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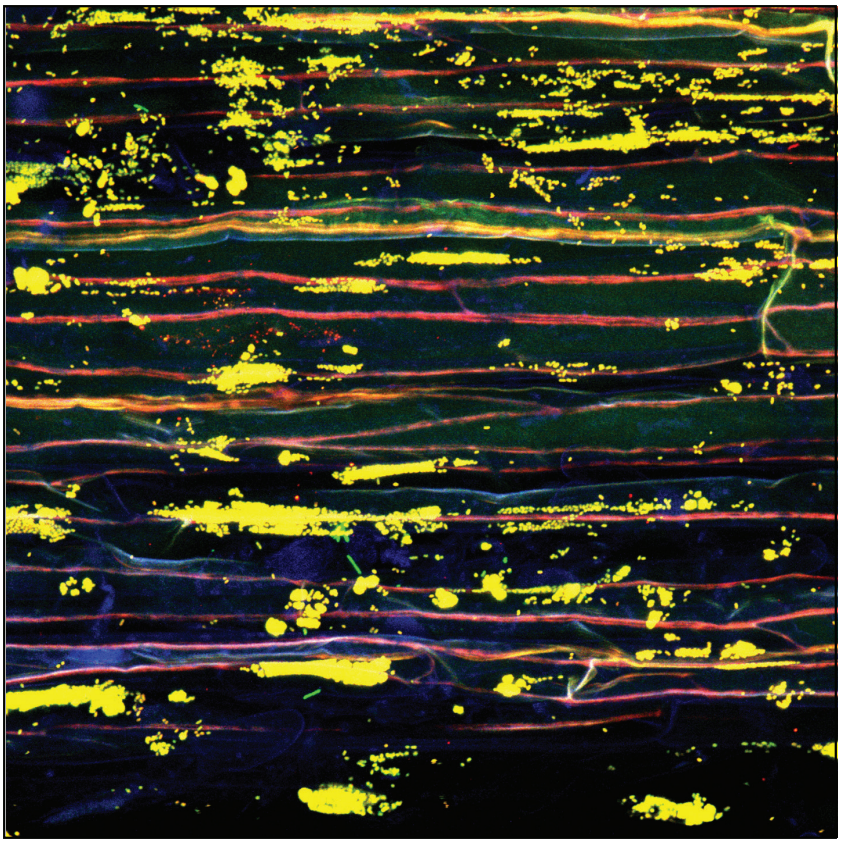
Nikoletta Galambos

Development of novel plant biofertilisers based on endophytic bacteria and innovative insect-mediated delivery strategies



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Development of novel plant biofertilisers based on endophytic bacteria and innovative insect-mediated delivery strategies

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1 SUMMARY

As a result of the constant demand to ensure food security and crop growth, there is an urgent need of a more sustainable and resilient agriculture. Chemical fertilisers and pesticides are widely used in conventional agriculture and they cause possible environmental impacts. Tomato (*Solanum lycopersicum*) is cultivated worldwide under field and greenhouse conditions, requires an extensive use of chemical fertilisers and is greatly affected by arthropod feeding damages. Microbial biofertilisers and non-microbial biostimulants can contribute to plant development. Among them, plant growth promoting endophytic bacteria can internally colonise plant tissues and promote plant growth. Likewise, humic acids (HA) are organic substances that improve soil structure and can facilitate plant nutrient uptake. However, scarce information is available on the synergistic effects of endophytic bacterial strains and HA on the bacteria-mediated plant growth promotion. Moreover, beneficial insects, including the generalist predators *Macrolophus pygmaeus* and *Nesidiocoris tenuis*, can be used in biological control for the management of crop arthropods. Despite the significance of insects as vectors of plant pathogens is unquestionable, the transmission of beneficial microbes by beneficial insects is unknown. The aims of this thesis were to get insight into the molecular basis of the interaction between endophytic bacterial strains and tomato plants in the presence of HA, in order to improve the understanding on the mechanism responsible for plant growth stimulation, and to investigate the possible use of beneficial insects as vectors to transmit selected endophytic bacterial strains for the further development of sustainable delivery strategies of bacterial biofertilisers.

In order to understand the complementation effects and cellular pathways activated by endophytic bacterial strains and HA, three bacterial strains (namely *Paraburkholderia phytofirmans* PsJN, *Pantoea agglomerans* D7G and *Enterobacter* sp. 32A) that were able to promote tomato shoot length in the presence of HA were selected. Double labelling of oligonucleotide probes for fluorescent *in situ* hybridisation was used to study tomato colonisation by endophytic bacterial strains. In particular, the colonisation intensity of tomato root and stem among the tested strains were comparable in the control and HA condition. Moreover, transcriptomics approach was applied to study the molecular mechanisms activated in tomato shoots and roots in response to endophytic bacteria and HA. Tomato genes were modulated by endophytic bacterial strains mainly in roots, indicating major transcriptional regulations in

belowground compared to aboveground tissues. The majority of DEGs was modulated by more than two strains, involving protein metabolism, transcription, transport, signal transduction and defence, representing possible common pathways modulated in response to bacterial endophytes. Moreover, strain-specific tomato responses involved signal transduction, transcription, hormone metabolism, protein metabolism, secondary metabolism and defence processes, highlighting specific traits of the endophyte-tomato interaction. In particular, the presence of HA enhanced the activation of signal transduction, hormone metabolism, transcription, protein metabolism, transport, defence and growth-related processes in response to *P. phytofirmans* PsJN, *P. agglomerans* D7G and *Enterobacter* sp. 32A inoculation, in terms of number of modulated genes and fold change values, indicating additive effects of bacterial endophytes and HA in plant growth promotion mechanisms.

In relation to the possibility to deliver endophytes by using insects, beneficial mirids of tomato (namely *M. pygmaeus* and *N. tenuis*) were tested as potential vectors to transmit *P. phytofirmans* PsJN and *Enterobacter* sp. 32A between tomato plants. Mirids feeding on seed-inoculated tomato plants were able to acquire the beneficial bacterial strains. In particular, after contact with bacterial-inoculated plants, *P. phytofirmans* PsJN and *Enterobacter* sp. 32A were detected on the majority of mirid epicuticle and inside surface-sterilised insects. Moreover, both tested mirids transmitted the selected bacterial strains between tomato plants and *P. phytofirmans* PsJN and *Enterobacter* sp. 32A could be re-isolated from tomato shoots after mirid-mediated transmission. Our study demonstrated that *P. phytofirmans* PsJN and *Enterobacter* sp. 32A can move within tomato plants, from shoots to roots after mirid-mediated transmission, indicating that *M. pygmaeus* and *N. tenuis* can acquire non-pytopathogenic microbes and the polyphagous and mobile nature of mirids could facilitate the transmission of beneficial endophytes among crops.

In summary, the overall work provide in-depth knowledge on the HA-dependent enhancement of growth-related processes stimulated by endophytic bacterial strains and demonstrates the potential of beneficial mirids in transmission of beneficial bacteria and paved the way for the further development of efficient HA- and mirid-mediated strategies for plant biofertilisation and beneficial bacteria delivery.

2 INTRODUCTION

2.1 SUSTAINABILITY IN AGRICULTURAL PRODUCTION

The constant need to feed the increasing global population and the environmental changes, including climate change, give rise to new challenges to the agricultural sector (Rouphael and Colla 2020a). Conventional agriculture largely depends on chemical fertilisers and pesticides to increase yield and ensure continuous productivity throughout the growing season, providing farmers with economic stability (Rouphael and Colla 2020a).

Among crop plants, tomato is cultivated under greenhouses and field conditions (Hobson and Grierson 1993) with 182 million tons harvested from 4.8 million hectares worldwide in 2018 (FAOSTAT Statistics Database 2018). However, tomato growth and yield is highly dependent on the soil nutrient composition and availability (Gould 1992), requires an extensive use of chemical fertilisers (Maham et al., 2020) and is greatly affected by arthropod feeding damages (Wakil et al., 2017). In particular, macronutrients (nitrogen, phosphorus and potassium) are required by plants in the greatest quantities (Sung et al., 2015). Moreover, the relatively low price of chemical fertilisers and growers' attitude to avoid yield loss through excessive fertilisation (Wang and Xing 2017) results in up to 50% of nitrogen and 90% of phosphate is not absorbed by the plants, accumulating millions of tons of synthetic nutrients that pollute the atmosphere, salinise the soil and cause eutrophication of aquatic ecosystems (da Costa et al., 2013; Ye et al., 2020). Besides nutrient availability, phytosanitary problems are limiting factors in tomato production (Gullino et al., 2020). Major pests affecting tomato include nematodes of *Meloidogyne* spp. that cause problems in nutrient transport, leafminers including *Tuta absoluta* and *Liriomyza* spp. reduce the photosynthetic area, aphids (*Myzus persicae, Aphis* spp.) thrips (*Frankliniella occidentalis, Thrips tabaci*) and whiteflies (*Bemisia tabaci, Trialeurodes vaporariorum*) cause direct and indirect damage due to virus transmission, mites (*Tetranychus* spp.) increase transpiration and unable plants to regulate the water balance (Gabarra and Besri 1999) (**Figure 1.1**). Beneficial insects and mites play essential roles in both native ecosystems and agriculture, including pollination and biological pest control (Redhead et al., 2020). Several specialist and generalist natural enemies were identified and applied with success against pests feeding on tomato, such as the parasitoid wasps *Encarsia formosa* and *Eretmocerus* spp. against whiteflies (Liu et al., 2015),

phytoseiid mites against spider mites (Drukker et al., 1997), the bug *Orius* spp. against thrips (de Puysseleyr et al., 2011) or beneficial mirid predators, including *Macrolophus pygmaeus*, against *T. absoluta* (Urbaneja et al., 2009), *Nesidiocoris tenuis* against *T. absoluta* (Calvo et al., 2012) and whiteflies (Calvo et al., 2009) (**Figure 1.1**). In addition, tomato pathogens causes severe diseases, such as bacterial speck (*Pseudomonas* spp.), bacterial spot (*Xanthomonas* spp.), powdery mildew (*Leveillula taurica, Oidium* spp., *Golovinomyces* spp.), leaf mould (*Passalora fulva*), late blight (*Phytophthora* spp.), early blight (*Alternaria* spp.) gray mould (*Botrytis cinerea*), septoria leaf spot (*Septoria lycopersici*), white mould (*Sclerotinia* spp.) and soilborne diseases such as Verticillium wilt (*Verticillium dahliae, V. albo-atrum*), fusarium wilt (*Fusarium oxysporum* f. sp. *lycopersici*) and fusarium crown and root rot (*F. oxysporum* f. sp. *radicis-lycopersici*) (Gabarra and Besri, 1999; Gullino et al., 2020).

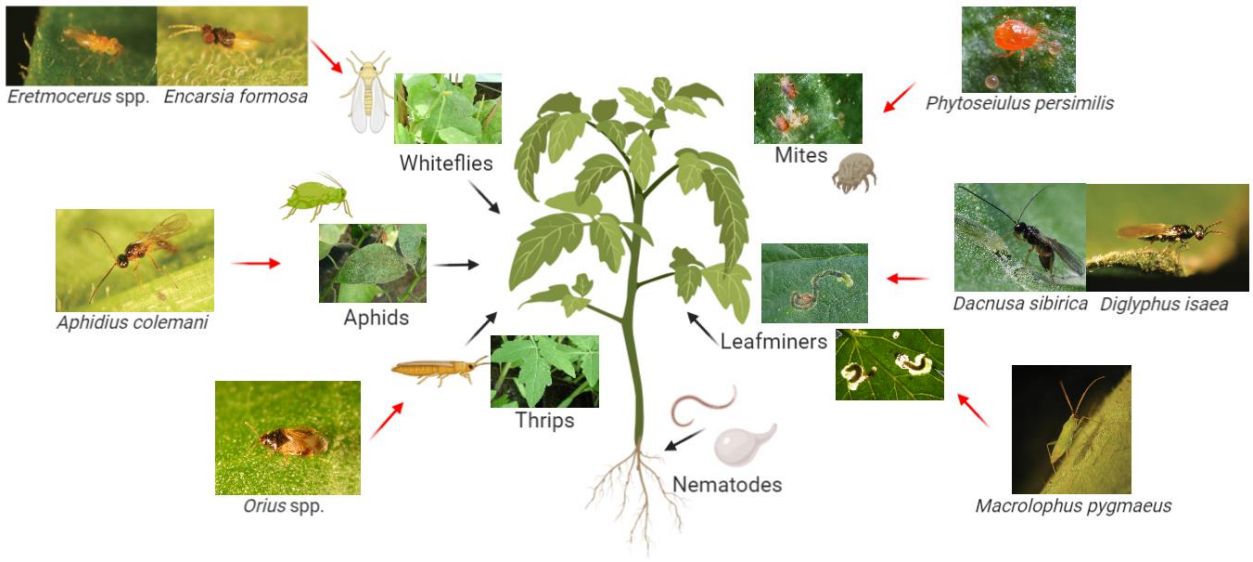

Figure 1.1 | Important pests and beneficial insects associated with tomato plants. Created with Biorender (https://biorender.com/). Source of images: https://www.biobestgroup.com.

Pest and disease management relies on the use of large amounts of chemical pesticides, which have numerous environmental drawbacks, including non-target species loss, ground and surface water contamination and residue accumulation in the final product (Iyaniwura 1991). In particular, unilateral reliance on pesticides leads to pest resistance (Ruberson et al., 1998). Therefore, seeking for new, ecologically and economically sustainable technologies is garnering interest for improving agricultural resource use efficacy, food security and preserving soil biodiversity (Frison et al., 2011; Jindo et al., 2020).

Microbial biofertilisers (arbuscular mycorrhiza and plant-growth promoting bacteria) and non-microbial biostimulants (humic substances, animal and plant-based extracts) are defined as fertilising products that improve nutrient use efficacy, abiotic stress tolerance, resistance induction and protection against pest and pathogens, product quality and availability of confined nutrients in the soil or rhizoshpere (Regulation of the European Parliament [EU] No 1069/2009; No1107/2009 and No 2003/2003). Therefore, microbial biofertilisers and non-microbial biostimulants are key alternative solutions to reduce the use of harmful synthetic fertilisers and pesticides in agriculture (Rouphael and Colla 2020b). As a result of the constant need to ensure food security and crop growth under abiotic stresses, caused by climate changes, biostimulant market is estimated to value 2.6 billion dollars in 2019 and projected to grow at a compound annual growth rate of 11.2% (Markets and Markets 2020), indicating the urgent need to render agriculture more sustainable and resilient (Rouphael and Colla 2020a).

2.2 MICROBIAL PRODUCTS FOR SUSTAINABLE AGRICULTURE

1.1 Plant growth-promoting bacteria

Soil serves as a reservoir for biodiversity and life forms, including bacteria, actinomycetes, fungi, algae and protozoa, that are unevenly distributed and mainly localised near the plant roots (rhizosphere) due to the presence of root exudates (Glick 2020) (**Figure 1.2**). Among the above mentioned taxa, bacteria are the most abundant (95%) and they form beneficial, neutral or harmful association with plants (Glick 2012). Plant growth-promoting bacteria (PGPB) can contribute to plant development, through direct (modulating plant hormone levels, facilitating nutrient acquisition) and indirect (alleviating biotic or abiotic stress) mechanisms or through the combination of multiple mechanisms (Bais et al., 2006; Glick 2012) (**Figure 1.3)**. In this cooperative interaction, plants provide carbon-rich root exudates as nutrient source for the plant-associated PGPB (Bais et al., 2006; Glick 2012). Bacteria use root exudates as chemical cues to locate and reach roots by chemotaxis (Walker et al., 2003). Thus, differences in the exuded compounds in time and space may affect the microbial composition and rate of colonisation (Compant et al., 2010). In addition, the composition of root exudates determines the gene

expression and metabolic activity of bacteria in the rhizosphere (Musilova et al., 2016; Haskett et al., 2020).

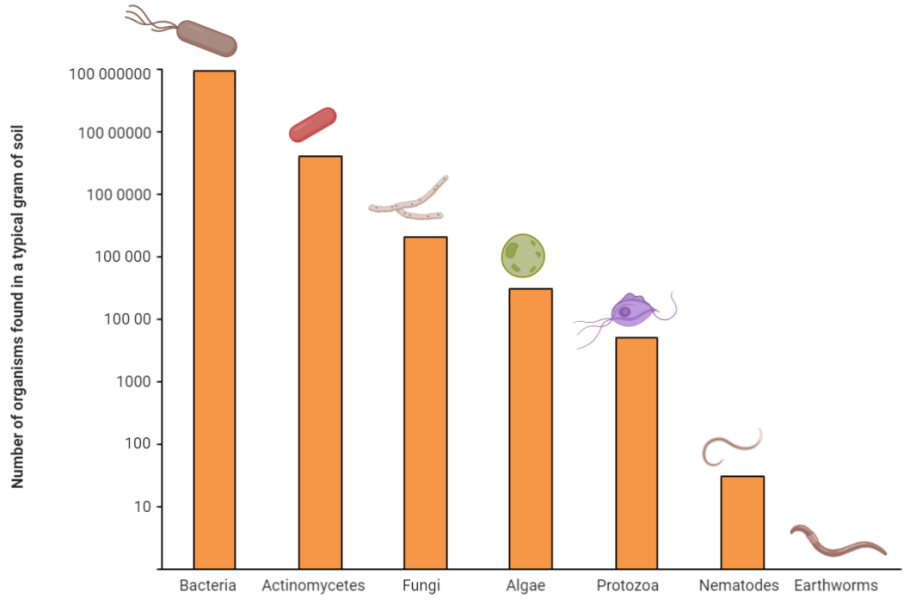

Figure 1.2 | Number of organisms found in a typical gram of soil. Created with Biorender (https://biorender.com/). Modified from Glick 2020.

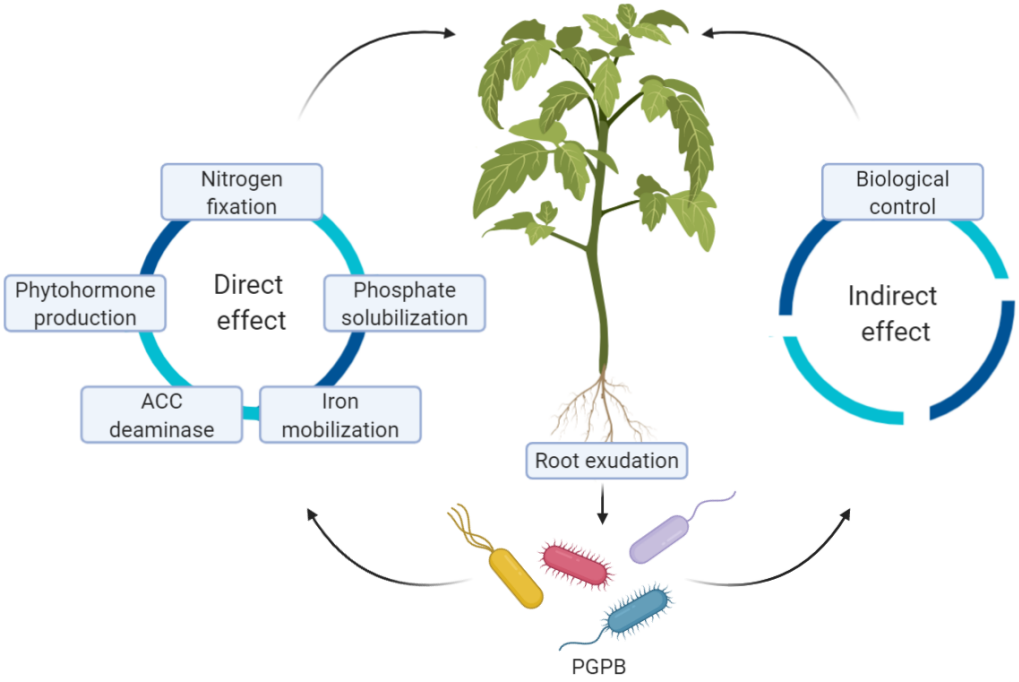

Figure 1.3 | Main direct and indirect mechanisms used by plant growth-promoting bacteria (PGPB) to facilitate plant growth and development. Created with Biorender (https://biorender.com/). Modified from Glick 2020.

Based on their life forms, PGPB can be free-living in the rhizosphere or colonisers of the interior of plant tissues (plant growth-promoting endophytes; Basu et al., 2017) (**Figure 1.4**). Endophytic colonisation is a finely tuned process, which is determined by several genetic and environmental factors (Brader et al., 2017). In more details, endophytes possess a set of genes, (e.g. encoding transporter proteins, secretion proteins, plant polymer degradation/modification enzymes, transcriptional regulator proteins and detoxification enzymes) that enable them to enter and alter their metabolism inside the plant tissues (Ali et al., 2014). Considering that rhizosphere is a highly competitive environment, bacterial endophytes could possess an ecological advantage, as internal tissues are thought to be more uniform and protective compared to the plant surfaces (Hallmann et al., 1997).

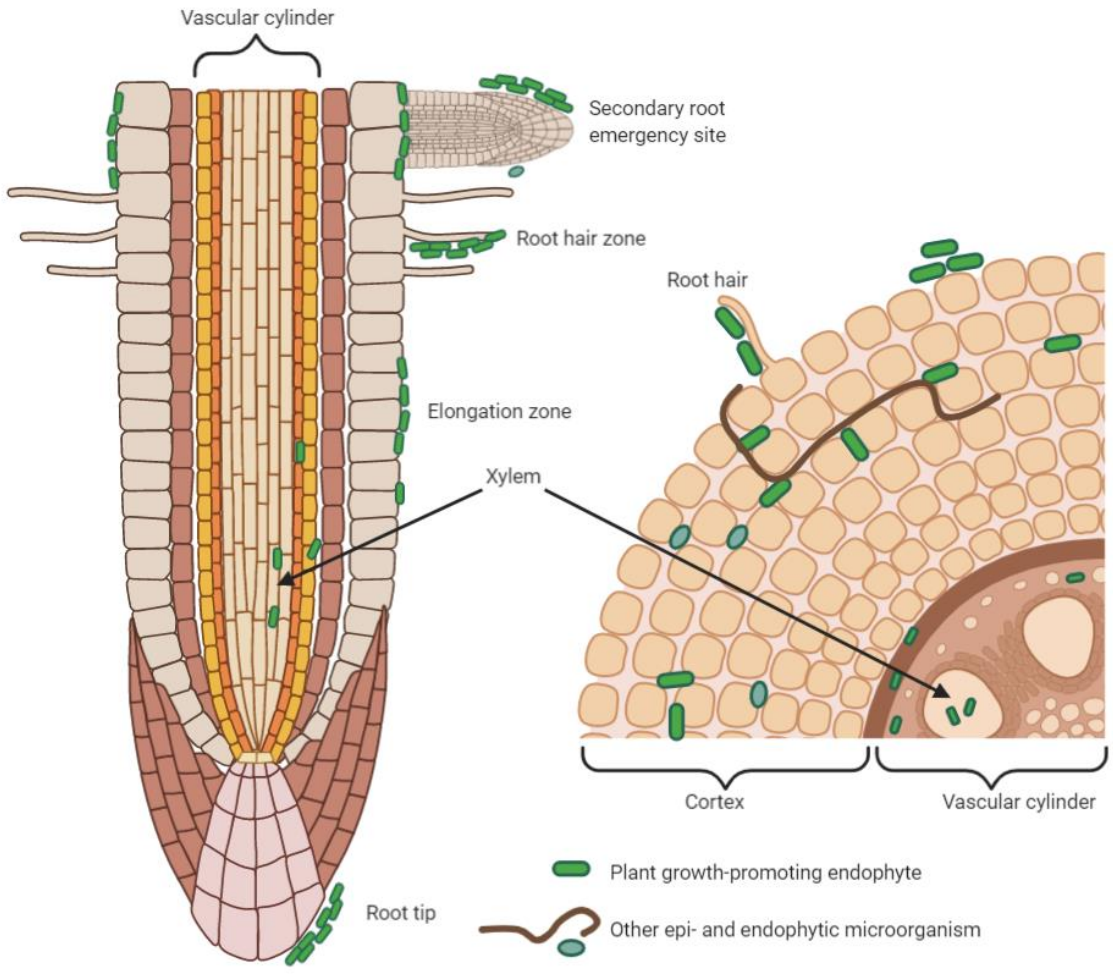

Figure 1.4 | Sites of plant colonisation by plant growth-promoting endophytes. Created with Biorender (https://biorender.com/). Modified from Compant et al., 2010.

1.1.1 Direct mechanisms of microbial-mediated plant growth-promotion

1.1.1.1 Nitrogen fixation by plant growth-promoting bacteria

Nitrogen is a key nutrient for plants as it has a central role in protein and nucleic acid metabolism (Novoa and Loomis 1981). Despite the fact that approximately 80% of the atmosphere consist of nitrogen gas that plants cannot assimilate, nitrogen is limiting factor in crop production (Santi et al., 2013). In particular, to find an optimal balance between nitrogen availability and plant nitrogen demand is a challenging task (Hoyle and Murphy 2011). For example, nitrogen excess in the first part of the plant growing season enhances vegetative growth and delays generative growth (Gould 1992). Moreover, anthropogenic nitrogen emission, mainly through nitrogen fertiliser use in intensive agriculture (Winiwarter et al., 2018), has negative impacts on biodiversity, ecosystem, human health and climate (Stevens 2019). In particular, increasing nitrate concentrations in groundwater due to leaching is responsible for eutrophication and degradation of water quality (Tribouillois et al., 2020). Nitrogen fixing bacteria (diazotrophic bacteria) are able to fix atmospheric nitrogen thanks to the activity of the nitrogenase enzyme, which converts nitrogen gas to ammonia (Nag et al., 2020) (**Figure 1.5**). Likewise, some PGPB can perform nitrogen fixation, such as the endophyte *Gluconacetobacter diazotrophicus* Pal5 that lives in association with sugarcane plants (Bertalan et al., 2009) and fixes atmospheric nitrogen inside the plant tissues (Cavalcante and Dobereiner 1988). Similarly, biological nitrogen fixation in symbiotic legume-rhizobia interactions is well documented (Andrews and Andrews 2017). More specifically, after endophytic colonisation the formation of root nodules occurs and they provide suitable environment for nitrogen fixation (Oldroyd et al., 2011). Even though, nitrogen fixation in nodules has the highest efficacy, it is mainly restricted to legumes (Werner et al., 2014; Nag et al., 2020). Recently, genetic engineering methods are under development for building PGPB strains that could deliver fixed nitrogen to other crops, such as cereals (Ryu et al., 2020).

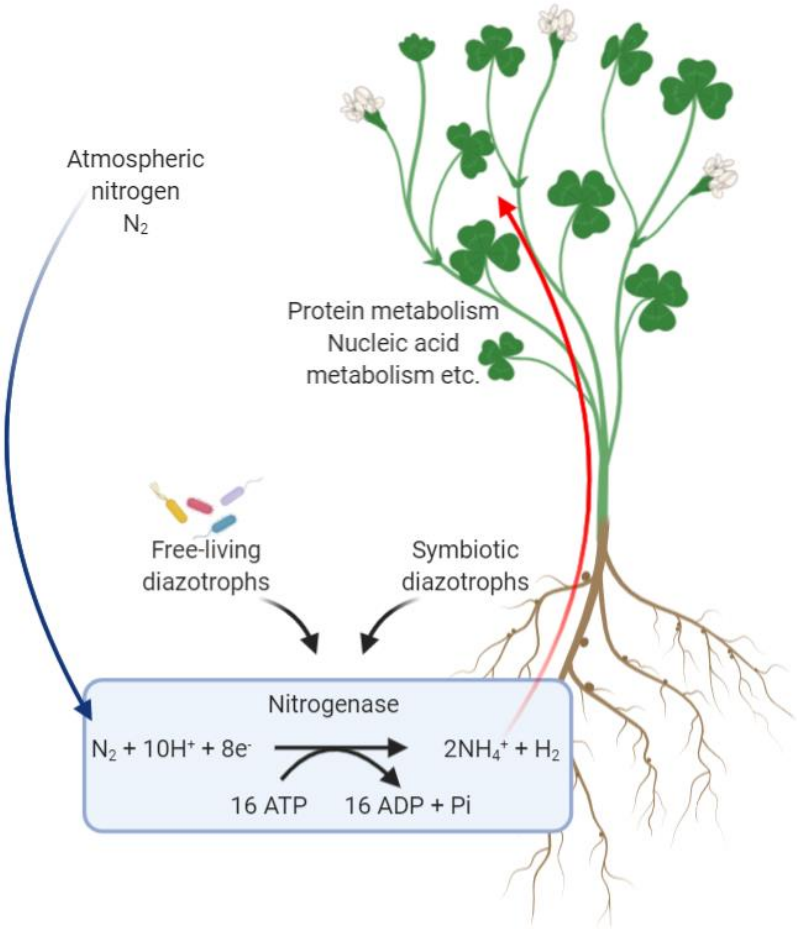

Figure 1.5 | Biological nitrogen fixation. Created with Biorender (https://biorender.com/). Modified from https://phys.org/news/2018-06-exploring-greener-approaches-nitrogen-fixation.html.

1.1.1.2 Phosphate solubilisation by plant growth-promoting bacteria

Phosphorus is a key component in cell membranes, nucleic acids and ATP, making up approximately 0.2% of plant dry weight (Schachtman et al., 1998). In particular, phosphorus influences tomato fruit quality, through increasing lycopene content (Liu et al., 2011). Even though phosphorus is abundant in soil, it is generally not available to plants due to precipitation (Alaylar et al., 2020). PGPB can solubilise phosphate by synthesising low molecular weight organic acids, such as *Azospirillum brasilense* Cd, *A. brasilense* 8-l and *A. lipoferum* JA4 that produce gluconic acid to reduce the pH of calcium phosphate medium and to release soluble phosphate (Rodriguez et al., 2004). Other PGPB promote the mineralization of organic phosphate, such as *Rhizobium leguminosarum* biovar. *viciae* TAL 1236 that produces acidic and alkaline phosphatases when grown on different organic phosphorus compounds (Abd-Alla 1994).

Recently, PGPB strains *Pseudomonas* sp. 42P4 and *Enterobacter* sp. 64S1, isolated from tomato rhizosphere, exhibited plant growth promoting traits, including phosphate solubilisation, suggesting that re-inoculation with native bacteria could increase tomato growth (Pérez-Rodriguez et al., 2020).

Currently, the market of biofertilisers based on phosphate solubilising strains, mainly *Bacillus* spp., is considerably lower (14%) compared to the market of biofertilisers based on nitrogen-fixing bacteria (79%), indicating the need for further research in mechanisms involved in phosphate solubilisation and appropriate formulation for quality and stability of the selected strains (Soumare et al., 2020).

1.1.1.3 Iron mobilization by plant growth-promoting bacteria

Iron is required for vital metabolic processes of plants, such as respiration, photosynthesis and amino acid biosynthesis (Hänsch and Mendel 2009). Although iron is abundant on earth's surface, it exists mainly in insoluble, oxidised ferric state (Guerinot and Yi 1994). For this reason, some graminaceous plants and PGPB synthesise low molecular weight siderophores that solubilise and sequester iron from the soil (Guerinot and Yi 1994) (**Figure 1.6**). Besides directly supplying iron to the plant, siderophore-producing PGPB compete with phytopathogens by reducing available iron from the rhizosphere (Loper and Buyer 1991). Moreover, the most effective PGPB competitors utilise a broad range of siderophores and produce specific siderophores, unavailable for other microbes (Lemanceau et al., 2009; Butaite et al., 2017). Recently, the application of microbial siderophores is increasing (Ghosh et al., 2020), for example pyoverdines or ferrioxamines produced by *Pseudomonas* spp. could be adapted in phytoremediation strategies (Thiem et al., 2018).

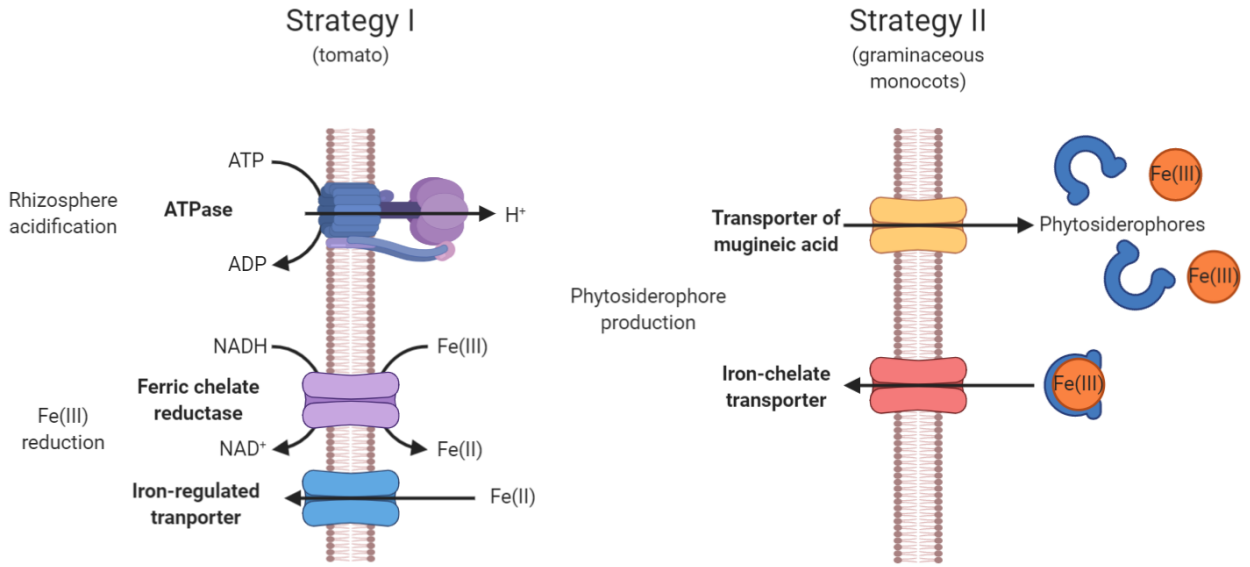

Figure 1.6 | Strategies for iron acquisition in higher plants. Created with Biorender (https://biorender.com/). Modified from Guerinot and Yi 1994.

1.1.1.4 Phytohormone production by plant growth-promoting bacteria

Most physiological activities in plants are regulated by phytohormones, including auxin, cytokinin, gibberellin, abscisic acid, ethylene, salicylic acid, jasmonic acid and brassinosteroids (Davies 2010) (**Figure 1.7**). Phytohormones are present in plants at very low concentrations and enable plants to regulate their growth or respond to the changing environment (Davies 2010; Glick 2020). In addition to plants, PGPB are capable of both synthesising and consuming phytohormones, thus interfering with plant phytohormone levels (Glick 2020).

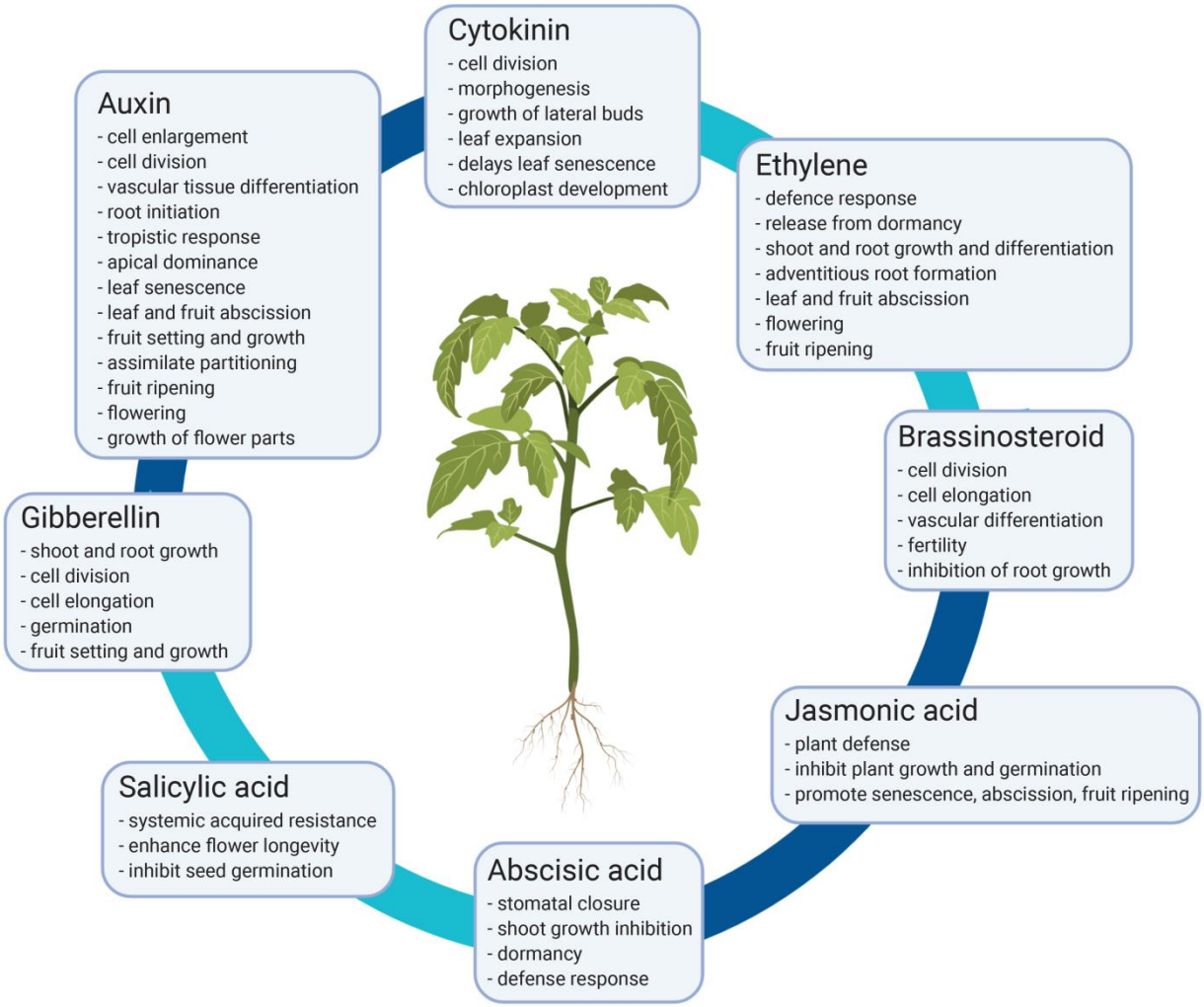

Figure 1.7 | Main roles of phytohormones in plants. Created with Biorender (https://biorender.com/). Modified from Davies, 2010.

Auxins are responsible for several physiological functions in plants, including cell enlargement, cell division, vascular tissue differentiation, root initiation, tropistic response, apical dominance, leaf senescence, leaf and fruit abscission, fruit setting and growth, assimilate partitioning, fruit ripening, flowering and growth of flower parts (Davies 2010). Among auxins, indole-3-acetic acid (IAA) is the most common, naturally occurring plant hormone (Keswani et al., 2020). IAA is synthesised in multiple pathways by plants and 80% of bacteria from the rhizosphere, mainly starting from the precursor tryptophan (Naveed et al., 2015; Keswani et al., 2020). Several PGPB, including the well-studied *Paraburkholderia phytofirmans* PsJN, are able to produce and degrade IAA and interfere with the auxin levels with direct effects on plant

growth processes (Zúñiga et al., 2013; Naveed et al., 2015). Moreover, the presence of auxin interferes with PGPB diversity and activity by increasing the amount of organic carbon exudation (Talboys et al., 2014).

Cytokinins modulate both developmental processes, including gametogenesis, seed germination, apical dominance, root elongation, xylem and chloroplast differentiation, transition to reproductive growth phase, flower and fruit development, leaf senescence and adaptive responses to a changing biotic and abiotic stress conditions, such as nutritional signalling, osmotic stress, cold stress and plant-pathogen interaction (Zwack and Rashotte 2015; Zürcher and Müller 2016; Glick, 2020). Likewise, PGPB can stimulate plant growth by cytokinins production, interacting with cytokinin-signalling pathway, as demonstrated in *Arabidopsis* plants inoculated with *Bacillus* sp. LZR2016 (Wang et al., 2018).

Gibberellins are involved in shoot and root growth, germination, flowering, leaf and fruit senescence (Davies 2010; Glick 2020). For example, *Sphingomonas* sp. LK11 enhanced tomato growth through production of physiologically active gibberellins and IAA (Khan et al., 2014). Likewise, *Bacillus pumilus* and *B. licheniformis* increased growth of *Alnus glutinosa* dwarf phenotype inhibited in gibberellin biosynthesis (Gutiérrez-Mañero et al., 2001), suggesting that growth promotion by PGPB can be explained, at least in part, by gibberellin production (Bottini et al., 2004).

1.1.1.5 ACC-deaminase activity of plant growth-promoting bacteria

The majority of environmental stresses (biotic and abiotic) trigger ethylene production in plants (Sapre et al., 2019). Ethylene interferes with metabolic processes including seed germination, tissue differentiation, root and shoot primordial formation, root branching and elongation and can be either stimulatory or inhibitory depending on its concentration in the tissue (Johnson and Ecker 1998; Glick, 2020). Thus, managing ethylene production is becoming an alternative solution to increase crop yield (Sapre et al., 2019). Moreover, it has been demonstrated that PGPB can improve plant growth under abiotic and biotic stress conditions by exhibiting 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity that reduces the ethylene concentration (Sapre et al., 2019). In more details, after successful colonisation, PGPB synthesise auxin (indole-acetic acid; IAA) that stimulate plant growth and/or induce the transcription of the gene encoding plant ACC-synthase, which converts the precursor of ethylene, S-

adenosylmethionine (SAM) to ACC (Glick et al., 1998). Since auxin increases root exudation, a portion of the newly synthesised ACC is exuded and taken up by the bacteria and subsequently degraded by the bacterial ACC-deaminase to ammonia and α-ketobutyrate (Glick et al., 1998) (**Figure 1.8**). The ACC deaminase activity lowers ethylene levels, thereby reducing the growth inhibition effects of ethylene and facilitating plant growth, especially under biotic or abiotic stress conditions (Glick et al., 1998). For example, ACC-deaminase producing *Pseudomonas* sp. UW4 and *Bacillus subtilis* Rhizo SF 48 mitigated salinity and drought stress by reducing the ethylene levels in tomato plants (Orozco-Mosqueda et al., 2019; Gowtham et al., 2020).

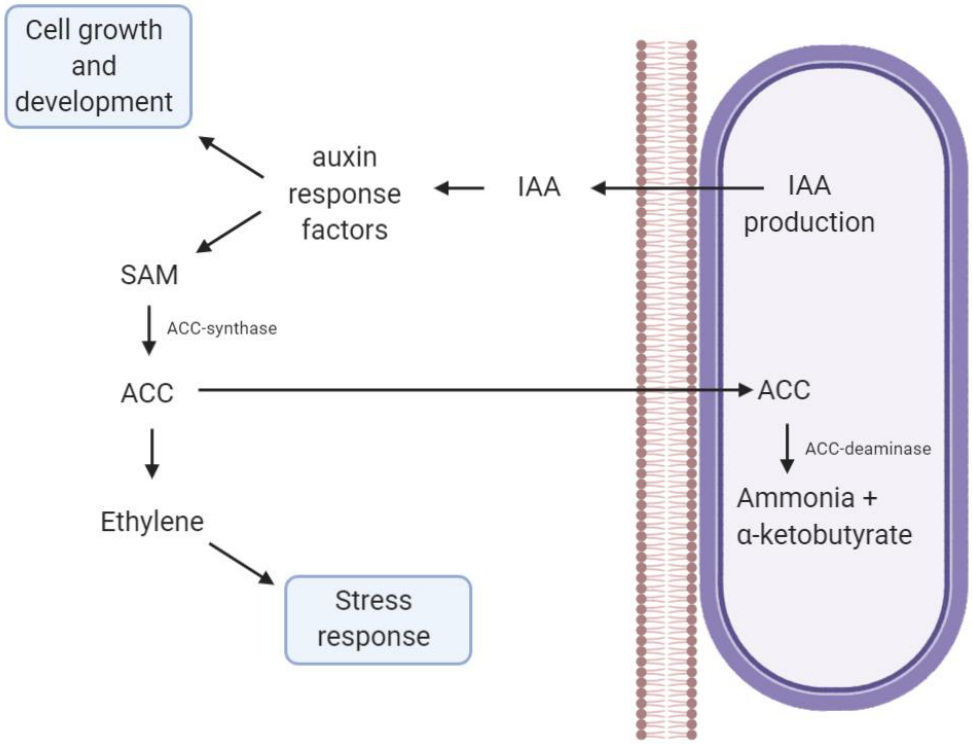

Figure 1.8 | Schematic representation of mechanisms involved in ACC deaminase-producing plant growth-promoting bacteria. Created with Biorender (https://biorender.com/). Modified from Glick 1998.

1.1.2 Indirect mechanisms of microbial-mediated plant growth-promotion

1.1.2.1 Biological control activity of plant growth-promoting bacteria

Several PGPB exhibit antagonistic properties against plant pathogens and arthropods (e.g. nematodes, insects, mites) through competition, protective biofilm formation or releasing bioactive compounds (Ruiu 2020). More specifically, PGPB produce allelochemicals that inhibit

pathogen growth, including antibiotics, hydrogen cyanide (HCN), cell wall degrading enzymes, lipopeptides or, as mentioned above, siderophores (Glick 2020). Thus, some PGPB (such as *Bacillus* spp., *Burkholderia* spp., *Pantoea* spp., *Pseudomonas* spp. or *Streptomyces* spp.) exhibited antagonistic effects against several tomato pathogens, including bacterial speck (*Pseudomonas* spp.; Romero et al., 2016), bacterial spot (*Xanthomonas* spp.; Abbasi and Weselowski 2015), bacterial wilt and canker (*Clavibacter michiganensis* subp. *michiganensis*; Aksoy et al., 2017), bacterial wilt (*Ralstonia solanacearum*; Nawangsih et al., 2011; Huang et al., 2013; Wei et al., 2013; Konappa et al., 2016), damping-off (*Pythium* spp.; Gravel et al., 2005), early blight (*Alternaria* spp.; Khan et al., 2012; Narendra Babu et al., 2015; Attia et al., 2020), gray mould (*Botrytis cinerea*; Mari et al., 1996; Li et al., 2012; Kilani-Feki et al., 2016; Romero et al., 2016), fusarium wilt (*Fusarium oxysporum* f. sp. *lycopersici*; Abdallah et al., 2016; Gowtham et al., 2016), fusarium crown and root rot (*F. oxysporum* f. sp. *radicis-lycopersici*; Omar et al., 2006), crown and stem rot (*Sclerotium rolfsii*; De Curtis et al., 2010) and tomato rot disease (*Rhizoctonia solani*; De Curtis et al., 2010; Goudjal et al., 2014; Ma et al., 2015). In addition, some PGPB possess biocontrol traits against insects, mites and nematodes. For example, *Bacillus thuringiensis*, producing Cry toxins, is a well-documented biocontrol agent, mainly against lepidopteran larvae (Azizoglu 2019). Likewise, *B. subtilis* BsDN delayed *Bemisia tabaci* development on tomato, through establishment of an induced systemic resistance (Valenzuela-Soto et al., 2010). Moreover, *Pseudomonas* sp. Q036B, produces hydrogen cyanide, siderophores, protease, cellulase and chitinase, causing larval mortality of *Bemisia tabaci* (Qessaoui et al., 2020) or *Tuta absoluta* (Qessaoui et al., 2019) and reducing survival rate of the two-spotted spider mite (*Tetranychus urticae*; Qessaoui et al., 2017) on tomato. *Pseudomonas putida* and *Rothia* sp. mitigated *Spodoptera litura* infestation in tomato, via proline production, enhanced activation of antioxidant enzymes, protease and polyphenol oxidases and increased phenolics, protein and chlorophyll content (Bano and Muqarab 2017). Similarly, *B. halotolerans* DDWA, *B. kochii* DDWB, *B. oceanisediminis* DDWC and *B. pseudomycoides* JNC suppressed root-knot nematode (*Meloidogyne incognita*) infestation in tomato in greenhouse and field experiments (Liu et al., 2020), suggesting the potential of PGPB as nonchemical alternatives for controlling plant pathogens and arthropods and for stimulating plant health and growth (Ruiu 2020).

2.3 NON-MICROBIAL PRODUCTS FOR SUSTAINABLE AGRICULTURE

2.1 Plant biostimulation by humic acid

Among non-microbial biostimulants, organic humic substances (e.g. humic acid, humin and fulvic acid) are major components of soil organic matter (Olivares et al., 2017). In particular, humic acid (HA) is abundant in soil, peat or lignite and derives from the decay of organic materials (Drobek et al., 2019). As a consequence, HA is a mixture of polymeric organic compounds, stabilised by weak forces (hydrophobic and hydrogen bonds) in a supramolecular arrangement that forms hydrophobic domains (Fischer, 2017). The underlying direct and indirect mechanisms of HA induced plant growth promotion is related to its chemical compositions (e.g. functional groups), hydrophobicity and flexible conformational structure (Garciá et al., 2016). More specifically, direct effects refer to the interaction of HA with plant cell membranes and metabolic pathways, while indirect effects are due to the modulation of chemical, physical and biological properties of the rhizosphere (Olaetxea et al., 2018) (**Figure 1.9**).

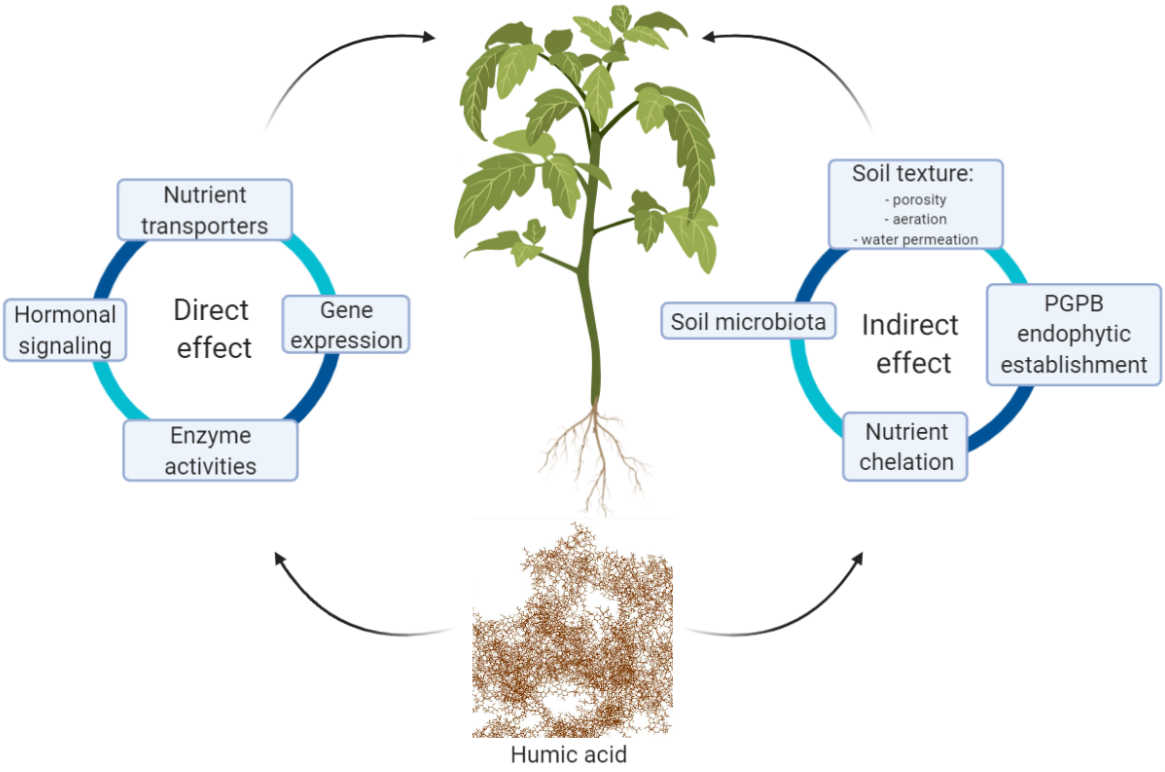

Figure 1.9 | Direct and indirect effect of humic acid on plant growth and development. Created with Biorender (https://biorender.com/). Modified from Olaetxea et al., 2018.

2.1.1 Direct mechanisms of humic acid-mediated plant growth promotion

HA is able to penetrate into plant tissues, activating signalling and metabolic pathways directly related to plant growth and development (Kulikova et al., 2016; Olaetxea et al., 2018). HA affects root growth, modifies root architecture, proliferation of lateral roots and root hairs by activating auxin- and nitric oxide-regulated signalling pathways (Zandonadi et al., 2010). More specifically, the presence of indoleacetic groups (Canellas et al., 2002) and N-isopropyldecanamide (Zandonadi et al., 2019), among the bioactive molecules forming the supramolecular structure of HA, activates root elongation, known as an auxin-like effect by inducing ATPase activity of the plasma membrane and accelerating root nutrient uptake (Zandonadi et al., 2007). Similarly, HA increases plant growth by interacting with jasmonic acid (De Hita et al., 2020) and abscisic acid (Olaetxea et al., 2015) regulation in roots or by inducing the phenylpropanoid pathway (Schiavon et al., 2010; Olivares et al., 2015). Moreover, HA interferes with reactive oxygen species (ROS) accumulation and metabolism, resulting in root growth promotion (Garciá et al., 2016) by calcium channel activation (Foreman et al., 2003). Shoot growth promoting effect of HA is strongly related to increased root plasma membrane ATPase (Mora et al., 2010) and aquaporin activity (Olaetxea et al., 2015). In particular, HA induces shoot growth promotion is associated with the increased activity of root ATPase and translocation of mineral nutrients, including nitrate from root to shoots (Mora et al., 2010). Moreover, this effect is associated with an increased concentration of active cytokinins and polyamines in shoots, which promote shoot growth (Mora et al., 2010).

HA was shown to enhance plant tolerance to abiotic stress, for example HA increased tomato growth and yield under salinity (Türkmen et al., 2004) and heat stress (Abdellatif et al., 2017). Moreover, HA inhibited egg hatching, delayed penetration and increased mortality of *Meloidogyne incognita* in tomato suggesting mitigation to biotic stresses (Jothi and Poornima 2018).

2.1.2 Indirect mechanisms of humic acid-mediated plant growth promotion

HA contributes indirectly to increased root growth and to provide higher nutrient uptake (Olk et al., 2018) by acting on the chemical, physical and biological soil properties, including structure, porosity, water infiltration rate, moisture holding capacity and functional properties of related organism (Nardi et al., 2009). More specifically, HA enhances soil microbial activity (Li et al.,

2019), interacts with plant pathogens (Zaller 2006) and provides physical protection to beneficial microbes (Kaiser et al., 2018). For example, HA can also contribute to the endophytic establishment of PGPB (Olivares et al., 2017) and it has been suggested as a suitable carrier for PGPB formulation (Young et al., 2006; Olivares et al., 2017; Ma 2019) (**Figure 1.10**). Moreover, HA is able to form stable complexes with micronutrients, mainly zinc, manganese, copper and iron, as a result of oxygen-, nitrogen- and sulphur-containing functional groups in its structure, thus increasing their solubility and bioavailability (Zanin et al., 2019). In particular, the acquisition of metals is favoured by HA in calcareous soil where these micronutrients form insoluble complexes (Olaetxea et al., 2018). Moreover, the presence of HA-metal complexes increase phosphate availability through binding phosphorus to HA with iron, magnesium or zinc bridges (Erro et al., 2009; Urrutia et al., 2014). Besides micronutrient chelation, HA also activates genes related to nitrogen (Jannin et al., 2012; Quaggiotti et al., 2004), phosphate (Jindo et al., 2016), sulphate (Jannin et al., 2012) and iron (Aguirre et al., 2009) transport and metabolism, suggesting a combination of direct and indirect mechanism on plant nutrient metabolism (Olaetxea et al., 2018).

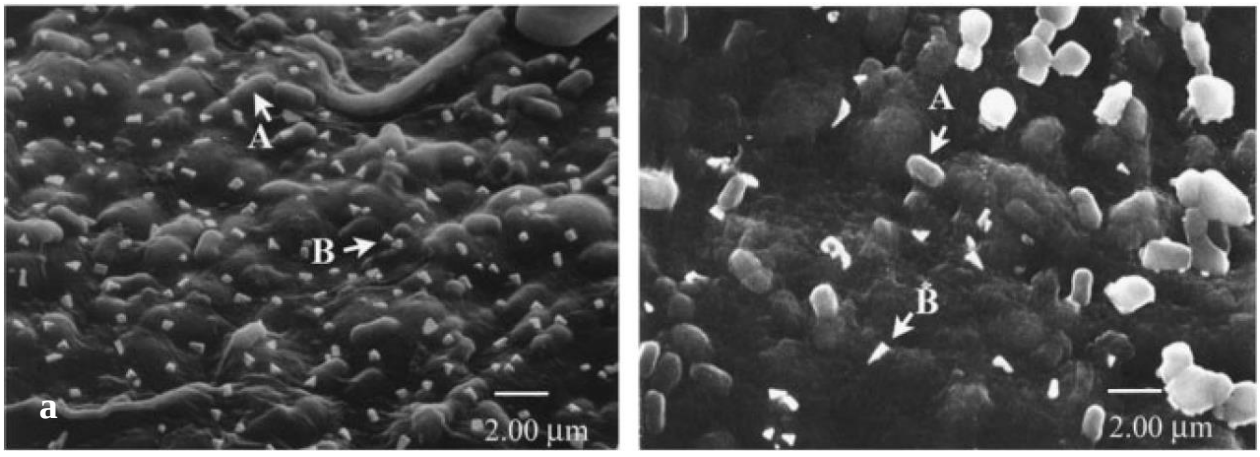

Figure 1.10 | ***Bacillus subtilis*** **CC-pg104 on the bead surface (a) and in the internal gel matrix (b) of humic acid enriched alginate beads as visualised by scanning electron microscopy. A:** ***B. subtilis*** **CC-pg104 B: humic acid. Adapted from Young et al., 2006.**

2.4 BENEFICIAL INSECTS FOR SUSTAINABLE AGRICULTURE

Beneficial insects (e.g. ladybugs, lacewings, hoverflies or bumblebees) are indispensable in both native ecosystems and agriculture and provide beneficial effects, including pollination and biological control (Redhead et al., 2020). Biological control in crop protection is the augmentation or introduction of beneficial organisms for the management of pests, pathogens or weeds (Heimpel and Cock 2018). A large number of beneficial insects and mites have been used with success against pests (Heimpel and Cock 2018), including the release of two mirid predators, *Macrolophus pygmaeus* and *Nesidiocoris tenuis* against the tomato leafminer *Tuta absoluta* in tomato (Urbaneja et al., 2009) . Meanwhile, in some instances biological control agent introductions led to ecologically damaging consequences, such as non-target species loss (Hoddle 2004), indicating the importance of risk assessment protocols prior to beneficial insects releases (Heimpel and Cock 2018). Despite its potential, cost-effectiveness and sustainability, the use of natural pest control still remains a small-scale solution of pest control (Barratt et al., 2018).

Insect-mediated transmission of plant associated microorganisms

Vectors are defined as organisms that can transmit pathogens (e.g. viruses, phytoplasmas, bacteria and fungi) into a plant, thus cause an infection (Purcell and Almeida 2004). Insects, in particular hemipterans (e.g. aphids, whiteflies, psyllids), are considered important vectors of plant pathogens (Heck 2018). Hemipterans possess specialised, elongate mouthparts, called stylets (Schaefer and Panizzi 2000), to penetrate the plant epidermal tissues (Perilla-Henao and Casteel 2016). Hemipteran mouthparts contain two channels, one is used for to pump salivary fluid into the organism while the other is used to suck fluids (Schaefer and Panizzi 2000). Thus, hemipterans interact with microbes inside the plant vascular system and can serve as vectors (Perilla-Henao and Casteel 2016). Meanwhile, plant-virus-vector interactions are widely studied, much less information is available on plant-bacterium-vector or plant-fungus-vector interactions (Perilla-Henao and Casteel 2016). Leafhoppers and psyllids are important vectors of bacterial phytopathogens and Cicadellidae vectors effectively spread the xylem coloniser (*Xylella fastidiosa*) among grapevines (Daugherty et al., 2011). Likewise, Psylliidae vectors transmit *Candidatus* Liberibacter *solanacearum* among tomato plants (Mas et al., 2014). Although the significance of insects as vectors of plant pathogens is unquestionable (Heck 2018), considerably

less information is available about the possible role of insects in transmitting beneficial microbes (Frank et al., 2017). Sap-feeders, pollinators and other arthropods may serve as vectors for endophytic bacterial strains (Frank et al., 2017). For example a hemipteran pest (*Scaphoideus titanus*) was able to transfer non-phytopathogenic bacterial communities between grapvines (Lòpez-Fernàndez et al., 2017). Likewise, plant-mediated transmission of insect symbionts, such as *Cardinium* (Gonella et al., 2015) or *Wolbachia* (Li et al., 2017), occurs in nature, but no information is available on the potential use of beneficial insects to transmit beneficial endophytic microorganisms.

2.5 TECHNIQUES COMMONLY USED TO CHARACTERISE PLANT-MICROBE AND PLANT-INSECT INTERACTIONS

Unravelling plant-microbe and plant-insect interaction by providing better insights can improve efficient strategies to increase productivity and stress alleviation in crops (Sharma et al., 2020). Next generation sequencing, transcriptomics, proteomics, metabolomics and their integration enable a rapid and high-throughput identification of numerous genes, proteins or metabolites related to a relevant characteristic (Li and Yan 2020) (**Figure 1.11**). For example, characterisation of the bioactive compounds of biostimulants and understanding the physiological and molecular mechanisms underlying growth promotion are needed for bioproduct development (Rouphael and Colla 2020a). Likewise, next-generation DNA sequencing allowed scientists to rapidly determine the sequence of large amount of DNA, thus sequencing entire bacterial and plant genomes (Glick 2020). Detailed analysis of PGPB genome reveals traits associated with PGPB activity (Glick 2020). For example, *P. phytofirmans* PsJN genome harbours genes important for endophytic life style and genes related for growth promotion and stress alleviation, such as ACC-deaminase, IAA-synthase, siderophore and secondary metabolite production (Esmaeel et al., 2018). Similarly, whole genome sequencing allowed scientist to study bacterial gene functions of plant- and insect-associated bacteria that are unculturable or difficult to culture (Perilla-Henao and Casteel 2016).

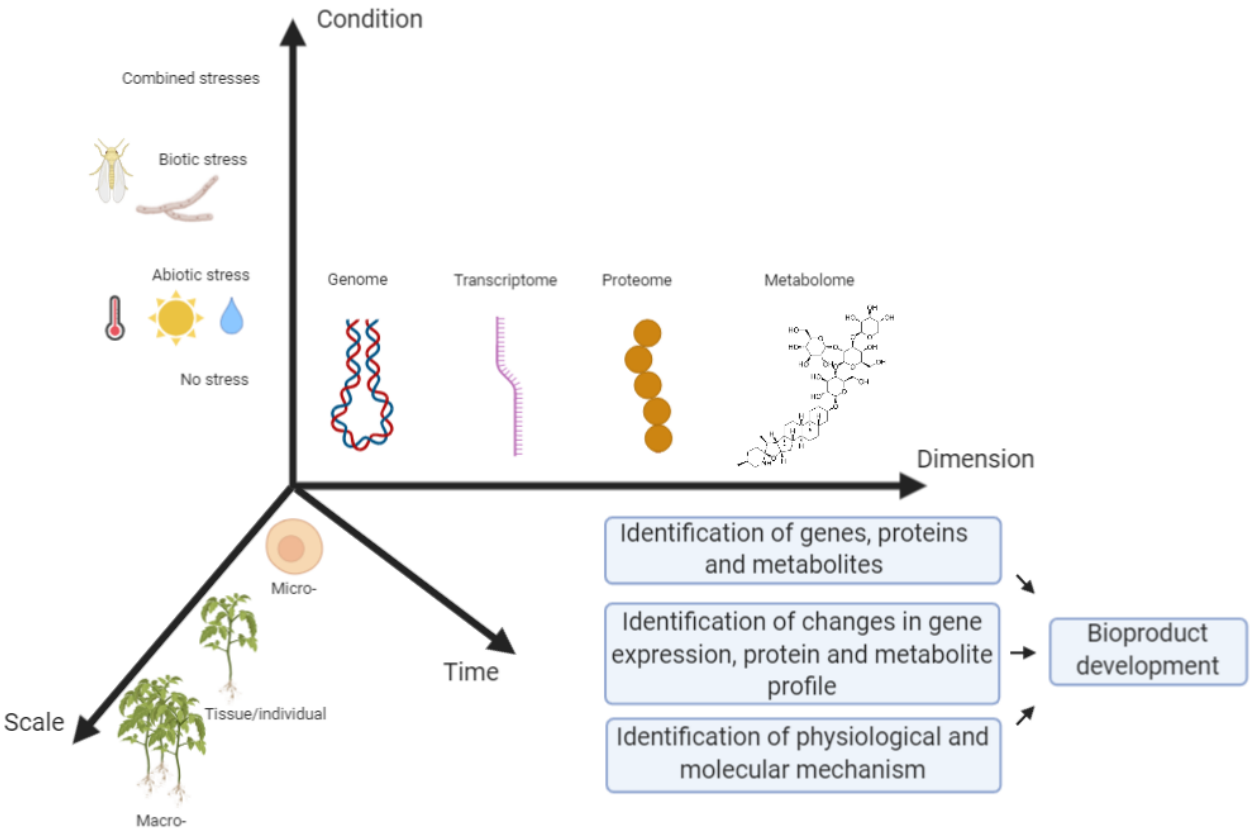

Figure 1.11 | Omics tools for characterisation of genes, proteins, metabolites and metabolic pathways important for sustainable agriculture. Created with Biorender (https://biorender.com/). Modified from Li and Yan 2020.

While genome- and metagenome-based studies reveal the presence of specific genes, transcriptomics, proteomics and metabolomics can be used to understand the changes in plant and/or bacterial regulations as a consequence of their interaction under different conditions (Kaul et al., 2016). Among the above mention techniques, transcriptomics studies the genes transcribed by an organism to provide information on the upregulation or downregulation of genes involved in key metabolic pathways following specific treatments (Glick 2020) (**Figure 1.12)**. In this manner, transcriptomics determines which genes are expressed, as well as, their level of expression (Glick 2020). For example, comparing the transcriptional response of tomato plants inoculated with *Enterobacter radicincitans* grown under different nitrogen fertilisation regimes allowed the identification of genes that play important roles in nitrogen-, phosphorus- and hormone-metabolism and regulation (Berger et al., 2013). Likewise, extracting total RNA from psyllid vectors was used to evaluate *Candidatus* Liberibacter *solanacearum* gene expression in association with its vector (Yao et al., 2016). Moreover, proteomics approaches are applied to

study the synthesised proteins by an organism (Glick 2020). For example strain- and genotype-dependent modifications related to stress response were determined by a proteomics study in ethylene-insensitive and wild-type tomato roots inoculated with *Bacillus megaterium* and *Enterobacter* sp. C7 (Ibort et al., 2018).

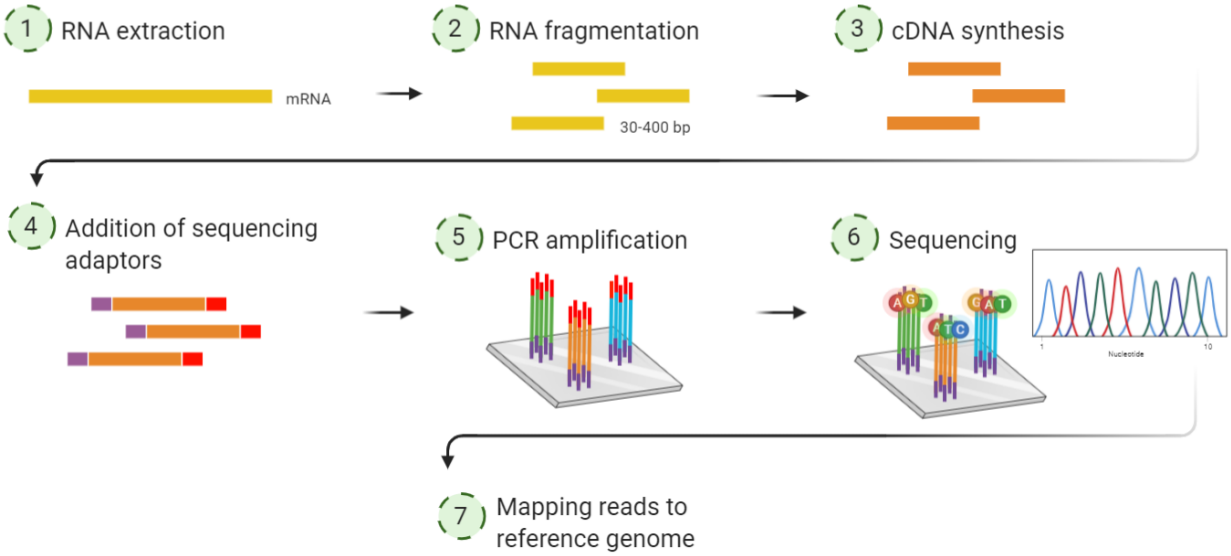

Figure 1.12 | Schematic representation of a transcriptomics workflow by RNA-Seq. Long RNAs are converted into a library of cDNA fragments with adaptors attached to one or both ends. Short sequences, typically 30-400 bp, from one end (single-end sequencing) or both ends (pair-end sequencing) are obtained through high-throughput sequencing technology. Following sequencing, the resulting reads can be aligned to a reference genome. Created with Biorender (https://biorender.com/). Adapted from Wang et al., 2009.

Metabolomics techniques are applied to study the synthesised metabolites and recent developments in mass spectrometry allowed scientists to rapidly and simultaneously measure thousands of different metabolites through targeted or non-targeted approaches (Tugizimana et al., 2013). In targeted approaches, a limited number of selected metabolites can be determined and quantified (Tugizimana et al., 2013). Meanwhile, untargeted studies measure the concentrations of metabolites present in the sample, including unknown or poorly characterised metabolites (Tugizimana et al., 2013). More specifically, metabolites are extracted from the biological samples, separated by liquid chromatography, analysed by high-resolution mass spectrometry and quantified in untargeted metabolomics (Nephali et al., 2020). For example, tomato inoculation with *Pseudomonas aeruginosa* M1 and *Burkholderia gladioli* M2 reduced root galls and decreased survival of the nematode, *Meloidogyne incognita*, through stimulation of

antioxidant machinery and increased level of plant pigments, photosynthesis related enzymes, phenolic compounds (phenols, flavonoids and anthocyanins), osmoprotectants (osmolytes, carbohydrates, reducing sugars, trehalose, proline, glycine betaine and free amino acids) and organic acids (fumaric, succinic, citric and malic acid; Khanna et al., 2019). Integrating omics approaches provides a holistic picture of plant response to different conditions (Li and Yan 2020). For example, citrus trees infected with *Candidatus* Liberibacter *asiaticus*, vectored by the Asian citrus psyllid, *Diaphorina citri*, showed dramatic differences in the transcriptome and proteome profile compared to control plants, supporting the development of diagnostic technologies for presymptomatic and early disease detection (Ramsey et al., 2020).

Besides, studying the interaction at gene, protein and metabolite level, visualisation techniques have greatly improved in the last decades (Compant et al., 2010; Glick 2020). For example, green fluorescent protein (GFP) exhibits green fluorescence when exposed to ultraviolet light, thus allows intact living cells to be monitored in real time (Fan et al., 2011; Glick, 2020) (**Figure 1.13**). Other methods include double labelling of oligonucleotide probes for fluorescent *in situ* hybridisation (DOPE-FISH) to visualise microbes both on the surface and inside plant tissue (Compant and Mathieu 2013) (**Figure 1.14**). For example, *P. phytofirmans* PsJN was detected inside soybean seed embryo after flower inoculation of parent plants (Mitter et al., 2017).

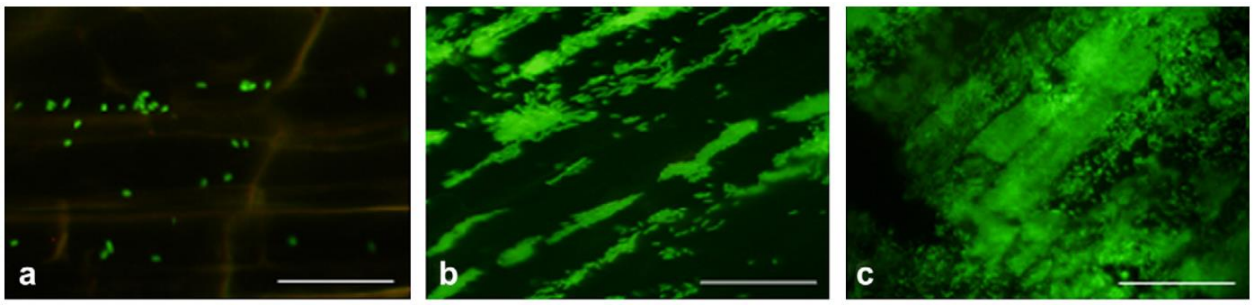

Figure 1.13 | Rhizoplane colonisation of GFP tagged *Paraburkholderia phytofirmans* PsJN on the root surface of grapevine. Scale bars: (a) 15 µm, (b-c) 30 µm. Adapted from Compant et al., 2010.

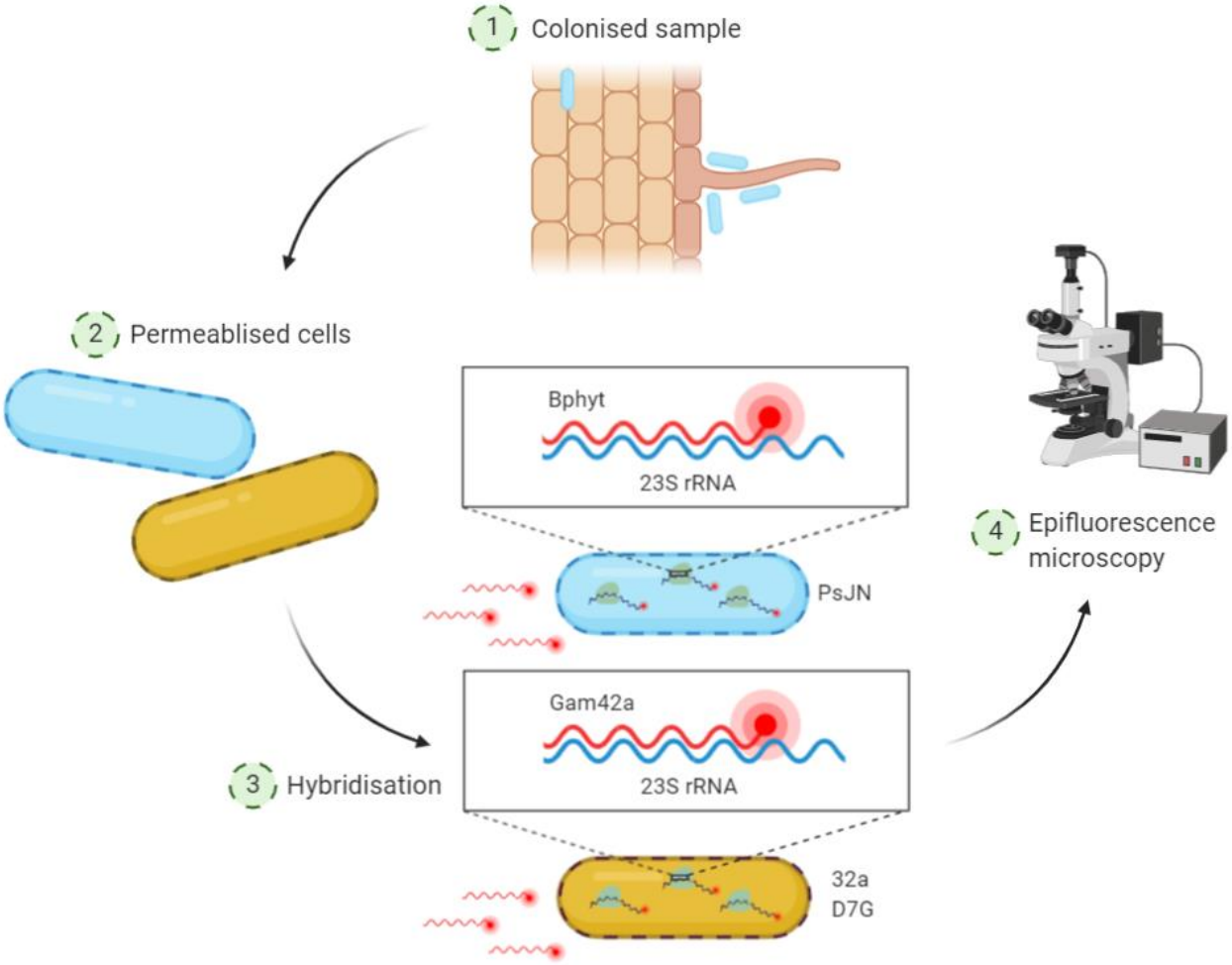

Figure 1.14 | Schematic representation of fluorescent *in situ* hybridisation of double-labelling oligonucleotide probes. Cell membranes are stabilised and permeabilised during the fixation step. Hybridisation is carried out with using double-labelled oligonucleotide probe that attach to its intracellular targets before the excess probe is washed away. The hybridised cells are detected and quantified with epifluorescence microscopy. Created with Biorender (https://biorender.com/). Modified from Amann and Fuchs 2008.

In summary, these techniques enable researchers to improve the understanding of the interaction between plant and PGPB for the further development of sustainable biofertilisers (Glick 2020). Even though, plant biostimulants are relatively new players in complementing synthetic fertilisers, there is a constant need to elucidate the molecular and physiological mechanisms to facilitate their distribution in agriculture (Rouphael and Colla 2020b).

3 AIM OF THE PROJECT

The aim of this thesis is to get insight into the molecular basis of the interaction between endophytic bacterial strains and tomato plants in the presence of HA, in order to improve the understanding on the mechanism responsible for plant growth promotion and to investigate the possible use of beneficial insects as vectors of selected endophytic bacterial strains for the further development of sustainable delivery strategies of bacterial biofertiliser for tomato production.

For this purpose, the specific objectives of this project, addressed in two studies, are:

1. to identify plant growth promoters and stable endophytic colonisers from a bacterial collection and to select the best performing biofertilisers on tomato plants;
2. to understand effects of HA as promoter of bacterial colonisation pattern by double labelling of oligonucleotide probes for fluorescent *in situ* hybridisation;
3. to identify transcriptional processes activated by selected endophytic bacterial strains in tomato plants in the presence and absence of HA;
4. to identify of metabolic pathways related to plant growth promotion activated by selected endophytic bacterial strains in tomato plants in presence and absence of HA;
5. to understand the capacity of beneficial mirids in the acquisition and transfer of selected endophytic bacterial strains.

4 Topic 1: HUMIC ACID ENHANCES THE GROWTH OF TOMATO PROMOTED BY ENDOPHYTIC BACTERIAL STRAINS THROUGH THE ACTIVATION OF HORMONE-, GROWTH-, AND TRANSCRIPTION-RELATED PROCESSES

Humic acid enhances the growth of tomato promoted by endophytic bacterial strains through the activation of hormone-, growth- and transcription-related processes

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Running title: Tomato response to endophytes and humic acid

ABSTRACT

Plant growth-promoting bacteria (PGPB) are promising alternatives in the reduction of the use of chemical fertilisers. Likewise, humic acid (HA) can improve plant growth and/or the establishment of endophytic PGPB. Although the effects of PGPB colonisation or HA treatment have been studied separately, little information is available on plant response to the combined applications of PGPB and HA. Thus, the aim of this work was to understand the physiological effects, bacterial colonisation and transcriptional responses activated by endophytic bacterial strains in tomato roots and shoots in the absence (control condition) and presence of HA (HA condition). Tomato shoot length was promoted by seed inoculation with *Paraburkholderia phytofirmans* PsJN, *Pantoea agglomerans* D7G or *Enterobacter* sp. 32A in the presence of HA, indicating a possible complementation of PGPB and HA effects. Tomato colonisation by endophytic bacterial strains was comparable in the control and HA condition. The main transcriptional regulations occurred in tomato roots and the majority of differentially expressed genes (DEGs) was upregulated by endophytic bacterial strains in the HA condition. Half of the DEGs was modulated by two or three strains as possible common reactions to endophytic bacterial strains, involving protein metabolism, transcription, transport, signal transduction and defence. Moreover, strain-specific tomato responses included the upregulation of signal transduction, transcription, hormone metabolism, protein metabolism, secondary metabolism and defence processes, highlighting specific traits of the endophyte-tomato interaction. The presence of HA enhanced the upregulation of signal transduction, hormone metabolism, transcription, protein metabolism, transport, defence and growth-related processes in terms of number of involved genes and fold change values. This study provides detailed information on HA-dependent enhancement of growth-related processes stimulated by endophytic bacterial strains in tomato plants and reports the optimised dosages, complementation properties and gene markers for the further development of efficient of PGPB- and HA-based biostimulants.

Keywords: plant growth-promoting bacterial endophytes, humic acid, transcriptomics, RNA sequencing, endophytic tomato colonisation

INTRODUCTION

Conventional agriculture largely depends on chemical fertilisers (e.g. nitrogen-, phosphorus-, potassium- and micro element-based fertilisers), which have numerous environmental drawbacks, such as surface and groundwater pollution and denitrification processes (Khan et al., 2018). Among crop plants, tomato (*Solanum lycopersicum*) is cultivated worldwide under field and greenhouse conditions (Hobson and Grierson 1993) and requires an extensive use of chemical fertilisers that cause a significant negative environmental impact (Maham et al., 2020).

Plant growth-promoting bacteria (PGPB) can improve plant development and increase nutrient supply, such as nitrogen and iron (Ferreira et al., 2019). PGPB application has been considered as a promising alternative to maintain agroecosystem health and productivity (Gouda et al., 2018). Some PGPB can colonise the internal tissues of numerous plant species (endophytes) and can positively influence plant growth through various mechanisms, including the production of hormones, the improvement of nutrient uptake and protection against biotic or abiotic stresses (Gaiero et al., 2013). In particular, species of the bacterial genera *Bacillus, Enterobacter, Microbacterium, Pantoea, Paraburkholderia* and *Sphingomonas* are known to establish this type of association with plants (Sessitsch et al., 2005; Campisano et al., 2014; Hardoim et al., 2015). For example, bacterial endophytes isolated from grapevine, such as *Microbacterium* sp. C9D (C9D), *Pantoea agglomerans* D7G (D7G), *P. eucalypti* 727 (727) and *Sphingomonas* sp. 11E (11E), were able to increase the seed germination of *Arabidopsis thaliana* and exhibited beneficial traits *in vitro*, such as 1-aminocyclopropane-1-carboxylic acid (ACC)-deaminase activity (Campisano et al., 2014; Lòpez-Fernàndez et al., 2015a). Other endophytic bacteria, such as *Bacillus* sp. 54A (54A) and *Enterobacter* sp. 32A (32A), inhibited the growth of plant pathogens (e.g. *Botrytis cinerea*, *Botryosphaeria dothidea* and *Botryosphaeria obtusa*) in dual-culture plate tests, suggesting that these strains can potentially protect plants against infections (Campisano et al., 2014; Lòpez-Fernàndez et al., 2015b). Among them, 32A affected the secondary metabolism and activated possible defence pathways in grapevine (Lòpez-Fernàndez et al., 2015a). A widely studied plant endophyte, *Paraburkholderia phytofirmans* PsJN (PsJN), previously classified as *Pseudomonas* and *Burkholderia* (Sessitsch et al., 2005; Sawana et al., 2014) is known to increase *A. thaliana* tolerance to salt stress through transcriptional and metabolic changes, such as proline accumulation, abscisic acid signalling and reactive oxygen species (ROS) scavenging (Pinedo et al., 2015). In particular, PsJN is able to improve the growth (Pillay and Nowak 1997; Sharma and Nowak 1998) and heat tolerance (Issa et

al., 2018) of tomato plants, increasing net photosynthesis rate, stomatal conductance and chlorophyll content. For these reasons, the use of PGPB could be a promising approach in tomato production to improve plant growth and to reduce the use of chemical fertilisers. However, limitations to the wide use of beneficial endophytes were often encountered, for example because of the variable and/or inconsistent effect on the plant, especially under field conditions (Martínez-Viveros et al., 2010; Timmusk et al., 2017). Although they have been relatively well studied, a better understanding on the bacterial colonization (e.g. colonization rate and stability, competition with other microorganisms) and effects on tomato physiology (e.g. transcriptional response) is needed, in order to develop more efficient PGPB-based biofertilisers.

In addition to PGPB, organic humic substances present in the soil (e.g. humic acid, humin and fulvic acid) can also improve plant growth and health and act as biostimulants (Olivares et al., 2017). Biostimulants are organic bioactive compounds that affect plant metabolism (Drobek et al. 2019). Among the natural biostimulants, humic acid (HA) is abundant in soil, peat or lignite and derives from the decay of organic materials (Drobek et al., 2019). HA improves nutrient uptake and the growth of tomato plants under hydroponic (Adani et al., 1998) and greenhouse conditions (Dursun et al., 2002), increasing electrolyte leakage, cell permeability and nutrient accumulation (David et al., 1994). HA is a mixture of polymeric organic compounds, stabilized by weak forces (hydrophobic and hydrogen bonds) in a supramolecular arrangement that forms hydrophobic domains (Fischer 2017). HA is refractory to degradation and its hydrophobic domains can provide protection for selected PGPB (Piccolo 1996; Canellas and Olivares 2014). The hydrophobic HA domain undergoes conformational changes in the presence of organic acids derived from root exudates and releases PGPB for the interaction with host plants (Nardi et al., 2009; Olivares et al., 2017). HA can also contribute to the endophytic establishment of PGPB (Olivares et al., 2017) and it has been suggested as a suitable carrier for PGPB formulation (Young et al., 2006; Olivares et al., 2017; Ma 2019). For example, *Herbaspirillium seropedicae* Z67 inoculation in the presence of HA increased root surface area, enhanced grain production and altered carbohydrate and nitrogen metabolism in maize plants (Canellas et al., 2012). In particular, in low fertility soils, *H. seropedicae* Z67 and HA increased maize production compared to non-inoculated plants through PGPB-driven hormone production and HA-stimulated changes in phenolic metabolism (Canellas et al., 2015). Likewise, tomato fruit biomass was increased by *H. seropedicae* HRC54 and HA through the stimulation of nitrogen and secondary metabolism (Olivares et al., 2015). A mixed inoculum of *H. seropedicae* HRC54 and *Gluconacetobacter diazotrophicus* PAL 5 in combination with HA changed the metabolite

fingerprints of amino acids, sugars and organic acids in maize and sugarcane seedlings, indicating that the activation of primary and secondary metabolism was partially responsible for the biostimulation effects (Aguiar et al., 2018; Canellas et al., 2019). Although considerable evidence of efficacy exists in literature, the molecular mechanisms of the combined applications of living PGPB and organic biostimulant on crops are less investigated (Bulgari et al., 2015). Our goal was to improve the understanding of the complementation effects and cellular pathways activated by endophytic bacterial strains and HA for the further development of sustainable biofertilisers for tomato production. More specifically, the present study aimed at understanding the colonisation, growth promotion effects and transcriptional responses in tomato plants inoculated with bacterial endophytes in the absence (control condition) and presence of HA (HA condition).

MATERIAL AND METHODS

Growth of bacterial strains and inoculum preparation

The bacterial strains *Microbacterium* sp. C9D (C9D; isolate MiVv2), *Bacillus* sp. 54A (54A; isolate BaVs16), *Pantoea eucalypti* 727 (727; isolate PaVv9), *Pantoea agglomerans* D7G (D7G; isolate PaVv7), *Enterobacter* sp. 32A (32A; isolate EnVs6) and *Sphingomonas* sp. 11E (11E) were previously isolated from the grapevine endosphere (Campisano et al., 2014), while *Paraburkholderia phytofirmans* PsJN (PsJN) was isolated from surface-sterilised onion roots (Sessitsch et al., 2005). Bacterial strains were stored in 80% glycerol at -80 °C and were grown in 5 mL nutrient broth (NB) in sterile 15 mL-tubes at 25°C for 24 h under orbital shaking at 220 rpm.

For seed inoculation, bacterial cells were collected by centrifugation at 3,500 g for 10 min and washed twice with sterile 10 mM $MgSO_4$. Bacterial cells were then suspended in sterile 10 mM $MgSO_4$ and the bacterial suspension was adjusted to 1.0×10^7 colony forming units (CFU) per unit of volume (CFU mL^{-1}) based on an optical density conversion table at 600 nm (OD_{600}) optimised for each strain (**Table S1**).

Since HA is poorly soluble in water, a stock solution (1 g L^{-1}) of HA (Sigma-Aldrich, St. Louis, Missouri, USA; code 53680) was prepared in 0.1 M NaOH and the pH was then adjusted to 6.8 with 70% HNO_3 (HA stock solution) to avoid acidification of the NB and half-strength Hoagland. Since $NaNO_3$ was formed in the HA preparation, a water solution with NaOH and HNO_3 at an equivalent

concentration to the HA stock solution was used as control in the bacterial compatibility, tomato seed inoculation and transcriptomic analyses (control stock solution).

Bacterial compatibility assay with humic acid

To assess the bacterial compatibility with HA, 20 µL of each bacterial suspension (1.0×10^7 CFU mL^{-1}) was inoculated in 200 µL NB supplemented with 50 mg L^{-1} HA (10 µL HA stock solution) in a 96-well microplate (Thermo Fisher Scientific, Waltham, MA, USA). NB supplemented with 10 µL control stock solution was used as control (0 mg L^{-1} HA). Samples were incubated at 25°C for 72 h under orbital shaking programmed at medium shaking speed and bacterial growth was monitored by measuring the OD_{600} every 30 min using a Synergy 2 Multi-Mode Microplate Reader (Biotek, Winooski, VT, USA). Six replicates (wells) were used for each treatment and the experiment was carried out twice.

Tomato seed inoculation and growth conditions in glass tube and square dish

Seeds of *S. lycopersicum* L. cv. Moneymaker (Justseed, Wrexham, UK) were treated with 70% ethanol for 1 min and 2% sodium hypochlorite containing 0.02% Tween 20 for 5 min in a 50 ml-tube (Subramanian et al., 2015) with vigorous shaking and washed three times with sterile distilled water (3 min each), in order to reduce the number of seed-associated microorganisms. Surface-sterilised seeds (50 seeds) were treated with 5 mL of sterile 10 mM $MgSO_4$ (mock-inoculated) or inoculated with 5 mL of the bacterial suspension (bacterium-inoculated) of the respective endophytic strain (1×10^7 CFU mL^{-1}) by overnight incubation at 25±1°C in a sterile 15 mL-tube under orbital shaking at 40 rpm. Seeds were transferred to Petri dishes (20 seeds for each dish) containing 1% water agar (Thermo Fisher Scientific) and incubated for 48 h in a growth chamber (Binder KBWF 720, Bohemina, NY, USA) at 25±1°C with a 16 h photoperiod (photon flux density of 0.033 mmol s^{-1} m^{-2}) to allow seed germination.

Germinated seeds with the same root length (1 mm) were selected and transferred to the growth medium in a glass tube or in a square dish as described below. To optimise the humic acid concentration for tomato plants, each germinated seed was transferred into a sterile 95 mL glass tube (Artiglass, Padova, Italy) containing 2.5 g sterile perlite and 10 mL half-strength Hoagland with 0, 25, 50 or 100 mg L^{-1} HA, and incubated in the growth chamber for six weeks. To assess the effect of HA on bacterium-inoculated plants, five seeds were transferred along a line in a central position of a 10cm-square dish (Sarstedt, Nümbrecht, Germany) containing 50 mL solid (14 g L^{-1} agar) half-

strength Hoagland with 0 mg L^{-1} (control condition; 2.5 mL control stock solution for each dish) or 50 mg L^{-1} HA (HA condition; 2.5 mL HA stock solution for each dish), as optimised HA concentration. Dishes were incubated in vertical position in the growth chamber, shoot and root length was measured with a ruler and the fresh weight of the whole plant was assessed with a precision balance at three and six days after incubation (DAI). Four and five replicates were analysed for each treatment in the experiment with glass tubes and square dishes, respectively, and each experiment was carried out twice.

Bacterial re-isolation from tomato plants

At the end of the incubation period, mock-inoculated and bacterium-inoculated plants were collected, and each plant was surface-sterilised in a 50 ml-tube with 70% ethanol for 1 min, 2% sodium hypochlorite for 1.5 min, followed by 70% ethanol for 1 min. Plants were washed three times with distilled water (2 min each), dried with a sterile filter paper before the assessment of the fresh weight. Plants were ground in a mixer-mill disruptor (MM 400, Retsch, Haan, Germany) at 25 Hz for 2 min in presence of 500 µL potassium phosphate buffer (1 mM, pH 7). Each suspension was serially diluted and 10 µL aliquots were plated in triplicates on nutrient agar (NA). Aliquots (10 µL) of the last washing solution were plated as the control of surface sterilisation. After incubation at 25°C for three days, CFU values of endophytic bacterial strains per unit of plant fresh weight (CFU g^{-1}) were calculated. Five replicates (plants) were analysed for each treatment and the experiment was carried out twice.

Fluorescence *in situ* hybridisation using double labelling of oligonucleotide probes

Double labelling of oligonucleotide probes for fluorescence *in situ* hybridisation (DOPE-FISH) was performed on mock-inoculated plants and PsJN-, D7G- or 32A-inoculated plants at 3 and 6 DAI in the control or HA condition in square dishes. Plants were aseptically cut into roots, stem and leaves and were sectioned transversally using razor blades. Samples were then fixed in a 4% paraformaldehyde in 1x phosphate-buffered saline (PBS) solution at 4°C for five hours and were rinsed three times with 1xPBS as previously reported (Compant et al., 2011). Plants were dehydrated in increasing concentrations of ethanol solution (25, 50, 75 and 99%; 20 min each step) and stored at 4°C. DOPE-FISH was carried out using probes from Eurofins (Germany) labelled at both the 5'and 3'positions. A probe mixture targeting eubacteria, composed of EUB338, EUB338II, EUB338III (EUBmix) coupled with a Cy3 fluorochrome and Bphyt probe targeting the 23S rRNA gene of PsJN

coupled with Cy5 (Amann et al., 1990; Daims et al., 1999; Mitter et al., 2017). For D7G and 32A, EUBmix and Gam42a probe targeting the 23S rRNA gene of D7G and 32A coupled with Cy5 was used (Manz et al., 1992). NONEUB probe coupled with Cy3 or Cy5 was used independently as negative control (Wallner et al. 1993). Fluorescent *in situ* hybridisation was carried out in sterile 1.5 mL-tubes at 46°C for 2 h in the dark with 60 µl hybridisation buffer for PsJN (containing 0.9 M NaCl; 0.02 M Tris HCl, 0.01% SDS, 10% formamide and 5 ng μL^{-1} of each probe) and with 60 µl hybridisation buffer for D7G and 32A (containing 0.9 M NaCl; 0.02 M Tris HCl, 0.01% SDS, 35% formamide and 5 ng μL^{-1} of each probe). Washing was conducted at 48°C for 30 min with a pre-warmed post-FISH solution containing 0.02 M Tris HCl, 0.01% SDS, NaCl and EDTA at a concentration corresponding to the formamide concentration. Samples were then rinsed with distilled water before overnight air-drying in the dark. Samples were observed under a confocal microscope (Olympus Fluoview FV1000 with multiline laser FV5-LAMAR-2 and HeNe (G) laser FV10-LAHEG230-2). Pictures were taken at 405, 488, 633 nm wavelengths with Cy3 assigned as green and Cy5 as red. Pictures were analysed using Imaris 8 software (BITPLANE, UK). Z-stacks were used to generate whole-stack pictures. Five replicates (plants) were analysed for each treatment and representative pictures were selected. Pictures were cropped and light/contrast balance improved in post process.

Sample collection, RNA extraction, and Illumina sequencing

Mock-inoculated plants and PsJN-, D7G- or 32A-inoculated plants in square dishes were collected at 3 DAI in the control and HA condition in square dishes. Five plants were randomly collected for each treatment (replicate), roots and shoots were cut, separately placed into 2 ml-tubes, immediately frozen in liquid nitrogen and stored at −80°C. A metal bead was added to each tube and samples were ground in a mixer-mill disruptor (MM200, Retsch) at 25 Hz for 1 min. Total RNA was extracted from 0.1 g of ground sample using a Spectrum Plant Total RNA Kit (Sigma-Aldrich) with an on-column DNase treatment with RNase-Free DNase Set (Qiagen, Hilden, Germany). Total RNA was quantified using a Qubit (Thermo Fisher Scientific) and RNA quality was checked using a Tapestation 2200 (Agilent Technologies, Santa Clara, CA, USA). For each treatment, three replicates (pool of five plants) were analysed. RNA samples were subjected to RNA-Seq library construction, using the TruSeq SBS v3 protocol (Illumina, SanDiego, CA, USA) and rRNA depletion with the RiboZero rRNA Removal Kit for plant according to the manufacturer's instructions (Illumina). Paired-end reads of 150 nucleotides were obtained using a NovaSeq 6000 S2 instrument (Illumina) at

the Institute of Applied Genomics (Udine, Italy) and sequences were deposited at the Sequence Read Archive of the National Center for Biotechnology (https://www.ncbi.nlm.nih.gov/sra) under the BioProject number PRJNA622763.

Bioinformatic analysis and identification of differentially expressed genes

Raw reads were cleaned and filtered using the programme Trimmomatic version 0.36 (Bolger et al., 2014) and low-quality bases with an average Phred quality score lower than 15 in a sliding window of four base were removed. Any resultant reads shorter than 36 bp in length were removed from the analysis and the quality check of filtered reads was performed using Fast QC version 0.11.7. Filtered read pairs were aligned and counted using STAR 2.7 (Dobin et al., 2013) to the *S. lycopersicum* genome release ITAG3.2 and counts of unambiguously mapped read pairs was obtained during the alignment with the STAR 2.7 program. Differentially expressed genes (DEGs) were identified with the Limma-Voom package (Law et al., 2014), which estimates the mean–variance relationship of Log_2-transformed counts, generating a precision weight for each observation that is fed into the Limma empirical Bayes analysis pipeline (Smyth 2006). A Volcano Plot was generated using the Python programming language and the matplotlib package (Hunter 2007) and a double cut-off on *P*-value ($P \leq 0.01$) and minimum Log_2 fold change (FC) of one [Log_2 (FC) ≥ 1 or Log_2 (FC) ≤ -1] were imposed to identify DEGs through pairwise comparisons. Three pairwise comparisons were analysed for shoots and roots: PsJN- vs. mock-inoculated, D7G- vs. mock-inoculated and 32A- vs. mock-inoculated plants. DEGs modulated by endophytic bacterial strains between the control and HA condition were compared in order to identify HA-dependent effects on processes activated by PsJN, D7G and 32A. Moreover, the pairwise comparison between the control and HA condition of mock-inoculated plants was included, in order to analyse the effects caused by HA in the absence of endophytic bacterial strains. The distribution of DEGs was summarised using the Venn diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/) and DEGs were grouped in upregulated and downregulated genes by at least two endophytic bacterial strains (DEGs modulated by two or three strains), to highlight possible common reactions in response to endophytic bacterial strains, or specifically by only one endophytic bacterial strain (PsJN-, D7G or 32A-specific tomato DEGs) in the control and HA condition. The heat map diagram of fold change values of DEGs was visualised using the Java Treeview tool (Saldanha 2004). Gene expression levels were then expressed as transcripts per million (TPM).

Gene Ontology (GO) terms and protein descriptions of tomato Heinz1706 genes (Sato et al., 2012) of the release ITAG3.2 were downloaded from the tomato genome browser (https://solgenomics.net/organism/Solanum_lycopersicum/genome). GO terms significantly overrepresented ($P \leq 0.05$, Benjamin and Hochberg FDR correction) in the DEG lists in comparison to the whole tomato transcriptome were identified using the Biological Networks Gene Ontology (BiNGO) tool (Maere et al., 2005) and the biological networks were visualised with Cytoscape version version 3.7.1 (Shannon et al., 2003). DEGs were further annotated on the basis of tomato protein description and grouped into 14 functional categories according to the previous literature. Genes that were not associated to any biological process were assigned to the unknown function category. Tomato cellular pathways were generated with Biorender (https://biorender.com/) according to literature search of functional annotation of DEGs.

Gene expression analysis by quantitative real-time RT-PCR

Tomato gene markers were selected for quantitative real-time PCR (qPCR) analysis (**Table S2**). The first strand of cDNA was synthesised from 1 µg of DNase-treated RNA using Superscript III (Invitrogen, Thermo Fisher Scientific) and oligo-dT primer. qPCR reactions were carried out with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Thermo Fisher Scientific) and specific primers using the Light Cycler 480 (Roche Diagnostics, Mannheim, Germany) as previously described (Perazzolli et al., 2016). Briefly, the PCR conditions were: 50°C for 2 min and 95°C for 2 min as initial steps, followed by 40 cycles of 95 °C for 15 s and 60°C for 1 min. Each sample was examined in three technical replicates and dissociation curves were analysed to verify the specificity of each amplification reaction. The Light Cycler 480 SV1.5.0 software (Roche) was used to extract Ct values based on the second derivative calculation and the LinReg software version 11.0 was used to calculate reaction efficiencies for each primer pair (Ruijter et al., 2009). For each gene, the relative expression level (fold change) was calculated according to the Pfaffl equation (Pfaffl 2001) for each pairwise comparison between bacterium-inoculated and mock-inoculated samples in the control and HA condition. Five housekeeping genes were analysed, such as genes encoding ankyrin repeat domain containing protein 2 (*ARD2*) (Pombo et al., 2017), kinesin light chain 2 isoform (*KLC*) (Pombo et al., 2017), vernalisation insensitive 3 (*VIN3*)(Pombo et al., 2017), small nuclear ribonucleoprotein family protein (*LSM7*) (Müller et al., 2015) and ubiquitin carboxyl-terminal hydrolase (*UCH*) (Müller et al., 2015), and their stability was validated using the ΔCt method (Silver et al., 2006). *ARD2* was then selected as constitutive gene for normalisation, because its expression

was not affected by the different conditions (**Table S2**). Three replicates (pool of five plants) were analysed for each condition.

Statistical analysis

All functional experiments were carried out twice and data were analysed with the Past 3.26 software (Hammer et al., 2001). After validating data for normal distribution (Shapiro-Wilk test, $P > 0.05$) and variance homogeneity of the data (Levene's tests, $P > 0.05$), each experimental repetition was analysed singularly and a two-way analysis of variance (ANOVA) was used to demonstrate non-significant differences between the two experiments ($P > 0.05$). Data from the two experimental repetitions were pooled and significant differences among treatments were assessed with the Student's t-test ($P \leq 0.05$) and the Tukey' test ($P \leq 0.05$) in case of pairwise and multiple comparisons, respectively. CFU values of bacterial resolution were Log_{10}-transformed and fold change values of gene expression analysis were Log_2-transformed. The Pearson's correlation coefficient between gene expression levels assessed by RNA-Seq and qPCR analysis was calculated with the Excel program.

RESULTS

Endophytic bacterial strains and humic acid enhance tomato growth

HA improved tomato growth (**Figure S1**), the maximum growth promotion of shoot and root length was obtained with 50 mg L^{-1} HA and this dosage was selected as optimised HA concentration for the subsequent experiments (HA condition). All endophytic bacterial strains grew in presence of 50 mg L^{-1} HA (**Figure S2** and **Table S1**). The tomato shoot length was longer in PsJN-, D7G-, 32A- and 11E-inoculated plants compared to mock-inoculated plants in the absence of HA (control condition; **Figure S3A**). Likewise, PsJN, D7G and 32A improved tomato shoot length in the HA condition and these three strains were selected for the subsequent experiments. Plants were colonised by the endophytic bacterial strains tested and PsJN, D7G and 32A were re-isolated from surface-sterilised tomato plants at 6 DAI at comparable levels in the control and HA condition (**Figure S3B**). Tomato shoot length was promoted by PsJN, D7G and 32A at 3 DAI in the control condition and it was also stimulated by HA in mock-inoculated plants, through a possible complementation of the endophytic PGPB and the organic biostimulant (**Figure 1**). Moreover, PsJN, D7G and 32A confirmed the

promotion of tomato shoot length at 6 DAI in the HA condition and indicated different effects of growth promotion according to the incubation time (**Figure S4**).

To better characterise the colonisation of tomato tissues by endophytic bacterial strains, the DOPE-FISH analysis was carried out in the control and HA condition using specific probes targeting the 23S rRNA gene and universal probes for bacteria. Yellow fluorescent PsJN (**Figure 2A-B)**, D7G (**Figure 2C-D**) and 32A (**Figure 2E-F**) single cells, aggregates and micro-colonies were found on the secondary root emergency site, root tip, root elongation zone, root hair and xylem of tomato roots in the control and HA condition. PsJN, D7G and 32A cells were also found on the tomato stem in the control and HA condition (**Figure S5**) and the colonization intensity of tomato roots among the tested strains were comparable in the control and HA condition at 3 DAI (**Figure 2**) and 6 DAI (**Figure S6**). In mock-inoculated plants only some native bacteria were present (**Figure S7**). The NONEUB probe was used as negative probe not targeting bacterial sequences and only a few green/blue-cyan autofluorescent microbes could be seen in mock-, PsJN-, D7G- and 32A-inoculated plants as indication of the rare presence of native microorganisms (**Figures S7 and S8**).

Endophytic bacterial strains and humic acid modulate tomato genes in roots and shoots

To further characterise the plant response to endophytic bacterial strains and HA, a transcriptomic analysis of tomato shoots and roots was carried out. From 11.7 to 23.8 million reads were obtained for each replicate of tomato shoots and roots collected from mock-inoculated plants and plants inoculated with PsJN, D7G and 32A at 3 DAI in the control and HA condition (**Table S3**). More than 80.0% of tomato genes were expressed in at least one condition (**Table S4**). A total of 6,135 and 623 DEGs were identified in tomato roots and shoots respectively, according to the pairwise comparisons between bacterium-inoculated (PsJN-, D7G- and 32A-inoculated) and mock-inoculated plants in the control and HA condition, while 4,227 and 422 genes were modulated by HA in mock-inoculated roots and shoots, with a *P*-value lower than 0.01 and minimum Log_2-transformed fold change of one (**Tables S5-S10**). The majority of DEGs was downregulated (79.4%) by endophytic bacterial strains in the control condition. Conversely, DEGs were mainly upregulated (80.0%) by endophytic bacterial strains in the HA condition, as a consequence of a possible HA-dependent enhancement of tomato reactions to endophytic bacterial strains (**Figure 3**). DEGs were grouped in genes modulated by at least two endophytic bacterial strains (DEGs modulated by two or three strains), to highlight possible common reactions to bacterial endophytes, or specifically by only one endophytic bacterial strain (PsJN-, D7G or 32A-specific DEGs), to highlight possible strain-specific reactions, in roots or shoots

in the control and HA condition (**Figures 3 and S9**). The RNA-Seq results were validated by a qPCR analysis of ten tomato genes (**Table S2**) that were selected according to their expression profiles [five genes modulated in roots and five in shoots; five modulated only in the control condition and three modulated only in the HA condition and belonging to one of the four different clusters (modulated by two or three strains, PsJN-specific, D7G-specific or 32A-specific)] and functional categories (e.g. defence, growth and development, hormone metabolism, oxidative stress, protein metabolism, secondary metabolism, transcription and transport). A close correlation (Pearson correlation coefficient, 0.93) between RNA-Seq and qPCR expression data was observed (**Figure S10**). In particular, expression profiles generated by qPCR and RNA-Seq agreed completely for eight genes and differed slightly for two genes (**Table S2**), possibly due to differences in the method sensitivity and discrimination capacity of multigene families (Perazzolli et al., 2016).

Endophytic bacterial strains activate a complex transcriptional response in tomato roots according to the presence of humic acid

In tomato roots, 539 and 3,688 genes were upregulated and downregulated by HA in mock-inoculated plants, respectively (**Figure S11A and Table S5**). A significant enrichment of GO categories related to regulation of metabolic process and regulation of transcription was found for genes upregulated by HA (**Figure S11D**), such as transcription factors (e.g. 12 MYB, seven WRKY, five NAC domain-containing and two ethylene-responsive transcription factors) and signal transduction-related genes (e.g. 14 kinases, eight calcium-binding proteins and four receptor kinases; **Figure S11C** and **Table S5**). Moreover, genes downregulated by HA in tomato roots indicated global repression of cellular metabolic processes and energy-related processes (**Figure S11E**).

There were 119 and 926 genes upregulated by two or three strains in the control and HA condition, respectively (**Tables S6 and S7**). Genes upregulated by two or three strains in tomato roots in the control condition were mainly involved in protein metabolism (e.g. one cysteine desulfurase, three F-box proteins and one tyrosine aminotransferase), transcription [e.g. two basic helix-loop-helix transcription factors, (bHLH), three zinc finger proteins and two WRKYs], transport (e.g. one heavy metal transport protein, one iron-regulated transporter, three potassium channels, one potassium transporter and two vacuolar iron transporters), signal transduction (e.g. four kinases, one receptor kinase and two serine/threonine-protein kinases) and defence [e.g. four defensin-like proteins, two nucleotide-binding site leucine-rich repeat proteins (NBS-LRRs) and three leucine rich repeat (LRR) receptorlike proteins; **Figure 4A** and **Tables S6**]. As a possible common reaction to

bacterial endophytes, a significant enrichment of GO categories related to defence response, response to stimulus and oxidative stress (e.g. five peroxidases and one glutaredoxin) was found for upregulated genes by two or three strains in the control condition (**Figure 4B and Table S6**). The presence of HA enhanced the transcriptional changes activated in response to two or three strains, in terms of number of genes and FC values. Thus, genes related to protein metabolism, transcription [e.g. one WRKY, one ethylene (ET) response factor and two ET responsive transcription factors], transport, signal transduction (e.g. six receptor kinases, 17 protein kinases, three calcium transporting ATPases, one calcium-dependent protein kinase, one calcium/calmodulin-dependent serine/threonine-kinase and 16 serine/threonine-protein kinases) and defence (e.g. four NBS-LRRs, four defensin-like proteins) were upregulated by two or three strains in the HA condition, together with genes implicated in the growth and development process (e.g. two cellulose synthases, six glycosyltransferases, one mannosyltransferase, two pectin lyases and three pectinesterases), in the hormone metabolism (e.g. four 1-aminocyclopropane-1-carboxylate synthases, three cytokinin riboside phosphoribohydrolases, one gibberellin oxidase, one gibberellin dioxygenase, one auxin efflux facilitator and eleven small auxin responsive proteins) and response to oxidative stress (e.g. seven peroxidases, four glutaredoxins and one glutathione S-transferase; **Figure 4A and Table S7**).

PsJN-specific genes revealed the upregulation of genes related to protein metabolism (e.g.one lysine-ketoglutarate reductase and one threonine aldolase), transcription [e.g. four basic helix loop helix transcription factors (bHLHs), two basic-leucine zipper family proteins (bZIP), six MYBs and three WRKY transcription factors and five zinc finger proteins], transport (e.g. two mannose transporter, one phosphate transporter and one potassium transporter), defence (e.g. two disease resistance proteins, four LRR receptor like proteins and six NBS-LRRs), signal transduction (e.g. nine kinases and eight receptor kinases) and hormone metabolism in the control condition (**Figure 4A**). As a consequence, the GO categories related to the chitin metabolic process and the aminoglycan and polysaccharide catabolic processes were enriched in the cluster of PsJN-specific genes in the control condition (**Figure 4C**). In addition, PsJN-specific genes upregulated in the HA condition were involved in protein metabolism (e.g. one cysteine desulfurase, one glutamate dehydrogenase and ten F-box proteins), transcription (e.g. two bHLHs, five zinc finger proteins, one MYB and two WRKYs), defence (e.g. one disease resistance proteins, three LRR receptor like proteins and one phenylalanine ammonia-lyase) and signal transduction (e.g. four serine/threonine-kinases, one histidine kinase, four protein kinases and one receptor kinase), as possible enhancement of tomato response in the HA condition (**Figure 4A**). D7G-specific genes upregulated in the control

condition were involved in transport, defence, growth and development (**Figure 4A**), while those upregulated in the HA condition were mainly involved in protein metabolism (e.g. one cysteine synthase and one glutamate dehydrogenase), transcription (e.g. one bHLH and two MYB transcription factors) and signal transduction (e.g. three protein kinases). In particular, D7G-specific genes involved in the response to hormone stimulus were upregulated in the HA condition (e.g. five small auxin responsive proteins and an ET receptor; **Figure 4D**) and control condition (e.g. 1-aminocyclopropane- 1-carboxylate synthase, auxin-regulated IAA protein, cytokinin hydrolase). Likewise, 32A-specific genes upregulated in the HA condition were mainly involved in protein metabolism, energy metabolism (e.g. nine NADH dehydrogenases, one cytochrome c oxidase and one ATPase) and transcription (e.g. three ankyrin repeat family proteins, seven bHLHs, eight zinc finger proteins; **Figure 4A**). In particular, the GO categories related to secondary metabolism and amino acid metabolism were enriched (**Figure 4E**) in the cluster of 32A-specific genes in the HA condition, together with genes related to oxidative stress response (e.g. 14 thioredoxins, three glutathione S-transferases, three superoxide dismutases, three glutaredoxins and two peroxidases; **Figure 4A and Table S7**). Thus, the cellular processes involved in tomato root response to endophytic bacterial strains in the control and HA condition revealed the activation of a complex recognition machinery that involves signal transduction pathways and the consequent activation of transcription-, protein-, transport- and defence-related pathways (**Figures 5 and S12**). Different recognition processes were activated by PsJN, D7G and 32A, and the presence of HA enhanced the upregulation of signal transduction, hormone metabolism, transcription, protein metabolism, transport, defence and growth-related process in terms of number of DEGs and fold change values. Conversely, genes downregulated by endophytic bacterial strains in tomato roots suggest fine regulation of protein metabolism, DNA metabolism and secondary metabolism in the control and HA condition (**Figure S13**).

Endophytic bacterial strains activate a complex transcriptional response in tomato shoots according to the presence of humic acid

HA incubation caused the upregulation and downregulation of 52 and 170 genes in tomato shoots of mock-inoculated plants, respectively (**Figure S11B** and **Table S8**). Tomato genes upregulated by HA were involved in primary metabolism (**Figure S11C**) and indicated the activation of the GO categories related to carbohydrate metabolism, alcohol metabolism and cell wall macromolecule

metabolism (**Figure S11F**). Conversely, genes related to ROS metabolism were mainly downregulated by HA (**Figure S11G**).

Genes upregulated by two or three strains in tomato shoots in the control condition were mainly related to protein metabolism, defence, growth and development (**Figure 6A; Tables S9 and S10**). A significant enrichment of the GO categories related to cell growth was found for upregulated genes by two or three strains in the control condition, such as cell wall-related processes (e.g. one glucan synthase and two xyloglucan endotransglucosylases; **Figure 6B**). In the HA condition, the enrichment of the GO categories related to aminoglycan metabolism and chitin metabolism was found for upregulated genes by two or three strains (**Figure 6C**). Genes associated to primary metabolism (e.g. two 2-oxoglutarate oxygenases and one lipase), protein metabolism (e.g. one calreticulin and one cysteine desulfurase) and transport (e.g. one calcium transporting ATPase) were upregulated by two or three strains in the HA condition (**Figure 6A**), in agreement with the shoot length promotion.

PsJN-specific genes modulated in tomato shoots were mainly related to protein metabolism, transcription and defence in the control condition (**Figure 6A**). Similarly, PsJN-specific genes upregulated in the HA condition were involved in transcription (e.g. one MYB and one zinc finger protein), defence and transport. Tomato processes related to transcription, growth and development (e.g. one cyclin and one cell division cycle protein), transport and defence were also upregulated by D7G in the control condition (**Figure 6A**), with the enrichment of the inositol and polyol GO processes (**Figure 6D**). In the HA condition, genes involved in transcription (e.g. one ET responsive transcription factor), growth and development (e.g. one expansin and two glycosyltransferases) and transport (e.g. one aluminium-activated malate transporter and two lipid transfer proteins) were upregulated by D7G. Moreover, 32A-specific genes upregulated in the control condition were mainly involved in the secondary metabolism and defence (**Figure 6A**) and the GO categories related to oxidative stress and phosphorylation were enriched (**Figure 6E**). 32A-specific genes related to transcription (e.g.one WRKY transcription factor), growth and development (e.g. one expansin, one xyloglucan hydrolase and one xyloglucan endoglucanase inhibitor), signal transduction (e.g. two LRR kinases) and oxidative stress response (e.g. one glutathione S-transferase and one peroxidase) were upregulated in the HA condition (**Figure 6A** and **Table S10**). In summary, cellular processes activated in tomato shoots in response to endophytic bacterial strains included recognition-, signal transduction- and transcription-related pathways, with an enhancement of transport- and growth-related processes in the HA condition (**Figure 7**). On the other hand, downregulated genes in tomato

shoots were related to stress response and DNA metabolism in the control condition, as well as lipid transport in the HA condition (**Figure S14**).

DISCUSSION

Some strains belonging to the bacterial genera *Enterobacter*, *Pantoea* and *Paraburkholderia* had already been previously recognized as PGPB (Sessitsch et al., 2005; Campisano et al., 2014; Hardoim et al., 2015) and this study demonstrated that seed inoculation with PsJN, D7G and 32A promotes tomato shoot growth. Inoculated tomato plants were efficiently colonised by the tested endophytic bacterial strains and HA did not increase the tissue colonisation compared to the control condition. Moreover, the addition of HA (at the optimal concentration of 50 mg L^{-1}) enhanced the tomato growth induced by the endophytic bacterial strains, suggesting some possible complementation effects of HA to the tested PGPBs. HA was known to improve nutrient uptake in tomato plants (Adani et al., 1998; Dursun et al., 2002), by increasing electrolyte leakage, cell permeability and nutrient accumulation (David et al., 1994) and activating primary and secondary metabolism (Aguiar et al., 2018; Canellas et al., 2019). HA incubation upregulated genes responsible for cellular regulations in mock-inoculated plants, such as transcription factors, receptors and kinases, and altered the transcriptional response of tomato plants to endophytic bacterial strains.

Tomato genes were modulated by endophytic bacterial strains mainly in roots (2,919 and 3,216 in the control and HA condition respectively) compared to shoots (355 and 268 in the control and HA condition, respectively), indicating major transcriptional regulations in belowground compared to aboveground tissues. The majority of DEGs was downregulated (79.4%) by endophytic bacterial strains in the control condition. Conversely, DEGs were mainly upregulated (80.0%) by endophytic bacterial strains in the HA condition, suggesting enhanced reactions of tomato plants to bacterial endophytes in the presence of HA. In particular, the majority of genes upregulated by the endophytic bacterial strains in roots in the HA condition was not modulated (64.2%) or downregulated (33.1%) in roots in the control condition, while only 2.7% was upregulated, but with lower extent, also in the control condition, indicating that specific genes are implicated in the tomato response to bacterial endophytes in the presence of HA. In particular, the presence of HA enhanced the activation of signal transduction, hormone metabolism, transcription, protein metabolism, transport, defence and growth-related processes in response to PsJN, D7G and 32A inoculation, as better discussed in the following

paragraphs. Moreover, half of the DEGs (45.5%) was modulated by at least two endophytic bacterial strains and they represent possible common pathways modulated in response to bacterial endophytes.

Transcriptional response of tomato roots and shoots to two or three endophytic bacterial strains and humic acid

Plant roots play a critical role in perception and recognition of the rhizosphere microorganisms (Palma et al., 2019) and the presence of HA enhanced the activation of signal transduction and transcription processes in response to endophytic bacterial strains. These functional categories were activated by two or three strains and they included genes encoding receptor kinases, protein kinases and NBS-LRR proteins, indicating the activation of a common recognition machinery to bacterial endophytes in tomato roots. In particular, serine/threonine kinases were upregulated by HA and two or three strains in roots, and protein kinases were also involved in HA-induced signalling in rice (Ramos et al., 2015) and *A. thaliana* (Trevisan et al., 2011). The elevation of intracellular calcium is also an indicator of plant response to beneficial microorganisms (Vadassery and Oelmüller 2009) and a modulation of calcium- and calmodulin-related genes was found in response to HA alone and two or three strains in the HA condition.

Since beneficial effects of endophytic bacterial strains can derive from multiple mode of action (Glick 2012; Ferreira et al., 2019), it is difficult to discriminate effects of microbial activities in providing nutrients to plants and/or direct stimulation of plant growth (e.g. modulation of the hormone levels). The increase of nutrient uptake was known as one of the mechanisms of plant growth promotion caused by PGPB (Glick 2012) and HA (Zanin et al., 2019). In this study, tomato genes related to potassium and iron transport were upregulated by two or three strains in the control condition and genes related to magnesium, nitrogen, phosphate, sulphate and zinc transport were upregulated in the HA condition, which makes them possible markers of tomato biostimulation. We found that ATPase-encoding genes were upregulated by two or three strains in the HA condition and by HA alone, and membrane pumps were previously found as activated by humic substances in tomato (Zandonadi et al., 2016) and maize (Quaggiotti et al., 2004), suggesting a positive effect of HA on tomato nutrient uptake.

Another mechanism of PGPB-dependent plant growth promotion is the modulation of the hormone levels (Glick 2012). Tomato genes related to jasmonic acid (JA) response (e.g. WRKY transcription factors and defensins) were upregulated by two or three strains in the control and HA condition, while those related to ET synthesis (e.g. 1-aminocyclopropane-1-caroxylate synthases) and

ET response (ET response factor and ET responsive transcription factors) were mainly upregulated in the HA condition. The interplay of auxin and ET signalling pathways was found also in the PsJN-dependent *A. thaliana* growth promotion (Poupin et al., 2016) and some auxin-responsive genes (e.g. auxin efflux facilitator and auxin responsive proteins) were upregulated by two or three strains in the HA condition and by HA alone. Likewise, the WAT1-related genes were upregulated by endophytic bacterial strains in both conditions and these genes are known to be involved in auxin transport and homeostasis, as well as in growth promotion and cell wall development (Irizarry and White 2018), indicating a complex hormonal response to endophytic bacterial strains in the presence of HA. In particular, some genes implicated in gibberellin biosynthesis (e.g. copalyl diphosphate synthase, gibberellin oxidase, gibberellin dioxygenase) and cytokinin metabolism (e.g. cytokinin riboside phosphoribohydrolases) were upregulated by HA alone and by two or three strains in the HA condition. PsJN, D7G and 32A were able to produce auxin (Poupin et al., 2013; Campisano et al., 2014) and PsJN was able to induce gibberellin synthesis in *A. thaliana* (Poupin et al., 2013). Likewise, humic substances upregulated auxin responsive genes in *A. thaliana* (Trevisan et al., 2010) and showed cytokinin-like (Pizzeghello et al., 2012) and gibberellin-like (Nardi et al., 2000) activity in maize plants, suggesting additive effects of endophytic bacterial strains and HA in the stimulation of growth-related hormone metabolism in tomato.

As a possible consequence of hormonal changes, genes upregulated by at least two endophytic bacterial strains in the HA condition were involved in cell growth and cell wall biosynthesis, such as cellulose synthases, glycosyltransferases, mannosyltransferases, pectin lyases, pectinesterases, glucan synthase and two xyloglucan endotransglucosylases as key markers of tomato biostimulation. Pectin and cellulose are implicated in cell wall expansion and the upregulation of genes encoding cell wall modification enzymes has been observed in growth promotion processes activated by *Pseudomonas fluorescens* in *A. thaliana* (Wang et al., 2005) and *Bacillus amyloliquefaciens* in cotton (Irizarry and White 2018). Thus, the upregulation of cell wall-loosening enzymes may be a common plant response to PGPB, in order to facilitate endophytic colonisation and plant growth promotion (Irizarry and White 2018). Markers of an attempted defence reaction and oxidative stress response were also upregulated by two or three strains in the control and HA condition. In particular, the upregulation of glutaredoxins, glutathione S-transferases and peroxidasesindicate the activation of the antioxidant machinery, as possible HA-dependent modulation of plant reaction to bacterial endophytes.

Transcriptional response of tomato roots and shoots specifically activated by *Paraburkholderia phytofirmans* PsJN, *Pantoea agglomerans* D7G or *Enterobacter* sp. 32A

Different signalling pathways were activated by PsJN, D7G or 32A, indicating a strain-specific response activated in tomato plants. Receptor kinases and transcription factors (e.g. bHLH, bZIP, MYB and WRKY) were upregulated specifically by PsJN in roots in the control and HA condition. Similarly, PsJN induced the expression of receptor-like kinase genes in swtichgrass (Lara-Chavez et al., 2015) and bZIP, MYB and WRKY transcription factors in *A. thaliana* (Timmermann et al., 2019) as possible key regulators of plant response to PsJN. Moreover, D7G- and 32A-specific genes upregulated in tomato roots and shoots included a distinctive signal transduction (e.g. protein kinases) and transcription (e.g. bHLH, MYB, WRKY and zinc finger transcription factors) process responsible for plant reaction to endophytic bacterial strains.

The strain-specific response of tomato involved the hormone metabolism. For example, the upregulation of salicylic acid (SA) biosynthesis (phenylalanine ammonia lyase) and SA responsive (e.g. pathogenesis-related genes) genes was found in PsJN-inoculated roots, suggesting SA accumulation in the HA condition, as previously shown in PsJN-inoculated switchgrass (Lara-Chavez et al., 2015). SA, JA and ET were implicated in PsJN-induced resistance (Timmermann et al., 2019) and the interplay of ET with the auxin signalling pathways was responsible for PsJN-dependent growth promotion in *A. thaliana* (Poupin et al., 2016). The auxin signalling- (indole-3-acetic acid inducible and dormancy associated/auxin-repressed) and transport-related (auxin efflux facilitator) genes were upregulated by PsJN in tomato roots in the control and HA condition, respectively. The hormone-related genes were upregulated also by D7G in the control and HA condition (e.g. auxin responsive genes, ET-related receptor and transcription factor, auxin and cytokinin metabolic genes) and this endophytic strain showed ACC-deaminase activity and auxin production activity *in vitro* (Campisano et al., 2014). As possible additive effect, the presence of HA can affect the auxin-related processes, as shown in *A. thaliana* (Canellas et al., 2010) and tomato (Canellas et al., 2011) plants, suggesting a complementation effect of endophytic bacterial strains and HA.

The protein metabolic pathways were activated by 32A in tomato roots in the HA condition, indicating the activation of nitrogen assimilation with upregulation of genes related to the metabolism of lysine, serine, glycine, cysteine, tyrosine, threonine, glutamine, alanine, arginine and methionine. Likewise, the nitrogen and secondary metabolism was activated in *H. seropedicae* HRC54-inoculated tomato plants in the presence of HA (Olivares et al., 2015) and the increased

concentration of amino acids and secondary metabolites was found in sugarcane plants inoculated with *H. seropedicae* HRC54 and *G. diazotrophicus* PAL 5 in the presence of HA (Aguiar et al., 2018; Canellas et al., 2019). In particular, 32A was able to fix atmospheric nitrogen *in vitro* (Campisano et al., 2014) and it caused the upregulation of a glutamine synthetase gene in tomato roots in the HA condition. Glutamine synthase encoding genes were also upregulated by endophytic diazotroph bacteria in sugarcane (Nogueira et al., 2005) and an increased amino acid content was found in sugarcane inoculated with the diazotroph *Pantoea* sp. 9C strain (Loiret et al., 2009), suggesting that the activation of the amino acid metabolism contributes to plant growth promotion. Amino acids are key precursors of secondary metabolites and genes related to secondary metabolism were induced by 32A in the HA condition. In accordance with these findings, a previous study had demonstrated that 32A affected the accumulation of secondary metabolites in grapevine plants as a possible mechanism for the successful host colonisation (Lòpez-Fernàndez et al., 2015a). Likewise, the precise tuning of the plant defence by the endophytic bacterial strains could contribute to a successful host colonisation. For example, the antioxidant machinery was activated in tomato roots mainly in the HA condition, indicating the activation of an attempted defence reaction against endophytic bacterial strains that is probably tuned by the endophytic bacterial strains to allow tissue colonisation.

CONCLUSIONS

Growth promotion effects and transcriptional responses activated by bacterial endophytes in tomato plants were affected by the presence of HA, indicating a complementation effect of PGPB and the organic biostimulant under controlled conditions. In particular, HA enhanced the activation of pathways responsible for signal transduction, hormone metabolism, transcription, protein metabolism, transport, defence and cell growth in response to the endophytic bacterial strains. Major transcriptional regulations occurred in tomato roots and involved global reactions activated by endophytic bacterial strains, including protein metabolism, transcription, transport, signal transduction and defence processes. The optimised HA dosage and an in-depth knowledge of tomato reaction to bacterial colonisation derived by this study represent key information for the further development of combined formulations of endophytic bacterial strains and HA as a tailored diet for tomato biostimulation. In addition, genes identified in this work may be the source of important markers of tomato biostimulation that can be used to monitor the plant response to bacterial endophytes and HA under field conditions.

DATA AVAILABILITY STATEMENT

RNA-Seq sequences were deposited at the Sequence Read Archive of the National Center for Biotechnology (https://www.ncbi.nlm.nih.gov/sra) under the BioProject number PRJNA622763.

AUTHOR CONTRIBUTIONS

NG, carried out the functional experiments, performed DOPE-FISH experiments, microscopy analyses and wrote the manuscript; SC, performed DOPE-FISH experiments and microscopy analyses; MM, analysed the RNA-Seq data; CS; helped in the functional experiments; AG and FW, revised the manuscript; IP, revised the manuscript and analysed the data; GP, conceived the study and revised the manuscript; MP, conceived the study, supervised the experiments, analysed the data and wrote the manuscript. All the authors revised and approved the manuscript.

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CONFLICT OF INTEREST

NG and FW are employed by Biobest NV. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPLEMENTARY DATA

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.582267/full#supplementary-material

REFERENCES

Adani, F., Genevini, P., Zaccheo, P., and Zocchi, G. (1998). The effect of commercial humic acid on tomato plant growth and mineral nutrition. Journal of Plant Nutrition. 21: 561–575. doi: 10.1080/01904169809365424.

Aguiar, N.O., Olivares, F.L., Novotny, E.H., and Canellas, L.P. (2018). Changes in metabolic profiling of sugarcane leaves induced by endophytic diazotrophic bacteria and humic acids. PeerJ. 6: e5445. doi: 10.7717/peerj.5445.

Amann, R.I., Binder, B.I., Olson, R.J., Chisholm, S.W., Devereux, R., and Stahl, D.A. (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Applied and Environmental Microbiology. 56: 1919–1925.

Bolger, A.M., Lohse, M., and Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics. 30: 2114–2120. doi: 10.1093/bioinformatics/btu170.

Bulgari, R., Cocetta, G., Trivellini, A., Vernieri, P., and Ferrante, A. (2015). Biostimulants and crop responses: A review. Biological Agriculture and Horticulture. 31: 1–17. doi: 10.1080/01448765.2014.964649.

Campisano, A., Pancher, M., Puopolo, G., Puddu, A., Lòpez-Fernàndez, S., Biagini, B., Yousaf, S., and Pertot, I. (2014). Diversity in endophyte populations reveals functional and taxonomic diversity between wild and domesticated grapevines. American Journal of Enology and Viticulture. 66: 12-21. doi: 10.5344/ajev.2014.14046.

Canellas, L.P., Balmori, D.M., Médici, L.O., Aguiar, N.O., Campostrini, E., Rosa, R.C.C., Façanha, A.R., and Olivares, F.L. (2012). A combination of humic substances and *Herbaspirillum seropedicae* inoculation enhances the growth of maize (*Zea mays* L.). Plant and Soil. 366: 119–32. doi: 10.1007/s11104-012-1382-5.

Canellas, L.P., Dantas, D.J., Aguiar, N.O., Peres, L.E.P., Zsogon, A., Olivares, F.L., Dobbss, L.B., Facanha, A.R., Nebbioso, A., and Piccolo, A. (2011). Probing the hormonal activity of fractionated molecular humic components in tomato auxin mutants. Annals of Applied Biology. 159: 202–211. doi: 10.1111/j.1744-7348.2011.00487.x.

Canellas, L.P., and Olivares, F.L. (2014). Physiological responses to humic substances as plant growth promoter. Chemical and Biological Technologies in Agriculture. 1: 3 doi: 10.1186/2196-5641-1-3.

Canellas, L.P., Piccolo, A., Dobbss, L.B., Spaccini, R., Olivares, F.L., Zandonadi, D.B., and Façanha, A.R. (2010). Chemical compositon and bioactivity properties of size-fractions separated from a vermicompost humic acid. Chemosphere. 78 (4): 457–466. doi: 10.1016/j.chemosphere.2009.10.018.

Canellas, L.P., Silva, S.F., Olk, D.C., and Olivares, F.L. (2015). Foliar application of *Herbaspirillum seropedicae* and humic acid increase maize yields. Journal of Food, Agriculture and Environment. 13 (1): 146–153.

Canellas, N.O.A., Olivares, F.L., and Canellas, L.P. (2019). Metabolite fingerprints of maize and sugarcane seedlings: Searching for markers after inoculation with plant growth‐promoting bacteria in humic acids. Chemical and Biological Technologies in Agriculture. 6: 14. doi: 10.1186/s40538-019-0153-4.

Compant, S., Mitter, B., Colli-Mull, J.G., Gangl, H., and Sessitsch, A. (2011). Endophytes of grapevine flowers, berries, and seeds: Identification of cultivable bacteria, comparison with other plant parts, and visualization of niches of colonization. Microbial Ecology. 62: 188–197. doi: 10.1007/s00248-011-9883-y.

Daims, H., Brühl, A., Amann, R., Schleifer, K.-H., and Wagner, M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all bacteria: Development and evaluation of a

more comprehensive probe set. Systematic and Applied Microbiology. 22: 434–444. doi: 10.1016/S0723-2020(99)80053-8.
David, P.P., Nelson, P. V, and Sanders, D.C. (1994). A humic acid improves growth of tomato seedling in solution culture. Journal of Plant Nutrition. 17: 173–184. doi: 10.1080/01904169409364717.
Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T.R. (2013). Star: Ultrafast universal RNA-seq aligner. Bioinformatics. 29: 15–21. doi: 10.1093/bioinformatics/bts635.
Drobek, M., Fraç, M., and Cybulska, J. (2019). Plant biostimulants: Importance of the quality and yield of horticultural crops and the improvement of plant tolerance to abiotic stress — A review. Agronomy. 9: 335. doi: 10.3390/agronomy9060335.
Dursun, A., Güvenç, I., and Turan, M. (2002). Effects of different levels of humic acid on seedling growth and macro and micronutrient contents of tomato and eggplant. Acta Agrobotanica. 56: 81–88. doi: 10.5586/aa.2002.046.
Ferreira, C.M.H., Soares, H.M.V.M., and Soares, E. V. (2019). Promising bacterial genera for agricultural practices: An insight on plant growth-promoting properties and microbial safety aspects. Science of the Total Environment. 682: 779–799. doi: 10.1016/j.scitotenv.2019.04.225.
Fischer, T. (2017). Humic supramolecular structures have polar surfaces and unpolar cores in native soil. Chemosphere. 183: 437–43. doi: 10.1016/j.chemosphere.2017.05.125.
Gaiero, J.R., McCall, C.A., Thompson, K.A., Day, N.J., Best, A.S., and Dunfield, K.E. (2013). Inside the root microbiome: Bacterial root endophytes and plant growth promotion. American Journal of Botany. 100: 1738–1750. doi: doi:10.3732/ajb.1200572.
Glick, B.R. (2012). Plant growth-promoting bacteria: Mechanisms and applications. Hindawi Publishing Corporation Scientifica. 2012: 963401. doi: 10.6064/2012/963401.
Gouda, S., Kerry, R.G., Das, G., Paramithiotis, S., Shin, H.-S., and Patra, J.K. (2018). Revitalization of plant growth promoting rhizobacteria for sustainable development in agriculture. Microbiological Research. 206: 131–140. doi: 10.1016/j.micres.2017.08.016.
Hammer, Ø., Harper, D.A.T., and Ryan, P.D. (2001). Past: Paleontological statistics software package for education and data analysis. Palaeontologia Electronica. 4: 1–9.
Hardoim, P.R., Overbeek, L.S. van, Berg, G., Pirttilä, A.M., Compant, S., Campisano, A., Döring, M., and Sessitsch, A. (2015). The hidden world within plants: Ecological and evolutionary considerations for defining functioning of microbial endophytes. Microbiology and Molecular Biology Reviews. 79: 293–320. doi: 10.1128/MMBR.00050-14.
Hobson, G., and Grierson, D. (1993). Tomato. In Biochemistry of Fruit Ripening., edited by G.B. Seymour, J.E. Taylor, and G.A. Tucker, 405–42. Springer, Dordrecht. doi: 10.1007/978-94-011-1584-1_14.
Hunter, J.D. (2007). Matplotlib: A 2D graphics environment. Computing in Science and Engineering. 9: 90–95. doi: 10.1109/MCSE.2007.55.
Irizarry, I., and White, J.F. (2018). *Bacillus amyloliquefaciens* alters gene expression, ROS production, and lignin synthesis in cotton seedling roots. Journal of Applied Microbiology. 124: 1589–1603. doi: 10.1111/jam.13744.
Issa, A., Esmaeel, Q., Sanchez, L., Courteaux, B., Guise, J.-F., Gibon, Y., Ballias, P., et al., (2018). Impacts of *Paraburkholderia phytofirmans* strain PsJN on tomato (*Lycopersicon esculentum* L.) under high temperature. Frontiers in Plant Science. 9: 1397. doi: 10.3389/fpls.2018.01397.
Khan, M.N., Mobin, M., Abbas, Z.K., and Alamri, S.A. (2018). Fertilizers and their contaminants in oils, surface and groundwater. Reference Module in Earth Systems and Environmental Sciences. 5: 225-240 doi: 10.1016/B978-0-12-409548-9.09888-2.
Lara-Chavez, A., Lowman, S., Kim, S., Tang, Y., Zhang, J., Udvardi, M., Nowak, J., Flinn, B., and

Mei, C. (2015). Global gene expression profiling of two switchgrass cultivars following inoculation with *Burkholderia phytofirmans* strain PsJN. Journal of Experimental Botany. 66: 4337–4350. doi: 10.1093/jxb/erv096.

Law, C.W., Chen, Y., Shi, W., and Smyth, G.K. (2014). Voom: Precision weights unlock linear model analysis tools for RNA-seq read counts. Genome Biology. 15: R29. doi: 10.1186/gb-2014-15-2-r29.

Loiret, F.G., Grimm, B., Hajirezaei, M.R., Kleiner, D., and Ortega, E. (2009). Inoculation of sugarcane with *Pantoea* sp. increases amino acid contents in shoot tissues; serine, alanine, glutamine and asparagine permit concomitantly ammonium excretion and nitrogenase activity of the bacterium. Journal of Plant Physiology. 166: 1152–1161. doi: 10.1016/j.jplph.2009.01.002.

Lòpez-Fernàndez, S., Compant, S., Vrhovsek, U., Bianchedi, P.L., Sessitsch, A., Pertot, I., and Campisano, A. (2015a). Grapevine colonization by endophytic bacteria shifts secondary metabolism and suggests activation of defense pathways. Plant and Soil. 405: 155–175. doi: 10.1007/s11104-015-2631-1.

Lòpez-Fernàndez, S., Sonego, P., Moretto, M., Pancher, M., Engelen, K., Pertot, I., and Campisano, A. (2015b). Whole-genome comparative analysis of virulence genes unveils similarities and differences between endophytes and other symbiotic bacteria. Frontiers in Microbiology. 6: 479. doi: 10.3389/fmicb.2015.00419.

Ma, Y. (2019). Seed coating with beneficial microorganisms for precision agriculture. Biotechnology Advances. 37: 107423. doi: 10.1016/j.biotechadv.2019.107423.

Maere, S., Heymans, K., and Kuiper, M. (2005). Bingo: A Cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks. Bioinformatics. 21: 3448–3449. doi: 10.1093/bioinformatics/bti551.

Maham, G.S., Rahimi, A., Subramanian, S., and Smith, D.L. (2020). The environmental impacts of organic greenhouse tomato production based on the nitrogen-fixing plant (*Azolla*). Journal of Cleaner Production. 245: 118679. doi: 10.1016/j.jclepro.2019.118679.

Manz, W., Amann, R., Ludwig, W., Wagner, M., and Schleifer, K.-H. (1992). Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: Problems and solutions. Systematic and Applied Microbiology. 15: 593–600. doi: 10.1016/S0723-2020(11)80121-9.

Martínez-Viveros, O., Jorquera, M.A., Crowley, D.E., Gajardo, G., and Mora, M.L. (2010). Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria. Journal of Soil Science and Plant Nutrition. 10: 293–319. doi: 10.4067/S0718-95162010000100006.

Nogueira, E.D., Olivares, F.L., Japiassu, J.C., Vilar, C., Vinagre, F., Baldani, I.J., and Hemerly, A.S. (2005). Characterization of glutamine synthetase genes in sugarcane genotypes with different rates of biological nitrogen fixation. Plant Science. 169: 819–32. doi: 10.1016/j.plantsci.2005.05.031.

Mitter, B., Pfaffenbichler, N., Flavell, R., Compant, S., Antonielli, L., Petric, A., Berninger, T., et al., (2017). A new approach to modify plant microbiomes and traits by introducing beneficial bacteria at lowering into progeny seeds. Frontiers in Microbiology. 8: 11. doi: 10.3389/fmicb.2017.00011.

Müller, O.A., Grau, J., Thieme, S., Prochaska, H., Adlung, N., Sorgatz, A., and Bonas, U. (2015). Genome-wide identification and validation of reference genes in infected tomato leaves for quantitative RT-PCR analyses. PLoS ONE. 10: e0136499. doi: 10.1371/journal.pone.0136499.

Nardi, S., Carletti, P., Pizzeghello, D., and Muscolo, A. (2009). Biological Activities of Humic Substances. In Biophysico-Chemical Processes Involving Natural Nonliving Organic Matter in Environmental Systems., edited by Nicola Sensei, Baoshan Xing, and Pan Ming Huang, 305–339. John Wiley and Sons, New Jersey. doi: 10.1002/9780470494950.ch8.

Nardi, S., Pizzeghello, D., Gessa, C., Ferrarese, L., Trainotti, L., and Casadoro, G. (2000). A low molecular weight humic fraction on nitrate uptake and protein synthesis in maize seedlings. Soil Biology and Biochemistry. 32: 415–419. doi: 10.1016/S0038-0717(99)00168-6.

Olivares, F.L., Aguiar, O.N., Rosa, R.C.C., and Canellas, L.P. (2015). Substrate biofortification in combination with foliar sprays of plant growth promoting bacteria and humic substances boosts production of organic tomatoes. Scientia Horticulturae. 183: 100–108. doi: 10.1016/j.scienta.2014.11.012.

Olivares, F.L., Busato, J.G., Paula, A.M. de, Lima, L. da S., Aguiar, N.O., and Canellas, L.P. (2017). Plant growth promoting bacteria and humic substances: Crop promotion and mechanisms of action. Chemical and Biological Technologies in Agriculture. 4: 30. doi: 10.1186/s40538-017-0112-x.

Palma, M. De, Salzano, M., Villano, C., Aversano, R., Lorito, M., Ruocco, M., Docimo, T., Piccinelli, A.L., D'Agostino, N., and Tucci, M. (2019). Transcriptome reprogramming, epigenetic modifications and alternative splicing orchestrate the tomato root response to the beneficial fungus *Trichoderma harzianum*. Horticulture Research. 6: 5. doi: 10.1038/s41438-018-0079-1.

Perazzolli, M., Herrero, N., Sterck, L., Lenzi, L., Pellegrini, A., Puopolo, G., Peer, Y. Van de, and Pertot, I. (2016). Transcriptomic responses of a simplified soil microcosm to a plant pathogen and its biocontrol agent reveal a complex reaction to harsh habitat. BMC Genomics. 17: 838. doi: 10.1186/s12864-016-3174-4.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acid Research. 29: e45. doi: 10.1093/nar/29.9.e45.

Piccolo, A. (1996). Humus and Soil Conservation. In Humic Substances in Terrestrial Ecosystems., edited by Alessandro Piccolo, 225–64. Elseviel Science B.V.

Pillay, V.K., and Nowak, J. (1997). Inoculum density, temperature, and genotype effects on in vitro growth promotion and epiphytic and endophutic colonization of tomato *(Lycopersicum esculentum* L.) seedlings inoculated with a *Pseudomonad* bacterium. Canadian Journal of Microbiology. 43: 354–361. doi: 10.1139/m97-049.

Pinedo, I., Ledger, T., Greve, M., and Poupin, M.J. (2015). *Burkholderia phytofirmans* PsJN induces long-term metabolic and transcriptional changes involved in *Arabidopsis thaliana* salt tolerance. Frontiers in Plant Science. 6: 466. doi: 10.3389/fpls.2015.00466.

Pizzeghello, D., Francioso, O., Ertani, A., Muscolo, A., and Nardi, S. (2012). Isopentenyladenosine and cytokinin-like activity of different humic substances. Journal of Geochemical Exploration. 129: 70–75. doi: 10.1016/j.gexplo.2012.10.007.

Pombo, M.A., Zheng, Y., Fei, Z., Martin, G.B., and Rosli, H.G. (2017). Use of RNA-seq data to identify and validate RT-qPCR reference genes for studying the tomato-*Pseudomonas* pathosystem. Scientific Reports. 7: 44905. doi: 10.1038/srep44905.

Poupin, M.J., Greve, M., Carmona, V., and Pinedo, I. (2016). A complex molecular interplay of auxin and ethylene signaling pathways is involved in *Arabidopsis* growth promotion by *Burkholderia phytofirmans* PsJN. Frontiers in Plant Science. 7: 492. doi: 10.3389/fpls.2016.00492.

Poupin, M.J., Timmermann, T., Vega, A., Zuñiga, A., and González, B. (2013). Effects of the plant growth-promoting bacterium *Burkholderia phytofirmans* PsJN throughout the life cycle of *Arabidopsis thaliana*. PLoS ONE. 8: e69435. doi: 10.1371/journal.pone.0069435.

Quaggiotti, S., Ruperti, B., Pizzeghello, D., Francioso, O., Tugnoli, V., and Nardi, S. (2004). Effect of low molecular size humic substances on nitrate uptake and expression of genes involved in nitrate transport in maize (*Zea mays* L .). Journal of Experimental Botany. 55: 803–813. doi: 10.1093/jxb/erh085.

Ramos, A.C., Dobbss, L.B., Santos, L.A., Fernandes, M.S., Olivares, F.L., Aguiar, N.O., and Canellas, L.P. (2015). Humic matter elicits proton and calcium fluxes and signaling dependent on Ca^{2+}-dependent protein kinase (CDPK) at early stages of lateral plant root development. Chemical and Biological Technologies in Agriculture. 2: 3. doi: 10.1186/s40538-014-0030-0.

Ruijter, J.M., Ramakers, C., Hoogaars, W.M.H., Karlen, Y., Bakker, O., Hoff, M.J.B. Van Den, and Moorman, A.F.M. (2009). Amplification efficiency: Linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acid Research. 37: e45. doi: 10.1093/nar/gkp045.

Saldanha, A.J. (2004). Java Treeview — extensible visualization of microarray data. Bioinformatics. 20: 3246–3248. doi: 10.1093/bioinformatics/bth349.

Sato, S., Tabata, S., Hirakawa, H., Asamizu, E., Shirasawa, K., Isobe, S., Kaneko, T., et al., (2012). The tomato genome sequence provides insights into fleshy fruit evolution. Nature. 485: 635–641. doi: 10.1038/nature11119.

Sawana, A., Adeolu, M., and Gupta, R.S. (2014). Molecular signatures and phylogenomic analysis of the genus *Burkholderia*: Proposal for division of this genus into the emended genus *Burkholderia* containing pathogenic organisms and a new genus *Paraburkholderia* gen. nov. harboring environmental species. Frontiers in Genetics. 5: 429. doi: 10.3389/fgene.2014.00429.

Sessitsch, A., Coenye, T., Sturz, A. V, Vandamme, P., Barka, E.A., Salles, J.F., Elsas, J.D. Van, et al., (2005). *Burkholderia phytofirmans* sp. nov., a novel plant-associated bacterium with plant-beneficial properties. International Journal of Systematic and Evolutionary Microbiology. 55: 1187–1192. doi: 10.1099/ijs.0.63149-0.

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B., and Ideker, T. (2003). Cytoscape: A software environment for integrated models of biomolecular interaction networks. Genome Research. 13: 2498–2504. doi: 10.1101/gr.1239303.

Sharma, V.K., and Nowak, J. (1998). Enhancement of *Verticillium* wilt resistance in tomato transplants by in vitro co-culture of seedlings with a plant growth promoting rhizobacterium (*Pseudomonas* sp. strain PsJN). Canadian Journal of Microbiology. 44: 528–536. doi: 10.1139/w98-017.

Silver, N., Best, S., Jiang, J., and Thein, S.L. (2006). Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. BMC Molecular Biology. 7: 33. doi: 10.1186/1471-2199-7-33.

Smyth, G.K. (2006). Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Statistical Applications in Genetics and Molecular Biology. 3: 1. doi: 10.2202/1544-6115.1027.

Subramanian, P., Mageswari, A., Kim, K., Lee, Y., and Sa, T. (2015). Psychrotolerant endophytic *Pseudomonas* sp. strains OB155 and OS261 induced chilling resistance in tomato plants (*Solanum lycopersicum* Mill .) by activation of their antioxidant capacity. Molecular Plant-Microbe Interactions. 28: 1073–1081. doi: 10.1094/MPMI-01-15-0021-R.

Timmermann, T., Poupin, M.J., Vega, A., Urrutia, C., Ruz, G.A., and González, B. (2019). Gene networks underlying the early regulation of *Paraburkholderia phytofirmans* PsJN induced systemic resistance in *Arabidopsis*. PLoS ONE. 14: e0221358. doi: 10.1371/journal.pone.0221358.

Timmusk, S., Behers, L., Muthoni, J., Muraya, A., and Aronsson, A.-C. (2017). Perspectives and challenges of microbial application for crop improvement. Frontiers in Plant Science. 8: 49. doi: 10.3389/fpls.2017.00049.

Trevisan, S., Botton, A., Vaccaro, S., Vezzaro, A., Quaggiotti, S., and Nardi, S. (2011). Humic substances affect *Arabidopsis* physiology by altering the expression of genes involved in primary metabolism, growth and development. Environmental and Experimental Botany. 74: 45–55. doi: 10.1016/j.envexpbot.2011.04.017.

Trevisan, S., Pizzeghello, D., Ruperti, B., Francioso, O., Sassi, A., Palme, K., Quaggiotti, S., and Nardi, S. (2010). Humic substances induce lateral root formation and expression of the early auxin-responsive IAA19 gene and DR5 synthetic element in *Arabidopsis*. Plant Biology. 12: 604–614. doi: 10.1111/j.1438-8677.2009.00248.x.

Vadassery, J., and Oelmüller, R. (2009). Calcium signaling in pathogenic and beneficial plant microbe interactions. Plant Signaling and Behaviour. 4: 1024–1027. doi: 10.4161/psb.4.11.9800.

Wallner, G., Amann, R., and Beisker, W. (1993). Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. Cytometry. 14: 136–143. doi: 10.1002/cyto.990140205.

Wang, Y., Ohara, Y., Nakayashiki, H., Tosa, Y., and Mayama, S. (2005). Microarray analysis of the gene expression profile induced by the endophytic plant growth-promoting rhizobacteria, *Pseudomonas fluorescens* FPT9601-T5 in *Arabidopsis*. Molecular Plant-Microbe Interactions. 18: 385–396. doi: 10.1094/MPMI-18-0385.

Young, C., Rekha, P.D., Lai, W., and Arun, A.B. (2006). Encapsulation of plant growth-promoting bacteria in alginate beads enriched with humic acid. Biotechnology and Bioengineering. 95: 76–83. doi: 10.1002/bit.20957.

Zandonadi, D.B., Santos, M.P., Caixeta, L.S., Marinho, E.B., Peres, L.E.P., and Façanha, A.R. (2016). Plant proton pumps as markers of biostimulant action. Scientica Agricola. 73: 24–28. doi: 10.1590/0103-9016-2015-0076.

Zanin, L., Tomasi, N., Cesco, S., Varanini, Z., and Pinton, R. (2019). Humic substances contribute to plant iron nutrition acting as chelators and biostimulants. Frontiers in Plant Science. 10: 675. doi: 10.3389/fpls.2019.00675.

Figures

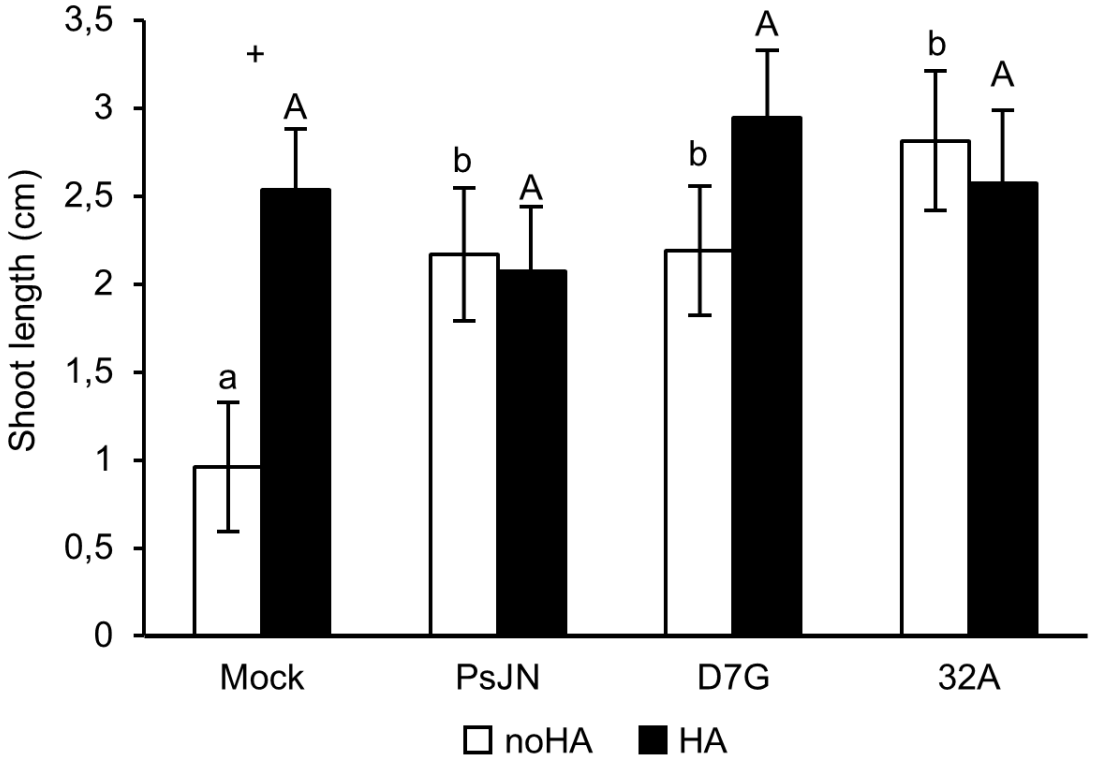

FIGURE 1 | Tomato growth promotion by endophytic bacterial strains. The shoot length (cm) of mock-inoculated plants (mock) and plants inoculated with *Paraburkholderia phytofirmans* PsJN (PsJN), *Pantoea agglomerans* D7G (D7G) or *Enterobacter* sp. 32A (32A) was assessed three days after incubation in half-strength Hoagland with 0 mg L^{-1} (white, control) and 50 mg L^{-1} humic acid (black, HA) in square dishes. Mean and standard error values of nine replicates (plants) are presented for each treatment. Different lowercase and uppercase letters indicate significant differences among treatments in the control and HA condition according to Tukey's test ($P \leq 0.05$), respectively. For each treatment, plus symbols indicate significant differences in the pairwise comparisons between the control and HA condition according to Student's t test ($P \leq 0.05$).

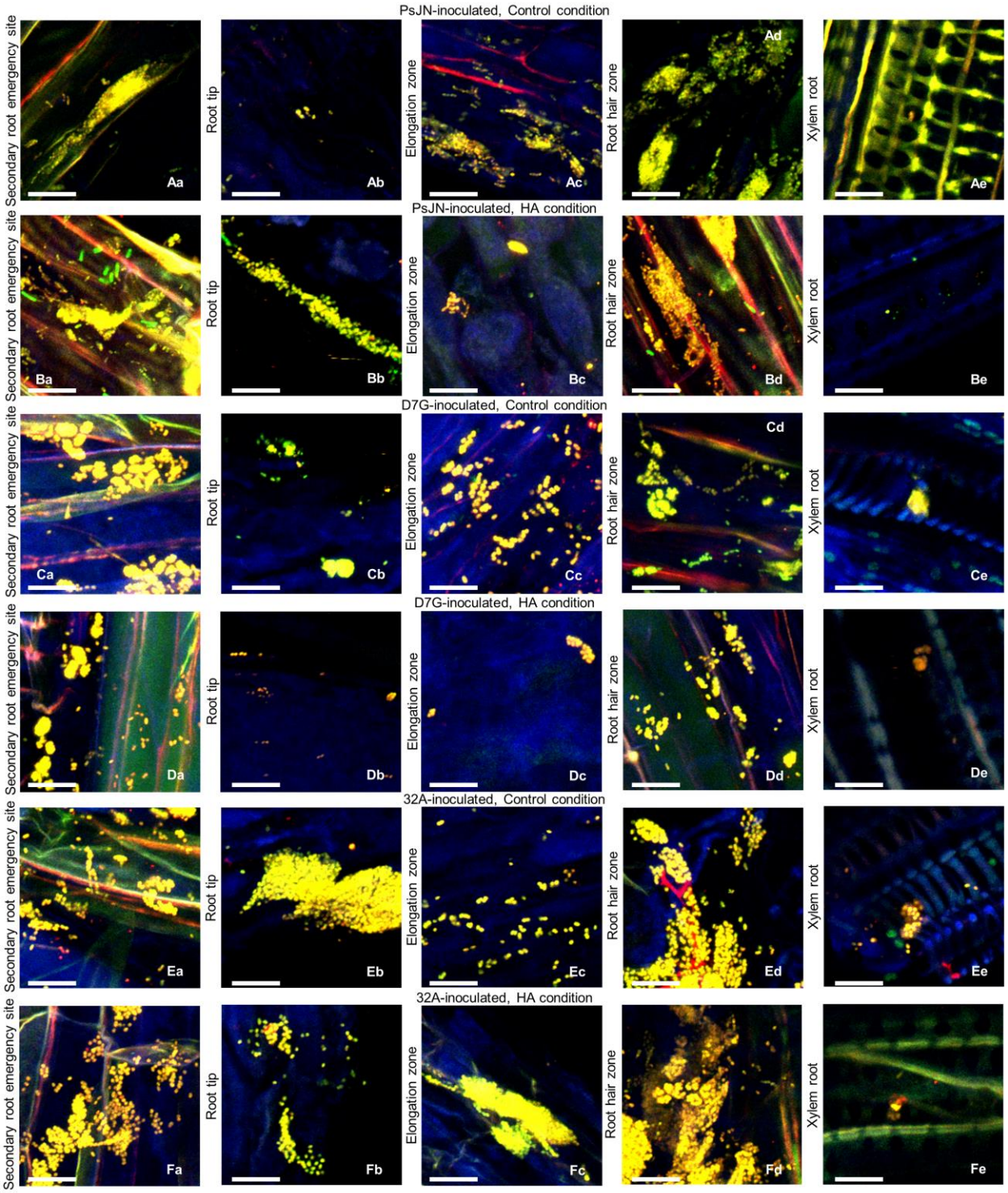

FIGURE 2 | Localization of endophytic bacterial strains in tomato roots. Bacterial cells of *Paraburkholderia phytofirmans* PsJN (PsJN) (A-B) were hybridised with the EUBmix and Bphyt probes, *Pantoea agglomerans* D7G (D7G) (C-D) or *Enterobacter* sp. 32A (32A) (E-F) were hybridised with the EUBmix and Gam42a probes on secondary root emergency sites (a), root tip (b), root elongation zone (c), root hair (d) and xylem (e) three days after incubation (DAI) in half-strength Hoagland with 0 mg L^{-1} (Control condition; A, C and E) and 50 mg L^{-1} humic acid (HA condition; B, D and F) in square dishes. Five replicates (plants) were analysed for each treatment and representative pictures were selected. Bars correspond to 10 µM.

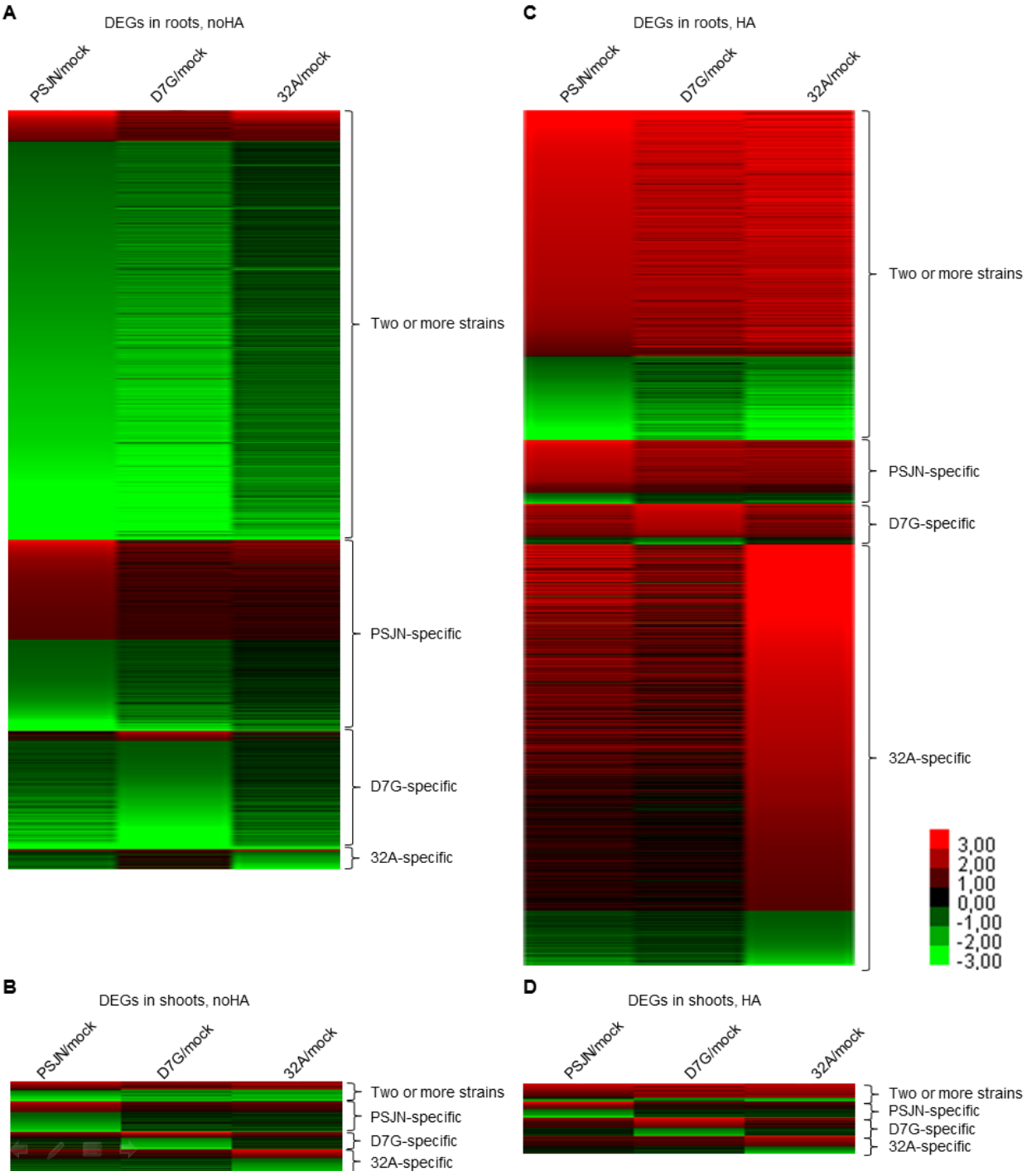

FIGURE 3 | Clustering of differentially expressed genes (DEGs) of tomato plants in response to endophytic bacterial strains and humic acid. Heat map diagram indicates the fold change values for DEGs identified in tomato roots (A, C) and shoots (B, D) three days after incubation with *Paraburkholderia phytofirmans* PsJN (PsJN), *Pantoea agglomerans* D7G (D7G) or *Enterobacter* sp. 32A (32A), calculated as compared to mock-inoculated plants (mock) in half-strength Hoagland with 0 mg L^{-1} (control; A, C) and 50 mg L^{-1} humic acid (HA; B, D). DEGs were classified as genes modulated by two or three strains or as genes modulated by only one bacterial strain (PsJN-, D7G- or 32A-specific). The heat map diagram was visualised using Java Treeview according to colour scale legend shown.

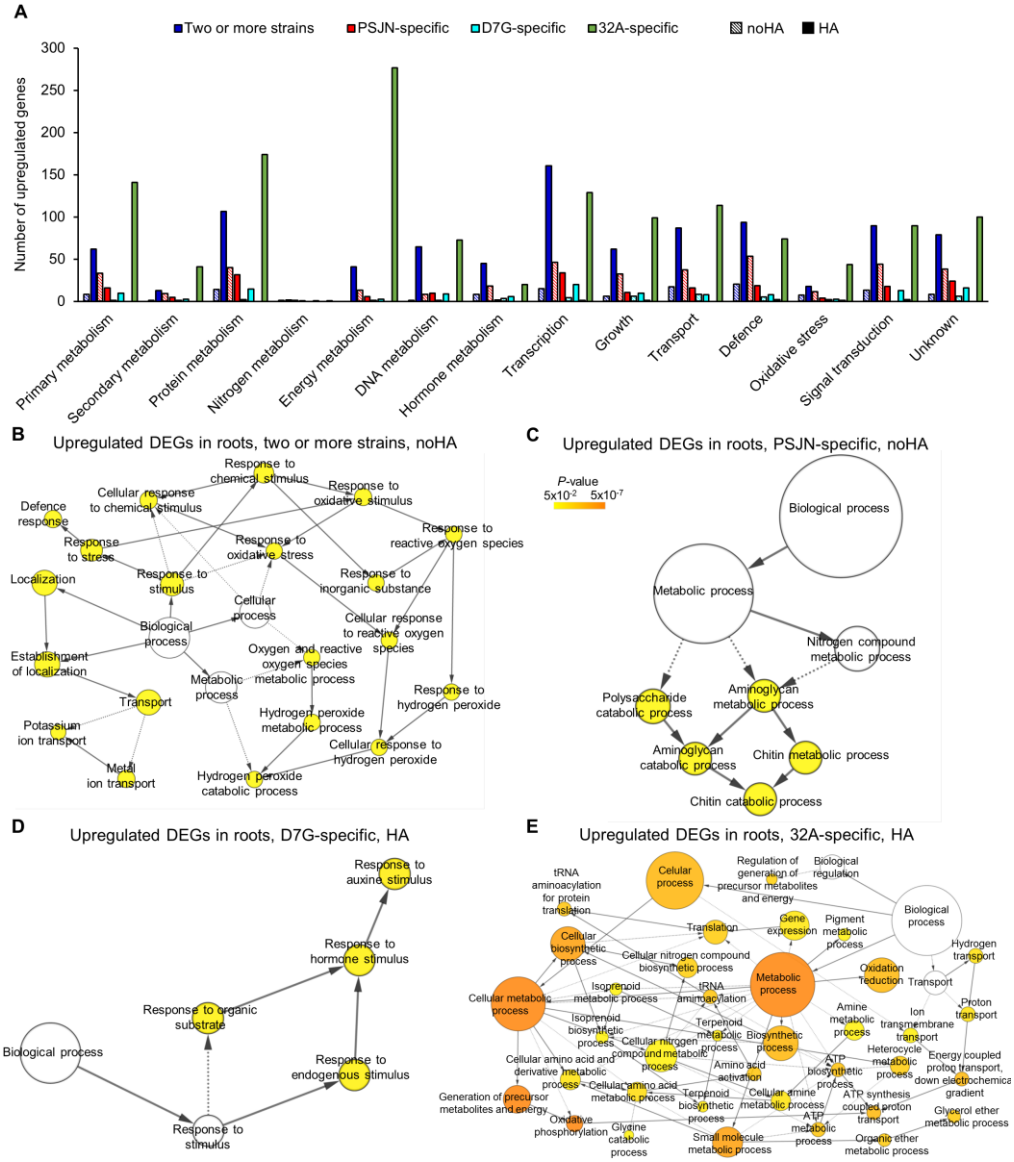

FIGURE 4 | Functional annotation of upregulated genes in tomato roots in response to endophytic bacterial strains. Functional classes (A) were assigned on the basis of the protein description of upregulated genes in tomato roots in response to two or three strains (blue) and specifically in response to *Paraburkholderia phytofirmans* PsJN (red), *Pantoea agglomerans* D7G (cyan) or *Enterobacter* sp. 32A (green) in half-strength Hoagland with 0 mg L^{-1} (control; stripped bars) and 50 mg L^{-1} humic acid (HA; solid bars). Biological networks of significantly enriched ($P \leq 0.05$) Gene Ontology (GO) terms of upregulated genes in tomato roots in response to two or three strains (B) or to PsJN (C) in the control condition and in response to D7G (D) or 32A (E) in the HA condition are reported. The colour scale legend indicates the level of significance for enriched GO terms and white nodes indicate not significantly overrepresented categories. Dotted lines indicate connection between biological process categories in the GO chart, where ancestor and child are omitted for simplicity. No significant GO enrichment was found for upregulated genes in response to two or three strains and to PsJN in the HA condition, as well as in response to D7G or 32A in the control condition.

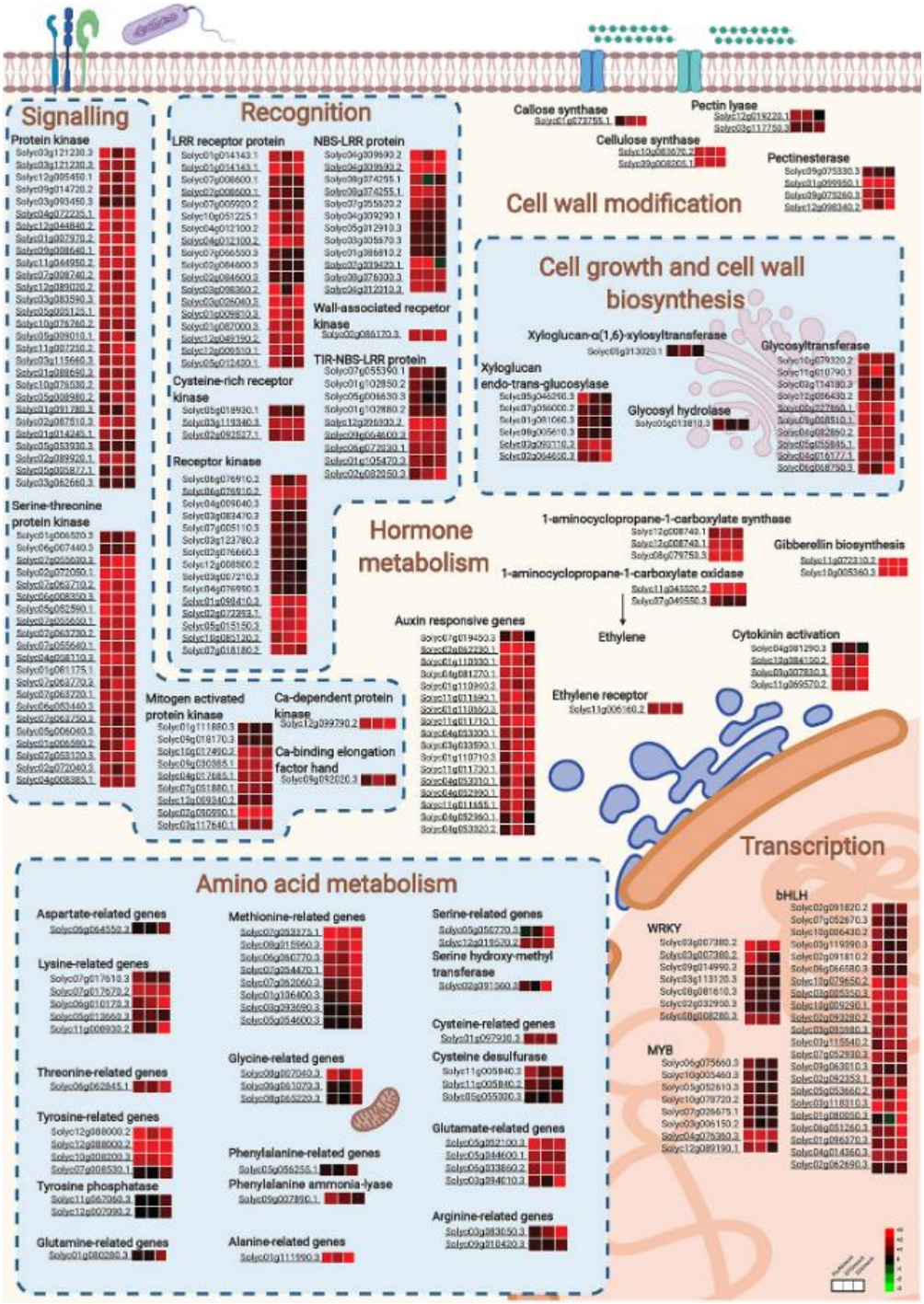

FIGURE 5 | Cellular processes activated by endophytic bacterial strains in tomato roots. Main cellular pathways of upregulated genes in tomato roots in response to *Paraburkholderia phytofirmans* PsJN (PsJN), *Pantoea agglomerans* D7G (D7G) or *Enterobacter* sp. 32A (32A) in half-strength Hoagland with 0 mg L^{-1} (control) and 50 mg L^{-1} humic acid (HA) were generated with Biorender. Not underlined and underlined gene codes indicate tomato genes modulated in the control and HA condition, respectively. For each gene, three squares represent the Log_2-transformed fold change values of PsJN-, D7G- or 32A-inoculated plants calculated as compared to mock-inoculated plants respectively, according to the colour scale reported. bHLH, basic helix-loop-helix; LRR, leucine-rich repeat; NBS-LRR, nucleotide-binding site leucine-rich repeat; TIR-NBS-LRR, non-toll-interleukin receptor nucleotide-binding site leucine-rich repeat; Ca, calcium.

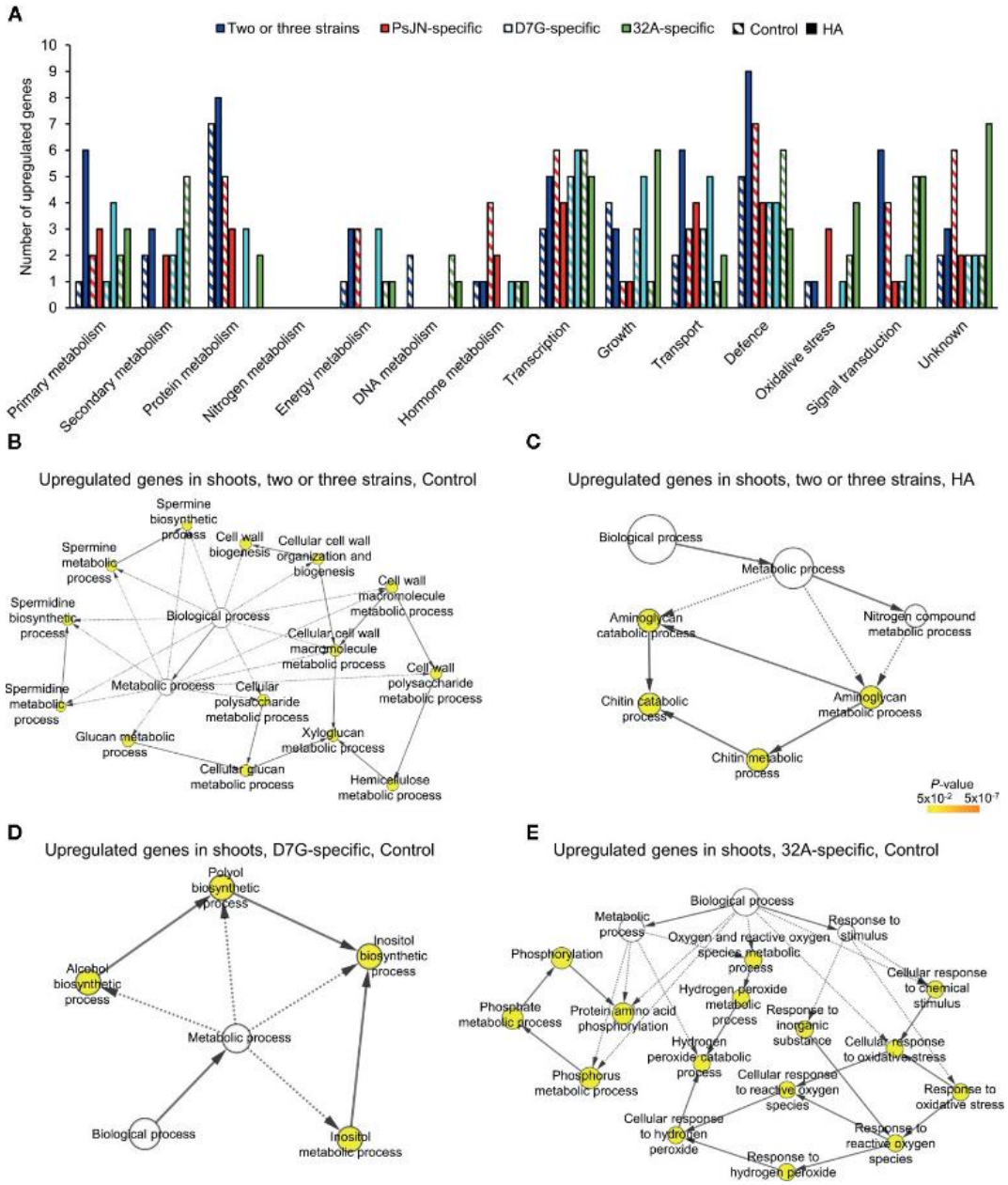

FIGURE 6 | Functional annotation of upregulated genes in tomato shoots in response to endophytic bacterial strains. Functional classes (A) were assigned on the basis of the protein description of upregulated genes in tomato shoots in response to two or three strains (blue) and specifically in response to *Paraburkholderia phytofirmans* PsJN (red), *Pantoea agglomerans* D7G (cyan) or *Enterobacter* sp. 32A (green) in half-strength Hoagland with 0 mg L^{-1} (control; stripped bars) and 50 mg L^{-1} humic acid (HA; solid bars). Biological networks of significantly enriched ($P \leq 0.05$) Gene Ontology (GO) terms of upregulated genes in tomato shoots in response to two or three strains in the control condition (B) and HA condition (C) and in response to D7G (D) or 32A (E) in the control condition are reported. The colour scale legend indicates the level of significance for enriched GO terms and white nodes indicate not significantly overrepresented categories. Dotted lines indicate connection between biological process categories in the GO chart, where ancestor and child are omitted for simplicity. No significant GO enrichment was found for upregulated genes in response to PsJN in the control and HA condition, as well as in response to D7G or 32A in the HA condition.

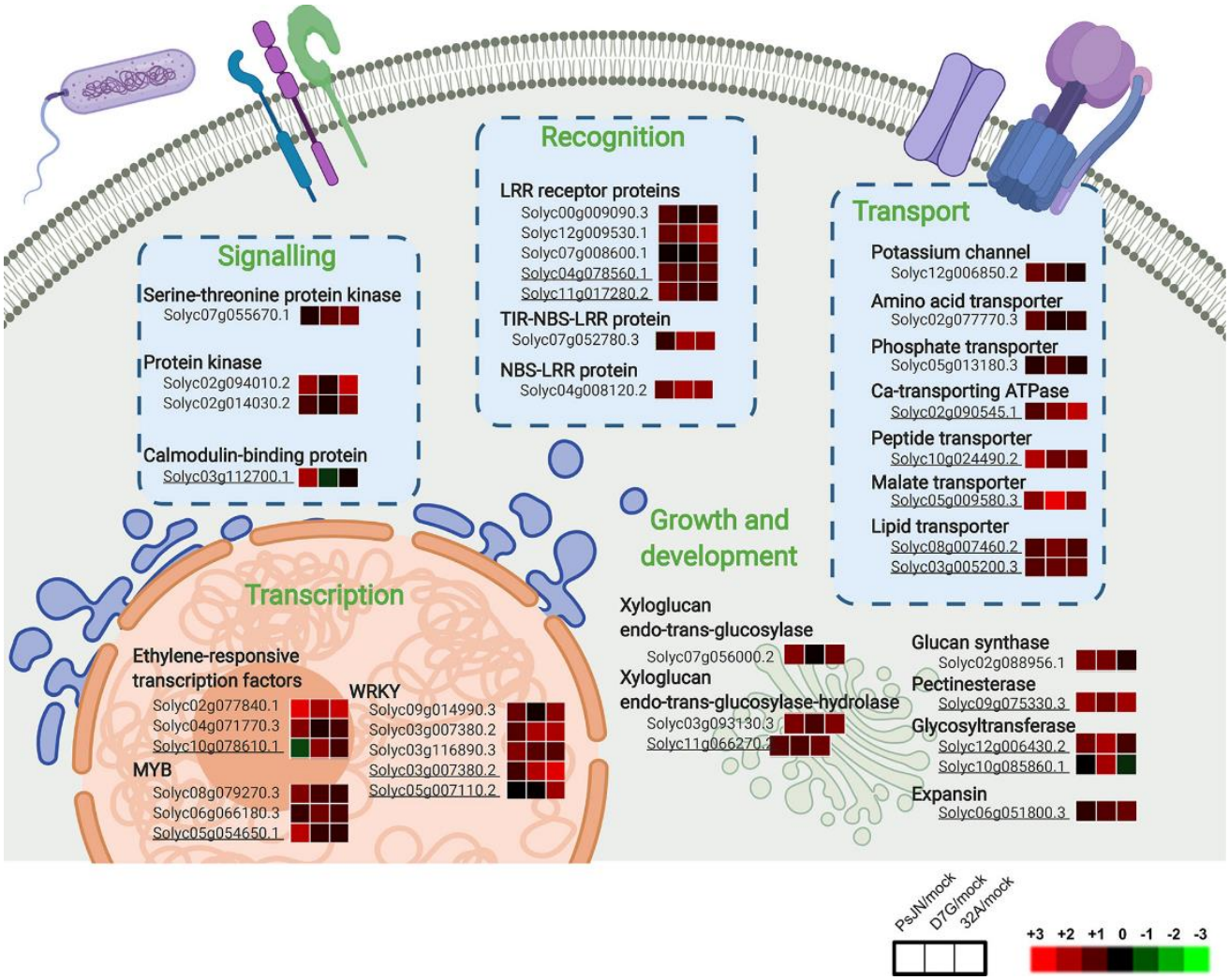

FIGURE 7 | Cellular processes activated by endophytic bacterial strains in tomato shoots. Main cellular pathways of upregulated genes in tomato shoots in response to *Paraburkholderia phytofirmans* PsJN (PsJN), *Pantoea agglomerans* D7G (D7G) or *Enterobacter* sp. 32A (32A) in half-strength Hoagland with 0 mg L^{-1} (control) and 50 mg L^{-1} humic acid (HA) were generated with Biorender. Not underlined and underlined gene codes indicate tomato genes modulated in the control and HA condition, respectively. For each gene, three squares represent the Log_2-transformed fold change values of PsJN-, D7G- or 32A-inoculated plants calculated as compared to mock-inoculated plants respectively, according to the colour scale reported. Ca, calcium; LRR, leucine-rich repeat receptor; NBS-LRR, nucleotide-binding site leucine-rich repeat; TIR-NBS-LRR, non-toll-interleukin receptor nucleotide-binding site leucine-rich repeat.

5 Topic 2: BENEFICIAL INSECTS DELIVER PLANT GROWTH-PROMOTING BACTERIAL ENDOPHYTES BETWEEN TOMATO PLANTS

Beneficial insects deliver plant growth-promoting bacterial endophytes between tomato plants

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ABSTRACT

Beneficial insects and mites, including generalist predators of the family Miridae, are widely used in biocontrol programs against many crop pests such as whiteflies, aphids, lepidopterans and mites. Mirid predators frequently complement their carnivory diet by feeding plant sap with their piercing-sucking mouthparts. This implies that mirids may act as vectors of phytopathogenic and beneficial microorganisms, such as plant growth-promoting bacterial endophytes. This work aimed at understanding the role of two beneficial mirids (*Macrolophus pygmaeus* and *Nesidiocoris tenuis*) in the acquisition and transmission of two plant growth-promoting bacteria, *Paraburkholderia phytofirmans* strain PsJN (PsJN) and *Enterobacter* sp. strain 32A (32A). Both bacterial strains were detected on the epicuticle and internal body of both mirids at the end of the mirid-mediated transmission. Moreover, both mirids were able to transmit PsJN and 32A between tomato plants and these bacterial strains could be re-isolated from tomato shoots after mirid-mediated transmission. In particular, PsJN and 32A endophytically colonised tomato plants and moved from shoots to roots after mirid-mediated transmission. In conclusion, this study provided novel evidence for the aquisition and transmission of plant growth-promoting bacterial endophytes by beneficial mirids.

Keywords: plant growth-promoting bacterial endophytes, beneficial mirids, *Macrolophus pygmaeus, Nesidiocoris tenuis*

INTRODUCTION

Beneficial insects (e.g. hymenopteran parasitoids, bumblebees, hoverflies lacewings, ladybugs and mirids) provide vital services in agricultural ecosystems, such as pollination and natural pest control (Pertot et al., 2017; Redhead et al., 2020). Among insect predators, the mirids *Macrolophus pygmaeus* (Rambur) and *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) are widely used for the biocontrol of whiteflies, aphids and moths among others (Calvo et al., 2009; Urbaneja et al., 2009; Castañé et al., 2011; Calvo et al., 2012). Tomato can be damaged by phytophagous arthropods worldwide (Wakil et al., 2018), both under field and greenhouse conditions (Hobson and Grierson, 1993). For example, leafminers (e.g. *Tuta absoluta* and the dipteran *Liriomyza* spp.) and phytophagous mites (e.g. *Tetranychus* sp. and *Aculops lycopersici*) reduce photosynthetic area and the latter also increase plant transpiration and alter water balance (Gabarra and Besri, 1999). Aphids (e.g. *Myzus persicae, Aphis* sp.), thrips (e.g. *Frankliniella occidentalis* and *Thrips tabaci*) and whitefly species (e.g. *Bemisia tabaci* and *Trialeurodes vaporariorum*) cause direct and indirect damage due to virus transmission (Gabarra and Besri, 1999). To counteract these pests, the generalist mirids (e.g. *M. pygmaeus* and *N. tenuis*) are increasingly used in many European and adjacent countries (Wakil et al., 2018), thanks to their ability to rapidly colonise tomato plants and to establish stable colonies early in the growing season (Gabarra et al., 2004; Put et al., 2012; Moerkens et al., 2017; Wakil et al., 2018).

Macrolophus pygmaeus and *N. tenuis* have piercing and sucking mouthparts that contain two channels: one to pump salivary fluid into plant tissues and the other to suck sap fluids from the host (Schaefer and Panizzi, 2000). These two species frequently complement their carnivory diet with plant sap feeding (Perdikis and Lykouressis 2000; Lykouressis et al., 2001), which could also lead to slight yield losses (Sánchez and Lacasa, 2008; Sanchez et al., 2018). For example, *M. pygmaeus* feeds mainly on the mesophyll of leaves, stems and fruits and high population densities caused fruit damage when plants are infected with pepino mosaic virus (PepMV) (Moerkens et al., 2015). On the other hand, *N. tenuis* feeds mainly within the vascular semi-ring on tomato plants (Wheeler, 2002; Chinchilla-Ramírez et al., 2021) and high population densities caused necrotic rings in both leaf and flower petioles, and whitish halos on fruits, independently of the presence of PepMV (Arnó et al., 2006; Moerkens et al., 2020).

Mouthparts morphology and trophic behaviour suggest possible exchange of microorganisms between insects and host plants as it has been demonstrated in many hemipteran species (Perilla-Henao and Casteel, 2016). Mirids are generally considered negligible vectors of plant pathogens

(Gibb and Randles, 1991), but *Erwinia amylovora* (Stahl and Leupschen, 1977) and *Lonsdalea quercina* pv. *lupinicola* (Myhre, 1988) can be transmitted by herbivorous *Lygus* spp. Likewise, *Pantoea* spp. and *Serratia marcescens* can be transmitted by *Lygus hesperus* (Cooper et al., 2014) and velvet tobacco mottle virus can be transmitted by *Cyrtopeltis nicotianae* (Gibb and Randles, 1988, 1991). In the case of *M. pygmaeus* and *N. tenuis*, they can both transmit parietaria mottle virus from *Parietaria officinalis* to tomato plants (Aramburu et al., 2010). Insect-mediated disease transmission can occur by direct feeding on infected plants (Gibb and Randles, 1988), through excretion products (Gibb and Randles, 1990) or by physical contact (Stahl and Leupschen, 1977), suggesting multiple mechanisms of possible transmission of plant-associated microorganisms by *M. pygmaeus* and *N. tenuis*. In addition to vectoring pathogens, insects can also transmit beneficial microbes as proved in the case of the leafhopper *Scaphoideus titanus* (Hemiptera: Cicadellidae), which was shown to transfer non-phytopathogenic bacterial communities between grapevine plants (Lòpez-Fernàndez et al., 2017). In this regard, no information is available for other insect/plant systems and in particular on the potential role of beneficial mirids in transmitting plant-associated beneficial microorganisms.

A multitude of bacteria, including those with beneficial effects for the plant host, live in the plant rhizosphere thanks to the presence of root exudates (Davison, 1988). Among them, the so called plant growth-promoting bacteria (PGPB) can improve plant growth and increase nutrient supply, including nitrogen, phosphorous and iron (Ferreira et al., 2019). Some PGPB can also colonise the internal tissues of numerous plant species. These beneficial endophytes can positively influence plant growth through various mechanisms, such as the production of hormones, improvement of nutrient uptake and protection against biotic or abiotic stresses (Gaiero et al., 2013). For example, the endophyte *Paraburkholderia phytofirmans* PsJN (PsJN), previously classified as *Pseudomonas* and then *Burkholderia* (Sessitsch et al., 2005; Sawana et al., 2014), improves plant growth (Pillay and Nowak 1997; Sharma and Nowak 1998; Galambos et al., 2020) and upregulates genes related to protein metabolism, transcription, transport, defence pathways, signal transduction and hormone metabolism in tomato plants (Galambos et al., 2020). Similarly, *Enterobacter* sp. 32A (32A), promotes plant growth, activates a complex transcriptional reprogramming in tomato plants (Galambos et al., 2020) and defence pathways in grapevine (Lòpez-Fernàndez et al., 2015).

In order to understand if beneficial mirids are able to acquire and transmit beneficial endophytes between tomato plants, we designed experiments to prove if PGPBs can be acquired from tomato plants previously inoculated and transmitted to non-inoculated plants by beneficial mirids. Moreover,

the ability of PGPB to reduce feeding damage of mirids was addressed, when tomato plants were offered as sole food source. This knowledge could greatly contribute to the development of combined approaches in biocontrol and biofertilisation.

MATERIAL AND METHODS

Bacterial strains and inoculum preparation

Two bacterial strains were used: *Paraburkholderia phytofirmans* PsJN (PsJN) isolated from surface-sterilised onion roots (Sessitsch et al., 2005) and *Enterobacter* sp. 32A (32A) isolated from the grapevine endosphere (Campisano et al., 2014). Bacterial strains were long term stored in 80% glycerol at -80 °C. To obtain the inoculum they were grown in 5 mL nutrient broth (NB) in sterile 15 mL-tubes at 25°C for 24 h under orbital shaking at 220 rpm. Bacterial cells were collected by centrifugation at 3,500 × g for 10 min and washed twice with sterile 10 mM $MgSO_4$. Bacterial cells were then suspended in sterile 10 mM $MgSO_4$ and the bacterial suspension was adjusted to 1.0×10^7 colony forming units (CFU) per unit of volume (CFU mL^{-1}) based on an optical density conversion at 600 nm (OD_{600}) optimised for each strain (OD_{600} 0.1 corresponded to 2.34×10^8 CFU ml^{-1} for PsJN and to 6.90×10^7 CFU ml^{-1} for 32A) (Galambos et al., 2020).

Tomato seed inoculation and growth conditions

Seeds of *S. lycopersicum* L. cv. Moneymaker (Justseed, Wrexham, UK) were disinfected with 70% ethanol for 1 min and 2% sodium hypochlorite plus 0.02% Tween 20 for 5 min in a 50 ml-tube with vigorous shaking and finally being washed three times (3 min each) with sterile distilled water (Galambos et al., 2020). Surface-disinfected seeds (120 seeds) were transferred to Petri dishes (100 mm diameter, 20 seeds for each dish) containing 1% water agar (Thermo Fisher Scientific, Waltham, MA, USA) and then incubated at 21±1°C in a growth chamber (Bertagnin, Bologna, Italy) for two days to allow seed germination. Seeds were treated with 1 mL of sterile 10 mM $MgSO_4$ (mock-inoculated) or inoculated with 1 mL of the bacterial suspension (bacterium-inoculated) using the respective bacterial strain (1×10^7 CFU mL^{-1} PsJN or 32A) by overnight incubation in the growth chamber (seed-inoculated plants) (Galambos et al., 2020).

Germinated seeds with the same root length (5 mm) were selected and each seed was transferred to a sterile 95 mL glass tube (Artiglass, Padova, Italy) containing 15 mL solid (7 g L^{-1} agar) half-

strength Hoagland (Galambos et al., 2020). Tubes were incubated in the growth chamber at 21±1°C with a 16 h photoperiod. Shoot length was measured with a ruler without mirid feeding (day 7 and day 11) or by image analysis before (day 7 and day 11) and after (day 11 and day 14) mirid feeding with using the software ImageJ version 1.50e (Schneider et al., 2012). The fresh weight was assessed of whole plants without mirid feeding (day 7 and day 11) or of shoots after mirid feeding (day 11 and day 14) with a precision balance (**Figure 1**). Twelve replicates (plants without mirid feeding) were analysed for each treatment and each experiment was carried out twice.

Mirid-mediated bacterial transmission assays

Beneficial mirids (*M. pygmaeus* and *N. tenuis*) were provided by Biobest NV (Westerlo, Belgium). Mirids had been mass-reared for several generations on tomato (*S. lycopersicum* L. cv. Moneymaker) under greenhouse conditions at 28 ± 5°C with a 16:8 light/dark photoperiod and were supplied with eggs of *Ephestia kuehniella* (Biobest Group NV, Westerlo, Belgium) as food source. To obtain freshly emerged adults, fifth instar nymphs were individually transferred to 100 mL plastic cups, covered with mesh, containing bean pods and *E. kuehniella* eggs (Sanchez et al., 2009). A freshly emerged mirid adult was placed in each glass tube containing a tomato plant that was either mock-inoculated or inoculated with PsJN or 32A (day 7) (**Figure 1**). Before transferring the mirid into the glass tube, plant shoot length was measured by image analysis. Plants were incubated for four days in the growth chamber at 21 ± 1°C (to avoid condensation on the inner glass surface) with a 16:8 light/dark photoperiod, in order to allow mirids to feed on tomato plants (acquisition period). At the end of the acquisition period (day 11), the shoot length was measured by image analysis after mirid feeding, five mirids for each treatment were sampled for double labelling of oligonucleotide probes for fluorescence *in situ* hybridisation (DOPE-FISH) analysis, and plants were collected for bacterial re-isolation and fresh weight assessment with a precision balance (day 11). Each remaining mirid was transferred to a new glass tube containing a mock-inoculated tomato plant and incubated for three days in order to allow mirids to feed on tomato plants (mirid-mediated transmission). Shoot length was measured by image analysis at the beginning (day 11) and at the end (day 14) of the mirid-mediated transmission. After mirid-mediated transmission, (day 14) mirids and plants were collected for bacterial re-isolation, the shoot length was measured by image analysis and the fresh weight of the whole plant was assessed with a precision balance. In addition, three glass tubes containing mock-inoculated plants were kept mirid-free for sterility control. Before transferring mirids into glass tubes, the surface of the half-strength Hoagland was overlaid with 1 mL of sterile melted paraffin

(Sigma-Aldrich, Merck, Darmstadt, Germany), in order to avoid contact of mirids with either the tomato roots or the growth medium, and to prevent contaminations (Lòpez-Fernàndez et al., 2017).

Bacterial re-isolation from tomato plants and mirids

Seed-inoculated plants and plants after mirid-mediated transmission were collected at day 11 and day 14, respectively (**Figure 1**). Mirids were collected at day 14 for bacterial re-isolation and each mirid was washed with 200 µL distilled water by vigorous vortexing in a 2 ml-tube to collect the majority of bacteria adhering to the mirid epicuticle (Lòpez-Fernàndez et al., 2017). Each plant was surface-sterilised in a 50 ml-tube and each mirid was surface-sterilised in a 2 ml-tube with 70% ethanol for 1 min, 2% sodium hypochlorite for 1.5 min, followed by 70% ethanol for 1 min (Galambos et al., 2020). Surface-sterilised plants or mirids were washed three times with distilled water (3 min each). After surface sterilisation, each seed-inoculated plant was placed in 2 ml-tube, while shoots and roots of plants after mirid-mediated transmission were cut and separately placed in 2 ml-tubes. Each plant or shoot was ground in 500 µL potassium phosphate buffer (1 mM, pH 7) in a mixer-mill disruptor (MM 400, Retsch, Haan, Germany) at 25 Hz for 2 min (Galambos et al., 2020). Conversely, each root or mirid was ground in 200 µL potassium phosphate buffer (1 mM, pH 7) in the mixer-mill disruptor (MM 400, Retsch). Each suspension was serially diluted and 10 µL aliquots were plated in triplicates on nutrient agar (NA). Aliquots of the last washing solution were plated as the control of surface sterilisation. After incubation at 25°C for three days, colonisation intensity was calculated as CFU values of bacteria per unit of plant fresh weight (CFU g^{-1}), per unit of mirid (CFU $mirid^{-1}$) or per unit of root (CFU $root^{-1}$). Nine replicates (seed-inoculated whole plants, shoots and roots after mirid-mediated transmission and mirids) were analysed for each treatment and the experiment was carried out twice.

PsJN and 32A colonies were recognised according to colony morphology and 16S rRNA gene sequencing. For bacterial identification by sequencing, three isolates were selected for each experiment and sample-type (seed-inoculated whole plants, shoots and roots after mirid-mediated transmission, bacteria adhering to the mirid epicuticle, bacteria in the mirid internal body) and a loopful of pure colonies were suspended into 1.5 ml-tubes containing sterile isotonic solution (0.85% NaCl in distilled water) and mixed with a vortex. Subsequently, the tubes were centrifuged for 2 min at 10,000 × g. Then, the pellet was suspended in 100 µL 0.05 M NaOH and incubated at 95°C for 15 min. After a centrifugation of 2 min at 10,000 × g, the supernatant was used as DNA template (Zhu et al., 2020). Amplicons of the 16S rRNA gene were obtained with PCR using the 27-forward (5′-

AGAGTTTGATCCTGGCTCAG-3′) and 1492-reverse (5′GGTTACCTTGTTACGACTT-3′) primer. PCR products were generated by amplifying 5 µL DNA with 0.1 µM of each primer, 12.5 µL Go Taq Green Master Mix (Promega GmbH, Mannheim, Germany) and 12 µL sterile deionized water. The amplification protocol consisted of denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, followed by a final extension at 72°C for 10 min in a thermal cycler (Biometra GmbH, Göttingen, Germany). Amplicons were purified by ExoProstar Kit (Illustra, Merck) and sequenced at the Sequencing and Genotyping Platform at Fondazione Edmund Mach. The resulting nucleotide sequences were compared to known sequences deposited in the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/nucleotide/) using BLASTN (Basic Local Alignment Tool) and aligned with the original sequences using the programme Mega X version 10.1.1 (Kumar et al., 2018), in order to confirm PsJN and 32A identity.

Fluorescence in situ hybridisation using double labelling of oligonucleotide probes

DOPE-FISH analysis was performed on mirids that fed on mock-inoculated plants and plants inoculated with PsJN or 32A for four days in glass tubes (**Figure 1**). Samples were fixed in a 4% paraformaldehyde in 1x phosphate-buffered saline (PBS) solution at 4°C for five hours and were rinsed three times with 1xPBS as previously reported (Compant et al., 2011). Mirids were dehydrated in increasing concentrations of ethanol solution (25, 50, 75 and 99%; 20 min each step) and stored at 4°C. DOPE-FISH was carried out using probes from Eurofins (Germany) labelled at both the 5' and 3' positions. A probe mixture targeting eubacteria, composed of EUB338, EUB338II, EUB338III (EUBmix) coupled with a Cy3 fluorochrome and Bphyt probe targeting the 23S rRNA gene of PsJN coupled with Cy5 (Amann et al., 1990; Daims et al., 1999; Mitter et al., 2017). For 32A, EUBmix and Gam42a probe targeting the 23S rRNA gene of 32A coupled with Cy5 was used (Manz et al., 1992). NONEUB probe coupled with Cy3 or Cy5 was used independently as negative control (Wallner et al., 1993). Fluorescent *in situ* hybridisation was carried out in sterile 1.5 mL-tubes at 46°C for 2 h in the dark with 60 µL hybridisation buffer for PsJN (containing 0.9 M NaCl; 0.02 M Tris HCl, 0.01% SDS, 10% formamide and 5 ng μL^{-1} of each probe) and with 60 µL hybridisation buffer for 32A (containing 0.9 M NaCl; 0.02 M Tris HCl, 0.01% SDS, 35% formamide and 5 ng μL^{-1} of each probe). Washing was conducted at 48°C for 30 min with a pre-warmed post-FISH solution containing 0.02 M Tris HCl, 0.01% SDS, NaCl and EDTA at a concentration corresponding to the formamide concentration. Samples were then rinsed with distilled water before overnight air-drying

in the dark. Samples were observed under a confocal microscope (Olympus Fluoview FV1000 with multiline laser FV5-LAMAR-2 and HeNe (G) laser FV10-LAHEG230-2). Pictures were taken at 405, 488, 633 nm wavelengths with Cy3 signal assigned as green and Cy5 as red. Pictures were analysed using Imaris 8 software (BITPLANE, UK). Z-stacks were used to generate whole-stack pictures. Five replicates (mirids) were analysed for each treatment and representative pictures were selected. Pictures were cropped and light/contrast balance improved in post process.

Statistical analysis

All experiments were carried out twice and data were analysed with the Past 3.26 software (Hammer et al., 2001). Successful bacterial colonisation, acquisition and transmission was calculated as percentage of positive samples (e.g. seed-inoculated whole plants, shoots and roots after mirid-mediated transmission, bacteria adhering to the mirid epicuticle, bacteria in the mirid internal body) with any amount of the respective bacteria present over the total number of samples. In order to estimate colonisation intensity after successful mirid-mediated transmission, CFU g^{-1}, CFU $mirid^{-1}$ or CFU $root^{-1}$ was calculated only on positive samples (Boucher et al., 2021). After validating data for normal distribution (Shapiro-Wilk test, $P > 0.05$) and variance homogeneity of the data (Levene's tests, $P > 0.05$), each experiment was analysed singularly and a two-way analysis of variance (ANOVA) was performed to assess the null hypothesis (i.e., non-significant differences between the two experiments, $P > 0.05$). Data from the two repeated experiments were pooled and significant differences among treatments were assessed with the Student's t-test ($P \leq 0.05$) and the Tukey' test ($P \leq 0.05$) in case of pairwise and multiple comparisons, respectively. CFU values of bacterial re-isolation were Log_{10}-transformed. If normal distribution (Shapiro-Wilk test, $P \leq 0.05$) or variance homogeneity (Levene's tests, $P \leq 0.05$) conditions were not satisfied, the Mann-Whitney test ($P \leq 0.05$) was used to assess significant differences in case of pairwise comparisons.

RESULTS

Beneficial mirids acquire endophytic bacteria by feeding on tomato plants

Tomato shoot length and fresh weight were improved in PsJN- and 32A-inoculated plants compared to mock-inoculated plants at seven days after seed inoculation (day 7) without mirid feeding (**Table 1**).

To characterise the acquisition of endophytic bacterial strains by beneficial mirids, the DOPE-FISH analysis was carried out on mirids at the end of the acquisition period (day 11) on bacterium- and mock-inoculated plants. Yellow fluorescent PsJN (**Figures 2A** and **2B**) and 32A (**Figures 2C** and **2D**) single cells, aggregates and micro-colonies were found on the abdomen, thorax and legs of *M. pygmaeus* and *N. tenuis*. Conversely, only some native bacteria were present on mirids fed on mock-inoculated plants (**Figures 2E** and **2F**). The NONEUB probe was used as a negative probe not targeting bacterial sequences and only a few green/blue-cyan autofluorescent microbes could be detected on mirids fed on mock-, PsJN- and 32A-inoculated plants, as an indicator of the rare presence of indigenous microorganisms colonising mirids (**Figure S1**).

Tomato plants were colonised by endophytic bacterial strains, PsJN and 32A were re-isolated at the end of the acquisition period (day 11) from 100% surface-sterilised seed-inoculated whole plants, at average levels of 10^6 CFU g^{-1} for both strains (**Figures 3A** and **3B**). At the end of the mirid-mediated transmission (day 14), 95.7% and 52.4% of tissue grinding of surface-sterilised *M. pygmaeus* (bacteria in the mirid internal body) had on average 10^3 and 10^4 CFU $mirid^{-1}$ of PsJN and 32A, respectively (**Figure 4A**). Meanwhile, 69.6% and 81.8% of washing suspension of *M. pygmaeus* (bacteria adhering to the mirid epicuticle) contained on average 10^4 and 10^2 CFU $mirid^{-1}$ of PsJN and 32A, respectively (**Figure 4A**). Likewise, following tissue grinding, 47.1% and 89.5% of surface-sterilised *N. tenuis* (bacteria in the mirid internal body) yielded on average 10^3 and 10^4 CFU $mirid^{-1}$ of PsJN and 32A, respectively (**Figure 4B**). Meanwhile, 23.5% and 75% of washing suspension of *N. tenuis* (bacteria adhering to the mirid epicuticle) yielded on average 10^2 and 10^3 CFU $mirid^{-1}$ of PsJN and 32A, respectively (**Figure 4B**). Moreover, PsJN and 32A were never detected in surface-sterilised mock-inoculated whole plants at the end of the acquisition period, in the washing suspension of *M. pygmaeus* and *N. tenuis* (bacteria adhering to the mirid epicuticle) nor in the tissue grinding of surface-sterilised *M. pygmaeus* and *N. tenuis* (bacteria in the mirid internal body), that were allowed to feed on mock-inoculated plants, at the end of the mirid-mediated transmission (**Figures 3** and **4**).

Beneficial mirids transmit endophytic bacteria by feeding on tomato plants

Both mirids species transmitted PsJN and 32A between tomato plants and bacterial strains were re-isolated from tomato shoots after mirid-mediated transmission (day 14). In particular, 91.7% and 45.5% of tomato shoots at the end of the *M. pygmaeus*-mediated transmission had on average 10^5 CFU g^{-1} of both PsJN and 32A (**Figure 3A**). Moreover, 62.5% and 22.7% of tomato roots at the end

of the *M. pygmaeus*-mediated transmission had on average 10^2 CFU g^{-1} of both PsJN and 32A (**Figure 3A)**. Likewise, 82.4% and 100% of tomato shoots at the end of the *N. tenuis*-mediated transmission had on average 10^4 and 10^5 CFU g^{-1} of PsJN and 32A, respectively (**Figure 3B**). Meanwhile, 17.6% and 55% of tomato roots at the end of the *N. tenuis*-mediated transmission had on average 10^3 CFU g^{-1} of both PsJN and 32A (**Figure 3B)**. Moreover, PsJN and 32A were never transmitted from mock-inoculated plants to mock-inoculated plants (**Figures 3** and **4**). Likewise, no colonies were detected in surface-sterilised mock-inoculated plants at seven days after seed inoculation (sterility control; data not shown).

Bacterial identity of re-isolated PsJN and 32A were confirmed by colony morphology and DNA sequencing of the 16S rRNA gene in surface-sterilised seed-inoculated plants at the end of the acquisition period, washing suspension of *M. pygmaeus* and *N. tenuis* (bacteria adhering to the mirid epicuticle), tissue grinding of surface-sterilised *M. pygmaeus* and *N. tenuis* (bacteria in the mirid internal body) and surface-sterilised tomato shoots and roots at the end of the mirid-mediated transmission.

The difference between tomato shoot length measured at the beginning (day 7) and at the end (day 11) of *M. pygmaeus* feeding on bacterium-inoculated plants by seed inoculation remained statistically similar to mock-inoculated plants (**Figures 1** and **5A**) but the difference measured on plants before (day 11) and after (day 14) *M. pygmaeus*-mediated transmission was greater in PsJN-inoculated plants compared to mock-inoculated plants (**Figures 1** and **5B**). Moreover, the difference between tomato shoot length measured at the beginning (day 7) and at the end (day 11) of *N. tenuis* feeding on seed-inoculated plants (**Figures 1** and **5C**) and on plants before (day 11) and after (day 14) *N. tenuis*-mediated transmission (**Figures 1** and **5D**) was greater in PsJN-inoculated plants compared to mock-inoculated plants.

DISCUSSION

Insects belonging to the suborder Heteroptera (also known as true bugs, which include mirids and stinkbugs) are poorly studied as potential vectors compared to those belonging to the former suborder Homoptera (e.g. leafhoppers, psyllids, aphids and whiteflies) (Gibb and Randles, 1991). Likewise, the significance of insects as vectors of plant pathogens is well characterised (Heck, 2018), while their role in the transmission of beneficial plant-associated microorganisms is much less studied and

in the case of mirids is unknown. This study assessed whether beneficial mirids, commonly used for biocontrol programs under greenhouse conditions, could acquire and transmit beneficial microorganisms. The results demonstrated that mirids were able to acquire the two beneficial bacterial strains, PsJN and 32A, both as bacteria adhering to the mirid epicuticle and bacteria in the mirid internal body through tissue feeding on seed-inoculated tomato plants. Bacterial strains were visualised on mirid abdomen, thorax and legs but not in the mirid internal body, due to technical limitations. However, bacterial presence in the mirid internal body was confirmed by re-isolation and 16S rRNA gene sequencing, supporting the mirid-mediated bacterial transmission from inoculated plants. This indicates that tomato tissues are suitable for the acquisition of endophytic bacteria, likely because PsJN and 32A are colonising the xylem (Gonella et al., 2015; Galambos et al., 2020). For insect symbionts, such as *Cardinium* spp. (Gonella et al., 2015) and *Wolbachia* spp. (Li et al., 2017), plant-mediated horizontal transmission has already been demonstrated between homopteran insects (e.g. leafhoppers and whiteflies), but for heteropterans experimental evidence is limited. For example, *Burkholderia* spp. was previously shown to be a highly prevalent in the bean bug *Riptortus clavatus* (Hemiptera: Alydidae) midgut, acquired from the soil at the nymphal stage (Kikuchi et al., 2007), indicating the potential complexity of insect-microbe and microbe-plant mutualistic associations.

Considering the mobile nature and piercing-sucking mouthparts of beneficial mirids, they could facilitate the introduction and spread of beneficial endophytes among crops (Gibb and Randle, 1991; Gonella et al., 2015), beside their principal role in pest control. Previously, it has been demonstrated that the two mirids have diverse feeding habits, *M. pygmaeus* feeds mainly on the mesophyll of leaves, stems and fruits, while *N. tenuis* feeds mainly within the vascular semi-ring on tomato plants (Wheeler, 2002; Chinchilla-Ramírez et al., 2021), indicating that the latter could be a more effective vector of microorganisms. In this study, PsJN was transmitted by 91.7% of *M. pygmaeus* and 82.4% of *N. tenuis* at the end of the mirid-mediated transmission. However, 32A was transmitted by *N. tenuis* (100%) with higher efficacy compared to *M. pygmaeus* (45.5%), suggesting that mirids could selectively transmit plant endophytes, as previously described for *S. titanus* on grapevine (Lòpez-Fernàndez et al., 2017), and indicating that specific studies must be carried out for each combination of mirid species and bacterial strain.

Successful plant colonisation is a crucial step for PGPB to promote growth (Compant et al., 2010). In this study, bacteria were re-isolated from surface-sterilised tomato plants (shoots and roots) at the end of the mirid-mediated transmission at levels similar to seed-inoculation, indicating their

potential to establish stable colonies after mirid-mediated transmission. Plant colonisation is a complex mechanism and endophytes can establish colonies inside the plant root then migrate to the stem (Compant et al., 2010). Likewise, some endophytes can penetrate from the leaves either through natural opening or by vector transmission (Frank et al., 2017). In this study, tomato roots, separated from the stems by a paraffin layer, were colonised by PsJN and 32A, indicating that both strains moved from stems to roots after transmission, as previously reported for 32A in grapevine (Lòpez-Fernàndez et al., 2017) and confirming their ability to systemically spread inside the plant (Hardoim et al., 2008).

In the case of increased predator density (Castañé et al., 2011), mirid feeding on plants could lead to slight yield losses (Sánchez and Lacasa, 2008; Sanchez et al., 2018). In this study mirids were provided with either mock-inoculated or bacterium-inoculated tomato plants as only food source. The results demonstrated that PsJN could mitigate *M. pygmaeus* feeding damage on plants after *M. pygmaeus*-mediated transmission. Moreover, PsJN mitigated *N. tenuis* feeding damage both on seed-inoculated plants and on plants after *N. tenuis*-mediated transmission. These results may indicate that PGPB either reduce plants' sensitivity response to insect feeding or exhibit anti-feeding properties that reduce insect feeding, although the underlying mechanisms of PsJN and 32A mitigating feeding damage remain to be elucidated. Likewise, several PGPB strains have been reported to exhibit antagonistic properties against arthropods. For example, *Pseudomonas putida* and *Rothia* sp. mitigated tobacco cutworm, *Spodoptera litura* infestation in tomato, via proline production, enhanced activation of antioxidant enzymes, protease and polyphenol oxidases and increased phenolics, protein and chlorophyll content (Bano and Muqarab, 2017), suggesting the potential of PGPB as nonchemical alternative for controlling arthropods and for stimulating plant health and growth (Ruiu, 2020).

Our findings provide evidence of mirid-mediated transmission of beneficial bacterial strains and assessed transmission efficacy, quantification and distribution pattern in tomato plant tissues (shoots and roots) and in mirid tissues (epicuticle and internal body of both mirids). In particular, PsJN and 32A can endophytically colonise tomato plants and move from shoots to roots after mirid-mediated transmission. In order to implement plant inoculation strategies with mirids, future steps must focus on the interactions between *M. pygmaeus* or *N. tenuis* and the PGPB strains in order to evaluate the persistence of beneficial bacteria on mirids, possible unknown harmful impact of PGPB on mirids' performance and efficacy as predators and to verify if PsJN and 32A can also be transmitted through feeding, physical contact, excretion and/or their combination.

DATA AVAILABILITY STATEMENT

All data obtained in this study can be found in the manuscript or in the supplementary materials.

AUTHOR CONTRIBUTIONS

NG, carried out the functional experiments, performed DOPE-FISH experiments, microscopy analyses and wrote the manuscript; SC, performed DOPE-FISH experiments and microscopy analyses; AS, GA, VM, IP and FW, revised the manuscript; MP, conceived the study, supervised the experiments, analysed the data and wrote the manuscript. All the authors revised and approved the manuscript.

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CONFLICTS OF INTEREST

NG and FW are employed by Biobest NV. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPLEMENTARY MATERIALS

Figure S1: Negative controls of double labelling of oligonucleotide probes for fluorescence in situ hybridisation.

REFERENCES

Amann, R. I., Binder, B. I., Olson, R. J., Chisholm, S. W., Devereux, R., and Stahl, D. A. (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Applied and Environmental Microbiology. 56 (6): 1919–1925. doi: 10.1128/AEM.56.6.1919-1925.1990

Aramburu, J., Galipienso, L., Aparicio, F., Soler, S., and López, C. (2010). Mode of transmission of Parietaria mottle virus. Journal of Plant Pathology. 92 (3): 679–684. doi: 10.4454/jpp.v92i3.313

Arnó J., Castañé C. R., Jordi & Roig, J. & Gabarra, Rosa. (2006). Characterization of damage to tomato plants produced by the zoophytophagous predator *Nesidiocoris tenuis*. IOBC/WPRS Bulletin. 29 (4): 249-254.

Bano, A., and Muqarab, R. (2017). Plant defence induced by PGPR against *Spodoptera litura* in tomato (*Solanum lycopersicum* L.). Plant Biology. 19 (3): 406–412. doi: 10.1111/plb.12535

Boucher, M., Collins, R., Harling, K., Brind'Amour, G., Hesler, S., Wentworth, K., Cox, K., Loeb, G. (2021). Field evaluation of interactions between insects and *Erwinia amylovora* in a New York apple orchard. Phytofrontiers. 1 (2): 94-103. doi: 10.1094/phytofr-10-20-0032-r

Calvo, F. J., Lorente, M. J., Stansly, P. A., and Belda, J. E. (2012). Preplant release of *Nesidiocoris tenuis* and supplementary tactics for control of *Tuta absoluta* and *Bemisa tabaci* in greenhouse tomato. Entomologia Experimentalis et Applicata. 143 (2): 111–119. doi: 10.1111/j.1570-7458.2012.01238.x

Calvo, J., Bolckmans, K., Stansly, P. A., and Urbaneja, A. (2009). Predation by *Nesidiocoris tenuis* on *Bemisia tabaci* and injury to tomato. Biological Control. 54 (2): 237–246. doi: 10.1007/s10526-008-9164-y

Campisano, A., Pancher, M., Puopolo, G., Puddu, A., Lòpez-Fernàndez, S., Biagini, B., Yousaf, S., and Pertot, I. (2014). Diversity in endophyte populations reveals functional and taxonomic diversity between wild and domesticated grapevines. American Journal of Enology and Viticulture. 66 (1): 12-21. doi: 10.5344/ajev.2014.14046

Castañé Cristina, C., Arnó, J., Gabarra, R., and Alomar, O. (2011). Plant damage to vegetable crops by zoophytophagous mirid predators. Biological Control. 59 (1): 22–29. doi: 10.1016/j.biocontrol.2011.03.007

Chinchilla-Ramírez, M., Garzo, E., Fereres, A., Gavara-Vidal, J., ten Broeke, C. J. M., van Loon, J. J. A., Urbaneja, A., and Pérez-Hedo, M. (2021). Plant feeding by *Nesidiocoris tenuis*: Quantifying its behavioral and mechanical components. Biological Control. 152 (3): 104402. doi: 10.1016/j.biocontrol.2020.104402

Compant, S., Clément, C., and Sessitsch, A. (2010). Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. Soil Biology and Biochemistry. 42 (5): 669-678. doi: 10.1016/j.soilbio.2009.11.024.

Compant, S., Mitter, B., Colli-Mull, J. G., Gangl, H., and Sessitsch, A. (2011). Endophytes of grapevine flowers, berries, and seeds: Identification of cultivable bacteria, comparison with other plant parts, and visualization of niches of colonization. Microbial Ecology. 62 (1): 188–197. doi: 10.1007/s00248-011-9883-y

Cooper, W. R., Nicholson, S. J., and Puterka, G. J. (2014). Potential transmission of *Pantoea* spp. and *Serratia marcescens* (Enterobacteriales: Enterobacteriaceae) to plants by *Lygus hesperus* (Hemiptera: Miridae). Journal of Economic Entomology. 107 (1): 63–65. doi: 10.1603/EC13056

Daims, H., Brühl, A., Amann, R., Schleifer, K.-H., and Wagner, M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all bacteria: Development and evaluation of a more comprehensive probe set. Systematic and Applied Microbiology. 22: 434–444. doi: 10.1016/S0723-2020(99)80053-8

Davison, J. (1988). Plant Beneficial Bacteria. Nature Biotechnology. 6 (3): 282–286. doi: 10.1038/nbt0388-282
Ferreira, C. M. H., Soares, H. M. V. M., and Soares, E. V. (2019). Promising bacterial genera for agricultural practices: An insight on plant growth-promoting properties and microbial safety aspects. Science of the Total Environment. 682: 779–799. doi: 10.1016/j.scitotenv.2019.04.225
Frank, A. C., Saldierna Guzmán, J. P. and Shay, J. E. (2017). Transmission of bacterial endophytes. Microorganisms. 5 (4): 70. doi: 10.3390/microorganisms5040070
Gabarra, R., and Besri, M. (1999). Tomatoes. In Integrated Pest and Disease Management in Greenhouse Crops. Developments in Plant Pathology., Eds. R. Albajes, M. L. Gullino, J. C. van Lenteren, and Y. Elad, 14: 420-434. (Springer, Dordrecht). doi: 10.1007/0-306-47585-5_30
Gabarra, R., Alomar, Ò., Castañé, C., Goula, M., and Albajes, R. (2004). Movement of greenhouse whitefly and its predators between in- and outside of Mediterranean greenhouses. Agriculture, Ecosystems and Environment. 102 (3): 341–348. doi: 10.1016/j.agee.2003.08.012
Gaiero, J. R., McCall, C. A., Thompson, K. A., Day, N. J., Best, A. S., and Dunfield, K. E. (2013). Inside the root microbiome: Bacterial root endophytes and plant growth promotion. American Journal of Botany. 100 (9): 1738–1750. doi: doi:10.3732/ajb.1200572
Galambos, N., Compant, S., Moretto, M., Sicher, C., Puopolo, G., Wäckers, F., Sessitsch, A., Pertot, I., and Perazzolli, M. (2020). Humic acid enhances the growth of tomato promoted by endophytic bacterial strains through the activation of hormone-, growth-, and transcription-related processes. Frontiers in Plant Science. 11 (9): 1–18. doi: 10.3389/fpls.2020.582267
Gibb, K. S., and Randles, J. (1991). Transmission of velvet tobacco mottle virus and related viruses by the mirid *Cyrtopeltis nicotianae*. Advances in Disease Vector Research. 7: 1–17. doi: 10.1007/978-1-4613-9044-2_1
Gibb, K. S., and Randles, J. W. (1988). Studies on the transmission of velvet tobacco mottle virus by the mirid, *Cyrtopeltis nicotianae*. Annals of Applied Biology. 112 (3): 427–437. doi: 10.1111/j.1744-7348.1988.tb02081.x
Gibb, K. S., and Randles, J. W. (1990). Distribution of velvet tobacco mottle virus in its mirid vector and its relationship to transmissibility. Annals of Applied Biology. 116 (3): 513–521. doi: 10.1111/j.1744-7348.1990.tb06634.x
Gonella, E., Pajoro, M., Marzorati, M., Crotti, E., Mandrioli, M., Pontini, M., Bulgari, D., Negri, I., Sacchi, L., Chouaia, B., Daffonchio, D., and Alma, A. (2015). Plant-mediated interspecific horizontal transmission of an intracellular symbiont in insects. Scientific Reports. 5: 1–10. doi: 10.1038/srep15811
Hammer, Ø., Harper, D. A. T., and Ryan, P. D. (2001). Past: Paleontological statistics software package for education and data analysis. Palaeontologia Electronica. 4 (1): 1–9.
Hardoim, P.R., van Overbeek, L.S., Elsas, J.D. (2008). Properties of bacterial endophytes and their proposed role in plant growth. Trends in Microbiology. 16 (10): 463-471. doi: 10.1016/j.tim.2008.07.008
Heck, M. (2018). Insect transmission of plant pathogens: a systems biology perspective. MSystems. 3 (2): e00168-17. doi: 10.1128/msystems.00168-17
Hobson, G., and Grierson, D. (1993). Tomato. In Biochemistry of Fruit Ripening., Eds. G. B. Seymour, J. E. Taylor, and G. A. Tucker, 405–442. (Springer, Dordrecht). doi: 10.1007/978-94-011-1584-1_14
Kikuchi, Y., Hosokawa, T., and Fukatsu, T. (2007). Insect-microbe mutualism without vertical transmission: A stinkbug acquires a beneficial gut symbiont from the environment every generation. Applied and Environmental Microbiology. 73 (13): 4308–4316. doi: 10.1128/AEM.00067-07
Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). MEGA X: Molecular evolutionary

genetics analysis across computing platforms. Molecular Biology and Evolution. 35 (6): 1547–1549. doi: 10.1093/molbev/msy096

Li, S. J., Ahmed, M. Z., Lv, N., Shi, P. Q., Wang, X. M., Huang, J. L., and Qiu, B. L. (2017). Plantmediated horizontal transmission of *Wolbachia* between whiteflies. ISME Journal. 11 (4): 1019–1028. doi: 10.1038/ismej.2016.164

Lòpez-Fernàndez, S., Compant, S., Vrhovsek, U., Bianchedi, P. L., Sessitsch, A., Pertot, I., and Campisano, A. (2015). Grapevine colonization by endophytic bacteria shifts secondary metabolism and suggests activation of defense pathways. Plant and Soil. 405: 155–175. doi: 10.1007/s11104-015-2631-1

Lòpez-Fernàndez, S., Mazzoni, V., Pedrazzoli, F., Pertot, I., and Campisano, A. (2017). A phloem-feeding insect transfers bacterial endophytic communities between grapevine plants. Frontiers in Microbiology. 8 (5): 1–17. doi: 10.3389/fmicb.2017.00834

Lykouressis, D. P., Perdikis, D., and Michalaki, M. (2001). Nymphal development and survival of *Macrolophus pygmaeus* Rambur (Hemiptera: Miridae) on two eggplant varieties as affected by temperature and presence/absence of prey. Biological Control. 20 (3): 222–227. doi: 10.1006/bcon.2000.0888

Manz, W., Amann, R., Ludwig, W., Wagner, M., and Schleifer, K. H. (1992). Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: Problems and solutions. Systematic and Applied Microbiology. 15 (4): 593–600. doi: 10.1016/S0723-2020(11)80121-9

Mitter, B., Pfaffenbichler, N., Flavell, R., Compant, S., Antonielli, L., Petric, A., Berninger, T., Naveed, M., Sheibani-Tezerji, R., Maltzahn, G. von, and Sessitsch, A. (2017). A new approach to modify plant microbiomes and traits by introducing beneficial bacteria at flowering into progeny seeds. Frontiers in Microbiology. 8 (1): 1–10. doi: 10.3389/fmicb.2017.00011

Moerkens, R., Berckmoes, E., Van Damme, V., Ortega-Parra, N., Hanssen, I., Wuytack, M., Wittemans, L., Casteels, H., Tirry, L., De Clercq, P., and De Vis, R. (2016). High population densities of *Macrolophus pygmaeus* on tomato plants can cause economic fruit damage: Interaction with Pepino mosaic virus? Pest Management Science. 72 (7): 1350–1358. doi: 10.1002/ps.4159

Moerkens, R., Berckmoes, E., Van Damme, V., Wittemans, L., Tirry, L., Casteels, H., and De Vis, R. (2017). Inoculative release strategies of *Macrolophus pygmaeus* Rambur (Hemiptera: Miridae) in tomato crops: Population dynamics and dispersal. Journal of Plant Diseases and Protection. 124 (3): 295–303. doi:10.1007/ s41348-017-0077-9

Moerkens, R., Pekas, A., Bellinkx, S., Hassen, I., Huysmans M., Bosmans L. and Wäckers F. (2020). *Nesidiocoris tenuis* as a pest in Northwest Europe: Intervention threshold and influence of Pepino mosaic virus. Journal of Applied Entomology. 144 (7): 566– 577. doi: 10.1111/jen.12789

Myhre, E. A. (1988). Effects of *Lygus* spp. (Hemiptera: Miridae) on Mediterranean white lupin (*Lupinus albus*). Washinton State University, Pullman, WA.

Perdikis, D. and Lykouressis, D. (2000). Effects of various items, host plants, and temperatures on the development and survival of *Macrolophus pygmaeus* Rambur (Hemiptera: Miridae). Biological Control. 17 (1): 55–60. doi: 10.1006/bcon.1999.0774

Perilla-Henao, L. M., and Casteel, C. L. (2016). Vector-borne bacterial plant pathogens: Interactions with hemipteran insects and plants. Frontiers in Plant Science. 7 (8): 1–15. doi: 10.3389/fpls.2016.01163

Pertot I., Caffi T., Rossi V., Mugnai L., Hoffmann C., Grando M. S., Gary C., Lafond D., Duso C., Thiery D., Mazzoni V., Anfora G. (2017). A critical review of plant protection tools for reducing pesticide use on grapevine and new perspectives for the implementation of IPM in viticulture. Crop Protection. 97: 70-84. doi: 10.1016/j.cropro.2016.11.025

Pillay, V. K., and Nowak, J. (1997). Inoculum density, temperature, and genotype effects on in vitro growth promotion and epiphytic and endophutic colonization of tomato (*Lycopersicum esculentum* L.) seedlings inoculated with a *Pseudomonad* bacterium. Canadian Journal of Microbiology. 43 (4): 354–361. doi: 10.1139/m97-049

Put, K., Bollens, T., Wäckers, F. L., and Pekas, A. (2012). Type and spatial distribution of food supplements impact population development and dispersal of the omnivore predator *Macrolophus pygmaeus* (Rambur) (Hemiptera: Miridae). Biological Control. 63 (2): 172–180. doi: org/10.1016/j.bioco ntrol.2012.06.011

Redhead, J. W., Powney, G. D., Woodcock, B. A., and Pywell, R. F. (2020). Effects of future agricultural change scenarios on beneficial insects. Journal of Environmental Management. 265: 110550. doi: 10.1016/j.jenvman.2020.110550

Ruiu, L. (2020). Plant-growth-promoting bacteria (PGPB) against insects and other agricultural pests. Agronomy. 10 (6): 1–12. doi: 10.3390/agronomy10060861

Sánchez, J. A., and Lacasa, A. (2008). Impact of the zoophytophagous plant bug *Nesidiocoris tenuis* (Heteroptera: Miridae) on tomato yield. Journal of Economic Entomology. 101 (6): 1864–1870. doi: 10.1603/0022-0493-101.6.1864

Sanchez, J. A., Lacasa, A., Arnó, J., Castañé, C., and Alomar, O. (2009). Life history parameters for *Nesidiocoris tenuis* (Reuter) (Het., Miridae) under different temperature regimes. Journal of Applied Entomology. 133 (2): 125–132. doi: 10.1111/j.1439-0418.2008.01342.x

Sanchez, J. A., López-Gallego, E., Pérez-Marcos, M., Perera-Fernández, L. G., and Ramírez-Soria, M. J. (2018). How safe is it to rely on *Macrolophus pygmaeus* (Hemiptera: Miridae) as a biocontrol agent in tomato crops? Frontiers in Ecology and Evolution. 6 (9): 1–10. doi: 10.3389/fevo.2018.00132

Sawana, A., Adeolu, M., and Gupta, R. S. (2014). Molecular signatures and phylogenomic analysis of the genus *Burkholderia*: Proposal for division of this genus into the emended genus *Burkholderia* containing pathogenic organisms and a new genus *Paraburkholderia* gen. nov. harboring environmental species. Frontiers in Genetics. 5: 429. doi: 10.3389/fgene.2014.00429

Schaefer, C. W., and Panizzi, A. R. (2000). Heteroptera of economic importance. (CRC Press). doi: 10.1201/9781420041859

Schneider, C., Rasband, W. and Eliceiri, K. (2012). NIH Image to ImageJ: 25 years of image analysis. Nature Methods. 9 (7): 671–675. doi: 10.1038/nmeth.2089

Sessitsch, A., Coenye, T., Sturz, A. V, Vandamme, P., Barka, E. A., Salles, J. F., Elsas, J. D. Van, Faure, D., Reiter, B., Glick, B. R., Wang-Pruski, G., and Nowak, J. (2005). *Burkholderia phytofirmans* sp. nov., a novel plant-associated bacterium with plant-beneficial properties. International Journal of Systematic and Evolutionary Microbiology. 55: 1187–1192. doi: 10.1099/ijs.0.63149-0

Sharma, V. K., and Nowak, J. (1998). Enhancement of verticillium wilt resistance in tomato transplants by in vitro co-culture of seedlings with a plant growth promoting rhizobacterium (*Pseudomonas* sp. strain PsJN). Canadian Journal of Microbiology. 44 (6): 528–536. doi: 10.1139/w98-017

Stahl, F. J., and Leupschen, N. S. (1977). Transmission of *Erwinia amylovora* to pear fruti by *Lygus* spp. Plant Disease Management Resports. 61: 936–939.

Urbaneja, A., Montón, H., and Mollá, O. (2009). Suitability of the tomato borer *Tuta absoluta* as prey for *Macrolophus pygmaeus* and *Nesidiocoris tenuis*. Journal of Applied Entomology. 133 (4): 292–296. doi: 10.1111/j.1439-0418.2008.01319.x

Wakil, W., Brust, G. E., and Perring, T. (2018). Sustainable management of arthropod pests of tomato. (Academic Press).

Wallner, G., Amann, R., and Beisker, W. (1993). Optimizing fluorescent *in situ* hybridization with

rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. Cytometry. 14 (2): 136–143. doi: 10.1002/cyto.990140205
Wheeler, A. G. (2002). Biology of the plant bugs (Hemiptera: Miridae): Pests, predators, opportunists. (Cornell University Press).
Zhu, Y., Kawai, H., Hashiba, S., Amarasiri, M., Kitajima, M., Okabe, S., and Sano, D. (2020). The effect of GD1a ganglioside-expressing bacterial strains on murine norovirus infectivity. Molecules. 25 (18): 4084. doi: 10.3390/molecules25184084

TABLE 1 | Tomato growth promotion by endophytic bacterial strains.

	Mock-inoculated		PsJN-inoculated		32A-inoculated	
	Day 7	**Day 11**	**Day 7**	**Day 11**	**Day 7**	**Day 11**
Shoot length (cm)	2.9 ± 0.15a	4.87 ± 0.23A	3.95 ± 0.16b	5.35 ± 0.17A	4.12 ± 0.19b	5.31 ± 0.12A
Fresh weight (g)	0.033 ± 0.002a	0.053 ± 0.002A	0.04 ± 0.001b	0.058 ± 0.002A	0.04 ± 0.001b	0.059 ± 0.002A

Shoot length (cm) and fresh weight (g) of mock-inoculated plants and plants inoculated with *Paraburkholderia phytofirmans* PsJN (PsJN) or *Enterobacter* sp. 32A (32A) was assessed without mirid feeding at seven (day 7) and eleven (day 11) days after seed inoculation. The two-way analysis of variance showed no significant differences between the two experimental repetitions ($P > 0.05$) and data from the two experiments were pooled. Mean and standard error values of 24 replicates (plants) are presented for each treatment. Different lowercase and uppercase letters indicate significant differences among treatments at three or seven days according to Tukey's test ($P \leq 0.05$), respectively.

Figures

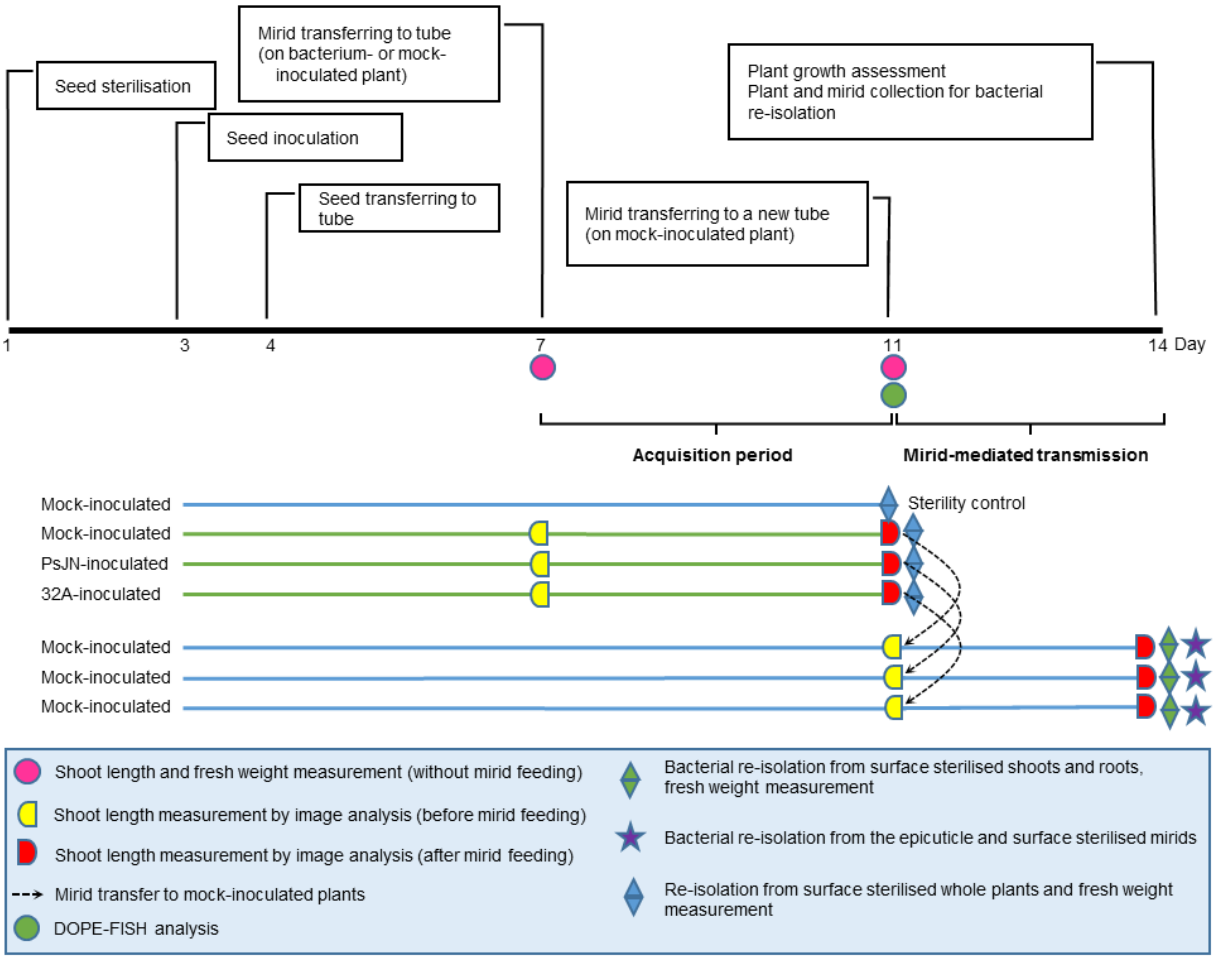

FIGURE 1 | Description of the experiment. Surface-sterilised seeds were transferred to Petri dishes containing 1% water agar and were incubated for two days in a growth chamber to allow seed germination (day 1). Seeds were treated with sterile $MgSO_4$ (mock-inoculated) or inoculated with a bacterial suspension of *Paraburkholderia phytofirmans* PsJN (PsJN) or *Enterobacter* sp. 32A (32A) by overnight incubation in the growth chamber (day 3). Germinated seeds with the same root length were selected and each seed was transferred to a sterile glass tube containing half-strength Hoagland (day 4). A freshly emerged mirid adult (*Macrolophus pygmaeus* or *Nesidiocoris tenuis*) was placed in each glass tube containing a seven days-old tomato plant that was either mock-inoculated or inoculated with PsJN or 32A (day 7). Before transferring the mirid in the glass tube shoot length was measured by image analysis (day 7). Tubes were incubated for four days in the growth chamber in order to allow mirids to feed on tomato plants (acquisition period). For double labelling of oligonucleotide probes for fluorescence *in situ* hybridisation (DOPE-FISH) analysis, mirids were collected four days after feeding (acquisition period) on mock-inoculated plants or plants inoculated with PsJN or 32A (day 11). Shoot length was measured by image analysis and plants were collected for bacterial re-isolation and fresh weight assessment after mirid feeding (day 11). Each mirid was transferred in a new glass tube containing a mock-inoculated tomato plant (day 11) and incubated for three additional days (mirid-mediated transmission), in order to allow mirids to feed on tomato plants. Mirids and plants after mirid-mediated transmission were collected for bacterial re-isolation (day 14), tomato shoot length was measured by image analysis and fresh weight was assessed (day 14).

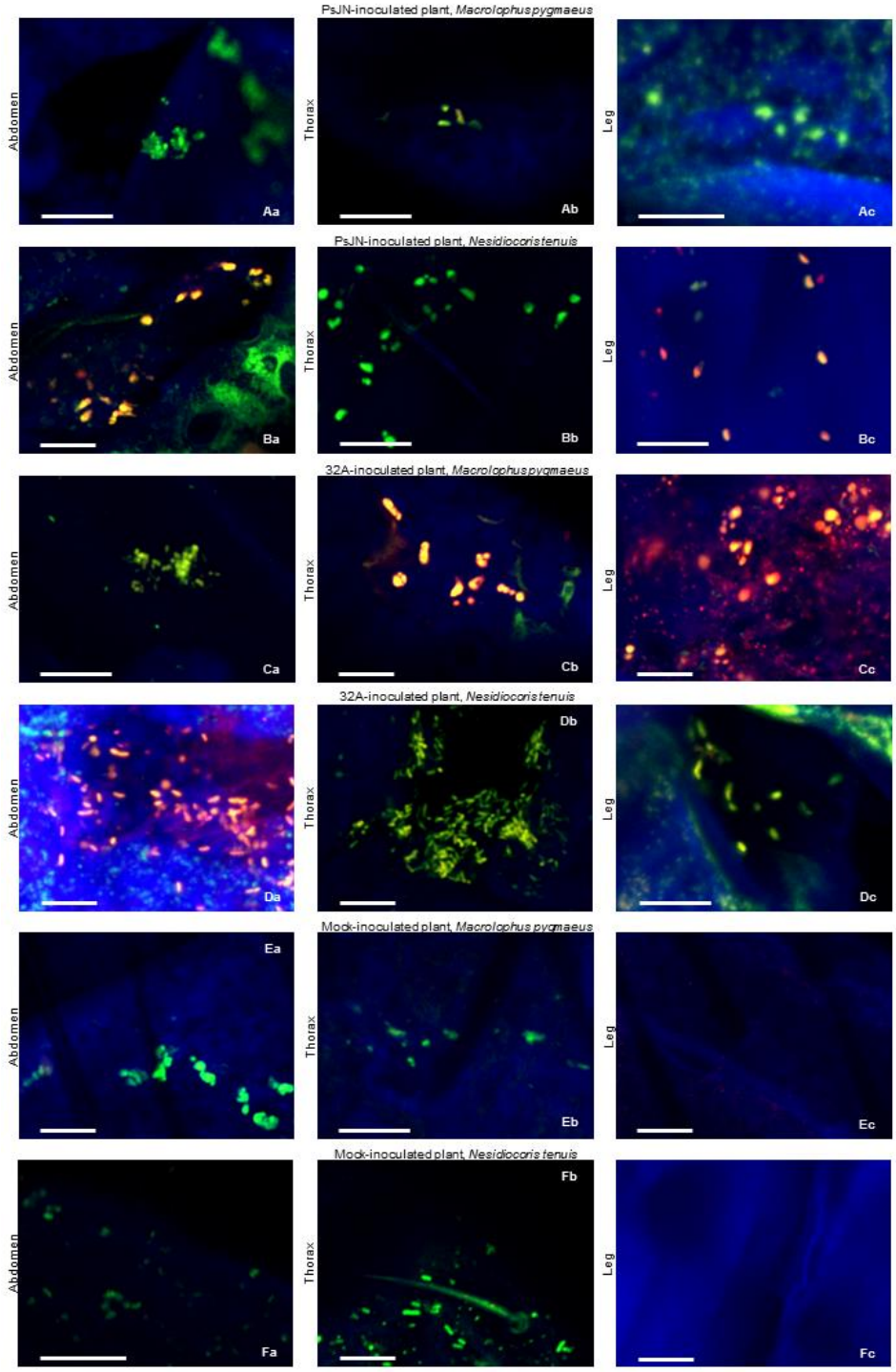

FIGURE 2 | Location of endophytic bacterial strains on mirids. *Macrolophus pygmaeus* (A, C and E) and *Nesidiocoris tenuis* (B, D and F) abdomen (a), thorax (b) and leg (c) samples were collected at the end of the acquisition period (day 11) on plants inoculated with *Paraburkholderia phytofirmans* PsJN (PsJN) (A, B) or *Enterobacter* sp. 32A (32A) (C, D) or mock-inoculated plants (E, F). PsJN cells were hybridised with the EUBmix and Bphyt probes (A, B) and 32A cells were hybridised with the EUBmix and Gam42a probes (C, D). Mirids fed on mock-inoculated plants (E, F) were hybridised with the EUBmix and Gam42a probes. Five replicates (mirids) were analysed for each treatment and representative pictures were selected. Bars correspond to 10 µm.

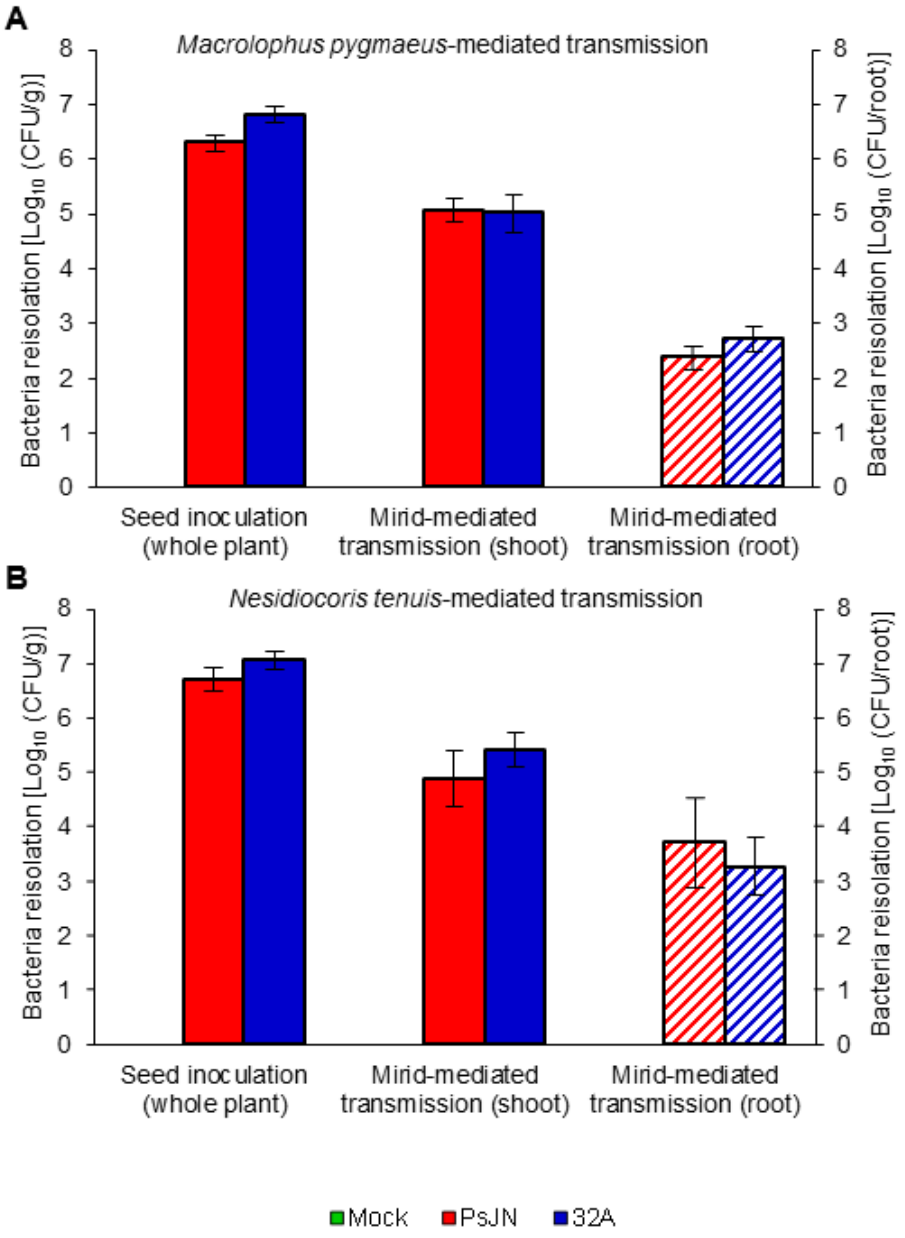

FIGURE 3 | Quantification of endophytic bacterial strains in tomato plants. Bacterial re-isolation was carried out from seed-inoculated whole plants at the end of the acquisition period (day 11) and from shoots or roots of plants at the end of the mirid-mediated transmission (day 14) with *Macrolophus pygmaeus* (A) or *Nesidiocoris tenuis* (B). The quantity of re-isolated bacteria is expressed as colony forming units (CFU) per gram of fresh weight of the whole plant (CFU g^{-1}) per gram of fresh weight of the plant shoot (CFU g^{-1}) or as CFU for each plant shoot (CFU g^{-1}) or root (CFU $root^{-1}$) of mock-inoculated plants (Mock, green) and plants inoculated with *Paraburkholderia phytofirmans* PsJN (PsJN, red) or *Enterobacter* sp. 32A (32A, blue). The two-way analysis of variance showed no significant differences between the two experimental repetitions ($P > 0.05$) and data from the two experiments were pooled. Figure only shows mean and standard error values for positive samples and at least nine replicates (plants) are presented for each treatment. For each treatment, no significant differences were found in the pairwise comparisons between *M. pygmaeus* or *N. tenuis* fed on PsJN- and 32A-inoculated plants according to Mann-Whitney test ($P \leq 0.05$). No PsJN and 32A bacterial colonies were isolated from mock-inoculated samples.

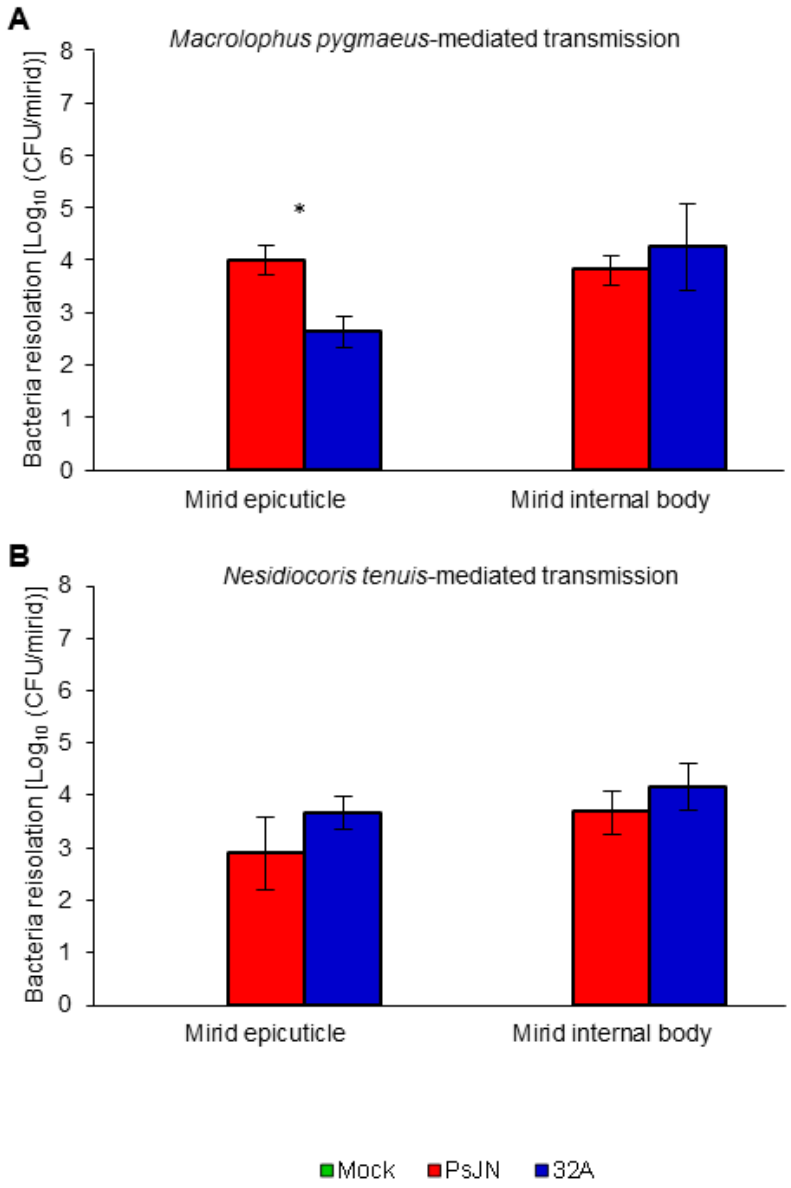

FIGURE 4 | Quantification of endophytic bacterial strains on mirid epicuticle and internal. Bacterial re-isolation was carried out from mirid washing suspension (bacteria adhering to the mirid epicuticle) or from tissue grinding of surface-sterilised mirid (bacteria in the mirid internal body) at the end of the mirid-mediated transmission (Mirid-mediated transmission; day 14) with *Macrolophus pygmaeus* (A) or *Nesidiocoris tenuis* (B). The quantity of re-isolated bacteria is expressed as colony forming units per mirid (CFU mirid^{-1}) fed on mock-inoculated plants (Mock, green) and plants inoculated with *Paraburkholderia phytofirmans* PsJN (PsJN, red) or *Enterobacter* sp. 32A (32A, blue). The two-way analysis of variance showed no significant differences between the two experimental repetitions ($P > 0.05$) and data from the two experiments were pooled. Figure only shows mean and standard error values for positive samples and at least nine replicates (mirids) are presented for each treatment. Asterisks indicate significant differences in the pairwise comparisons between *M. pygmaeus* fed on PsJN- and 32A-inoculated plants according to Mann-Whitney test ($P \leq 0.05$). For each treatment, no significant differences were found in the pairwise comparisons between *N. tenuis* fed on PsJN- and 32A-inoculated plants according to Mann-Whitney test ($P \leq 0.05$). No PsJN and 32A bacterial colonies were isolated from mirids fed on mock-inoculated samples.

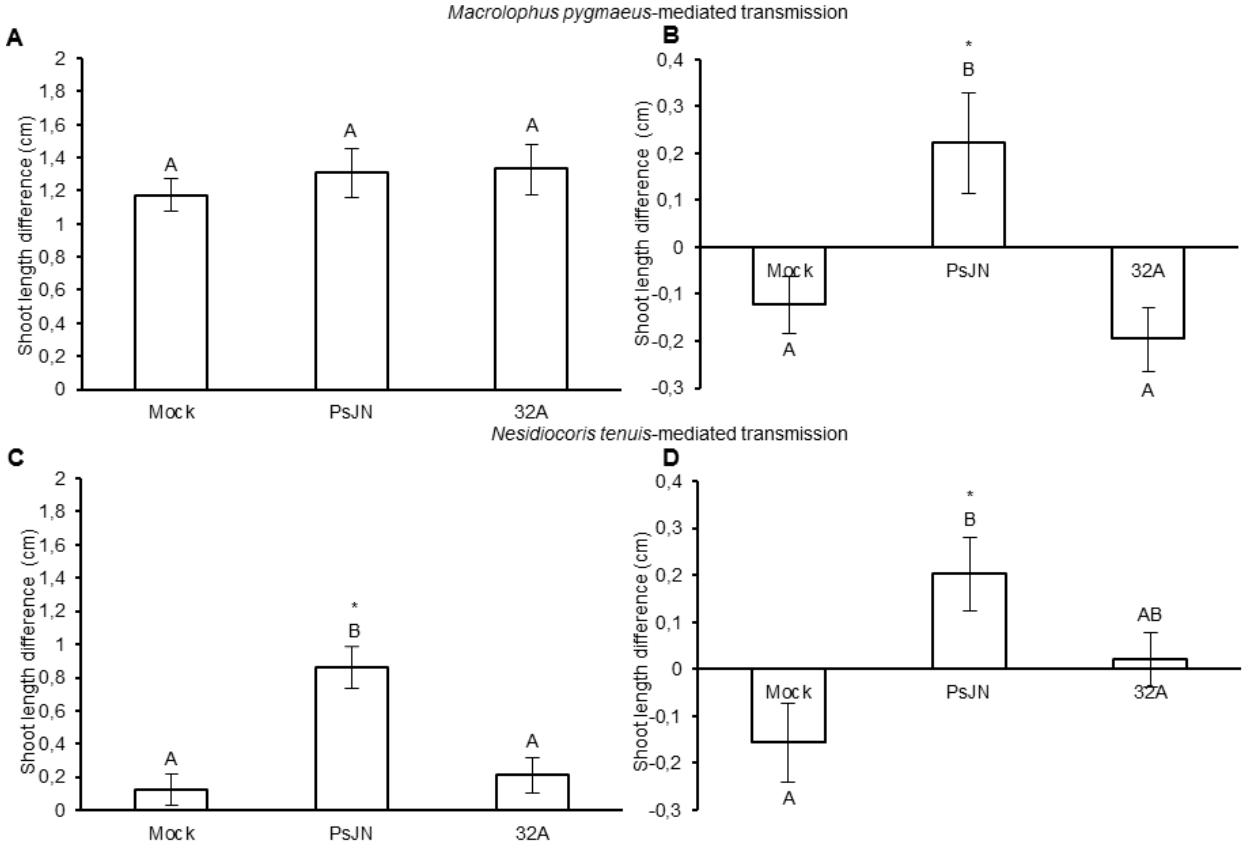

FIGURE 5 | Effects of mirid feeding on tomato shoot length. Changes in shoot length caused by *Macrolophus pygmaeus* (A and B) or *Nesidiocoris tenuis* (C and D) feeding were assessed on mock-inoculated plants (Mock) and plants inoculated with *Paraburkholderia phytofirmans* PsJN (PsJN) or *Enterobacter* sp. 32A (32A) and calculated as the difference between shoot length measured before (day 7) and at the end of the acquisition period (day 11; A and C) or before (day 11) and after the mirid-mediated transmission (day 14; B and D). The two-way analysis of variance showed no significant differences between the two experimental repetitions ($P > 0.05$) and data from the two experiments were pooled. Figure only shows mean and standard error values for positive samples and at least nine replicates (plants) are presented for each treatment. Different letters indicate significant differences among treatments according to Tukey's test ($P \leq 0.05$). Asterisks indicate significant differences in the pairwise comparisons between mock-inoculated and bacterium-inoculated plants, according to Student's t test ($P \leq 0.05$).

SUPPLEMENTARY MATERIAL

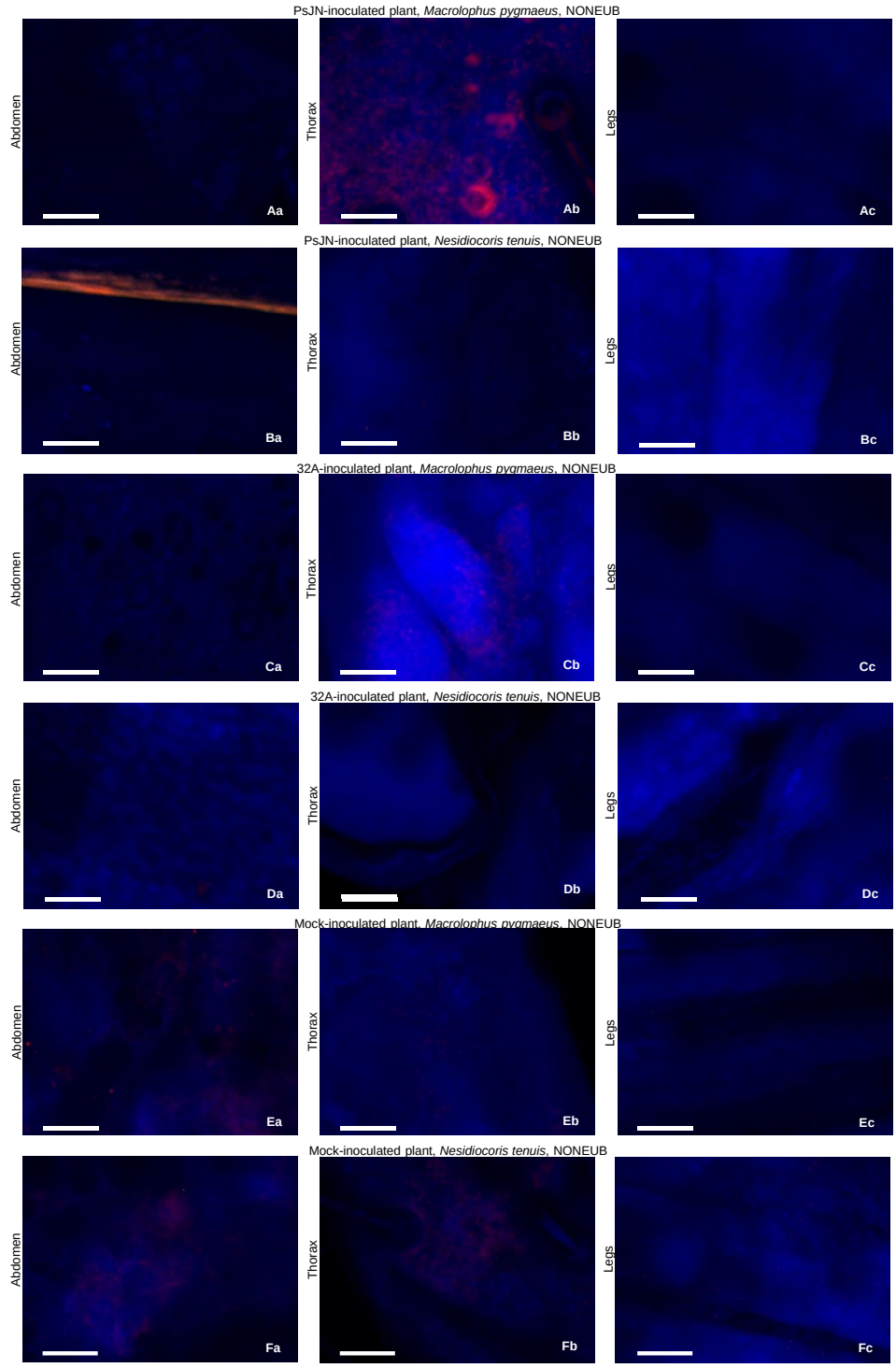

FIGURE S1 | Negative controls of double labelling of oligonucleotide probes for fluorescence *in situ* hybridisation. *Macrolophus pygmaeus* (A, C and E) and *Nesidiocoris tenuis* (B, D and F) abdomen (a), thorax (b) and legs (c) samples were collected four days after feeding (acquisition period) on plants inoculated with *Paraburkholderia phytofirmans* PsJN (PsJN) (A, B) or *Enterobacter* sp. 32A (32A) (C, D) or mock-inoculated plants (E, F) and hybridised with the NONEUB probe as negative probe not targeting bacterial sequences. Five replicates (plants) were analysed for each treatment and representative pictures were selected. Bars correspond to 10 µm.

6 DISCUSSION

Conventional agriculture largely depends on the use of synthetic chemical fertilisers and pesticides, which have numerous environmental drawbacks, such as the contamination of soil and aquatic ecosystems, non-target species loss and residue accumulation in the final product (Iyaniwura 1991; Khan et al., 2018). Among crop plants, tomato is cultivated under greenhouses and field conditions (Hobson and Grierson 1993), requires an extensive use of chemical fertilisers and is greatly affected by arthropod feeding damages (Wakil et al., 2017; Maham et al., 2020). Microbial biofertilisers (e.g. plant growth-promoting bacteria; PGPB) and non-microbial biostimulants (e.g. humic acids; HA) are key alternative solutions to reduce the use of harmful synthetic fertilisers and pesticides in agriculture (Rouphael and Colla 2020b). Although PGPB interaction with plants have been relatively well studied, the combined applications of living PGPB and organic biostimulant on crops remains poorly investigated.

Among alternative solutions, biological control in crop protection is the augmentation or introduction of beneficial organisms (including generalist predators of the family Miridae) for the management of crop pests, pathogens or weeds (Heimpel and Cock 2018). However, the wide host range, winged adults and piercing-sucking mouthparts indicate the potential of beneficial mirids predators as vector of phytopathogenic or beneficial microbes, such as PGPB (Gibb and Randle, 1991).

In this PhD project, the molecular basis of the interaction between endophytic bacterial strains and tomato plants in the presence of HA was investigated, in order to improve the understanding on the mechanism responsible for plant growth promotion. Some strains belonging to the bacterial genera *Enterobacter*, *Pantoea* and *Paraburkholderia* had already been previously recognised as PGPB (Sessitsch et al., 2005; Campisano et al., 2014; Hardoim et al., 2015) and seed inoculation with *P. phytofirmans* PsJN, *P. agglomerans* D7G and *Enterobacter* sp. 32A increased tomato shoot growth. Inoculated tomato plants were efficiently colonised by the tested endophytic bacterial strains and HA did not increase the tissue colonisation intensity compared to the control condition. Moreover, the addition of HA (at the optimal concentration of 50 mg L^{-1}) enhanced the tomato growth induced by the endophytic bacterial strains, suggesting some possible additive effects of HA to the tested PGPBs, as reported in previous studies for maize (Canellas et al., 2012; Canellas et al., 2015). Half of the DEGs was modulated by at least two

endophytic bacterial strains and they represent possible common pathways modulated in response to bacterial endophytes, involving protein metabolism, transcription, transport, signal transduction and defence. Moreover, tomato genes were modulated by endophytic bacterial strains mainly in roots compared to shoots, indicating major transcriptional regulations in belowground compared to aboveground tissues. Likewise, plant roots play a critical role in perception and recognition of the rhizosphere microorganisms (De Palma et al., 2019) and the presence of HA enhanced the activation of signal transduction and transcription processes in response to endophytic bacterial strains. Since beneficial effects of endophytic bacterial strains can derive from multiple mode of action (Glick 2012; Ferreira et al., 2019), it is difficult to discriminate effects of microbial activities in providing nutrients to plants and/or stimulation of plant growth (e.g. modulation of the hormone levels). The increase of nutrient uptake is one of the direct mechanisms through which PGPB (Glick 2012) and HA (Zanin et al., 2019) contribute to plant development. In this study, several transporter-encoding tomato genes were upregulated suggesting a positive effect of PGPB and HA on tomato nutrient uptake, in agreement with previous findings (Zandonadi et al., 2016; Ibort et al., 2018). Another direct mechanism of PGPB-dependent plant growth promotion is the modulation of the hormone levels (Glick 2012). Previous studies demonstrated the ability of *P. phytofirmans* PsJN, *P. agglomerans* D7G and *Enterobacter* sp. 32A to produce auxin (Poupin et al., 2013; Campisano et al., 2014) and the capacity of HA to upregulate auxin responsive genes in *A. thaliana* (Trevisan et al., 2010) and induce cytokinin-like (Pizzeghello et al., 2012) and gibberellin-like (Nardi et al., 2000) activity in maize plants, suggesting complementation effects of endophytic bacterial strains and HA in the stimulation of hormone metabolism. As a possible consequence of hormonal changes, genes upregulated by at least two endophytic bacterial strains in the HA condition were involved in cell growth and cell wall biosynthesis. Thus, the upregulation of cell wall-loosening enzymes may be a common plant response to PGPB, in order to facilitate endophytic colonisation and plant growth promotion (Irizarry and White 2018). In addition, the antioxidant machinery was activated in tomato roots mainly in the HA condition, indicating an attempted plant defence reaction against endophytic bacterial strains that is probably precisely tuned by the endophytic bacterial strains to allow tissue colonisation (van Loon et al., 2007). In addition, strain-specific tomato responses involved signal transduction, transcription, hormone metabolism, protein metabolism, secondary metabolism and defence processes, highlighting specific traits of the

endophyte-tomato interaction. These findings demonstrate the complementation properties of HA on the activation of pathways responsible for signal transduction, hormone metabolism, transcription, protein metabolism, transport, defence and cell growth in response to the endophytic bacterial strains. Moreover, this study reports the optimised HA dosage and gene markers for the further development of efficient of PGPB- and HA-based biostimulants. Moreover, the results of the study contribute to a better understanding of the molecular mechanisms and metabolic pathways activated in tomato root and shoot by *P. phytofirmans* PsJN, *P. agglomerans* D7G and *Enterobacter* sp. 32A.

This PhD project verified the role of beneficial mirids, *M. pygmaeus* and *N. tenuis* in the transmission of PGPB between tomato plants. The majority of mirids fed on *P. phytofirmans* PsJN- and *Enterobacter* sp. 32A-inoculated tomato plants were able to acquire the bacterial strains both as epicuticle-adhering bacteria and insect internal bacteria. This evidence indicates that tomato tissues are suitable media for the acquisition of the selected endophytic bacteria, possibly because *P. phytofirmans* PsJN and *Enterobacter* sp. 32A are colonising the xylem (Gonella et al., 2015; Galambos et al., 2020). Both mirid species transmitted *P. phytofirmans* PsJN and *Enterobacter* sp. 32A between tomato plants and bacteria could be re-isolated from tomato shoots after mirid-mediated transmission. In particular, plant roots, separated from the stems by a paraffin layer in order to prevent the contamination of the growth medium and to avoid insect contact with media and roots, were colonised by *P. phytofirmans* PsJN and *Enterobacter* sp. 32A, indicating that both strains moved from stems to roots after transmission, as previously reported in grapevine for *Enterobacter* sp. 32A (Lòpez-Fernàndez et al., 2017). Moreover, considering the mobile nature of mirids, they have the potential to facilitate the transmission of beneficial endophytes among crops (Gibb and Randle, 1991; Gonella et al., 2015). These findings provide direct evidence of mirid-mediated transmission of beneficial bacterial strains, including the transmission efficiency, quantification and distribution pattern in tomato plant tissues (shoots and roots) and in insect tissues (epicuticle-adhering bacteria and insect internal bacteria). In order to implement plant inoculation strategies with mirids, future steps must focus on the interactions between *M. pygmaeus* or *N. tenuis* and the PGPB strains in order to evaluate the persistence of beneficial bacteria on mirids, possible unknown harmful impact of PGPB on mirids' performance and efficacy as predators and to verify if PsJN and 32A can also be transmitted through feeding, physical contact, excretion and/or their combination.

7 CONCLUSIONS

Three bacterial strains (namely *P. phytofirmans* PsJN, *P. agglomerans* D7G and *Enterobacter* sp. 32A) that endophytically colonise tomato plants were selected and they were able to promote tomato shoot length in the presence of HA, indicating a possible complementation of PGPB and HA effects. Tomato colonisation by endophytic bacterial strains was comparable in the control and HA condition and molecular mechanisms activated in tomato plants in response to endophytic bacteria were clarified by a transcriptomic study. In particular, the main transcriptional regulations occurred in tomato roots and the majority of DEGs was upregulated by endophytic bacterial strains in the HA condition. Half of the DEGs was modulated by more than two strains, as possible common reactions to endophytic bacterial strains, involving protein metabolism, transcription, transport, signal transduction and defence. Moreover, strain-specific tomato responses included the upregulation of signal transduction, transcription, hormone metabolism, protein metabolism, secondary metabolism and defence processes, highlighting specific traits of the endophyte-tomato interaction. The presence of HA enhanced the upregulation of signal transduction, hormone metabolism, transcription, protein metabolism, transport, defence and growth-related processes in terms of number of involved genes and fold change values, indicating additive effects of bacterial endophytes and HA in plant growth promotion mechanisms. Beneficial mirids (namely *M. pygmaeus* and *N. tenuis*) act as efficient vectors to transmit *P. phytofirmans* PsJN and *Enterobacter* sp. 32A between tomato plants. Tomato shoot and root colonisation by endophytic bacterial strains was found in tomato plants after mirid-mediated transmission of *P. phytofirmans* PsJN and *Enterobacter* sp. 32A. These results provide information on acquisition and transmission of PGPB by beneficial insects and reports a novel method for biofertiliser delivery. Knowledge on the HA-dependent enhancement of growth-related processes stimulated by endophytic bacterial strains and on the insect-mediated transmission of beneficial bacterial endophytes obtained in this work represent key information for the further development of combined formulations of endophytic bacterial strains and HA and for the possible use of beneficial insects as delivery strategies of bacterial biofertilisers for tomato production. Further studies could add knowledge on the interaction of endophytes and HA with the plant microbiome and their combined effect on plants' fitness. Likewise, future steps must focus on the interactions between *M. pygmaeus* or *N. tenuis* and the

PGPB strains in order to evaluate the persistence of beneficial bacteria on mirids, possible unknown harmful impact of PGPB on mirids' performance and efficacy as predators. Genes identified in this work may be the source of important markers to monitor the tomato response to bacterial endophytes and HA under field conditions. Although the data presented in this thesis does not specifically seek to understand how the combined application of PGPB and HA affect mineral nutrition and hormone metabolism in plants, further molecular and biochemical studies would help to elucidate their complementary role in plant growth promotion. More trials are needed under commercial greenhouse or field conditions over multiple years with employing omics technologies followed by analysis of targeted genes, proteins and metabolites to confirm plant growth promotion effects of endophytic bacteria and HA, to understand changes in both plants and PGPB and to verify the successful transmission mediated by mirids.

8 ABBREVIATIONS

ACC	1-aminocyclopropnae-1-carboxylate
DOPE-FISH	Double labelling of oligonucleotide probes for fluorescent *in situ* hybridisation
GFP	Green fluorescent protein
HA	Humic acid
HCN	Hydrogen cyanide
IAA	Indole-3-acetic acid
PGPB	Plant growth-promoting bacteria
ROS	Reactive oxygen species
SAM	S-adenosylmethionine

9 REFERENCES

Abbasi, P. A., and Weselowski, B. (2015). Efficacy of *Bacillus subtilis* QST 713 formulations, copper hydroxide, and their tank mixes on bacterial spot of tomato. Crop Protection. 74: 70–76. doi: 10.1016/j.cropro.2015.04.009

Abd-Alla, M. H. (1994). Phosphatases and the utilization of organic phosphorus by *Rhizobium leguminosarum* biovar *viceae*. Letters in Applied Microbiology. 18 (5): 294–296. doi: 10.1111/j.1472-765X.1994.tb00873.x

Abdallah, R. A. B., Mokni-Tlili, S., Nefzi, A., Jabnoun-Khiareddine, H., and Daami-Remadi, M. (2016). Biocontrol of Fusarium wilt and growth promotion of tomato plants using endophytic bacteria isolated from *Nicotiana glauca* organs. Biological Control. 97: 80–88. doi: 10.1016/j.biocontrol.2016.03.005

Abdellatif, I. M. Y., Abdel-Ati, Y. Y., Abdel-Mageed, Y. T., and Hassan, M. A. M. M. (2017). Effect of humic acid on growth and productivity of tomato plants under heat stress. Journal of Horticultural Research. 25 (2): 59–66. doi: 10.1515/johr-2017-0022

Aguirre, E., Leménager, D., Bacaicoa, E., Fuentes, M., Baigorri, R., Zamarreño, A. M., and García-Mina, J. M. (2009). The root application of a purified leonardite humic acid modifies the transcriptional regulation of the main physiological root responses to Fe deficiency in Fe-sufficient cucumber plants. Plant Physiology and Biochemistry. 47 (3): 215–223. doi: 10.1016/j.plaphy.2008.11.013

Aksoy, H. M., Kaya, Y., Ozturk, M., Secgin, Z., Onder, H., and Okumus, A. (2017). Pseudomonas putida – Induced response in phenolic profile of tomato seedlings (*Solanum lycopersicum* L.) infected by *Clavibacter michiganensis* subsp. *michiganensis*. Biological Control. 105: 6–12. doi: 10.1016/j.biocontrol.2016.11.001

Alaylar, B., Egamberdieva, D., Gulluce, M., Karadayi, M., and Arora, N. K. (2020). Integration of molecular tools in microbial phosphate solubilization research in agriculture perspective. World Journal of Microbiology and Biotechnology. 36 (7). doi: 10.1007/s11274-020-02870-x

Ali, S., Duan, J., Charles, T. C., and Glick, B. R. (2014). A bioinformatics approach to the determination of genes involved in endophytic behavior in *Burkholderia* spp. Journal of Theoretical Biology. 343: 193–198. doi: 10.1016/j.jtbi.2013.10.007

Amann, R., and Fuchs, B. M. (2008). Single-cell identification in microbial communities by improved fluorescence *in situ* hybridization techniques. Nature Reviews Microbiology. 6 (5): 339–348. doi: 10.1038/nrmicro1888

Andrews, M., and Andrews, M. E. (2017). Specificity in legume-rhizobia symbioses. International Journal of Molecular Sciences. 18 (4). doi: 10.3390/ijms18040705

Attia, M. S., El-Sayyad, G. S., Abd Elkodous, M., and El-Batal, A. I. (2020). The effective antagonistic potential of plant growth-promoting rhizobacteria against *Alternaria solani*-causing early blight disease in tomato plant. Scientia Horticulturae. 266 (9): 109289. doi: 10.1016/j.scienta.2020.109289

Azizoglu, U. (2019). *Bacillus thuringiensis* as a biofertilizer and biostimulator: a mini-review of the little-known plant growth promoting properties of Bt. Current Microbiology. 76 (11): 1379–1385. doi: 10.1007/s00284-019-01705-9

Bais, H. P., Weir, T. L., Perry, L. G., Gilroy, S., and Vivanco, J. M. (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. Annual Review of Plant Biology. 57: 233–266. doi: 10.1146/annurev.arplant.57.032905.105159

Bano, A., and Muqarab, R. (2017). Plant defence induced by PGPR against *Spodoptera litura* in

tomato (*Solanum lycopersicum* L.). Plant Biology. 19 (3): 406–412. doi: 10.1111/plb.12535
Barratt, B. I. P., Moran, V. C., Bigler, F., and van Lenteren, J. C. (2018). The status of biological control and recommendations for improving uptake for the future. Biological Control. 63 (1): 155–167. doi: 10.1007/s10526-017-9831-y
Basu, S., Rabara, R., and Negi, S. (2017). Towards a better greener future - an alternative strategy using biofertilizers. I: Plant growth promoting bacteria. Plant Gene. 12: 43–49. doi: 10.1016/j.plgene.2017.07.004
Berger, B., Brock, A. K., and Ruppel, S. (2013). Nitrogen supply influences plant growth and transcriptional responses induced by *Enterobacter radicincitans* in *Solanum lycopersicum*. Plant and Soil. 370 (1–2): 641–652. doi: 10.1007/s11104-013-1633-0
Bertalan, M., Albano, R., de Pádua, V., Rouws, L., Rojas, C., Hemerly, A., Teixeira, K., Schwab, et al.,(2009). Complete genome sequence of the sugarcane nitrogen-fixing endophyte *Gluconacetobacter diazotrophicus* Pal5. BMC Genomics. 10 450. doi: 10.1186/1471-2164-10-450
Bottini, R., Cassán, F., and Piccoli, P. (2004). Gibberellin production by bacteria and its involvement in plant growth promotion and yield increase. Applied Microbiology and Biotechnology. 65 (5): 497–503. doi: 10.1007/s00253-004-1696-1
Brader, G., Compant, S., Vescio, K., Mitter, B., Trognitz, F., Ma, L. J., and Sessitsch, A. (2017). Ecology and genomic insights into plant-pathogenic and plant-nonpathogenic endophytes. Annual Review of Phytopathology. 55: 61–83. doi: 10.1146/annurev-phyto-080516-035641
Butaite, E., Baumgartner, M., Wyder, S., and Kümmerli, R. (2017). Siderophore cheating and cheating resistance shape competition for iron in soil and freshwater *Pseudomonas* communities. Nature Communications. 8 (1). doi: 10.1038/s41467-017-00509-4
Calvo, F. J., Lorente, M. J., Stansly, P. A., and Belda, J. E. (2012). Preplant release of *Nesidiocoris tenuis* and supplementary tactics for control of *Tuta absoluta* and *Bemisa tabaci* in greenhouse tomato. Entomologia Experimentalis et Applicata. 143 (2): 111–119. doi: 10.1111/j.1570-7458.2012.01238.x
Calvo, J., Bolckmans, K., Stansly, P. A., and Urbaneja, A. (2009). Predation by *Nesidiocoris tenuis* on *Bemisia tabaci* and injury to tomato. Biological Control. 54 (2): 237–246. doi: 10.1007/s10526-008-9164-y
Campisano, A., Pancher, M., Puopolo, G., Puddu, A., Lòpez-Fernàndez, S., Biagini, B., Yousaf, S., and Pertot, I. (2014). Diversity in endophyte populations reveals functional and taxonomic diversity between wild and domesticated grapevines. American Journal of Enology and Viticulture. 66: 12-21. doi: 10.5344/ajev.2014.14046
Canellas, L. P., Olivares, F. L., Okorokova-Façanha, A. L., and Façanha, A. R. (2002). Humic acids isolated from earthworm compost enhance root elongation, lateral root emergence, and plasma membrane H^+-ATPase activity in maize roots. Plant Physiology. 130 (4): 1951–1957. doi: 10.1104/pp.007088
Canellas, Luciano Pasqualoto, Balmori, D. M., Médici, L. O., Aguiar, N. O., Campostrini, E., Rosa, R. C. C., Façanha, A. R., and Olivares, F. L. (2012). A combination of humic substances and *Herbaspirillum seropedicae* inoculation enhances the growth of maize (*Zea mays* L.). Plant and Soil. 366 (1–2): 119–132. doi: 10.1007/s11104-012-1382-5
Canellas, Luciano P, da Silva, S. F., Olk, D. C., Olivares, F. L., Silva, S. F. da, Olk, D. C., and Olivares, F. L. (2015). Foliar application of plant growth-promoting bacteria and humic acid increase maize yields. Journal of Food, Agriculture & Environment. 13 (1): 131–138.
Cavalcante, V. A., and Dobereiner, J. (1988). A new acid-tolerant nitrogen-fixing bacterium associated with sugarcane. Plant and Soil. 108 (1): 23–31. doi: 10.1007/BF02370096

Compant, S., Clément, C., and Sessitsch, A. (2010). Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. Soil Biology and Biochemistry. 42 (5): 669–678. doi: 10.1016/j.soilbio.2009.11.024

Compant, S., and Mathieu, F. (2013). Use of DOPE-FISH tool to better visualize colonization of plants by beneficial bacteria? An example with *Saccharothrix algeriensis* NRRL B-24137 colonizing grapevine plants. Molecular Microbial Ecology of the Rhizosphere. 2: 929–931. doi: 10.1002/9781118297674.ch87

da Costa, P. B., Beneduzi, A., de Souza, R., Schoenfeld, R., Vargas, L. K., and Passaglia, L. M. P. (2013). The effects of different fertilization conditions on bacterial plant growth promoting traits: Guidelines for directed bacterial prospection and testing. Plant and Soil. 368 (1–2): 267–280. doi: 10.1007/s11104-012-1513-z

Daugherty, M. P., Rashed, A., Almeida, R. P. P., and Perring, T. M. (2011). Vector preference for hosts differing in infection status: Sharpshooter movement and *Xylella fastidiosa* transmission. Ecological Entomology. 36 (5): 654–662. doi: 10.1111/j.1365-2311.2011.01309.x

Davies, P. (2010). The plant hormones: Their nature, occurrence, and functions. In Plant Hormones: Physiology, Biochemistry and Molecular Biology., Eds. Davies, P. (Springer, Dordrecht). doi: 10.1007/978-1-4020-2686-7_1

De Curtis, F., Lima, G., Vitullo, D., and De Cicco, V. (2010). Biocontrol of Rhizoctonia solani and *Sclerotium rolfsii* on tomato by delivering antagonistic bacteria through a drip irrigation system. Crop Protection. 29 (7): 663–670. doi: 10.1016/j.cropro.2010.01.012

De Hita, D., Fuentes, M., Fernández, V., Zamarreño, A. M., Olaetxea, M., and García-Mina, J. M. (2020). Discriminating the short-term action of root and foliar application of humic acids on plant growth: emerging role of jasmonic acid. Frontiers in Plant Science. 11 (4): 1–14. doi: 10.3389/fpls.2020.00493

De Palma, M., Salzano, M., Villano, C., Aversano, R., Lorito, M., Ruocco, M., Docimo, T., Piccinelli, A. L., D'Agostino, N., and Tucci, M. (2019). Transcriptome reprogramming, epigenetic modifications and alternative splicing orchestrate the tomato root response to the beneficial fungus *Trichoderma harzianum*. Horticulture Research. 6 (1): 1–15. doi: 10.1038/s41438-018-0079-1

de Puysseleyr, V., Höfte, M., and de Clercq, P. (2011). Ovipositing *Orius laevigatus* increase tomato resistance against *Frankliniella occidentalis* feeding by inducing the wound response. Arthropod-Plant Interactions. 5 (1): 71–80. doi: 10.1007/s11829-010-9117-0

Del Carmen Orozco-Mosqueda, M., Duan, J., DiBernardo, M., Zetter, E., Campos-García, J., Glick, B. R., and Santoyo, G. (2019). The production of ACC deaminase and trehalose by the plant growth promoting bacterium *Pseudomonas* sp. UW4 synergistically protect tomato plants against salt stress. Frontiers in Microbiology. 10 (6): 1–10. doi: 10.3389/fmicb.2019.01392

Drobek, M., Fraç, M., and Cybulska, J. (2019). Plant biostimulants: Importance of the quality and yield of horticultural crops and the improvement of plant tolerance to abiotic stress — A review. Agronomy. 9 (6): 355. doi: 10.3390/agronomy9060335

Drukker, B., Janssen, A., Ravensberg, W., and Sabelis, M. W. (1997). Improved control capacity of the mite predator *Phytoseiulus persimilis* (Acari: Phytoseiidae) on tomato. Experimental and Applied Acarology. 21: 507–518. doi: 10.1023/b:appa.0000018885.35044.c6

Erro, J., Zamarreño, A. M., Garcia-Mina, J. M., and Yvin, J. C. (2009). Comparison of different phosphorus-fertiliser matrices to induce the recovery of phosphorus-deficient maize plants.

Journal of the Science of Food and Agriculture. 89 (6): 927–934. doi: 10.1002/jsfa.3530
Esmaeel, Q., Miotto, L., Rondeau, M., Leclère, V., Clément, C., Jacquard, C., Sanchez, L., and Barka, E. A. (2018). *Paraburkholderia phytofirmans* PsJN-plants interaction: From perception to the induced mechanisms. Frontiers in Microbiology. 9 (8): 1–14. doi: 10.3389/fmicb.2018.02093
E.U. Regulation of the European Parliament and of the Council Laying Down Rules on the Making Available on the Market of EU Fertilising Products and Amending Regulations (EC) No 1069/2009 and (EC) No1107/2009 and Repealing Regulation (EC) No 2003/2003. 2019. Available online: https://eurlex.europa.eu/legalcontent/EN/TXT/?uri=OJ:L:2019:170:TOC (accessed on 6 December 2020).
Fan, B., Chen, X. H., Budiharjo, A., Bleiss, W., Vater, J., and Borriss, R. (2011). Efficient colonization of plant roots by the plant growth promoting bacterium *Bacillus amyloliquefaciens* FZB42, engineered to express green fluorescent protein. Journal of Biotechnology. 151 (4): 303–311. doi: 10.1016/j.jbiotec.2010.12.022
FAOSTAT Statistics Database (2018). Food and Agriculture Organization of the United Nations. Available online: http://www.fao.org/faostat/en/#data/QC/visualize. (accessed on 9 December 2020).
Ferreira, C. M. H., Soares, H. M. V. M., and Soares, E. V. (2019). Science of the total environment promising bacterial genera for agricultural practices: An insight on plant growth-promoting properties and microbial safety aspects. Science of the Total Environment. 682: 779–799. doi: 10.1016/j.scitotenv.2019.04.225
Fischer, T. (2017). Humic supramolecular structures have polar surfaces and unpolar cores in native soil. Chemosphere. 183: 437–443. doi: 10.1016/j.chemosphere.2017.05.125
Foreman, J., Demidchik, V., Bothwell, J. H. F., Mylona, P., Miedema, H., Angel Torres, M., Linstead, P., Costa, S., Brownlee, C., Jones, J. D. G., Davies, J. M., and Dolan, L. (2003). Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature. 422 (6930): 442–446. doi: 10.1038/nature01485
Frank, A., Saldierna Guzmán, J., and Shay, J. (2017). Transmission of bacterial endophytes. Microorganisms. 5 (4): 70. doi: 10.3390/microorganisms5040070
Frison, E. A., Cherfas, J., and Hodgkin, T. (2011). Agricultural biodiversity is essential for a sustainable improvement in food and nutrition security. Sustainability. 3 (1): 238–253. doi: 10.3390/su3010238
Gabarra, R., and Besri, M. (1999). Tomatoes. In Integrated Pest and Disease Management in Greenhouse Crops. Developments in Plant Pathology., Eds. R. Albajes, M. L. Gullino, J. C. van Lenteren, and Y. Elad, 14: 420-434. (Springer, Dordrecht). doi: 10.1007/0-306-47585-5_30
Galambos, N., Compant, S., Moretto, M., Sicher, C., Puopolo, G., Wäckers, F., Sessitsch, A., Pertot, I., and Perazzolli, M. (2020). Humic acid enhances the growth of tomato promoted by endophytic bacterial strains through the activation of hormone-, growth-, and transcription-related processes. Frontiers in Plant Science. 11 (9): 1–18. doi: 10.3389/fpls.2020.582267
Garciá, A. C., De Souza, L. G. A., Pereira, M. G., Castro, R. N., Garciá-Mina, J. M., Zonta, E., Lisboa, F. J. G., and Berbara, R. L. L. (2016). Structure-property-function relationship in humic substances to explain the biological activity in plants. Scientific Reports. 6 (1): 1–10. doi: 10.1038/srep20798
Ghosh, S. K., Bera, T., and Chakrabarty, A. M. (2020). Microbial siderophore – A boon to

agricultural sciences. Biological Control. 144 (1): 104214. doi: 10.1016/j.biocontrol.2020.104214
Gibb, K. S., and Randles, J. (1991). Transmission of velvet tobacco mottle virus and related viruses by the mirid *Cyrtopeltis nicotianae*. Advances in Disease Vector Research. 7: 1–17. doi: 10.1007/978-1-4613-9044-2_1
Glick, B. R. (2012). Plant growth-promoting bacteria: Mechanisms and applications. Scientifica. 963401. doi: 10.6064/2012/963401
Glick, B. R. (2020). Beneficial plant-bacterial interactions. (Springer International Publishing). doi: 10.1007/978-3-030-44368-9
Glick, B. R., Penrose, D. M., and Li, J. (1998). A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. Journal of Theoretical Biology. 190 (1): 63–68. doi: 10.1006/jtbi.1997.0532
Gonella, E., Pajoro, M., Marzorati, M., Crotti, E., Mandrioli, M., Pontini, M., Bulgari, D., Negri, I., Sacchi, L., Chouaia, B., Daffonchio, D., and Alma, A. (2015). Plant-mediated interspecific horizontal transmission of an intracellular symbiont in insects. Scientific Reports. 5: 1–10. doi: 10.1038/srep15811
Goudjal, Y., Toumatia, O., Yekkour, A., Sabaou, N., Mathieu, F., and Zitouni, A. (2014). Biocontrol of *Rhizoctonia solani* damping-off and promotion of tomato plant growth by endophytic actinomycetes isolated from native plants of Algerian Sahara. Microbiological Research. 169 (1): 59–65. doi: 10.1016/j.micres.2013.06.014
Gould, W. A. (1992). Tomato Production, Processing and Technology. (Woodhead Publishing, Cambridge). doi: 10.1533/9781845696146
Gowtham, H. G., Hariprasad, P., Nayak, S. C., and Niranjana, S. R. (2016). Application of rhizobacteria antagonistic to *Fusarium oxysporum* f. sp. *lycopersici* for the management of Fusarium wilt in tomato. Rhizosphere. 2: 72–74. doi: 10.1016/j.rhisph.2016.07.008
Gowtham, H. G., Singh, S., Murali, M., Shilpa, N., Melvin, P., Aiyaz, M., Amruthesh, K., and Niranjana, S. R. (2020). Induction of drought tolerance in tomato upon the application of ACC deaminase producing plant growth promoting rhizobacterium *Bacillus subtilis* Rhizo SF 48. Microbiological Research. 234: 126422. doi: 10.1016/j.micres.2020.126422
Gravel, V., Martinez, C., Antoun, H., and Tweddell, R. J. (2005). Antagonist microorganisms with the ability to control Pythium damping-off of tomato seeds in rockwool. Biological Control. 50 (5): 771–786. doi: 10.1007/s10526-005-1312-z
Guerinot, M. L., and Yi, Y. (1994). Iron: Nutritious, noxious, and not readily available. Plant Physiology. 104 (3): 815–820. doi: 10.1104/pp.104.3.815
Gullino, M. L., Albajes, R., and Nicot, P. C. (2020). Integrated Pest and Disease Management in Greenhouse Crops. (Springer International Publishing). doi: 10.1007/978-3-030-22304-5
Gutiérrez-Mañero, F. J., Ramos-Solano, B., Probanza, A., Mehouachi, J., Tadeo, F. R., and Talon, M. (2001). The plant-growth-promoting rhizobacteria Bacillus pumilus and Bacillus licheniformis produce high amounts of physiologically active gibberellins. Physiologia Plantarum. 111 (2): 206–211. doi: 10.1034/j.1399-3054.2001.1110211.x
Hallmann, J., Quadt-Hallmann, A., Mahaffee, W. F., and Kloepper, J. W. (1997). Bacterial endophytes in agricultrual crops. Canadian Journal of Microbiology. 43: 895–914. doi: 10.1139/m97-131
Hänsch, R., and Mendel, R. R. (2009). Physiological functions of mineral micronutrients (Cu, Zn, Mn, Fe, Ni, Mo, B, Cl). Current Opinion in Plant Biology. 12 (3): 259–266. doi: 10.1016/j.pbi.2009.05.006
Hardoim, P. R., van Overbeek, L. S., Berg, G., Pirttilä, A. M., Compant, S., Campisano, A.,

Döring, M., and Sessitsch, A. (2015). The hidden world within plants: Ecological and evolutionary considerations for defining functioning of microbial endophytes. Microbiology and Molecular Biology Reviews. 79 (3): 293–320. doi: 10.1128/MMBR.00050-14
Haskett, T. L., Tkacz, A., and Poole, P. S. (2020). Engineering rhizobacteria for sustainable agriculture. ISME Journal. doi: 10.1038/s41396-020-00835-4
Heck, M. (2018). Insect transmission of plant pathogens: a systems biology perspective. MSystems. 3 (2): e00168-17. doi: 10.1128/msystems.00168-17
Heimpel, G. E., and Cock, M. J. W. (2018). Shifting paradigms in the history of classical biological control. Biological Control. 63 (1): 27–37. doi: 10.1007/s10526-017-9841-9
Hobson, G., and Grierson, D. (1993). Tomato. In Biochemistry of Fruit Ripening., Eds. G. B. Seymour, J. E. Taylor, and G. A. Tucker, 405–442. (Springer, Dordrecht). doi: 10.1007/978-94-011-1584-1_14
Hoddle, M. S. (2004). Restoring balance: using exotic species to control invasive exotic species. Conservation Biology. 18 (1): 38–49. doi: 10.1111/j.1523-1739.2004.00249.x
Hoyle, F. C., and Murphy, D. V. (2011). Influence of organic residues and soil incorporation on temporal measures of microbial biomass and plant available nitrogen. Plant and Soil. 347 (1): 53–64. doi: 10.1007/s11104-011-0922-8
Huang, J., Wei, Z., Tan, S., Mei, X., Yin, S., Shen, Q., and Xu, Y. (2013). The rhizosphere soil of diseased tomato plants as a source for novel microorganisms to control bacterial wilt. Applied Soil Ecology. 72: 79–84. doi: 10.1016/j.apsoil.2013.05.017
Ibort, P., Imai, H., Uemura, M., and Aroca, R. (2018). Proteomic analysis reveals that tomato interaction with plant growth promoting bacteria is highly determined by ethylene perception. Journal of Plant Physiology. 220 (10): 43–59. doi: 10.1016/j.jplph.2017.10.008
Irizarry, I., and White, J. F. (2018). *Bacillus amyloliquefaciens* alters gene expression, ROS production and lignin synthesis in cotton seedling roots. Journal of Applied Microbiology. 124 (6): 1589–1603. doi: 10.1111/jam.13744
Iyaniwura, T. T. (1991). Non-target and environmental hazards of pesticides. Reviews on Environmental Health. 9 (3): 161–176. doi: 10.1515/REVEH.1991.9.3.161
Jannin, L., Arkoun, M., Ourry, A., Laîné, P., Goux, D., Garnica, M., Fuentes, M., Francisco, S. S., Baigorri, R., Cruz, F., Houdusse, F., Garcia-Mina, J. M., Yvin, J. C., and Etienne, P. (2012). Microarray analysis of humic acid effects on *Brassica napus* growth: Involvement of N, C and S metabolisms. Plant and Soil. 359 (1–2): 297–319. doi: 10.1007/s11104-012-1191-x
Jindo, K., Olivares, F. L., Malcher, D. J. da P., Sánchez-Monedero, M. A., Kempenaar, C., and Canellas, L. P. (2020). From lab to field: Role of humic substances under open-field and greenhouse conditions as biostimulant and biocontrol agent. Frontiers in Plant Science. 11 (5): 1–10. doi: 10.3389/fpls.2020.00426
Jindo, K., Soares, T. S., Peres, L. E. P., Azevedo, I. G., Aguiar, N. O., Mazzei, P., Spaccini, R., Piccolo, A., Olivares, F. L., and Canellas, L. P. (2016). Phosphorus speciation and high-affinity transporters are influenced by humic substances. Journal of Plant Nutrition and Soil Science. 179 (2): 206–214. doi: 10.1002/jpln.201500228
Johnson, P. R., and Ecker, J. R. (1998). The ethylene gas signal transduction pathway: A molecular perspective. Annual Review of Genetics. 32: 227–254. doi: 10.1146/annurev.genet.32.1.227
Jothi, G., and Poornima, K. (2018). Effect of potassium humate on hatching , mortality and plant infectivity of *Meloidogyne incognita* in tomato. Journal of Entomology and Zoology Studies. 6 (4): 1105–1108.

Kaul, S., Sharma, T., and Dhar, M. K. (2016). "Omics" tools for better understanding the plant–endophyte interactions. Frontiers in Plant Science. 7 (6): 1–9. doi: 10.3389/fpls.2016.00955

Keswani, C., Singh, S. P., Cueto, L., García-Estrada, C., Mezaache-Aichour, S., Glare, T. R., Borriss, R., Singh, S. P., Blázquez, M. A., and Sansinenea, E. (2020). Auxins of microbial origin and their use in agriculture. Applied Microbiology and Biotechnology. 104 (20): 8549–8565. doi: 10.1007/s00253-020-10890-8

Khan, A. L., Waqas, M., Kang, S. M., Al-Harrasi, A., Hussain, J., Al-Rawahi, A., Al-Khiziri, S., Ullah, I., Ali, L., Jung, H. Y., and Lee, I. J. (2014). Bacterial endophyte *Sphingomonas* sp. LK11 produces gibberellins and IAA and promotes tomato plant growth. Journal of Microbiology. 52 (8): 689–695. doi: 10.1007/s12275-014-4002-7

Khan, N., Mishra, A., and Nautiyal, C. S. (2012). *Paenibacillus lentimorbus* B-30488 r controls early blight disease in tomato by inducing host resistance associated gene expression and inhibiting *Alternaria solani*. Biological Control. 62 (2): 65–74. doi: 10.1016/j.biocontrol.2012.03.010

Khan, M. N., Mobin, M., Abbas, Z. K., and Alamri, S. A. (2018). Fertilizers and their contaminants in soils, surface and groundwater. Reference Module in Earth Systems and Environmental Sciences. 5: 225-240 doi: 10.1016/B978-0-12-809665-9.09888-8

Khanna, K., Jamwal, V. L., Kohli, S. K., Gandhi, S. G., Ohri, P., Bhardwaj, R., Wijaya, L., Alyemeni, M. N., and Ahmad, P. (2019). Role of plant growth promoting bacteria (PGPRs) as biocontrol agents of *Meloidogyne incognita* through improved plant defense of *Lycopersicon esculentum*. Plant and Soil. 436 (1–2): 325–345. doi: 10.1007/s11104-019-03932-2

Kilani-Feki, O., Ben Khedher, S., Dammak, M., Kamoun, A., Jabnoun-Khiareddine, H., Daami-Remadi, M., and Tounsi, S. (2016). Improvement of antifungal metabolites production by *Bacillus subtilis* V26 for biocontrol of tomato postharvest disease. Biological Control. 95 73–82. doi: 10.1016/j.biocontrol.2016.01.005

Konappa, N. M., Maria, M., Uzma, F., Krishnamurthy, S., Nayaka, S. C., Niranjana, S. R., and Chowdappa, S. (2016). Lactic acid bacteria mediated induction of defense enzymes to enhance the resistance in tomato against *Ralstonia solanacearum* causing bacterial wilt. Scientia Horticulturae. 207: 183–192. doi: 10.1016/j.scienta.2016.05.029

Kulikova, N. A., Abroskin, D. P., Badun, G. A., Chernysheva, M. G., Korobkov, V. I., Beer, A. S., Tsvetkova, E. A., Senik, S. V., Klein, O. I., and Perminova, I. V. (2016). Label distribution in tissues of wheat seedlings cultivated with tritium-labeled leonardite humic acid. Scientific Reports. 6 (3): 1–10. doi: 10.1038/srep28869

Lemanceau, P., Bauer, P., Kraemer, S., and Briat, J. F. (2009). Iron dynamics in the rhizosphere as a case study for analyzing interactions between soils, plants and microbes. Plant and Soil. 321 (1–2): 513–535. doi: 10.1007/s11104-009-0039-5

Li, Qili, Ning, P., Zheng, L., Huang, J., Li, G., and Hsiang, T. (2012). Effects of volatile substances of *Streptomyces globisporus* JK-1 on control of *Botrytis cinerea* on tomato fruit. Biological Control. 61 (2): 113–120. doi: 10.1016/j.biocontrol.2011.10.014

Li, Qing, and Yan, J. (2020). Sustainable agriculture in the era of omics: Knowledge-driven crop breeding. Genome Biology. 21 (1): 5–9. doi: 10.1186/s13059-020-02073-5

Li, S. J., Ahmed, M. Z., Lv, N., Shi, P. Q., Wang, X. M., Huang, J. L., and Qiu, B. L. (2017). Plantmediated horizontal transmission of *Wolbachia* between whiteflies. ISME Journal. 11 (4): 1019–1028. doi: 10.1038/ismej.2016.164

Li, Y., Fang, F., Wei, J., Wu, X., Cui, R., Li, G., Zheng, F., and Tan, D. (2019). Humic acid fertilizer improved soil properties and soil microbial diversity of continuous cropping

peanut: A three-year experiment. Scientific Reports. 9 (1): 1–9. doi: 10.1038/s41598-019-48620-4
Liu, G., Lin, X., Xu, S., Liu, G., Liu, F., and Mu, W. (2020). Screening, identification and application of soil bacteria with nematicidal activity against root-knot nematode (*Meloidogyne incognita*) on tomato. Pest Management Science. 76 (6): 2217–2224. doi: 10.1002/ps.5759
Liu, K., Zhang, T. Q., Tan, C. S., and Astatkie, T. (2011). Responses of fruit yield and quality of processing tomato to drip-irrigation and fertilizers phosphorus and potassium. Agronomy Journal. 103 (5): 1339–1345. doi: 10.2134/agronj2011.0111
Liu, T. X., Stansly, P. A., and Gerling, D. (2015). Whitefly parasitoids: Distribution, life history, bionomics, and utilization. Annual Review of Entomology. 60: 273–292. doi: 10.1146/annurev-ento-010814-021101
Loper, J. E., and Buyer, J. S. (1991). Current review siderophores in microbial interactions on plant surfaces. Molecular Plant-Microbe Interactions. 4 (1): 5–13. doi: 10.1094/mpmi-4-005
Lòpez-Fernàndez, S., Mazzoni, V., Pedrazzoli, F., Pertot, I., and Campisano, A. (2017). A phloem-feeding insect transfers bacterial endophytic communities between grapevine plants. Frontiers in Microbiology. 8 (5): 1–17. doi: 10.3389/fmicb.2017.00834
Ma, X., Wang, X., Cheng, J., Nie, X., Yu, X., Zhao, Y., and Wang, W. (2015). Microencapsulation of *Bacillus subtilis* B99-2 and its biocontrol efficiency against *Rhizoctonia solani* in tomato. Biological Control. 90: 34–41. doi: 10.1016/j.biocontrol.2015.05.013
Ma, Y. (2019). Seed coating with beneficial microorganisms for precision agriculture. Biotechnology Advances. 37 (7): 107423. doi: 10.1016/j.biotechadv.2019.107423
Maham, S. G., Rahimi, A., Subramanian, S., and Smith, D. L. (2020). The environmental impacts of organic greenhouse tomato production based on the nitrogen-fixing plant (*Azolla*). Journal of Cleaner Production. 245: 118679. doi: 10.1016/j.jclepro.2019.118679
Mari, M., Guizzardi, M., Brunelli, M., and Folchi, A. (1996). Postharvest biological control of grey mould (*Botrytis cinerea* pers.: Fr.) on fresh-market tomatoes with *Bacillus amyloliquefaciens*. Crop Protection. 15 (8): 699–705. doi: 10.1016/s0261-2194(96)00042-7
Markets and Markets (2020). Biostimulants Market by Active Ingredient (Humic Substances, Amino Acids, Seaweed Extracts, Microbial Amendments), Crop Type (Fruits & Vegetables, Cereals, Turf & Ornamentals), Application Method, Form, and Region—Global Forecast to 2025. Available online: https://www.marketsandmarkets.com/Market-Reports/biostimulant-market1081.html?gclid=CjwKCAjw4_H6BRALEiwAvgfzq1LVX47L4C4O0v0leN5GfYGuk0xW2oF25JDZhWGs03E3I2rL1kEwGxoCnsAQAvD_BwE (accessed on 6 December 2020).
Mas, F., Vereijssen, J., and Suckling, D. M. (2014). Influence of the pathogen *Candidatus* Liberibacter *solanacearum* on tomato host plant volatiles and Psyllid vector settlement. Journal of Chemical Ecology. 40 (11–12): 1197–1202. doi: 10.1007/s10886-014-0518-x
Mitter, B., Pfaffenbichler, N., Flavell, R., Compant, S., Antonielli, L., Petric, A., Berninger, T., Naveed, M., Sheibani-Tezerji, R., Maltzahn, G. von, and Sessitsch, A. (2017). A new approach to modify plant microbiomes and traits by introducing beneficial bacteria at flowering into progeny seeds. Frontiers in Microbiology. 8 (1): 1–10. doi: 10.3389/fmicb.2017.00011
Mora, V., Bacaicoa, E., Zamarreño, A. M., Aguirre, E., Garnica, M., Fuentes, M., and García-Mina, J. M. (2010). Action of humic acid on promotion of cucumber shoot growth involves nitrate-related changes associated with the root-to-shoot distribution of cytokinins,

polyamines and mineral nutrients. Journal of Plant Physiology. 167 (8): 633–642. doi: 10.1016/j.jplph.2009.11.018

Musilova, L., Ridl, J., Polivkova, M., Macek, T., and Uhlik, O. (2016). Effects of secondary plant metabolites on microbial populations: Changes in community structure and metabolic activity in contaminated environments. International Journal of Molecular Sciences. 17 (8):. doi: 10.3390/ijms17081205

Nag, P., Shriti, S., and Das, S. (2020). Microbiological strategies for enhancing biological nitrogen fixation in nonlegumes. Journal of Applied Microbiology. 129 (2): 186–98. doi: 10.1111/jam.14557

Nardi, S., Pizzeghello, D., Gessa, C., Ferrarese, L., Trainotti, L., and Casadoro, G. (2000). A low molecular weight humic fraction on nitrate uptake and protein synthesis in maize seedlings. Soil Biology and Biochemistry. 32 (3): 415–419. doi: 10.1016/S0038-0717(99)00168-6

Nardi, S., Carletti, P., Pizzeghello, D., and Muscolo, A. (2009). Biological Activities of Humic Substances. In Biophysico-Chemical Processes Involving Natural Nonliving Organic Matter in Environmental Systems., Eds. N. Sensei, B. Xing, and P. M. Huang, 305–39. (John Wiley and Sons, New Jersey). doi: 10.1002/9780470494950.ch8

Narendra Babu, A., Jogaiah, S., Ito, S. ichi, Kestur Nagaraj, A., and Tran, L. S. P. (2015). Improvement of growth, fruit weight and early blight disease protection of tomato plants by rhizosphere bacteria is correlated with their beneficial traits and induced biosynthesis of antioxidant peroxidase and polyphenol oxidase. Plant Science. 231: 62–73. doi: 10.1016/j.plantsci.2014.11.006

Naveed, M., Qureshi, M. A., Zahir, Z. A., Hussain, M. B., Sessitsch, A., and Mitter, B. (2015). L-Tryptophan-dependent biosynthesis of indole-3-acetic acid (IAA) improves plant growth and colonization of maize by *Burkholderia phytofirmans* PsJN. Annals of Microbiology. 65 (3): 1381–1389. doi: 10.1007/s13213-014-0976-y

Nawangsih, A. A., Damayanti, I. K. A., Wiyono, S., and Kartika, J. G. (2011). Selection and characterization of endophytic bacteria as biocontrol agents of tomato bacterial wilt disease. HAYATI Journal of Biosciences. 18 (2): 66–70. doi: 10.4308/hjb.18.2.66

Nephali, L., Piater, L. A., Dubery, I. A., Patterson, V., Huyser, J., Burgess, K., and Tugizimana, F. (2020). Biostimulants for plant growth and mitigation of abiotic stresses : A metabolomics perspective. Metabolites. 10 (12): 505. doi: 10.3390/metabo10120505

Novoa, R., and Loomis, R. S. (1981). Nitrogen and plant production. Plant and Soil. 58 (1): 177–204. doi: 10.1007/BF02180053

Olaetxea, M., De Hita, D., Garcia, C. A., Fuentes, M., Baigorri, R., Mora, V., Garnica, M., Urrutia, O., Erro, J., Zamarreño, A. M., Berbara, R. L., and Garcia-Mina, J. M. (2018). Hypothetical framework integrating the main mechanisms involved in the promoting action of rhizospheric humic substances on plant root- and shoot- growth. Applied Soil Ecology. 123 (7): 521–537. doi: 10.1016/j.apsoil.2017.06.007

Olaetxea, M., Mora, V., Bacaicoa, E., Garnica, M., Fuentes, M., Casanova, E., Zamarreño, A. M., Iriarte, J. C., Etayo, D., Ederra, I., Gonzalo, R., Baigorri, R., and García-Mina, J. M. (2015). Abscisic acid regulation of root hydraulic conductivity and aquaporin gene expression is crucial to the plant shoot growth enhancement caused by rhizosphere humic acids. Plant Physiology. 169 (4): 2587–2596. doi: 10.1104/pp.15.00596

Oldroyd, G. E. D., Murray, J. D., Poole, P. S., and Downie, J. A. (2011). The rules of engagement in the legume-rhizobial symbiosis. Annual Review of Genetics. 45: 119–144. doi: 10.1146/annurev-genet-110410-132549

Olivares, F. L., Aguiar, N. O., Rosa, R. C. C., and Canellas, L. P. (2015). Substrate

biofortification in combination with foliar sprays of plant growth promoting bacteria and humic substances boosts production of organic tomatoes. Scientia Horticulturae. 183 (1): 100–108. doi: 10.1016/j.scienta.2014.11.012

Olivares, F. L., Busato, J. G., de Paula, A. M., da Silva Lima, L., Aguiar, N. O., and Canellas, L. P. (2017). Plant growth promoting bacteria and humic substances: crop promotion and mechanisms of action. Chemical and Biological Technologies in Agriculture. 4 (1): 1–13. doi: 10.1186/s40538-017-0112-x

Olk, D. C., Dinnes, D. L., Rene Scoresby, J., Callaway, C. R., and Darlington, J. W. (2018). Humic products in agriculture: potential benefits and research challenges—a review. Journal of Soils and Sediments. 18 (8): 2881–2891. doi: 10.1007/s11368-018-1916-4

Omar, I., O'Neill, T. M., and Rossall, S. (2006). Biological control of fusarium crown and root rot of tomato with antagonistic bacteria and integrated control when combined with the fungicide carbendazim. Plant Pathology. 55 (1): 92–99. doi: 10.1111/j.1365-3059.2005.01315.x

Pérez-Rodriguez, M. M., Piccoli, P., Anzuay, M. S., Baraldi, R., Neri, L., Taurian, T., Lobato Ureche, M. A., Segura, D. M., and Cohen, A. C. (2020). Native bacteria isolated from roots and rhizosphere of *Solanum lycopersicum* L. increase tomato seedling growth under a reduced fertilization regime. Scientific Reports. 10 (1): 1–14. doi: 10.1038/s41598-020-72507-4

Perilla-Henao, L. M., and Casteel, C. L. (2016). Vector-borne bacterial plant pathogens: Interactions with hemipteran insects and plants. Frontiers in Plant Science. 7 (8): 1–15. doi: 10.3389/fpls.2016.01163

Pizzeghello, D., Francioso, O., Ertani, A., Muscolo, A., and Nardi, S. (2012). Isopentenyladenosine and cytokinin-like activity of different humic substances. Journal of Geochemical Exploration. 129 70–75. doi: 10.1016/j.gexplo.2012.10.007

Poupin, M. J., Timmermann, T., Vega, A., Zuñiga, A., and González, B. (2013). Effects of the plant growth-promoting bacterium *Burkholderia phytofirmans* PsJN throughout the life cycle of *Arabidopsis thaliana*. PLoS ONE. 8 (7): 22–24. doi: 10.1371/journal.pone.0069435

Purcell, A. H., and Almeida, R. P. P. (2004). Insects as vectors of disease agents. Encyclopedia of Plant and Crop Science. 1–14. doi: 10.1081/e-epcs-120010496

Qessaoui, R., Amarraque, A., Lahmyed, H., Ajerrar, A., Mayad, E. H., Chebli, B., Walters, A. S., and Bouharroud, R. (2020). Inoculation of tomato plants with rhizobacteria suppresses development of whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae): Agro-ecological application. PLoS ONE. 15 (4): 1–9. doi: 10.1371/journal.pone.0231496

Qessaoui, R., Bouharroud, R., Amarraque, A., Ajerrar, A., El Hassan, M., Chebli, B., Dadi, M., Elaini, R., El Filali, F., and Walters, A. S. (2017). Ecological applications of *Pseudomonas* as a biopesticide to control two-spotted mite *Tetranychus urticae*: Chitinase and HCN production. Journal of Plant Protection Research. 57 (4): 409–416. doi: 10.1515/jppr-2017-0055

Qessaoui, R., Rachid, B., Abderahim, A., Hind, L., Abdelhadi, A., Naima, A. A., Abdelghani, T., El Hassan, M., and Bouchra, C. (2019). Effect of *Pseudomonas* as a preventive and curative control of tomato leafminer *Tuta absoluta* (Lepidoptera: Gelechiidae). Journal of Applied Sciences. 19 (5): 473–479. doi: 10.3923/jas.2019.473.479

Quaggiotti, S., Ruperti, B., Pizzeghello, D., Francioso, O., Tugnoli, V., and Nardi, S. (2004). Effect of low molecular size humic substances on nitrate uptake and expression of genes involved in nitrate transport in maize (*Zea mays* L.). Journal of Experimental Botany. 55 (398): 803–813. doi: 10.1093/jxb/erh085

Ramsey, J. S., Chin, E. L., Chavez, J. D., Saha, S., Mischuk, D., Mahoney, J., Mohr, J., Robison, F. M., Mitrovic, E., Xu, Y., Strickler, S. R., Fernandez, N., Zhong, X., Polek, M. Lou, Godfrey, K. E., Giovannoni, J. J., Mueller, L. A., Slupsky, C. M., Bruce, J. E., and Heck, M. (2020). Longitudinal transcriptomic, proteomic, and metabolomic analysis of Citrus limon response to graft inoculation by *Candidatus* Liberibacter *asiaticus*. Journal of Proteome Research. 19 (6): 2247–2263. doi: 10.1021/acs.jproteome.9b00802

Redhead, J. W., Powney, G. D., Woodcock, B. A., and Pywell, R. F. (2020). Effects of future agricultural change scenarios on beneficial insects. Journal of Environmental Management. 265: 110550. doi: 10.1016/j.jenvman.2020.110550

Rodriguez, H., Gonzalez, T., Goire, I., and Bashan, Y. (2004). Gluconic acid production and phosphate solubilization by the plant growth-promoting bacterium *Azospirillum* spp. Naturwissenschaften. 91 (11): 552–555. doi: 10.1007/s00114-004-0566-0

Romero, F. M., Marina, M., and Pieckenstain, F. L. (2016). Novel components of leaf bacterial communities of field-grown tomato plants and their potential for plant growth promotion and biocontrol of tomato diseases. Research in Microbiology. 167 (3): 222–233. doi: 10.1016/j.resmic.2015.11.001

Rouphael, Y., and Colla, G. (2020a). Editorial: Biostimulants in agriculture. Frontiers in Plant Science. 11 (2): 1–7. doi: 10.3389/fpls.2020.00040

Rouphael, Y., and Colla, G. (2020b). Toward a sustainable agriculture through plant biostimulants: From experimental data to practical applications. Agronomy. 10 (10). doi: 10.3390/agronomy10101461

Ruberson, J., Nemoto, H., and Hirose, Y. (1998). Pesticides and conservation of natural enemies in pest management. Conservation Biological Control. 207–220. doi: 10.1016/b978-012078147-8/50057-8

Ruiu, L. (2020). Plant-growth-promoting bacteria (PGPB) against insects and other agricultural pests. Agronomy. 10 (6): 1–12. doi: 10.3390/agronomy10060861

Ryu, M. H., Zhang, J., Toth, T., Khokhani, D., Geddes, B. A., Mus, F., Garcia-Costas, A., Peters, J. W., Poole, P. S., Ané, J. M., and Voigt, C. A. (2020). Control of nitrogen fixation in bacteria that associate with cereals. Nature Microbiology. 5 (2): 314–330. doi: 10.1038/s41564-019-0631-2

Santi, C., Bogusz, D., and Franche, C. (2013). Biological nitrogen fixation in non-legume plants. Annals of Botany. 111 (5): 743–767. doi: 10.1093/aob/mct048

Sapre, S., Gontia, I., and Tiwari, S. (2019). ACC deaminase-producing bacteria: A key player in alleviating abiotic stresses in plants. In Plant Growth Promoting Rhizobacteria for Agricultural Sustainability., Eds. A. Kumar and V. S. Meena, 267–291. (Springer, Singapore). doi: 10.1007/978-981-13-7553-8_14

Schachtman, D. P., Reid, R. J., and Ayling, S. M. (1998). Phosphorus uptake by plants: From soil to cell. Plant Physiology. 116 (2): 447–453. doi: 10.1104/pp.116.2.447

Schaefer, C. W., and Panizzi, A. R. (2000). Heteroptera of economic importance. (CRC Press). doi: 10.1201/9781420041859

Schiavon, M., Pizzeghello, D., Muscolo, A., Vaccaro, S., Francioso, O., and Nardi, S. (2010). High molecular size humic substances enhance phenylpropanoid metabolism in maize (*Zea mays* L.). Journal of Chemical Ecology. 36 (6): 662–669. doi: 10.1007/s10886-010-9790-6

Sessitsch, A., Coenye, T., Sturz, A. V, Vandamme, P., Barka, E. A., Salles, J. F., Elsas, J. D. Van, Faure, D., Reiter, B., Glick, B. R., Wang-Pruski, G., and Nowak, J. (2005). Burkholderia phytofirmans sp. nov., a novel plant-associated bacterium with plant-beneficial properties. International Journal of Systematic and Evolutionary Microbiology. 55: 1187–

1192. doi: 10.1099/ijs.0.63149-0
Sharma, M., Sudheer, S., Usmani, Z., Rani, R., and Gupta, P. (2020). Deciphering the omics of plant-microbe interaction: Perspectives and new insights. Current Genomics. 21 (5): 343–362. doi: 10.2174/1389202921999200515140420
Soumare, A., Boubekri, K., Lyamlouli, K., Hafidi, M., Ouhdouch, Y., and Kouisni, L. (2020). From isolation of phosphate solubilizing microbes to their formulation and use as biofertilizers: Status and needs. Frontiers in Bioengineering and Biotechnology. 7 (1): 1–14. doi: 10.3389/fbioe.2019.00425
Stevens, C. J. (2019). Nitrogen in the environment. Science. 363 (6427): 578–580. doi: 10.1126/science.aav8215
Sung, J., Lee, S., Lee, Y., Ha, S., Song, B., Kim, T., Waters, B. M., and Krishnan, H. B. (2015). Metabolomic profiling from leaves and roots of tomato (*Solanum lycopersicum* L.) plants grown under nitrogen, phosphorus or potassium-deficient condition. Plant Science. 241 55–64. doi: 10.1016/j.plantsci.2015.09.027
Talboys, P. J., Owen, D. W., Healey, J. R., Withers, P. J. A., and Jones, D. L. (2014). Auxin secretion by *Bacillus amyloliquefaciens* FZB42 both stimulates root exudation and limits phosphorus uptake in *Triticum aestivium*. BMC Plant Biology. 14 (1): 1–9. doi: 10.1186/1471-2229-14-51
Thiem, D., Złoch, M., Gadzała-Kopciuch, R., Szymańska, S., Baum, C., and Hrynkiewicz, K. (2018). Cadmium-induced changes in the production of siderophores by a plant growth promoting strain of *Pseudomonas fulva*. Journal of Basic Microbiology. 58 (7): 623–632. doi: 10.1002/jobm.201800034
Trevisan, S., Pizzeghello, D., Ruperti, B., Francioso, O., Sassi, A., Palme, K., Quaggiotti, S., and Nardi, S. (2010). Humic substances induce lateral root formation and expression of the early auxin-responsive IAA19 gene and DR5 synthetic element in *Arabidopsis*. Plant Biology. 12 (4): 604–614. doi: 10.1111/j.1438-8677.2009.00248.x
Tribouillois, H., Constantin, J., Guillon, B., Willaume, M., Aubrion, G., Fontaine, A., Hauprich, P., Kerveillant, P., Laurent, F., and Therond, O. (2020). AqYield-N: A simple model to predict nitrogen leaching from crop fields. Agricultural and Forest Meteorology. 284 (4): 107890. doi: 10.1016/j.agrformet.2019.107890
Tugizimana, F., Piater, L., and Dubery, I. (2013). Plant metabolomics: A new frontier in phytochemical analysis. South African Journal of Science. 109 (5–6): 18–20. doi: 10.1590/sajs.2013/20120005
Türkmen, Ö., Dursun, A., Turan, M., and Erdinç, Ç. (2004). Calcium and humic acid affect seed germination, growth, and nutrient content of tomato (*Lycopersicon esculentum* L.) seedlings under saline soil conditions. Acta Agriculturae Scandinavica Section B: Soil and Plant Science. 54 (3): 168–174. doi: 10.1080/09064710310022014
Urbaneja, A., Montón, H., and Mollá, O. (2009). Suitability of the tomato borer *Tuta absoluta* as prey for *Macrolophus pygmaeus* and *Nesidiocoris tenuis*. Journal of Applied Entomology. 133 (4): 292–296. doi: 10.1111/j.1439-0418.2008.01319.x
Urrutia, O., Erro, J., Guardado, I., San Francisco, S., Mandado, M., Baigorri, R., Claude Yvin, J., and Ma Garcia-Mina, J. (2014). Physico-chemical characterization of humic-metal-phosphate complexes and their potential application to the manufacture of new types of phosphate-based fertilizers. Journal of Plant Nutrition and Soil Science. 177 (2): 128–36. doi: 10.1002/jpln.201200651
Valenzuela-Soto, J. H., Estrada-Hernández, M. G., Ibarra-Laclette, E., and Délano-Frier, J. P. (2010). Inoculation of tomato plants (*Solanum lycopersicum*) with growth-promoting

Bacillus subtilis retards whitefly *Bemisia tabaci* development. Planta. 231 (2): 397–410. doi: 10.1007/s00425-009-1061-9
Van Loon, L. C. (2007). Plant responses to plant growth-promoting rhizobacteria. European Journal of Plant Pathology. 119 (3): 243–254. doi: 10.1007/s10658-007-9165-1
Wakil, W., Brust, G. E., and Perring, T. (2017). Sustainable management of arthropod pests of tomato. (Academic Press).
Walker, T. S., Bais, H. P., Grotewold, E., and Vivanco, J. M. (2003). Root exudation and rhizosphere biology. Plant Physiology. 132 (3): 44–51. doi: 10.1104/pp.102.019661
Wang, J., Zhang, Y., Jin, J., Li, Q., Zhao, C., Nan, W., Wang, X., Ma, R., and Bi, Y. (2018). An intact cytokinin-signaling pathway is required for *Bacillus* sp. LZR216-promoted plant growth and root system architecture altereation in *Arabidopsis thaliana* seedlings. Plant Growth Regulation. 84 (3): 507–518. doi: 10.1007/s10725-017-0357-1
Wang, X., and Xing, Y. (2017). Evaluation of the effects of irrigation and fertilization on tomato fruit yield and quality: A principal component analysis. Scientific Reports. 7 (1): 1–13. doi: 10.1038/s41598-017-00373-8
Wang, Z., Gerstein, M., and Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. Nature Reviews. Genetics. 10 (1): 57–63. doi: 10.1038/nrg2484
Wei, Z., Huang, J., Tan, S., Mei, X., Shen, Q., and Xu, Y. (2013). The congeneric strain Ralstonia pickettii QL-A6 of *Ralstonia solanacearum* as an effective biocontrol agent for bacterial wilt of tomato. Biological Control. 65 (2): 278–285. doi: 10.1016/j.biocontrol.2012.12.010
Werner, G. D. A., Cornwell, W. K., Sprent, J. I., Kattge, J., and Kiers, E. T. (2014). A single evolutionary innovation drives the deep evolution of symbiotic N2-fixation in angiosperms. Nature Communications. 5 (5): 1–9. doi: 10.1038/ncomms5087
Winiwarter, W., Höglund-Isaksson, L., Klimont, Z., Schöpp, W., and Amann, M. (2018). Technical opportunities to reduce global anthropogenic emissions of nitrous oxide. Environmental Research Letters. 13 (1). doi: 10.1088/1748-9326/aa9ec9
Yao, J., Saenkham, P., Levy, J., Ibanez, F., Noroy, C., Mendoza, A., Huot, O., Meyer, D. F., and Tamborindeguy, C. (2016). Interactions "*Candidatus* Liberibacter *solanacearum*"-*Bactericera cockerelli*: Haplotype effect on vector fitness and gene expression analyses. Frontiers in Cellular and Infection Microbiology. 6: 62. doi: 10.3389/fcimb.2016.00062
Ye, L., Zhao, X., Bao, E., Li, J., Zou, Z., and Cao, K. (2020). Bio-organic fertilizer with reduced rates of chemical fertilization improves soil fertility and enhances tomato yield and quality. Scientific Reports. 10 (1): 1–11. doi: 10.1038/s41598-019-56954-2
Young, C., Rekha, P. D., Lai, W., and Arun, A. B. (2006). Encapsulation of plant growth-promoting bacteria in alginate beads enriched with humic acid. Biotechnology and Bioengineering. 95: 76–83. doi: 10.1002/bit.20957
Zaller, J. G. (2006). Foliar spraying of vermicornpost extracts: Effects on fruit quality and indications of late-blight suppression of field-grown tomatoes. Biological Agriculture and Horticulture. 24 (2): 165–180. doi: 10.1080/01448765.2006.9755017
Zandonadi, D. B., Santos, M. P., Dobbss, L. B., Olivares, F. L., Canellas, L. P., Binzel, M. L., Okorokova-Façanha, A. L., and Façanha, A. R. (2010). Nitric oxide mediates humic acids-induced root development and plasma membrane H^+-ATPase activation. Planta. 231 (5): 1025–1036. doi: 10.1007/s00425-010-1106-0
Zandonadi, D. B., Santos, M. P., Caixeta, L. S., Marinho, E. B., Peres, L. E. P., and Façanha, A. R. (2016). Plant proton pumps as markers of biostimulant action. Scientia Agricola. 73 (1): 24–28. doi: 10.1590/0103-9016-2015-0076

Zandonadi, D. B., Matos, C. R. R., Castro, R. N., Spaccini, R., Olivares, F. L., and Canellas, L. P. (2019). Alkamides: a new class of plant growth regulators linked to humic acid bioactivity. Chemical and Biological Technologies in Agriculture. 6 (1): 1–12. doi: 10.1186/s40538-019-0161-4

Zandonadi, D. B., Canellas, L. P., and Façanha, A. R. (2007). Indolacetic and humic acids induce lateral root development through a concerted plasmalemma and tonoplast H^+ pumps activation. Planta. 225 (6): 1583–1595. doi: 10.1007/s00425-006-0454-2

Zanin, L., Tomasi, N., Cesco, S., Varanini, Z., and Pinton, R. (2019). Humic substances contribute to plant iron nutrition acting as chelators and biostimulants. Frontiers in Plant Science. 10 (5): 1–10. doi: 10.3389/fpls.2019.00675

Zúñiga, A., Poupin, M. J., Donoso, R., Ledger, T., Guiliani, N., Gutiérrez, R. A., and González, B. (2013). Quorum sensing and indole-3-acetic acid degradation play a role in colonization and plant growth promotion of *Arabidopsis thaliana* by *Burkholderia phytofirmans* PsJN. Molecular Plant-Microbe Interactions. 26 (5): 546–553. doi: 10.1094/MPMI-10-12-0241-R

Zürcher, E., and Müller, B. (2016). Cytokinin synthesis, signaling, and function-Advances and new insights. International Review of Cell and Molecular Biology. 324: 1–38. doi: 10.1016/bs.ircmb.2016.01.001

Zwack, P. J., and Rashotte, A. M. (2015). Interactions between cytokinin signalling and abiotic stress responses. Journal of Experimental Botany. 66 (16): 4863–4871. doi: 10.1093/jxb/erv172