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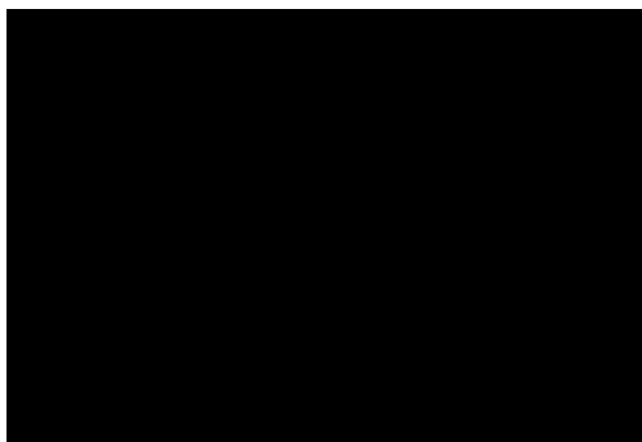
Evaluation of Biostimulants activity in mitigating the N and drought stress in tomato

PH.D. THESIS



Tutor
Prof.ssa Maria Rosa Abenavoli

Co-Tutor
Prof. Francesco Sunseri

Ph.D. Coordinator
Prof. Leonardo Schena

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Abstract

Plant biostimulants (PBs) represent a sustainable and efficient strategy to achieve stable crop yields under optimal or sub-optimal conditions, reducing chemical inputs. The new EU Regulation 2019/1009 has defined them as "substances and/or microorganisms that applied to the plant or rhizosphere stimulate natural processes, improve nutrient uptake and assimilation efficiency, tolerance to abiotic stresses, and product quality". They include a large variety of biological products such as plant extracts, humic and fulvic acids, protein hydrolysates, phosphites, seaweed extracts, and living microorganisms, typically bacteria and fungi. They can promote plant growth, increase crop yields and quality, nutrient uptake, nitrogen (N) fixation, phosphorus (P), potassium (K) and micronutrient solubilization, biotic and abiotic stress tolerance.

In this PhD thesis, the effect of *Kocuria rhizophila* and *Streptomyces violaceoruber*, two actinomycete strains, and Eranthis (Green Has Italia), a commercial non-microbial biostimulant, was evaluated to highlight their potential for improving growth performance of tomato (*Solanum lycopersicum* L., UC82, a commercial variety), used as plant model, grown under nutritional and drought stress.

First, twenty-two bacterial strains were preliminary characterized for multiple PGPB traits. Among them, *K. rhizophila* and *S. violaceoruber* showed the best PGP activities, such as indole acetic acid production, phosphate solubilization, N_2 fixation, as well as drought and salt tolerance. The secondary metabolites and volatile organic compounds (VOCs) and the PGP activity of these actinomycete strains were evaluated on tomato. Their secreted and cellular metabolome revealed a rich arsenal of bioactive molecules, including antibiotics and siderophores, with *S. violaceoruber*, being the most effective PGPB strain. In particular, it was able to improve the seed germination index, hypocotyl and epicotyl growth and seedling growth. Furthermore, the produced VOCs showed antimicrobial activity, being able to modulate volatilome and exert control on the global DNA methylation of tomato seedlings. For these reasons, both *K. rhizophila* and *S. violaceoruber* were deeply studied for their PGPB activities. By a multi -omics approach, differential expressed genes as well as accumulated proteins and metabolites involved in the plant growth promotion in *K. rhizophila*-treated tomato plants compared to untreated control. Furthermore, eight gene modules based on their correlation with differential accumulated proteins and metabolites were identified by a weighted gene co-expression network analysis (WGCNA) approach. In particular, two modules showed the highest correlation with nine proteins, among which a nucleoside diphosphate kinase, and several

metabolites, mainly belonging to amino acids and tricarboxylic acids (TCA). Our findings highlighted that sugars and amino acids, key nutrients for improving plant growth and yield, were strongly modulated by plant-*K. rhizophila* interaction.
Furthermore, the ability of *S. violaceoruber* to mitigate N and/or drought stress condition was evaluated in different tomato growth systems: hydroponics, pots and *plate-on-plate*. In hydroponics, *S. violaceoruber* was able to increase morpho-physiological parameters such as fresh and dry root weight, root diameter and lateral root numbers, chlorophyll content, net photosynthesis rate, transpiration in tomato N stressed plants. Interestingly, it was able to restore these morpho-physiological parameters to the high N values. The undecylprodigiosin, mycelial red pigment produced by *S. violaceoruber* along tomato roots, confirmed root-bacteria interaction. In pots, the *S. violaceoruber* treatment increased the root fresh weight, shoot dry weight and SPAD index as well as net photosynthetic rate, transpiration rate and stomatal conductance of tomato treated plants. More interestingly, treated plants showed also an improved nitrogen use efficiency (NUE) and its component, nitrogen utilization efficiency (NUtE), useful traits to maintain high plant growth and yields. In *plate-on-plate* system, volatiles, produced by *S. violaceoruber*, applied by indirect inoculation, increased plant biomass, number and length of lateral roots, in control conditions, but they were not able to overcome drought stress effect.
Finally, Eranthis, a commercial non-microbial biostimulant based on brown seaweed (*Ascophylum nodosum* and *Laminaria digitata*) and yeast extracts (Green Has Italia S.p.a (Canale, Italy), was evaluated for its ability to mitigate N stress. Under low N, Eranthis was able to increase fresh and dry weight, and lateral root number in tomato plants, grown in hydroponic system. This effect could be due to its composition rich in antioxidant molecules, such as flavonoids and flavanols, which contribute to reactive oxygen species (ROS) scavenging.
In conclusion, both *S. violaceoruber* and *K. rizophila* and the no-microbial Eranthis could be considered as new candidates for developing novel biofertilizers with low environmental impact in a more sustainable cropping system. Further researches are needed to understand their mode of action to improve plant growth and stress tolerance, as well as their correct and efficient use in agriculture.

Keywords: Plant Growth Promoting bacteria (PGPB), Actinomycetes, seaweed extract (SE), nitrogen deficiency, tomato, metabolomics, transcriptomics.

Riassunto

I biostimolanti rappresentano una strategia sostenibile ed efficiente per ottenere rese colturali stabili in condizioni ottimali o sub-ottimali, riducendo gli input chimici. Il nuovo Regolamento UE 2019/1009 li definisce come "sostanze e/o microrganismi che applicati alla pianta o alla rizosfera stimolano i processi naturali, migliorano l'efficienza di assorbimento e assimilazione dei nutrienti, la tolleranza agli stress abiotici e la qualità del prodotto". Essi comprendono un'ampia varietà di prodotti biologici, come estratti vegetali, acidi umici e fulvici, idrolizzati proteici, fosfiti, estratti di alghe marine, e microrganismi vivi, tipicamente batteri e funghi. I biostimolanti possono promuovere la crescita delle piante, aumentare la resa e la qualità delle colture, l'assorbimento dei nutrienti, la fissazione dell'azoto (N), la solubilizzazione di fosforo (P), potassio (K) e micronutrienti, la tolleranza agli stress biotici e abiotici. In questo progetto di tesi, è stato valutato l'effetto di *Kocuria rhizophila* e *Streptomyces violaceoruber*, due ceppi di actinomiceti, e di Eranthis (Green Has Italia), un biostimolante commerciale non microbico, nel migliorare le *performance* di crescita di pomodoro (*Solanum lycopersicum* L., UC82, varietà commerciale), usato come pianta modello, in diverse condizioni di stress nutrizionale ed idrico. In primo luogo, 22 ceppi batterici sono stati caratterizzati per le molteplici caratteristiche di batteri promotori di crescita delle piante (PGPB). Tra questi, *K. rhizophila* e *S. violaceoruber*, sono risultati i ceppi migliori per le loro attività PGPB, quali la produzione di acido indoloacetico, la solubilizzazione dei fosfati, la fissazione di N_2 e la tolleranza alla siccità e al sale. Sono stati valutati i loro metaboliti secondari, i composti organici volatili (VOCs) e la loro attività PGP sul pomodoro. Il loro metaboloma secreto e cellulare ha rivelato un ricco arsenale di molecole bioattive, tra cui antibiotici e siderofori, e tra questi, *S. violaceoruber* si è dimostrato il ceppo più efficace. In particolare, esso era in grado di migliorare l'indice di germinazione, la crescita dell'ipocotile e dell'epicotile e la crescita delle piantine. Inoltre, i VOCs prodotti mostravano attività antimicrobica, essendo in grado di modulare il volatiloma e di esercitare un controllo sulla metilazione del DNA delle piantine di pomodoro. Alla luce di questi risultati, sia *K. rhizophila* che S. violaceoruber sono stati studiati più a fondo per le loro attività PGPB.

Mediante un approccio multi-omico, sono stati identificati geni, proteine e metaboliti, coinvolti nella promozione della crescita delle piante, attivati da *K. rhizophila* in piante di pomodoro. Inoltre, con un approccio WGCNA, sono stati identificati otto moduli genici correlati con proteine e metaboliti accumulati in modo differenziale. In particolare,

due moduli hanno mostrato la più alta correlazione con nove proteine, tra cui una nucleoside difosfato chinasi, ed una serie di metaboliti appartenenti ad amminoacidi e TCA. I risultati hanno evidenziato che zuccheri ed aminoacidi, nutrienti chiave per la crescita e resa delle piante, sono stati fortemente modulati dall'interazione pianta-*K. rhizophila*. Successivamente, è stata valutata la capacità di *S. violaceoruber* di mitigare le condizioni di stress da N e/o idrico in pomodoro uitlizzando diversi sistemi di crescita: idroponica, vaso e *plate-on-plate*. In idroponica, *S. violaceoruber* era in grado di aumentare i parametri morfo-fisiologici come il peso delle radici fresche e secche, il diametro, il numero di radici laterali, il contenuto di clorofilla, il tasso di fotosintesi netta e la traspirazione delle piante di pomodoro sottoposte ad N stress. Inoltre, il trattamento era in grado di ripristinare questi parametri morfo-fisiologici alla condizione di alto N. L'undecilprodigiosina, pigmento rosso miceliare prodotto da *S. violaceoruber* lungo le radici del pomodoro, confermano l'interazione radice-batterio. In vaso, il trattamento con *S. violaceoruber* aumentava il peso fresco delle radici, il peso secco dei germogli, l'indice SPAD, il tasso fotosintetico netto, il tasso di traspirazione e la conduttanza stomatica delle piante di pomodoro. Il trattamento ha anche influenzato lo sviluppo della pianta migliorando l'efficienza d'uso dell'azoto (NUE) e l'utilizzo dell'azoto (NUtE), utile per mantenere alte le rese. Nel sistema *plate-on-plate*, i composti volatili prodotti da *S. violaceoruber*, applicato tramite inoculazione indiretta, aumentavano la biomassa della pianta, il numero e la lunghezza delle radici laterali, in condizioni di controllo, ma non sono stati in grado di mitigare l'effetto dello stress idrico.
Infine, Eranthis, un biostimolante commerciale di natura non microbica, costituito da estratti di alghe brune ed (*Ascophylum nodosum* and *Laminaria digitata*) e lieviti (Green Has Italia S.p.a (Canale, Italy), è stato valutato per la sua capacità di mitigare lo stress l'N stress. Il trattamento con Eranthis ha influenzato i parametri morfologici delle radici, aumentando il peso fresco e secco e il numero di radici laterali nelle piante di pomodoro, coltivate in un sistema idroponico, in condizioni di limitazione di N stress. Questo effetto potrebbe essere dovuto alla sua composizione ricca di molecole antiossidanti, come flavonoidi e flavanoli, che sono in grado di contribuire alla rimozione dei ROS.
In conclusione, *S. violaceoruber* e *K. rizophila* potrebbero essere considerati come nuovi candidati per lo sviluppo di nuovi biofertilizzanti a basso impatto ambientale per un sistema colturale più sostenibile. Sono necessarie ulteriori ricerche per meglio comprendere le modalità d'azione dei biostimolanti, microbici e non, nel migliorare la

crescita delle piante e la tolleranza agli stress, per un loro più efficace utilizzo in un'agricoltura sostenibile.

Parole chiave: Batteri promotori della crescita delle piante (PGPB), Attinomiceti, carenza di azoto, pomodoro, metabolomica, trascrittomica.

General Introduction

1. An overview of the use of plant biostimulants in crops

In recent decades, intensive agricultural activity has relied on inputs from non-renewable energy sources (e.g. use of chemical fertilisers and pesticides) that have increased the crop yield, while at the same time, caused a sharp decline in environmental assets such as deforestation, soil erosion, air pollution, declining surface and groundwater quality and loss of biodiversity. To date, these negative effects continue to occur at an alarming rate (Altieri, 2002), further exacerbated by the impacts of global climate change, leading to increased uncertainly in food security (Tilman et al., 2011).

Today, the challenges of environmental sustainability are the following: i) feeding a growing population; ii) protecting the environment; and iii) producing renewable energy sources. Indeed, the food demand is expected to increase 2-5 times by 2030 and food production by 60% in the coming decades (Clair and Lynch, 2010). Therefore, the innovative agronomic techniques must aim to improve the quality and yield of crops with minimal inputs in order to safeguard the environment and human health. An interesting turning point for an agricultural sustainability is the Fork to Farm Strategy (F2F), published in May 2020 by the European Union, whose aim is to make food systems fair, healthy and environmentally friendly by accelerating the transition to a more sustainable production system in which the dependence on pesticides, antimicrobials and fertilisers is drastically reduced by 2050. The COVID-19 pandemic has further underlined the importance of a robust and resilient food system that can guarantee the demand for affordable food for all [European Commission. Available online: https://ec.europa.eu/food/system/files/2020-05/f2f_action-plan_2020_strategy-info_en.pdf (accessed on 26 July 2021)].

In addition to increasing population, food security and environmental pressure, modern agriculture faces the challenges of soil degradation and reduced arable land. Approximately 24 billion tonnes of fertile soil have been depleted worldwide due to poor farming practices and erosion. In addition, drought, salinisation of cultivated land and natural disasters have had a negative impact on agriculture. About 60-70% of the yield gap is due to abiotic stresses such as salinity, drought, nutritional deficiency, temperature and hypoxia (Rouphael and Colla, 2020).

The new challenge is therefore to build food production systems based on 'ecological intensification' strategies that promote nutrient and water use efficiency, restore soil

fertility, protect biodiversity and reduce chemical control of diseases and pests (Tittonell, 2014).

In this context, plant biostimulants (PBs) represent an innovative and supportive tool to address the future challenges of agriculture and environmental sustainability. They are substances and/or microorganisms that enhance plant performance in terms of growth, metabolic activities, tolerance to biotic and abiotic stresses, and ultimately improve crop yields (Tilman et al., 2011).

2. Biostimulants

General concept

The term "biostimulant", first proposed by Zhang and Schmidt (1997), referred to "substances" used in “minute quantities”, which promoted plant growth without falling into the nutrient and fertilizer categories, soil conditioners or crop protection agents. Initially, PBs included the humic substances (HS) and seaweed extracts (SEs), whose stimulatory effect was essentially due to a hormone-like and antioxidant activity able of increasing plant growth and protection against environmental stresses. Subsequently, Kauffman et al. (2007) proposed a biostimulant definition introducing a possible taxonomy that included "highly heterogeneous materials" classifiable into eight categories such as HS, complex organic materials, beneficial chemical elements, inorganic salts, SEs, chitin and chitosan derivatives, antitranspirants, free amino acids, and nitrogen-containing substances". Later, Basak (2008) grouped biostimulants on the basis of single or multicomponent formulations classifying them on the origin of the active components. First, Du Jardin (2012) developed a scientific rationale of classification considering 7 categories of PBs, in which did not include those of microbial origin, avoiding any conflict with existing categorization of microorganisms as biopesticides and sources of plant hormones. Later Bulgari et al. (2015) proposed a biostimulant classification based on their mode of action rather than their composition. In 2015, Du Jardin provided the first in-depth analysis of PBs science with an emphasis on their categorization based on biochemical and physiological function, mode of action and origin. Later, Colla and Rouphael (2015) included three microbial biostimulants [arbuscular mycorrhizal fungi (AMF), plant growth-promoting rhizobacteria (PGPR) and *Trichoderma* spp.] together with six non microbial (i.e., chitosan, humic and fulvic acids, protein hydrolysates, phosphites, SEs, and silicon) able to promote and modulate growth, development and tolerance to environmental stresses in plants. Therefore, the PB definition was broadened as "applied substances and/or microorganisms that enhance plant productivity due to novel or emergent properties of the constituent complex

and not as a consequence of the presence of essential nutrients, growth regulators, or plant protective compounds" (Yakhin et al., 2017). (Table 1).

Table .1 Classification of plant biostimulants (from Shahrajabian et al., 2021)

Plant Biostimulants	Key Points
Protein hydrolysates (PHs) and other *N*-containing compounds (amino acids)	a. Mixtures of peptides and amino acids which are produced via enzymatic, chemical or thermal hydrolysis of animal- or plant-derived proteins. b. Effective in increasing yield and quality of various crop products. c. Categorization based on proteins, sources and the hydrolysis system; PHs boost both primary and secondary plant metabolism biochemical and physiological procedures. d. Effective in alleviating negative abiotic stress effects.
Humic substances	a. Include fulvic acids and humic acids which they differ in color, molecular weight, carbon content and the degree of polymerization. b. They could increase cationic exchange capacity (CEC) of the soil and interact with root membrane transporters.
Seaweed extracts	a. Extracts from brown seaweeds, e.g., *Ascophyllum*, *Fucus*, and *Laminaria* genera. b. They are rich in polysaccharides, polyphenols and compounds with hormonal activity that affect plant growth and development.
Biopolymers (Chitosans and other polymers)	a. Chitosans are naturally occurring components in fungi nematodes, insects and crustaceans. b. They regulate plant-defense mechanisms related to phytoalexins biosynthesis, reactive oxygen species, and pathogenesis-related proteins making plants more resistant to biotic and abiotic stressors.
Microbial biostimulants (Mycorrhizal and non-mycorrhizal fungi, *Rhizobium*, *Trichoderma*, and Plant Growth-Promoting Rhizobacteria (PGPR))	a. Symbiotic fungi, especially arbuscular mycorrhizal fungi (AMF) within the genus *Glomus*. b. *Trichoderma* genus c. Beneficial bacteria with plant growth promoting properties also known as PGPBs (*Bacillus*, *Rhizobium*, *Pseudomonas*, *Azospirillum*, *Azotobacter*, and many others).
Phosphite (Phi)	a. A phosphate ($H_2PO_4^-$) analog which affects various plant growth and development processes. b. Several beneficial effects have been reported in various vegetable crops. c. Biostimulatory impacts on fruit such as avocado, banana, citrus, peach, raspberry and strawberry.
Silicon	a. Effective against abiotic and biotic stressors.
Vermicomposts	a. Hormonal activity of vermicompost leachates due to content in trace elements of hormones such as cytokinins, indolo-acetic acid (IAA), eighteen gibberellins (GAs)

The PBs definition has been rigorously debated over the past decade until the new EU Regulation 2019/1009, which defines them as “substances and/or microorganisms that applied to the plant or rhizosphere stimulate natural processes, improve nutrient uptake and assimilation efficiency, tolerance to abiotic stresses, and product quality”. The PBs have no direct effects on pests and pathogens and therefore do not fall into the category of pesticides. [European Commission. (EC) No. 1069/2009 and (EC) No. 1107/2009 and repealing Regulat. Off. J. Eur. Union 2019, 2019, 1-114. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32019R1009&from=EN (accessed on July 26, 2021)].

The proposed definition has become a benchmark for the acceptance of the PB category in non-EU regulations as well. For example, in the United States, the 2018 Farm Bill, which became law, defined biostimulant as "a substance or microorganism that, when applied to seeds, plants, or the rhizosphere, stimulates natural processes to enhance or stimulate nutrient uptake, use efficiency, tolerance to abiotic stresses, or crop quality and yield."

Noteworthy, since 2011, the "European Biostimulants Industry Council" (EBIC) and the "Biostimulant Coalition" consortia have been formed in Europe and United States, respectively, which dialogue with stakeholders, regulatory bodies and the research world, helping to create an international market for PBs contributing to sustainable agricultural production, green innovation, economic growth and other social goals recognized.

Currently, the PBs global market is estimated at about $3.0 billion (2021) with an annual growth rate of 10-12% (Dunhamtrimmer.com, 2018). Based on the Marketsandmarkets.com database (2017), Europe is the largest market for PBs, accounting for 34% of the global share, followed by North America and Asia-Pacific, which have 23% and 22%, respectively. The main factors driving its rapid growth can be attributed to the (i) increasing availability of new PBs that address specific agronomic needs; (ii) need to promote more efficient and effective use of synthetic chemicals and mineral fertilizers; and (iii) increasing frequency of environmental conditions adverse to crop growth and productivity.

2.1. Biostimulant Classification

Based on the EU definition, PBs are classified as two major groups: non-microbial such as (i) humic acids, (ii) protein hydrolysates, (iii) seaweed extracts (iv) silicon, (v) biopolymers such as chitosan; and microbial such as (a) arbuscular mycorrhizal fungi (AMF), (b) N-fixing bacteria, and (c) plant growth promoting bacteria (PGPR) (du Jardin, 2015; Rouphael et al. 2018) (Figure 1).

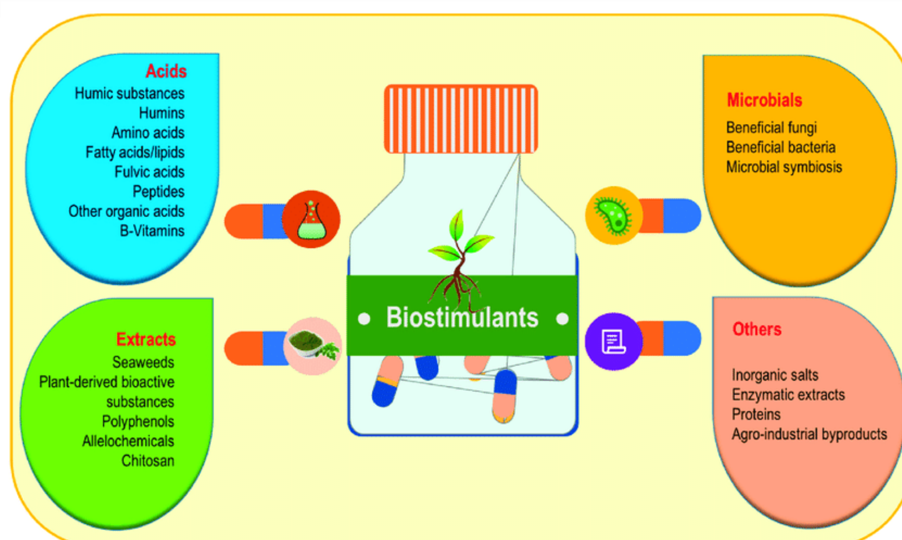

Figure 1. The major biostimulant categories (Hasanuzzaman et al. 2021)

2.1.1 Humic acids

Humic substances (HS) are natural constituents of soil organic matter, resulting from the decomposition of plant, animal and microbial residues, and soil microbial metabolic activity. The HSs are a collection of heterogeneous compounds, originally classified according to their molecular weight and solubility in humins, humic and fulvic acids. These compounds exhibit complex association/dissociation dynamics in supramolecular colloids, a process influenced by both microorganisms and roots through the release of protons and exudates. Therefore, the HS effects are due to the nature of the complex, environmental conditions, plants, dose and mode of application (Rose et al., 2014). The HSs can be commercially extracted from a wide variety of substrates, including the organic matter of plants, animals or soil origin, peats, soft-coals and industrial or urban by-products and residues. However, the source and manner in which HSs are extracted, isolated and purified, deeply affect HS composition and their physiological effects on plants that also depend on the concentration and molecular weight of the fractions applied (Calvo et al., 2014).

The effects of HSs on plant growth and productivity have long been studied (Nardi et al, 2016). The HSs: 1) increase the expression of genes encoding aquaporins, thereby increasing root hydraulic conductance (Olaetxea, et. al, 2015); 2) stimulate root elongation, lateral root number, and proton pump H^+-ATPase activity, promoting nutrient uptake (Cannellas, et al. 2002); 3) contribute to improving the soil chemical-physical and biological characteristics and consequently its fertility, increasing plant nutrient availability and agronomic efficiency (AE) (Brown et al, 2019); 4) contribute to cell wall loosening, cell enlargement, and organ growth (Jindo et al., 2022); 5) increase stress tolerance by activating phenylpropanoid metabolism, a central pathway involved in stress responses (Olivares et al, 2015; Schiavon et al., 2010); 6) increase the genes expression involved in nitrogen and sulfur uptake, assimilation and distribution, improving their use efficiency (Jannin et al., 2012); 7) increase the photosynthetic pigments, carotenoids, total phenols, flavonoids content, and NPK concentration (Bayat et al., 2021) (Figure 2).

When applied in excess, HS can influence plant growth through the direct provision of nutrients or enhanced tolerance to stress (Diacono and Montemurro, 2010). De Pascale et al. (2017) suggested that HS have a significant influence on secondary metabolism and stress alleviation in plants, directly altering NUE. These HS effects may be the result of induced or inhibited protein synthesis, increased enzyme activities and induced morpho-functional changes in plants.

However, at low rates, HS affects biochemical and molecular pathways or physiological processes as well as root growth and development, plant productivity (Rose et al., 2014), nutrient uptake (Billard et al., 2014; Sánchez-Sánchez et al., 2006), chlorophyll content (Anjum et al., 2011) and gene expression (Aguirre et al., 2009; Billard et al., 2014).

Concerning nutrient uptake, HSs application increased nitrate (NO_3^-) and iron (Fe) uptake, by increasing the expression of genes involved in their transport (Jannin et al., 2012; Tomasi et al., 2014). Recently, Zanin et al. (2019) summarized the multiple mechanisms of HSs stimulation on Fe nutrition claiming that: (1) HSs may increase the Fe pool available for plant through the formation of HS-Fe complexes and by altering the normal soil-Fe precipitation and oxidation reactions; (2) HS-Fe complexes may also act directly on physiological processes by enhancing within-plant transport and delivery to developing tissues and by enhancing the activity of identified root and shoot Fe-uptake mechanisms including Fe(III)-chelate reductase (LeFRO1), Fe transporter genes (*LeIRT1* and *LeIRT2*) (Tomasi et al., 2014) and leaf transporters (CsFRO1, CsIRT1, CsNRAMP) (Zanin et al., 2015); (3) HS materials induced changes in root morphology, which, depending upon existing soil conditions, and may enhance not merely Fe uptake but the uptake of all nutrient elements (Zanin et al., 2019; Olaetxea et al., 2018). In relation to NO_3^-, HSs enhanced the expression of two major nitrate transporters (NRT1.1 and NRT2.1) and nitrate reductase (NR) activity in shoots and roots, without increasing NO_3^- accumulation in both tissues, suggesting that all supplemental N taken up by the treated roots was directly assimilated in relation to the growth rate without being stored. In contrast, Quaggiotti et al. (2004) showed that the nitrate content of leaves increased by 50% in seedlings treated with HSs in comparison with control plants. The authors suggested that HSs may exert direct effects on gene transcription in roots, and this may implicate a more efficient translocation of NO_3^- to its metabolic sinks, reflecting a better internal nitrogen utilization efficiency (IE).

Finally, the HS were also combined with beneficial microorganisms as plant growth promoter or biological control agents (Naidu et al., 2013; Olivares et al., 2015), acting as a carrier to introduce beneficial microorganisms in field. Therefore, the PGPB and HSs combination is a promising approach for improving plant performance and metabolic processes, reducing environmental costs for agricultural production (Olivares et al., 2017). Recently, da Silva et al. (2021) addressed the PGPB and HSs effect on plant responses to abiotic stresses, discussing the role of HSs in protecting plants against pathogens.

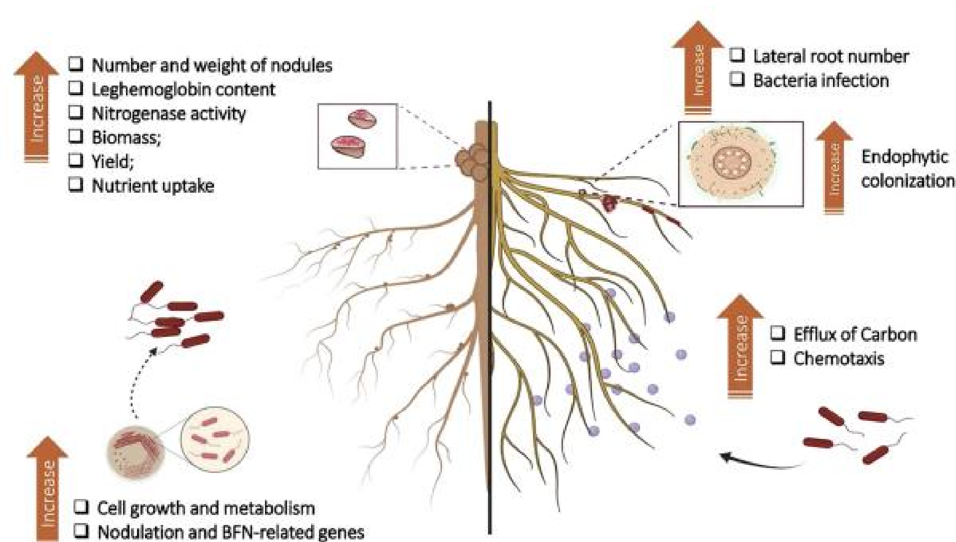

Figure 2. Summary of literature on the humic substances (HSs) effects on plant growth-promoting bacteria (PGBP) and interaction with plant root systems (from Silva et al., 2021).

Although there are numerous HS-based formulations, which can be applied as biologically active natural products in advanced sustainable agriculture, more research is still needed on their effects and mode of action (Zanin et al., 2019).

2.1.2 Protein hydrolysates

Protein hydrolysates (PHs) include two main categories: 1) mixtures of peptides and amino acids (AA) of animal or plant origin, and 2) single amino acids such as glutamate, glutamine, proline, and glycine betaine. They are produced by the enzymatic, chemical or thermal hydrolysis from animal and plant protein, including epithelial or connective tissues, collagen and elastin of animal origin (Cavani et al., 2006; Ertani et al, 2009, 2013), carob proteins (Parrado et al., 2008) and alfalfa plants (Schiavon et al., 2008; Ertani et al., 2009, 2013). The different extraction methodologies lead to the formation of diverse hydrolysates in terms of composition and activity: very stringent hydrolysis produces exclusively amino acids and oligopeptides, while the milder ones gives higher molecular weight polypeptides. Generally, chemical hydrolysis breaks amino acid chains in an irregular manner resulting in more dextrorotatory (D-) form mixtures, a configuration not easily absorbed by plants, and sometimes phytotoxic. On the contrary, enzymatic hydrolysis favors the production of mixtures of levorotatory (L-) amino acids, naturally occurring and produced by plants and therefore nontoxic and active. Therefore, the molecular size of peptides, constituting a protein hydrolysate, can be extremely variable, ranging from a few hundred to several thousand daltons (Da). Commonly, the PH biostimulant activity is fundamentally related to the fraction

with smaller molecular size (Quartieri et al., 2002), approximately lesser than 5,000 Da, as well as to free amino acids (Cavani and Ciavatta, 2007), which are able to permeate through the cell wall and membrane, acting directly or indirectly on plant metabolism (Borecky and Vercesi, 2005). Furthermore, following the Tsetse/Bse (mad cow disease) emergence, 10,000 Da represents the maximum value to safely employ animal-derived PHs. Single amino acids include all the twenty ones involved in protein and non-protein amino acids, which are adsorbed by both roots and leaves, and then translocated into the plants (Vranova et al., 2011; Zhang et al., 2015).

The first effect of PH on plants is the stimulation of root and leaf biomass (Schiavon et al., 2008; Ertani et al., 2009). Indeed, short-term PHs application increases the maize root dry weight compared to untreated ones, facilitating plant transplanting and resulting in higher plant biomass and yield (Zhang et al., 2003; Ertani et al., 2009). The PH treatment also modifies root morphology in a manner similar to IAA, suggesting that they induce greater "nutrient acquisition" by increasing uptake surface area (Ertani et al., 2012). Kauffman et al. (2007) showed that PH foliar treatment improves the photochemical efficiency and thermostability of membranes of *Lolium perenne*, suggesting a beneficial effect of these PBs on heat stress tolerance. A positive effect is also induced on alfalfa plants under salt stress, in which PHs increase plant biomass by stimulating nitrogen metabolism and antioxidant systems (Ertani et al., 2013). Therefore, the PH mechanism of action is to promote nitrogen assimilation via a coordinated regulation of C and N plant metabolism. For instance, a PH, derived from alfalfa plants, enhances shoot biomass production, soluble sugar accumulation and nitrogen assimilation in hydroponically grown maize plants (Schiavon et al., 2008). In particular, the authors demonstrated that PHs increase the activity and gene expression of three enzymes of tricarboxylic acid cycle (TCA) (malate dehydrogenase, isocitrate dehydrogenase and citrate synthase) and five enzymes (nitrate reductase, nitrite reductase, glutamine synthetase, glutamate synthase and aspartate aminotransferase) involved in N reduction and assimilation. Later, Ertani et al. (2013) showed that the same PB improves the maize growth cultivated under salinity stress, by increasing the Na^+ and K^+ ratio in leaves, and the synthesis of flavonoids. The application of a legume-based PH (containing amino acids and soluble peptides), as foliar and drench substrate, mitigates the drought stress in tomato grown in controlled environment, by (i) increasing transpiration use efficiency, (ii) improving ROS-mediated tolerance, (iii) modulating phytohormones and lipids profiles (Paul et al., 2019). Similar hormonal effects of an animal-based PH (containing L-a amino acids, free amino acids, organic-nitrogen, iron, and potassium) on water-stressed tomato plants were also

observed by Casadesus et al. (2020). In particular, the application of this PH confers, in tomato grown in greenhouse, an antioxidant protection, exerting a major hormonal effect in water-stressed tomato leaves by increasing the endogenous content of auxin, cytokinin, and jasmonic acid (Figure 3).

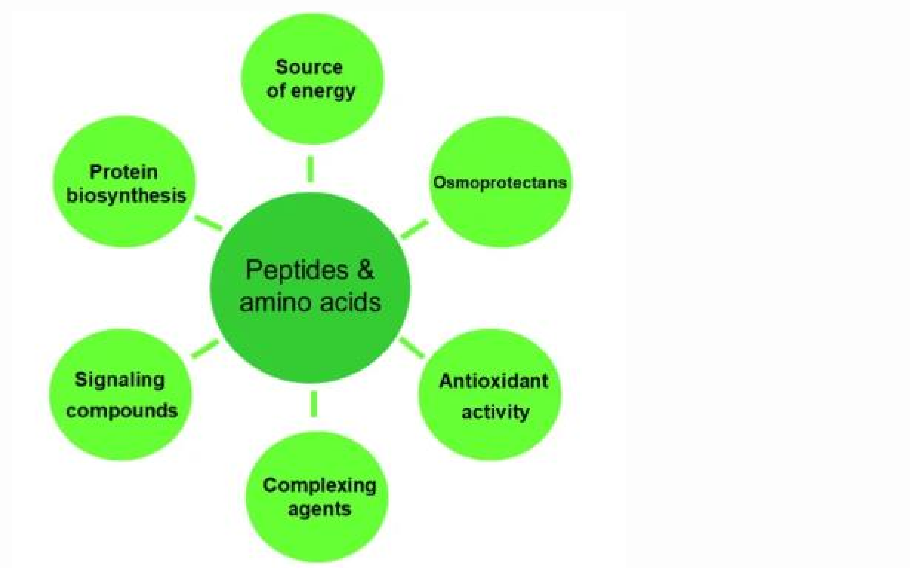

Figure 3. Physiological effects of protein hydrolisates (PHs) on plants (from Colla et al., 2015).

Besides these positive effects, the foliar applications of commercial PHs (Cerdán et al., 2009; Lisiecka et al., 2011), from animal origin, can cause phytotoxicity and plant growth inhibition, and no- or negative responses in plants, especially at high concentrations and in field experimental conditions (Calvo et al., 2014).

2.1.3 Seaweed extracts

Seaweed extracts (SEs) have always been used in agriculture as soil conditioners to improve soil fertility, and are the first materials used to produce PBs (Craigie et al., 2011), constituting more than 33% of the total biostimulant market worldwide (Eef et al., 2018). These numerous products based on green, red or brown algae extracts (mainly *Ascophyllum nodosum*, *Ecklonia maxima*, *Laminaria digitata* and *Fucus spp*) are inexpensive, easy to prepare and use (Hernández-Herrera et al., 2014). However, the type of SEs, harvest period and extraction method greatly influence the chemical characteristics of the extracts and thus their properties. Goni et al. (2016) confirmed the importance of extraction methods in relation to the efficacy of different *Ascophyllum nodosum* extracts, showing that the matrix origin is not sufficient to guarantee biostimulant results, but it is the combination between the components and extraction procedure fundamental for their effects on target plants. Generally, the SE active

components are macro- and micro-nutrients, amino acids, vitamins, sugars (carbohydrates and oligo- and polysaccharides), and hormones (cytokinins, auxins, gibberellins and abscisic acid, polyamines, brassinosteroids), which can influence cell metabolism improving plant growth and yield (Khan et al., 2009; Battacharyya et al., 2015; Shukla et al., 2019). In addition, the presence of polyphenols (such as floroglucinol and eckol) or polysaccharides (e.g., alginate, fucoidan, laminarin, carrageenan and their derived oligosaccharides), are responsible for the increased tolerance of plants to stresses (Battacharyya et al., 2015; Hernández-Herrera et al., 2016; Mzibra et al., 2021). The beneficial effects are already evident at very low concentrations (diluted to 1:1,000 or more; Crouch and van Staden, 1993).
The SE effects on the root system underlie increased root functionality in terms of growth, soil exploration, transport activity and efficient nutrient utilization (Brown et al., 2019). In this regard, Shukla et al. (2019) demonstrated that SEs influence genes involved in nutrient acquisition, increasing their uptake and consequently plant growth (Figure 4).

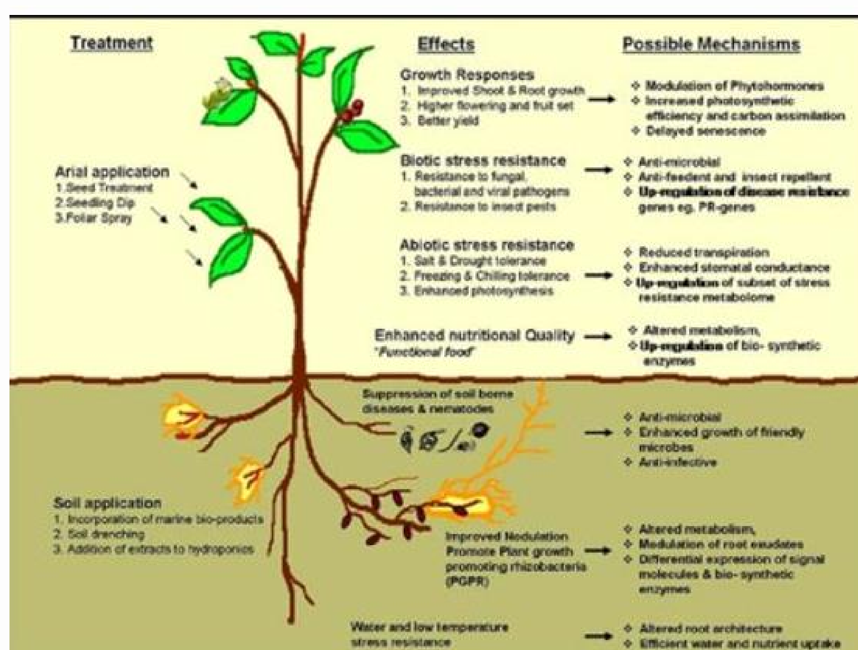

Figure 4. Physiological effects of seaweed extracts (SEs) on plants (from Khan et al., 2009).

The SE applications may induce tolerance against abiotic stressors and boost crop performance, improving the shelf-life of various crop products (Battacharyya et al., 2015). Recent studies highlight the protective effects of SEs against oxidative stress in plants subjected to environmental stress, thus reducing electrolyte leakage and lipid peroxidation (Shukla et al., 2021). Foliar application of *Ascophyllum nodosum* increased photosynthetic activity, stomatal conductance associated with a higher antioxidant capacity under moderate

water stress compared to untreated plants, leading to improved water status (Xu and Leskovar, 2015). Recently, Campobenedetto et al. (2021) showed that tomato plants, grown under drought conditions and treated with the SE-based biostimulant, exhibited lower abscisic acid (ABA), malondialdehyde (MDA) and proline, which correlated with lower ROS scavenger enzyme activity than untreated plants. These data, together with higher stem water potential and photosynthetic pigment levels suggested that SEs can mitigate the effects of water stress in tomato (Figure 5).

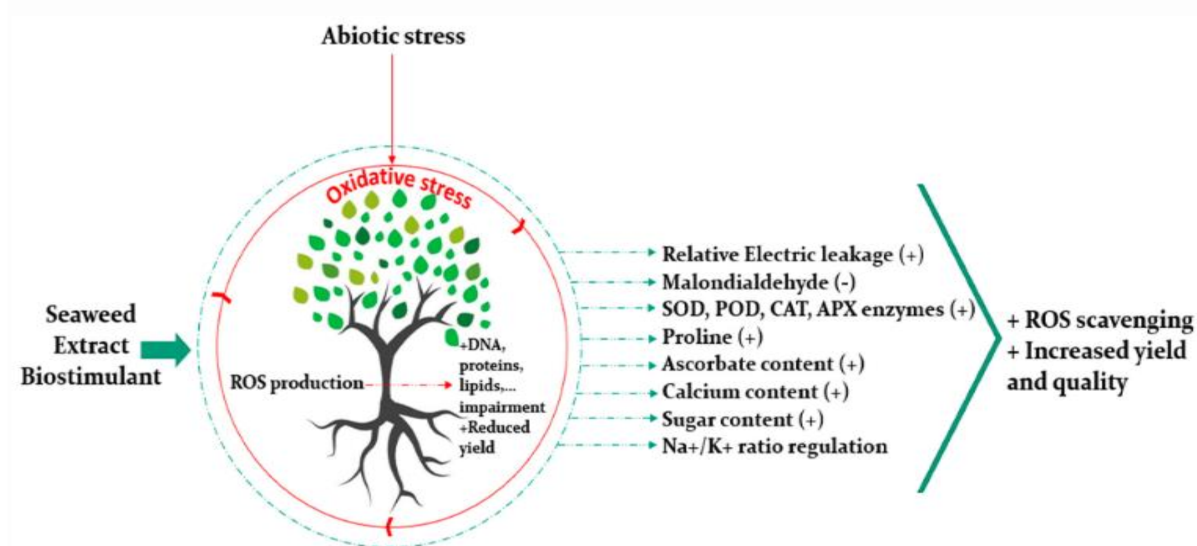

Figure 5. Beneficial effect of seaweed extracts under abiotic stress (from El Boukhari et al., 2020).

Among SEs, *Ascophyllum nodosum* extracts are among the most commonly PBS studied. They increased: yield and nutritional quality of spinach (Rouphael et al., 2018), nutritional status and shelf-life of lettuce (Chrysargyris et al., 2018), drought tolerance in tomato plants (Goñi et al., 2018), plant growth and yield in carrot and strawberry (Alam et al., 2013), or alleviated the water stress effects on common bean (Galvão et al., 2019). The mechanisms behind these beneficial effects of *A. nodosum* extracts are still under investigation, although various studies postulated hormonal effects on plant growth through the up- or down-regulation of auxin-responsive genes.

2.1.4 Plant Growth Promoting Microrganisms (PGPMs)

The PGPMs include various species of fungi (both mycorrhizal, AMF, and non-mycorrhizal), endosymbiotic bacteria and plant growth-promoting rhizobacteria (PGPR) (du Jardin, 2015) (Figure 6). In particular, the PGPMs consist of a microorganism or a consortium of microorganisms listed in the CMC-7 (Component Material Categories, number 7).

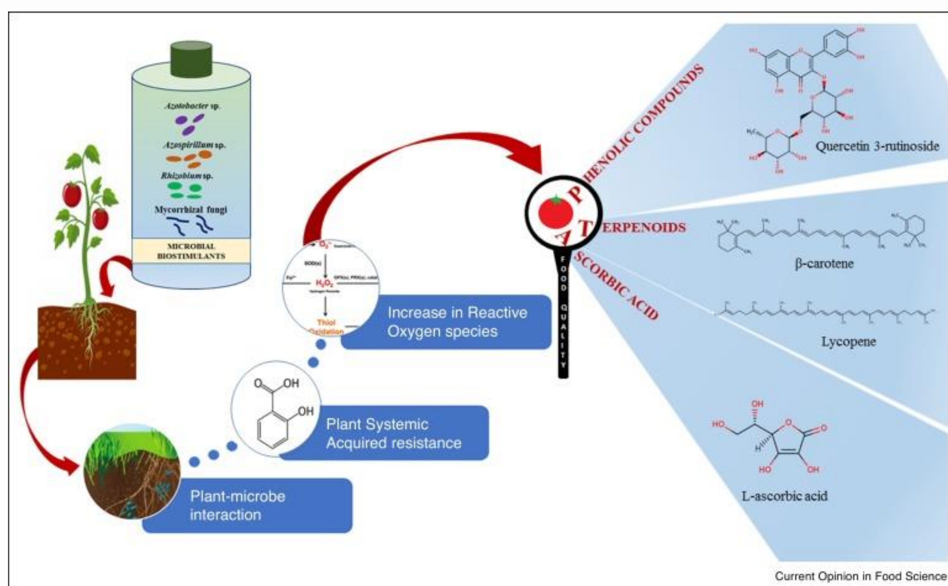

Figure 6. Microbial biostimulants: effects and mechanisms of action (Ganugi et al. 2021)

Currently, there are more than hundred products on the market based on live microorganisms, such as fungi and beneficial bacteria, which have been isolated and characterized from soil, plants, plant residues, water and composted manures (Calvo et al., 2014). The PGPMs can colonize both plant root and aerial organs: a) they can reside near the root (rhizosphere), b) on the root surface (rhizoplane), c) in the aerial part (phyllosphere), or d) they can exhibit endophytic behavior by colonizing the plant tissues (Khan et al., 2015).

2.1.5. Arbuscular mycorrhizal fungi (AMF)

Among beneficial microorganisms, arbuscular mycorrhizal fungi (AMF) constitute an important PB category, which positively influence productivity, supporting the ecosystem sustainability (Rouphael et al., 2015) (Figure 7).

Arbuscular mycorrhizal symbiosis is a mutually beneficial interaction between soil arbuscular mycorrhizal fungi (AMF) and more than 80 % of vascular plant families (Wang and Qiu, 2006) including agronomical important crops (Rillig et al., 2016). It is also an important integral part of natural ecosystems (Garg and Chandel, 2010). The AMF comprise more than 150 species belonging to the orders *Glomerales, Diversisporales, Gigasporales, Archaeosporales and Paraglomerales* (Oehl et al., 2011). They can only be cultivated in the presence of host plants (i.e. obligate symbionts; Owen et al., 2015), whose symbiosis develops between the fungus hyphae and plant roots (Gutjahr et al., 2009; Gutjahr and Paszkowski, 2013). The photosynthetic products obtained by the host plant are utilised by the fungus, which

in turn provides the plant with soil nutrients such as phosphorus, nitrogen, copper and zinc (Ferrol et al., 2019). However, the symbiosis goes far beyond the nutritional benefits, because of their ubiquity and physiological characteristics, the AMF play several very important roles in plant ecology, particularly, in salt stress mitigation (Giri et al., 2007), water supply and drought resistance (Ruiz-Lozano et al., 2012), protection against pathogens (Akhtar and Siddiqui, 2008a-b), bioremediation (Garg and Chandel, 2010), phyto-hormone production (Johansson et al., 2004), adaptation to various adverse environmental conditions (Miransari et al., 2008; Birhane et al., 2012), and ecosystem and ecological balance (Oehl et al., 2011). It has also been shown that AMF can improve crop quality by increasing antioxidant and vitamin content in their edible parts (Albrechtova et al., 2012).

Figure 7. Arbuscular mycorrhizal fungi (AMF) (Rouphael et al. 2015)

The mechanisms underlying tolerance depend on stress characteristics and, in most cases, are finely regulated by phytohormones (Pozo et al., 2015). Omics technologies have elucidated the important mechanisms of plant-AMF interactions in the response to abiotic stress (Bernardo et al., 2017; Bernardo et al., 2019). The metabolomic analysis demonstrated an accumulation of antioxidant compounds such as phenolics, terpenoids and ascorbic acid (Rouphael et al., 2020), a stimulation of phenylalanine ammonia lyase (PAL), a key enzyme in the phenylpropanoids biosynthesis, in various crops (Kolega et al., 2020).

Recently, through next-generation sequencing (NGS), it has been possible to identify mycorrhizal genes involved in symbiosis and nutrient transfer (Tisserant et al., 2013). This has allowed to identify new and potentially more beneficial strains (Igiehon and Babalola, 2017), to understand the factors influencing the mycorrhizal community composition in the

soil (Thonar et al., 2012), the establishment and persistence of inoculated strains over time (Pellegrino et al., 2012).

2.1.6. Plant growth-promoting bacteria (PGPB)

Plant growth-promoting bacteria (PGPB) are a heterogeneous group of non-pathogenic, soil-dwelling beneficial bacteria that can efficiently colonize plants, seeds and root systems improving their growth, nutrition and stress tolerance (Vacheron et al., 2013). Initially, the term PGPB was used to describe soil bacteria strains of the *Pseudomonas* genus, today the term includes the *Bacillu*s, *Azospirillum*, *Paenibacillu*s, *Enterobacter*, *Klebsiella*, *Paraburkholderia*, *Serratia*, *Gluconacetobacter*, *Azoarcus*, *Herbaspirillum* and *Arthrobacter* (Spaepen et al., 2009) genera. The PGPBs can induce different plant benefits depending on the microorganism, cultivar and growth medium (du Jardin, 2015). Since their capabilities, several hundred PGPB strains have been evaluated in laboratory, greenhouse and field studies worldwide (Martinez-Viveros et al., 2010); however, their mechanisms of action are not fully elucidated yet. The stimulating effect of PGPBs, under both optimal and suboptimal conditions, could be attributed to several direct and indirect mechanisms, including an increase of: (i) uptake and translocation of nutrients, particularly N and P and some micronutrients such as Fe, Zn and Mn; (ii) root system vigour (increased biomass, surface area and number of lateral roots), especially in crops with a taproot system (e.g. carrot) or a shallow root system (e.g. onion); (iii) water relations and photosynthetic capacity; (iv) an antioxidant defence system; (v) plant hormones such as auxins, ABA, cytokinins, ethylene and gibberellins); (vi) nutrient transporters such as NRT1 1, NRT2, NAR2.2 (nitrogen), AMT (ammonium), Pht1 and PT2-1(phosphate); and (vii) enzymes (phosphatases) and/or exudation of low levels of amino acids, sugars, organic acids and phenols, and high molecular weight organic compounds (mucilage and proteins) in the rhizosphere (Colla et al., 2015; Rouphael et al., 2015; Bitterlich et al., 2018) (Figure 8).

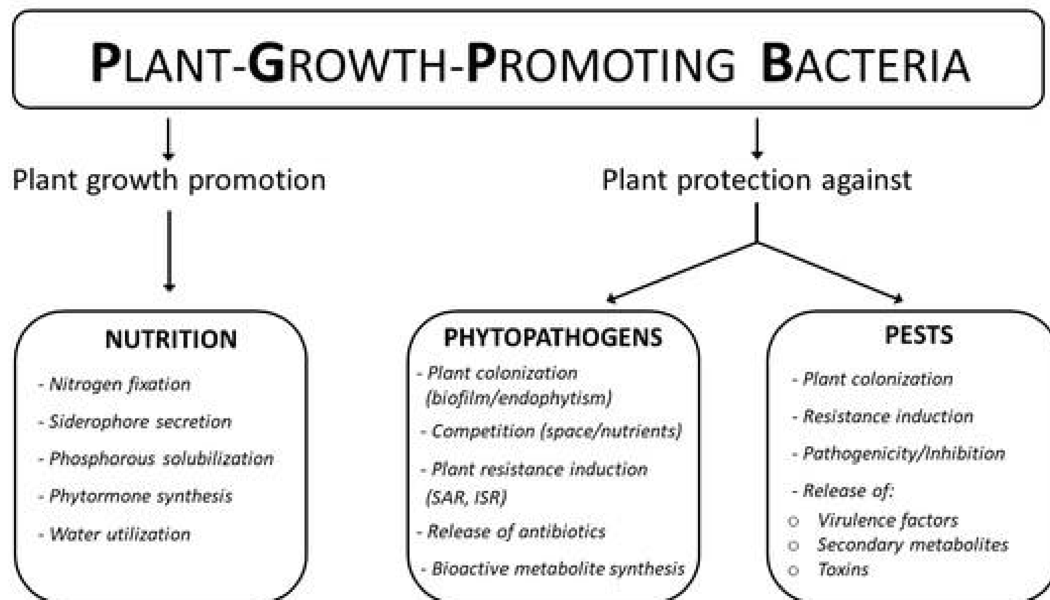

Figure 8. Multiple effects of PGPB and their mechanisms of action (Ruiu 2020)

In particular, PGPBs play a key role in the biogeochemical cycles of mineral nutrients, modulating their bioavailability in the soil (Alegria Terrazas et al, 2016). They stimulate root growth by increasing the surface area responsible for nutrient uptake (Adesemoye et al., 2009), the plasma membrane proton pump H^+-ATPase activity, a driving force in the ion secondary transport such as nitrate, phosphate and sulphate (Pii et al., 2015). This positive effect has been observed in roots of some species after inoculation with different PGPR strains (Canellas et al., 2002; 2013) and also under nutrient deficiency such as phosphorus (P) (Wang et al., 2017) or salt stress (Singh and Jha, 2016). In an indirect manner, PGPBs are able to prevent and reduce the deleterious effects of several phytopathogenic organisms through the synthesis of numerous antimicrobial compounds and/or the induction of systemic resistance (Olenska et al., 2020). Antimicrobial metabolites that are antagonistic towards phytopathogenic microorganisms include siderophores, antibiotics, cyanides, fungal cell wall-degrading enzymes and gaseous products such as ammonia (Idris et al., 2007). Antagonistic effects are caused by cytolysis, leakage of potassium ions, disruption of membrane structural integrity, inhibition of mycelial growth and protein biosynthesis (Quan et al., 2010).

3. A focus on *Streptomyces* and *Kocuria* species as PGPB

In recent years, the *Actinomycetes* use, in agricultural practices, has increased due to their potential action as PGPB, metabolic versatility, drought resistance, bioactive metabolites production such as vitamins, antibiotics, plant growth factors, enzymes (Manigundan et al.,

2022) and their ubiquitous repartition in plants (Yadav et al., 2018). Usually, they are soil inhabitants where they conduct a saprophytic life style (Bonaldi et al. 2015; Thilagam and Hemalatha, 2019). Isolated from rhyzosphere and root tissues, they represent a major component of rhizospheric microbial populations, with economic importance for humans, agriculture and forest productivity (Yadav et al., 2017), significantly influencing nutrient cycling (Halder et al., 1991; Elliott and Lynch, 1995), plant growth and health.

Among these soil bacteria, *Streptomyces* species are the most prolific source of bioactive metabolites able to improve plant development and growth, nutrient uptake and biotic and abiotic stress resistance either by producing indole-3-acetic acid (IAA) and siderophores and/or by inhibiting soil-borne fungal pathogens (Olanrewaju et al., 2017; Suárez-Moreno et al., 2019; Manigundan et al.,2022). For example, the main biocontrol ability is attributed to the strong production of antibiotics, volatile compounds, and other metabolites which help in its role as antipathogens. For example, they produce siderophores (*S. coelicolor*) (Som et al. 2017), chitinase (*S. violaceusniger* YH27A strain) (Gherbawy et al. 2012), antifungal nigericin, and antibiotic geldanamycin (*S. violaceusniger* YCED-9) (Shrivastava and Kumar, 2018). In addition, some *Streptomyces*, enhancing phytohormones and siderophores production in saline soil conditions, relieving plants from stress and improving their health and development (Sadeghi et al. 2012). The PGP potential of *Streptomyces sp*. has been demonstrated on tomato, wheat, rice, bean and pea (Tokala et al. 2002; Nassar et al. 2003; El-Tarabily 2008; Sadeghi et al. 2012; Gopalakrishnan et al. 2013). Furthermore, they are able to grow in a nitrogen-free medium using atmospheric nitrogen, due to their nitrogen fixing properties (Manigundan et al., 2022; Sellstedt and Richau, 2013).

Aside from the exploratory growth mechanism, PGPB use volatile organic compounds (VOCs) to modulate environmental conditions through their actions as signals. These signals can result in regulating gene expression of surrounding microbes. They can act as elicitors of gene activation or repression as well as determine the response of other microbes in the soil environment (Oluwaseyi and Olubukola, 2019).

Beside *Streptomyces sp*., also *Kocuria* genus is gaining a lot consideration as PGPB due to their metabolic versatility, resistance to harsh conditions and indole-3-acetic acid (IAA) production (Marchawala and Amin, 2018). Their role as biocontrol agent against fungal pathogen, to protect vineyards from the common vine pest, has been demonstrated (miotto-Vilanova et al., 2016; Jindo et al., 2022). The genus *Kocuria,* belonging to the family *Micrococcaceae,* order *Actinomycetales* (Kocur et al., 2006), includes *Kocuria rhizophila,* a Gram-positive bacterium, characterized by coccoid cells, grouped in pairs, chains, tetrads,

cubical arrangements of eight, or irregular clusters (Stackebrandt et al. 1995). The *Kocuria rhizophila* is a soil dwelling bacterium, commonly used in industry for antimicrobial testing and food preparation. Recently, Guesmi et al. (2022) identified in this strain new genes potentially involved in molecules biosynthesis with antifungal activities, such as bacilysin and cycloserine. Furthermore, Li et al. (2020) demonstrated that the inoculation with *K. rhizophila* Y1 strain significantly contributes to mitigate salt stress tolerance by regulating plant hormones and nutrient acquisition, maintaining ion homeostasis and improving plant growth performance in maize.

4. Biostimulant activity

The PB activity is multiple and very complex influencing all the stages of agricultural production from seed germination to plant growth and harvested products. The PBs offer a potentially novel approach for the regulation/modification of physiological processes in plants to stimulate growth, to mitigate stress-induced limitations, and to increase yield. However, despite the efforts made to understand their effects, the mode/mechanisms action of PBs is not well defined yet. They include many different biochemical, physiological and molecular processes such as nitrogen metabolism, phosphorus release from soils, soil microbial activity, seed germination and root growth, plant metabolism, photosynthesis, nutrient uptake and abiotic stress tolerance (Figure 9). Furthermore, their beneficial effects occur often when plants are challenged by abiotic and biotic stress such as drought, heat, salinity, chilling, oxidative, mechanical, and chemical stress.

A wide array of molecular methods has been used to discern the chemical composition of PBs and their activity, including chemical and non-chemical characterization, morpho-physiological analysis and yield studies (Yakhin et al., 2017). More recently, multi *omics* approaches, including microarray, transcriptomic, genomic, phenomic, proteomic and metabolomic analysis, allowed to elucidate deeply the PB molecular mode of action and utilization and their important mechanisms protecting plants from abiotic stress (Rouphael et al., 2020). Only through a combination of methodologies, the progress in PB research will be possible.

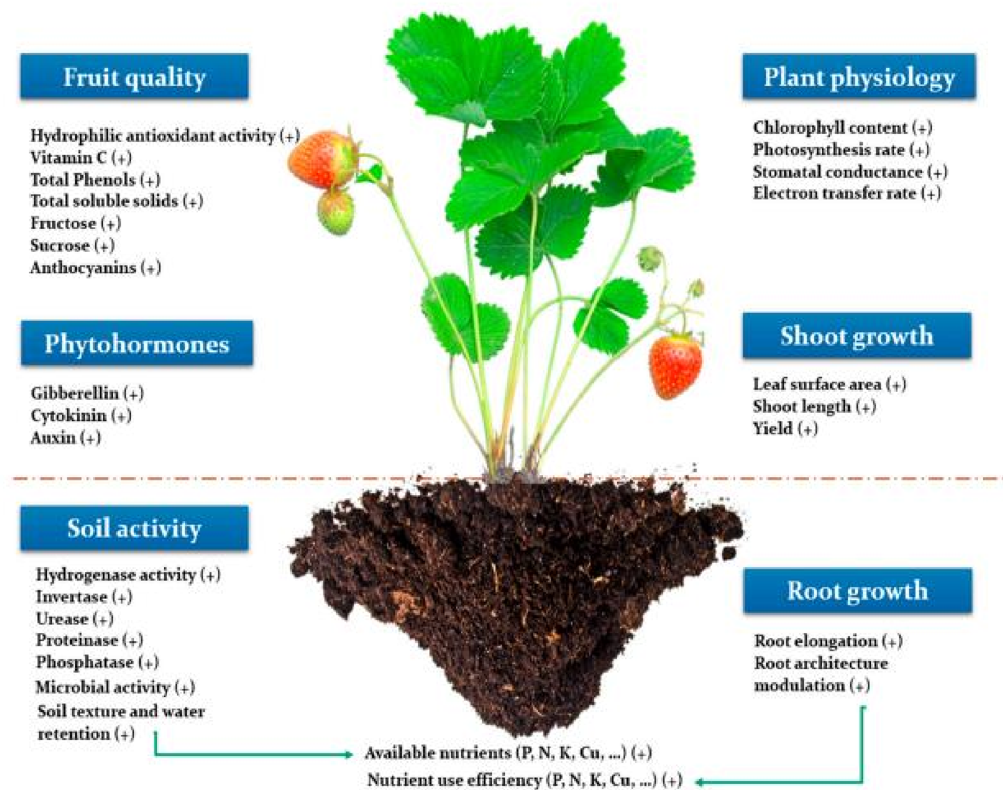

Figure 9. Conceptual illustration highlighting the positive impact of biostimulants on the whole soil–plant system. Such effects encompass improving fruit quality, and plant phytohormone content, increasing soil enzymatic activity, improving the rooting system and the overall physiological features of plants (EL Boukhari et al. 2020).

4.1 Biostimulants and seed germination

Seed germination is a critical phase for the plant life cycle, growth, survival and production. Germination is a complex process including imbibition, respiration, protein synthesis, and phytohormone production. All of these processes can be manipulated by extrinsic factors including the PB application, which have the potential to release dormancy and enhance seed germination in many plant species. They are able to affect the seed physiological processes, early radicle protrusion, emergence (appearance of a seedling through the soil), seedling establishment, metabolic efficiency, hormone metabolism even under abiotic stress (Makhaye et al. 2021; Gupta et al., 2021).

For example, reygrass seeds sowed with humic extracts, from peat, showed the highest germination percentage after 7 days of treatment (Asenjo et al., 2000). The humic extracts, containing humic substances extracted from leonardite, stimulated barley seedling growth (Szczepanek and Wilczevski, 2011); the humic acids, extracted from vermicompost, increased shoot and root dry weigth of rice (Garcia et al., 2012); rice seeds primed with Vimpel, containing humic substances, showed a higher seedling growth (Adetumbi et al., 2019).

The microalgae such as *Acutodesmus dimorphus*, *Chlorella sp*., *Dunaliella salina*, *Haematoccocus sp*., and *Scenedesmus sp*. can be used as PBs in the early stages of crop cultivation to improve seed germination traits (Puglisi et al. 2020). They contain high levels of micronutrients, macronutrients and phytohormones (gibberellins, auxin, and cytokinin) which are essential for plant growth and development (Stirk et al. 2013).

The SEs isolated from brown macroalgae (or seaweeds) (e.g. *Ascophyllum nodosum*, *Ecklonia maxima*) and green macroalgae (e.g. *Caulerpa sp.*, *Ulva sp.*) and red macroalgae (e.g. *Kappaphycus alvarezii*) enhanced seed germination and seedling establishment in many crops (Pramanick et al. 2017). Their effect depends mainly on the dose of application (Hernández-Herrera et al. 2016), being inhibitory at high concentrations (extend seed germination time) for the presence of high salt concentrations (Layek et al. 2018). Conversely, at low concentrations, the seed germination and seedling vigor promotion could be attributed to the presence of phytohormones (such as gibberellins and cytokinins) and micronutrients (Kulkarni et al. 2021).

Besides, PBs regulate phytohormones, in the seeds or seedlings, for the presence of hormone-mimicking compounds (Zandonadi et al. 2019); or by interacting with plant hormone signaling pathways (Jindo et al. 2020). For example, wheat peptides mimic gibberellins like activity (Ghosh et al. 2010); PHs stimulate auxin and gibberellin-like activities (Ertani et al. 2009, 2019; Colla et al. 2014); SEs promote the germination of photosensitive Grand Rapids lettuce seeds under dark, red and far-red light conditions by reducing the ABA levels and modulating cytokinins (Gupta et al. 2019).

Furthermore, PBs, such as SEs, KAR1 and vermicompost leachate, containing gibberellins or compounds with gibberellins like activity, alleviated dormancy in various crops (Nemahunguni et al. 2020), stimulating dormant seeds to produce enzymes that break stored reserves and convert them to energy required for respiration (Aremu et al. 2015). Moreover, putrescine and spermidine alleviated dormancy in apple (*Malus domestica*) embryos by enhancing ROS (H_2O_2 and O_2 •−) and RNS (NO and ONOO−) (chiefly NO) production (Krasuska et al. 2014). By contrast, the abnormal or excessive ROS accumulation in germinating seeds and developing embryos can be detrimental negatively affecting the germination rate, which is controlled by antioxidant mechanisms (Jeevan Kumar et al. 2015). Several PBs induce ROS-detoxifcation enzyme system and elicit/contain non-enzymatic antioxidant compounds, which are helpful for ROS detoxification, preventing their accumulation in germinating seeds, at cellular and subcellular levels (Khaliq et al. 2015).

Finally, the PB active compounds influence enzyme involved in post-germination process that control plant growth rate. For example, SEs enhanced the α-amylase and lipase activity (which aided in mobilizing stored reserves), promoting the germination of lettuce seeds under dark, red and far-red light (Gupta et al. 2019). The positive effect of SEs on seed germination and vigor cultivars was also correlated with enhanced dehydrogenase activity of weakly germinating narrowleaf lupine (*Lupinus angustifolius*), providing energy for de novo synthesis of metabolic compounds and hydrolysis of food reserve (Płażek et al. 2018).

4.2 Biostimulants and plant growth and metabolism

In recent years, PBs have been used widely in agriculture to promote crops productivity since they stimulated different physiological processes involved in plant growth and development (Figure 10). These positive effects are based on the increasing of chlorophyll content in leaves, thus raising the photosynthesis efficiency. For example, the *Hibiscus* cultivation treated with biowaste soluble hydrolysates, showed a 15% increase in the chlorophyll content, resulting in a 24% increase in the photosynthesis rate compared to the control (Massa et al., 2018). Recently, the positive effect of foliar feeding with four PBs (*Ecklonia maxima* algae extract, titanium, humic substances, auxin and cytokinin) on the chlorophyll content and plant height of three *Solanum tuberosum* cultivars was reported (Zarzecka et al., 2022). After the foliar application of the *Moringa oleifera* leaf extract (MLE), classified as one of the current PB, a 34.6% increase in the chlorophyll content of *Cucurbita pepo* L. leaves was recorded compared to the control (Abd El-Mageed et al., 2017). In particular, MLE-treated plants had higher growth and yield characteristics, harvest index (HI),WUE, chlorophyll fluorescence (Fv/Fm and PI), photosynthetic pigments, soluble sugars and free proline, leaf anatomy, relative water content (RWC%) and membrane stability index (MSI%) and had lower electrolyte leakage (EL%) compared to MLE-untreated plants, especially under the irrigation deficiency. The aqueous garlic extracts (AGE) in conjunction with acetyl salicylic acid (ASA) were applied to eggplant and pepper seedlings as foliar application and fertigation methods. The results revealed stimulatory responses in the growth of the vegetables with improved plant height, number of leaves, root growth, fresh and dry weight. Moreover, significant alterations were indicated in plant metabolites such as chlorophyll, carotenoids, and soluble sugars, and an antioxidant enzyme stimulation such as superoxide dismutase (SOD) and peroxidase (POD) (Hayat et al., 2018).

Some of PHs contain many enzymes, such as nitrate reductase, malate dehydrogenase, leucino-amino peptidase, phosphorilase and phosphatase, which improve plant nutrient

utilization and consequently leading to a better plant growth (Maini, 2006). Furthermore, they contain bioactive peptides able to elicit hormone-like activities (auxin and gibberellin), influencing shoot and root growth, root architecture (e.g. length, number, density, and surface of lateral roots) thus improving crop productivity. Moreover, PHs can add complex nutrients to the soil solution potentially rendering them more available for plant uptake and enhancing microbial activity (Colla et al., 2017). In addition, tropical plant extract (PE) also exert indirect effects on plants, as they modify root architecture increasing their hair surface expansion, thus enhancing macro- and microelement uptake, which result in enhancement of growth (Colla, et al., 2017), production and quality. Notably, PE or PHs also encourage plant activity of key enzymes involved either in N or C metabolism (Sestili et al., 2018). For example, two protein hydrolyzate–based fertilizers (PHFs), one from alfalfa (AH) and one from meat flour (MFH) increased root and leaf growth inducing morphological changes in root architecture. Besides, they increased nitrate reductase (NR) and glutamine synthetase (GS) activities, suggesting a positive role of the two hydrolyzates in the induction of nitrate conversion into organic nitrogen (Ertani et al., 2009).

The SEs exhibit growth-stimulating activities, promoting root growth and development and improving plant mineral uptake by roots (Vernieri et al., 2005) and leaves (Mancuso et al., 2006). The *Ascophyllum nodosum* affects carbon and nitrogen metabolism (Jannin et al., 2013) also containing osmolytes such as mannitol, which play a protective role in plants exposed to stress (Reed et al., 1985).

Finally, the increased production of antioxidants by PBs in plants decrease their sensitivity to stress conditions (Ertani et al., 2011).

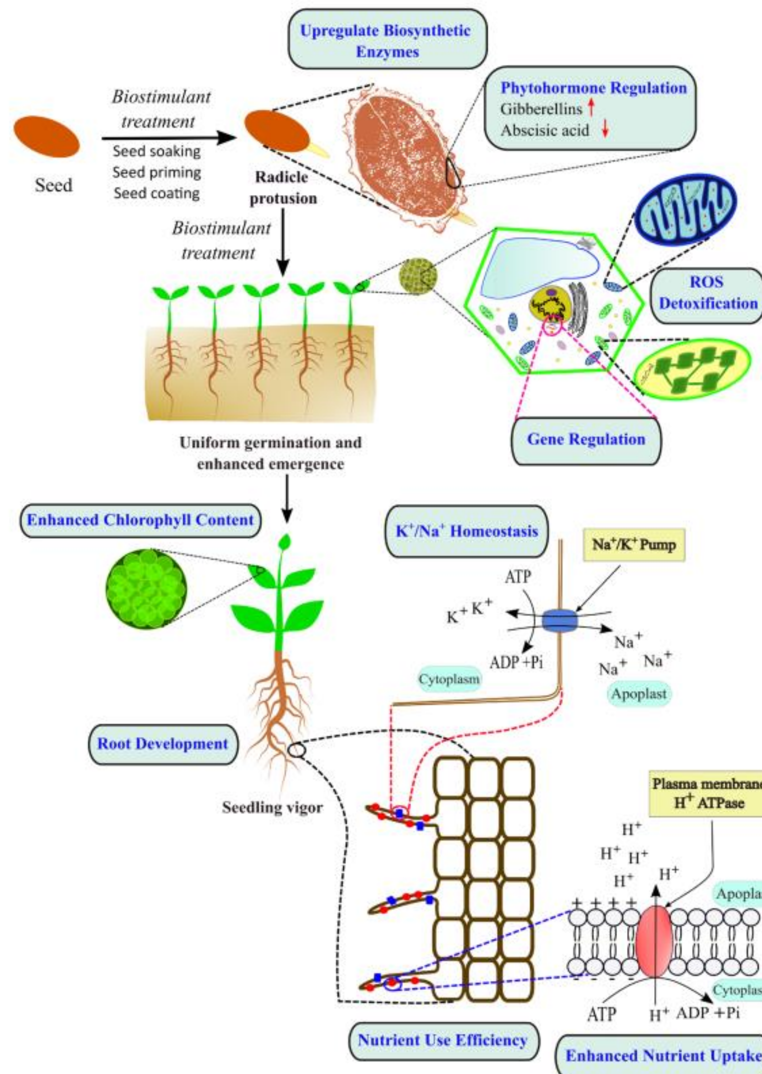

Figure 10. Role of biostimulants in enhancing the metabolic activity of seed and seedlings (from Gupta et al., 2021)

4.3 Biostimulant, yield and fruit quality

The PBs can be used in vegetable production to improve productivity, yield, and fruit quality. For example, the biostimulant Actiwavew (Valagro s.p.a., Atessa, Chieti, Italy), increased yield of rocket (*E. sativa*) grown in a floating system, even if the nutrient concentration was reduced (Vernieri et al. 2006). This effect was also observed in baby leaf lettuce (*Lactuca sativa* var. acephala) grown in a plastic tunnel (Amanda et al. 2009) and in strawberry where the PB stimulated the vegetative growth, leaf chlorophyll content, stomata density, photosynthetic activity, yield and fruit weight (Spinelli et al. 2010). The effect of foliar application of SE Primo, an organic biostimulant, showed a significant improvement in potato (*Solanum tuberosum* cv. Sante) growth, yield and tuber quality, in terms of nitrogen, total soluble solids and protein contents (Haider et al., 2012). An increase in the average length and

diameter of cucumbers was attained after humic acids application in the first and second growing season compared to the control (Fawzy, 2012).

The PE- and PH-based biostimulants gave higher tomato yield and nutritional quality in both conventional and organic farming system in different way. The PH treatment led to higher fruit number than the control, whereas PE incurred significant increase in yield only under organic farming. The mean fruit weight attained the highest value upon PE application under conventional management. However, both PB applications resulted in higher total phenol and ascorbic acid as well as in lycopene content, and lipophilic antioxidant activity (Caruso et al., 2019). Norrie and Keathley (2006) reported that *A. nodosum* extracts showed positive effects on the yield of 'Thompson seedless' grape (*Vitis vinifera* L.) consistently over a 3-year period. They observed that the treated plants always outperformed (in terms of berries per bunch, berry size, berry weight, rachis length, and the number of primary bunches per plant) the controls maintained under the regular crop management program, and resulted in improved fruit size, weight, and yields.

An eco-friendly microbial-based biostimulant effect was tested on the biometric parameters, yield and nutritional value of *Capsicum annuum* fruits. After its application, an increase in leave pigment content agreed well with the higher total and commercial yields of treated pepper cultivars, together with a higher vitamin C and total phenolic content in fruits, during the hot summer season, were observed (Majkowska-Gadomska et al., 2021).

Furthermore, the consortium of arbuscular mycorrhizal fungi such as *Rhizophagus* spp., *Rhizophagus aggregatus*, *Septoglycus viscosum*, *Claroideoglomus etunicatum*, *Claroideoglomus claroideum* with the plant growth-promoting bacteria (PGPB) increased weight, fruit diameter and elongation in tomato (Bona et al., 2018).

Recently, Li et al. (2022) demonstrated that PBs: (1) add a yield benefit about of 17.9%, reaching the highest potential via soil treatment; (2) applied in arid climates and vegetable cultivation had the highest impact on crop yield; and (3) were more efficient in low soil organic matter content, non-neutral, saline, nutrient-insufficient, and sandy soils. They also compared the effectiveness of PBS application among different crop types such as cereals, fruits, legumes, root/tubers, vegetables, and other crops demonstrating that vegetable crops showed the highest yield benefit and roots/tubers differ by more than two-fold (+22.8% *vs* +10.6%). Legumes were significantly better at responding to PBs applications than fruits, cereals, and other crops. Finally, they analyzed the yield effectiveness in relation to climate and soil properties showing that the PBs effect was most positive in climates with seriously

limited water availability (arid and desert), which was considered as a critical factor for the PBs effectiveness (Li et al., 2021).

4.4 Biostimulants and abiotic stress

The PB are able to counteract environmental stress such as water deficit, soil salinization, nutrient deficiency, and sub-optimal growth temperatures in several ways (Van Oosten et al., 2017; Yamauchi, 2018). They improve plant performance, enhance plant growth and productivity, interact with several processes involved in plant responses to stress, and increase the accumulation of antioxidant compounds that allow decrease plant stress sensitivity. The PB effectiveness to counteract the stressful condition depends on several factors, such as timing of application, plant stages (seeds, early growth stages and fully developed plants) and mode of action. They may be applied at different timings: before, during or after the stress depending on their components. For example, PB containing anti-stress compounds, such as proline or glutamic acid, can be applied when the stress occurs or during stress conditions, while those involved in the activation of bioactive compound biosynthesis must be applied before the stress. Proper timing of application during crop development differs from species to species and depends on the most critical phases for crop productivity. The PBs may be applied on seeds, in early stages of growth, when crops are fully developed, during blooming or fruit setting (Kunicki et al., 2010). Another interesting approach to induce tolerance to abiotic stresses is soaking plant seeds with different compounds, synthetic or natural. This strategy called "seed priming" has been deeply reviewed by Ashraf et al. (2018).

4.4.1 Biostimulants and salinity stress

Salinity is one of the main harmful stress affecting plant growth, metabolism and crop yield. It causes a significant reduction of both fresh weight and chlorophyll content; nutrient imbalance and availability; solubility reduction of micronutrients such as Cu, Fe, Mn, Mo and Zn; water potential decrease (Yarsi et al., 2017). Salinity may also alter photosynthesis (Sayyad-Amin et al., 2016), respiration (Moud and Maghsoudi, 2008), phytohormone regulation, protein biosynthesis, nitrogen assimilation (Flores et al., 2004), total phenolics and total soluble protein reduction, and catalase, superoxide dismutase and peroxidase activity suppression (Bano et al., 2012). Many studies have been performed to verify the effects of the PB applications under salt stress on lettuce plants, a crop moderately sensitive to salinity. Lucini et al. (2015) showed that a plant-derived protein hydrolysate improved lettuce tolerance

to salinity, increasing yield and dry weight. Similar results were observed in response to the Retrosal® application, an organic commercial biostimulant (Bulgari et al., 2019). The *Azospirillum brasilense* inoculation caused positive results on lettuce fresh weight, dry weight, ascorbic acid content, and germination percentage, followed by a better visual appearance due to higher chlorophyll levels (Fasciglione et al., 2015). Mayak et al. (2004) tested several strains of rhizobacterium on tomato seedlings, finding that plants inoculated with *Achromobacter piechaudii* and irrigated with saline water had a higher fresh and dry weight and an increased water use efficiency. The Super Fifty® and Acadian, two seaweed-based plant biostimulants containing *Ascophyllum nodosum*, applied respectively on lettuce (Guinan et al., 2013) and strawberry (Ross and Holden, 2010), were associated with a significant increase in yield and root dry weight, despite the adverse salinity condition. The SE application from *Sargassum muticum* and *Jania rubens* significantly alleviated salt negative effects through regulation of amino acid metabolism, ionic content balance and improved antioxidant defense in chickpea plants. Amino acids such as serine, threonine, proline and aspartic acid were identified in roots as responsible for salt stress amelioration (Abdel Latef et al., 2017). Several plant extracts based on licorice roots have been tested on common bean, a salt sensitive plant (Rady and Mohamed, 2015). It improved the seed germination percentage, stability of cell membrane and relative water potential and antioxidant system under saline conditions. A recent study highlighted the ability of a bee-honey based biostimulant to improve the onion plants tolerance to salinity stress. Treated plants showed higher biomass, bulb yield, photosynthetic pigments together with the osmoprotectans content as proline, soluble sugars and total free amino acids, the membrane stability index and the enzymatic and non-enzymatic antioxidant activity (Semida et al., 2019). In particular, the accumulation of osmolytes enhances the cell osmotic potential and the level of protective molecules against oxidative stress induced by salinity.

4.4.2 Biostimulants and drought stress

Drought stress is increasing due to the negative impacts of global climate change, remaining the main critical growing factor for food production in numerous countries (Lesk et al., 2016). Drought stress negatively affects plant growth and development by inducing many changes at morpho-physiological and molecular levels (Osakabe et al., 2014) and diminishing yields of important crops by over 10% (Elliott et al., 2014).

The PB application could be a possible strategy to improve water use efficiency, reducing the amount of water added to crops (Calvo et al., 2014). The *Ascophyllum nodosum* application

on broccoli (Kałuzewicz et al., 2017) enhanced gas exchange through the stomatal closure reduction, resulting in an increased plant resistance to water stress. Similar results were obtained in responses to Megafol treatments in tomato (Petrozza et al. 2014) revealing that treated plants were healthier than non-treated ones in terms of biomass and chlorophyll fluorescence. Romero et al. (2014) demonstrated that the *Azospirillum brasilense* treatments, a strain isolated in arid environments, delayed wilting of tomato plants, which showed a high xylem vessels area, resulting in a more efficient water transport. Furthermore, there are several PGPR strains that produced exopolysaccharides, phytohormones, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, volatile compounds, inducing osmolyte and antioxidant accumulation, or up or down regulation of stress responsive genes and an alteration in root morphology leading to water stress tolerance (Kumar and Verma, 2018). Goupta et al. (2022) observed that PGPR containing ACC-deaminase, a precursor of ethylene, significantly decreased the water stress effects on pea (*Pisum sativum*) growth and yield too. Recently, Lephatsi et al. (2022) demonstrated that a microbial-based biostimulant, a *Bacillus* consortium, induced differential morpho-physiological, epigenetic, genetic and metabolic changes to promote drought stress tolerance in maize plants.

A micro-algae-based biostimulant application reduced the damaging effects of drought stress, increasing plant height, root length, and enhanced the number and area of tomato leaves (Oancea et al., 2013). Recently, Campobenedetto et al. (2021) demonstrated that the foliar application of Eranthis, a biostimulant based on seaweed and yeast extracts, was able to mitigate water stress in tomato enhancing ROS scavenging, especially at flowering time.

Hence, biostimulants are capable of reducing drought injures, being able to enhance the biosynthesis of osmolytes and antioxidants against ROS and plant hormones, like ABA, regulating transpiration and avoiding excessive water losses.

4.4.3 Biostimulants and nutrient deficiency

The PB application can represent a valuable tool to enhance soil nutrient availability, plant nutrient uptake and assimilation through different strategies (De Pascale et al., 2017). Nowadays, soil nutrient imbalance is an increasing problem for farmers that often applied an excessive dose of fertilizers to resume soil fertility with negative consequence for the environment and human health. For instance, PBs are able to change soil structure or nutrient solubility, modify root morphology directly or ameliorate nutrient uptake and transport in plants, resulting in an increase of agronomic nutrient use efficiency (AE) (Halpern et al., 2015). Higher AE due to the application of PBs makes them interesting for sustainable and

environment-friendly plant production, reducing fertilization, especially for nitrogen. Interestingly, biostimulant application boosted nitrogen use efficiency with a 23% increase over untreated plants. Furthermore, their application might be useful in poor soil conditions and in low input horticultural cultivation systems (Toscano et al., 2019).

Mora et al., (2010) hypothesized that the effects of PBs, such as HS in plants, may involve a primary effect on the root H^+-ATPase activity and nitrate root–shoot distribution that, in turn, would cause changes in the root-shoot distribution of certain cytokinins, polyamines and abscisic acid, thus affecting shoot growth.

Koleška et al. (2017) studied the foliar application of biostimulant Viva® in tomato plants exposed to reduced NPK fertilization, demonstrating that its application help to counteract the negative effects of nutrient deficiency. In particular, lycopene and chlorophyll content was preserved in treated plants grown with NPK reduction. Moreover, biostimulant application helped maintain cell homeostasis and prevent oxidative stress.

A seaweed-based product (Kelpak®) has been tested on okra seedlings grown with different nutrient deficiencies (Papenfus et al., 2013). They were applied three times a week and were compared with a polyamine solution treatment. Plants treated with Kelpak® showed an increase in growth parameters, such as shoot length, stem thickness, leaves and roots numbers, and fresh weight under phosphorous and potassium deficiency. It also significantly increased the Zn, Cu, Fe and MN content in the grass species. The Kelpak® efficacy might be due to the combination of auxins, cytokinins and polyamines contained in the product (Goldlewska and Cipiela, 2016). Spinelli et al. (2010) measured the effects of another commercial seaweed extract, named Actiwave® on the vegetative and productive performance of strawberry plants grown under iron deficiency. They found that vegetative growth, chlorophyll content, stomatal density, photosynthetic rate, and fruit production and weight were enhanced after PB treatment. The positive effect could be ascribed to the more developed root system, which increases the nutrient uptake. Furthermore, the betaine contained in this product, also contrasted the negative effects of iron chlorosis, while its polysaccharide content helps the soil structure. Nevertheless, Vernieri et al. (2005) obtained good results also by applying Actiwave in a hydroponic system of rocket plants with different concentrations of nutrient solutions. Yield and leaf area were higher in plants grown with the lowest nutrient concentration, indicating a better nutrient use efficiency.

Most of the PBs contains a mixture of different amino acids and short peptides that have a positive effect on plant growth under nutrient deficiency (Cerdán et al. 2013). For example, tomato plants grown under iron deficiency conditions and treated with two products

containing amino acids from plant and animal origin showed different responses. Plant-derived amino acids promoted growth and chlorophyll content both in controlled and iron deficiency conditions. This effect might be ascribed to glutamic acid content, which plays an important role in nitrogen metabolism (Miflin and Lea, 1976) and chlorophyll biosynthesis (Porra, 1997).

Nutrient imbalance might be the cause of several disorders during plant growth and development. For example, calcium deficiency usually caused blossom-end rot in pepper young fruits. Paradikovi´c et al. (2013) tested four different biostimulant products for their effects on yield and blossom-end rot (BER) incidence on pepper. The results obtained revealed that PB application helped to reduce the BER occurrence and increase yield. Moreover, nutrient accumulation in fruits and leaves was promoted by the treatments.

Sánchez-Sánchez et al. (2009) applied humic acids, extracted and purified from a commercial product obtained from lignites, to Fe-deficient young tomato plants grown in hydroponics. Their biostimulant effect on Fe uptake mechanism alleviated symptoms of Fe deficiency as well as improved plant growth.

However, these results revealed that PB cannot totally replace fertilizers but they could be really useful to reduce and/or help in nutrient deficiency and imbalanced situations, increasing nutrient uptake by increasing root biomass, nutrient transport/translocation, and enzyme activities involved in nutrient assimilation. Moreover, the difficulty in truly assessing the PB benefits on NUE is complicated since the preponderance of studies were conducted under no-field and no-commercial conditions and in very short-term experiments. Valuable information on PB effects can be reached only by examining the full cropping cycle and in the environment in which the crop would be commercially grown.

Another important aspects in the PB application is the reduction in N inputs in leafy vegetables for containing the phenomenon of nitrate accumulation in edible leaves. Several PB stimulate biomass formation and elicits enzyme action regulating nitrogen use (nitrate reductase, nitrite reductase, glutamine synthetase (GS), and glutamine oxoglutarate aminotransferase (GOGAT)), thus reducing excess nitrate in plant tissues, a main feature of leafy green vegetables. In addition, it can improve vegetable quality and increase resistance to climatic stresses (Ottaiano et al., 2021).

5. Biostimulant regulations

The PB inclusion in the legislation of fertilizers always showed numerous problems, sometimes insuperable, until July 5, 2006, the date of Legislative Decree No. 217 of April 29,

2006 (Legislative Decree No. 217/06) "Revision of the regulations on fertilizers," published in Official Gazette No. 141 of June 20, 2006 - Ordinary Suppl. No. 152. The difficulty of their inclusion was the lack of an official method for the ascertainment and quali - quantitative determination of PB activity, by the competent authority. When Legislative Decree No. 217/06 came into force, biostimulant properties were declarable for only two products: (i) Protein Hydrolysate of Alfalfa and (ii) Hydrolyzed Animal Epithelium (solid and fluid). Subsequently, the Italian Legislation (Legislative Decree No. 75/2010 and the subsequent amendment of July (10, 2013) dedicated a special section to "Products with specific action-Biostimulants" (entire All. 6), categorizing them, and according to the elements underlying the formulations, defining them as "products that contribute to another fertilizer or to the soil or plant, substances that favor or regulate the nutrients uptake or correct several physiological anomalies."

The need to reshape the Fertilizer Regulation was aimed at promoting the internal market for these products in order to establish a common legal framework for PB, which is currently fragmented among member states. The new EU Regulation 2019/1009 (OJEU, June 25) of the European Parliament and Council, together with the amending Regulations (EC) No. 1069/2009 and (EC) No. 1107/2009 and repealing Regulation (EC) No. 2003/2003" defines biostimulants as reported in the previous §, including compounds of microbial and non-microbial nature. Annex I of EU Reg. No. 2019/1009 describes the Functional Categories of Products (PFCs) fertilizers in the EU, and, PFC 6, which provides the category of Biostimulants, is divided into two subcategories: PFC 6 (A) and (B) microbial and non-microbial, respectively. Annex II includes the Categories of Constituent Materials (CMCs) that must exclusively constitute the above fertilizer products.

Regarding microbial biostimulants, the regulations stipulate that it must consist of a microorganism or a consortium of microorganisms included in CMC No. 7, and specifically refers to four different genera of microorganisms: *Azotobacter* spp., *Mycorrhizal Fungi* (AMF), *Rhizobium* spp., and *Azospirillum* spp.

From July 2022 onwards, there will be two options for placing biostimulants on the market.

It will still be possible to follow the national regulations. Therefore 1) a product will be legally placed on the market in one Member State following national rules and requirements. Afterwards it is possible to use the principle of mutual recognition in accordance with Regulation (EU) 2019/515 to request an authorization in another Member State. The procedures and/or requirements, with regard to the assessment of risks to human health and the environment, will be made on a case-by-case basis, depending on products and countries,

where the Mutual Recognition is sought; 2) it will be possible to follow a EU harmonized marketing process for biostimulants following the framework established in the new Regulation (EU) 2019/1009. Once the EU-type certificate will be granted to a biostimulant, the marketer will be able to affix the CE mark and the product will have the access to the whole EU market.

6. Biostimulant limitations

Besides the positive effects of PB, there are also several studies reporting that their application might induce no-response or negative responses in plants, especially when they are applied in excess or in field conditions (Ruiz et al., 2000; Cerdán et al., 2009; Lisiecka et al., 2011). Foliar applications of commercial PH products from animal origin can cause phytotoxicity and plant growth depression. For instance, Asli and Neumann (2010) reported that multiple applications of HAs inhibited the maize shoot growth, grown hydroponically. No-positive effects were also reported by Kirn et al. (2010) in field soil experiments with okra (*Abelmoschus esculentus*), where no significant increases in fruits per plant were observed when the recommended dose was not applied. In this respect, Colla et al. (2015) recommended lowering the PBs dosage to avoid growth inhibition, caused by overdose. In general, the PBs efficiency depends on crop and uptake rate, which is highest prior to maximum growth rates (Jones et al., 2011; Nguyen et al., 2019). For example, SE application was best during the tilling (Stamatiadis et al., 2021) and seedling stage of sugarcane (Chen et al., 2021). Moreover, plants sensitivity is regulated by their daily circadian clock that also may influence the effectiveness of PB (Belbin et al., 2019), suggesting that is better to spray PBs in the early morning or late afternoon because of the open stomata (Specialty Fertilizers, 2015). It is also necessary to carry out several trials that take into account the factors that may potentially influence the PB efficacy and their specific mechanism of action, together with the data about their exact composition. All these aspects are a mandatory requirements to register a product as a plant biostimulant and to optimize their application in crop management.

Furthermore, despite their beneficial effects on plants, the PBs microbial application may pose a risk to other living organisms, especially human beings. Although most PGPB do not have a negative effect, some genera are involved in causing infections in animals and humans. Bacteria belonging to the genera *Serratia*, *Acinetobacter*, *Bacillius cereus*, *Stenotrophomon*a, *Enterobacter*, *Herbaspirillum*, *Ochrobactrum*, and *Pseudomonas* are not only powerful candidates for plant-growth promotion but may also cause disease in humans (Berg et al., 2013; Selvakumar et al., 2014). For example, although *Bacillus* sp. has a wide variety of

applicability in agriculture, industry, and the pharmaceutical sector, it is associated with many types of illness in humans and animals. It can cause disease in immunocompromised as well as in healthy individuals. Many bacteria isolated from the rhizosphere, soil, and water, besides having PGP activity, are also involved in causing diseases in immunocompromised and healthy individuals (Berg et al, 2005). Hence, there is a huge need to develop a systemic and polyphasic approach through which, beside their PGP activity and bioinoculant development, the disease-causing ability of microbes isolated from an environmental niche is checked (Tindall et al., 2010). For example, Kim et al. (2014) suggested checking for the presence of genes involved in the virulence or pathogenicity of novel bacteria isolates, before their application as PBs, to determine their safety level concerning humans and plants.

Therefore, collaborative efforts of different expertise and technological advancements are needed. Recently, Kumari et al (2023) undelined that biochemical, immunological, proteomics, and genomics approach unraveling the characters and identification of microbes needed to rapidly and accurately address safety concerns, such as pathogenicity, of biostimulant microbes following a suitable strategic plan before releasing the inoculant for field application.

7. Tomato as model plants

Tomato (*Solanum lycopersicum* L.) as well as potato, tobacco, pepper and eggplant belongs to the Solanaceae, extensively studied family among the Euasterids, with high economic importance. Among them, tomato is the most considered member of this family, mainly due to its short generation time, elementary diploid genetics, a well-known genetic transformation methodology, inbreeding tolerance, and a vast well-characterized genetic resource (Van der Hoeven et al., 2002; Barone et al., 2008). Originated from South America, it was spread around the world to become one of the most consumed vegetables worldwide also owing to the development of products such as soups, juices, purees, and sauces (Del Giudice et al., 2016). Tomato is an essential component of the Mediterranean diet and of other traditional diets. Besides its economic value, it has interesting developmental features, such as compound leaves, fleshy fruits, and sympodial shoot branching (Townsley and Sinha, 2012). For its behavior, tomato has known an exciting phase of research development, in which huge amount of genomic, proteomic and metabolomics datasets have been gathered. These advantages make tomato an excellent model plant for both basic and applied research.

Many researches have been conducted on tomato plants to understand the effects of both microbial and no-microbial PBs on plant metabolism and growth, yield, fruit quality and on

their ability to confer abiotic stress tolerance. Recently, Mannino et al. (2020) demonstrated that the Expando application, a biostimulant based on seaweed extract, reduced tomato ripening times causing a concomitant enhancement of production during the earliest ripening times, in terms of fruit yield (+110%), size (+85%), and, to a lesser extent, fruit quality. The CycoFlow, a novel plant-based biostimulant, was able to stimulate growth (plants up to 48.5% taller), number of fruits (up to 105.3%), antioxidant content in both tomato leaves and fruits, at elevated temperatures (up to 42 °C) (Francesca et al., 2020). The effects of five biostimulants, based on biocompost and biofertilizer compounds, were studied on tomato plants grown under salt stress. Among these, Biocompost 70%/Biofertilizer 30 % (Bc70/Bf30) showed the best growth performance and defense responses at the time of leaf harvesting compared with the other treatments and controls, since it activated the plant antioxidant mechanisms in an earlier stage by ensuring plant growth and development under stress condition (Gedeon et al., 2022). Two biostimulants, RutfarmMaxifol (*Ascophyllum nodosum* extract 17.5%, amino acids, macro- and microelements, Agromaster, Russia) and Radifarm (polysaccharides, glycosides, amino acids, and microelements; Valagro, Italy), were applied at different concentrations on tomato plants (hybrid cv 'Merlice') grown in hydroponic system. All biostimulant treatments resulted in higher values of growth parameters and yield productivity in relation to the control. They improved fruit quality by increasing the total soluble solids and antioxidants, ascorbic acid, and carotenoid contents (Abdelkader et al., 2021). Foliar applications of a novel calcium-based biostimulant (SOB01) increased the photosynthetic rate and the chlorophyll content under water deficiency compared to the standard fertilizer leding to a higher yield in terms of fruit dry matter and a reduction in the number of cracked fruits (Della Lucia et al., 2022). The application of humic acid (HA), at low NPK supply, improved tomato yield and plant ability to cope with nutritional stress due to the stimulation of defense mechanisms by reprogramming plant development status (Monda et al., 2021). Koleska et al. (2017) already observed similar effects on two tomato hybrids (Ombeline F1 and Bostina F1) under reduced nitrogen, phosphorus and potassium (NPK). They demonstrated that the foliar applied Viva® biostimulant decreased superoxide dismutase (SOD, EC 1.15.1.1) and peroxidase (POD, EC 1.11.1.7) activity in tomato leaves even when recommended NPK nutrition was reduced at 40%.

In the Figure 11 are reported specific effects of PBs on different part of tomato plants and the corresponding authors/references.

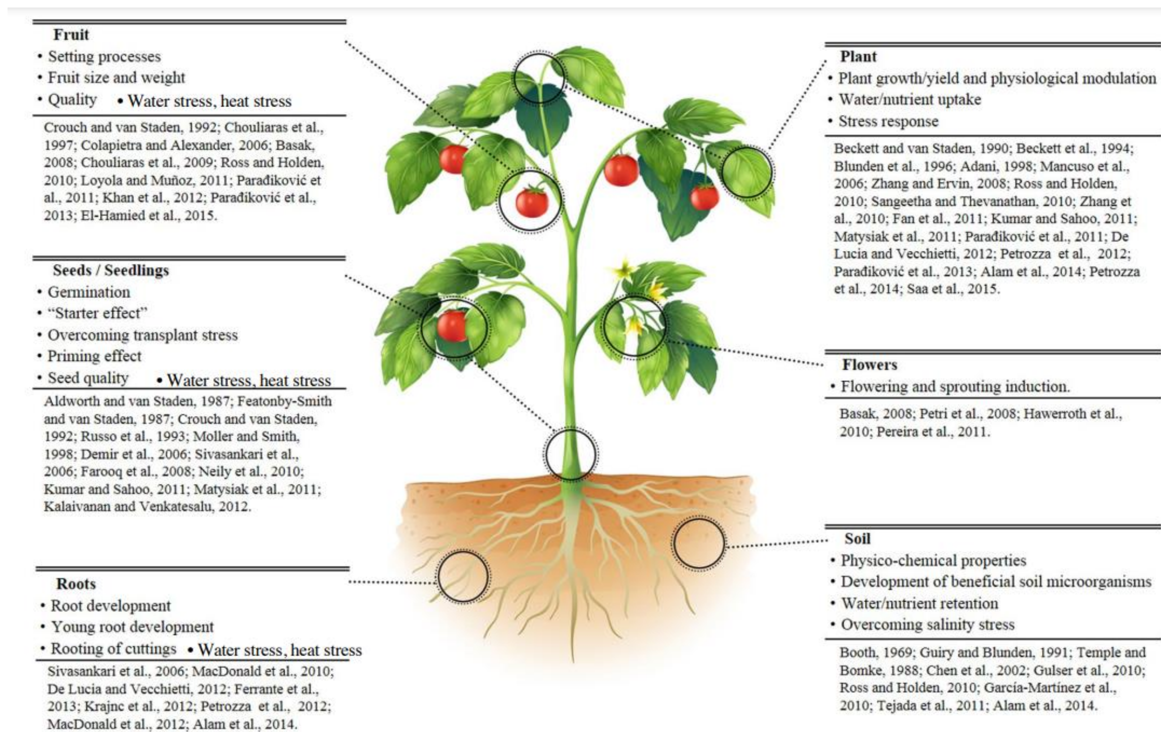

Figure 11. Specific effects of PBs on different tomato organs (Povero et al. 2016)

8. Objectives and organization of the thesis

The excessive use of fertilizers and pesticides has been a useful strategy to boost crop production and to meet growing food demand (Guo et al., 2010; McGuire, 2015). Otherwise, it caused harmful impacts on the environment, animals, human health, and economic costs (Sponsler, et al., 2019). Recently, the use of plant biostimulants (PBs) has become a promising and sustainable strategy to reduce fertilizer rates, gaining a worldwide interest (De Pascale et al, 2017). Their introduction in biofertilizer formulations and programs by agriculture industry might improve a sustainable crop yield, and a healthier environment (Calicioglu et al., 2019). PBs include a large variety of biological products such as plant extracts, humic and fulvic acids, protein hydrolysates, phosphites and seaweed extracts, or living microorganisms, typically bacteria and fungi, able to improve plant growth and yield, as well as tolerance to biotic and abiotic stresses (Sible et al., 2021). In particular, plant growth- promoting bacteria (PGPB) and seaweed extracts (SEs), a microbial and a no-microbial PBs, respectively, have gained a special attention by agricultural industry. Among PGPB, soil bacteria, belonging to the actinobacteria, are considered very promising due to their metabolic versatility, drought resistance and bioactive metabolite production. They can colonize plant roots of several species, showing their positive impact on plants at different physiological and molecular levels (Kandel et al., 2017), being also an important tool for

protecting plant health in an ecological manner. Among SEs, those obtained from brown (*Ascophyllum nodosum*, *Laminaria* spp., *Macrocystis* spp., etc.), red (*Kappaphycus alvarezii*, *Palmaria* spp., *Gracilaria* spp., etc.), and green seaweeds (*Ulva* spp. and *Enteromorpha* spp.) (Goñi et al., 2020; Sujeeth et al., 2022) represent an eco-friendly alternative to synthetic fertilizers and growth regulators (Carvalho et al., 2019). They are constituted by complex polysaccharides, fatty acids, vitamins, phytohormones and mineral nutrients and are able to affect soil physical, biochemical and biological properties, root architecture, improving nutrient uptake, yield and quality (Shukla et al., 2019).

This project aimed to investigate the ability of both PGPB and SE biostimulants to affect plant growth and development to mitigate nitrogen and/or drought stress in tomato and to understand the physiological and molecular mechanisms activated upon their application.

In this respect, UC82, a commercial variety of tomato (*Solanum lycopersicum* L.), belonging to the Solanaceae family, has been used as model plant. It is one of the most intensively cultivated and studied horticultural crop, mainly due to its short generation time and elementary diploid genetics. Furthermore, tomato is highly susceptible to drought, nutrient deficiency and therefore required large N fertilizer amount making it a useful model for both basic and applied research on PBs.

In Chapter I, twenty-two bacterial strains of selected microorganisms were characterized for multiple PGPB traits, such as IAA, organic and inorganic phosphate solubilization, N_2-fixation, and drought and salt tolerance. According to the scientific literature, one of these traits needed to define a microorganism as PGPB. Among these, two Actinobacteria, namely *Streptomyces violaceoruber* and *Kocuria rhizophila*, were selected for possessing multiple PGP traits and were then deeply studied for their properties and in vitro plant promoting activities. They were also analyzed for the secreted and cellular metabolome in axenic cultivations and actinobacterial co-cultures to identify possible bioactive compounds exerting stimulatory effects on plant growth. Furthermore, the in vivo ability of *S. violaceoruber* to affect tomato germination and growth was evaluated. The results indicated that PGPB did not affect the seed germination but it was able to cause the increase of Germination Index (IG), as well as the hypocotyl and epicotyl growth. Finally, the DNA methylation levels and the volatilome of PGPB-treated tomato plant were evaluated to establish the possible pleiotropic effects on plant physiology. Our results confirmed the efficacy of the selected actinobacteria strains in promoting plant growth and development by producing volatile and non-volatile bioactive molecules. For these reasons, both *K. rhizophila* and *S. violaceoruber* were deeply studied for their PGPB activities under stressed and control conditions.

In Chapter II, by a multi -omics approach, a powerful tool to deeply understand the PGPB-plant interaction (Rouphael et al., 2020), the first molecular evidence on the pathways activated by *K. rhizophila* application on tomato were investigated, identifying several genes, proteins and metabolites involved in the plant growth promoted by PGPB. By using a WGCNA approach, eight gene modules based on their correlation with differential accumulated proteins and metabolites (DAPs and DAMs) were identified. In particular, two modules showed the highest correlation with nine proteins, among which a nucleoside diphosphate kinase, a conserved protein family involved in the energy homeostasis and development process (Ye et al., 2020), a cytosolic ascorbate peroxidase, a scavenging enzyme in plant, which plays a critical role in plant growth and development (Wu et al., 2019; Li et al., 2020), and several metabolites, mainly belonging to amino acids and TCA. Our findings highlighted that plant nutrients, including sugars and amino acids, key factors for improving plant yield, are strongly modulated by plant-PGPB interaction.

In Chapter III, we focused on the ability of *S. violaceoruber* to mitigate nitrogen and/or drought stress conditions, in tomato plants, grown in different systems: hydroponic, pot and plate-on-plate systems. In hydroponics, once identified the effective dose of PGPB able to alleviate low nitrate (LN) condition, its ability to restore plant growth to control condition (high N, HN) was evaluated. The results indicated that PGPB was able to increase fresh and dry root weight, diameter, volume and surface area, as well as the numbers of lateral roots in tomato. Moreover, it was able to enhance the chlorophyll content, net photosynthesis rate, transpiration in the LN treated plants, restoring the values to the high N condition. The undecylprodigiosin mycelial red pigment produced by *S. violaceoruber* along tomato roots confirms the root-bacteria interaction.

After evaluating the effectiveness of PGPB treatments in the hydroponic system, S. violaceoruber was applied on tomato plants grown in pots. After 70 days after treatment (DAT), the treated plants showed a significant increase in root fresh weight (FW), shoot dry weight (DW), and SPAD index compared to the control. Furthermore, the PGPB increased the exchange parameters, such as net photosynthetic rate, transpiration rate and stomatal conductance of tomato plants. The effect of PGPB on nitrogen use efficiency (NUE) and its components, nitrogen uptake efficiency (NUpE) and nitrogen utilization efficiency (NUtE) were evaluated. The results indicated that PGPB increased both NUE and NUtE values.

Finally, to assess the PGPB ability to mitigate drought stress its effects were investigated on tomato plant treated by inoculum and dipping methods in a plate-on-plate system. In this condition, the volatiles produced by *S. violaceoruber*, applied by indirect inoculation,

increased plant biomass, the number and length of lateral roots, but it was not able to overcome the drought stress effect.
In Chapter IV, the ability of Eranthis, a commercial no-microbial biostimulant based on brown seaweed (*Ascophylum nodosum* and *Laminaria digitata*) and yeast extracts (Green Has Italia S.p.a (Canale, Italy), to mitigate N stress in tomato plants grown in hydroponic system was evaluated. Once identified the effective dose, the morpho-physiological parameters were measured. The Eranthis treatment was able to mitigate N stress increasing both root fresh and dry weights, lateral root numbers and SPAD index, in tomato plants under N limited condition. This effect could be due to Eranthis composition rich in antioxidant molecules, such as flavonoids and flavanols, which are able to contribute to ROS scavenging.
In conclusion, both *S. violaceoruber* and *K. rizophila* and the no-microbial Eranthis could be considered as new candidates for developing novel biofertilizers with low environmental impact for a more sustainable cropping system. Further researches are needed to understand their mode of action to improve plant growth and stress tolerance in tomato, for their correct and efficient use in a more sustainable agriculture.

9. Chapter I:

Article

Bioactive Metabolite Survey of Actinobacteria Showing Plant Growth Promoting Traits to Develop Novel Biofertilizers

Teresa Faddetta [1], Giulia Polito [1], Loredana Abbate [2], Pasquale Alibrandi [3], Marcello Zerbo [2], Ciro Caldiero [4], Chiara Reina [5], Guglielmo Puccio [2], Edoardo Vaccaro [3], Maria Rosa Abenavoli [4], Vincenzo Cavalieri [1], Francesco Mercati [2], Antonio Palumbo Piccionello [1,*] and Giuseppe Gallo [1,6,*]

[1] Dipartimento STEBICEF, Università degli Studi di Palermo, Viale delle Scienze, 90128 Palermo, Italy
[2] Consiglio Nazionale delle Ricerche, Institute of Biosciences and Bioresources (IBBR), Corso Calatafimi 414, 90129 Palermo, Italy
[3] Mugavero Teresa S.A.S., Corso Umberto e Margherita n. 1/B, Termini Imerese, 90018 Palermo, Italy
[4] Dipartimento AGRARIA, Università Mediterranea di Reggio Calabria, località Feo di Vito, 89122 Reggio, Italy
[5] Istituto di Candiolo–Fondazione del Piemonte per l'Oncologia—IRCCS Strada Provinciale, 142-KM 3.95, 10060 Candiolo, Italy
[6] National Biodiversity Future Center, Piazza Marina 61, 90133 Palermo, Italy
* Correspondence: antonio.palumbopiccionello@unipa.it (A.P.P.); giuseppe.gallo@unipa.it (G.G.); Tel.: +39-09123897544 (A.P.P.); +39-09123897212 (G.G.)

Abstract: The use of chemical fertilizers and pesticides has caused harmful impacts on the environment with the increase in economic burden. Biofertilizers are biological products containing living microorganisms capable of improving plant growth through eco-friendly mechanisms. In this work, three actinobacterial strains *Streptomyces violaceoruber*, *Streptomyces coelicolor*, and *Kocuria rhizophila* were characterized for multiple plant growth promoting (PGP) traits such as indole acetic acid production, phosphate solubilization, N_2-fixation, and drought and salt tolerance. Then, these strains were investigated for their secreted and cellular metabolome, revealing a rich arsenal of bioactive molecules, including antibiotics and siderophores, with *S. violaceoruber* being the most prolific strain. Furthermore, the in vivo assays, performed on tomato (*Solanum lycopersicum* L.), resulted in an improved germination index and the growth of seedlings from seeds treated with PGP actinobacteria, with a particular focus on *S. violaceoruber* cultures. In particular, this last strain, producing volatile organic compounds having antimicrobial activity, was able to modulate volatilome and exert control on the global DNA methylation of tomato seedlings. Thus, these results, confirming the efficacy of the selected actinobacteria strains in promoting plant growth and development by producing volatile and non-volatile bioactive molecules, can promote eco-friendly alternatives in sustainable agriculture.

Keywords: PGP traits; bioactive metabolites; VOCs; actinobacteria; streptomycetes; tomato seeds and seedlings; biofertilizers

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9.1. Introduction

The use of chemical fertilizers and pesticides has been a strategy to boost agricultural production and meet global food demand for the increasing world population (Baweja et al. 2020). However, their excessive utilization has caused harmful impacts on the environment, animals and human health, and economic costs (Baweja et al. 2020). Their negative effects are also exacerbated by climate change scenario, which increases the multiple and concurrent abiotic stresses (i.e., drought, salinity, low and high temperature, waterlogging, metal toxicity, etc.) representing an additional serious threat for crop yield and food security (Hatfield et al. 2013; Hatfield et al. 2019). Therefore, the search for eco-friendly alternatives is a crucial challenge in sustainable agriculture.

A promising strategy would be the use of biofertilizers that are biological products containing living microorganisms, typically bacteria and fungi, able to improve plant growth and yield through eco-friendly mechanisms (Mahanty et al. 2017). Biofertilizers, usually applied to soil, seeds, plant surface, are often capable to colonize the rhizosphere and even to migrate into the interior part of plant tissue increasing plant growth and protecting them from abiotic and biotic stress (Bhardwaj et al. 2014). The mechanisms of microbial stimulation on plant growth can be either direct or indirect, involving the regulation of hormonal, nutritional and water balance, the solubilization of organic and inorganic phosphates, the nitrogen fixation, the pathogen protection by induction of plant systemic resistance and/or through the microbial production of antibiotic compounds or enzymes (Calvo et al. 2014; Elnahal et al. 2022). Therefore, biofertilizers are generally formulated using plant growth-promoting (PGP) bacteria, which deemed to mostly contribute to increased crop yields and soil fertility. The ability of exerting plant growth promotion seems widespread among different kinds of bacteria genera including *Azospirillum*, *Azotobacter*, *Bacillus*, *Enterobacter*, *Pseudomonas*, *Kocuria*, *Klebsiella*, *Streptomyces*, and *Rhizobium* (Compant et al. 2005; Afridi et al. 2021). Although many aspects of biofertilizer utilization have to be elucidated, such as the plant-specific dosages and long-term effects on microbial biodiversity, currently, the market of biofertilizers is greatly increasing and it is expected to reach 3.8$ billion by 2025 (Elnahal et al. 2022; Riaz et al. 2021). Thus, the formulation of commercial biofertilizers containing the most effective PGP bacterial strains is desirable. In particular, soil bacteria belonging to the actinobacteria are considered very promising due to their metabolic versatility, drought resistance, capability of supplying nutrients (such as phosphate) for better growth and potential to produce a vast array of bioactive metabolites - such as vitamins, antibiotics,

plant growth factors - and enzymes (Kaari et al. 2022). Among these bacteria, *Streptomyces* species are the most prolific source of bioactive metabolites being capable to improve the plant development and growth, and the biotic and abiotic stress resistance (Compant et al. 2005; Doolotkelvieva et al. 2015; Kaari et al. 2022). Beside, also *Kocuria* genus is gaining a lot consideration as PGP bacterium due to their metabolic versatility, resistance to harsh conditions and production of indole-3-acetic acid (IAA) (Marchawala et al. 2018).

In the within of a collaborative project involving an academic-industrial consortium aiming at the development of innovative biofertilizers, three actinobacteria, namely *Streptomyces coelicolor*, *Streptomyces violaceoruber* and *Kocuria rhizophila* where selected for possessing at least one PGP trait or for being related to bacterial genus showing PGP properties, according to scientific literature. In order to verify their possible utilization for the development of actinobacteria -based biofertilizers, these microorganisms were preliminary characterized for multiple PGP traits, such as IAA, organic and inorganic phosphate solubilization, N_2-fixation, and drought and salt tolerance. Then, they were analyzed for the secreted and cellular metabolome in axenic cultivations and co-cultures to identify possible bioactive compounds exerting stimulatory effects on plant growth according to scientific literature. Finally, to evaluate their *in vivo* PGP capability, tomato (*Solanum lycopersicum* L.) plants were used as model of study. In addition, global DNA methylation levels and volatilome (Mansurova et al. 2018) of the model plant, treated with *S. violaceoruber*, were assayed in comparison with the untreated plants to establish the possible pleiotropic effects on plant physiology.

9.2. Materials and Methods

9.2.1 Bacterial strains and culturing conditions

Actinobacterial strains were used for this study. *Streptomyces coelicolor* M145 and *Kocuria rhizophila* were from our laboratory collection; *Streptomyces violaceoruber* DSM 40783 was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures GmbH). The spore production of *Streptomyces* strains was obtained as previously described by (Kieser et al. 2000). In particular, cultures were performed on mannitol soy adding sterile distilled water into the plates. The mixtures were filtered through a syringe containing hydrophilic cotton to eliminate the mycelia. The cell biomass of *K. rhizophila* was obtained in cultures obtained from a single colony inoculated into tryptone soy broth (TSB) and incubated for 24-48h at 30 °C and 180 rpm.

Spore and cell concentrations were evaluated by colony forming unit (CFU) method on tryptone soy agar (TSA) medium. For a long storage time, each bacterial strain was stored at -80°C in a 20% glycerol solution.

9.2.2 Estimation of PGP traits and abiotic stress tolerance

The actinobacterial strains were studied for different PGP traits and for tolerance to abiotic stresses (drought and salt resistance/tolerance), phosphate solubilization, indole acetic acid (IAA) production, nitrogen fixing. All the assays were conducted in triplicate.

9.2.3 Indolic compound production

The IAA production by the selected strains was estimated with a colorimetric assay using the Salkowski's reagent (0.5 M $FeCl_3$ in 35% $HClO_4$ aqueous solution) that is able to reveal in the presence of indole compounds like IAA (Glickmann et al. 1995). In particular, the strains were grown in 10 mL of R5A (Tischler et al.2018) and incubated for 24, 48 and 72 h (30 °C, 180 rpm). After incubation, the cultures were centrifuged and the supernatants were mixed with Salkowski's reagent (1:2). The optical density (OD) was recorded at 530 nm after 30 min of incubation, a standard curve with known concentrations (0.5–100 µg/ml) of IAA (Sigma-Aldrich) was used to determine the amount of IAA produced.

9.2.4 Organic and inorganic phosphate solubilization

The ability to solubilize organic and inorganic phosphates was investigated as previously described (Faddetta et al. 2021). In particular, the strains were plated on NBRIP agar media (Nautiyal et al. 1999) containing different sources of phosphate: $AlPO_4$ (5 g L^{-1}), $Ca_3P_2O_8$ (5 g L^{-1}); $FePO_4$ (5 g L^{-1}) to characterize their ability to solubilize inorganic phosphates; phytate (2 g L^{-1}) to characterize their ability to solubilize organic phosphates. In particular, 5 µL of microbial suspension (10^7-10^8 CFU/ mL) were plated on NBRIP agar media and incubated at 30 °C for 5 days to check the development of a solubilization halo around the colonies. To highlight phosphate solubilization by clearance halo formation, the growth media were added with bromophenol blue (0.05 g $L^{-}1$) but $Ca_3P_2O_8$ containing one since halos can be visible (Nautiyal et al. 1999).

9.2.5 Growth under drought and salt stress

All strains were tested for drought resistance and salt tolerance by adding 5% and 10% v/w of Polyethylene Glycol 8000 (PEG) or 7.5% v/w of NaCl to the R5A medium,

respectively. The cultures were incubated at 30 °C for 48 and 72 h at 180 rpm. The growth was by measuring the optical density at 600 nm and compared with that of the strains incubated in the same conditions but NaCl or PEG addition.

9.2.6 Growth in nitrogen-free medium

For the evaluation of the nitrogen fixation activity, the bacteria were inoculated into Derxia medium, *i.e*. a nitrogen-free mineral medium used as an element of discrimination for the cultivated strains (Matthews et al. 2010). For all the samples, the ability to use and transform atmospheric molecular nitrogen was confirmed, thus dealing with putative diazotrophic strains. To carry out this test, an amount of $10^{\wedge 7}$-10^{8} CFU (*i.e.* cells and spores for *K. rhizophila* and streptomycetes, respectively), taken from a 20% v/v glycerol solution in a rich growth medium, was inoculated into the test tubes containing 2 mL of Derxia medium. At the same time, with the same procedure described above, cultures were set up in 2 mL of Derxia medium supplemented with the addition of $(NH_4)_2SO_4$ as an inorganic source of nitrogen (growth control) For the evaluation of the growth of the strains, two parameters were taken into consideration, such as the culture turbidity and pH variation (given by the bromothymol blue present in the medium).

9.2.7 Metabolite extraction and HPLC/MS/Q-TOF analysis

Axenic cultivations or co-cultivations of *S. coelicolor*, *S. violaceoruber* and *K. rhizophila* were performed by inoculating a suspension of spores (in the case of the two streptomycete strains) or cells (for *K. rhizophila*) at a concentration of 10^{7}-10^{8} CFU/mL in 50 mL flasks containing 10 mL of R5A medium. The cultures were incubated at 30 °C at 180 rpm for 72 h. Then, 1 mL of cultivations was collected and centrifuged (12000 x *g*, 5 min., 4 °C) to separate the bacterial cells and the spent media that were stored at -20 °C until use.

HPLC/MS analysis was performed adapting previously reported methods (Emanuele et al. 2018; Raimondo et al. 2022). Samples for HPLC (Agilent 1260 Infinity) were prepared collecting spent media from cultures (1 mL). The liquid was filtered, diluted with 1 mL of MeOH and directly injected. Analyses were performed in triplicate. Water and acetonitrile were of HPLC/MS grade. Formic acid was of analytical quality. A reversed-phase Phenomenex Luna C18(2) column (150 mm × 4.6 mm, particle size 3 µm) with a Phenomenex C18 security guard column (4 × 3 mm) was used. Injection volume was 25 µL. The eluate was monitored through Mass Total Ion Count (MS TIC) and UV (270

nm). Mass spectra were obtained on an Agilent 6540 UHD accurate-mass Quadrupole-Time of flight (Q-TOF) spectrometer equipped with a Dual AJS Electrospray Inonization (ESI) source working in positive or negative mode. Nitrogen N_2 was employed as desolvation gas at 300 °C and a flow rate of 8 L min^{-1}. The nebulizer was set to 45 psig. The sheath gas temperature was set at 400 °C and a flow of 12 L min^{-1}. A potential of 2.6 kV and 3.2 kV was used on the capillary for negative and positive ion mode, respectively. The fragmentor was set to 75 V. MS spectra were recorded in the 150–1000 m/z range. Quality control was performed prior to analysis by means of mass calibration in the range 100-3000 Dalton (Q-TOF calibration mix) and solvent delay calibration for retention time. An in-house quality check mix containing known compounds (phenylalanine, sucrose, benzoic acid and rutin) was injected during batch of analysis. Mass spectrum data were analyzed for metabolite annotation by using MassHunter Qualitative Analysis B.06.00, and the Metlin database (Metlin, 2022).

9.2.8 Evaluation of in vivo plant growth promotion by PGP actinobacteria

Seeds of *Solanum lycopersicum*, L., commercial tomato variety (Red Cherry), were surface-sterilized with 70 % (v/v) ethanol for 1 min, followed by a treatment with 2.5 % (w/v) sodium hypochlorite solution for 2 min, as reported in Faddetta et al. 2021. Afterwards, three rinses in sterile distilled water (5 min each one) were carried out. Surface-sterilized seeds were immersed for 45 min into a dilution (1:10 in distilled water) of PGP actinobacterial cultivations (about 3% v/v packed mycelium and 10^9-10^{10} CFU mL^{-1} for the two strepomycetes and *K. rhizophila*, respectively) performed in R5A growth medium at 30 °C for 72 h. In parallel, control condition experiments were performed using sterile distilled water instead of diluted PGP actinobacterial cultures. Five replicates, which included 10 seeds each, were aseptically transferred to Petri dishes with filter paper (Whatman), soaked in 3 mL of distilled sterile water. Plates were incubated in dark at 25-26 °C for six days for germination and after they were moved in a growth-controlled chamber (25 ± 2 °C, 65–75% relative humidity, 16 hours of daylight with a light intensity 3000 lux) for other six days. The plant responses (root and shoot growth at 12 days after treatment - DAT) to each thesis were analyzed with one-way ANOVA using R-packages [24], considering at least three replicates (30 plants) for each treatment. Tukey's test ($p < 0.05$) was applied to test the significance among different treatments (R-Packages, 2024).

9.2.9 Effect of S. violaceoruber culture seed-priming treatment on germination

For this experiment, seeds of *Solanum lycopersicum*, L., commercial variety UC82, were sterilized as above described. Finally, seeds were washed with distilled water to remove residual ethanol and NaOCl. After sterilization, seeds were treated with three different conditions: Milli-Q water (control condition), 1:5 (T1) and 1:10 (T2) dilute water solution of *S. violaceoruber* cultures (about 3% v/v packed mycelium) performed in R5A growth medium at 30 °C for 72 h. The treatment was applied drop by drop to dried seeds shaking until the complete e visible distribution of product on seed surface was obtained (Campobenedetto et al. 2020). Following the treatment, seeds were dried at room temperature and then placed in Petri dishes (9 cm Ø) (10 seeds for plate) containing two filter papers saturated with 3,5 ml distilled water. Five replicates were performed for each treatment. The Petri dishes were then placed in the dark in a growth chamber at 24°C temperature, 65% relative humidity and a photoperiod of 14h with 350 µmol light intensity, for 7 days. After this period, the germination percentage, germination index (GI %), root and hypocotyl length were evaluated as reported above:

$$\%\ \text{Germination} = \frac{Germinated\ seeds\ number}{total\ seed\ number} \cdot 100$$

$$\text{Germination Index}\ (IG\%) = \frac{average\ number\ of\ Germinated\ seeds\ (T) * average\ L_R(T)}{average\ number\ of\ Germinated\ seeds\ (C) * average\ L_R(C)} * 100$$

L_R = Root length

(T) = Treated

(C) = Control

Morphological measurements of root and hypocotyl length were made by placing the seedlings on graph paper and analyzed with ImageJ software.

9.2.10 Identification of VOCs by Means of SPME-GC/MS

GC/MS analysis was performed adapting previously reported methods (Polito et al. 2022). In particular, vials for solid-phase microextraction (SPME) were partially filled either with i) *S. violaceruber* mycelial biomass on the excised surface of 2 g R5A-agar growth medium plugs obtained after 3 days of incubation at 30 °C or ii) with freshly collected *S. lycopersicum* seedlings, regenerated on filter paper in Petri dishes and either treated with *S. violaceruber* cultures or untreated (control condition) as above described. Then, volatile organic compounds (VOCs) were extracted from the sealed vial headspace

and concentrated by SPME before desorption in the GC injection port. Headspace extraction was performed with a 2.5 mL Syringe-HS (0.64-57-R-H, PTFE, GERSTEL) conditioned and held at 40 °C from sample collection to injection.

In the SPME, one Fibre Assembly was evaluated and used: 50/30 μm divinylbenzene (DVB)/ carbowax (CAR)/ polydimethylsiloxane (PDMS) (Supelco, Bellefonte, PA, USA). Fiber was exposed to bacterial culture in 20 mL SPME vial (75.5 × 22.5 mm) for 30 min at 40 °C, after 30 min of equilibration time. The desorption time was 5 min. Before use, fibre was conditioned and cleaned at 270 °C for 30 min, following instructions from Supelco®. Splitless injection was used.

Gas chromatographic analysis was performed using an Agilent 7000C GC (Agilent Technologies, Inc., Santa Clara, CA, USA) system equipped with a split/splitless injector, fitted with an Agilent HP5-MS UI capillary column (30 m × 250 μm; 0.25 μm film thickness) coupled to an Agilent triple quadrupole Mass Selective Detector MSD 5973 (Agilent Technologies, Inc., Santa Clara, CA, USA), with ionization voltage, 70 eV; electron multiplier energy, 2000 V; transfer line temperature, 270 °C. Solvent Delay: 0 min. Helium was used as the carrier gas (1 mL min^{-1}). The oven program was as follows: temperature was initially kept at 40 °C for 5 min and then gradually increased to 250 °C at a rate of 2 °C/min, which was held for 15 min and finally raised to 270 °C at 10 °C/min. Samples were injected at 250 °C automatically. Interval scan: 35–450 m/z; Scan speed: 10,000 amu·s^{-1} (25 Hz).

The GC–MS mass spectrum data were analyzed using MassHunter Qualitative Analysis B.06.00, and the database of National Institute Standard and Technology (NIST) was used to interpret analyzed data. Comparison of the mass spectrum of the unidentified components released by the bacterial isolates was carried out against the mass spectrum of already-known components available in the NIST 11 MS library.

9.2.11 Quantification of global DNA methylation

A total of nine individual *S. lycopersicum* seedlings, regenerated on filter paper in Petri dishes and either treated with *S. violaceruber* cultures or untreated as above described, were hand-dissected. Then shoots were pooled into three independent biological samples. The DNA isolation was carried out with the Plant Genomic DNA Purification Kit (Macherey-Nagel).

Global DNA methylation levels, referred to the total level of 5-methylcytosine (5-meC) content in a sample, were quantified by using 100 ng of genomic DNA from each sample

and MethylFlash Methylated DNA Quantification Kit (Epigentek), according to the manufacturer's instructions. Briefly, 100 ng of genomic DNA samples were bound to an ELISA plate and fluorescently labeled for 5-methyl Cytosine (5-meC) presence using specific antibodies. Each sample was run in duplicates along with internal controls provided by the kit, and the optical density (OD) intensity was measured for the plate based on the amount of 5-meC absorbance at 450 nm. The slope of the standard curve generated by positive controls was determined using linear regression and used to identify the global 5-meC amount of each sample. The percentage of global DNA methylation was then calculated as a ratio of its OD relative to the OD of positive controls, after subtracting the negative control OD values. Data are presented as a mean ± SE.

9.3. Results

9.3.1. Looking for multiple PGP traits of three selected actinobacteria

As preliminary characterization, the actinobacteria strains *S. coelicolor*, *S. violaceoruber* and *K. rhizophila* showed to be positive for all (*S. violaceoruber*) or most (*S. coelicolor* and *K. rhizophila*) of the assayed PGP traits consisting in i) producing IAA, ii) solubilizing organic and inorganic phosphate salts, iii) using atmospheric nitrogen to grow, iv) growing under saline and drought stresses (Table 1; Figure S1).

Table 2. Characterization of multiple PGP traits of actinobacteria strains investigated.

Strain	IAA production	Growth using $FePO_4$	Growth using $Ca_3P_2O_8$	Growth using $AlPO_4$	Growth using fitate	Growth using N_2	Growth under drought stress	Growth under saline stress
S. coelicolor	+	g^+	g^+	g^+	g^+	N.A.[1]	+	N.D.[2]
S. violaceoruber	+	g^+	g^+	g^+	g^+	+	+	+
K. rhizophila	+	g^+	g^+	g^+	g^+	N.A.[1]	+	+

+, positive result. -, negative result. g, growth. g^+, growth and solubilization halo. N.A., unable of growing in the medium with the addition of nitrogen source. N.D., not investigated in this study.

These results confirm and expand those obtained in previous works concerning some PGP traits of the same or close related strains thus highlighting the potential application of actinobacteria to develop novel biofertilizers due their different metabolic capabilities, which can directly or indirectly improve plant growth, nutrient acquisition and abiotic stress tolerance of plants (Afridi et al. 2021; Boubekri et al. 2022; Kol et al. 2010).

9.3.2 Bacterial metabolomic analyses of single and mixed actinobacterial cultures

K. rhizophila, S. coelicolor and *S. violaceoruber* were cultivated in R5A growth medium as axenic- or co-cultivation to analyze the bacterial secreted and cellular metabolome. *S. coelicolor*, included in this assay, is a model strain for streptomycetes with its genome sequenced (Bentley et al. 2002) and whose secretome has been characterized (Faddetta et al. 2022; Nodwell et al. 2019). Metabolites from liquid cultures were identified by means of HPLC/ESI/MS/Q-TOF. In particular, compounds release into the supernatant were directly analysed, after culture centrifugation, while intracellular metabolites were extracted from the pellet with methanol. A total of 33 metabolites was identified and quantified from 7 different experiments including axenic cultures, double and triple co-cultivations, and spent medium mixes (all data are reported in Table 3). Figure 12 reports most of the secreted metabolites with quantitative variations. Identified compounds belongs to different classes of bacterial metabolites, among these various compound were annotated related to tryptophan metabolism (3-methyl-indole, L-tryptophan, kynurenic acid, *N*-acetyl-L-tryptophan), various polyketides related to the biogenesis of actinorhodin (frenolicin E, frenolicin E isomer, nanaomycin E, nanaomycin A, nanaomycin E isomer, nanaomycin A isomer, 3,8-Dihydroxy-1- methylanthraquinone-2-carboxylic acid (DMAC), aloesaponarin, actinorhodin), four alkaloid (coelimycin P1, carbazomycin F, pimprinethine, streptazolin), two siderophores (desferrioxamine B, nocardamin), one inosine (futalosine), one diterpenoid (anthranoyllycoctonine), two phenezine alkaloids (streptophenazine F, streptophenazine A), two sesquiterpene lactones (arenaemycin E, pentalenolactone E), one isoflavone (3',8-Dihydroxy-4',6,7-trimethoxyisoflavone), three pyranone polyketides (Germicidin A, B and D), one antibiotic polypeptide (Calcium-dependent antibiotic CDA4b), one Manumycin (Colabomycin E), and two prodiginine antibiotic (streptorubin B, undecylprodigiosin) (Table 3). It is interesting to note that the most prolific strains in terms of number of produced metabolite is *S. violaceoruber* and, in some cases, co-cultivations determine stimulation of metabolite production, like in the case of colabomycin E and carbazomycin E and F, and in other cases a decrease in a metabolite-specific manner (Figure 12; Table 3). Anyhow, *Streptomyces*-*Kocuria* co-cultivations resulted in a similar production or a production decrement in the respect of axenic cultures.

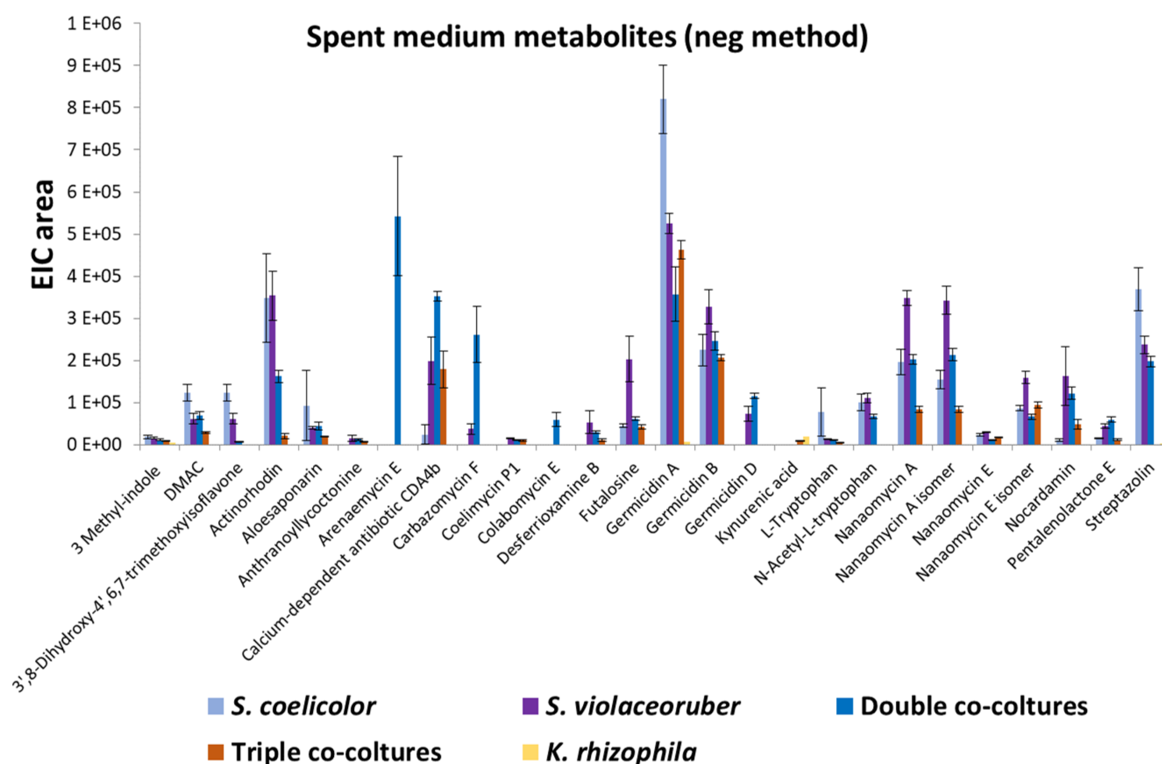

Figure 12. Quantitative profiles of extracellular metabolites reported as extracted ion chromatogram (EIC) area and identified in *K. rhizophila, S. coelicolor* and *S. violaceoruber* cultivations, in *S. coelicolor* and *S. violaceoruber* (double) co-cultures and in *S. coelicolor*, *S. violaceoruber* and *K. rhizophila* (triple) co-cultures. The values are reported as the mean of three cultivations; standard deviations are also reported.

Table 3. Metabolites identified by HPLC/MS/ESI/Q-TOF from spent media of bacterial cultivations and their occurrence in different conditions.

t_R (min)	Compounds	Molecular Formula	ESI⁻ $[M-H]^-$ (m/z) Exp.	ESI⁺ $[M+H]^+$ (m/z) Exp.	Classes	Occurrence[1]	
1.22	3 Methyl-indole[a]	C_9H_9N	130.0869	-	Tryptophan metabolism	1-7 neg	-
2.41	L-Tryptophan[a]	$C_{11}H_{12}N_2O_2$	203.0822	205.0980	Amino Acid	1-6 neg	1-6 pos
2.69	Kynurenic acid	$C_{10}H_7NO_3$	188.0332	190.0509	Tryptophan metabolism	5-7 neg	5-7 pos
3.41	Frenolicin E	$C_{18}H_{20}O_8$	-	365.1190	Polyketide	-	2-6 pos
3.68	Frenolicin E isomer	$C_{18}H_{20}O_8$	-	365.1190	Polyketide	-	2-6 pos
4.20	Coelimycin P1	$C_{17}H_{20}N_2O_4S$	347.1033	349.1251	Alkaloid	2-5 neg	2-6 pos
4.27	Desferrioxamine B	$C_{25}H_{48}N_6O_8$	559.3428	561.3649	Siderophore	2-6 neg	2-6 pos
4.62	Carbazomycin F	$C_{16}H_{15}NO_4$	284.0938	-	Alkaloid	2-4,6 neg	-

4.71	Futalosine	$C_{19}H_{18}N_4O_7$	413.1090	415.1260	Inosine	1-6 neg	1-6 pos
4.72	Streptophenazine F	$C_{25}H_{30}N_2O_5$	-	439.2264	Phenazine	-	3,4,6 pos
4.82	Pimprinethine[a]	$C_{13}H_{12}N_2O$	-	213.1034	Alkaloid		1-7 pos
4.84	Anthranoyllycoctonine	$C_{32}H_{46}N_2O_8$	585.3142	587.332	Diterpenoid	2-6 neg	2-6 pos
5.07	Nocardamin	$C_{27}H_{48}N_6O_9$	599.3336	601.3565	Siderophore	1-6 neg	1-6 pos
5.15	Streptazolin[a]	$C_{11}H_{13}NO_3$	206.0820	-	Alkaloid antibiotic	1-4,6,7 neg	-
5.30	Nanaomycin E	$C_{16}H_{14}O_7$	317.0659	319.0833	Polyketide	1-6 neg	1,2,3,5 pos
5.46	N-Acetyl-L-tryptophan	$C_{13}H_{14}N_2O_3$	245.0922	-	Tryptophan metabolism	1-4,6 neg	-
5.47	Nanaomycin A[a]	$C_{16}H_{14}O_6$	301.0707	303.0880	Polyketide	1-6 neg	1-6 pos
5.48	Nanaomycin E isomer	$C_{16}H_{14}O_8$	317.0658	-	Polyketide	1-6 neg	-
5.56	Streptophenazine A	$C_{24}H_{28}N_2O_5$	-	425.2081	Phenazine	-	2-6 pos
5.69	Arenaemycin E[a]	$C_{15}H_{16}O_5$	275.0906	277.1078	Sesquiterpene lactone	3-6 neg	3-6 pos
5.87	3',8-Dihydroxy-4',6,7-trimethoxyisoflavone	$C_{18}H_{16}O_7$	343.0700	-	Isoflavone	1-4,6 neg	-
6.20	Nanaomycin A isomer	$C_{16}H_{14}O_6$	301.0709	303.0880	Polyketide	1-6 neg	1-6 pos
6.40	Germicidin B[a]	$C_{10}H_{14}O_3$	181.0866	183.1027	Pyranone Polyketide antibiotic	1-6 neg	1-6 pos
6.55	Pentalenolactone E	$C_{15}H_{18}O_4$	261.1118	-	Sesquiterpene lactone	1-6 neg	-
7.15	Germicidin A[a]	$C_{11}H_{16}O_3$	195.1027	197.1184	Pyranone Polyketide antibiotic	1-7 neg	1-7 pos
7.20	Calcium-dependent antibiotic CDA4b	$C_{67}H_{80}N_{14}O_{26}$	1495.5206	1497.5536	Polypeptide antibiotic	1-6 neg	2-6 pos
7.72	Colabomycin E	$C_{32}H_{32}N_2O7$	555.2174	-	Manumycin	3,4,6 neg	-
7.85	Germicidin D[a]	$C_{11}H_{16}O_4$	211.0962	-	Pyranone Polyketide antibiotic	2-4,6 neg	-
7.98	3,8-Dihydroxy-1-methylanthraquinone-2-carboxylic acid (DMAC)[a]	$C_{16}H_{10}O_6$	297.039	-	Anthracene polyketide	1-6 neg	-
9.39	Aloesaponarin[a]	$C_{15}H_{10}O_4$	253.0501	-	Anthracene (polyketide)	1-6 neg	-
9.56	Actinorhodin[a]	$C_{32}H_{22}O_{14}$	629.0917	631.1143	Polyketide antibiotic	1-6 neg	1-6 pos

10.21	Streptorubin B[a]	$C_{25}H_{33}N_3O$	-	392.2730	Prodiginine antibiotic	-	1-6 pos
11.12	Undecylprodigiosin[a]	$C_{25}H_{35}N_3O$	392.2692	394.2881	Prodiginine antibiotic	1-6 neg	1-6 pos

[1] Experiments were referred from 1 to 7 as following: 1, *S. coelicolor*; 2. *S. violaceoruber*; 3, *S. coelicolor* and *S. violaceoruber* co-cultivations; 4, *S. coelicolor* and *S. violaceoruber* spent mediun mix; 5, *S. coelicolor*, *S. violaceoruber* and *K. rhizophila* co-cultivations; 6, *S. coelicolor*, *S. violaceoruber* and *K. rhizophila* spent medium mix; 7, *K. rhizophila* cultivations. pos and neg refers to positive or negative mode for ion mode, respectively.

[a] Compound also present in the extract from cell biomass.

9.3.3 Biostimulant effects on S. lycopersicum *seedlings from PGP actinobacteria treated seeds*

Overall, the seed treatments performed using actinobacteria cultivations resulted in a general trend of increased root and shoot length of the regenerated seedlings in comparison to the control condition (Figure 2S). Anyhow, the effect of the PGP actinobacteria slightly differed on root and shoot length and not all the increments were significant. Indeed, although in shoot a general increase was observed with all PGP actinobacterial cultivations, showing an increment of a total shoot length ranging from 5.05% to 43.44% for *S. violaceoruber* and *K. rhizophila* culture mixture (VIOL+KOC) and *S. coelicolor* cultures (M145), respectively, significant values were recorded for *S. violaceoruber S. coelicolor* and *K. rhizophila* single culture treatments ((VIOL, M145 and KOC, respectively) (Figure 2; Table 3). Among the mixtures, only *S. violaceoruber* and *S. coelicolor* culture mixture (VIOL+M145) had a significant increase of shoot length (Figure 2). In agreement, all PGP actinobacteria culture treatments displayed a clear growth promoting effect on root, with a growth rate from 38.03% to 78.24% (Table 3). All treatments had significant values of increasing in comparison to the control (Figure 3; Table 3), with a higher effect on root length for M145 and VIOL+M145 that showed root nearly two times greater than untreated plants.

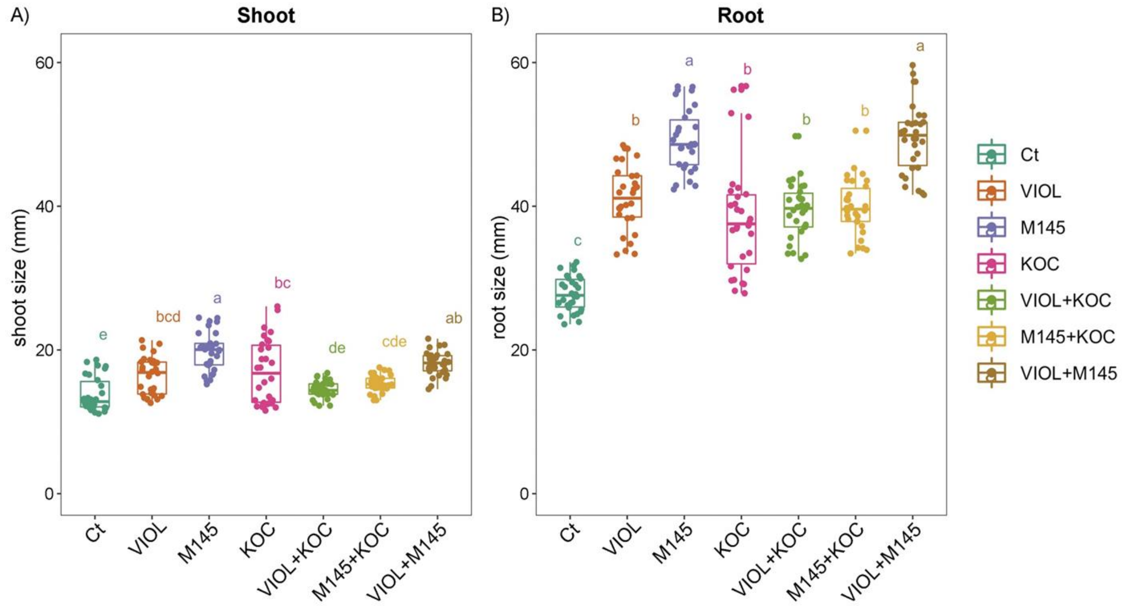

Figure 13 Tomato shoot (A) and root (B) responses to the PGPB treatments after 12 DAT analyzed with one-way ANOVA following Tukey's post-hoc test ($p < 0.05$). Different letters indicate statistically significant differences among treatments in pairwise comparisons. Ct: control; VIOL: *S. violaceoruber*; M145: *S. coelicolor*; KOC: *K. rhizophila*; VIOL+KOC: *S. violaceoruber* and *K. rhizophila* mixes; M145+KOC: *S. coelicolor* and *K. rhizophila* mixture; VIOL+M145: *S. violaceoruber* and *S. coelicolor* mixture.

Table 4 Effect on root and shoot of *S. lycopersicum* seedlings due PGP actinobacteria treatments on seeds**.**

Thesis[1]	tissue	mean value[2]	standard deviation	Q_3[3]	growth rate (%)[4]
Ct	Root	27.81	2.43	29.82	-
	Shoot	13.80	2.38	15.61	-
VIOL	Root	41.16	4.51	44.25	47.97
	Shoot	16.41	2.62	18.32	18.90
M145	Root	49.24	4.35	52.01	77.04
	Shoot	19.79	2.71	20.92	43.44
KOC	Root	38.39	7.97	41.58	38.03
	Shoot	16.92	4.50	20.66	22.61
VIOL+KOC	Root	39.40	3.91	41.81	41.65
	Shoot	14.49	1.18	15.28	5.05
M145+KOC	Root	39.87	3.88	42.48	43.33
	Shoot	15.36	1.19	16.06	11.35
VIOL+M145	Root	49.58	4.90	51.68	78.24
	Shoot	18.14	1.66	19.22	31.45

[1] Ct: control; VIOL: *S. violaceoruber*; M145: *S. coelicolor*; KOC: *K. rhizophila*; VIOL+KOC: *S. violaceoruber* and *K. rhizophila* mixes; M145+KOC: *S. coelicolor* and *K. rhizophila* mixture; VIOL+M145: *S. violaceoruber* and *S. coelicolor* mixture.

[2] Mean value calculated on 3 biological replicates per thesis, consisting of 10 plants each (in total 30 plants).

[3] Third quartile (Q3), the middle value between the median and the highest value (maximum) of the data set. It is known as the upper or 75th empirical quartile, as 75% of the data lies below this point.

[4] Relative growth rate evaluated by the comparison between the mean value of Ct and each PGPB treatment, in both tissues.

9.3.4 Effect of PGP seed-priming treatment on germination

The *S. violaceoruber* culture seed-priming treatment, at both concentrations, did not significantly affect tomato seed germination (data not shown), which not differ to the control values. Conversely, at the highest concentration, the *S. violaceoruber* culture seed-priming treatment significantly increased seed germination index %, root and hypocotyl length compared to the control seeds (Table 4).

Table 5. Effect *S. violaceoruber* culture seed-priming treatment on the germination index (%), root and hypocotyl length of tomato seedlings.

Treatments*	Germination index %#	Root length (cm) #	Hypocotyl length (cm) #
T1	4.75 ab	1.11 ab	1.01 ab
T2	5.11 a	1.41 a	1.18 a
CTRL	4.61 b	1.06 b	0.89 b

* Treatments were carried out for 7 days with three doses: 0 (CTRL), 1:5 and 1:10 (T1 and T2, respectively) dilutions in distilled water of *S. violaceruber* cultures.

\# Mean values (n=5) and standard error bar (SE). Different letters indicate statistically significant differences (Tukey's test, $P < 0.05$).

9.3.5 Volatile organic compounds produced by S. violaceoruber *and* S. lycopersicum

In order to have a deeper picture of biological mechanisms involved in the bacterium-plant interaction, VOCs produced by *S. violaceoruber* and *S. lycopersicum* plants treated or not with *S. violaceoruber* were identified by means of SPME GC/MS. Eight bacterial metabolites were revealed and are reported in Table 6. Among the identified compounds there are some of particular interest such as sulfur-containing compounds antibiotic dimetildisulfide or dimethyltrisulfide (Ossowicki et al.2017; Ross et al. 2001) and characteristic terpenes produced by streptomycetes such as 2-methylisoborneol (MIB) and geosmin (Becher et al. 2020). Concerning *S. lycopersicum* metabolites, all nine identified compounds were common monoterpenes produced by tomato plants (Table 7) (Zhou et al. 2020). The quantitative evaluation of tomato VOCs revealed a reduction of all the identified compounds upon the treatments (Figure 14).

Table 6. VOCs produced by *S. violaceoruber* identified by SPME-GC/MS.

t_R (min)	Compounds	Area %
7.75	Disulfide, dimethyl	76.89
20.09	Dimethyl trisulfide	1.09
25.60	Hexanoic acid, 2-ethyl-, methyl ester	14.74
36.41	2-Methylisoborneol	0.35
39.24	1*H*-Indene, 1-ethylideneoctahydro-7a-methyl	0.89
39.49	1*H*-Indene, 1-ethylideneoctahydro-7a-methyl-, isomer	0.41
51.52	Geosmin	4.02
53.69	Cadinene	1.61

Table 7. VOCs produced by *S. lycopersicum* identified by SPME-GC/MS.

t_R (min)	Compounds	% remaining on treated leaves vs Ctrl
15.9	α-pinene	10.39
16.7	2-carene	10.93
17.4	α-terpinene	9.17
21.4	p-cymene	7.17
21.9	o-cymene	2.33
22.5	menthatriene	3.34
23.9	limonene	1.45
24.1	β-phellandrene	1.09
24.5	γ-terpinene	1.96

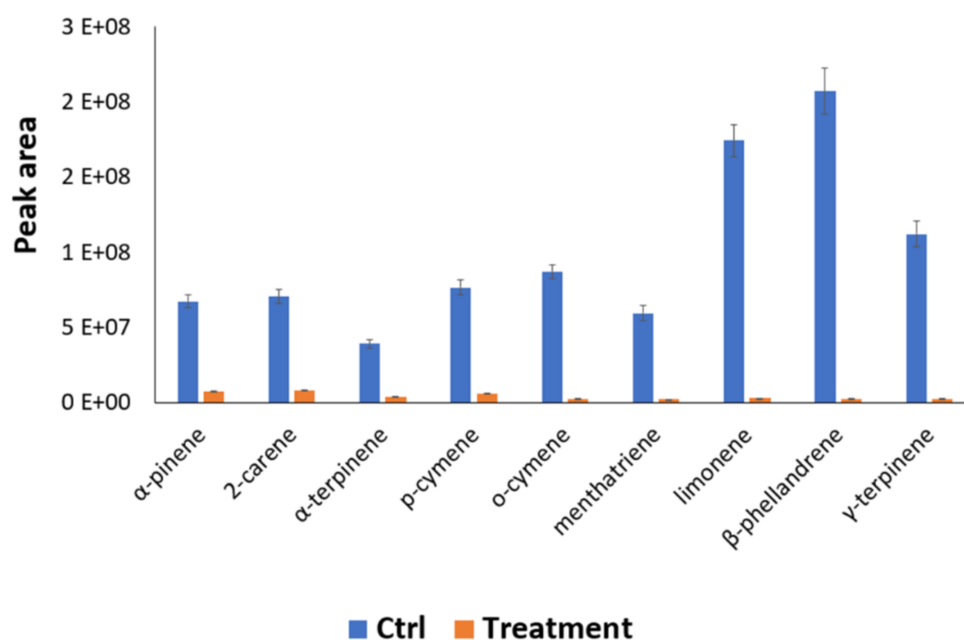

Figure 14. Quantitative profiles of VOCs reported as peak area and produced by *S. licopersicum* treated (Treatment) and untreated (Ctrl, control condition) identified by SPME-GC/MS. Group difference is statistically significant ($p < 0.05$) according to the One-Way ANOVA test.

9.3.6 Effect of S. violaceoruber cultivation on global DNA methylation amount of S. lycopersicum *shoots*

Global DNA methylation analysis in shoots from tomato plants grown on filter paper in Petri dishes revealed that PGP treatment elicited hypermethylation (Figure 15). In particular, averaged 5-meC percentages increased from 2.1 ± 0.74 to 2.73 ± 0.17 following PGP exposure. Worth mentioning, compared to sibling control untreated plants, shoots from PGP-treated plants exhibited quite similar methylation levels each other, suggesting that PGP exposure elicited higher inter-individual uniformity in global 5-meC amounts.

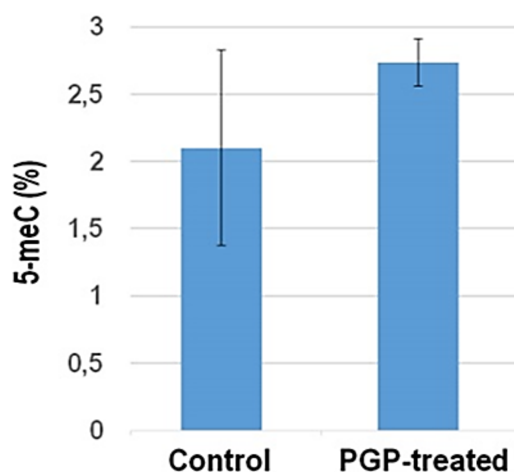

Figure 15. Histogram representing the global 5-meC level measured by ELISA-based assays in shoots from tomato plants either untreated (control) or exposed to *S. violaceoruber* cultures (PGP-treated). Data are presented as a mean ± SE. Group difference is statistically significant ($p < 0.05$) according to the One-Way ANOVA test.

9.4. Discussion

The characterization of PGP bacteria that positively influence plant growth and development has been widely discussed in recent works to develop strategies for ecosystem friendly and sustainable agriculture. The PGP bacteria can promote plant growth directly - involving the production of phytohormones, facilitating the uptake of nutrients like phosphorus, from the environment, mitigating drought and saline stress - or indirectly - by preventing the deleterious effects of phytopathogens producing bioactive

metabolites. Many studies have been conducted on actinobacteria, highlighting the ability of these microorganisms to promote plant growth and their synergistic effects on plant growth and protection (Merzaeva et al.2006).
The actinobacteria usually are soil inhabitants where they conduct a saprophytic life style and they are often isolated from rhizosphere and root tissues (Thilagam et al. 2019; Bonaldi et al. 2015). Rhizospheric actinobacteria represent a major component of rhizospheric microbial populations, with economic importance for humans. In fact, both productivity of agricultural and forest fields depend on their contributions to soil systems (Yadav et al. 2017), significantly influencing nutrient cycling, and improving plant health and growth. In this study we characterized the three actinobacteria *S. coelicolor*, *S. violaceoruber* and *K. rhizophila*. As it has been previously described (Vessey 2003), they can be considered PGP rhizobacteria, since they satisfy at least two of the following three criteria: plant colonization, plant growth stimulation and biocontrol. Indeed, the results of this study revealed that as the rhizosphere bacteria possessing different PGP traits - such as bioactive compound production, growing using different inorganic or organic phosphate sources and under drought and saline stress- these actinobacterial strains can enhance plant growth by: improving the availability of mineral nutrients through siderophore production, phosphate solubilization and putative diazotrophic activity; stimulating plant development by phytohormone, antimicrobial and VOC production; increasing plant tolerance to drought and salt stresses. The *Streptomyces* tolerance to abiotic stresses is essential not only for the survival of the microorganism itself but also because some streptomyces enhance PGP traits such as production of phytohormones and siderophores in saline soil conditions, relieving at the same time the plants from stress and, thus, improving its health, growth and development (Sadeghi et al. 2012). In addition, the possible occurrence of the ability to in a nitrogen-free medium by *S. violaceoruber* is interesting since it suggests that *S. violaceoruber* is able to grow using atmospheric nitrogen. This result is in agreement with other studies on nitrogen fixing properties, which revealed this ability in some actinobacteria including *Streptomyces* species (Kaari et al. 2022; Sellstedt et al. 2012). Although this point is really crucial and fascinating also for an ecological perspective concerning the importance and spreading of this microbial process, it deserves further analyses such as the nitrogen isotope uptake coupled with MS analysis together with an elucidation on the possible metabolic pathways and enzymes involved in this process. Nevertheless, concerning the

development of novel biofertilizers, the eventual contribution of this strain in relieving nitrogen limitation stress on plant has to be analysed.
For in vivo assays, tomato -that is one of the most important crops in the world and a model plant for the study of growth and fruit development - was used (Kimura et al. 2008). The assay was performed using surface sterilized seeds regenerated in filter papers in Petri dishes after treatment using different bacterial cultivations or cultivation mixture. The PGP actinobacteria were cultivated in the R5A growth medium that it is suitable for stimulating bioactive metabolite production in streptomycetes (Tischler et al.2018) and, at same time, it has not any stimulatory effect on *S. lycopersicum* seed germination and/or growth and development according to our preliminary investigations. In general, the treatment using bacterial cultures provided for an improvement of rooting development, as compared to the untreated control. In addition, tomato seed germination was not improved by *S. violaceoruber* culture priming treatment. Similar results were found in tomato seeds treated with mugwort aqueous extract (Pannacci et al. 2022). However, the increase of the IG %, root and hypocotyl length underlined the positive effect of PGP-priming treatment on seedlings growth. This effect may be due to the high capacity of this strain to produce IAA, an important growth promoter (Duca et al. 2014). Therefore, the *S. violaceoruber* culture seed-priming treatment is not harmful to seeds and has not shown phytotoxicity as biostimulants used in other studies (Mesa-Marin et al. 2019). Furthermore, it could be used to enhance the initial tomato seedling growth, in both open field and protected cultivation, also increasing its performance in the transplanting of seedlings in a horticultural nursery.
The use of actinobacteria, with *Streptomyces* spp. the most studied particularly as biocontrol agents to contrast various bacteria and fungi causing plant diseases (Cheng et al. 2010; Le et al.2021), has been described. These studies highlighted the production of an arsenal of different bacterial volatile and non-volatile bioactive molecules that can promote plant growth by increasing tolerance against abiotic and biotic stresses and by stimulating plant development as inferred from scientific literature describing their action mechenisms (El-Tarabily et al. 2019; Arunachalam Palaniyandi et al. 2013; Al Raish et al. 2021). For, example, the fermentation broth of *Streptomyces* sp. AN090126 has been demonstrated exhibiting antimicrobial activity against bacterial and fungal pathogens. In this contest regarding biological activities of PGP compounds, this study highlighted the production of different kinds of interesting bioactive molecules such as: germicidins (antibiotics, spore germination regulators) (Aoki et al. 2011); siderophores like

desferrioxamine E (iron uptake and transport) (Powell et al. 1982) and nocardamin (prevents plant defence agains infections) (Smits et al. 2011); many antibiotics such as calcium-dependent antibiotic (CDA), prodiginines, and actinorhodin (Vassallo et al. 2020); tryptophan metabolites are related to IAA (PGP compound) (Revelou et al. 2019), while kynurenic acid plays a role on ethylene/auxin balance (He et al. 2011).

The results concerning the quantitative variations of tomato VOCs due to PGP bacterial treatment are interesting since they highlight an interaction between actinobacteria and plants which may control different aspect of plant physiology, thus suggesting a pleiotropic level of regulation. The identified tomato VOCs are terpenes of which numerous studies have reported the pharmacological properties including antioxidant, anti-inflammatory, antiparasitic, antidiabetic, antiviral, antitumor, antibacterial, and antifungal activities (Guimarães et al. 2019; Leyva-Lòpez et al. 2017). As an example, the antimicrobial effect of monoterpenoids such γ-terpinene has been ascribed to a perturbation of the lipid layer of microbial plasma membrane (Oyedemi et al. 2009). A possible interpretation of the reduction of terpene levels in the treated plants could be ascribed to the bacterial strain and plant interaction to avoid any damage to the PGP strain due to the antimicrobial effect of terpenes that can be replaced by the plethora of bioactive compounds produced by the bacterial strain, including the VOCs that were identified considering *S. violaceoruber* alone. Indeed, it has been reported that dimethyl disulfide and dimethyl trisulfide inhibit growth of *Rhizoctonia solani* and *Pythium ultimum* and many Gram-negative and Gram-positive bacteria (Ossowicki et al. 2017; Ross et al. 2001). In addition, the abundance of dimethyl disulfide positively correlated with the antimicrobial activity of *Arthrobacter* sp. OVS8 against *Burkholderia cepacia* complex strains (Polito et al. 2022) and VOCs produced by *Streptomyces* sp. AN090126 and including dimethyl sulfide and trimethyl sulfide inhibited the growth of pathogenic bacteria and fungi in vitro (Le et al. 2022). Interestingly, it has been recently shown that the interaction of *Streptomyces. rochei* with *Fusarium moniliforme* and *Curvularia lunata*, two sorghum grain mold pathogens, affects the production of microbial mVOCs suggesting also a stimulatory role on plant growth (Sudha et al. 2022). In addition, another study reveals the potential contribution of bacterial endophytes on the production of plant essential oils (Polito et al. 2022), Thus, all together this data suggest that the bacteria may have a key role on the production of terpenes from plants and *vice versa* and that VOC production is an interesting aspect of plant and prokaryotic interaction and coevolution that still deserves further investigations. In this context, it should be emphasized that plant

epigenomes are heavily susceptible to environmental variation, essentially because plants are sessile organisms that must respond to or endure continuous challenges in their surrounding environment (Cavalieri et al. 2020). Indeed, the observed change in global DNA methylation elicited by PGP exposure represents a valuable starting point for further studies aimed to define correlations among epigenotype, gene expression, and phenotype after exposure of tomato plants to PGP bacterial strains.

9.5. Conclusions

In this study, three actinobacterial strains *Streptomyces violaceoruber*, *Streptomyces coelicolor* and *Kocuria rizophila* were characterized for multiple PGP traits such as IAA production, organic and inorganic phosphate solubilization, N_2-fixation, and drought and salt tolerance. Then, these strains were also investigated for their secreted and cellular metabolome, revealing a rich arsenal of bioactive molecules, including antibiotics and siderophores, with the *S. violaceoruber* the most prolific strain. The actinobacterial PGP trait characterization and metabolomic investigations paralleled the *in vivo* assay on *S. lycopersicum* seeds and seedlings, confirming the efficacy of the selected strains in promoting plant growth and development. In fact, these *in vivo* assays, performed on tomato (*Solanum lycopersicum* L.), resulted in improved germination index, growth of seedlings from seeds treated with PGP actinobacteria, with a particular focus on *S. violaceoruber* cultures. This actinobacterial strain, producing volatile organic compounds having antimicrobial activity, was also able to modulate volatilome and to exert a control on global DNA methylation of tomato seedlings.

Thus the results of this study suggests the possible use of these three promising actinobacterial strains as biofertilizers potentially able to preserve PGP traits even under adverse conditions such as salinity and drought, thus promoting plant growth and increasing tolerance against abiotic and biotic stresses. Detailed investigations of the metabolome, proteome and VOCs, as well as epigenetic insights on mature plants, will be able to provide insights on novel plant-bacterial interaction mechanisms and will allow to identify new active biomolecules to be applied in eco-sustainable agriculture, reducing the use of pesticides.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Characterization of PGP traits in the selected

actinobacteria; Figure S2: Effect of the selected PGP actinobacteria on seedlings regenerated from *S. lycopersicum* seeds on filter paper.

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10. Chapter II:

A multi-omics approach unravels tomato- *Kocuria rhizophila* interaction toward a sustainable agriculture

10.1. Introduction

The very fast-growing world population and, consequently, the increasing demand for food, require an intensification of crop production, which is related to an excessive use of chemical inputs, often responsible of the environmental pollution and the loss of soil biodiversity (Panfili et al., 2019). Tomato (*Solanum lycopersicum* L.) is one the most spread vegetables worldwide (FAOSTAT 2020) due to nutritional, health-promoting, and economical value of its fruits. The improvement of yield, size, shape, firmness, color, taste, and solid content of tomato fruits represents important goals to increase tomato commercial value (Kimura and Sinha, 2008; Del Giudice et al., 2016). However, an intensive application of chemical fertilizers to the plants is needed to reach high product quality and yield and also to increase resistance to abiotic/biotic stresses (Kalt, 2005; Flores et al., 2009), thus causing alterations to cropping systems and to the environment (Villarreal-Sanchez et al., 2003). Therefore, it is mandatory to rethink a novel and more sustainable agriculture for preserving the environment – by reducing the usage of primary resources, such as water and land – and, at the same time, for the increase of crop yield as well as food nutritional values. In this respect, the use of plant biostimulants (PBs) may represent an eco-friendly and innovative tool for a sustainable agriculture, diminishing the environmental pressure of the cropping systems (Rouphael and Colla, 2020).

PBs have been defined as fertilizers able to promote plant growth and development (EU, 2019). They can be classified as i) non-microbial ones, including bioactive substances (*e.g.*, protein hydrolysates, seaweed extracts, and humic and fulvic acids), and ii) microbial ones, comprising plant growth-promoting bacteria (PGPB) as well as arbuscular mycorrhizal fungi (AMF) (Du Jardin, 2015; Puccio et al., 2023). Among the beneficial effects of PGPB and AMF, worth mentioning is their ability to promote plant growth, efficiency in the use of nutrients, tolerance to abiotic/biotic stresses, quality traits, and availability of nutrients in soil and/or rhizosphere (Xu and Geelen, 2018; Drobek et al., 2019; Guerrieri et al., 2021).

Currently, the use of PGPB-based biofertilizers pertain to the general strategy of plant microbiome modulation that is considered as a valuable and alternative strategy to genetic

manipulation and traditional breeding (Ma et al., 2022; Ray et al., 2020) for stimulating plant growth. In particular, PGPB, usually belonging to *Bacillus*, *Pseudomonas*, *Kocuria* and *Azospirillum* (Ruzzi and Aroca, 2015), can promote diverse morpho-physiological, cellular and molecular processes that improve crop yield and quality including: *i)* production/regulation of levels of phytohormones, secondary metabolites, and/or volatile organic compounds (VOCs) (Bitas et al., 2013); *ii)* rise in the availability of plant nutrients through nitrogen (N) fixation, phosphorus (P) solubilization and Fe supply, via production of organic acids, acid phosphatases, and siderophores (Ruzzi and Aroca, 2015; White et al., 2019; Cabello et al., 2005; Pii et al., 2015; Sharon et al., 2016; Saha et al., 2016); *iii)* enhancement of photosynthetic efficiency (Rossi et al. 2021); *iv)* modulation of plant root growth and architecture assisting uptake of nutrients (Lim and Kim, 2009; Ortiz-Castro et al., 2020; Bavaresco et al. 2020); *v)* enhancement of resistance to abiotic and biotic stresses such as drought and pathogens, respectively (Agliassa et al., 2021; Kerchev et al. 2020).

The genus *Kocuria,* belonging to the family *Micrococcaceae,* order *Actinomycetales* (Kocur et al*.,* 2006), includes *Kocuria rhizophila,* which is a Gram-positive bacterium, presenting coccoid cells grouped in pairs, chains, tetrads, cubical arrangements of eight, or irregular clusters (Stackebrandt et al. 1995). It is a soil dwelling bacterium, commonly used for antimicrobial testing and food preparations. Recently, new genes potentially involved in the biosynthesis of antifungal molecules, such as bacilysin and cycloserine in this microorganism have been identified (Guesmi et al. 2022).

Although *K. rhizophila* has been poorly studied for its plant growth promoting (PGP) their PGPB activities, recently some strains for multiple PGP traits have been characterized (Faddetta et al., 2023) as well as for the capability of improving maize growth under salt stress by regulating hormone synthesis and nutrient uptake, and by uptake maintaining ion homeostasis (Li et al., 2020).

Due to the complexity of PGPB-plant interactions, the molecular mechanisms underlying these positive effects are not yet deeply elucidated, limiting the use of PGPB in novel formulation of biofertilizers for agronomic practices.

Recently, multi-omics approach has been shown as a valuable tool to elucidate the molecular mechanisms and the physiological processes underlying the activity of these microorganisms as well as their protective action on plants toward abiotic stresses [i.e., transcriptomics (Salvioli et al., 2012), proteomics (Bernardo et al., 2017), and metabolomics (Bernardo et al., 2019)].

In the present study, we originally provided the first molecular evidence on the metabolic and biosynthetic pathways activated by the application to tomato of a *K. rhizophila*-based biofertilizer. By using a multi-omic approach, differential genes, proteins and metabolites affected by PGPB treatment and involved in biological processes relevant for promoting tomato plant growth were highlighted.

10.2. Materials and methods

10.2.1 Bacterial growth conditions

The cell biomass of *K. rhizophila* was obtained in cultures from a single colony inoculated into Luria Bertani (LB) agar and incubated overnight at 30 °C and 180 rpm. Subsequently, 1.5% (v/v) of the culture was transferred into a bioreactor (Applikon Ez Control, Getinge) for 24h, containing 9.85 liters of Triptone Soy Broth (TSB), at pH 6.8, 28°C, aeration rate of 1.0 vvm, and agitation rate from 250 to 350 rpm, to ensure that the dissolved oxygen in the solution was ≥ 30%. Cell concentrations were evaluated by colony forming unit (CFU) method on tryptone soy agar (TSA) medium. For a long storage time, each bacterial strain was stored at -80°C in a 20% v/v glycerol solution.

10.2.2 Plant material and treatment

Four-week tomato plants (*Solanum lycopersicum* L.) of uniform size were transplanted in plastic pots (Ø 22 cm, h 22 cm, 7 L) filled with coconut fiber and placed in a growth-controlled chamber (25 ± 2 °C, 65–75% relative humidity, 12 h of daylight with a light intensity 18-20 kilolux).

After transplanting, plantlets were inoculated by drenching with 100 mL of *K. rhizophila* suspension (PGPB; 10^7 - 10^8 CFU/ml; Faddetta et al., 2023) (K), while the same amount of distilled water was used for the control (Ct), following a randomized design. A second PGPB treatment was carried out after 14 days. All the tomato plants (30 for each thesis) were irrigated with a standard nutrient solution (50 mg HNO_3 65%, 242 mg $Ca(NO_3)_2$, 210 mg KNO_3, 147 mg $Mg(NO_3)_2$, 38 mg NH_4NO_3, 88 mg KH_2PO_4, and microelements including Fe-EDTA); and three fertigations per day were performed. Tissues from each treatment (Ct and K plants) were harvested at 14 (T1) and 42 (T2) days from the first PGPB inoculation (Figure S1) and stored at -80 °C until use. Three biological replicates (each consisting of a pool of three plants bulked), for each time sampling and treatment, were freeze-dried and ground to a fine powder. Aliquots of the same material were used for the *multi-omics* approach. The PGPB effect on tomato plant growth was preliminary

assessed by evaluating fresh and dry weight of Ct and K plants at each time sampling. Tukey's test ($p < 0.05$) was applied to assay the significance among treatments.

10.2.3 Transcriptomic analysis

Leaf total RNA was isolated using a NucleoSpin RNA Plant (Macherey-Nagel GmbH & Co. KG, 52355 Düren, Germany) and treated with RNase-free DNase. RNA integrity was assessed using an Agilent Bioanalyzer RNA nanochip (Agilent, Wilmington, DE). Sequence libraries were prepared as already reported (Puccio et al., 2021). Quality, quantity, and insert size distribution were assessed using an Agilent Bioanalyzer DNA 1000 chip. Sequence libraries were pooled in equimolar concentration and analyzed on an Illumina NextSeq500 generating 2x100 nt paired end (PE) reads.

Raw reads obtained from all samples were assessed for quality by FastQC v0.11.8 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adapter were removed and the trimming was performed using Trimmomatic v0.38.0 (http://www.usadellab.org/cms/?page =trimmomatic). Reads were filtered by length and only those longer than 20 bp were selected. The filtered reads were mapped on the latest available tomato reference genome (SL4.0) (Hosmani et al., 2019) using RNA STAR v2.7.8a (Dobin et al., 2012).

Transcript quantification was performed using htseq-count v0.9.1 (Anders et al., 2014). All the sampling times (T1, and T2) within and between each thesis (Ct and K) were compared to identify the Differentially Expressed Genes (DEGs) ($\log_2$FoldChange $\geq \pm$ 1.0 and Padj < 0.05). DEGs isolation, Principal Component Analysis (PCA), and an heatmap were carried out by Deseq2 (http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html) and ClustVis (https://biit.cs.ut.ee/clustvis/), respectively, using default settings. Gene ontology (GO) enrichment analysis of specific gene functions across treatments was assessed with AgriGOv2.0 software (Tian et al., 2017) by using *S. lycopersicum* Transcript ID (ITA4.0 version). Finally, the MapMan tool (http://gabi.rzpd.de/projects/MapMan/) was used to link specific metabolic pathways to isolated DEGs.

10.2.4 RT-qPCR analysis

Reverse transcription quantitative PCR (RT-qPCR) was performed to target and validate the transcript abundance of candidate genes (primer sequences are listed in Table S1), using the *actin7-like* (Joseph et al., 2018) as housekeeping gene, following the procedure

previously described in Cavalieri et al. (2017). RT-qPCR experiments were performed on three biological replicates for each thesis and all reactions were run in triplicate on a Step OnePlus Real-Time PCR System (Thermo Fisher Scientific) using SYBR Green detection chemistry. Relative expression levels were calculated as described in Livak and Schmittgen (2001).

10.2.5 DNA methylation level

Global DNA methylation levels, referred to as the total level of 5-methylcytosine (5-meC) content in a sample, were quantified using MethylFlash Methylated DNA Quantification Kit (Epigentek), as described (Faddetta et al., 2023). Briefly, 100 ng of genomic DNA samples were bound to an ELISA plate and fluorescently labelled for 5-methyl Cytosine (5-meC) presence using specific antibodies. Each sample was run in duplicates along with internal controls provided by the kit, and the optical density (OD) intensity was measured for the plate based on the amount of 5 meC absorbance at 450 nm. The slope of the standard curve generated by positive controls was determined using linear regression and used to identify the global 5 meC amount of each sample. The percentage of global DNA methylation was then calculated as a ratio of its OD relative to the OD of positive controls, after subtracting the negative control OD values. Data are presented as a mean ± standard error (SE). The significance among treatments was evaluated by Tukey's test ($p < 0.05$).

10.2.6 Shotgun proteomics

Whole protein extracts from 100 mg of tomato leaves using the procedures already described (Faddetta et al. 2018) with some modifications were obtained. In particular, leaf samples were washed five times with 1 mL 10% v/v TCA/acetone, one with 0.1 M ammonium acetate/methanol, one with acetone and twice with 1 mL of extraction buffer (10 mM Tris–HCl pH 7.5, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 4 mg/mL leucopeptin©, 0.7 mg/mL pepstatin, 5 mg/mL benzamidine) by vortexing, and then were centrifuged (15,000×*g*, 10 min, at 4 °C). Cells were disrupted by sonication on ice (8 times, 15 sec at setting 4 with 10 sec break in-between each pulse, Vibra Cell, USA) in 1 mL of extraction buffer with 0.3% w/v sodium dodecylsulfate. The samples were boiled for 5 min, and rapidly cooled down on ice for 15 min; then they were treated with DNase (100 µg/mL) and RNase (50 µg/mL) in ice, for 15 min. Cell debris and non-broken cells were separated by centrifugation (15,000×*g*, for 15 min, at 4 °C). Protein extracts were

treated with 1 vol of phenol/chloroform/isoamyl alcohol (25/24/1, v/v; Sigma-Aldrich) for 5 min, at room temperature, mixing by vortex. After centrifugation (15,000×*g*, 5 min, at 4 °C), they were recovered from the interface and organic phases by discharging the aqueous phase. Proteins were precipitated with 0.1 M ammonium acetate/methanol, at −20 °C, overnight, and protein precipitate samples were recovered by centrifugation (15,000×*g*, 10 min, at 4 °C). The protein pellets were washed with 1 mL of methanol and then with 1 mL of acetone, and finally were dried under vacuum.

For quantitative proteomics, protein samples were dissolved in 8 M urea, 50 mM triethylammonium bicarbonate, 1% w/v a protease inhibitor mix (Sigma-Aldrich), pH 8.5 and treated as previously reported (Rosina et al., 2022). Briefly, protein concentration values were determined using the Pierce BCA Protein Assay Kit™ (ThermoFisher Scientific, USA). Equal amounts of protein samples (100 µg) were reduced with 5 µL of 200 mM tris (2-carboxyethyl) phosphine for 60 min at 55°C and then alkylated with iodoacetamide, precipitated with cold acetone, pelleted by centrifugation and then vacuum dried. Each sample was independently digested with trypsin (enzyme to protein ratio, 1:50), at 37 °C, overnight. Resulting peptides were labeled with TMT10plex Isobaric Label reagent kit (Thermo-Fisher Scientific). For a set of comparative experiments, tagged peptides were mixed in equal molar ratios (1:1) and dried under vacuum. Pooled TMT-labeled peptide mixtures were solved in 0.1% v/v trifluoroacetic acid and fractionated with a high pH reversed-phase peptide fractionation kit (ThermoFisher Scientific) into eight fractions, which were analyzed on a NanoLC-ESI-Q-Orbitrap-MS/MS platform consisting of a HPLC UltiMate™ 3000 RSLCnano System coupled to a Q-ExactivePlus mass spectrometer through a Nanospray Flex Ion Source (ThermoFisher Scientific). Peptides were loaded onto an Acclaim™ PepMap™ RSLC C18 column (150 mm × 75 µm ID, 2 µm particles, 100 Å pore size) (ThermoFisher Scientific) and eluted with a gradient of solvent B (19.92/80/0.08 [v/v/v] water/acetonitrile/formic acid) in solvent A (99.9/0.1 [v/v] water/formic acid), at a flow rate of 300 nL/min. Gradient and mass spectrometer settings were already reported (Rosina et al., 2022). Raw data MS files for three technical replicates of each fraction were merged for protein identification and relative protein quantification into Proteome Discoverer (PD) software v. 2.4 (Thermo Scientific), allowing a database search by Mascot algorithm v. 2.4.2 (Matrix Science, UK) using the following criteria: UniProtKB protein database (*S. lycopersicum*, 36,951 protein sequences, *K. rhyzophila* 2,352 protein sequences, 03/2022), including the most common protein contaminants;

carbamidomethylation of Cys and TMT10plex modification of lysine and peptide N-terminal were set as fixed modifications; all other settings were previously reported (Rosina et al., 2022). Protein candidates were considered as confidently identified when assigned based on at least two sequenced peptide spectra matchings (PSMs) with an individual Mascot Score ≥25. For relative protein quantification, PD software calculated abundance ratios between experimental samples from the ratios of TMT reporter ion intensities in the MS/MS spectra from raw datasets. Results were filtered to a false discovery rate of 1%. Proteomic data were deposited to the ProteomeXchange consortium (Perez-Riverol et al., 2022) within the PRIDE partner repository with the dataset identifier PXD038137.

10.2.7. Metabolomic analysis

Leaf samples for HPLC were prepared suspending 100 mg of freeze-dried material with MeOH (5 mL) and sonicating for 1 h. The solvent was filtered and injected. HPLC/MS analysis was performed on an Agilent 1260 Infinity as previously reported (Raimondo et al., 2022), using LC-MS grade water and acetonitrile, and analytical-grade formic acid. A reversed-phase Phenomenex Luna C18(2) column (150 mm × 4.6 mm, 3 µm particles) with a Phenomenex C18 security guard column (4 × 3 mm) was used. Injection volume was 25 µL. The eluate was monitored through Mass Total Ion Count (MS TIC) and UV (270 nm). Mass spectra were obtained with an Agilent 6540 UHD accurate-mass Quadrupole-Time of flight (Q-TOF) spectrometer equipped with a Dual AJS Electrospray Ionization (ESI) source working in positive or negative mode. Nitrogen was used as desolvation gas at 300 °C and a flow rate of 8 L/min. The nebulizer was set to 45 psig. The sheath gas temperature was set at 400 °C and a flow of 12 L/min. A potential of 2.6 kV and 3.2 kV was used on the capillary for negative and positive ion mode, respectively. The fragmentor was set to 75 V. MS spectra were recorded in the 150–1000 *m*/*z* range.
Metabolomic data were normalized (Van den Berg et al., 2006) and analyzed using MetaboAnalyst 4.0 (Xia et al., 2009), highlighting qualitative and quantitative variation of metabolites between both treatments and times. The differential metabolites in the K *vs* Ct comparison at each time were screened by combining the Fold Change (FC; ≥ 1) and *T*-tests ($p < 0.05$). The metabolites pathway analysis was developed, and the paths with p <0.05 and higher impact value (≥ 0.5) were considered statistically significant.

10.2.8 Integrated weighted gene co-expression network analysis by using multi-omics data

A Weighted Gene Co-expression Network Analysis (WGCNA) was performed on the DESeq2 variance stabilizing transformed (VST) expression data of the previously identified DEGs using the WGCNA package in R language (Langfelder and Horvath, 2008). The WGCNA soft threshold power parameters were defined based on the approximate preconditions of the unscaled topology and the cut-off criteria of Edge Threshold = 0.80. All the variable sets were used to build the weighted gene co-expression network and visualize the gene modules using WGCNA. Topological Overlap Matrix (TOM) and the corresponding dissimilarity (1-TOM) were calculated using the adjacency matrix (Yip and Horvath, 2007). After a hierarchical clustering, the highly correlated genes were included in the same module by using the Dynamic Tree Cut algorithm (minimum module size = 10). This analysis identified modules representing highly interconnected genes represented by different color and defined as Module Eigengene (ME; threshold = 11). The *multi-omics* relationships were presented by a heatmap based on Pearson's correlation between module expression profile (ME) and the other -*omics* variables (proteins and metabolites) with the aim to identify module membership (MM). The chromatic scale (red, blue) represents the strength of the correlation while each box included the correlation *p*-value. To identify the hub genes network, the VisANT 5.53 tool was used (Hu et al., 2017).

10.3. Results

10.3.1 Effects of *K. rhizophila*-treatment on tomato plant growth

Tomato plants grown in a phytotron by setting-up the K and C conditions were sampled at two times (T1 and T2) after PGPB treatment. The *K. rhizophila* inoculated (K) plants showed a significant increase ($p < 0.05$) in both fresh and dry weight of shoot at each time sampling compared to control (Ct) (Figure 16A; Figure S2). By contrast, in root, a significant increase in K plants was recorded only in fresh weight at T1 ($p < 0.05$), while any changes in dry weight at T1 and in both fresh and dry weight at T2 were observed (data not shown). A general trend of increasing in root and shoot length, at each time sampling in K plants, was observed (data not shown). These results suggested to focus our attention on shoot, for dissecting the *K. rhizophila* mode action by using a multi-omics approach.

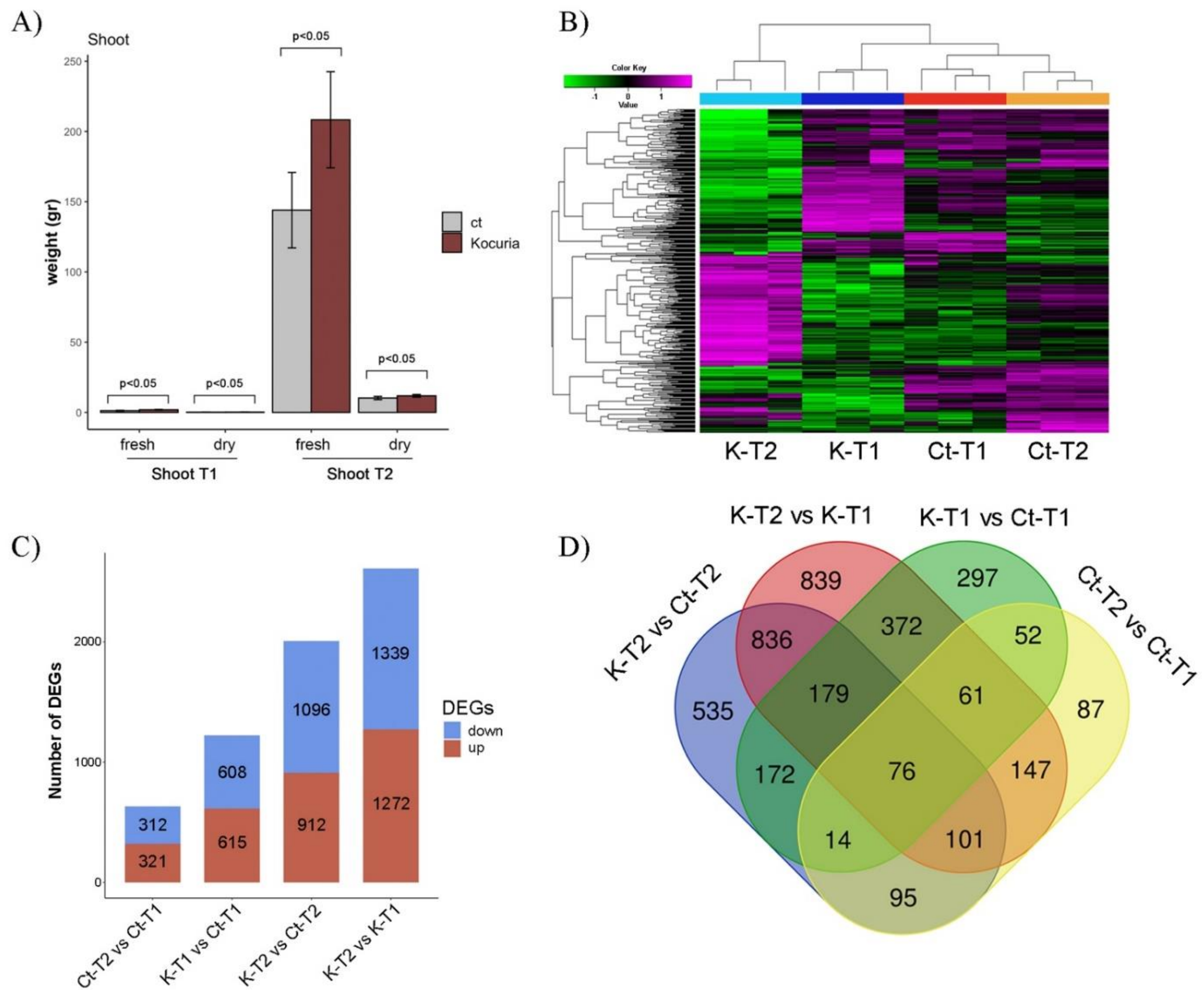


Figure 16. Morphological and transcriptome comparison of PGPB inoculated (K) and non-inoculated (Ct) tomato plants. **A)** Morphological effects (fresh and dry shoot weight) of *K. rhizophila* treatment on tomato plants. The significant differences between K and Ct (based on the Student's t test) were indicated ($P < 0.05$). **B)** Heatmap expression pattern of five hundred most variable genes detected between K and Ct at each time point (T1 and T2). **C)** Numbers of differentially expressed genes (DEGs) detected between each comparison. **D)** The DEGs Venn diagram for different treatments of each comparison group and the unique and common pathways of DEGs enrichment between theses.

10.3.2 Differentially expressed genes (DEGs)

The leaf transcriptomic profiles of K and C tomato plants grown in phytotron at two sampling times (T1 and T2) were compared. The statistics of this transcriptomic analysis was performed on the whole differentially expressed gene (DEG) dataset (Table S2). The PCA highlighted the main source of variation between the inoculated samples (K-T1 *vs*. K-T2); by contrast, Ct-T1 and Ct-T2 resulted very closed and distinguished from the inoculated samples. PC1 and PC2 explained 25.8% and 18.5% of the total variance,

respectively (Figure S3). In agreement, correlation coefficient heatmap of the expression patterns for the most variable genes displayed a clear separation between K and C plants, with K-T2 more distant from the controls (Ct) compared to K-T1 (Figure 16B). The differentially expressed genes (DEGs), obtained by two pairwise comparisons, at the same time sampling between K and Ct plants showed: 615 and 608 up- and down-regulated in K-T1 *vs.* C-T1; 912 and 1,096 up- and down-regulated in K-T2 *vs.* C- T2; 321 and 312 up- and down-regulated in C-T2 *vs.* C-T1; 1272 and 1339 up- and down-regulated in K-T2 *vs*. K-T1 (Figure 16C). A significant lower number of unique DEGs (87) was identified in the Ct-T2 *vs.* Ct-T1 comparison (Figure 1D). By contrast, the highest number of DEGs was observed in the K-T2 *vs*. K-T1 (839), K-T2 *vs*. Ct-T2 (535) and K-T1 *vs*. Ct-T1 (297) comparisons (Figure 16D). Furthermore, 179 DEGs were shared in the comparisons that included K-T2, while 172 DEGs were identified in both K *vs*. Ct comparisons, regardless sampling time (Figure 16D). Finally, 95 DEGs were common to all pairwise comparisons.
To validate the transcriptomic results, nine randomly chosen key isolated DEGs (Table S1) were tested by RT-qPCR. Overall, their relative gene expression trend was comparable to the RNASeq data (Figure S4).

10.3.3 Enriched GO Terms, DEGs and methylation profiles related to K. rhizophila treatment

To obtain more information about the molecular mechanisms induced by *K. rhizophila* on tomato, a GO enrichment analysis, including up- and down-regulated genes distinctly, was performed for the main three GO categories (Molecular Function MF; Cellular Component CC; and Biological Process BP; (Figure 17A; Figure S5; Table S3). GO functional annotation results highlighted that up-regulated DEGs in the K plants belonging to BP (FDR <0.05) were mainly related to *i)* response to external factors or involved in interaction with host (such as “response to stress”, “cellular response to stimulus”, and “cellular response to stress”), *ii)* catabolic processes (e.g. “macromolecule catabolic process” or “polysaccharide catabolic process”), *iii)* transport (like “intercellular transport” or “plasmodesmata−mediated intercellular transport”), and *iv)* steroid and sterol metabolic process. Furthermore, among significantly enriched GO terms for down-regulated genes, many BP categories were included in photosynthetic pathways (Figure 17A; Table S3), and metabolism processes, (such as “tetrapyrrole metabolic process”, “serine family amino acid metabolic process”, “flavonoid metabolic

process”, “flavonoid biosynthesis process”, and “small molecule metabolic process”). DEG metabolic categories within these groups differed in response to *K. rhizophila*-treatment (Figure 17A; Tables S3), highlighting a variation of DEGs number and their biological function between K and Ct during time.

MapMan analysis showed that the *K. rhizophila*-specific DEGs were mainly related to amino acid metabolism, cell wall organization, lipid and secondary metabolism (Figure 17B). At lesser extent, C1-metabolism, linking to long-term development, light reaction, nucleotide metabolism, oxidative pentose phosphate, and tricarboxylic acid cycle (TCA) were also significantly influenced by *K. rhizophila*-treatment (Figure 17B; Table S3). Functional annotation highlighted an expression level increase in several genes related to plant growth and development induced by *K. rhizophila*-treatment. Indeed, few genes (Solyc09G090680.3.1, Solyc03G005690.3.1 and Solyc06G083750.3.1) all related to plant development or stress responses in the “host interaction” categories were identified (Table S3), such as a cysteine-rich repeat secretory protein, and plasmodesmal proteins (PD), included also in the “plasmodesmata-mediated intercellular transport” category (Sager et al., 2020). In the “catabolic processes” category several aspartic proteases (APs, e.g., Solyc07g006470.1.1, Solyc08g067100.2.1 and Solyc06g069220.1.1), involved in plant growth and development (Cao et al., 2019), were found. Furthermore, in the “steroid and sterol metabolic process”, the allene oxide synthases (AOS; Solyc04g079730.1.1 and Solyc11g069800.1.1) that produce the allene oxide, a jasmonic acid precursor, and a cytochrome P450 enzyme resulted up-regulated in the K samples. Among the genes included in the “amine metabolic process”, worth mentioning is a S-adenosylmethionine decarboxylase (SAMDC; Solyc06g054460.1.1), with a key role in plant growth and development (Tassoni et al., 2007), was up-regulated by *K. rhizophila*-treatment. A similar profile was observed for the inositol transporter (Solyc12g099070.1.1), identified in the “inorganic anion transport" and related to important plant signaling pathways (Zhou et al., 2022).

By contrast, several genes translating proteins belonging to Calvin cycle, photosystem I and II (Qiu et al., 2021), were down regulated by *K. rhizophila* treatment. In agreement, a geranylgeranyl reductase (GGR; Solyc03g115980.1.1), included in both “chlorophyll metabolic process" and “Tetrapyrrole metabolic process”, involved in the plant chlorophyll biosynthesis (Ryouichi et al., 1999), was down regulated by *K. rhizophila* treatment. Finally, in the “flavonoid metabolic process", genes catalyzing the formation of vinorine (vinorine synthase; Solyc07g006670.1.1, Solyc07g006680.1.1 and

Solyc12g096800.1.1), which is a precursor of the monoterpenoid indole alkaloid related to stone cell development (Sheng et al., 2021), and the agmatine coumaroyl-transferases (Solyc11g071470.1.1 and Solyc11g071480.1.1), involved in plant defense (Muroi et al., 2012), showed a lower expression in K plants compared to control (Ct).

The different expression pattern highlighted between K and Ct samples agreed with the global DNA methylation profiling recorded. As expected, significant differences in the shoot methylation pattern were highlighted between treatments, where higher levels of methylation ($p<0.05$) were observed in the inoculated samples at both time samplings (Figure S6).

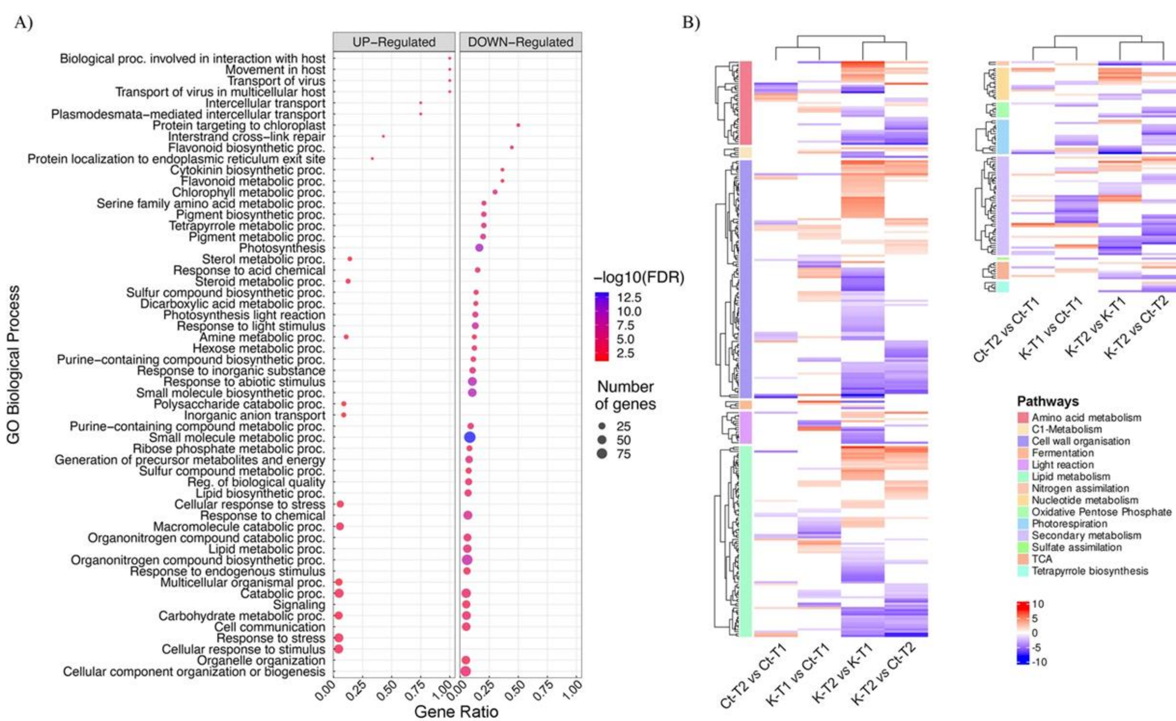


Figure 17. A) GO enrichment analysis, showing up- and down-regulated genes, for Biological Process (BP), one of the three main categories was performed for the main three GO categories. Molecular Function (MF) and Cellular Component (CC) were included in Figure S7. **B)** DEGs related to the main pathways extracted between each comparison.

10.3.4 Differentially Accumulated Proteins (DAPs) and KEGG ontology

Proteomic analysis identified proteins associated with 3,177 non-redundant sequence entries. Overall, 193 and 287 differentially accumulated proteins (DAPs) were identified in K-T1 *vs* C-T1 and K-T2 *vs* C-T2 comparisons, respectively (Table S4). Among these, 85 and 142 DRPs were exactly identified from the K/Ct comparisons at T1 and T2,

respectively (Figure 18A). Along time, eighty proteins showed different expression in Ct samples, while any DRPs were identified in the K-T2/K-T1 comparison, and consequently any DAPs were shared between K and Ct samples (Figure 18A).

According to their sequence homology and similarities, DAPs were classified into more wide functional groups. GO analysis included these proteins in 120 functional groups belonging to the three main GO categories BP, CC, and MF (Table S5). Among the BP enriched categories, several groups of DAPs were involved in response to oxidative stress, transport, catabolic, and metabolic processes. Among them, many categories related to photosystem ("plastid", "thylakoid membrane", "thylakoid", "chloroplast stoma", "respiratory chain") and organelles were identified in both CC and MF categories (Figure S7). In addition, several categories involved in carbohydrate and amino acids were also extracted.

KEGG analysis of DAPs in the K/Ct comparison identified 11 and 5 main KEGG categories at T1 and T2, respectively (Figure 18B; Figure 18C; Table S6). KEGGs isolated at T2 were also found at T1, showing compared fold enrichment values, except "glutathione metabolism" (Fold Enrichment 16.72) (Tables S6).

Among DAPs isolated (Table S4), several proteins involved in photosynthesis, cell division, and plant growth were high represented in the samples inoculated with *K. rhizophila*. In detail, a photosynthetic NDH subcomplex (Solyc05G007780.3), three photosystem II reaction center Psb family protein (Solyc06g065490.3; Solyc08g067840.3; Solyc09g064500.3), a photosystem I reaction center subunit IV and V family protein (Solyc07g066150.1 and Solyc09g063130.3), a Thylakoid membrane phosphoprotein (Solyc10g005050.3), a Chlorophyll a-b binding protein (Solyc12g011450.2), and an ATP synthase delta-subunit protein (Solyc12g056830.1) were found higher translated in PGPB treated samples than control. In addition, a succinyl-CoA ligase (SCoAL), related to TCA, two hexokinase involved in plant development and stress resistance, namely HXK1 (Solyc03g121070.3) and HXK4 (Solyc04G081400.3), two enolases (Solyc06G076650; Solyc10g085555.1.1) affecting metabolism and growth in plants, an alpha-ketoglutarate dehydrogenase E2 (Solyc12G005080.2) involved in the basal immune response against bacterial in tomato (Ma et al., 2020), a cytosolic ascorbate peroxidase (APX1; Solyc06g005160.3) able to mitigate oxidative stress, a cytochrome c oxidase subunit Vb (Solyc12G042900.2) belonging to the oxidative phosphorylation pathway, often related to abiotic stress tolerance (Zhou et al., 2013), and phosphofructokinases (PFKs) (Solyc12G095880.2;

Solyc07g045160.3) showing a role in the cell division (Beauvoit et al., 2014), were extracted in the *K. rhizophila*-inoculated/control plant comparison. Finally, *K. rhizophila* treatment caused a differential accumulation of some proteins involved in signaling pathways of important biological mechanisms, such as the up-represented ubiquitin carboxyl-terminal hydrolase (UCH; Solyc07g063650.3), the UBX domain-containing protein (Solyc08g080410.3.1) and nucleoside diphosphate kinase (Solyc03g110960.3) which were down- and up-represented, respectively.

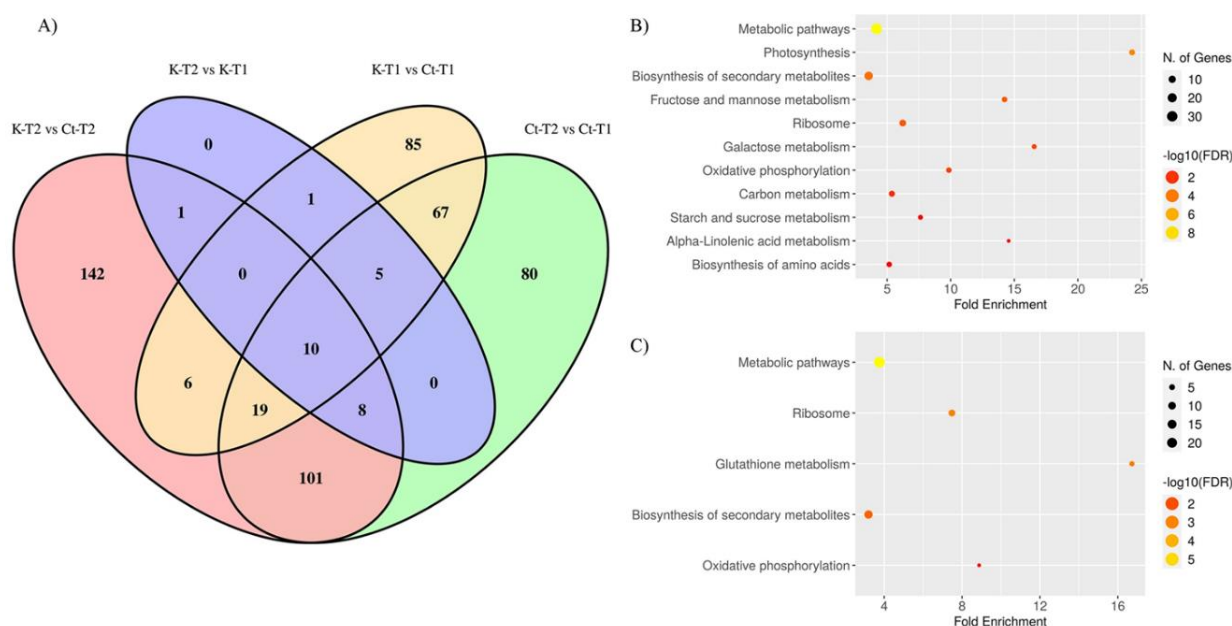


Figure 18. Proteomics analysis of PGPB inoculated (K) and non-inoculated (Ct) tomato plants. **A)** Venn diagram of Differentially Represented Proteins (DRPs) between each comparison group (Ct-T2 vs Ct-T1, K-T2 vs K-T1, K-T1 vs Ct-T1, and K-T2 vs Ct-T2). **B)** KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis of DRPs in the K/Ct comparison at T1, and **C)** T2).

10.3.5. Metabolite variation and Pathway Analysis

To investigate the metabolites variation in tomato due to *K. rhizophila*-treatment, metabolome for K and Ct plants at each time sampling was developed by HPLC/MS. Seventy-three (73) metabolites were detected, with significant differences in their amount between treatment and times (Figure 19A). At T1, the number of compounds related to TCA significantly higher mainly in the control (Ct-T1) than K-T1 samples, while metabolites belonging to amino acids (e.g., valine, serine, tryptophan, ornithine), dopamine, carbohydrates, and some antioxidant substances (e.g., L-ascorbate) were increased in *K. rhizophila*-treated samples (K-T1) in comparison to Ct-T1 plants (Figure 19A). At T2, a similar trend between treatments (Ct *vs* K) was observed. Indeed,

asparagine, lysine, tryptophan, leucine, and other amino acids were significantly increased in K-T2 compared to Ct-T2 samples. By contrast, the concentration of fructose, and raffinose increased in the Ct; however, the amount of other carbohydrates, such as mannitol, maltitol, and saccharate, showed a common trend for K and Ct plants at T2 (Figure 19A). Finally, proline showed a constant concentration in the PGPGB-treated samples at both time points and up accumulated in the K/Ct comparison at T1, while in control plant its concentration increased at T2.

By the enrichment analysis, fifteen classes were identified, among which "amino acid and peptides", "monosaccharides", and "TCA" were the most significant (Figure 19B; Table S7). Among the metabolite classes, significant Differentially Accumulated Metabolites (DAMs) were found between pairwise comparisons (Figure 19A, 4C). By comparing the DAMs of the two theses (Ct and K) across time (K-T1 *vs* Ct-T1 and K-T2 *vs* Ct-T2), the up-set analysis highlighted 3 (caffeoylglycoside feruloylglycoside, and uracil) and 1 (maltose) unique DAMs, at T1 and T2 respectively, induced by *K. rhizophila* treatment (Figure 4C; Table S8); whereas comparing time-dependent metabolites variation (K-T2 *vs* K-T1 and Ct-T2 *vs* Ct-T1) 7 DAMs and only 4 DAMs were extracted in PGPB-treated and control plants, respectively (Figure 19C; Table S8). The most impact pathways extracted were the "Alanine, aspartate and glutamate metabolism", with an over accumulation of L-Aspartate and Succinate, and "Isoquinoline alkaloid biosynthesis", showing a greater amount of L-Tyrosine and Dopamine due to the PGPB-treatment (Table S9).

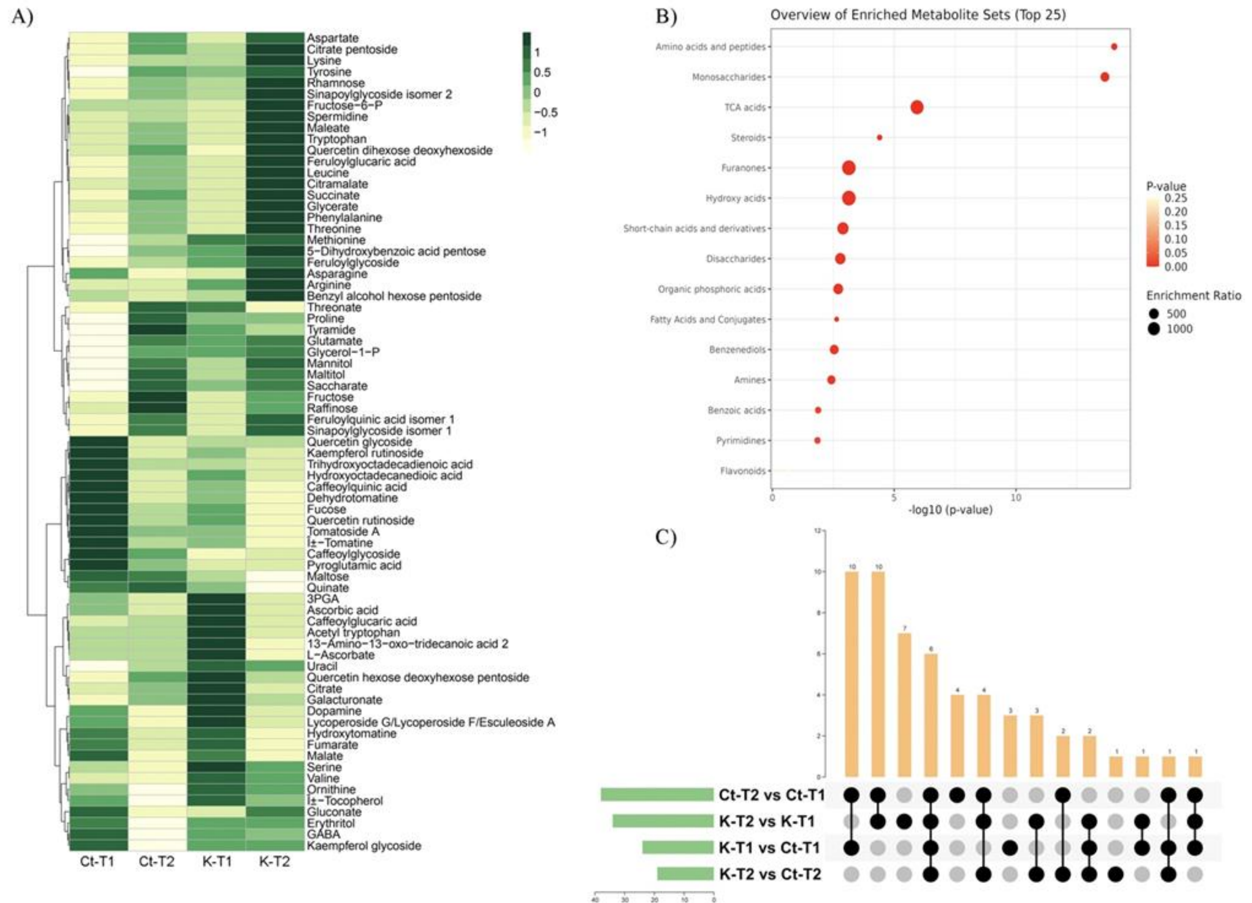


Figure 19. Metabolome comparison of PGPB inoculated (K) and non-inoculated (Ct) tomato plants. **A)** Heatmap of metabolites amount recorded at each treatment and time. **B)** Enriched metabolites analysis. The data for each class were reported in Table S7. **C)** UpSet Venn diagram of Differentially Accumulated Metabolites (DAMs) under different treatments and times (Ct-T2 vs Ct-T1, K-T2 vs K-T1, K-T1 vs Ct-T1, and K-T2 vs Ct-T2). The horizontal bar chart on the left shows the element (count of differential metabolites; up numbers indicate the elements for each comparison) statistics for each comparison group. A single black dot represents a grouping specific element, and the lines between points represent the intersection specific to different groupings. The vertical bar chart represents the number of corresponding intersection elements.

10.3.6 Weighted gene co-expression network analysis (WGCNA)

Based on differentially expressed gene profiles, as well as represented proteins and accumulated metabolites identified, a co-expression network analysis by using WGCNA tool was carried out to identify the most relevant modules and relative pathways associated to *K. rhizophila* treatment in tomato. Eight modules were isolated through a hierarchical linkage clustering and three out of eight modules were strongly correlated to several variables (Figure 20A, 5B). The "turquoise" module showed the highest correlation coefficient ($r > 0.80$; $P < 0.01$), followed by the "green" ones ($r > 0.70$, $P < 0.01$). These two modules exhibited a comparable correlation between genes and the same set of proteins, such as UBX domain-containing protein (Solyc08g080410.3.1),

nucleoside diphosphate kinase (NDPK; Solyc03g110960.3), cytosolic ascorbate peroxidase 1 (Solyc06g005160.3.1), Likely structural component of ribosome (Solyc12g098890.2), and metabolites, that included amino acids, TCA compounds, and dopamine (Figure 20B). By contrast, the "blue" module was strongly correlated with proteins mainly accumulated at T1 in *K. rhizophila*-treated samples ($r > 0.80$, $P < 0.01$), showing a negative correlation ($r > -0.90$, $P < 0.01$) to other proteins (Figure 20B). In the same module, only three out of thirty-seven metabolites (dopamine, lycoperoside, ornithine) showed high positive and significant correlation ($r > 0.72$, $P < 0.01$) (Figure 20B).

To explore the biological relevance of genes and pathways included in the three main modules identified (turquoise, green, and blue), a Gene Ontology (GO) enrichment analysis was performed. The "turquoise" module consisted of 575 genes, whose functions were mainly included in the Biological Process (BP) "response to biotic stimulus" (GO: 0009607), the Molecular Function (MF) "protein binding" (GO: 0005515), and the Cellular Component (CC) "cell periphery" (GO: 0071944) (Table S10). In this module the cytochrome P450 (Solyc02g070580.1.1) and AOS (Solyc04g079730.1.1) were included, and six hub genes with higher connectivity have been identified: the kinesin family member (kinesin family member C1) (Solyc11g071730.3.1), the ribosomal RNA-processing protein 7 (RRP7) (Solyc02g089600.3.1), the translation initiation factor (eIF4E) (Solyc03g005870.4.1), the glycosyl hydrolases (β-galactosidase) (Solyc04g080840.3.1), the zinc finger protein CONSTANS 1 (CO1) (Solyc02g089540.3.1), and the peroxisomal membrane protein (Pex16) (Solyc01g091900.3.1) (Table S10; Figure S8). One hundred two genes were included in the "green" module such as "root hair cell differentiation" (BP, GO: 0048765), "trichoblast maturation" (BP, GO: 0048764) and "cellular response to chemical stimulus" (BP, GO: 0070887), and "membranes" (CC, GO: 0016020). A serine/threonine-protein kinase (Solyc12g005290.2.1), a purine nucleobase transmembrane transport (PUNUT) (Solyc08g077370.4.1), a ferredoxin [2Fe-2S] (Solyc08g077050.3.1), a SIG5 14-3-3 family protein (Solyc04g012120.3.1), a multi antimicrobial extrusion (MATE, Solyc03g026230.1.1), and a subtilase family member (Solyc01g087840.3.1) were the hub genes identified in the green module (Table S10; Figure S8). Finally, the "blue" module comprised 457 genes, many of which correlated to the important biological processes, such as "metal ion homeostasis" (GO:0055065), "potassium ion transmembrane transport" (GO:0071805), "response to karrikin" (GO:0080167; a class of germination

stimulants), "post-embryonic development" (GO:0009791), "stomatal complex patterning" (GO:0010374) (Table S10; Figure S8). Here, the SAMDC gene (Solyc06g054460.1.1) and a plasmodesmal protein (Solyc09g090680.3.1) were found, as well as six hub genes with a high degree of connectivity: a hydroxyphenylpyruvate reductase (HPPR) (Solyc12g044250.2.1), a KDEL-tailed cysteine endopeptidase (CysEP) (Solyc03g111730.3.1), a zinc-finger component (E3 ubiquitin protein ligase) (Solyc03g032060.1.1), a BRO1-like domain component (BRO1) (Solyc03g019950.3.1), a PI-3-kinase-related kinase (SMG-1) (Solyc01g109080.3.1), and a peptide-methionine (R)-S-oxide reductase / Selenoprotein R (SelR) (Solyc01g080410.3.1) (Table S10; Figure S8).

Figure 20. WGCNA (Weighted Gene Co-expression Network Analysis) of genes modulated by *K. rhizophila* treatment on tomato shoot. **A)** Establishment of a Gene clustering tree (dendrogram) obtained via hierarchical clustering of topological overlapping dissimilarity. Dendrogram showing the co-expression

modules identified by WGCNA across time in the PGPB-treated plants. The major tree branches constitute eight modules labelled with different colors (see also Table S9). **B)** Correlation heatmap among genes, belonging to the eight high significant modules extracted, and the other factors (proteins and metabolites) investigated: each row corresponds to a module, and each column represents a specific compound isolated from proteomic and metabolomic analysis. The color of each cell at the row-column intersection indicates the Pearson's correlation coefficient (*p-value* was indicated in bracket) between module and trait. Red indicates the positive correlation between module and trait, while blue shows the negative correlation.

10.4 Discussion

Plant Biostimulants (PBs) are products exerting beneficial effect on plants by the improvement of nutrient use efficiency, fruit quality, and tolerance to abiotic stress (EU, 2019). The beneficial properties of PBs on tomato yield and fruit quality have been already reported (Colla et al., 2017), as well as the growth-promoting and stress-ameliorating effects under salt stress (Gedeon et al., 2022). However, the PBs mode of action is still largely unknown. Here, the key molecular players activated by the plant growth promoting bacterium *K. rhizophila* on tomato have been identified through a multiple omics approach, highlighting their role in the tomato responses correlated to plant growth and development.

DEGs enrichment analysis revealed the most impacted pathways by K. rhizophila treatment

The *K. rhizophila* treatment determined distinct transcriptomics responses compared to untreated samples on tomato leaves at both sampling times that differ for specific pathways elicited by PGPB. As expected, a significant number of Differentially Expressed Genes (DEGs) were identified in all the pairwise comparisons with the highest number of DEGs (912 up- and 1,096 down-regulated) by comparing K treated and untreated (Ct) plants at T2 (K-T2 *vs*. Ct-T2).

K. rhizophila applications were able to trigger transcriptionally changes and the GO enrichment analysis of DEGs underlined the significant increase of expression levels involved in cellular response to stimulus, cellular response to stress, macromolecule catabolic process, polysaccharide catabolic process, intercellular transport, plasmodesmata–mediated intercellular transport, steroid, and sterol metabolic process. In particular, the PGPB treatment increased the expression level of plasmodesmal proteins (PDs), a class of cytoplasmic membrane-lined channels involved in the molecules transport across cells (Burch-Smith et al. 2011). Interestingly, PDs mediate the transport of brassinosteroids (BRs), a class of steroidal plant hormones involved in a wide range of functions related to plant growth and development regulation (Ali, 2019). In turn, the

intracellular BR higher content is able to modulate the PD permeability to optimize the molecules mobility (Wang et al. 2023). In agreement, the GO category "steroid and sterol metabolic process" resulted upregulated by PGPB treatment, including a cytochrome P450 (Solyc02g070580.1.1) that is involved in the BR biosynthesis (Enoki et al., 2023). Furthermore, allene oxide synthases (*AOS*; Solyc04g079730.1.1; Solyc11g069800.1.1), key enzymes for the Jasmonic Acid (JA) biosynthesis, were also detected. Interestingly, JA is reported as plant growth regulator (Wasternack and Strnad, 2016), as well as a crosstalk between JA and other hormones in regulating plant growth and defense response was recently reported (Yang et al. 2019).

SAMDC (Solyc06g054460.1.1), included in the "amine metabolic process", was found up-regulated by *K. rhizophila*-treatment. *SAMDC* seemed to play a role in plant growth and development, also under stress condition. Indeed, high levels of *SAMDC1* enhanced vegetative growth and induced an early flowering in tobacco (Zhu et al. 2020), as well as its overexpression improved cold tolerance in maize and turfgrass species (Luo et al. 2017). SAMDC is able to decarboxylate the S-adenosylmethionine (SAM) resulting in the spermidine or spermine production. In alternative, SAM can be converted into 1-Aminocyclopropane 1-carboxylic acid (ACC) and then to ethylene, a key plant growth hormone (Borges et al., 2019). The *SAMDC* transcriptomic levels are driven by several metabolites, included the dopamine, a catecholamine neurotransmitter widely present in plants (Chakraborty et al., 2022). Recently, dopamine has been reported to play a key role in mediating plant growth by increasing the carbon and nitrogen metabolism activity and related enzymes (Lan et al., 2020).

Furthermore, an inositol transporter (Solyc12g099070.1.1), belonging to "inorganic anion transport", was up-regulated by PGPB-treatment. These transporters are known to transport several metabolites, such as lipids, minerals, and sugars, playing an important role in plant signaling pathways that regulate the message transduction from hormones, neurotransmitters, and growth factors (Zhou et al., 2022).

Otherwise, among the down regulated genes by the PGPB-treatment, transcripts belonging to "small molecule biosynthesis processes" and involved in the photosynthesis, the electron transport chain, the light harvesting complex, and the tetrapyrrole pathway, were identified. The down regulation of genes involved in the photosynthesis process could cause a reactive oxygen species (ROS) accumulation in the cell organelles. Indeed, the PGPB treatment attends in the ROS balance by up-regulating genes involved in the "cellular response to stress" category, such as the mitogen-activated protein kinases

(*MAPKs*; Solyc02g090990.1.1; Solyc08g076490.2.1) and a heat stress transcription factor (*HSF*; Solyc02g079180.1.1). The important role of *MAPKs* and *HSFs* in the transduction of environmental and developmental signals through the phosphorylation of downstream targets, included also in the ROS balancing, has been reported (Jagodzik et al., 2018; Hoang et al., 2019).

Finally, different genes commonly involved in plant defense were down-regulated by *Kocuria*-treatment, and similar evidence have been already reported (Mukherjee, 2022). Recently, transcriptomic analysis focused on plant-PGPB interactions in rice showed the down regulation of pathogenesis-related (PR) genes, as well as chitinases, thionins, cinnamoyl-CoA-reductases, all genes encoding for well-known disease resistance mechanisms (Wiggins et al., 2022). Therefore, also our study supported the idea that the host plant adjusts its transcripts profile in response to the beneficial effects of microbes on plant health.

Our finding was also supported by the overall levels of methylation associated to PGPB-treatment. DNA methylation is a major epigenetic modification driving the expression of genes associated to key biological mechanisms, including plant growth and development (Zhang et al., 2018). The methylation status of DNA in plants is easily affected by physiological factors, developmental stages, and the environment, and its changes cause the difference of gene expression levels. Therefore, the significant higher DNA methylation triggered by *K. rhizophila*-treatment agreed with the modified transcriptomic profiles observed.

Proteomic analysis revealed the most impacted pathways by PGPB treatment in tomato

Shotgun proteomic analysis revealed differentially accumulation of several proteins (DAPs) involved in the nutritional (carbohydrate and amino acids) and energetic (photosystem) metabolisms, related to plant growth promoted by *Kocuria* inoculation.

In our study several proteins playing a key role in photosynthesis (photosystem I reaction center subunit V and photosystem I subunit G chloroplastic, PSI-G, both related to the light-harvesting, *LHC*, or antenna complex) resulted higher represented in the *Kocuria*-treated plants. PSI-G protein was already reported over accumulated in plants whose salt and drought tolerance were increased by a mutualistic interaction with the root microbiome (Roy et al., 2021). Our results showed also that there a significant upregulation of photosystem-II-related proteins after inoculation with *K. rhizophila*, which are beneficial to photosynthesis and plant growth, such as the Psb family proteins

(involved in the Photosystem II reaction center) determinant for the plant growth rate (Justin et al., 2017). In agreement to our observations, the higher expression of proteins, such as LHC-I, LHC-II, PSI-K (photosystem I reaction center subunit K) and PSI-G (photosystem I reaction center subunit V), has been already observed in barley interacting with fungal endophyte (Ghaffari et al., 2016; 2019). Therefore, also in our experiments, PGPB appeared able to affect plant photosynthetic capacity, thereby promoting plant growth (Mathivanan et al., 2017).

Furthermore, K-treatment on tomato induced the over accumulation of proteins (DAPs) related to abiotic stress response, such as the cytosolic ascorbate peroxidase (APX1) able to mitigate the effects of oxidative bursts playing a key role in hydrogen peroxide removal and managing the reactive oxygen gene network (Davletova et al., 2005). Interestingly, the Nucleoside diphosphate kinase (NDK1) involved in the synthesis of nucleoside triphosphates other than ATP were found over accumulated in K-treated plants. Plants over-translating NDK1 protein resulted tolerant to paraquat pesticide by increasing the ability to remove H_2O_2 (Fukamatsu et al. 2003). In addition, the ubiquitin carboxyl-terminal hydrolase (UCH), over accumulated in the K-treated plants, participates in key cellular processes including signal transduction and transcription (Hayama et al., 2019). Among others, the UCHs are involved in the regulation of AUX/IAA protein stability, able to influence auxin-dependent developmental pathways in *Arabidopsis* through their deubiquitylation activities (Tian et al., 2012; Hayama et al., 2019).

Finally, in agreement to the results previously described, different phosphofructokinases (PFKs) were over accumulated in the K-treated plants. The activity of these enzymes of sugar metabolism were already reported strongly increases during cell expansion and plant growth (Beauvoit et al., 2014).

Overall, the over accumulated *K. rhizophila*-induced proteins and their role in the key physiological mechanisms can sustain the PGPB fortifying role for plant growth and development and/or to mitigate plant abiotic stress.

Metabolite profiling changes by PGPB treatment in tomato

Significant changes on metabolite accumulation were also observed in the tomato PGPB inoculated plants. Interestingly, DAM induced by K-treatment mainly belonged to amino acids and carbohydrates. Pathways related to “alanine, aspartate and glutamate” metabolism as well as “isoquinoline alkaloid biosynthesis” were significantly affected by K-treatment at both sampling times. Significant changes in the “alanine, aspartate and

glutamate" metabolism to enhance plant tolerance to abiotic stress were recently reported (Abd El-Daim et al., 2020), as well as in the "isoquinoline alkaloid biosynthesis" to mitigate the oxidative stress by removing ROS in plant cells (Gong et al., 2020).
Higher levels of dopamine in the K-treated than control plants were also found. Dopamine was recently reported as compound produced by plants, providing evidence of its role into stress responses and plant growth and development. Indeed, endogenous dopamine affected the expression of many genes related to abiotic stresses such as drought, salt, nutrient stress, including *SAMDC*, upregulated in K-treated plant, highlighting its multi-regulatory role able to coordinate many aspects of the plant growth and development (Liu et al., 2020).
Finally, the levels of feruloyl glycoside, a phenolic compound, resulted increased by K-treatment. Feruloyl glucoside is a ferulic acid with functional properties as antioxidant and anti-inflammatory, and its accumulation has been recently highlighted during the plant growth cycle of the cactus *Turbinicarpus* (Solis-Castañeda et al. 2020).
Metabolomic profiles of K-treated plants agreed to the findings provided by transcriptomics and proteomics, further underlining that *K. rhizophila* modulates carbon sources and energy, and increased the levels of messengers, like dopamine, affecting the expression of genes involved in plant growth and development.

Integrative omics approaches through Weighted Gene Co-expression Network Analysis (WGCNA)

Plant responses to PGPBs are a complex of connected pathways driving specific responses of genes, proteins and metabolites, involved in important biological processes, such as plant growth and development. We identified eight gene modules based on their correlation with differential accumulated proteins and metabolites (DAPs and DAMs) showing significant interactions by using a WGCNA approach. According to the module correlation, the turquoise and green modules showed the highest correlation with nine proteins, among witch a nucleoside diphosphate kinases, a conserved family of protein involved in the energy homeostasis and development process (Ye et al., 2020), and a cytosolic ascorbate peroxidase, enzymes family that plays a key role in plant growth and development (Wu et al., 2019; Li et al., 2020), and several metabolites, mainly belonging to amino acid metabolism and TCA compounds.
In the turquoise module, six hub genes significantly up-regulated by K-treatment, including a member of the Kinesin family (KINESIN FAMILY MEMBER C1), a

Ribosomal RNA-processing protein 7 (RRP7), a Translation initiation factor (eIF4E), a Glycosyl hydrolases (β-galactosidase), a Zinc finger protein (CONSTANS 1 - CO1) and a Peroxisomal membrane protein (Pex16), were identified.

Kinesins are motor proteins able to regulate the gibberellin biosynthesis and cell growth by transcription activity (Li et al., 2012; Nebenführ and Dixit, 2018). They affect microtubule organization, organelle distribution and vesicle transport; overall, kinesins contribute directly or indirectly to cell division and cell growth in several tissues (Wang et al., 2014).

RRP7 is a ribosome biogenesis factor required for 18S ribosomal RNA (rRNA) maturation. Interestingly, *rrp7 Arabidopsis* mutant showed slow growth, altered shoot phyllotaxy, and abscisic acid hypersensitivity (Micol-Ponce et al., 2018). The eIF4E regulation under abiotic stress conditions was reported as a mechanism involved in the recognition of mRNA cap, ATP-dependent unwinding of 5'-terminal secondary structure and mRNA recruitment to the ribosome (Echevarría-Zomeño et al., 2013; Zhu et al., 2020).

The β-galactosidase is believed to play key roles in the modification of cell wall components during several processes such as fruit ripening, the loosening of the cell wall during growth and the flower senescence (Ahn et al., 2007). CO1 transcription factor has been known to regulate a series of cellular processes including the transition from the vegetative growth to flower development in plants, however, their role in regulating fruit yield in tomato is poorly understood (Cui et al., 2022).

Finally, Pex16 appeared responsible for importing peroxisomal membrane proteins whose targeting signal is not yet well-defined (Akther et al., 2022); *pex16 Arabidopsis* mutant is defective in the formation of peroxisomes and the transportation of plasma membrane- and cell wall-associated proteins, revealing the Pex16 role in seed protein storage (Lin et al., 1999). Among the other genes, the cytochrome P450 and AOS, involved in BR and JA biosynthesis respectively, were included in the turquoise module.

The hub genes in the green module included a serine/threonine-protein kinase, a Purine nucleobase transmembrane transport (PUNUT), a Ferredoxin [2Fe-2S], a SIG5 14-3-3 family protein, a multi antimicrobial extrusion (MATE) and a Subtilase family (Table S9 B).

The serine/threonine kinases phosphorylate the amino-acid residues serine or threonine determining posttranslational modification with a significant role in a wide range of cellular processes such as the regulation of cell proliferation, cell differentiation and the

embryonic development. Interestingly, it appears involved in abiotic stress responses as well as the abscisic acid response, calcium signaling and antioxidant defense (Ye et al., 2017; Ramachandiran et al., 2018).

PUNUT is a large plant and fungal gene family of purine and cytokinin transporters. The plant vascular system transports nucleobases and their derivatives such as cytokinins and caffeine by a common H^+-coupled high-affinity purine transport system (Bürkle et al., 2003). The Ferredoxin [2Fe-2S] play an important role in electron transfer processes and in various enzymatic reactions which act as electron carriers in photosynthesis and it is involved in the control of multiple processes, ranging from oxidative stress, cell proliferation to nitrogen fixation (Nechushtai et al., 2020).

The SIG-5 14-3-3 family protein belongs to a ubiquitous regulatory class, phosphoserine/threonine-binding proteins, that plays important roles in many biological processes which are regulated by phosphorylation, including cell cycle regulation, protein trafficking, plant growth and development, cell elongation and division. They mediate plant response to environmental stresses such as salt, alkaline, osmotic, drought and cold stress (Huang et al., 2022). The MATE transporters are involved in plant growth and stress responses. Interestingly, allelic forms and the expression patterns of MATE are reported to be associated with favorable agronomic traits in domesticated crops (Ku et al., 2022).

Finally, the Subtilase family is involved in the plant life cycle, such as the development of seeds and fruits, cell wall modification, processing of peptide growth factors and epidermal development, but also in the response to biotic and abiotic stress (Figueiredo et al., 2018).

In the blue module hub genes with a high degree of connectivity with the other genes of the module, a Hydroxyphenylpyruvate reductase (HPPR), a KDEL-tailed cysteine endopeptidase (*CysEP*), a Zinc finger (E3 ubiquitin protein ligase), a BRO1-like domain (BRO1), a PI-3-kinase-related kinase (SMG-1) and a Peptide-methionine (R)-S-oxide reductase / Selenoprotein R (SelR), were identified. Interestingly, all the hub genes appeared involved in plant growth and development as well as the plant adaptation to abiotic stresses.

The HPPR is the key enzyme in the biosynthesis of 4-hydroxyphenyllactic acid (pHPL) from tyrosine, which have showed diverse functions in plant growth, development, and adaptation (Xu et al., 2018). The *CysEP* also plays a role in regulating plant growth and

development, and in addition tomato plants highly tolerant to abiotic stress showed a high expression of this gene (Wen et al., 2021).
The E3 ubiquitin protein ligase can possess different biological functions and plays important roles in various plant growth stages and several plant abiotic stress responses (Shu and Yang, 2017). The BRO1 involved in the endosomal sorting complex required for transport (ESCRT) machinery to control the homeostasis of membrane proteins by selective vacuolar degradation and it is determinant for multiple physiological functions in growth and differentiation of eukaryotic cells (Shen et al., 2018). The SMG1 regulate several eukaryotic cellular processes, for instance, cell-signaling cascades related to DNA damage and repair, cell growth and proliferation (Angira et al., 2020). SelR mediates the methionine sulfoxide reduction, an important process by which cells regulate biological processes and cope with oxidative stress. This gene encodes for the peptide methionine sulfoxide reductase A (MsrA) and its overexpression resulted in the increase of plant protection against abiotic and biotic environmental constraints (Rey and Tarrago, 2018). Finally, *SAMDC* and a plasmodesmal protein were also grouped in the blue module that are modulated by dopamine, a metabolite strongly correlated to this module, and involved in the BR transport.

10.5 Conclusion

Although further studies are required to confirm the key roles of the most interesting hub genes identified related to the plant growth-promoting (PGP) action of *K. rhizophila*, the multi-omics approach here described allowed to shedding light about the mechanisms induced by our PGPB on tomato. During plant growth and development, the nutrient acquisition and transport are essential for cellular signaling in the plant and to activate the growth-promoting gene expression. Our findings highlighted that plant nutrients, including sugars and amino acids, energy and related regulators, such as dopamine, are the key factors necessary for improving plant yield and are strongly modulated by plant-PGPB interaction.

11. Chapter III:

Streptomyces violaceoruber as plant growth promoting bacteria in different growth systems

11.1 Introduction

A more sustainable and efficient agriculture needs to increase crop yield for providing food supply in adequate quantity and quality, limiting excessive fertilizers and pesticides use, for a healthy life population and environment. However, plants are steadily subjected to many abiotic stresses, throughout their whole life cycle, which limited their productivity, now intensified by climate change (Hatfield et al., 2019). In particular, water and nitrogen (N) availability are two of the most critical factor limiting plant growth and crop yield worldwide (Plett et al., 2020). For this reason, in over the past decades, N fertilization has been applied in excess compared to crop requirement, which takes only 40-50%, and the remaining is lost into the environment, causing water and atmosphere pollution (Wang et al., 2019). At the same time, in many regions of the world, severe drought and dwindling natural water resources cause a negative impact on crop yield and food security (Liu et al., 2021). A promising and sustainable innovation would be the use of plant biostimulants (PBs), able to reduce the use of chemical fertilizers increasing NUE (Fiorentino et al., 2018), to mitigate drought stress (Di Stasio et al., 2018), thus improving the cropping system sustainability (Olaniyan and Adetunji, 2021).

Among PBs, plant growth promoting bacteria (PGPB) can be applied to the soil to increase nutrient availability, promoting plant growth, and controlling pathogens (Yadav and Sarkar, 2019; Olaniyan and Adetunji, 2021). They can also alleviate abiotic stress and improve the soil biological properties, in turns resulting in higher crop yield (Yadav et al., 2020; Olaniyan and Adetunji, 2021).

Among bacteria, *Streptomyces* spp., belonging to the *Actinobacteria* genus, they are considered promising beneficial microorganisms. They constitute to a large part of the rhizosphere microbiota, where they may live saprophytically and endophytically, colonizing plant roots (Saleem et al., 2016). Widely used in the pharmaceutical industry for the antibiotics production, their potential application in agriculture as growth promoter and biocontrol agents have been recently demonstrated (Puppala et al., 2019; Al-Ansari et al., 2020; Sharma et al., 2020; El-Naggar, 2021; Nazari et al., 2022; Nazari et al., 2023).

Streptomyces produce several phytohormones, such as auxins, gibberellins, ethylene, cytokinin-like chemicals, salicylic acid, jasmonic acid, abscisic acid, which play a key role in promoting plant growth and dealing biotic and abiotic stresses (Aallam et al., 2021; Fu et al., 2022; Kumar et al., 2022; Silambarasan et al., 2022; Tran et al., 2021). According to the plant developmental stages, their metabolite production might regulate plant metabolism for plant adaptation to adverse conditions, promoting hormones that sustain a more virtuous nutrient cycling to remedy nutrient imbalance occurring under stressful conditions (Pang et al., 2022).

In the Chapter I, we identified three actinomycete strains: *Streptomyces violaceoruber*, *Streptomyces coelicolor* and *Kocuria rhizophila* characterized for multiple plant-growth promoting traits such as indole acetic acid (IAA) production, phosphate solubilization, N_2-fixation, drought and salt tolerance. Among these, *Streptomyces violaceoruber* was the most prolific strain for its secreted and cellular metabolome, revealing a rich arsenal of bioactive molecules, including antibiotics and siderophores. Furthermore, *in vivo* assays on tomato (*Solanum lycopersicum* L.), proved its ability to improve germination index and seedlings growth, as well as to produce volatile organic compounds with antimicrobial activity by modulating plant volatilome and finally, to exert a control on global DNA methylation (Faddetta et al., 2023).

Here, the effect of *S. violaceoruber* on tomato plant grown in different culture systems, such as petri dishes, hydroponics and pots, under nutritional and/or drought stress was investigated. The aim of this study was to assess the biostimulant ability to stimulate plant growth and to mitigate stress conditions.

11.2 Materials and methods

11.2.1 PGPB

Streptomyces violaceoruber DSM 40783, an actinomycete strain, was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures GmbH). The spore production was obtained as described by Kieser et al. (2000). In detail, *S. violaceoruber* cultures were growth on mannitol soy flour (MSF) medium at 30°C, for 5-7 days. The spore suspensions were prepared by adding sterile distilled water into the plates. The mixtures were then filtered through a syringe containing hydrophilic cotton to eliminate the mycelia. Spore and cell concentrations were evaluated by colony forming unit (CFU) method on tryptone soy agar (TSA) medium. For culture long storage, each bacterial

strain was stored at -80°C in a 20% (v/v) glycerol solution until used. Before using, the bacterial culture was activated by incubating at 30 °C in a shaker at 180 rpm for 4 hours.

11.2.2 Hydroponic assay experiment

11.2.2.1 Plant material

The UC82 tomato (*Solanum lycopersicum* L.) seeds, a commercial variety, were sterilized with 70% (v/v) EtOH and subsequently with 25% (v/v) NaOCl solution for 2 and 6 min, respectively. Then, seeds were rinsed three times with autoclaved Milli-Q water to remove EtOH and NaOCl residues. Sterilized tomato seeds were germinated in Magenta boxes containing 1% (v/w) agarose gel diluted in 0,5 mM $CaSO_4$ and placed in a growth chamber at 24 ° C, 65% relative humidity, in the dark, for 3 days. Later (about 3 days), they were exposed to 14/10 light/dark photoperiod (350µmol m^2 s^{-1} light intensity), for 7 days. Uniform selected seedlings (10 days old) were transferred into an aerated hydroponic system (4.5 L plastic boxes, 12 seedlings per box) containing a modified Hoagland solution composed by macronutrients (1mM KH_2PO_4, 2.5 mM K_2SO_4, 2 mM $MgSO_4$, 9 µM Fe-EDTA) and micronutrients (4.6 µM H_3BO_3, 9 µM $MnCl_2$, 0.76 µM $ZnSO_4$, 0.32 µM $CuSO_4$, 0.11µM Na_2MoO_4), pH 5.8 (Mauceri et al., 2020). The nutrient solution was continuously aerated and renewed every 3 days and the pH was adjusted with 1N KOH solution. Finally, tomato seedlings were placed in the growth-controlled chamber, at the same conditions reported before, up to the 4th true leaf stage.

11.2.2.2 Curve-dose plant response

As reported in the schematic representation (Fig.21), at the 4th true leaf stage, tomato seedlings (15/20 days old) were N-starved for 3 days (using a modified Hoagland solution without N source), and then resupplied with low NO_3^- (LN; 0.5 mM) and inoculated, directly into the solution, with four different PGPB volumes, 0, 5, 10, 20 and 40 ml (control, T5, T10, T20 and T40, respectively) of bacterial culture containing 3% (v/v) of packed mycelium. The growing systems were placed in the growth chamber, at the same condition reported before, for 7 days. At the end of the experiment, relative chlorophyll content, fresh and dry biomass of shoots and roots collected separately, and root system morphology were evaluated.

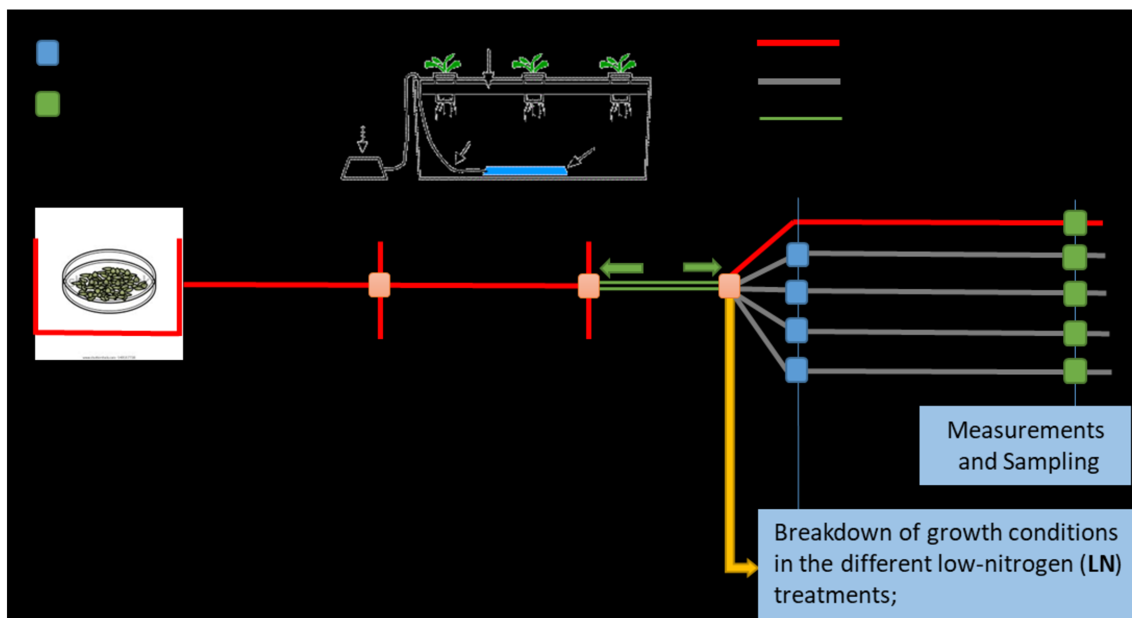


Fig 21. Schematic representation of the hydroponic assay experiment.

11.2.3 Chlorophyll content

For each experimental condition, the relative chlorophyll content was determined by a SPAD 502 chlorophyll meter (Konica Minolta Sensing, Inc., Japan) in fully expanded tomato leaves (30 d) placed in different stakes.

11.2.4 Plant biomass

For each experimental condition, plants were collected, shoots and roots were separated and their fresh weight was measured (SFW and RFW, respectively). Then, shoots and roots were dried at 72°C for 48 h to determine their dry weight (SDW and RDW, respectively).

11.2.5 Root morphological analysis

For each experimental condition, roots were immersed in a 0.1% (w/v) toluidine solution (Sigma Aldrich, 89160) for 5 min, and then washed in running water to remove excess dye for morphological analysis. Root images were acquired at 600 dpi resolution by a scanner (WinRhizo STD 1600, Instruments Règent Inc., Quebec, Canada) to determine primary (PRL; cm), lateral (LRL; cm), total root length (TRL; cm), and volume (cm^3) by

using WinRhizo Pro System v. 2002a software. Based on the above measurements. Lateral root number (LRN) was manually counted as reported by Lupini et al. (2014).

11.2.6 Application of the effective dose of Streptomyces on N limiting conditions

After identifying the effective PGP dose (T10), tomato seedling (15/20 days old), grown in the same conditions reported before, were N-starved for 3 days (using a modified Hoagland solution without N source) and then resupplied with low (LN; 0.5 mM) and high (HN) NO_3^-. The seedlings grown at LN were then treated with T10. The growing system were placed in the growth chamber, at the same reported conditions, for 7 days. Afterwards, roots and shoots were collected (as reported above) and the morpho-physiological analyses were carried out.

11.2.7 Photosynthetic activity

To measure the net CO_2 assimilation rate (A, µmol(CO_2 m^{-2} s^{-1}), stomatal conductance (gs, mol H_2O m^{-2} s^{-1}) and transpiration rate (T, mmol H_2O m^{-2} s^{-1}) on the leaf surface, a calibrated portable photosynthesis system (LI-6400; LI-COR, Inc.; Lincoln, NE) was used. These exchange parameters were measured at a flow rate of 500 cm^3 min^{-1}, a leaf temperature of 26 °C, a CO_2 concentration of 400 µmol(CO_2) mol(air)$^{-1}$ (controlled by a CO_2 canister) and 1200 µmol $m^{-2}s^{-1}$ of photosynthetically active radiation provided by the LED light source in the leaf chamber. Each measurement was performed with minimum and maximum waiting time of 120 and 200 s, respectively, and by matching the infrared gas analyzers to 50 µmol (CO_2) mol(air$^{)-1}$ of CO_2 concentration between the sample and reference, before each plant change. The vapor pressure difference (VPD) between leaf and air was set at 1.5 kPa, and constantly monitored around the leaf during all the measurements and maintained at a constant level by manipulating the humidity of the incoming air as required. All the measurements were performed in the growth chamber.

11.2.8 Pigment content

A hand-held spectrophotometer equipped with photodiode array capturing the range 450–1100 nm was used for non-destructive measurement of quality parameters (CP Pigment Analyzer PA1101 produced by Control in Applied Physiology GbR., Germany). This

instrument consists of 9 LEDs (660–780 nm) and a PDA (Photodiode Array Detector) that measures photons within the range of 450–1100 nm, remitted from the plant's epidermis, in fully expanded tomato leaves (30 d). Pigment contents are displayed on the screen as NDVI (Normalized Difference Vegetation Index) and NAI (Normalized Anthocyanin Index) (Piwowarczyk et al. 2020). Indices are optimised for the pigments determination and calculated according to the following equations (Herold et al., 2009; Ochmian et al., 2013):

$$NDVI = (I780 - I660)/(I780 + I660)$$

$$NAI = (I780 - I550)/(I780 + I550)$$

11.2.9 Pot assay experiment

The UC82 tomato seeds were germinated in a 90-well plateau, filled with a loamy sand soil, Typic Xerorthent, coarse loamy, mixed, thermic (Soil Survey Staff - USDA, 2010) produced by Chiodo farm, and then placed in a growth-controlled chamber. As described by the schematic representation (Fig.22), at the 4th true leaf stage (15/20 DAS, day after sowing), seedlings, selected for uniform size, were transferred to the pots (Ø16cm), filled with 360g of the same soil type mixed with 20% perlite. They were placed, in a completed randomized design, in the growth chamber at 24°C, 65% relative humidity, with 14/10 light/dark photoperiod (350 μmol m^2 s^{-1} of light intensity). After 24h from transplanting, 500 μL containing 10^7 CFU/ml culture of *S. violaceoruber* was directly inoculated into the rhizosphere. At 30 and 45 days after transplanting (DAT), treatment was repeated. Control plants (CTRL) were treated with the same volume of water. Plants were irrigated every other day with a half strength modified Hoagland solution (Mauceri et al. 2020). Pots were then placed in the growth chamber at the same condition reported before, for 60 days. Every 15 days after each treatment, physiological measurements were carried out (as described above). At harvesting time (60 d), plants (roots and shoots) were collected and physiological measurements and nitrogen content were carried out (as reported below).

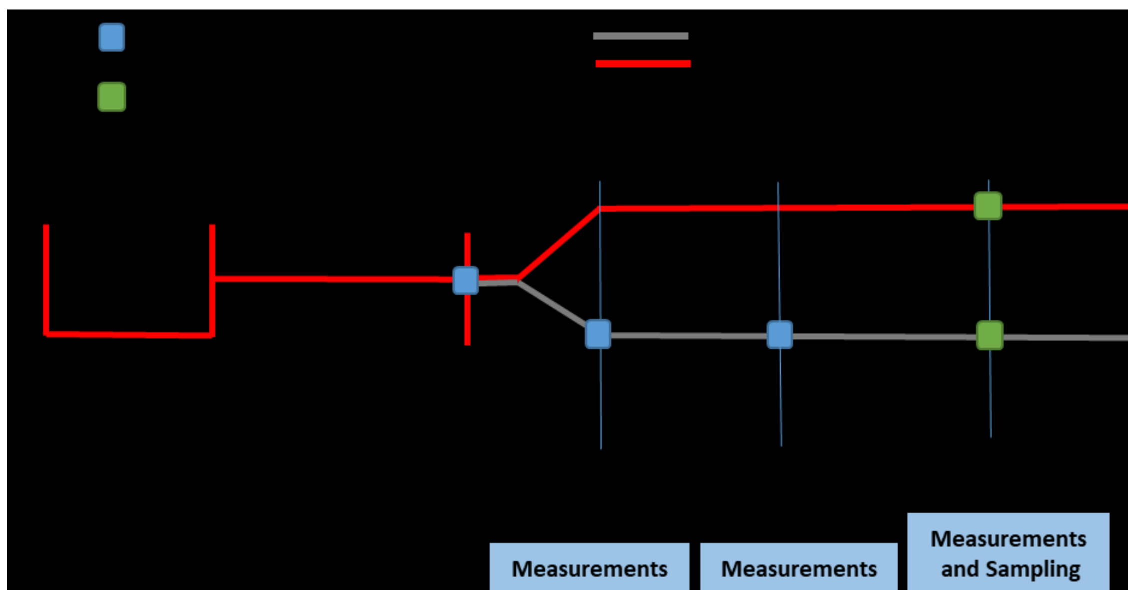


Fig. 22. Schematic representation of the pot assay experiment.

11.2.10 Nitrogen Content and Nitrogen Use Efficiency

Total nitrogen content (Nc, mg N) was determined by combustion method through a LECO-CNS-1000 analyzer (LECO Instruments Ltd., Mississauga, ON) as reported by Lupini et al. (2017). Nitrogen Use Efficiency (NUE, SDW N%$^{-1}$, where N% is the g N (100 g DW)$^{-1}$) (Chardon et al., 2010) and Nitrogen Utilization Efficiency (NUtE, SDW2 Nc^{-1}) (Siddiqi and Glass, 1981) were calculated. Nitrogen Uptake Efficiency (NUpE) was also estimated as total (shoot + root) dry weight (TDW) x N concentration (g N g TDW^{-1}) (Chardon et al., 2010).

11.2.11 Plate on plate assay

Tomato seeds were surface sterilized with a 30% (v/v) bleach + 0.02% (v/v) Triton X-100 solution for 10 minutes, and then washed with sterile Milli-Q water 3 times, for 3 minutes. Seeds were placed in the dark at 24°C for two days, on square Petri dishes containing half-strength Murashige and Skoog medium (½MS) including vitamins (Duchefa Biochemie), 0.1% (v/w) 2-(N-morpholino)ethanesulfonic acid monohydrate (MES)(Duchefa Biochemie), and 10 g L^{-1} plant agar (Duchefa Biochemie), pH was adjusted to 5.8. New small round Petri dishes (35mm) containing trypton soy agar (TSA) were prepared and placed in the middle of one side, in new square Petri dishes (120x120mm) filled with the same growth media as above. To impose drought stress

150mM sorbitol was added to the growth medium. After germination, two seedlings were transferred to each plate-on-plate system, placing them on the opposite side of the small Petri dishes, as reported in the next image. Bacterial treatment was carried out in two ways: 1) inoculum of 100 µL containing 10^7 CFU culture of *S. violaceoruber* into the small Petri dishes; 2) dipping of the germinated seeds with radicles produced in a solution containing 10^7 CFU/ml culture of *S. violaceoruber*. TSA medium was used as the control. Plates were then placed in a growth chamber in 70° angle-racks, at constant temperature of 24°C and 16h photoperiod.

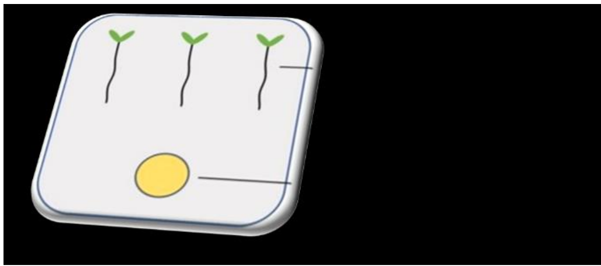


Fig. 23 Plate on plate system

Before the experiment, strains, stored at -80°C, were first transferred on solid trypton soy broth (TSB) medium, supplemented with 50 mg/ml Rifampicin, at 28°C for 72h. Biomass was then harvested by scraping it off from the growth medium with an inoculation loop and washed twice with 10 mM $MgSO_4$ by centrifugation (5 min. at 4,000g). Lastly, the cells were resuspended in 10 mM $MgSO_4$, after which the strain suspensions could be used for the experiment.

After seven days and fourteen days from transfer, photo scans of seedlings were obtained. The PRL and NLR were measured using the Rootnav software. At the end of the experiment, seedlings were harvested and fresh and dry weight of shoot, root and total plants were determined per plate.

The experiment included six conditions: ½ MS (control); ½ MS + Sorbitol 100mM; ½ MS + *S.violaceoruber*-I (Inoculum); ½ MS + Sorbitol 100mM + *S.violaceoruber*-I; ½ MS + *S.violaceoruber*-D (Dipping); ½ MS + Sorbitol 100mM + *S.violaceoruber* D.

11.2.12 Statistical analysis

The hydroponic experiments were set up in a completely randomized experimental design with at least three replications (six plants per replica) adopted for each experiments. Normality (Kolmogorov-Smirnov test) and homogeneity of variance (Levene test) for each dataset were assessed. The statistical significance of the differences between means was determined by a two-way ANOVA (PB treatment and nitrate as main factors), and

the means were separated by the Tukey's Honest Significant Difference (HSD) test, using Minitab 17 statistical software and Past 4.03 statistical software. The significance was set at $p < 0.05$.

Pot experiment was set up as completely randomized blocks with six replicates for each condition. After checking for normality and homogeneity of variance, the data were analyzed by two-way ANOVA (bacterial treatment as main factor), and means separated by Tukey's honest significant difference (HSD) test ($p < 0.05$). Data were analyzed by two-way ANOVA based on three biological replicates for each treatment by using Minitab 17 statistical software.

Plate-on-plate assay was carried out as completely randomized blocks with five replicates (2 plants per replica) for each treatment condition. The data have been checked for normality and homogeneity of variance and analyzed by two-way ANOVA (bacterial inoculum, dipping of seeds and drought stress as main factors), and means separated by Tukey's honest significant difference (HSD) test ($p < 0.05$). Data were analyzed by two-way ANOVA based on three biological replicates for each treatment by using Minitab 17 statistical software.

11.3 Results

11.3.1 Hydroponic assay results

11.3.1.1 Curve-dose plant response

In order to identify the optimal dose of PGPB able to mitigate N stress, tomato plants at the fourth true leaf stage were treated with different doses of bacteria 0, 5, 10, 20, 40 ml (Ctrl, T5, T10; T20, T40, respectively) and low nitrate (LN, 0.5 mM NO_3^-) for one week. Figure 25 shows the most significant morphological data of the PGPB-treated plants exposed to LN stress. All the doses, except for T20, resulted in a significant increase, about 20 %, of root fresh weight compared to the control (Fig.25 A). The root diameter was significantly increased at all PGPB concentrations by about 20% on average compared to the control (Fig.25 B). A large increase of about 77% was observed in the surface area of primary root at all the doses, except for the T20. Interestingly, the lateral roots number which showed a positive trend compared to the control at all doses applied, significantly increasing, by about 49%, at the T10 dose only (Fig.25 D).

Fig 24. Tomato plants grown hydroponically untreated (CTRL) and treated with 5 (T5), 10 (T10), 20 (T20) and 40 (T40) ml PGPB and low nitrogen (LN).

A
Fresh Weight Root (g)
0,2000 0,1500 0,1000 0,0500 0,0000
B A A AB A
Ctrl T5 T10 T20 T40
Treatments

B
Total Root diameter (mm)
0,500 0,400 0,300 0,200 0,100 0,000
B A A A A
Ctrl T5 T10 T20 T40
Trattamento

C
Surface Area(cm^2) primary root
30,000 25,000 20,000 15,000 10,000 5,000 0,000
B A A AB A
Ctrl T5 T10 T20 T40
Treatment

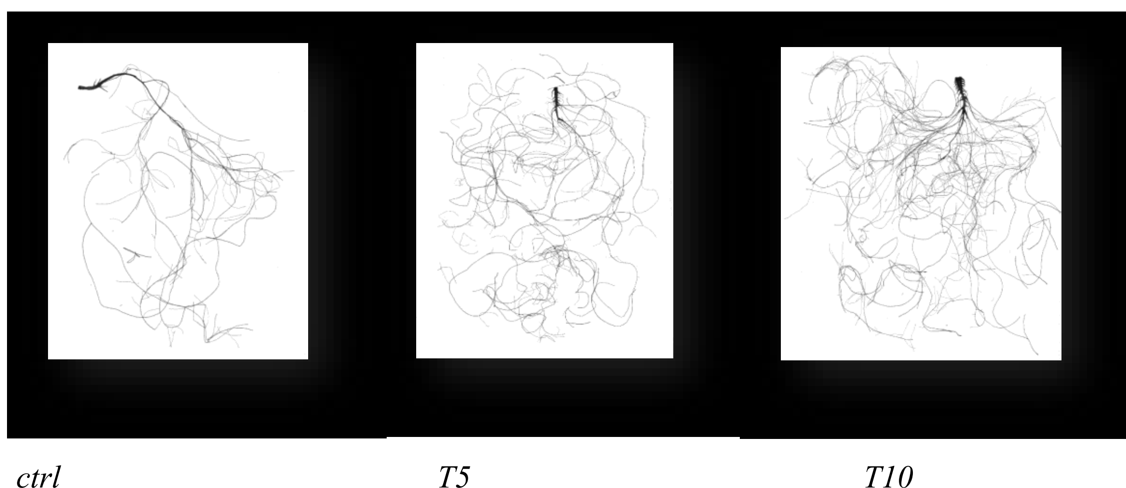
D
Num. Lateral Roots
50,0 40,0 30,0 20,0 10,0 0,0
B AB A AB AB
Ctrl T5 T10 T20 T40
Treatments

E
SPAD
40,0 30,0 20,0 10,0 0,0
B AB A AB AB
Ctrl T5 T10 T20 T40
Treatments

Fig 25. Effect of PGPB on fresh root weight (A), total root diameter (B), primary root surface area (C), lateral root number (D) and SPAD index (E) of tomato seedlings treated with increasing PGPB doses (5, 10, 20 and 40 ml) of a *Streptomyces violaceoruber* mother culture and exposed to low NO_3^- (0.5 mM, LN) for 7 days**.** The values are means ± SE (n = x). Different letters indicate means that significantly differ according to Tukey's HSD test at P < 0.05. N=3.

Finally, a trend of increase in the SPAD index was also observed after PGPB treatment, but only at the T10 dose, which showed a significant increase compared to the control (Fig.25 E).

On Fig.26 the root morphology of treated plants with T5 and T10 PGPB is shown, confirming their stimulatory effect.

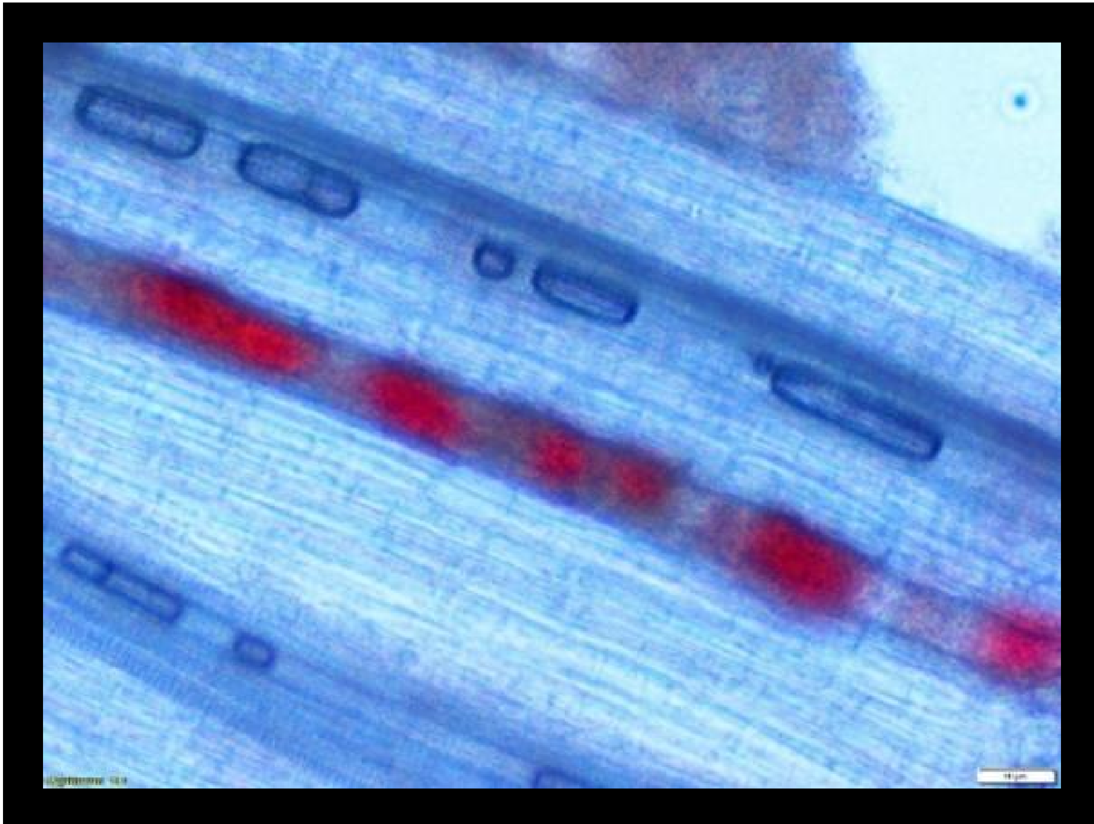


ctrl *T5* *T10*

Fig 26. Root systems of tomato plants grown hydroponically untreated (CTRL) and treated with 5 (T5) and 10 (T10) ml PGPB and low nitrogen (LN). Images were scanned and analyzed using WinRHIZO.

11.3.1.2 Root system colonization

Fig 27 clearly demonstrates that *S.violaceoruber* was able to colonize the tomato root system (Fig.27 top) thus confirming its interaction with the seedlings and consequently its potential effect on growth even in a hydroponic system. Glomerular structures typical of *S. violaceoruber*, exhibit pinkish-purple staining due to the production of actinorhodin compounds (Fig.27 bottom).



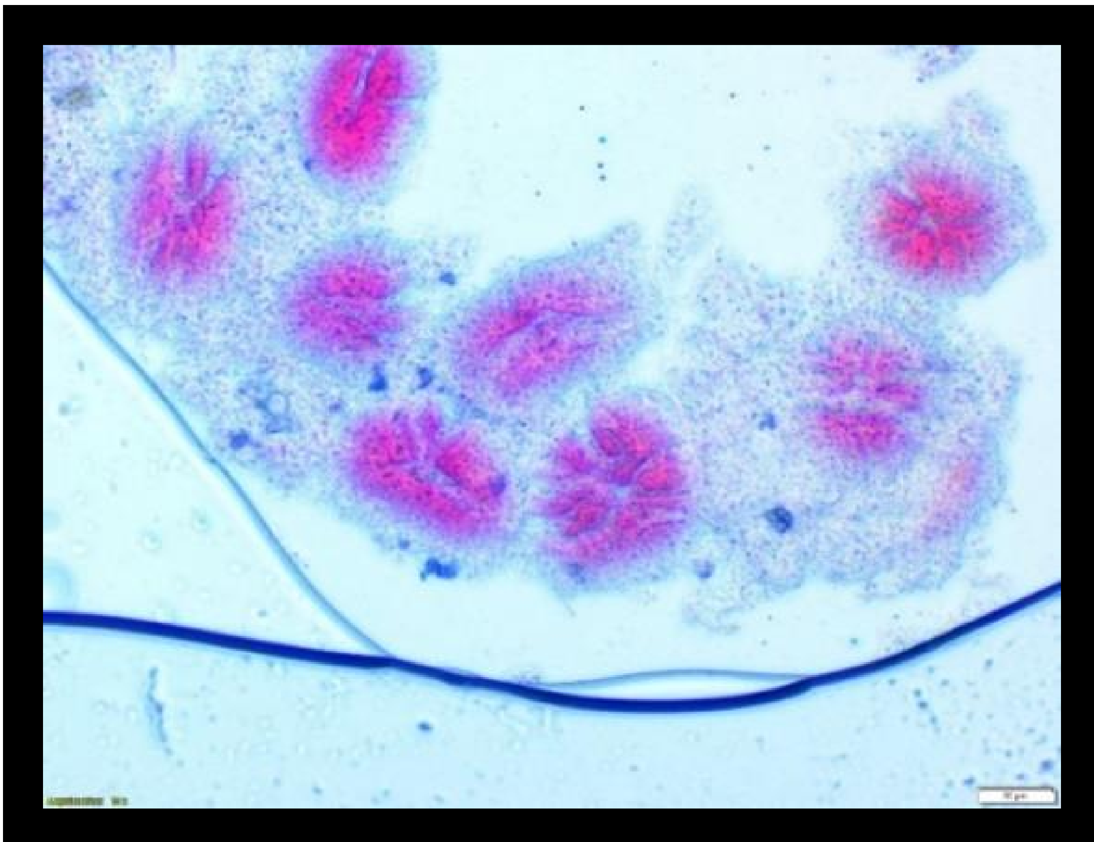
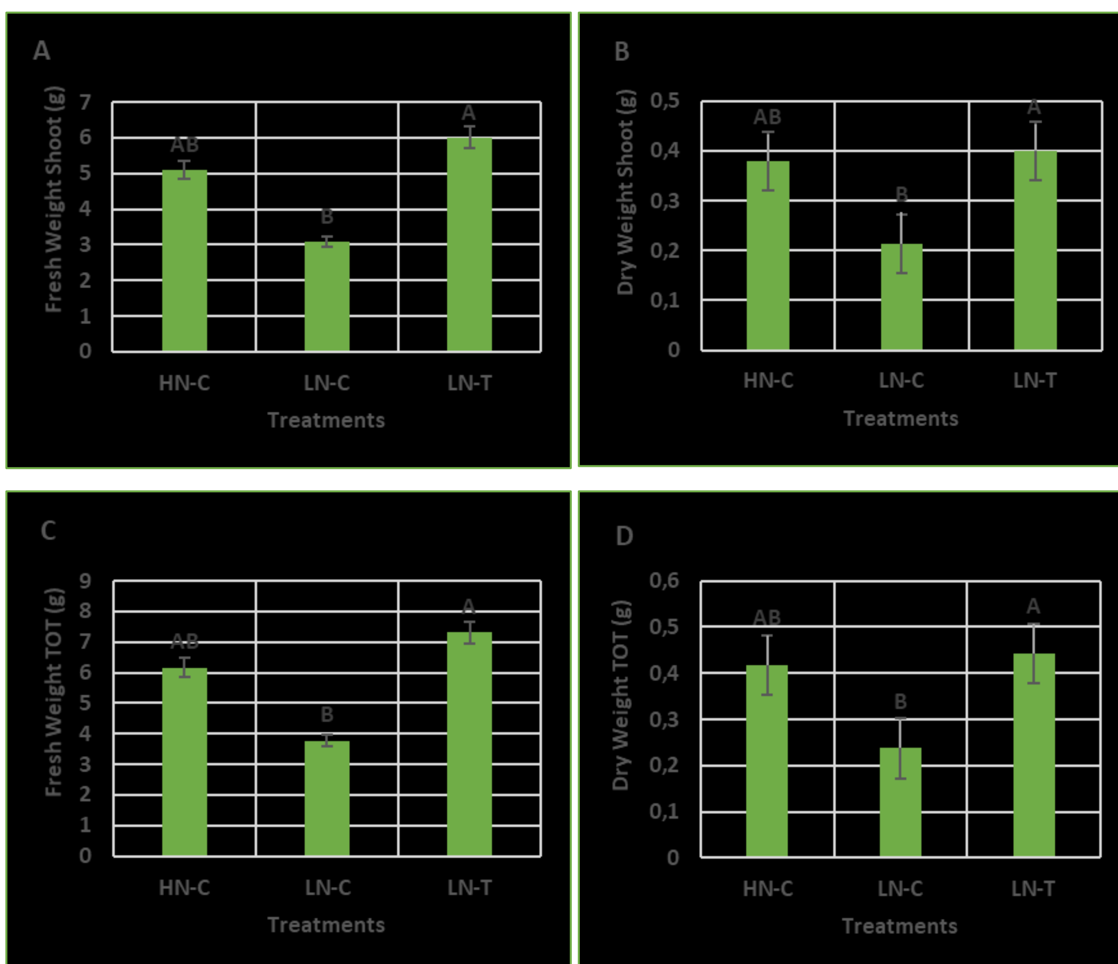


Fig. 27 Root system staining shows the ability of *S.violaceoruber* to colonize the roots. Optical microscope; magnification 10x, bar 50µm.

11.3.1.3 Effective PGPB dose effects plant growth under low and high N condition

Once the T10 has been identified as the most effective dose under LN, we performed a second trial aimed at understanding the PGPB ability to restore the N stressed plants to the optimal nutritional condition (high nitrogen, HN). For this purpose, tomato plants were grown at low (LN, 0,5 mM NO_3^-) and high N (HN, 10 mM NO_3^-) and exposed to T10 (10 ml of bacterial culture containing 3% (v/v) of packed mycelium) in the same conditions reported before. Interestingly, the results showed that the PGPB treatment not only significantly increases all the morphological parameters compared to the LN control condition, but also significantly restores them to the optimal nutritional condition (HN) (Fig.28). In detail, the treatment with PGPB significantly increased fresh and dry shoot weight (A and B), the increase compared to low nitrogen, for both parameters, was by 48%. Similarly, the results showed an increase in fresh and dry total weight (C and D), as well as a significant increase in the SPAD index (E).



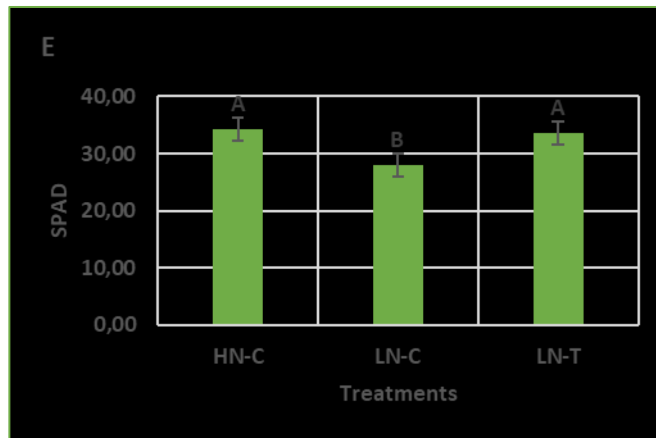


Fig. 28 Effect of PGPB on fresh and dry shoot weight (A, B), total fresh and dry weight (C, D) and SPAD index (E) of tomato seedlings treated with T10 dose and exposed to LN (0.5 mM NO_3^-, LN-T), HN (10 mM NO_3^-;HN-C) and control (LN-C) for 7 days**.** The values are means ± SE (n = x). Different letters indicate means that are significantly different, according to Tukey's HSD test at $P < 0.05$. N=6

Tomato plants exposed to T10 dose PGPB showed also an increase in normalized difference vegetation index (NDVI) and normalized anthocyanin index (NAI) (Fig.29 A and B), with an increase by about 6% in NDVI and a decrease of NAI by about 45%.

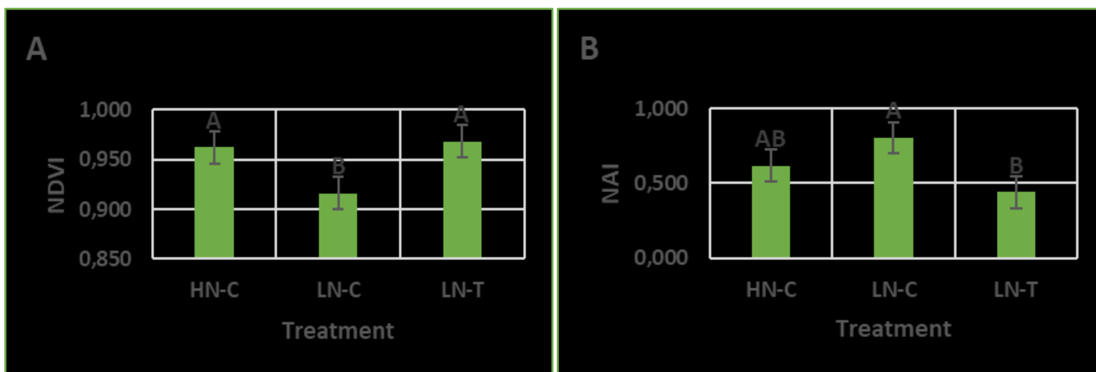


Fig. 29 Effect of PGPB on normalized difference vegetation index (A) and normalized anthocyanin index (B) of tomato seedlings treated with T10 dose and exposed to LN (0.5 mM NO_3^-, LN-T), HN (10 mM NO_3^-;HN-C) and control (LN-C) for 7 days**.** The values are means ± SE (n = x). Different letters indicate means that significantly differ according to Tukey's HSD test at $P < 0.05$. N=6

Furthermore, the WinRhizo analysis of primary roots confirmed the effect of T10 dose. As shown on Fig. 30, the primary root increment in both length (A) and surface area (B) was observed by about 50% and 90%, respectively and not significantly different from HN-C. The primary diameter values were not statistically significant, although they showed a slight increase by about of 16%, while the primary root volume were highly increased by PGPB treatment, with a 82% of increase.

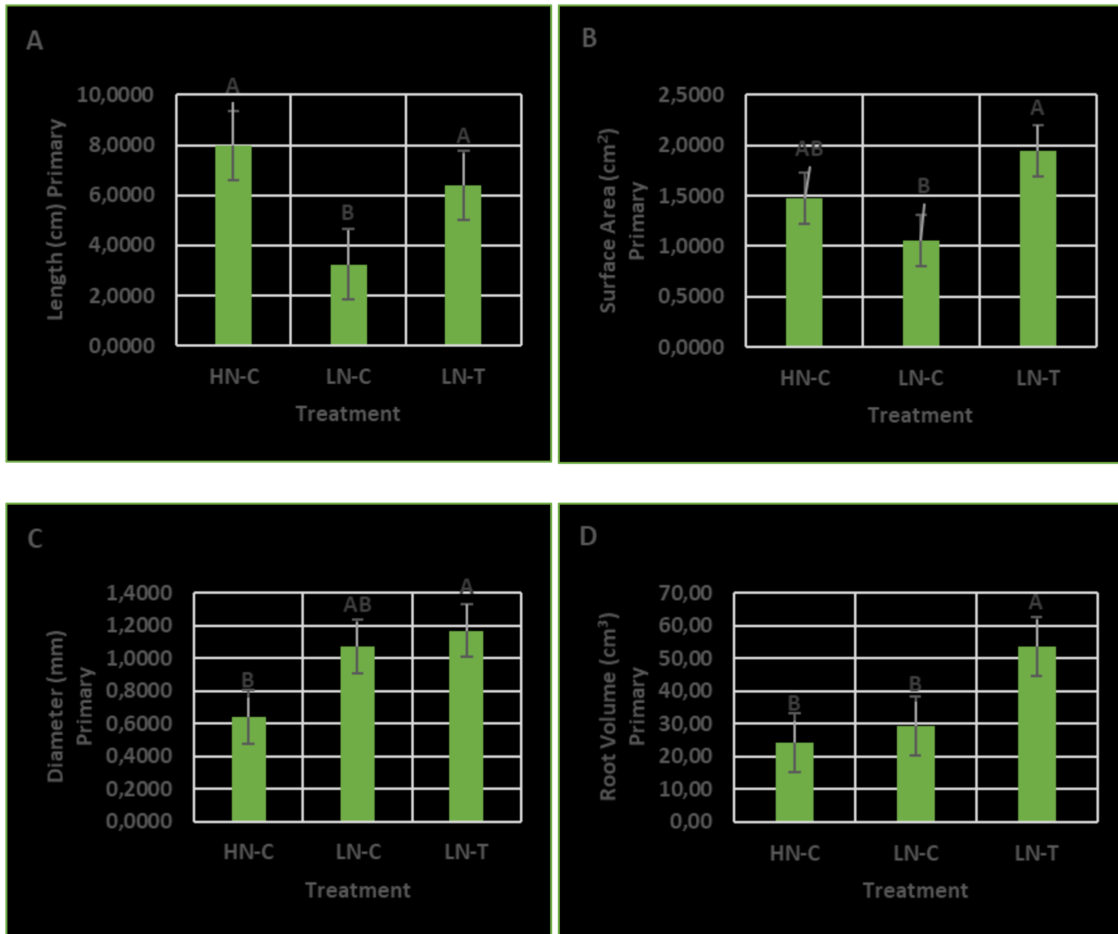


Fig. 30 Effect of PGPB on primary root length (A), surface area (B), diameter (C) and volume (D) of tomato seedlings treated with T10 dose and exposed to LN (0.5 mM NO_3^-, LN-T), HN (10 mM NO_3^-;HN-C) and control (LN-C), for 7 days**.** The values are means ± SE (n = x). Different letters indicate means that significantly differ according to Tukey's HSD test at P < 0.05. N=6

Afterwards, the PGPB effect on gas exchange parameters, such as net photosynthetic rate, maximal PSII efficiency for photochemistry (*F*v'/*F*m'), transpiration rate and stomatal conductance was evaluated. Plants treated with T10 dose (LN-T) showed a significant

increase in all these parameters compared to the LN-C plants, reaching values similar to HN-C plants (Fig.31 A, B, C and D).

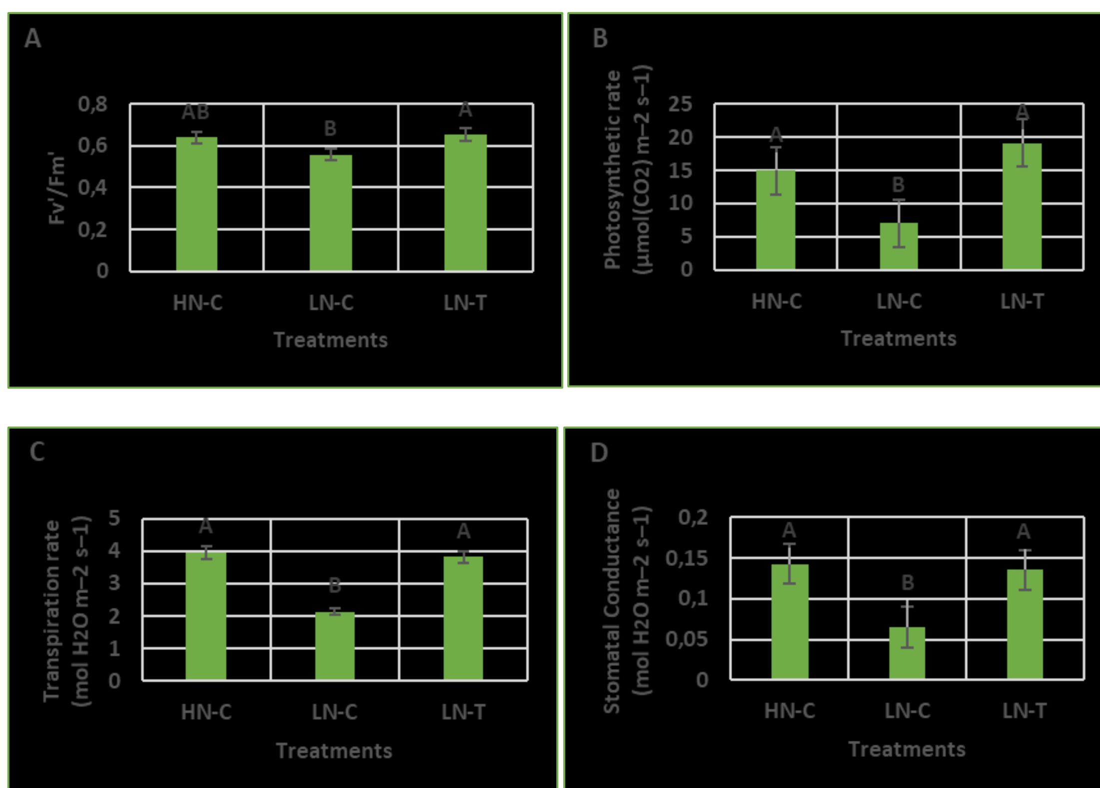


Fig. 31 Effect of PGPB on maximal PSII efficiency for photochemistry (Fv'/Fm') (A), net photosynthetic (B), transpiration rate (C) and stomatal conductance (E) of tomato seedlings treated with T10 dose and exposed to LN (0.5 mM NO_3^-, LN-T), HN (10 mM NO_3^-;HN-C) and control (LN-C), for 7 days**.** The values are means ± SE (n = x). Different letters indicate means that significantly differ according to Tukey's HSD test at $P < 0.05$. N=6.

11.3.2 Pot assay experiment

After evaluating the effectiveness of treatments in the hydroponic system, PGPB was applied on tomato plants grown in pots. The rhizosphere inoculation was repeated three times (after 24 h from transplanting, 30 and 45 DAT). Plants were collected after 60 DAT for the analyses. The inoculated plants (T) showed a significant increase in both root FW and DW, shoot DW and SPAD index compared to the uninoculated plants (C) (Fig. 32 A, B, C, D and E), with an increase of 50%, 82% and 29,4%, respectively. Conversely, although shoot FW increased by about of 65%, it not was statistically significant compared to the control plants (C).

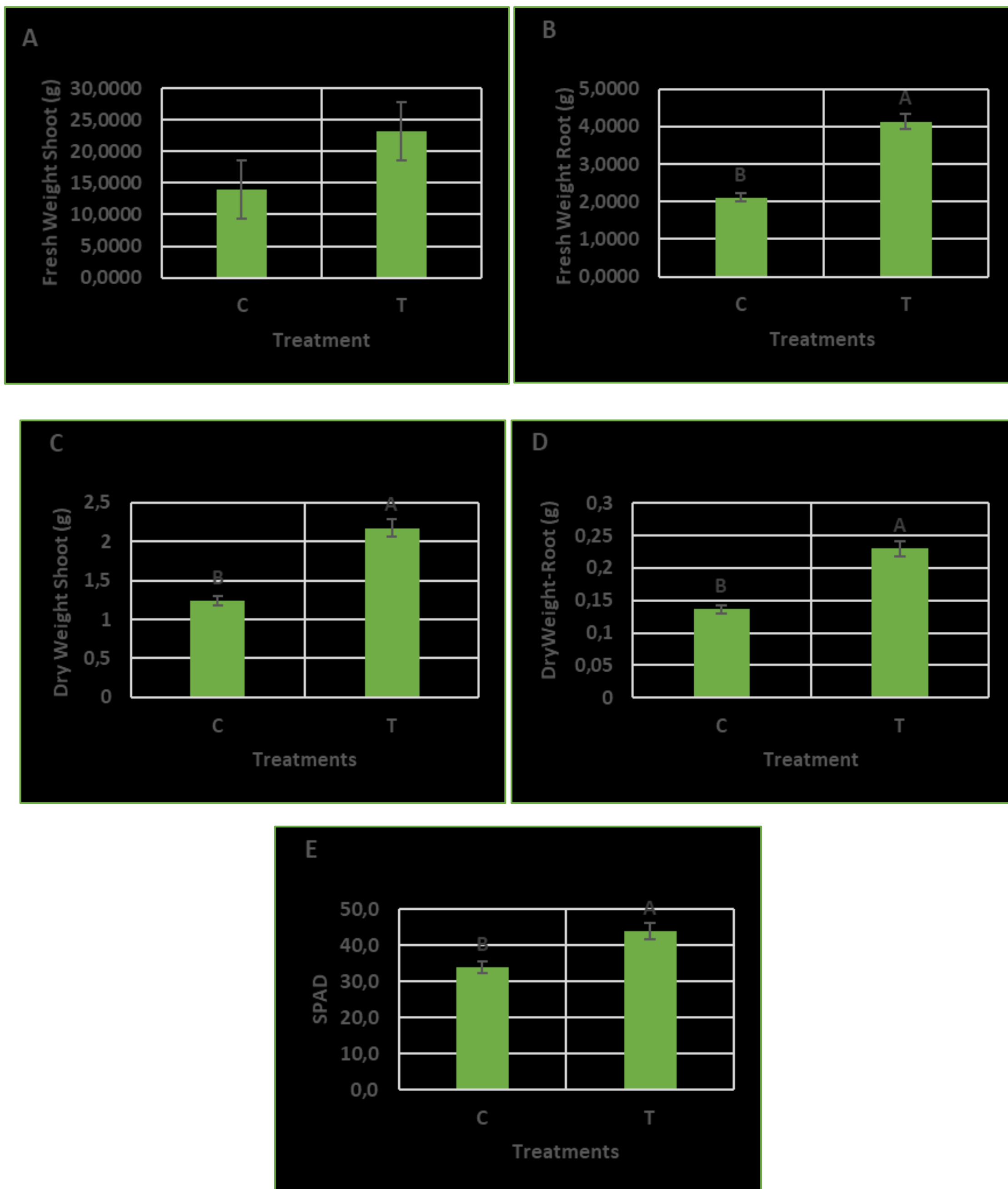


Fig. 32 The PGPB effects on shoot and root fresh weight (A, B), shoot and root dry weight (C, D) and SPAD (E) in tomato plants treated with PGPB (T) compared with untreated (C) grown in pots, for 60 days. The values are means ± SE (n = x). Different letters indicate means that significantly differ according to Tukey's HSD test at $P < 0.05$. N=6.

Furthermore, the PGPB effect on gas exchange parameters, such as net photosynthetic rate, transpiration rate and stomatal conductance of tomato plants was evaluated (Fig. 33

A, B and C). The T plants showed a significant increase in net photosynthesis, stomatal conductance and transpiration rate compared to the C ones (Fig 33 A, B, C)

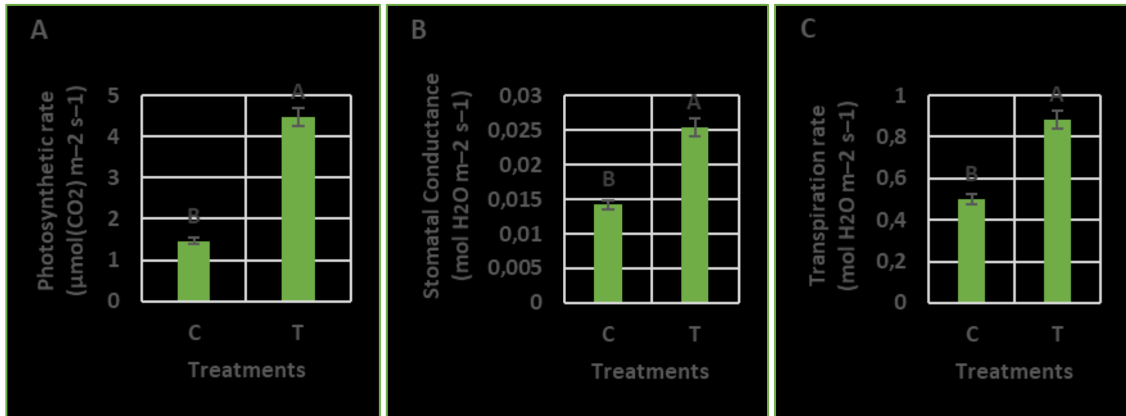
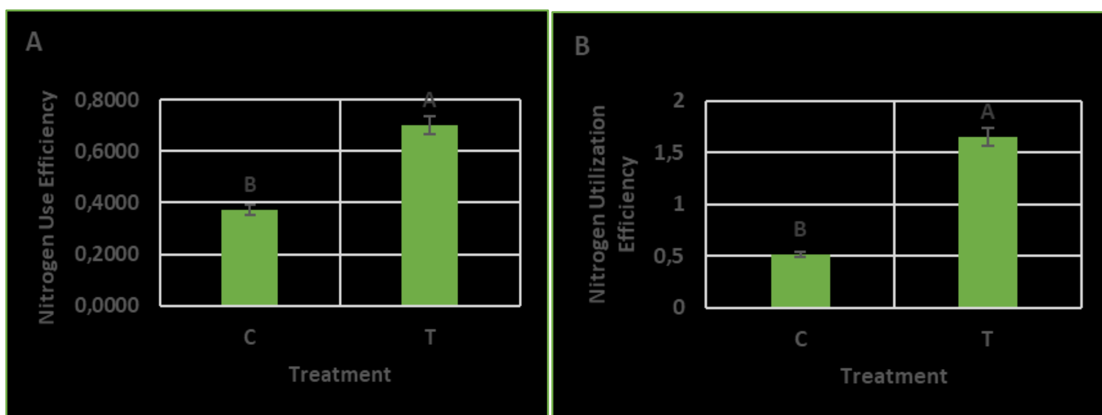


Fig. 33 PGPB effects on net photosynthetic rate (A), stomatal conductance (B) and transpiration rate (C) of tomato plants treated with PGPB (T) compared with untreated (C) grown in pots, for 60 days. The values are means ± SE (n = x). Different letters indicate means that significantly differ according to Tukey's HSD test at $P < 0.05$. N=6.

Afterwards, the effect of PGPB on NUE and its components, NUtE and NUpE (Fig. 34) was evaluated. Plants treated (T) showed a significant increase in both NUE and NUtE parameters compared to the C plants, with an increment of 87% and 220%, respectively. By contrast, the NUpE value was not statistically significant, although an increase by about of 50% compared to the control plants (C) was observed.



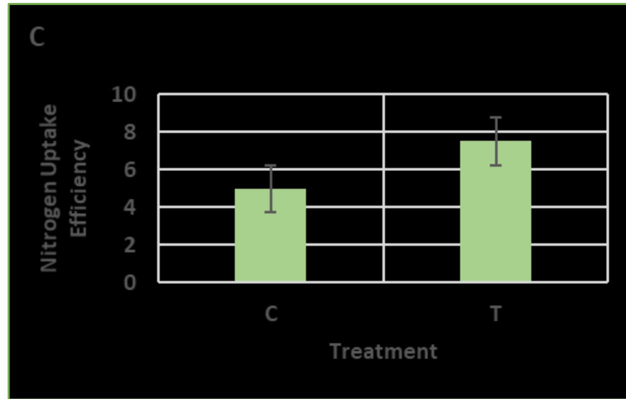


Fig. 34 PGPB effects on NUE (A), NUtE (B) and NUpE (C) of tomato plants grown in pots for 60 days were reported. Treated plants with PGPB (T) were compared with untreated plants (C). The values are means ± SE (n = x). Different letters indicate means that significantly differ according to Tukey's HSD test at $P < 0.05$. N=6.

Fig 35 shows the root morphology of treated plants with the inoculum of PGPB, confirming its stimulatory effect, compared to the control plants (CTRL).

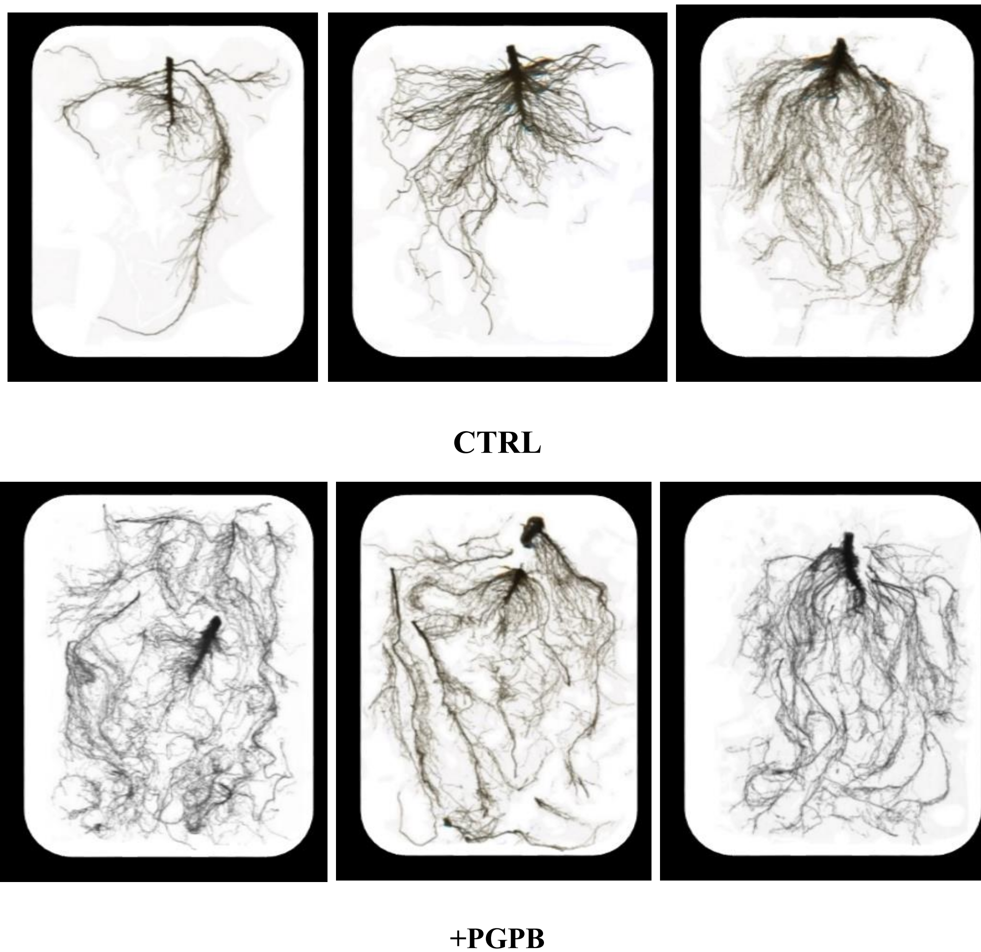


Fig. 35 Root systems of tomato plants grown in pots, untreated (CTRL) and treated (+PGPB) by inoculum of 500 μL containing 10^7 CFU/ml PGPB. Images scanned using WinRHIZO.

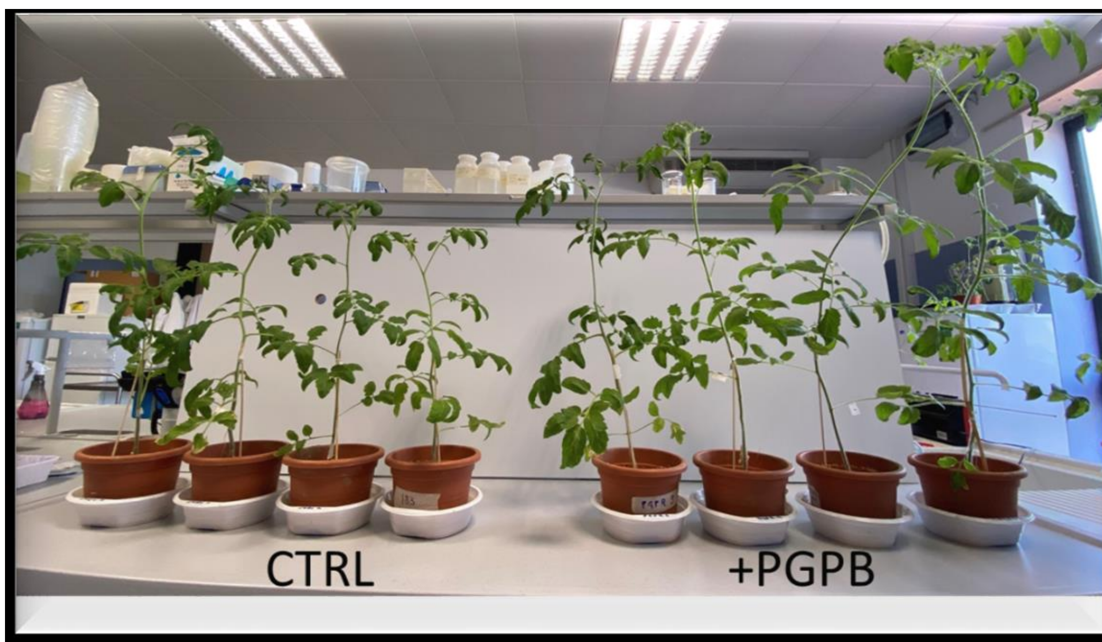
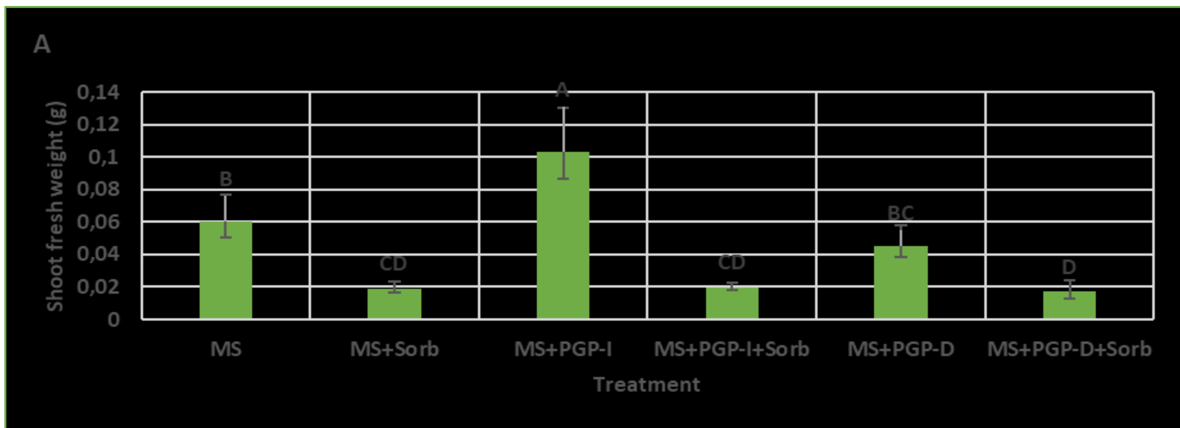
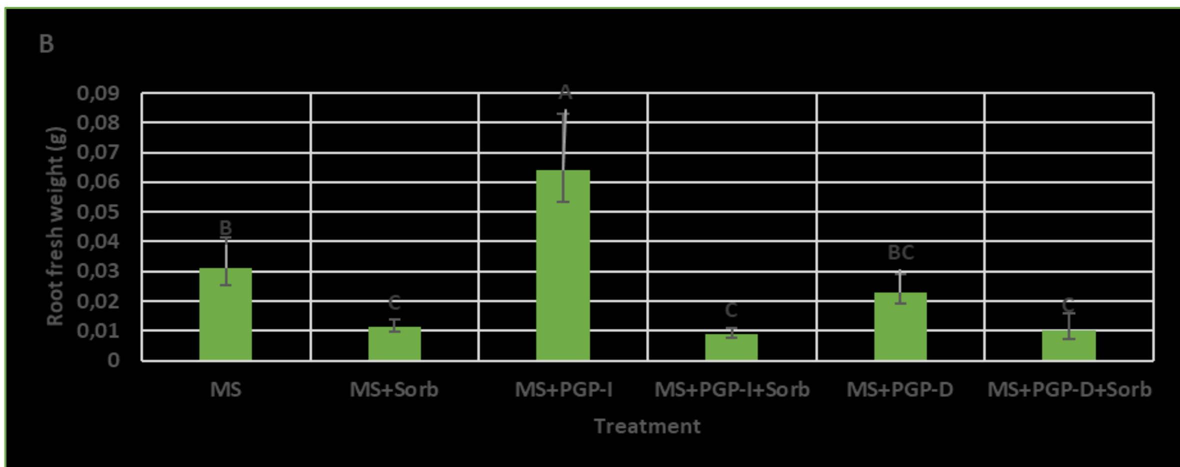


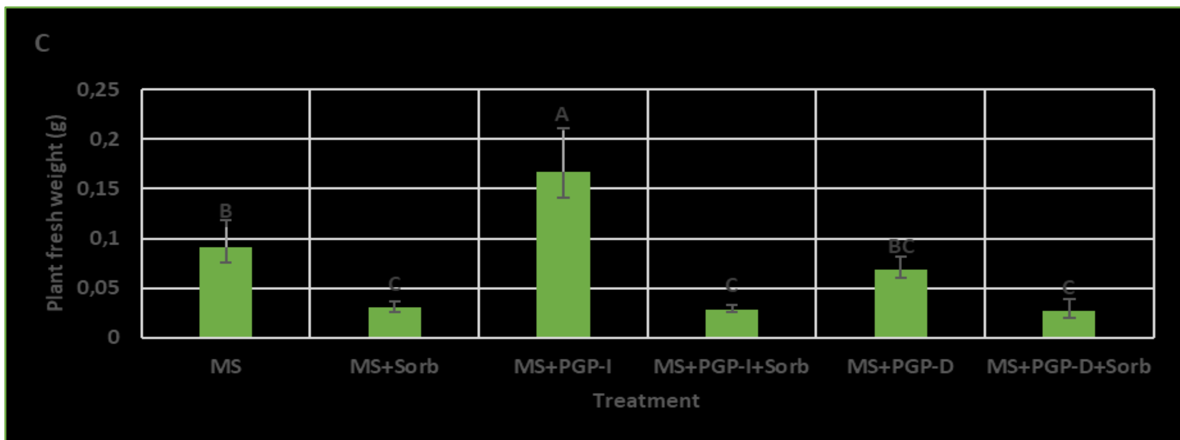
Fig. 36 Tomato plants grown in pot system, untreated (CTRL) and treated (+PGPB) by inoculum of 500 μL containing 10^7 CFU/ml PGPB.

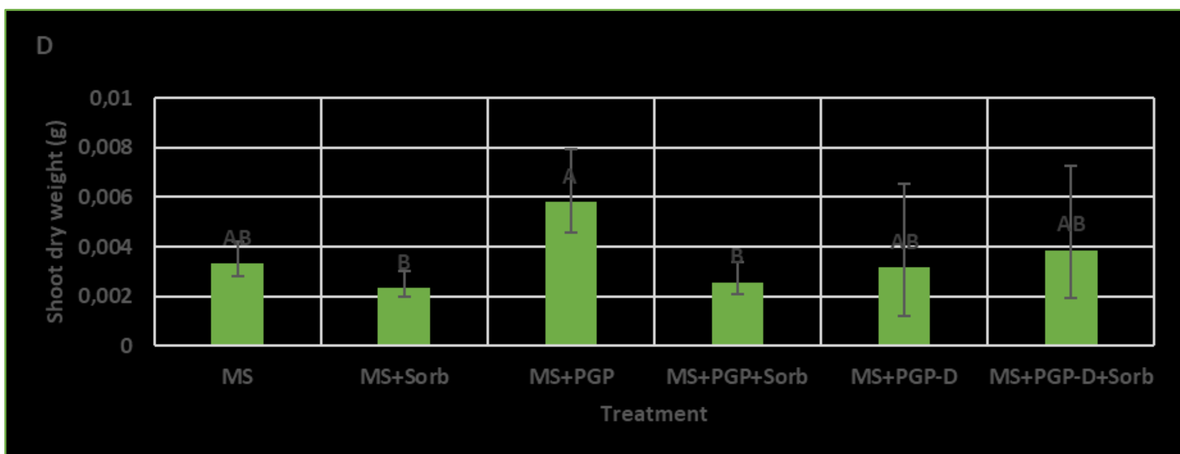
11.3.3 *In vitro* effect of PGPB on tomato seedlings

An *in vitro* assay was carried out to assets the effects of two different applications of the PGPB treatment, respectively inoculum and dipping of the radicles. The treatments were carried out in control and under drought stress conditions. The presence of the bacterial inoculum has proven capable of improving the growth performance of tomato seedlings in control condition, increasing FW of shoot, root and plant, as well as DW of root and plant (Fig.37 A, B, C, E and F), except for the shoot DW (Fig. D), not statistically significant, although increased by 75%. By contrast, the treatment by inoculum under drought stress showed no positive effect on seedling growth performance, which was similar to the values of the drought stress control in all morphological parameters analyzed. In addition, the treatment by dipping would appear to slightly worsen biomass levels of shoot, root and plant fresh weight (A, B and C) in control condition, with a decrease of ~33% for each parameters. Similarly, the treatment by dipping, in drought stress conditions, slightly decreased shoot FW (A), while it showed no effect on root and plant FW (B and C), as well as in root and plant DW (E and F). Interestingly, the treatment did show a slightly increase in shoot DW (D) of by 30%, although not statistically significant.


A
Shoot fresh weight (g)
0,14
0,12
0,1
0,08
0,06
0,04
0,02
0
B
CD
A
CD
BC
D
MS
MS+Sorb
MS+PGP-I
MS+PGP-I+Sorb
MS+PGP-D
MS+PGP-D+Sorb
Treatment


B
Root fresh weight (g)
0,09
0,08
0,07
0,06
0,05
0,04
0,03
0,02
0,01
0
B
C
A
C
BC
C
MS
MS+Sorb
MS+PGP-I
MS+PGP-I+Sorb
MS+PGP-D
MS+PGP-D+Sorb
Treatment


C
Plant fresh weight (g)
0,25
0,2
0,15
0,1
0,05
0
B
C
A
C
BC
C
MS
MS+Sorb
MS+PGP-I
MS+PGP-I+Sorb
MS+PGP-D
MS+PGP-D+Sorb
Treatment


D
Shoot dry weight (g)
0,01
0,008
0,006
0,004
0,002
0
AB
B
A
B
AB
AB
MS
MS+Sorb
MS+PGP
MS+PGP+Sorb
MS+PGP-D
MS+PGP-D+Sorb
Treatment

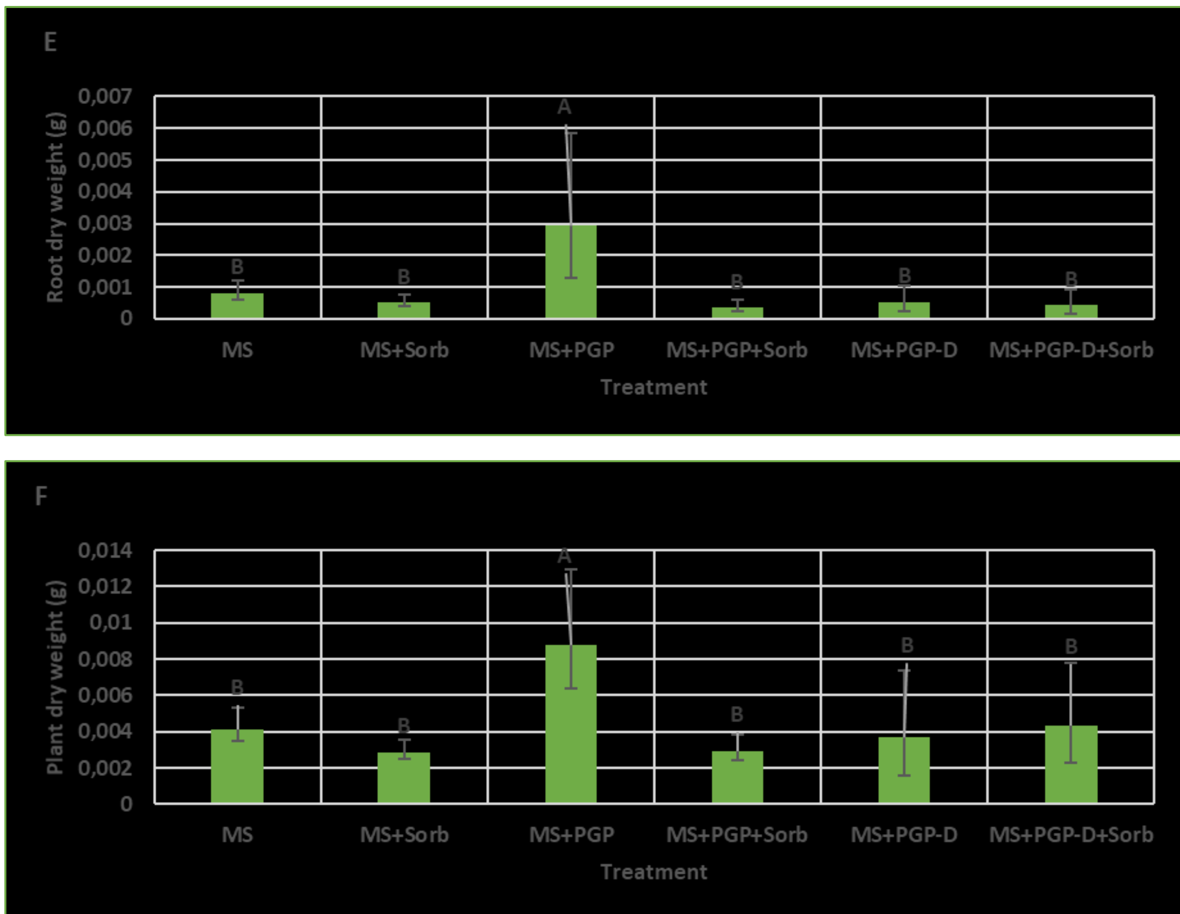
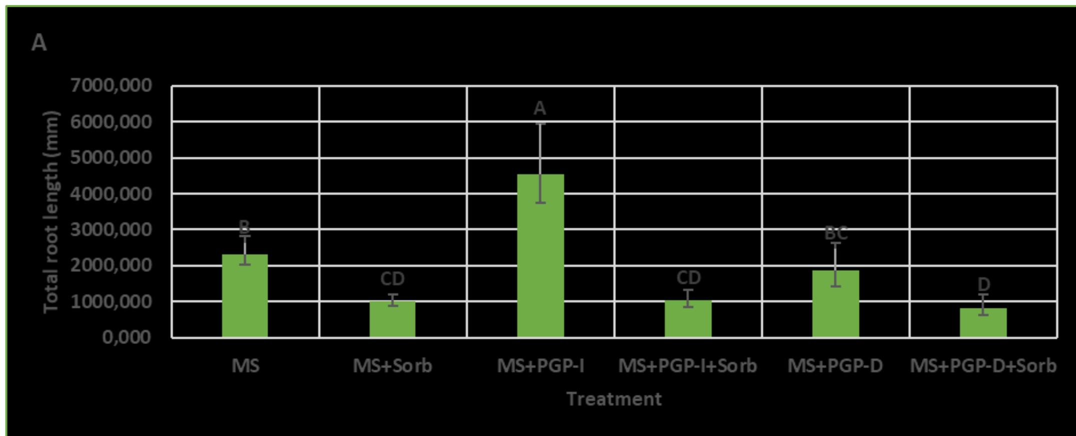
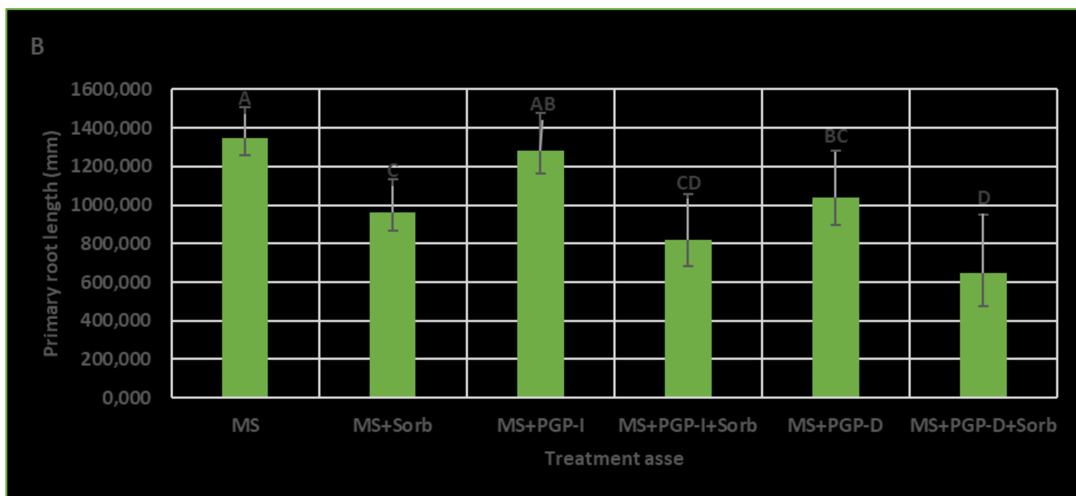


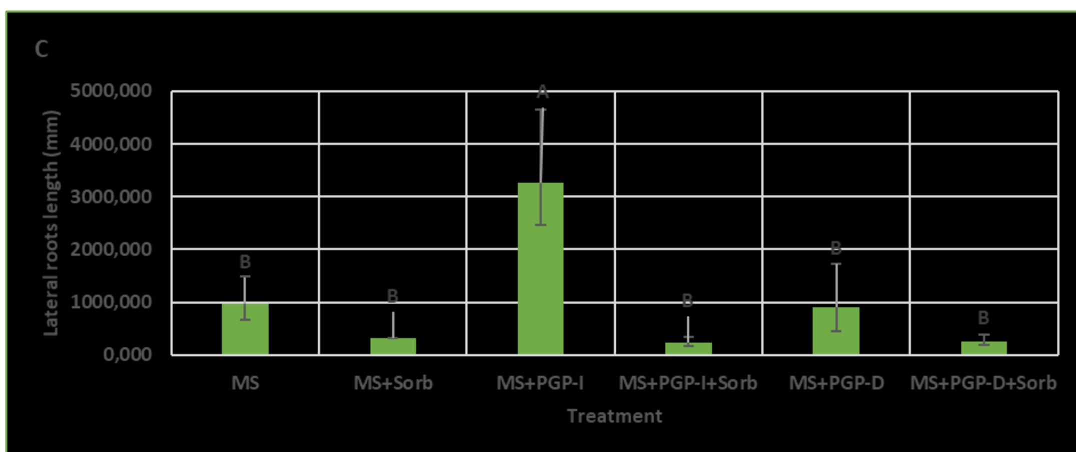
Fig. 37 Effect of the PGPB VOC's on shoot, root and plant fresh weight (A, B, C) and on shoot, root and plant dry weight (D, E, F) of tomato seedlings treated -on-plate growth system for 7 days**.** Control (MS), drought stress (MS+Sorb), bacterial treatment by inoculum (MS+PGP), bacterial treatment under drought stress (MS+PGP+Sorb), bacterial treatment by dipping (MS+PGP-D) and bacterial treatment by dipping under drought stress (MS+PGP-D+Sorb) were the different growth conditions. The values are means ± SE (n = x). Different letters indicate means that significantly differ according to Tukey's HSD test at $P < 0.05$. N=6.

Moreover, morphological investigation (Fig.38) of the root system showed the following results. The treatment by inoculum increased TRL by 96% (A), LRL by 239% (C) and LRN by 198% (D), values in control conditions, and no significant difference on PRL (B) value; in drought stress conditions, decreased significantly TRL (A) and primary root length (B), while no significant effect on LRL (C) and LRN (D). The treatment by dipping slightly decreased TRL (A) and significantly decreased PRL (B), while no effect were observed in LRL (C) and LRN (D); in drought stress conditions, dipping treatment

slightly increase LRN (D), TRL (A) and had no effect on PRL (B) and LRL (C), although no significant difference was observed.







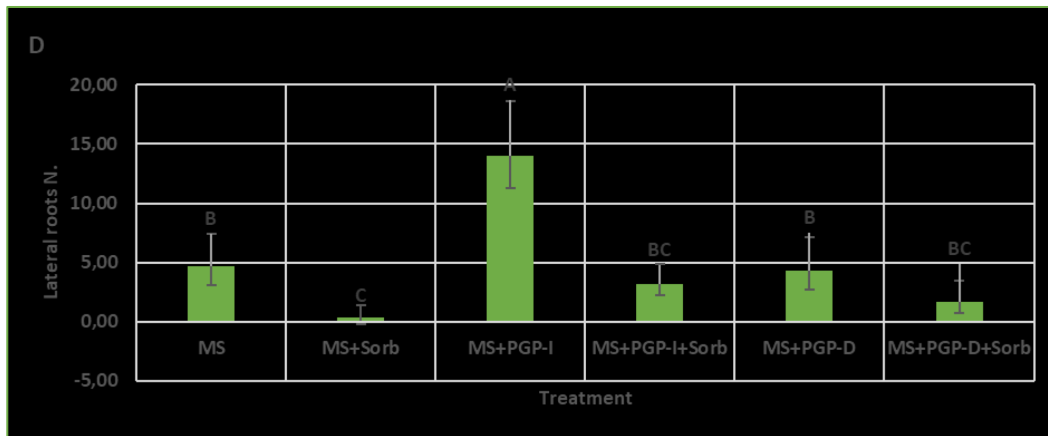


Fig. 38 Effect of the PGPB VOC's on total (A) primary (B) and lateral (C) root length and lateral roots number (D) of tomato seedlings treated in plate-on-plate growth system for 7 days**.** Control (MS), drought stress (MS+Sorb), bacterial treatment by inoculum (MS+PGP), bacterial treatment under drought stress (MS+PGP+Sorb), bacterial treatment by dipping (MS+PGP-D) and bacterial treatment by dipping under drought stress (MS+PGP-D+Sorb) were the different growth conditions. The values are means ± SE (n = x?). Different letters indicate means that significantly differ according to Tukey's HSD test at P < 0.05. N=6.

Next we tested if the VOCs of the bacteria can also induce plant growth promoting effect. As shown on Fig. 39 a clear differences in the morphology of the root system of the plants was observed under the effect of PGPB VOC's, in control conditions and under drought stress.

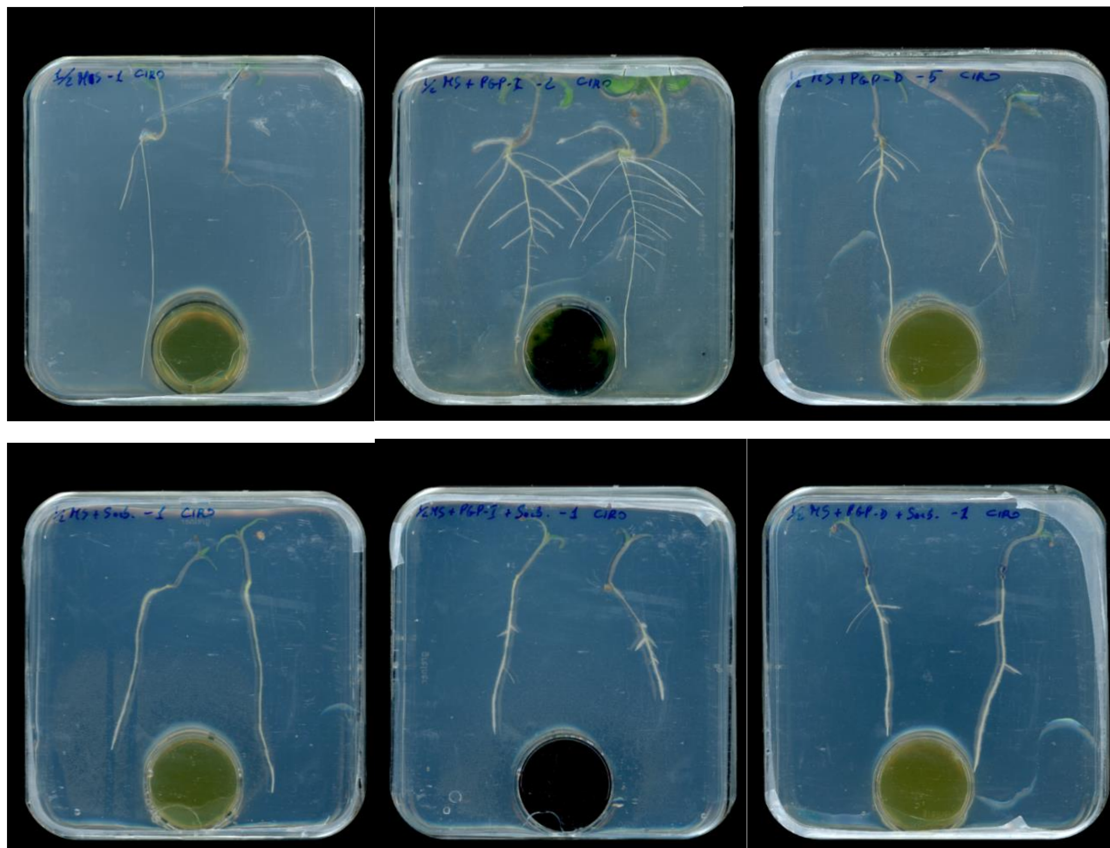


Fig. 39 Root system of under the effect of the PGPB VOC's in a plate-on-plate growth system after 7 days**.** Control (MS), drought stress (MS+Sorb), bacterial treatment by inoculum (MS+PGP), bacterial treatment under drought stress (MS+PGP+Sorb), bacterial treatment by dipping (MS+PGP-D) and bacterial treatment by dipping under drought stress (MS+PGP-D+Sorb).

11.4 Discussion

The use of beneficial microorganisms in agriculture is recently subjected to wide interest and debate (Camaille et al., 2021), as a promising alternative to chemical fertilizers, useful to increase crop yield in more sustainable cropping systems (Mahanty et al., 2017; Koskey et al., 2021). Applied to soils, seeds, and plant surfaces, they are able to colonize the rhizosphere and/or migrate into the inner plant tissues, where they improve soil physical-chemical and biological properties, plant growth and development, nutrient availability, contribute to mitigate abiotic and biotic stresses, resulting in higher crop yields (Yadav et al., 2020; Koskey et al., 2021; Olaniyan and Adetunji, 2021).

Among the different genera, *Streptomyces* spp are considered the most interesting plant growth-promoting bacteria (PGPB), as a promising tool to be used in agriculture, for their metabolic versatility, drought resistance, and bioactive metabolite production such as vitamins, antibiotics, plant growth regulators and enzymes (Manigundan et al., 2022; Nazari et al., 2023). In the genus *Streptomyces*, we identified *S. violaceoruber,* characterized for multiple PGPB traits, bioactive molecule production, including antibiotics and siderophores, and volatile organic compound (VOCs) emission with antimicrobial activity (Faddetta et al., 2023).

Here, the ability of *S. violaceoruber* to stimulate plant growth and to mitigate N and /or drought stress conditions in tomato, grown in different culture systems, such as hydroponics, pots and petri dishes, was evaluated. To date, *Streptomyces* spp. are well known to alleviate abiotic stress, such as salinity, drought, and contaminants, which reduced crop growth and yields (Nazari et al., 2022, 2023).

For the first time, the ability of this strain to alleviate N stress condition in tomato plants grown under limited N supply (LN), in hydroponic system, and to restore their morpho-physiological parameters to the high N conditions (HN) was demonstrated. In particular, *S. violaceoruber* alleviated NO_3^- limitation, the predominant source of N supply to plants and an important signal for several developmental processes (Asim et al., 2020). The *S. violaceoruber* positive effect was observed on root traits by the increased biomass,

diameter, volume and surface area, number of lateral roots compared to both controls (LN and HN). The ability to modulate root traits has been recently proposed as a crucial criterion for the selection of potential PGPB strains to be used in agriculture (Grover et al., 2021). The red pigment along the tomato roots, due to the production of the bioactive mycelial compound, undecylprodigiosin, together with the presence of the hyphal glomeruli, constituting the actinomycete colonies, confirmed the root-*S. violaceoruber* colonization. This process is essential for enhancing PGPB activity to improve root biomass as well as tolerance to biotic and abiotic stress, as reported in other successful host-microbe interaction (Berendsen et al., 2012; Gopalakrishnan et al, 2015). Once the colonization occurred, *S. violaceoruber* stimulated root growth and development, helping tomato plants to overcome NO_3^- limitation. This effect could be due to its ability to produce and secrete IAA (Faddetta et al., 2023), which integrates the information conserning the nitrate status inside and outside the plant to reasonably distribute resources and sustainably construct the plant root system (Hu et al., 2021). The *Streptomyces* genus is a leader in producing indole-3-acetic acid (IAA), via the indole-3-acetamide pathway from L-tryptophan (Fitriani et al., 2022; Keyeo et al., 2011). In addition, IAA secretion in the growth media can also facilitate the root exudates production that benefit root growth by relaxing its cell wall and, according to the developmental stages and metabolism, to adapt to the adverse conditions (Pang et al., 2022). In particular, IAA produced by *S. violaceoruber* could be also responsible for the higher lateral roots number observed in treated plants, contributing to a more efficient root system to take up nutrients and water from the soil (Tsegaye et al. 2022, Hungria et al. 2021). However, the root growth stimulation could also be due to the secondary metabolites produced by *S. violaceoruber*, such as prodiginines, antibiotics and tryptophan related to IAA formation, as well as the kynurenic acid that plays a role in ethylene/auxin balance, previously identified with metabolomics (Faddetta et al., 2023). Several *Streptomyces* strains promote plant growth and biocontrol pests, diseases, weeds and phytopathogenic microorganisms by producing siderophores, enzymes, volatile organic compound (VOCs), antibiotics, and other secondary metabolites (Nazari et al., 2023).

The positive effect of *S. violaceoruber* application was also evident on the areal part of tomato plants, by the increased SPAD, NDVI, and the decreased NAI indexes along with all the physiological parameters correlated to photosynthesis and transpiration processes. Since large amount of leaf N content was localized in chlorophyll (Chl), a significant relationship between leaf N and leaf Chl contents, which is determined by N availability,

was demonstrated (Han et al., 2001). Therefore, the SPAD and NDVI indexes could provide an estimate of N and health plant status. Our results underlined that *S. violaceoruber* treatment, increasing the relative leaf chlorophyll content (SPAD) and seedling greenness (NDVI), maintained a good nutritional status and health of tomato plants under LN limitation. These effects were also supported by the low NAI index, an indicator of the plant stress severity, observed in *S. violaceoruber* treated plants. The NAI index measures the anthocyanin accumulation (Askey et al., 2019), which is positively correlated with different stresses, including high-intensity light, pathogens, wounding, drought, and nutrient deficiency (Shan et al., 2009). Therefore, the low NAI values confirmed the *S. violaceoruber* effectiveness in protecting plant under LN condition and improving its growth performance. These results were also accompanied by an increase of the net photosynthetic rate and maximal PSII efficiency for photochemistry (*F*v'/*F*m') in treated plants. Under stress condition, the PGPB-root symbiosis mitigates the negative stress effect by counteracting the degradation of photosynthetic pigments and increasing the maximum quantum yield of photosystem II (Duc et al., 2018). Several studies sustained that PGPB-root colonization by P-solubilizer strains, such as *S. violaceoruber,* increased chlorophyll concentrations, gas exchange parameters (Liu et al., 2020; Borowiak et al., 2021), carotenoid synthesis (Vafadar et al., 2014; Chen et al., 2017), and resistance to biotic and abiotic stress (Vafadar et al., 2014) by protecting the photosynthetic apparatus, reducing photodamage and photoinhibition effects (Uarrota et al., 2018). These P-solubilizer microorganisms can also influence some growth-associated compounds, such as cytokinins (Abbamondi et al., 2016; Kudoyarova et al., 2017), which have a positive effect on chlorophyll biosynthesis, delaying senescence, and programmed cell death processes (Zhang et al., 2021). Furthermore, the high stomatal conductance in *S. violaceoruber* treated plants, under LN, allows a higher CO_2 uptake during photosynthetic processes, although at the expense of a high transpiration rate.

In conclusion, these results demonstrated the ability of *S. violaceoruber* to stimulate tomato plant growth and mitigate N stress. This ability may justify its future use as bio-fertilizer, allowing for reduction of chemical N fertilizer application, especially on tomato that needs high N inputs. However, to better understand the pathways and genes involved in N stress-*S. violaceoruber* interaction and the signals/metabolites intercepted and/or intersected these pathways, both metabolomics and transcriptomics analyses have been carried out.

The *S. violaceoruber* efficacy was also evaluated in tomato seedlings grown in pots under optimal growth conditions. Here, we applied a soil inoculation, introducing the strain directly into the soil, by drenching, added as close as possible to the host roots, as previously reported (Romeiro, 2007; Lopes et al., 2018). This application was useful for PGPB to perform several critical functions for promoting plant development, such as phosphate solubilization, siderophore and phytohormone synthesis, in the rhizosphere (Gouda et al., 2018). At the end of the experiment, the results indicated that the bacterial strain was able to improve tomato growth increasing all the morphological parameters, such as plant height, fresh and dry weight, as well as the root system architecture parameters. Further, *S. violaceoruber* caused a significant increase in net photosynthesis, stomatal conductance and transpiration rate, confirming its effectiveness on tomato growth. These positive effects perfectly mirrored what we have already observed in hydroponics, confirming once again the positive role of *S. violaceoruber* as PGPB. These effects could be due to its ability to produce phytohormones, siderophores, antibiotics, antioxidant compounds able to improve plant growth, as already hypothesized. Similar results were reported with other PGPBs, which improved the photosynthetic efficiency, rate and pigment content, resulting in higher carbohydrate and biomass accumulation under both optimal and abiotic stress conditions (Zhang et al., 2018). More recently, Nephali et al. (2020) demonstrated that the PGPR-based biostimulant application on maize plants, under normal conditions, induced a global reprogramming of primary and secondary metabolism, such as TCA intermediates, ascorbic acid, amino acids, phytohormones, lipids and flavonoids, which are directly involved in plant growth and development. The *Streptomyces*-treatment also resulted in a significant increase in nitrogen use efficiency (NUE) and nitrogen utilization efficiency (NUtE), but not in the other component, nitrogen uptake efficiency (NUpE), compared to uninoculated plants. In agreement, recent studies have shown a positive correlation between PGPR application and nitrogen uptake and utilization efficiency in different plant model (Lan et al. 2022; Wang et al. 2021). For example, Fiorentino et al. (2018) demonstrated that two strains of *Trichoderma* (*T. virens* GV41 or *T. harzianum* T22), under suboptimal, optimal, and supra-optimal N levels improved Nitrogen Use Efficiency (NUE) in two leafy vegetables, lettuce and rocket, improving the uptake of native N present in the soil. Very interestingly, the results confirmed NUtE as the main component in contributing to the NUE increase in tomato plants, under LN condition (Abenavoli et al., 2016). However, NUE is a complex trait, whose steps are tightly controlled at gene transcriptional, translational, and

post-translational level. Therefore, the pathways activated by *S. violaceoruber* in plants for increasing NUE need to be deeply understood, since, its integration with current crop practices to enhance N use efficiency may represent key challenges for many cropping systems.

Finally, in the plate-on-plate experiments, the ability of *S. violaceoruber* to mitigate drought stress was evaluated. *Streptomyces* spp. are capable of alleviating drought stress in different plants (Nazari et al., 2023), increasing several enzymatic activities in roots, stems, and leaves, and the amino acid production, main factors responsible for greater tolerance to abiotic stress (Manullang and Chuang, 2020; Niu et al., 2022). However, in our experimental condition, *S. violaceoruber,* applied by indirect inoculation or dipping, was not able to recover drought stress condition, but changed, in different way, tomato root morphology. In particular, by the inoculation method, the root system showed higher biomass, number of lateral roots modifying tomato root morphology. For example, co-culturing of *Serratia marscescens* with *Arabidopsis* resulted in inhibition of elongation of primary roots, while inducing lateral roots (Shi et al., 2010). Furthermore, an increased root branching was also observed in alder (*Alnus glutinosa* L.) due to root colonization by *Frankia* UGL010708 (Orfanoudakis et al., 2010). Thus, PGPR capable of modulating root traits can play important role in agricultural sustainability. *In vitro* studies on the effect of PGPR inoculation reveal that many PGPR reduce the growth of main root, increase the number, and/or length of lateral roots and stimulate root hair elongation thus enhancing the uptake of water and nutrients and resulting in increased plant growth and development (Vacheron et al., 2013; Cassán et al., 2020). These effects may be due to VOCs, produced by *S. violaceoruber* and/or tomato plants in response to bacteria strain inoculation. Similar results were reported by Gutiérrez-Luna et al. (2010) using a divided Petri plate assay, where positive effect of certain rhizobacteria isolated from lemon (*Citrus limon* L.) on root morphogenesis and biomass production in *A. thaliana* seedlings was observed, indicating the role of VOCs in plant growth modulation. Microbes secrete VOCs for a variety of reasons like, crosstalk and protection (Kai and Piechulla, 2009), and plant root system can quickly and efficiently sense the VOCs released and modulate its architecture. Thus, VOCs are considered effective mediators of chemical crosstalk as, attracting, repelling or warning signals. Many PGPR, by producing VOCs and secondary metabolites, play important role in influencing the root architecture and growth, resulting in increased surface area for nutrient exchange and other rhizosphere effects. The VOCs

analyses produced by both *S. violaceoruber* and tomato plants could help to understand the beneficial mutual interactions between plants and bacteria.

Overall, the results obtained on the effect of *S. violaceoruber* on tomato grown under different growth conditions highlights the potential of this bacterial strain as novel candidate to develop biofertilizers for low environmental impact and sustainable agriculture. Further studies could help to deeply understand the effect/role of PGPB-root interactions.

12. Chapter IV:

Can seaweeds help to mitigate N stress in tomato plants?

12.1 Introduction

In recent years, the scientific interest on the algae and yeast utilization increased due to several discoveries and advances for their biostimulant properties. They have a great ecological role along with a high economic value with an attractive business opportunity in agronomy and agro-industries (Kapoor et al., 2021). Nowadays, their applications are very broad, including antibiofilm action, biofuel processing, bioremediation, fertilizer, and fish feed (Gomez-Zavaglia, 2019). In particular, brown seaweeds (SEs) are the second most abundant group, comprising about 2.000 species, which reach their maximum biomass levels on the rocky shores in the temperate zones (Al-Juthery et al. 2020). Among them, *Ascophyllum nodosum* together with *Fucus* spp., *Laminaria* spp., *Sargassum* spp., and *Turbinaria* spp. (Khan et al. 2009) are the seaweed extracts (SEs) widely used as biostimulants in agriculture (Di Stasio et al., 2018). Their application in soils, in hydroponics, or sprayed, improves nutrient uptake, water efficiency, tolerance to abiotic and biotic stress, leading to increased plant growth, vigor and yield (Stirk and van Staden, 2020; Ali et al. 2021). SE effects may be ascribed to the presence of endogenous auxins as well as carbohydrates, amino acids, small amounts of phytohormones, osmoprotectants, and proteins in their complex mixture (Khan et al. 2009; Stirk and van Staden, 2020). In addition, SEs contributed to reduce seed dormancy and to improve root system, flowering, and fruit quality and taste (Ali et al. 2019; Kapur et al. 2018; Li et al. 2015). Recently, it has been demonstrated that the Expando, which also contains seaweed extracts, application was able to reduce the ripening times and fruit size, while slightly increasing nutritional and nutraceutical values, leading to more marketable tomato fruits (Mannino et al., 2020). Sometimes SE are used in combination with yeast extracts (Mannino et al., 2022; Campobenedetto et al., 2021). On the other hand, yeast-based plant biostimulant effects were observed on yield and abiotic stress tolerance of various plants such as wild rocket (*Diplotaxis tenuifolia* L.) (Schiattone et al., 2021), tomato (*Solanum lycopersicum* L.) (Lonhienne et al., 2014; Mannino et al., 2020; Campobenedetto et al., 2021), rice (*Oryza sativa* L.) (Johnson and Puthur, 2022) and chia (*Salvia hispanica* L.) (Esmail et al., 2022).

The aim of this study was to assess the ability of Eranthis (SE), a seaweed and yeast extract biostimulant, to mitigate N stress in tomato plants grown in hydroponic system.

12.2 Materials and Methods

12.2.1 Biostimulant

A commercial biostimulant, ERANTHIS® (SE), based on brown seaweed extract (*Ascophyllum nodosum* and *Laminaria digitata*) and selected yeasts with organic matrices of exclusively plant origin, was provided by Green Has Italia S.p.a (Canale, Italy). The label of the products claims to contain 2.5% (w/w) of organic nitrogen and 14% (w/w) of organic carbon. It had a density of 1.2 gmL^{-1}, a pH (in 1% w/w water solution) of 5.0 ± 0.5 (measured on three different replicates and expressed as mean ± standard deviation) and an electric conductivity (water solution 1 gL^{-1}) of 250 $\mu S\ cm^{-1}$. It includes many compounds belonging to the flavonoid, flavan-3-ol and flavanol families, showing a stronger radical scavenging activity (Campobenedetto et al. 2021).

12.2.2 Hydroponic system experiment

12.2.2.1 Plant material.

The UC82 tomato (*Solanum lycopersicum*) seeds, a commercial variety, were sterilized with 70% (v/v) EtOH and then with 25% (v/v) NaOCl solution for 2 and 6 min, respectively. Afterwards, seeds were rinsed three times with autoclaved Milli-Q water to remove EtOH and NaOCl residues. Tomato seeds were germinated in Magenta boxes containing 1% (v/w) agarose gel diluted in 0,5 mM $CaSO_4$ and placed in a growth chamber at 24 ° C, 65% relative humidity, in the dark, for 3 days. Later, they were exposed to 14/10 light/dark photoperiod (350µmol m^2 s^{-1} light intensity), for 7 days. Finally, uniform selected seedlings (10 days old) were transferred an aerated hydroponic system (4.5L plastic boxes) (12 seedlings per box) containing a modified Hoagland containing macronutrients (1mM KH_2PO_4, 2.5 mM K_2SO_4, 2 mM $MgSO_4$, 9 µM Fe-EDTA) and micronutrients (4.6 µM H_3BO_3, 9 µM $MnCl_2$, 0.76 µM $ZnSO_4$, 0.32 µM $CuSO_4$, 0.11µM Na_2MoO_4), pH 5.8 (Mauceri et al, 2020). The nutrient solution was continuously aerated and renewed every 3 days and the pH was maintained with 1N KOH solution. Seedlings were then placed in the growth chamber, at the same conditions reported before, up to the 4th true leaf stage.

12.2.2.2 Dose response-curve

At the 4th true leaf stage, tomato seedlings (20 days old) were N-starved for 3 days (grown in a modified Hoagland solution without N source) and then resupplied with low (LN; 0.5 mM) and high (HN; 10mM) NO_3^-concentrations. The LN seedlings were exposed to

different SE concentrations: 0, 2, 7, 1,33, 0,675 and 0,3375 ml/L (control, T1, T2, T3 and T4 respectively). The growing systems were then placed in the growth chamber, at the same condition reported before, for 7 days. At the end of the experiment, relative chlorophyll content, fresh and dry biomass of shoots and roots collected separately, and root morphology were evaluated.

12.2.3 Root Morphological analysis

For each experimental condition, roots were immersed in a 0.1% (w/v) toluidine solution (Sigma Aldrich, 89160) for 5 min, and then washed to remove excess dye. Root images were acquired by scanner at 600 dpi resolution (WinRhizo STD 1600, Instruments Règent Inc., Quebec, Canada) to determine primary (PRL; cm), lateral (LRL; cm) and total root length (TRL; cm), and volume (cm^3) using WinRhizo Pro System v. 2002a software (Lupini et al. 2016, 2017). Lateral root number (LRN) was manually counted as reported by Lupini et al. (2014).

12.2.4 Relative chlorophyll content

After SE treatment, the relative chlorophyll content was determined by means of SPAD 502 chlorophyll meter (Konica Minolta Sensing, Inc., Japan), placed in different stakes, in fully expanded tomato leaves (30 d), and treated with T3 dose.

12.2.5 Statistical analysis

The hydroponic experiments were set up in a completely randomized experimental design, and at least three replications (six plants per replica) was adopted in all the experiments. Before each analysis, the normality (Kolmogorov-Smirnov test) and the homogeneity of variance (Levene test) of data were assessed. The statistical significance of the differences between means was determined by testing the homogeneity of variance and normality of distribution by ANOVA. The means were separated by Tukey's Honest Significant Difference (HSD) test, using Minitab 17 statistical software. The significance was set at $p < 0.05$.

12.3 Results

12.3.1 Dose response-curve

The SE treatment, at T3 and T4 doses, to tomato plants, grown under LN (0,5 mM NO_3^-), significantly increased root FW of about 450% and 393%, respectively (Fig.40 A).

Similar results were observed in root dry weight (DW) where the T3 and T4 doses caused a significant increase in DW of about 130% and 115%, respectively (Fig. 40 B). By contrast, no significant difference were observed in both shoot FW and DW, at all doses applied (Fig.40 C and D).

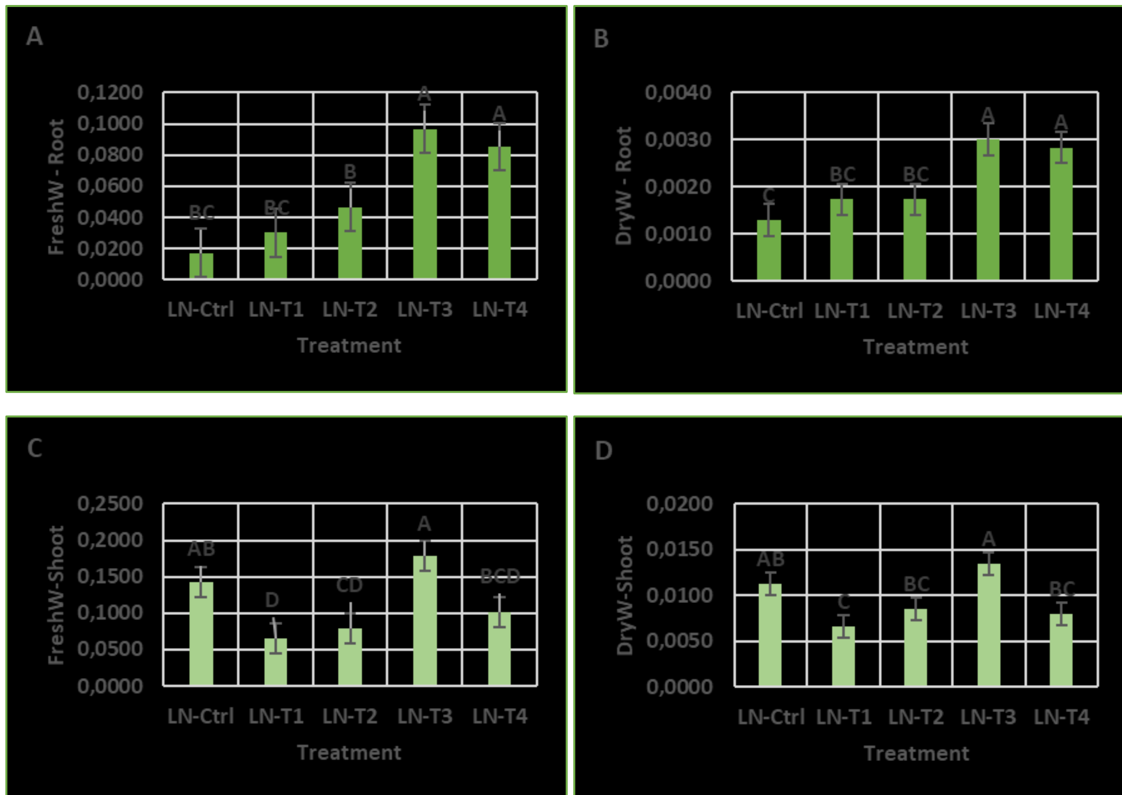


Fig. 40 SE effects on tomato fresh (A, C) and dry (B, D) weight of root (A, B) and shoot (C, D) of LN stressed plants treated with increasing doses of SE and grown at LN (LN-Ctrl, LN-T1, T2, T3, T4). The values are means ± SE (n = x). Different letters indicate means that significantly differ according to Tukey's HSD test at $P < 0.05$. N=3.

The root morphological analysis showed that both T3 and T4 treatments increased total surface area and root volume, by about 59% and 51% (T3) and 146% and 159% (T4) respectively, compared with LN-Ctrl condition (Fig.41 A, B). By contrast, T3 and T4 treatments did not show any significant difference in total root length compared to LN-Ctrl condition (Fig.41 C). The SE treatment also increased surface area and root volume of lateral roots (Fig. 41 E and F) of tomato plants with an increase by about 81% (T3 – surface area), 442% and 500% (T3 and T4 – root volume) respectively.

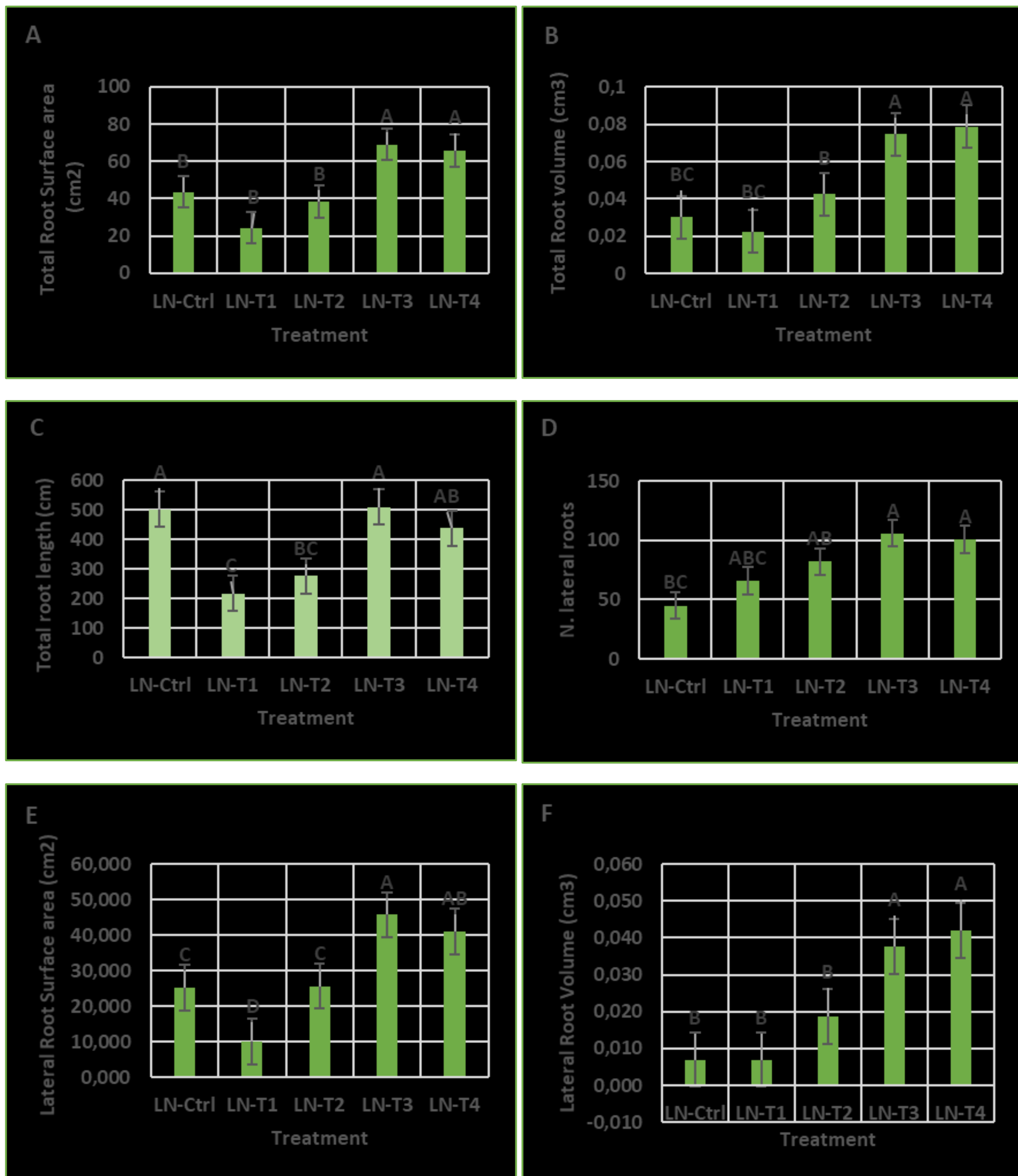


Fig. 41 SE effects on tomato total root surface area and volume(A, B), total root length (C), lateral root number (D) and lateral root surface area and volume (E, F) of LN stressed plants treated with increasing doses of PB (LN-Ctrl, LN-T1, T2, T3, T4). The values are means ± SE (n = x). Different letters indicate means that significantly differ according to Tukey's HSD test at $P < 0.05$. N=3.

Similar trend was observed for the lateral root number, where T3 and T4 treatments caused a significant increase compared to LN-Ctrl condition (Fig.41 D). These results suggested that both T3 and T4 were the effective doses of PB under low NO_3^- condition. Finally, we evaluated the effect of T3 (0,675 ml/L), the most effective dose, on SPAD index. The results indicated that SE was able to increase relative chlorophyll content compared to LN-Ctrl plants (Fig.42).

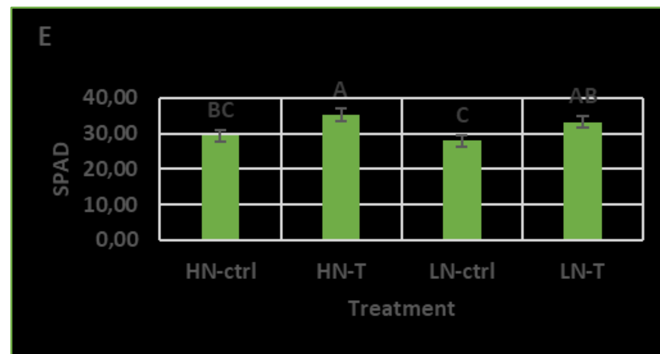


Fig. 42 SE effects on SPAD index (E) of tomato treated plants (LN-T) were compared with plants grown at low (LN-ctrl), high (HN) and HN-T plants. The values are means ± SE (n = x). Different letters indicate means that significantly differ according to Tukey's HSD test at P < 0.05. N=6.

12.4 Discussion

Plant Biostimulants (PBs) are complex products, derived from plant extracts or processing wastes that include useful active compounds such as polyphenols, carbohydrates, amino acids, vitamins, macro and micronutrients, cytokinins, auxins, and abscisic acid (Pereira et al. 2020; Rajendran et al. 2021; Villa and Vila et al. 2023), able to promote positive effects on plants (Cristofano et al. 2021). Seaweed extracts (SEs), included in PBs category, are classified in three classes based on their color, brown, red, and green, and widely used in agriculture. They show phytostimulatory properties able to increase plant growth and yield in several important crops; and phytoelicitor activity that contributes to confer resistance to biotic, and abiotic stresses including drought, salinity, and cold (Ali et al., 2021). In particular, PBs extracted from the brown seaweed, *Ascophyllum nodosum*, have gained attention due to their ability to improve to abiotic stress tolerance (Carmody et al., 2020; Goñi et al., 2021), crop quality traits (Frioni et al., 2018; Łangowski et al., 2019, 2021), and nutrient use efficiency in diverse crops (Jannin et al., 2013; Billard et al., 2014; Stamatiadis et al., 2015; Łangowski et al., 2022). Recently, it has been demonstrated that an engineered biostimulant, PSI-362, derived from *A. nodosum*, was capable of increasing NUE in barley under field conditions. The targeted application of PSI-362 as a coating on a granular N fertilizer enhanced N uptake, transport and assimilation markers at phenotypic, metabolic, enzymatic and genetic levels in a coordinated manner (Goñi et al., 2021). Similar positive results on NUE were already demonstrated in lettuce (Ottaiano et al., 2021), barley (Goñi et al., 2020), tomato (Koleška et al., 2017) and rocket leaves (Di Mola et al., 2019). Here, the ability of Eranthis, a commercial PB (Green Has, Italy), containing seaweed

(*Ascophyllum nodosum* and *Laminaria digitate*) and yeast extracts, to mitigate low N availability was evaluated. This PB, already chemically characterized via UV/Vis spectrometry, resulted rich in phenolic compounds belonging to the flavonoid and flavonol family, together with a strong radical scavenging and a low reducing metal activity (Campobenedetto et al., 2021). Furthermore, it cannot be excluded that the positive effect of Eranthis is due to the combination of the seaweed and the yeasts extract. Recently Chambard et al. (2023) showed that living *Saccharomyces cerevisiae*-based biostimulant reproducibly impacted in several pathways such as abiotic stress tolerance and cell wall/carbohydrate synthesis, protecting the plant against various abiotic stresses during plant development and maintaining a higher level of sugars. In this preliminary study, different Eranthis concentrations were applied by inoculum, in tomato plants grown in hydroponic system, and exposed to low nitrate condition (LN). NO_3^- is usually the most abundant N source in aerobic soils, and the predominant N form taken up by plants from the soil. Unfortunatly, this anionic form is readily dissolved in soil water and very mobile in the soil (Jin et al., 2015; Zarabi and Jalali, 2012), causing environmental pollution (Singh et al., 2022). Indeed, NO_3^- pollution has become a serious environmental concern all over the world and PB application could be an eco-friendly strategy to reduce N input for achieving sustainable cropping systems (Ma et al., 2023).
In our experimental condition, Eranthis, at lower doses, was able to mitigate LN stress condition in tomato plants by increasing total root fresh and dry weight, surface and volume compared to untreated plants. By contrast, it did not affect total and primary root length, but very interestingly, Eranthis was able to increase lateral root number and volume. The positive effects on many root traits could be due to the bioactive molecules present in its formulation, such as phenolic compounds amino acids, peptides, glycine betaine, mannitol, alginates, fucodians, and polyphenols., which generally confer the ability to mitigate abiotic stress providing strong protection against oxidative stress in crop plants (Van Oosten et al., 2017; Campobendetto et al, 2021). However, a hormonal effect might not be ruled out since its application did not affect primary root growth but stimulated lateral root formation, typical of an auxin-like behavior (Fukaki et al., 2007). Indeed, many reports indicated that seaweeds and their extracts contain plant hormones, including abscisic acid (ABA), gibberellins, brassinosteroids, ethylene, auxins, cytokinins (De Saeger et al., 2020). More interestingly, an increase in auxin and cytokinin signaling in *Ascophyllum nodosum* extract-treated plants, implying enhanced levels of these hormones, was observed (Rayorath et al., 2008; Khan et al., 2011). Recently, Ertani

et al. (2018) demonstrated that an *A. nodosum* extract was the most effective in promoting root morphological traits in maize, because of its elevate auxin content. Currently, no information on the hormonal composition and/or the auxin-like effect of Eranthis was provided. However, we demonstrated its positive effect on lateral root formation, trait of large interest to improve plant nutrient uptake. Indeed, lateral roots are important to forage plants for nutrients due to their ability to increase the uptake area of a root system (Pellissier et al., 2021). Finally, the treatment also showed a significant increase in the SPAD index. According to our results, the *A. nodosum* application, in tomato and sweet pepper, led to the increased chlorophyll content, probably due to the inhibition of chlorophyll degradation. These compounds in the seaweed extracts sustain high photosynthetic activity by the inhibition of chlorophyll degradation (Ali et al. 2019). Similarly, a significant increase in chlorophyll content, stomatal conductance, photosynthetic rate, and transpiration rates were recorded in asparagus plants treated with *A. nodosum* (Al-Ghamdi and Elansary, 2018).

These preliminary promising results supported the hypothesis that Eranthis can mitigate low N availability stress and the derived plant benefits made it useful for a more sustainable agriculture. These benefits might be mainly explained by SE chemical composition, rich in bioactive compounds, by the presence of yeast extract, that aid plant development, by reducing N fertilization rates. However, further studies need to deeply understand the physiological and molecular mechanisms in mitigating N stress.

13. Chapter V:

Conclusions and future perspectives

In last decades, the increasing world population and the need to reduce chemical fertilizers in agriculture require more sustainable cropping-systems. For this purpose, plant biostimulants (PBs) have been demonstrated useful to sustain and maintain crop yield, product quality and plant tolerance to biotic and abiotic stresses, reducing the agriculture negative impact on the environment. Indeed, PBs can increase plant growth, nutrient use efficiency.

In this PhD thesis, we investigated the effects of three different biostimulants, two microbial strains from the Actinomycetales, *Kocuria rhizopphila* and *Streptomyces violaceoruber*, and a commercial seaweed extract, Eranthis (Green Has, Italy), on tomato growth performances under N and/or drought stress, using different growth systems. The *S. violaceoruber* and *K. rhizophila*, were selected and considered PGP for their characteristics, such as production of indolacetic compounds, solubilization of organic and inorganic phosphate, N2 fixation, and drought and salt tolerance. Then, the analyses of the secreted metabolome showed a high production of several bioactive molecules, including antibiotics and siderophores, with *S. violaceoruber* being the most performant strain. In vivo assays on tomato seeds and seedlings, confirmed the efficacy of the selected strains in promoting plant growth and development by an improved germination index and seedling growth from seeds treated with PGP actinobacteria. In particular, *S. violaceoruber* produces volatile organic compounds with antimicrobial activity, and was also able to modulate volatilome and exert control over global DNA methylation of tomato seedlings. For this reasons, *K. rhizophila* and *S. violaceoruber* were extensively studied for their PGP activities on tomato plants, under N and/or drought stress.

First, the "omics" (transcriptomics, proteomics and metabolomics) changes and their integration pathways, activated by *K. rhizophila* application on tomato, were investigated. The treatment with *K. rhizophila* caused wide transcriptional changes, and the analysis of differential expressed genes (DEGs) highlighted the significant increase in the expression of genes involved in the cellular response to stimulus and stress, macromolecule and polysaccharide catabolic process, intercellular transport, plasmodesmal-mediated intercellular transport, and steroid and sterol metabolic process. Proteomic analysis revealed differentially accumulation of several proteins (DAPs) involved in the nutritional (carbohydrates and amino acids) and energetic (photosystem I, PSI-G, and II, Psb family protein) metabolisms, related to plant growth (PFKs) and abiotic stress responses (APX1)

promoted by *Kocuria* inoculation. Moreover, metabolomics analysis underlined that *K. rhizophila* modulates carbon sources and energy, and increase the levels of messengers, like dopamine. More interestingly, we identified eight gene modules based on their correlation with differentially accumulated proteins and metabolites (DAPs and DAMs) that showed significant interactions. Based on the correlation of the modules, the highest correlation was found with nine proteins, including a nucleoside diphosphate kinase, a conserved family of proteins involved in energy homeostasis and the developmental process, and a cytosolic ascorbate peroxidase, a family of enzymes that plays a key role in plant growth and development, and several metabolites, mainly belonging to amino acid metabolism and TCA compounds. Therefore, our study supported the idea that the host plant adjusts its transcripts, proteins and metabolites profiles in response to *Kocuria* treatment that exert beneficial effects on plant performances.
The effects of *Streptomyces violaceoruber* application on tomato growth parameters, and its ability to mitigate N deficiency (LN) and/or drought stress, in different growth systems, were then evaluated. A preliminary screening in hydroponics identified the best dose (10 ml containing 3% (v/v) of packed mycelium of *S. violaceoruber* bacterial culture inoculum) of PGPB inoculum that was able to sustain the development of tomato plants, mitigating N deficiency, by improving morpho-physiological growth parameters. We also observed that *S. violaceoruber* established root-bacteria interaction confirmed by the red pigment along the root, due to the production of the bioactive mycelial bacterial compound undecylprodigiosin. The glomerular structures composed of the bacterial hyphae, constituting the colonies were identified by microscopy analysis. In pot system, the PGPB inoculation resulted in the increase of the main growth morpho-physiological parameters in the PGPB-treated tomato plants compared to control, such as biomass values as well as an improvement in the root system. More interestingly, the biostimulant effect of the bacterial inoculum positively influenced both the photosynthesis activity of the treated plants and their chlorophyll content, this enhancement is likely to be due to an increase in the nitrogen use efficiency (NUE) and in the nitrogen utilization efficiency (NUtE). In addition, I delved into the effect of *S. violaceoruber*, when applied by indirect inoculation in control and drought stress conditions, its VOC's produced significantly influenced the tomato development, improving growth performance, and in particular led to the increase of lateral roots, positively changing the root architecture of treated seedlings. Unfortunately, the bacterial treatment was unable to mitigate the drought stress. Based on these results, *S. violaceoruber* can be considered as a novel PGPB, being able to increase

the performances of tomato, resulting an excellent proposal for the formulation of microbial-based biostimulants.

Finally, the biostimulant effects of a commercial seaweed-based (*A. nodosum* and *L. digitata*) and yeast extract, Eranthis, in mitigating N-stress deficiency, were evaluated in hydroponic system. Our results showed the potential of this seaweed and yeast extracts to enhance plant growth and development, as well as promote nutrient uptake in tomato under nitrogen stress. A dose response curve screening experiment allowed us to identify the effective dose of Eranthis (0,675 ml/L). When applied under both HN and LN conditions, the Eranthis treatment increased morpho-physiological parameters and positively affect root size and architecture, in particular enhancing lateral growth root. Moreover, chlorophyll content of treated plants was also positively affected by Eranthis inoculation. This effect could be related to the biostimulant formulation, including antioxidant molecules, such as flavonoids and flavanols, which are able to contribute to ROS scavenging, probably derived from seaweed extracts. Although other investigations are needed for this product, our results highlighted significant potential of this extract in enhancing plant growth and promoting nutrient uptake in N-stressed tomato crops.

In conclusion, the three biostimulants under study, the two actinomycetes, *Streptomyces violaceoruber* and *Kocuria rhizophila*, and the algal extract Eranthis, can represent three effective candidates for the formulation of products for a more sustainable cropping system based on their biostimulant effects. Based on the interesting results on tomato performances of *S. violaceoruber* application, the transcriptomic and metabolomic analyses as well as an in-depth investigation of its volatilome are actually underway. Further studies and investigations will be needed to elucidate yet unresolved mechanisms of action, further enhancing understanding for their possible use and application.

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	(MS+PGP), bacterial treatment under drought stress (MS+PGP+Sorb), bacterial treatment by dipping (MS+PGP-D) and bacterial treatment by dipping under drought stress (MS+PGP-D+Sorb).	
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Supplementary fugures and tables

Table S1. List of gene-specific oligonucleotides used in the qPCR.

Gene	Gene ID	Forward (5'-3')	Reverse (5'-3')
CSD	Solyc02g032330.3	TGAAGGTCTTGGTTACGGAAG	TAGGAGTCTGATTCTTGACGG
OSB	Solyc09g065840.3	TTGGACTCGTCTTGCTGTAAG	TCATCACCTTCAACTGTATCTG
PAE	Solyc03g025600.3	AGATATTGTGATGGAGCCTCAT	CATAGCCTTCTTTGCGTGCC
Fabacaea N70	Solyc01g111350.3	GGCTCCGTCTCCGAATACTT	CAATGTAGAGTGATCCTGGCA
FRO	Solyc03g112320.4	GGAAGGTGGACAAGCACTCT	GAAGTAGACTATCGTATCTCAG
Zn-CDF	Solyc06g076440.3	CTCAGAGCAAGAGCACAGTC	GCATCAGTTAGAACCGCAAGA
SIB	Solyc10g078440.2	CTTTAGTCCAACAACTCACTG	GTACCACTTGATGATGTATAGTA
NSP1	Solyc03g123400.1	GTTCGCCTGACCGTTATTACA	GATTGATGTTGATAGCCTTTGC
CML	Solyc01g010020.3	CCTCACACAACTAGAGTTAGC	CAGGCATTATAGCATTTACGAG
Actin7-like	Solyc11g005330.2	CGTATGAGCAAGGAAATCACC	CAGACTCGTCGTACTCGCC

Table S2. Overview of sequencing and assembly output of PGPB-inoculated and not inoculated tomato plants transcriptome.

Sample Name	Duplicate Reads	GC contents	Average Sequences Length	Number of reads
Ct-T1_R1	6,12%	42,00%	75 bp	42353738
Ct-T1_R2	5,58%	42,50%	75 bp	53062887
Ct-T1_R3	5,51%	42,00%	75 bp	41625240
Ct-T2_R1	5,76%	42,50%	75 bp	53304888
Ct-T2_R2	6,15%	42,00%	75 bp	79335645
Ct-T2_R3	5,41%	42,50%	75 bp	36382479
K-T1_R1	5,91%	41,00%	75 bp	44522551
K-T1_R2	5,79%	42,50%	75 bp	48391458
K-T1_R3	5,46%	41,00%	75 bp	51840263
K-T2_R1	5,84%	41,50%	75 bp	52120642
K-T2_R2	1,78%	42,00%	75 bp	49724563
K-T2_R3	6,10%	42,00%	75 bp	46025278

Table S6. List of KEGGs (Kyoto Encyclopedia of Genes and Genomes) grouping the DRPs extracted from K/Ct comparison at each time (T1 and T2; see Figure 3).

Enrichment FDR	*nGenes*	*Pathway Genes*	*Fold Enrichment*	*Pathway*	*URL*	*Genes*
3,93E+04	32	1229	416.950.063.390.542	Metabolic pathways	http://www.genome.jp/kegg-bin/show_pathway?sly01100	SOLYC01G007910.3 SOLYC01G080280.3 SOLYC01G097970.3 SOLYC02G036350.3 SOLYC02G080570.3 SOLYC02G081340.3 SOLYC03G005230.3 SOLYC03G095190.3 SOLYC03G097470.3 SOLYC03G113800.3 SOLYC03G121070.3 SOLYC04G015750.3 SOLYC04G081400.3 SOLYC05G007080.3 SOLYC05G007780.3 SOLYC06G005150.3 SOLYC06G005490.3 SOLYC06G036350.3 SOLYC06G068090.3 SOLYC07G007870.3 SOLYC07G063040.3 SOLYC07G064160.3 SOLYC07G066150.1 SOLYC09G063130.3 SOLYC09G064500.3 SOLYC10G007600.3 SOLYC11G006340.2 SOLYC12G011450.2 SOLYC12G042900.2 SOLYC12G056830.1 SOLYC12G095880.2 SOLYC12G096190.2
4,10E+09	5	33	242.628.611.698.379	Photosynthesis	http://www.genome.jp/kegg-bin/show_pathway?sly00195	SOLYC05G007780.3 SOLYC07G066150.1 SOLYC09G063130.3 SOLYC09G064500.3 SOLYC12G056830.1
0.000206316015446789	16	722	354.869.548.412.034	Biosynthesis of secondary metabolites	http://www.genome.jp/kegg-bin/show_pathway?sly01110	SOLYC01G007910.3 SOLYC02G036350.3 SOLYC02G080570.3 SOLYC03G005230.3 SOLYC03G121070.3 SOLYC04G015750.3 SOLYC04G081400.3 SOLYC05G007080.3 SOLYC06G005490.3 SOLYC06G068090.3 SOLYC07G007870.3 SOLYC07G049690.3 SOLYC10G007600.3 SOLYC11G006340.2 SOLYC12G095880.2 SOLYC12G096190.2
0.00158634707042621	4	45	142.342.118.863.049	Fructose and mannose metabolism	http://www.genome.jp/kegg-bin/show_pathway?sly00051	SOLYC03G121070.3 SOLYC04G081400.3 SOLYC06G005490.3 SOLYC12G095880.2
0.00158634707042621	7	180	62.274.677.002.584	Ribosome	http://www.genome.jp/kegg-bin/show_pathway?sly03010	SOLYC03G044000.1 SOLYC04G008810.3 SOLYC06G069860.3 SOLYC06G071870.3 SOLYC07G063910.3 SOLYC09G075430.3 SOLYC12G098890.2
0.00489256272101607	3	29	165.656.776.263.031	Galactose metabolism	http://www.genome.jp/kegg-bin/show_pathway?sly00052	SOLYC03G121070.3 SOLYC04G081400.3 SOLYC12G095880.2
0.00489256272101607	4	65	985.445.438.282.648	Oxidative phosphorylation	http://www.genome.jp/kegg-bin/show_pathway?sly00190	SOLYC03G095190.3 SOLYC06G036350.3 SOLYC12G042900.2 SOLYC12G056830.1
0.0137797293359799	5	149	53.736.538.161.386	Carbon metabolism	http://www.genome.jp/kegg-bin/show_pathway?sly01200	SOLYC01G007910.3 SOLYC03G121070.3 SOLYC04G081400.3 SOLYC06G005490.3 SOLYC12G095880.2

0.0330337569331035	3	63	762.547.065.337.763	Starch and sucrose metabolism	http://www.genome.jp/kegg-bin/show_pathway?sly00500	SOLYC02G080570.3 SOLYC03G121070.3 SOLYC05G007080.3
0.0330337569331035	2	22	145.577.167.019.027	Alpha-Linolenic acid metabolism	http://www.genome.jp/kegg-bin/show_pathway?sly00592	SOLYC07G007870.3 SOLYC07G049690.3
0.0330337569331035	4	124	516.564.141.035.259	Biosynthesis of amino acids	http://www.genome.jp/kegg-bin/show_pathway?sly01230	SOLYC01G080280.3 SOLYC06G005490.3 SOLYC12G095880.2 SOLYC12G096190.2

Table S7. List of metabolite sets enriched at the K/Ct comparison (see figure 4B).

Metabolite Set	Total	Hits	Expect	P value	Holm P	FDR
Amino acids and peptides	723	9	0,131	9,27E-15	2,26E-12	2,26E-12
Monosaccharides	97	6	0,0176	2,24E-14	5,45E-12	2,73E-12
TCA acids	9	2	0,00163	0,00000115	0,000278	0,0000936
Steroids	1050	4	0,19	0,0000397	0,00956	0,00242
Furanones	4	1	0,000726	0,000725	0,174	0,0295
Hydroxy acids	4	1	0,000726	0,000725	0,174	0,0295
Short-chain acids and derivatives	7	1	0,00127	0,00127	0,302	0,0442
Disaccharides	9	1	0,00163	0,00163	0,387	0,0498
Organic phosphoric acids	11	1	0,002	0,00199	0,47	0,054
Fatty Acids and Conjugates	3090	4	0,56	0,00231	0,544	0,0565
Benzenediols	16	1	0,0029	0,0029	0,678	0,0643
Amines	21	1	0,00381	0,0038	0,886	0,0773
Benzoic acids	74	1	0,0134	0,0133	1	0,245
Pyrimidines	78	1	0,0141	0,0141	1	0,245
Flavonoids	5300	2	0,961	0,25	1	1

Table S8. List of compounds (metabolites) included in the Upset analysis (see Figure 4C). Compounds shared only between one specific comparison were highlighted in red.

	Ct-T2 vs Ct-T1	K-T2 vs K-T1	K-T1 vs Ct-T1	K-T2 vs Ct-T2
Ct-T2 vs Ct-T1	α-Tocopherol Erythritol Gluconate Feruloylquinic acid isomer 1 Dehydrotomatine Hydroxyoctadecanedioic acid α-Tomatine Threonate Tomatoside A Caffeoylquinic acid Hydroxytomatine Lycoperoside G/Lycoperoside F/Esculeoside A GABA Ornithine Pyroglutamic acid Kaempferol rutinoside	3PGA Glycerate Dopamine Threonine Phenylalanine Feruloylglucaric acid Quercetin rutinoside Sinapoylglycoside isomer 1 Malate Fumarate	Tyrosine Trihydroxyoctadecadienoic acid Citrate pentoside Methionine Glutamate Quercetin glycoside Rhamnose Asparagine Glycerol-1-P 5-Dihydroxybenzoic acid pentose	Tyramide Raffinose
K-T2 vs K-T1				

		Ascorbic acid 13-Amino-13-oxo-tridecanoic acid 2 Citrate Citramalate L-Ascorbate Maleate Galacturonate Dehydrotomatine Hydroxyoctadecanedioic acid α-Tomatine Threonate Tomatoside A Caffeoylquinic acid Hydroxytomatine Lycoperoside G/Lycoperoside F/Esculeoside A GABA Ornithine Caffeoylglucaric acid Acetyl tryptophan Kaempferol rutinoside	Quercetin hexose deoxyhexose pentoside	Quinate Spermidine Tryptophan
K-T1 vs Ct-T1			Caffeoylglycoside Feruloylglycoside Uracil Dehydrotomatine Hydroxyoctadecanedioic acid α-Tomatine Threonate Tomatoside A Caffeoylquinic acid Caffeoylglucaric acid Acetyl tryptophan	

			Pyroglutamic acid Kaempferol rutinoside	
K-T2 vs Ct-T2				Maltose Dehydrotomatine Hydroxyoctadecanedioic acid α-Tomatine Threonate Tomatoside A Caffeoylquinic acid Hydroxytomatine Lycoperoside G/Lycoperoside F/Esculeoside A GABA Ornithine Caffeoylglucaric acid Acetyl tryptophan Pyroglutamic acid

Table S9. Pathway analysis and impact using the metabolites extracted from K-T1 *vs*. Ct-T1 and K-T2 *vs*. Ct-T2 comparison

Comparing	Pathway Name	Match Status	p	-log(p)	Holm p	FDR	Impact
K-T1 *vs*. Ct-T1	Isoquinoline alkaloid biosynthesis	2/6	1.9181E-4	3.7171	0.0038427	3.3758E-4	1
K-T1 *vs*. Ct-T1	Alanine, aspartate and glutamate metabolism	6/22	5.3928E-6	5.2682	2.0493E-4	3.0583E-5	0.58274
K-T1 *vs*. Ct-T1	Phenylalanine metabolism	1/11	1.0425E-4	3.9819	0.0028149	2.2936E-4	0.47059
K-T1 *vs*. Ct-T1	Arginine and proline metabolism	6/34	3.3693E-6	5.4725	1.3477E-4	2.97E-02	0.40342
K-T1 *vs*. Ct-T1	Arginine biosynthesis	5/18	3.6926E-5	4.4327	0.0010708	1.0126E-4	0.30972
K-T1 *vs*. Ct-T1	Tyrosine metabolism	3/16	1.3106E-4	3.8825	0.0031455	2.75E-01	0.22298
K-T1 *vs*. Ct-T1	Citrate cycle (TCA cycle)	4/20	4.5486E-4	3.3421	0.0076778	6.6713E-4	0.21839
K-T1 *vs*. Ct-T1	Glyoxylate and dicarboxylate metabolism	5/29	4.3397E-6	5.3625	1.6925E-4	3.0583E-5	0.16264
K-T1 *vs*. Ct-T1	Pyruvate metabolism	2/22	9.3308E-4	3.0301	0.013063	0.0013244	0.15462
K-T1 *vs*. Ct-T1	Glycine, serine and threonine metabolism	4/33	2.8032E-4	3.5523	0.0053262	4.74E-01	0.14292
K-T1 *vs*. Ct-T1	Butanoate metabolism	3/17	7.8854E-6	5.1032	2,78E-01	3.4696E-5	0.13636
K-T1 *vs*. Ct-T1	Cysteine and methionine metabolism	2/46	1.3475E-6	5.8705	5.7945E-5	2.6736E-5	0.12832
K-T1 *vs*. Ct-T1	Tryptophan metabolism	1/28	0.099163	1.0037	0.30194	0.099163	0.12037
K-T1 *vs*. Ct-T1	Galactose metabolism	1/27	0.075484	1.1221	0.30194	0.081007	0.07998
K-T1 *vs*. Ct-T1	beta-Alanine metabolism	3/18	2.6078E-5	4.5837	8.8666E-4	9.69E-02	0.0754
K-T1 *vs*. Ct-T1	Carbon fixation in photosynthetic organisms	3/21	0.0029951	2.5236	0.032946	0.003876	0.06468
K-T1 *vs*. Ct-T1	Glutathione metabolism	3/26	1.7352E-7	6.7606	7,64E-03	7.64E-03	0.06248
K-T1 *vs*. Ct-T1	Pyrimidine metabolism	1/38	3.9124E-5	4.4076	0.0010955	1.0126E-4	0.05766
K-T1 *vs*. Ct-T1	Sulfur metabolism	1/15	0.013168	1.8805	0.092176	0.014856	0.03315
K-T1 *vs*. Ct-T1	Phenylalanine, tyrosine and tryptophan biosynthesis	3/22	3.5677E-5	4.4476	0.0010703	1.0126E-4	0.02152
K-T1 *vs*. Ct-T1	Glycerolipid metabolism	1/21	1.4676E-4	3.8334	0.0033756	2.9353E-4	0.00426
K-T1 *vs*. Ct-T1	Starch and sucrose metabolism	1/22	0.090975	1.0411	0.30194	0.095307	0.00401
K-T2 *vs*. Ct-T2	Isoquinoline alkaloid biosynthesis	2/6	0.004464	2.3503	0.058032	0.006138	1
K-T2 *vs*. Ct-T2	Alanine, aspartate and glutamate metabolism	6/22	2.1939E-6	5.6588	9.6531E-5	7.9618E-5	0.58274
K-T2 *vs*. Ct-T2	Phenylalanine metabolism	1/11	1.8584E-5	4.7309	7.6193E-4	9.9876E-5	0.47059
K-T2 *vs*. Ct-T2	Arginine and proline metabolism	6/34	5.4285E-6	5.2653	2.28E-4	7.9618E-5	0.40342
K-T2 *vs*. Ct-T2	Arginine biosynthesis	5/18	6.3907E-5	4.1944	0.0019172	1.7814E-4	0.30972
K-T2 *vs*. Ct-T2	Tyrosine metabolism	3/16	9.2139E-4	3.0356	0.014742	0.001398	0.22298
K-T2 *vs*. Ct-T2	Citrate cycle (TCA cycle)	4/20	5.2365E-5	4.281	0.0016233	1.6458E-4	0.21839
K-T2 *vs*. Ct-T2	Glyoxylate and dicarboxylate metabolism	5/29	1.8588E-4	3.7308	0.0042752	3.5706E-4	0.16264
K-T2 *vs*. Ct-T2	Pyruvate metabolism	2/22	0.49412	0.30617	0.98824	0.50561	0.15462
K-T2 *vs*. Ct-T2	Glycine, serine and threonine metabolism	4/33	1.9231E-5	4.716	7.6193E-4	9.9876E-5	0.14292
K-T2 *vs*. Ct-T2	Butanoate metabolism	3/17	8.2773E-5	4.0821	0.0020693	1.821E-4	0.13636
K-T2 *vs*. Ct-T2	Cysteine and methionine metabolism	2/46	1.005E-4	3.9978	0.002412	2.1057E-4	0.12832
K-T2 *vs*. Ct-T2	Tryptophan metabolism	1/28	2.083E-5	4.6813	7.7071E-4	9.9876E-5	0.12037
K-T2 *vs*. Ct-T2	Galactose metabolism	1/27	3.6477E-4	3.438	0.0069306	6.173E-4	0.07998
K-T2 *vs*. Ct-T2	beta-Alanine metabolism	3/18	1.8664E-4	3.729	0.0042752	3.5706E-4	0.0754

K-T2 *vs.* Ct-T2	Carbon fixation in photosynthetic organisms	3/21	0.0010618	2.974	0.015927	0.0015573	0.06468
K-T2 *vs.* Ct-T2	Glutathione metabolism	3/26	5.2067E-6	5.2834	2.2389E-4	7.9618E-5	0.06248
K-T2 *vs.* Ct-T2	Pyrimidine metabolism	1/38	0.092552	1.0336	0.46276	0.10181	0.05766
K-T2 *vs.* Ct-T2	Sulfur metabolism	1/15	0.0059227	2.2275	0.071072	0.0076647	0.03315
K-T2 *vs.* Ct-T2	Phenylalanine, tyrosine and tryptophan biosynthesis	3/22	7.6924E-5	4.1139	0.0020131	1.7814E-4	0.02152
K-T2 *vs.* Ct-T2	Glycerolipid metabolism	1/21	2.5791E-5	4.5885	8.769E-4	9.9876E-5	0.00426
K-T2 *vs.* Ct-T2	Starch and sucrose metabolism	1/22	4.8885E-4	3.3108	0.0087992	7.9664E-4	0.00401

Figure S1. Experimental design carried out to dissect the mechanism induced by PGPB *K. rhizophyla* on tomato.

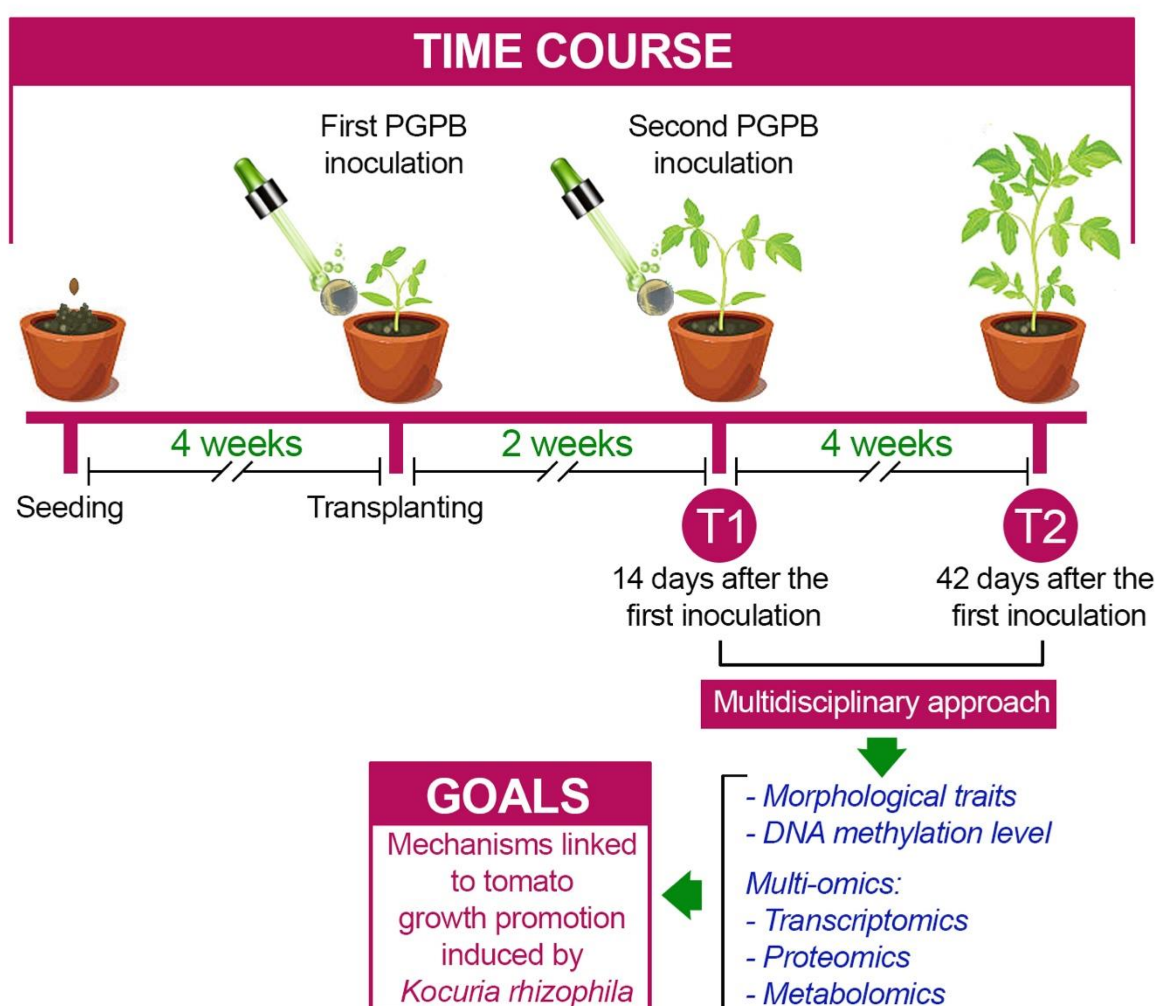


Figure S2. PGPB-inoculated (K) and not inoculated (Ct) tomato plants at T2

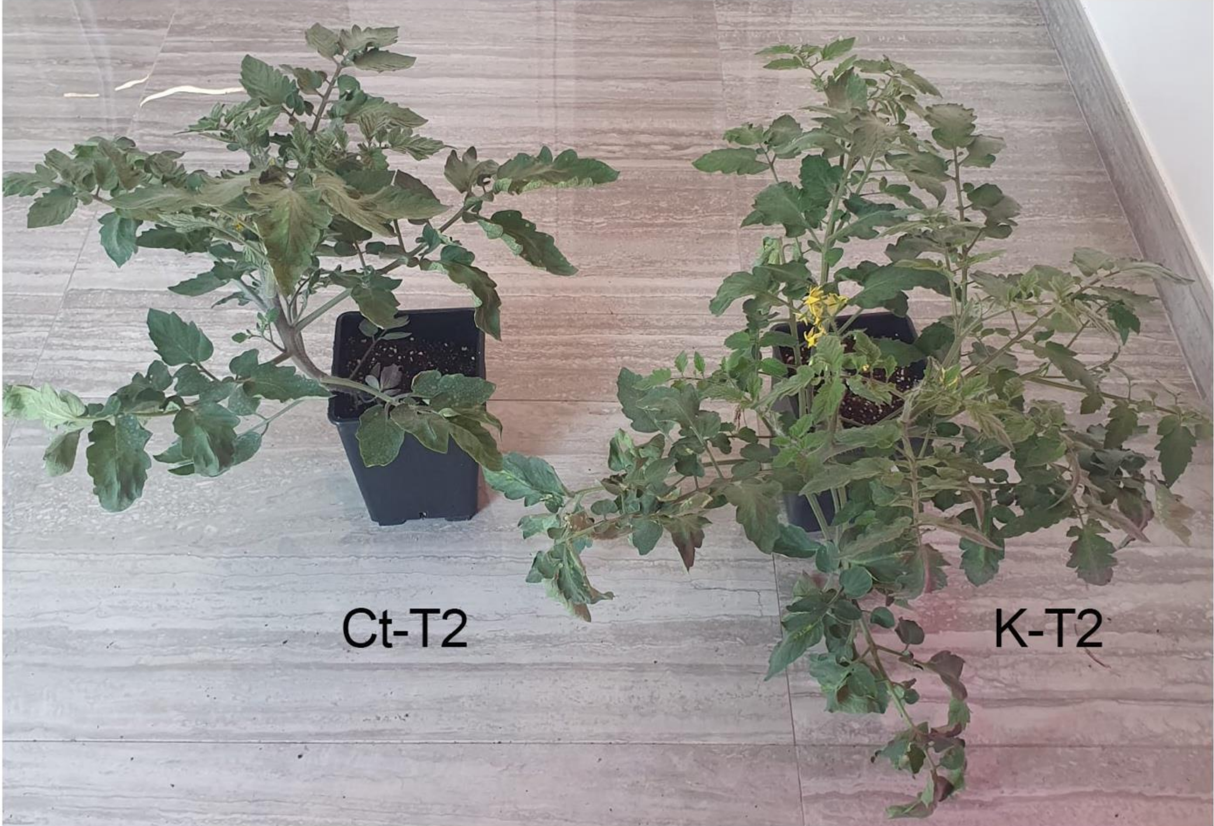


Figure S3. PCA (Principal Component Analysis) of transcriptome analysis of PGPB inoculated (K) and non-inoculated (Ct) tomato plants at different time points (T1 and T2). X and Y axis show principal component 1 (PC1) and principal component 2 (PC2), explaining 25.8% and 18.5% of the total variance, respectively.

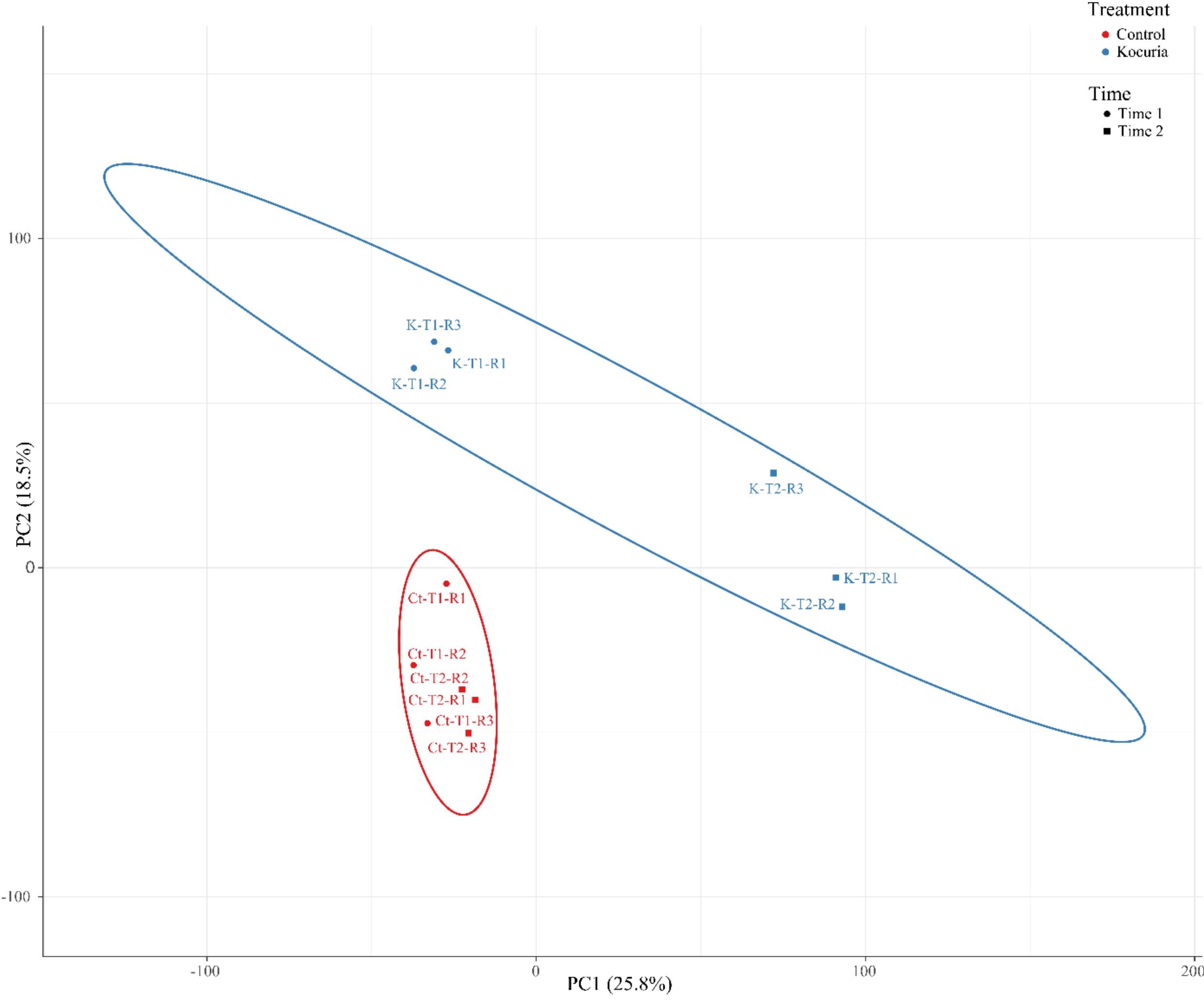


Figure S4. RT-qPCR validation. The relative expression profiles by qPCR of nine key isolated genes randomly chosen (see Table S1) at T1 (A) and T2 (B) of PGPB inoculated (K) and non-inoculated (Ct) tomato plants.

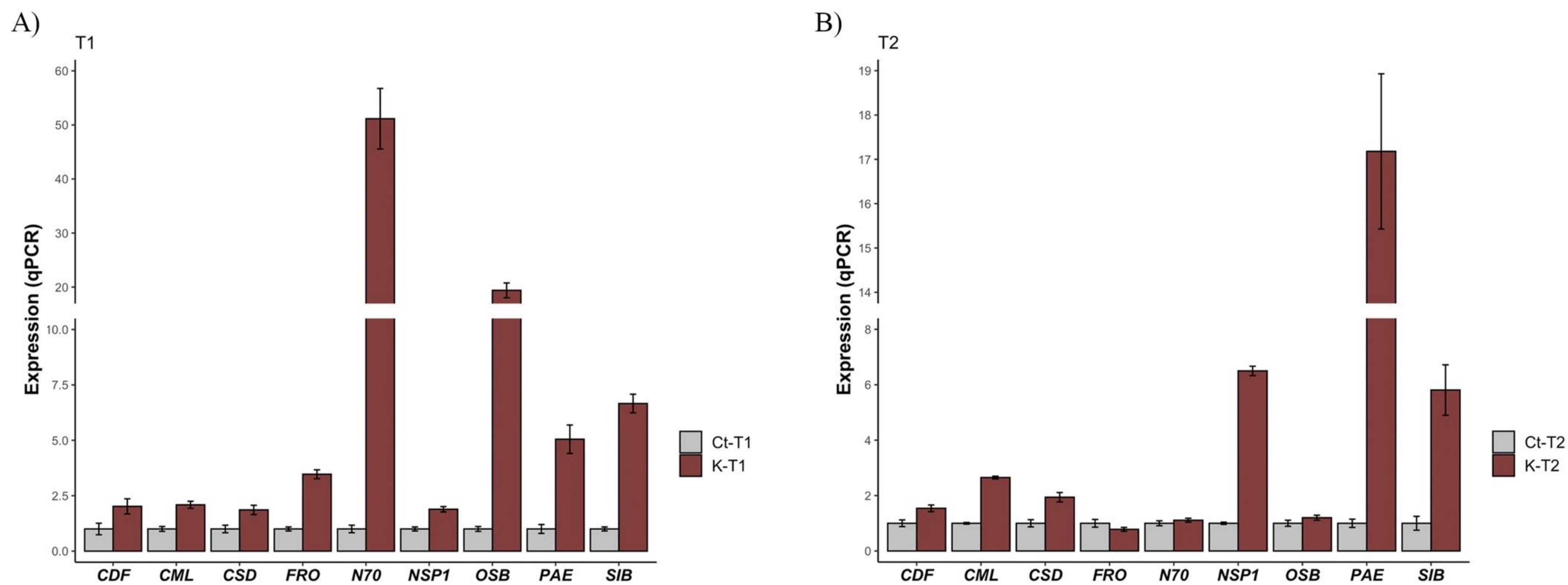


Figure S5. GO enrichment analysis of the up- and down-regulated genes at the Molecular Function (MF) and Cellular Component (CC) categories.

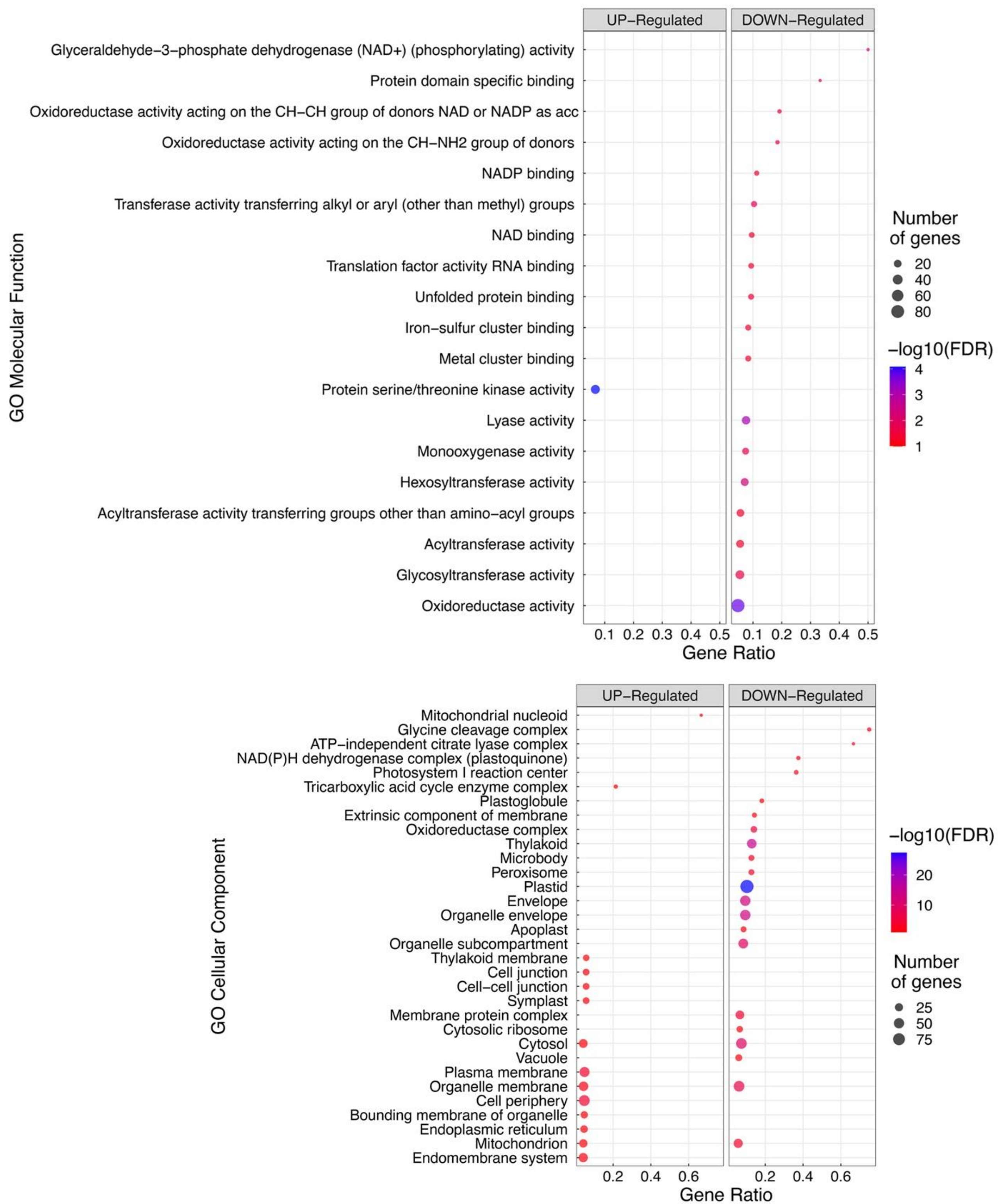


Figure S6. Methylation level of PGPB-inoculated (K) and not inoculated (Ct) tomato plants at T1 and T2.

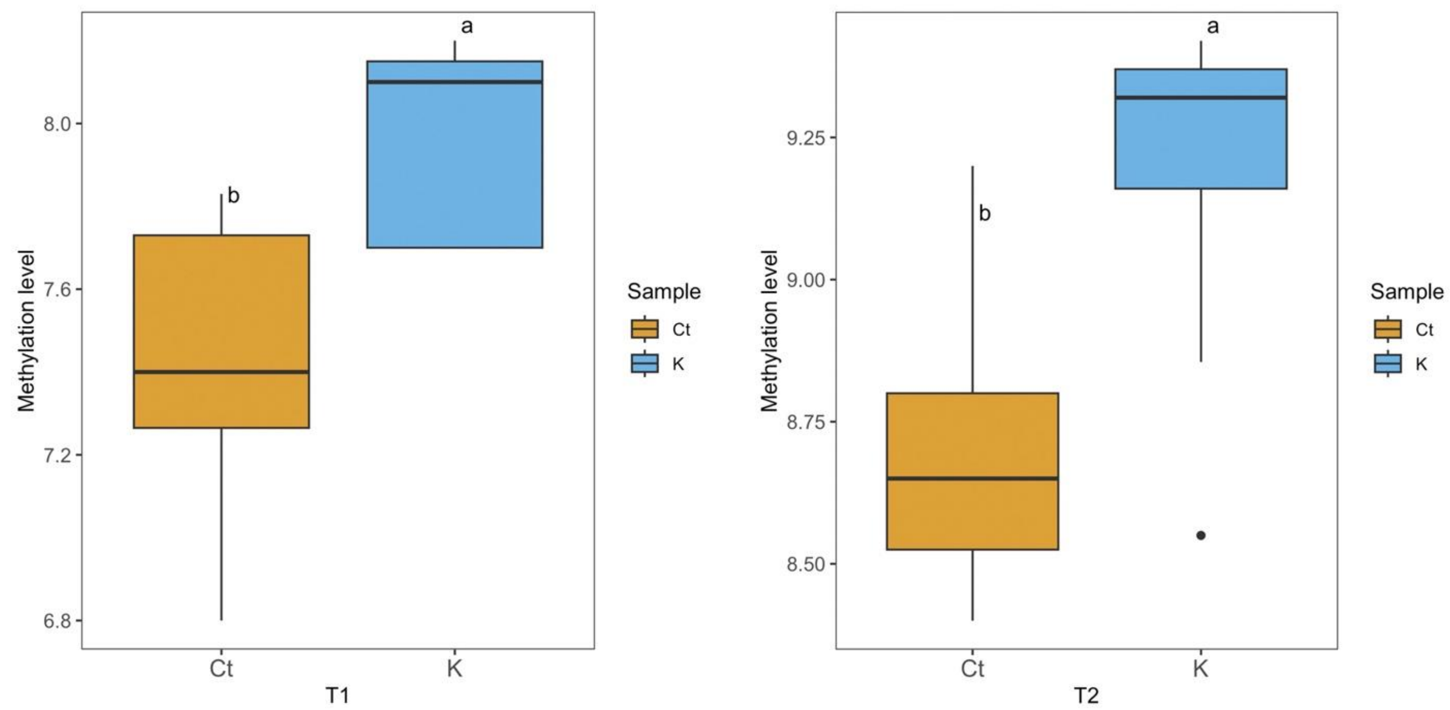


Figure S7. Networks for DRPs extracted from PGPB inoculated (K) and non-inoculated (Ct) tomato plants comparison classified into the three main GO categories. Blue: Biological Process (BP); red: Molecular Function (MF); green: Cellular Component (CC). involved in the lactation process. Each node represents a GO and each line refers an interaction.

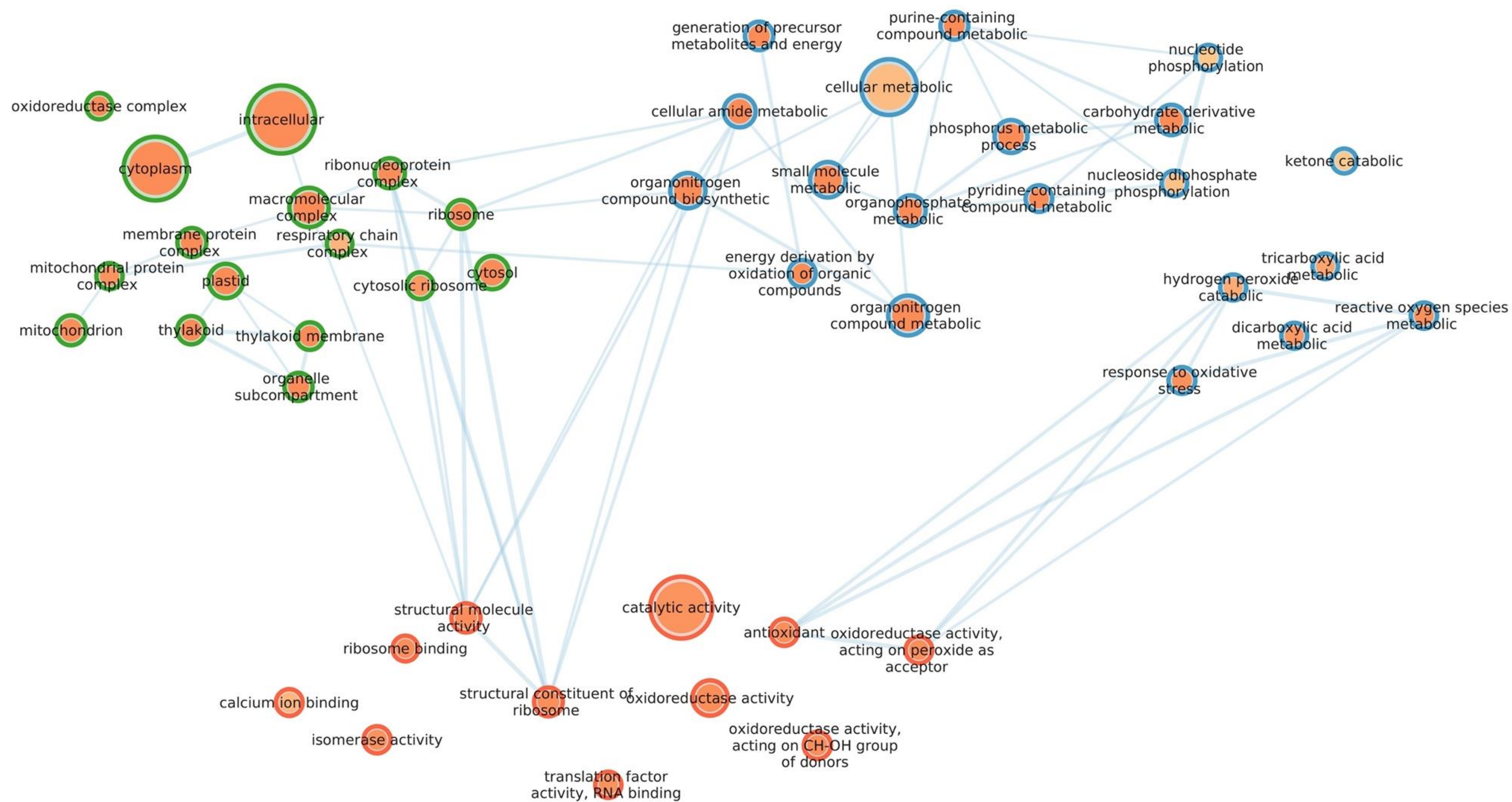
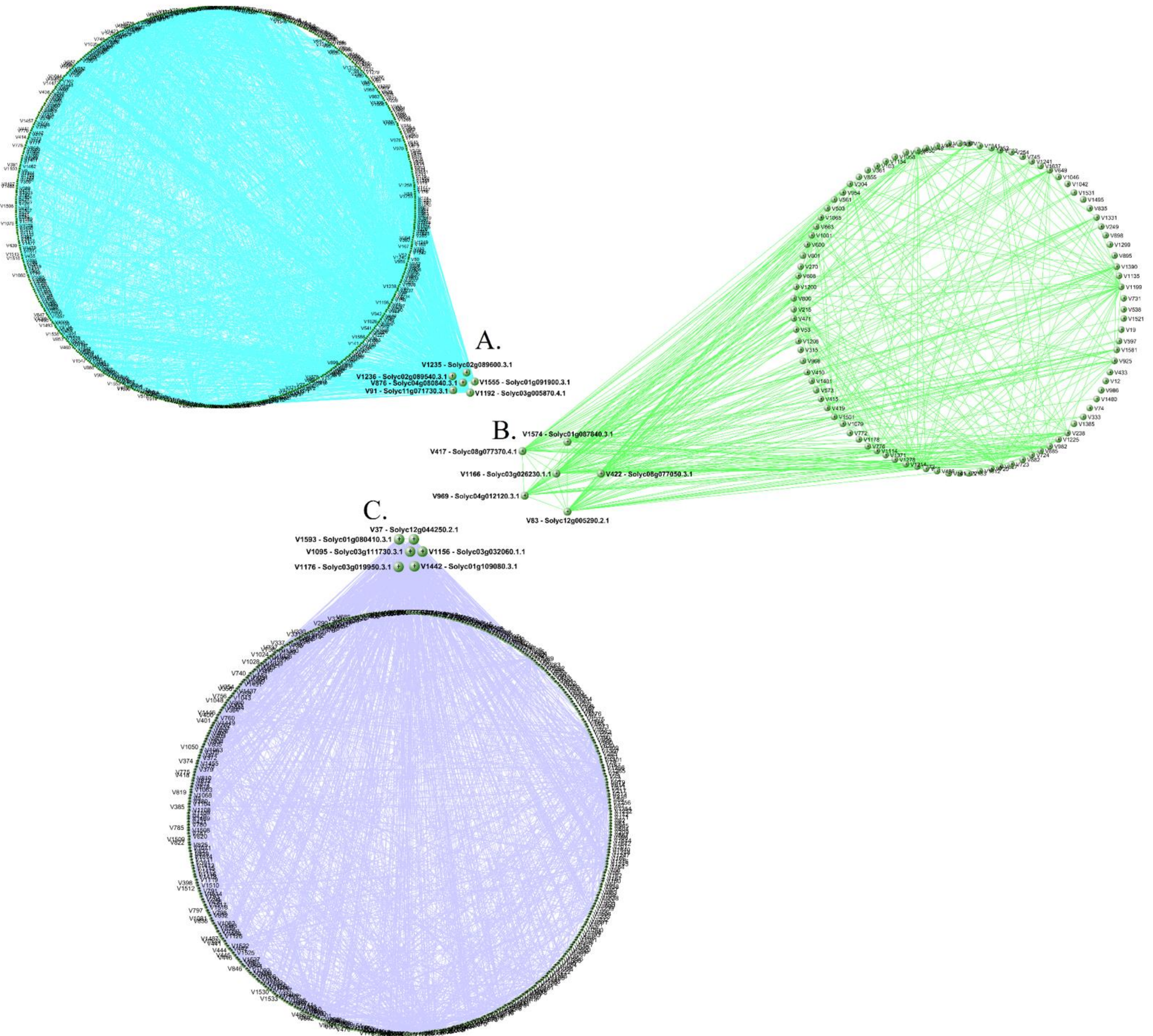


Figure S8 Gene networks using the hub genes extracted in co-expression analysis by VisANT 5.0 in the turquoise (A), green (B), and blue (C) modules. The annotation for each module is reported in Table S10.



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