less than 1-fold both in uninoculated and inoculated plants under 150 mM NaCl treatment compared to the corresponding controls and no significant differences were observed (**Figure 26A**). In the leaves, after 24 hrs, PVr_9 induces a decrease in *SOS1* expression both under control and stress conditions. Instead, a slight increase in *SOS1* transcript was observed in uninoculated stressed seedlings. After 48 hrs, there was still a decrease in *SOS1* expression in PVr_9 inoculated plants relative to those uninoculated

ones under control conditions. Salt induced a 2-fold up-regulation of *SOS1* in the leaves of uninoculated *A. thaliana*, while a more-evident up-regulation of 3-fold was observed in inoculated plants (**Figure 26B**). At 24 hrs, *NHX1* gene transcription was not modulated by PVr_9 in roots under control conditions, while an increase in the steady state level less than 2-fold was observed at 24 hrs in roots of inoculated and salt stressed plants. A surprising down-regulation of *NHX1* transcription was observed in uninoculated stressed seedlings roots. No differences were observed after 48 hrs in *NHX1* expression levels in the four conditions analysed (**Figure 26A**). In the leaves of seedlings treated for 24 hrs a down-regulation was observed in PVr_9 inoculated plants grown in the absence or in the presence of 150 mM NaCl with respect to the control condition and to non-inoculated plants treated with NaCl. At 48 hrs, in the leaves, the decrease in *NHX1* expression in PVr_9 inoculated plants was maintained, but salt treatment induced a significant increase of 1.5-fold and 2.5-fold of the *NHX1* transcript in stressed plants, in the absence or presence of PVr_9, respectively (**Figure 26B**). Regarding *HKT1*, no differences were observed at 24 hrs in the roots between uninoculated and inoculated plants under control condition. On the other hand, a decrease in the level of *HKT1* transcript was observed in plants treated with 150 mM NaCl, in fact, the uninoculated plants showed a 1-fold reduction in transcription, while the inoculated plants showed about a 4-fold reduction. At 48 hrs, a significant reduction in the level of *HKT1* transcript was observed in the roots of uninoculated and inoculated plants with a 1-fold and a 4- fold reduction, respectively (**Figure 26A**). In the leaves of plants grown under 150 mM NaCl, at 24 and 48 hrs, the *HKT1* transcript is always lower than in the other conditions examined both in the roots and in the leaves, and no differences were observed between inoculated and uninoculated seedlings. PVr_9 induces a significant down-regulation of *HKT1* compared to the control, excluding the observation made in the leaves after 48 h of stress, where the transcription level is the same as the control (**Figure 26B**).

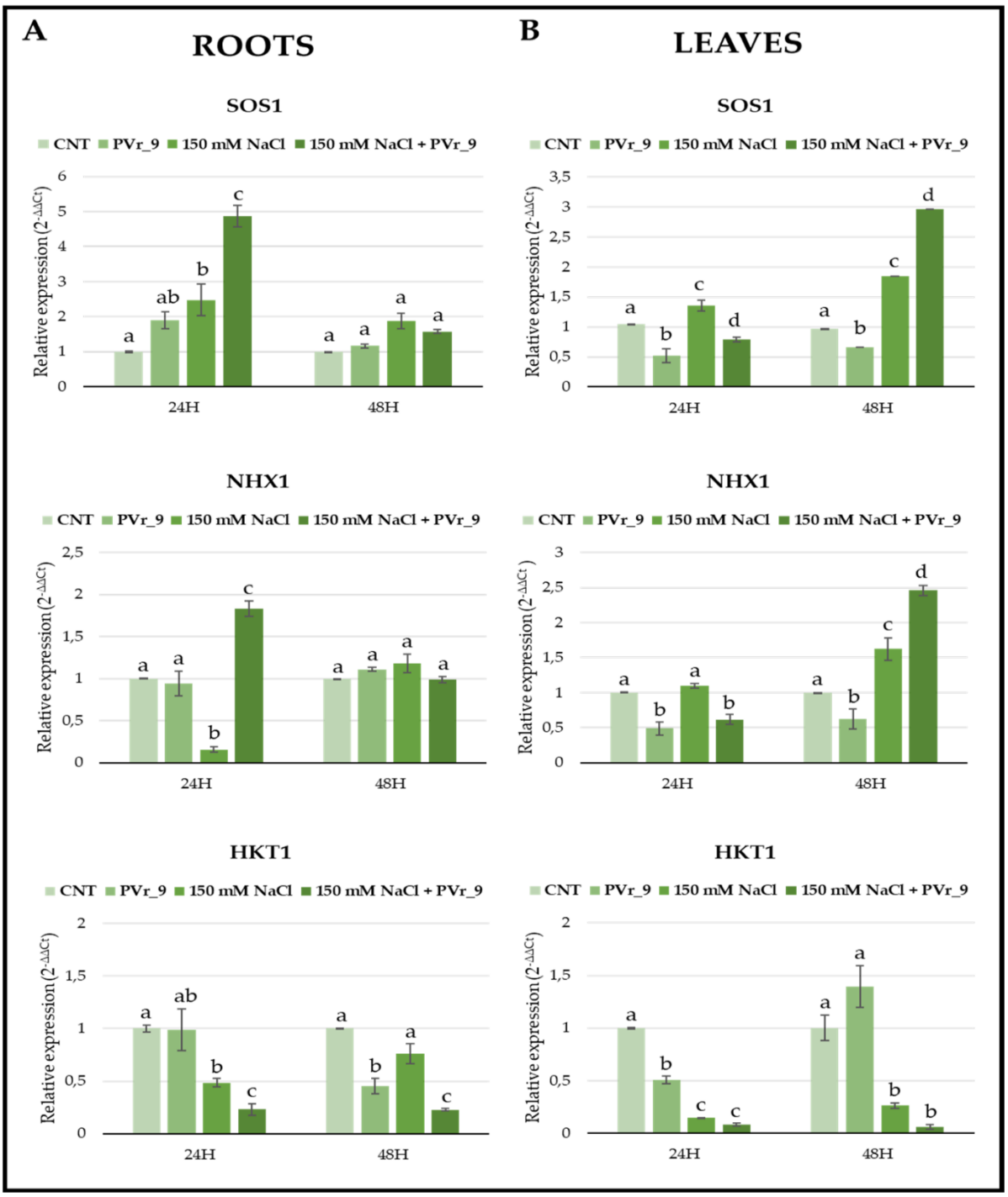

Figure 26. qRT-PCR determinations of relative expression levels of A. thaliana genes: SOS1 *(Salt Overly Sensitive 1);* ***HKT1*** *(High-Affinity K+ Transporter1);* ***NHX1*** *(Sodium Hydrogen Exchanger 1) in roots* ***(A)*** *and leaves* ***(B)*** *of A. thaliana plants treated with 0 mM NaCl or 150 mM NaCl inoculated or not inoculated with PVr_9. RNA was extracted after 24h and 48 h of treatment. Data are means of n=3 biological replicates ± S.D Different letters represent significant differences between treatments (Two-way ANOVA and Tukey's test, p < 0.05).*

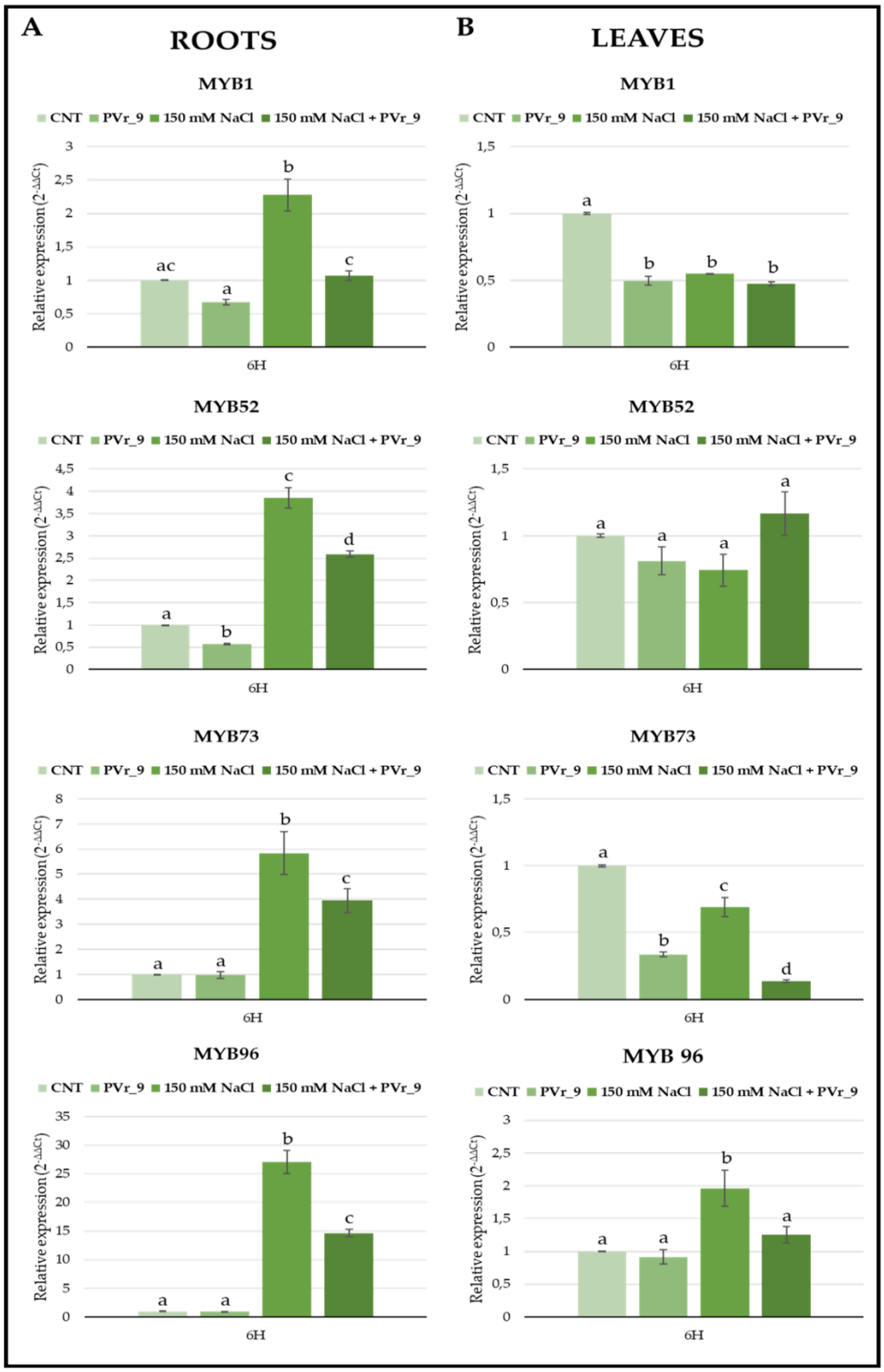

Figure 27. qRT-PCR determinations of relative expression levels of A. thaliana MYB (MYB domain protein) genes: *MYB1; MYB52; MYB73; MYB96 in roots* ***(A)*** *and leaves* ***(B)*** *of A. thaliana plants treated with 0 mM NaCl or 150 mM NaCl inoculated or not inoculated with PVr_9. RNA was extracted after 6 h of treatment. Data are means of n=3 biological replicates ± S.D Different letters represent significant differences between treatments (Two-way ANOVA and Tukey's test, $p < 0.05$).*

The expression analysis of four different *MYB* genes involved in the salt stress response was also analysed in both roots and in shoots in PVr_9 inoculated and non-inoculated plants in control condition and after 6 hrs of 150 mM NaCl treatments.

In roots, analysis of gene expression reveals a common trend in *MYB1*, *MYB73* and *MYB96* genes. Indeed, except for *MYB52*, where PVr_9 inoculation induced a 1-fold decrease in the transcription level with respect to the control, the *MYB1*, *MYB73* and *MYB96* transcripts showed the same abundance in both PVr_9 inoculated plants and uninoculated ones under control conditions (**Figure 27A**). An increase in the transcript level was observed always in roots of plants treated with 150 mM NaCl and 2-fold for *MYB1*, 4-fold for *MYB52*, 6-fold for *MYB73* and 25-fold for *MYB96* with respect to control condition. Furthermore, in PVr_9 inoculated salt stressed plants, the level of *MYB1* is the same of the control while the other *MYB* genes showed a decrease in expression levels with respect to plants grown on 150 mM NaCl which were significantly lower than those of non-inoculated stressed plants (*MYB52* 2.5-fold; *MYB73* 4-fold; *MYB96* 15-fold) (**Figure 27A**). *MYB1* showed a 1-fold decrease in expression respect to control in all the other condition (**Figure 27B**) while in the leaves no differences in expression levels were found for *MYB52* (**Figure 27B**). PVr_9 inoculation induced a down regulation of *MYB73* both in absence (2.5-fold) and in presence of salt stress (10-fold) while the transcript in uninoculated plants grown on 150 mM NaCl decreased 1.4-fold (**Figure 27B**). The *MYB96* transcript was not modulated by PVr_9 in the absence of salt stress and in PVr_9 inoculated salt stressed plants but increased 2-fold in the leaves of uninoculated plants in the presence of 150 mM NaCl (**Figure 27B**).

5. In-soil analyses of PVr_9 effects in *A. thaliana* under salt stress conditions

The last part of the research was dedicated to the evaluation of PVr_9 ability to determine a reduction in salt stress even in the soil with long-term stress. After 1 month of treatment of plants with 150 mM NaCl, the fresh weight (FW), dry weight (DW), and primary root length were evaluated. Fresh and dry weight were evaluated only for the rosette and the roots were excluded since it was very complicated to remove all the soil residues from the roots that would distort the measurement. As shown in **Figure 28A** no differences were observed for all the treatments, neither in leaves fresh weight nor in leaves dry weight. Leaves of plants grown under not stressed conditions and inoculated with PVr_9 appear to have a higher fresh weight, although this finding is not significant. The primary root of plants grown under 150 mM NaCl and inoculated with PVr_9 appears to be longer than plants not inoculated, even if also in this case the difference is not significant (**Figure 28B**). On the other hand, a significant difference was observed in the primary root length of plants grown in not stressed condition; indeed, the primary root of inoculated plants was longer than that of uninoculated plants, reinforcing the *in vitro* finding of the role of PVr_9 as PGPR in *Arabidopsis* (**Figure 28B, C**).

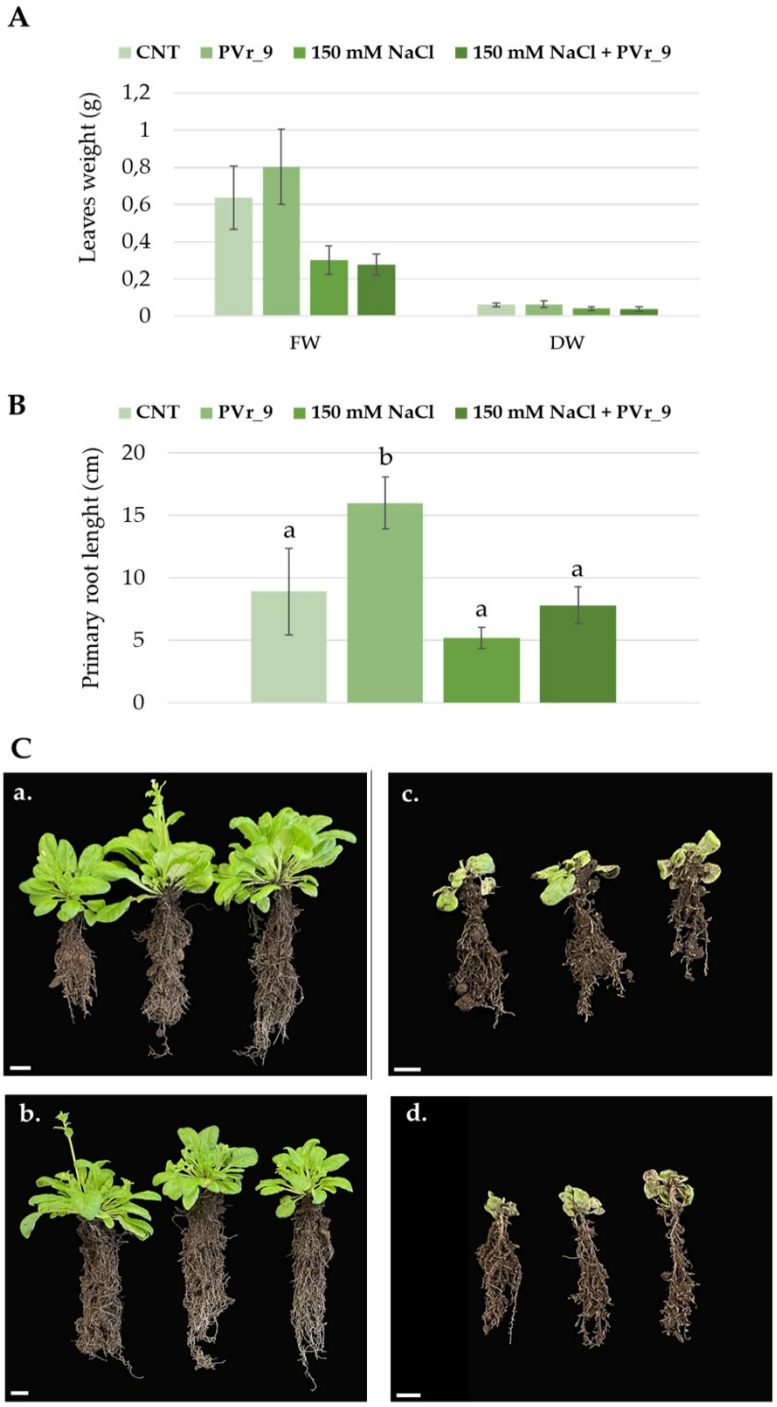

Figure 28. In-soil effects of PVr_9 capacity in reducing Arabidopsis NaCl detrimental effects. ***(A)*** *Fresh and dry weight of A. thaliana leaves inoculated and uninoculated treated with 150 mM NaCl after one month of treatment.* ***(B)*** *Primary root length of A. thaliana inoculated and uninoculated treated with 150 mM NaCl after one month of treatment.* ***(C)*** *Representative photographs of A. thaliana plants inoculated or not with PVr_9;* ***(a)*** *CNT,* ***(b)*** *PVr_9,* ***(c)*** *150 mM NaCl,* ***(d)*** *150 mM NaCl + PVr_9. Data were means of n=6 plants per treatment ± S.D. Different letters represent significant differences between treatments (Two-way ANOVA and Tukey's test, $p < 0.05$). White bars in photographs correspond to 1 cm.*

DISCUSSION

1. Important features of soil PGP bacteria for plants health

The beneficial interaction between plants and rhizosphere bacteria has long been recognized as they perform multiple growth-promoting, health-protecting, and phytopathogen-protecting activities in various economically relevant crops. Many PGPR show similar activities, but some have more than one beneficial characteristic for the plant. Since different species of PGPR from the same genus often exhibit different interactions with the phytosphere, and the nature and mechanisms of these beneficial interactions have not yet been fully elucidated (Olanrewaju et al., 2017; Sessitsch et al., 2019), it is very desirable to study and characterize new strains, as well as to study the different mechanisms that govern any beneficial effect.

With this aim, in this study six bacterial strains were selected and analysed from a previously described panel of bacterial isolates from the rhizosphere of different plants grown in harsh and stressful environments or the surrounding soil. The search for plant-beneficial bacteria in harsh environments holds great promise because bacterial populations in these areas are under evolutionary pressure and have evolved adaptive characteristics that result in more efficient host plant responses to stress than populations of plants grown in more favourable environments (Fierer, 2017). Furthermore, the screening of drought-tolerant plants and halophytes inhabiting arid and salty areas allowed the identification of bacterial isolates with interesting biocontrol and contrasting abiotic stress traits. (Leontidou et al., 2020), validating the exploration of similar, extreme environments as a rewarding strategy for the individuation of PGP strains.

The panel of bacteria used in this study was composed of bacteria isolated from different harsh environments, identified at the genera and species level by 16S rDNA sequencing, and only partially characterized for some putative PGPR properties: PVr_5 and PVr_9 homologous to *Paenarthrobacter ureafaciens* and *Beijerinckia fluminensis* respectively, were isolated from the rhizosphere of *Pteris*

vittata, an As-hyperaccumulating fern that naturally grows in arsenic contaminated soils (Antenozio et al., 2021); PHA_1, homologous to *Pseudomonas protegens*, from a soil rich in hydrocarbons (Rizzo et al., 2020); NCr-1, belonging to *Arthrobacter* genera and found to be an endophyte, from the roots of the Ni hyperaccumulator *Noccaea caerulescens* (Visioli et al., 2014); Bioch_2 and Bioch_7, homologous to *Arthrobacter defluii* and *Arthrobacter nicotinovorans*, respectively, were isolated from a third-year biochar amended soil (Bertola et al., 2019). The first part of this work focused on a deeper characterization of selected bacterial strains. Research into their plant-protective/promoting characteristics and potentials was conducted, observing the direct effects on the growth parameters of the model plant *A. thaliana* and on selected phytopathogenic fungi. In addition, an attempt was made to identify interesting molecules such as siderophores.

Among the tested strains, Pvr_9 was considered the most interesting due to the PGP characteristics, the important effects shown as both a plant growth promoter and a biocontrol agent against some phytopathogen fungi. Previous molecular identification showed a homology with the bacterial specie *Beijerinckia fluminensis* (Antenozio et al., 2021), belonging to a genus that was still poorly characterized for its putative PGPR properties. Recently, Al-Shwaiman and colleagues (Al-Shwaiman et al., 2022) published a work in which a *Beijerinckia fluminensis* strain BFC-33 was characterised, offering us important confirmation about our previous finding and about the data obtained in this thesis. BFC-33 was found to be a multi stress tolerant bacteria, surviving to NaCl stress and heavy metal (Pb and Cd) stress. In our work PVr_9 tolerated NaCl and was also found to grow up to 100 mM As(V) (Antenozio et al., 2021). Moreover, similar to PVr_9, BFC-33 showed SA production, ACC deaminase activity, siderophores, IAA, antifungal activity, as well as showed PGP activity and induction of tolerance to abiotic stress in *Triticum aestivum*.

On the contrary, PHA_1 belongs to the well-known genus *Pseudomonas*, which includes various interesting species that show microbial biocontrol features

and PGP properties, and has proven to be very versatile and of great agronomic potential. PHA_1 showed a significant increase in the formation of secondary roots of *Arabidopsis.* This data from *A. thaliana* is consistent with recent observation of maize roots inoculated with the *Pseudomonas* PS01 strain (Chu et al., 2020). In some *P. protegens* strains, different PGP traits were also reported, such as IAA production, siderophores and ammonia production, and solubilization of phosphate (Andreolli et al., 2019). PHA_1 also showed interesting properties as a biocontrol agent against tested phytopathogenic fungi. PHA_1 proved to be highly inhibitory on fungal growth, especially when the conidia were forced to germinate in presence of the bacterial cells in co-inoculation assays; in fact, the inhibition reached 100%, regardless of the concentration of bacterial cells. The ability of *Pseudomonas protegens* strains to produce antimicrobial compounds may be suggested, for example, by the presence, in the genome of the strain FD6 of 12 putative gene clusters for secondary metabolites production, including the antibiotics 2,4-diacetylphloroglucinol (2,4-DAPG), pyoluteorin (PLT), and pyrrolnitrin (PRN) (Zhang et al., 2020).

In addition, there are many works that have described *Pseudomonas protegens* as an effective antimicrobial agent. For example, Cesa-Luna and co-workers (Cesa-Luna et al., 2020) evaluated the ability of the *P. protegens* strain EMM-1 against several fungal species, reporting significant activity against *Fusarium* spp. and *Aspergillus* spp. The antimicrobial activity of *P. protegens* strain AS15 was demonstrated against *A. flavus*, whose growth and aflatoxin production were reduced in rice grains after bacterial co-inoculation (Mannaa et al., 2017).

NCr-1, Bioch_2, and Bioch_7 belong to the *Arthrobacter* genus, and PVr_5 to the *Paenarthrobacter* genus, in which many plant endophytes are grouped.

As well as the *Pseudomonas* genus, also the *Arthrobacter* genus is well characterized for the plant growth promoting activity of its relative species; its ability to produce auxins, siderophores, and ACC deaminase, as well as to exert a

P-solubilizing activity, are widely reported, and often associated with a reduction in plant stress when *Arthrobacter* is inoculated.

As an example, *A. nitroguajacolicus* was able to act on maize under salt stress conditions as a PGPR (Safdarian et al., 2019); 29 different isolates belonging to *Arthrobacter* genus were isolated by Tchuisseu Tchakounté et al. (Tchuisseu Tchakounté et al., 2018) and many of them possessed at least one of the tested PGP traits. Kumar et al. (2014) confirmed the presence of PGP traits within the *Arthrobacter* genus because characterization of bacterium *A. chlorophenolicus* has shown its ability to produce NH_3, HCN, N_2 fixation and P-solubilizing capability.

There is increasing interest in siderophore-producing bacteria and siderophore molecules, not only because they play a possible role in the bioavailability of for plant nutrition but also because of their suppressive activity against phytopathogens. All bacteria analysed in our study were siderophore producers and were more or less able to interfere with the development of mycelium. IAA and soil microbial siderophores play an important role in plant iron absorption (Jin et al., 2006).

Pyoverdine, a siderophore produced by *P. fluorescens*, was shown to have an important role in iron uptake of *A. thaliana* (Vansuyt et al., 2007), while Masalha et al. (Masalha et al., 2000) showed the importance of microbial activity for iron acquisition in *Helianthus annuus* and *Zea mays*. The importance of siderophores in inhibiting fungal development was reported in many works. For example, as reported by Ruiz and colleagues (Ruiz et al., 2015), siderophores produced by the *Pseudomonas protegens* Pf-5 can mitigate the toxic effect of fusaric acid produced by the *Fusarium* genus. Suppressing sporulation and fungal growth, siderophores produced by *Pseudomanas syringae* are biologically active against pathogenic plant fungi, including *Fusarium oxysporum* (Yu et al., 2017). For this purpose, the identification of the siderophores produced by bacterial strains could help to better investigate possible molecules involved not only in plant nutrition, but also in bacterial antimicrobial activity against the phytopathogenic fungi tested.

Among the identified molecules showing hydroxamate functional group, desferrioxamine B and asperchrome B were identified. Desferrioxamine B, produced by PVr_9, is the most known and is produced by various bacterial species and fungi. Desferrioxamine B is a linear trilhydroxamic acid siderophore (Codd et al., 2018), able to chelate, not only Fe (III), but also Cu (II), Se (II), Pb (II), Co (III), Mn (III), and Bi (III) (Bellotti and Remelli, 2021). Desferrioxamine B and its chemical derivatives have received much attention because of their biological activity, especially for the applications in the medical field. The utilization of this molecule is useful, for example, in antimalarial prophylaxis, in a strategy based on the use of siderophore-bound antibiotics to facilitate its entry into cells (trojan horse strategy), its use as a fluorescent sensor, and in the treatment of patients with metal poisoning and iron overload (Nagoba and Vedpathak, 2011). PVr_9 was found to produce catecholate siderophore aminochelin. A characterization of the chemical properties of aminochelin was carried out by (Khodr et al., 2002). Aminochelin is a triprotic acid with two chatecol protons and one amine proton, with a simple bidentate structure and high hydrophobicity. This structure enables Fe (III) chelation and to solubilize ferric hydroxides. Finally, the carboxylate siderophore quinolobactin was found, an 8-hydroxy-4-methoxy-2-quinoline carboxylic acid, produced by Bioch_2.

Quinolobactin was identified as a siderophore for *Pseudomonas fluorescens* ATCC 17400 (Mossialos et al., 2000).

Interestingly, salicylic acid (SA) was found to be produced by most of the strains tested. In addition to its use by bacteria to maintain iron-limiting growth conditions (Bakker et al., 2014), SA production was reported to also exert an inhibitory potential against several postharvest pathogens, including *Botrytis cinerea*, *Fusarium oxysporum*, *Penicillium expansum* and *Rhizopus stolonifer* (Da Rocha Neto et al., 2015; Mandal et al., 2009; Panahirad et al., 2012; Zhang et al., 2020).

2. Alleviation of plant salt stress symptoms by plant growth promoting bacteria: the case of PVr_9

The results obtained identified PVr_9 as the most promising bacterium, as it was able to increase *A. thaliana* growth to a greater extent, as well as to interfere with the growth of all the fungi tested, with both methods used. For this reason, the PVr_9 strain was selected to evaluate its ability to reduce salt stress in *A. thaliana* and to identify possible physiological and molecular mechanisms involved in the response.

The ability of the Pvr_9 strain to grow in a NaCl containing medium was also confirmed in our study (**Figure 19**) since 100 mM and 200 mM NaCl did not affect its growth in a minimal liquid medium, also showing greater growth than the control condition. Recently, Al-Shwaiman et al. (2022) demonstrated that the BFC-33 strain homologous to *Beijerinckia fluminensis* acts as a multi-stress tolerant soil bacterium.

Salinity impacts plant development in different ways, such as nutritional impairments, osmotic effects, and Na^+ toxicity. The first macro-effect of salt stress results in growth stunting; in fact, because of salt shock, cells become dehydrated and shrink, although in a short period of time these regain their original volume. Despite this restoration, cell elongation and to a lesser extent cell division are reduced, resulting in a decrease in the growth rate of leaves and roots (Läuchli and Grattan, 2007). As mentioned above, under normal growth conditions, PVr_9 inoculation results in an increased *A. thaliana* growth. Our experimental design was based on the inoculation of the bacterium on the root tip of 7-day-old seedlings and further growth for another 7 days in MS ½ + 1% sucrose medium that allowed proper colonization by PVr_9 of the root; during this time, the bacterium exerted its growth-promoting action on the plants, producing seedlings that were already more developed before transfer to NaCl-containing medium

than those not inoculated. For this reason, to assess the impact of PVr_9 on salt stress tolerance, was assessed the inhibition of primary root growth and the reduction in projected rosette area during the growth period in saline medium, rather than measuring primary root length or rosette growth between control and treatment conditions.

Thus, in an in-plate experiment (**Figure 20A, 20B**) inoculation of PVr_9 results in a significant reduction in *A. thaliana* root growth inhibition induced by 150 mM NaCl compared to that induced in uninoculated plants. In fact, compared to a growth inhibition of about 30% in uninoculated seedlings, the inhibition induced by 150 mM NaCl in seedlings inoculated with PVr_9 is only about 5%. **Figure 21B (c)** shows the visible effects that salt induces on rosettes, which result in remarkable morphological changes. 150 mM NaCl determines a strong folding of the leaves on uninoculated plants, probably due to the strong hardening and loss of flexibility of the leaves, as well as an onset of chlorosis in some leaves. Rosettes of inoculated plants grown in the presence of 150 mM NaCl appear to be less impacted by salt (**Figure 21B (d)**). In these rosettes, although there are some closed leaves in this condition, salt impact is significantly less strong. Indeed, compared to the reduction in the projection of the rosette area between 40% and 50% of inoculated stressed plants, 30% of reduction is detected in those inoculated. In addition, no signs of chlorosis are evident or present to a lesser extent. Therefore, Pvr_9 is capable of supporting the growth of the primary root of *A. thaliana* on 150 mM NaCl as well as reducing the deleterious impact of the salt on the rosettes.

The ability of PGPR to increase plant growth under normal conditions as well as under various abiotic stresses, including salinity, is well documented (Ha-Tran et al., 2021; Ma et al., 2020). A growing body of evidence agrees that plant-PGPR interaction is instrumental in inducing salt stress resistance. The mechanisms underlying salt stress resistance usually involve water and nutrient

uptake, modulation of stomatal conductance, antioxidant enzymes, ion transport, and signal translation proteins (Ilangumaran et al., 2017).

Among PGPR capable of increasing salt stress tolerance, it is not uncommon to find among the features the ability to produce IAA and ACC deaminase. IAA producing bacteria were found to increase the fitness of plants grown under salt stress (Egamberdieva and Kucharova, 2009; Ilangumaran and Smith, 2017; Saghafi et al., 2018). Ethylene, a gaseous hormone, is produced by plants in response to stress conditions, and because it acts as a negative regulator of plant growth, ethylene induces its effects by reducing the growth of roots, causing chlorosis and leaf abscission, suppresses leaf expansion, or promotes epinasty (Abeles et al., 1992; Gamalero and Glick, 2015). PGPR with the ability to produce ACC deaminase use plant ACC as a source of nitrogen and energy in the rhizosphere, divide it into ammonia and α-ketobutyrate and prevent the accumulation of ethylene. For example, ACC deaminase-producing strains increase the yield of maize and improve salt resistance in *Arachis hypogea* plants, as well as in tomato and rice (Bal et al. 2013; Mayak et al. 2004; Nadeem et al., 2009; Saravanakumar and Samiyappan, 2006). Siddikee et al. (2010) reported that the salinity resistant bacteria accompanied by different ACC deaminase producing strains of *Bacillus*, *Brevibacterium*, *Planococcus*, *Zhihengliuella*, *Halomonas*, *Exiguobacterium*, *Oceanimonas*, *Corynebacterium*, *Arthrobacter* and *Micrococcus*, increase plant growth potential under salinity stress (Saghafi et al., 2019).

Salt stress induces an increase in ROS, which can exceed the normal scavenging capacity of the plant, causing an alteration of the cellular redox balance (Noctor et al., 2018; Sharma et al., 2019). In green plants, chloroplast is the major production site of ROS, including superoxide anion (O2•-), hydroxyl radical (HO•), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2). Na+ negatively affects the photosynthetic components and therefore reduces enzyme activities and pigment synthesis. These stressful conditions as well as ROS themselves decrease the CO_2 assimilation rate, and the excess of light absorbed that is not used by the

plant can lead to an increase in ROS production and consequently oxidative stress (Asada, 2006; Ma et al., 2020; Mignolet-Spruyt et al., 2016). ROS react with all biological macromolecules and cause membrane lipid peroxidation, DNA and RNA damage, protein oxidation, and enzymes inhibition (Vinocur and Altman, 2005). Accumulation of ROS can cause crosslinking of phenolics and cell-wall glycoproteins, resulting in cell-wall stiffening (Tenhaken, 2015). PVr_9 inoculated seedlings accumulated fewer ROS under salt stress compared to the uninoculated (**Figure 22A**). The trend between lower ROS accumulation after PGPR inoculation and a less stressed phenotype is well documented, both in *Arabidopsis thaliana* and in crops. Application of PGPR has been shown to reduce ROS accumulation in different crops species such as peanut, canola, and *Capsicum annum* (Neshat et al., 2022; Sharma et al., 2016; Yasin et al., 2018).

The most common forms of cellular oxidative DNA damage are the guanine oxidation products, 8-oxoguanine (8-oxoG) and its nucleotide 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxodG). The guanine (G) base is more easily oxidised than adenine (A), cytosine (C) and thymine (T) and the high mutagenic capacity of 8-oxoG is related to its ability to form G:C–C:G and G:C–T:A transversions during DNA replication or repair. The oxidation of the DNA bases by ROS can occur either directly in the genomic DNA strands or indirectly in the nucleotide pool (Chiorcea-Paquim, 2022). Because 8-oxodG is an important biomarker used to quantify oxidative DNA damage in cells, we also measured the amount of 8-oxodG to relate it to the ROS content. In our study, the application of PVr_9 significantly reduces the content of 8-oxodG in plants grown under salt conditions, compared to the uninoculated, indicating less DNA damage in stressed inoculated plants (**Figure 22B**).

Ion imbalance and water deficiency cause osmotic stress in the plant under salinity conditions. This results in biophysical changes, including the reduction in cell turgor pressure, shrinkage of the plasma membrane, and physical alteration of the cell wall (Park et al., 2016). In response to salt stress, non-toxic molecules

that do not interfere with normal metabolism called osmolytes are produced. Among the most important osmolytes produced by plants are proline, glycine-betaine, and sugars (Chen and Murata, 2002; Sahi et al., 2006). Osmolytes are mainly involved in the regulation of the osmotic pressure by lowering the osmotic potential in the cytosol, but they could also act as signalling molecules to induce ABA accumulation or alter related gene expression (Marusig and Tombesi, 2020). Osmotic adjustments are essential as an adaptive response to salt stress, but they require an enormous energy cost that can impair plant growth (Munns, 1988; Munns, 2005; Munns and Tester, 2008). Proline is considered one of the most important osmolytes among all. Under stress, in addition to its role in osmotic regulation, proline plays a role in stabilizing subcellular structures, stabilizing proteins and protein complexes, detoxifying ROS, buffering the cellular redox potential (Ashraf and Foolad, 2007; Muchate et al., 2016). As expected, salt induces an increase in both proline and total soluble sugars. The content of proline and total soluble sugars is not influenced by the presence of PVr_9 in *A. thaliana* seedlings under control conditions, but the quantity of both proline and total soluble sugars in *A. thaliana* seedlings inoculated with PVr_9 grown on a saline medium appears to be lower than those of non-inoculated plants (**Figure 23A, 23B**). Taken together, these two data confirm the ability of PVr_9 to reduce the stress induced by NaCl. The reduction in proline accumulation by PGPR is in agreement with other studies in *A. thaliana* (Liu et al., 2020; Shi et al., 2022). Furthermore, the lower energy cost associated with a lower osmolyte production could be functional for the growth enhancement observed in inoculated plants vs. non inoculated ones under salt stress conditions. Given the role of proline in ROS detoxification, the smaller amount of proline may be due to a smaller amount of ROS accumulated in the seedlings. In inoculated *Avena sativa* grown in a saline medium, a reduction in proline accumulation and ROS accumulation was observed (Sapre et al., 2018) and similar results were also obtained in salt-stressed radish and *Arachis hypogea* inoculated with PGPR. In the cases just mentioned, the

content of proline detected in inoculated plants was lower than in uninoculated ones, as was the content of another oxidative stress marker such as MDA (Alexander et al., 2020; Shahid et al., 2022).

In response to salinity, endogenous ABA levels increase rapidly and, as one of the most important stress hormones, it plays an indispensable role to salt stress (Yu et al., 2020). In our work, PVr_9 reduces ABA accumulation under salt stress conditions in both leaves and roots (**Figure 24A, B**). The reduction in ABA content is also consistent with the observation made in other works (Khan et al., 2021a; Khan et al., 2021b; Wei et al., 2022). ABA is known to mediate signals in plant cells subjected to environmental stresses. These signals allow for the expression of stress-related genes followed by the synthesis of compatible osmolytes such as proline (Kavi Kishore et al., 2005; Thomashow, 1999; Zhu, 2001). In this context, the observed reduction in the accumulation of proline could also be associated with the reduction in ABA production. Furthermore, the two *MYB* genes, *MYB1* which regulates the biosynthesis of ABA (Wang et al., 2015b), and *MYB96* which is induced by ABA (Baldoni et al., 2015; Seo et al., 2009), are both downregulated due to PVr_9 inoculation in salt stressed seedlings. The downregulation of *MYB1* suggests an active role for PVr_9 in determining a reduction in the ABA content in stressed plants.

High Na^+ concentration induces nutrition disorders that reduce the activity of many essential nutrients in the soil making them less available to the plants (Shabala and Cuin, 2008). Läuchli and Grattan (2007) have well summarized the impact of Na^+ on plant uptake of Ca^{2+}. Salinity also reduces K^+, P and Fe uptake and translocation (Ben Abdallah, 2017; Clarkson and Marschenner, 1995). Analysis of K^+, Ca^{2+}, P and Fe revealed that *A. thaliana* subjected to salt stress undergoes a strong reduction in the content of these nutrients, but no significant differences in their amount were observed between inoculated and uninoculated seedlings (**Figure 25A**). Furthermore, observing the data on Na^+ accumulation in the seedlings, it can be stated that PVr_9 is not involved in reducing Na^+ uptake,

nor in increasing the content of the other ions examined (**Figure 25A**). In Giannelli et al., (2022), Pvr_9 was found to be effective in siderophores production. Iron ions can be abundant in the soil, but unavailable to plants and different bacteria increase the availability of Fe through the production of siderophores (Backer et al., 2018). Although PVr_9 had shown the production of siderophores *in vitro*, this did not translate into an increase in Fe within *A. thaliana* seedlings. In fact, both under normal conditions and under salt stress, the inoculum of Pvr_9 does not cause an increase in iron uptake. Interestingly, analysing the content of Na^+ separately in the leaves and in the roots, a significant, albeit slight, decrease in Na^+ is observed in the leaves of the inoculated plants compared to the uninoculated plants, while the opposite trend was observed in the roots (**Figure 25B**). These data can lead us to suggest that PVr_9 could induce tolerance to Na^+ by determining a greater compartmentalization of Na^+ in the roots compared to the leaves, decreasing its toxicity in the most sensible aerial part of the plants, as also showed by Niu and colleagues (2016) analysing the effects of a rhizobacteria in *Puccinellia tenuiflora*.

A multitude of genes are involved in the plant response to salt stress and, among these, those that contribute to tolerance regulating ion homeostasis (Yang and Guo, 2018). Strategies by which the plant can reduce sodium accumulation in the cytosol are diverse, and include counteracting Na^+ entry into the cell, increasing Na^+ efflux from the cell and, the in the cell, maximizing Na^+ compartmentalization in the vacuole, or more generally, reducing Na^+ entry into the plant. (Chen et al., 2014).

One of the essential genes for salt tolerance is represented by the *SOS1* gene, which in *A. thaliana* encodes for a plasma membrane Na^+/H^+ antiporter. Its importance is demonstrated as *sos1* mutants are more sensitive to growth inhibition by high Na^+ and low K^+ environments, typical of saline media (Shi et al., 2000). SOS1 is involved in several processes such as Na^+ efflux from roots, control of long-distance Na^+ transport in the plant through Na^+ loading and unloading in and from the xylem, slowing Na^+ accumulation in the cytosol and facilitating its storage in the vacuole (Almeida et al.,

2017; Conde et al., 2011; Zhu, 2003). It has been shown that under salt stress conditions SOS1 is specifically involved in the transport of Na^+ out of cells, and depending on the intensity of stress, mediating active Na^+ loading in the xylem of *A. thaliana* under mild salinity conditions and recovering Na^+ from the xylem of *A. thaliana* grown under high salinity conditions (Qiu et al., 2002; Shi et al., 2002). Another gene that is up-regulated by NaCl treatment is the one coding for tonoplast-localized protein NHX1 (Na^+/H^+ exchanger 1), a key protein in maintaining low cytoplasmatic Na^+ levels (Blumwald and Poole, 1985; Yokoi et al., 2002). Overexpression of *Arabidopsis* vacuolar *NHX1* was found to increase the salinity tolerance of *A. thaliana*, cotton, and tomato (Apse et al., 1999; He et al., 2005; Zhang and Blumwald, 2001), as well as the overexpression of a cereal *NHX1* homologous was found to improve salt tolerance in *Arabidopsis thaliana* and rice (Brini et al., 2007; Fukuda et al., 2004; Zhao et al., 2006). In *Arabidopsis thaliana,* HKT1 (high-affinity K+ transporters 1) protein is involved in the low affinity uptake of Na^+ (Rus et al., 2001; Wang et al., 2015a) and a selective uniporter that controls Na^+ influx in *Saccharomyces cerevisiae* and *Xenopus laevis* oocytes (Uozumi et al., 2000). Another role of HKT1 is to remove sodium from the xylem by transferring it to the parenchyma cells of the xylem, protecting the leaves from sodium toxicity. *HKT1* mutants show a hypersensitivity to sodium in the leaves despite a lower quantity of sodium in the roots (Berthomieu et al., 2003; Davenport et al., 2007; Mäser et al., 2002; Sunarpi et al., 2005).

In this study, the expression analyses showed that PVr_9 is able to modulate genes involved in Na^+ homeostasis; indeed, PVr_9 enhances *SOS1* and *NHX1* expression both in roots at 24 hrs and in leaves at 48 hrs of NaCl stress with respect to the uninoculated condition following a space time modulation (**Figure 26A, B**). These results agree with a less stressed phenotype. Given the roles indicated for the two respective proteins, the up- regulation of *SOS1* by PVr_9 could allow for a greater Na^+ loading in the xylem reducing its accumulation and toxicity in the leaves and a higher extrusion of Na^+ in the apoplast. Similarly, *NHX1* induction by PVr_9 could help to maintain a lower cytoplasmic level of Na^+, carrying it into the vacuole of both roots

and leaves cells. The lower cytosolic Na^+ accumulation could also explain the lower proline, soluble sugars, and ROS accumulation in the PVr_9 inoculated compared to the uninoculated plants. **Figures 26A** and **26B** show a general down-regulation of the *HKT1* gene under salt stress conditions, but a higher down-regulation is showed in PVr_9 inoculated plants compared to the uninoculated. A similar result was observed by Pinedo et al. (2015). In stressed and unstressed plants of *A. thaliana* inoculated and uninoculated with *Burkholderia phytofirmans* PsJN, *HKT1* was found to be down-regulated in the roots after 24 h of stress as well as in the leaves after 24h and 72h compared to the control condition. Despite this down-regulation, in our work the lower expression of *HKT1* is not correlated with a reduction in Na^+ uptake. It is possible that, in our case, although *HKT1* is down- regulated, the input of Na^+ is determined by other proteins, as also suggested by Wang et al. (2015a). Furthermore, *HKT1* in our case does not seem to be a determining gene in reducing saline stress by PVr_9.

MYB genes encode for transcription factors involved in plants biological processes such as primary and secondary metabolism, cell fate and identity, and developmental processes (Dubos et al., 2010). Many studies have indicated the critical role of MYB TFs in plant abiotic stress responses; indeed, MYB TFs also contribute to salt tolerance and many of their downstream targets have been identified (Baldoni et al., 2015; Li et al., 2019; Roy, 2016). MYB proteins are also involved in the ABA response and regulate salt tolerance through the regulation in ABA signalling pathways (Wang et al., 2021). The largest MYB family in plants is represented by the R2-R3-MYB gene family, of which *MYB1*, *MYB52*, *MYB73,* and *MYB96* are all members (Stracke et al., 2001). *MYB1* expression is induced by drought and salt but is not induced by ABA. It regulates ABA biosynthesis, signalling-related genes, and influences plant growth during salt stress. Knock-out *Arabidopsis* mutant lines are less sensitive to salt compared to wild type or overexpressing lines (Wang et al., 2015b). AtMYB52 is involved in response to drought and salt stress; overexpressing lines are ABA-hypersensitive and show salt sensitivity (Park et al., 2011). AtMYB73 is a transcription factor that functions during salt-specific response and is a negative regulator of salt

stress signalling and the *Atmyb73* knock-out mutants have high salt tolerance. Furthermore, *atmyb73* mutant lines showed increased *SOS1* transcription (Kim et al., 2013). *AtMYB96* expression is significantly induced by drought, salt, and ABA (Baldoni et al., 2015; Seo et al., 2009). In *AtMYB96-ox* lines, in response to ABA, the stomata opening is reduced, while in *myb96-1* mutant leaves this reduction is reduced. ABA signals, mediated by AtMYB96, induce plant tolerance to water deficit by reducing stomatal opening (Seo et al., 2009). Plants overexpressing *MYB1, MYB52, MYB73,* and *MYB96* are reported to be more sensitive to salt stress. In the root cells, where the presence of salt in the soil is sense earlier and transmits the signal to other plant parts, the inoculation of PVr_9 induces a reduction in the expression of all the *MYB* genes with respect to uninoculated stressed plants (**Figure 27A).** In the leaves, only *MYB73* is strongly down-regulated by PVr_9 under both control conditions and in stressed plants. Given its role as a negative regulator of the salt stress response, its down-regulation following PVr_9 inoculation confirms the role of PVr_9 in inducing salt tolerance in *Arabidopsis*. The expression of *MYB1*, for which a down-regulation in the presence of PVr_9 was observed in control conditions, suggests a priming effect of the bacterium that allows a better response of the plant under salt stress conditions.

The growth promoting activity of PVr_9 was also demonstrated in soil under control conditions. Indeed, after one month of growth PVr_9, inoculated plants showed an increase in the primary root length (**Figure 28B**) and a root system more developed with respect to those uninoculated (**Figure 28C (b)**). However, by analysing the effect of PVr_9 on a long-term stress in soil, PVr_9 was unable to determine an increased tolerance in plants grown under 150 mM NaCl (**Figure 28A, B, C**). This could be due to the stress conditions applied, which could be too extreme in terms of duration and salt concentration. For these reasons future analyses will be necessary to evaluate the effect of PVr_9 on *A. thaliana* in soil.

CONCLUSIONS

This study evaluated PGPR properties of different bacterial isolates and demonstrated the capacity of PVr_9 strain, homologous to *B. fluminensis,* to act as PGPR in *Arabidopsis thaliana* and as an antimicrobial biocontrol agent against phytopathogenic fungi. The PVr_9 *in vitro* potential is translated into an effective plant growth promotion, indeed PVr_9 induces an increase in the primary root length as well as the rosette area. The antimicrobial activity is proved by the strong inhibition induced against all the three different phytopathogenic fungal species tested, especially in the co-inoculation test, but was also evident with the administration of the filtered culture broth. Furthermore, PVr_9 ameliorates salt stress in *A. thaliana* seedlings. PVr_9 reduced root growth inhibition and the reduction of the deleterious effect that salt has on the rosette of *A. thaliana* seedlings. PVr_9 reduced the accumulation of ROS, 8-oxodG, proline, soluble osmolytes, and ABA content in salt stressed plants, by stimulating a redistribution of Na^+ in the plant tissues, reducing its amount in the leaves which are the most salt sensible tissues. At this purpose, PVr_9 modulates the expression of different *MYB* genes involved in the salt signal transduction pathway and in ABA production, and genes regulating Na^+ compartmentation and Na^+ recycling in plant tissues and sub-cellular compartments. These data obtained on the model plant species *Arabidopsis thaliana*, open the possibility to use *Beijerinckia fluminensis* as a viable bio-fertilizer and bio-pesticide also in field trials of agronomical important crops.

This study also provides further confirmation in favour of the need to go beyond the classical PGPR screening that is limited to pure culture analysis. In fact, as it is evident from our analyses, although all the bacteria examined possess characteristics that can be attributed to PGPR, some of them in association with a plant do not produce appreciable effects, and others even have effects that are not properly determinative of increased plant growth.

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Scientific production

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Poster

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