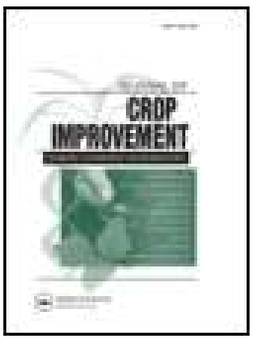

Journal of Crop Improvement

ISSN: 1542-7528 (Print) 1542-7536 (Online) Journal homepage: https://www.tandfonline.com/loi/wcim20

Root exudation of organic acids as affected by plant growth-promoting rhizobacteria *Bacillus subtilis* RR4 in rice

Kandaswamy Rekha, Mohankumar Ramasamy & Balasundaram Usha

To cite this article: Kandaswamy Rekha, Mohankumar Ramasamy & Balasundaram Usha (2020): Root exudation of organic acids as affected by plant growth-promoting rhizobacteria *Bacillus subtilis* RR4 in rice, Journal of Crop Improvement, DOI: 10.1080/15427528.2020.1746719

To link to this article: https://doi.org/10.1080/15427528.2020.1746719

Published online: 13 Apr 2020.

JOURNAL OF CROP IMPROVEMENT
https://doi.org/10.1080/15427528.2020.1746719

Taylor & Francis
Taylor & Francis Group

Root exudation of organic acids as affected by plant growth-promoting rhizobacteria *Bacillus subtilis* RR4 in rice

Kandaswamy Rekha[a,b], Mohankumar Ramasamy[c], and Balasundaram Usha[a]

[a]Department of Genetic Engineering, SRM Institute of Science and Technology, Chennai, India; [b]Department of Chemical Engineering, Pennsylvania State University, State College, Pennsylvania, USA; [c]Interdisciplinary School of Indian Systemic Medicine, SRM Institute of Science and Technology, Chennai, India

ABSTRACT

Bacillus subtilis possesses plant growth-promoting traits and enhances the level of essential metabolites in plants for improved growth. Included among the metabolites are the primary metabolite malic acid (MA) and the secondary metabolite salicylic acid (SA). The impact of plant growth-promoting rhizobacteria (PGPR) on root exudation/secretion of metabolites (like MA and SA) remains unexplored, however. In this study, we aim to analyze the impact of *B. subtilis* RR4 on the root exudation pattern of MA and SA in rice (*Oryza sativa* L.). For this, rice plants were treated with the PGPR *B. subtilis* RR4 in hydroponics and the root exudates were collected at different periods to analyze the changes in the levels of MA and SA through high-performance liquid chromatography (HPLC). Analysis of HPLC chromatograms showed that progressive colonization by RR4 enhanced root exudation of MA 3-fold and of SA 7-fold at 96 h post-inoculation (hpi), as compared to the control. This study, thus, highlights the potential of *B. subtilis* RR4 of inducing/enhancing root exudation of MA and SA, which are stress alleviators, plant growth-promoting agents, and a source of carbon for microbes. With this knowledge, bioformulations, comprising the PGPRs and specific metabolites as PGPR stimulants, can be developed.

ARTICLE HISTORY
Received 10 November 2019
Accepted 20 March 2020

KEYWORDS
Bioformulation; high performance liquid chromatography; malic acid; *Oryza sativa*; salicylic acid

Introduction

Rhizobacteria colonize the rhizosphere and help in promoting plant growth through several mechanisms, which include bacterial synthesis of essential phytochemicals, such as phytohormones and siderophores, mobilization of phosphate, biocontrol against pathogens, fixation of nitrogen, and production of antimicrobials, such as hydrogen cyanide (Richardson et al. 2009; Vacheron et al. 2013). On the other hand, metabolites secreted through plant roots, called root exudates, act as a source of nutrition for the root-colonizing bacteria. Root exudation thus acts as a driving force between the plant and bacteria to form a stable association (Lugtenberg and Dekkers 1999; Bacilio-Jiménez et al. 2003;

CONTACT Balasundaram Usha sundaram.usha@gmail.com Department of Genetic Engineering, SRM Institute of Science and Technology, Chennai 603203, India

Kamilova et al. 2006). Of the total exuded carbon, 90% is reported to be metabolized by the rhizobacteria and hence the microbial diversity is enriched in the rhizosphere soil relative to the bulk soil (non-rhizosphere soil) (Lynch and Whipps 1990; Badri and Vivanco 2009).

The major components in the root exudates reported to play a key role in plant-microbe symbiosis include organic acids, phenolics, and flavonoids. For instance, malic acid (MA), the major organic acid component of root exudates, was shown to act as a carbon source and a chemoattractant for the rhizobacteria, such as *Azospirillum* and *Bacillus subtilis* (Bashan and De-Bashan 2002; Rudrappa et al. 2008). Similarly, phenolics, such as benzoxanoids and salicylic acid (SA), from the root exudates of plants, such as maize and Arabidopsis, reportedly play roles in recruitment and enhancement of *Pseudomonas putida* population in the rhizosphere (Harwood, Rivelli, and Ornston 1984; Neal et al. 2012; Lebeis et al. 2015).

Besides regulation of soil microbiota, root exudation of MA has roles in plant-growth promotion itself. MA mitigates the abiotic stresses in plants by alleviating the heavy metal toxicity (by conjugation with metals like aluminum), by reducing the soil alkalinity (being acidic in nature, MA reduces the toxicity attributable to high soil pH); and by enhancing the bioavailability of limiting essential minerals, such as phosphate (through solubilization of complex forms of phosphate) (Lipton, Blanchar, and Blevins 1987; Wu and Zhao 2013; Gao, Zhang, and Hoffland 2009). There are also reports demonstrating the role of MA in biotic stress tolerance (Bashan and De-Bashan 2002; Rudrappa et al. 2008). In the presence of MA, proliferation of rhizobacteria, such as *Azospirillum* and *Bacillus subtilis*, was accelerated, which ultimately reduced the incidence of *Pseudomonas syringae* infection in Arabidopsis leaves (Bashan and De-Bashan 2002; Rudrappa et al. 2008; Meyer et al. 2011). As an intermediate compound in the Krebs cycle, MA plays a role in the regulation of pH, assimilation of nutrients, and functioning of stomata, as well as it acts as a transient carbon-storage molecule in C3 plants (Fernie and Martinoia 2009).

Similarly, SA plays diverse roles in direct plant-growth promotion in addition to the regulation of microbial community (Vlot, Dempsey, and Klessig 2009; Robert-Seilaniantz, Grant, and Jones 2011; Pieterse et al. 2012). It serves as a potent phytohormone and enhances seed germination, flowering, and fruit yield (Klessig and Malamy 1994). As a signaling molecule, SA regulates chloroplast biogenesis, photosynthesis, and gravitropism (Medvedev and Markova 1991; Uzunova and Popova 2000; Fariduddin, Hayat, and Ahmad 2003;). However, SA concentrations greater than 50 μM are detrimental to plant growth and hence, its biosynthesis is tightly regulated (Ishihara et al. 2008; Pajerowska-Mukhtar et al. 2012; Chandran et al. 2014; Seyfferth and Tsuda 2014; Vidhyasekaran 2015).

B. subtilis, a gram-positive bacterium, has emerged as a promising plant growth-promoting rhizobacterium (PGPR) for a broad range of host plants. Mechanisms of direct plant-growth promotion by *B. subtilis* include phosphate solubilization, production of auxins, such as indole acetic acid (IAA), and emission of volatile compounds (Asaka and Shoda 1996; Toro, Azcon, and Barea 1997; Krebs et al. 1998; Ryu et al. 2003). Besides direct growth promotion, mechanisms of indirect growth promotion by *B. subtilis* strains majorly involve the production of antimicrobials (such as iturin A, surfactin, fengycin, bacillomycin, difficidin, and bacilysin) and lytic enzymes (Chen et al. 2006; Ramarathnam et al. 2007).

Past research on plant-PGPR symbiosis has mainly focused on signaling by flavonoids (Parmer and Dadarwal 1999; Peters, Frost, and Long 1986). Despite their multifaceted nature, the effect of PGPRs in root exudation of MA and SA remains unexplored. Earlier studies on plant–PGPR interaction have shown increased levels of MA (a primary metabolite) in root tissues of rice plants in response to PGPRs, such as *Herbaspirillum seropedicae* and *B. subtilis* RR4 (Curzi et al. 2008; Rekha et al. 2018a). As for secondary metabolites, there are a few reports under pathogenic conditions where root colonization by a PGPR was shown to stimulate SA biosynthesis in plants, which eventually led to the induction of plant systemic resistance, thereby boosting the plant's basal defense mechanisms (Malamy, Henning, and Klessig 1992; Schilirò et al. 2012). Studies exploring the impact of PGPR on root exudation of compounds will further our understanding of the plant–microbe interaction and pave the way for enhancing the effectiveness of bioformulations. Our previous reports have demonstrated enhanced biosynthesis of MA and SA in root tissues of rice plants in response to colonization by *B. subtilis* RR4 (Rekha et al. 2018a,b). Here we investigated the influence of RR4 on root exudation of MA and SA in rice plants at different stages to get a better understanding of the molecular interaction between the PGPR (RR4) and rice roots, from its initial stages of colonization through its establishment in the rhizosphere. Further, the effect of SA on bacterial growth (as a nutrient source or an antimicrobial) was analyzed. This knowledge should help in effective exploitation of the root exuding potential of PGPRs in bioformulations that can serve as an eco-friendly approach to enhance crop production and sustenance of beneficial microbes, and thus help mitigate the ill effects of the chemical fertilizers and pesticides.

Materials and methods

Plant material and growth conditions

Rice seeds (TKM 9) were obtained from Rice Research Station, Tirur, Thiruvallur, Tamil Nadu, India. TKM 9 seeds were dehusked and surface

sterilized with Tween 80 (2 min) and 0.1% mercuric chloride (2 min). Following sterilization, the seeds were placed in culture tubes containing half-strength Murashige and Skoog (MS) media, incubated under dark conditions (2 days) for germination and transferred to a plant-growth chamber maintained at 25 ± 2 °C with 16 h light/8 h dark photoperiod for 2 weeks (Rekha et al. 2018a).

Bacterial culture and treatment of rice plants with the PGPRs

B. subtilis RR4 was previously isolated from rice rhizosphere (NCBI accession no.: KU974922) and was identified to possess plant growth-promoting traits (Rekha et al. 2018a,b). For treatment of rice plants with *B. subtilis* RR4 [tagged with rifampicin resistance (Rekha et al. 2018a)], culture of RR4 was prepared in nutrient broth with a cell density of 10^7 cells mL^{-1}, pelleted at 5000 g for 7 min, and resuspended in the same volume of sterile deionized water. From this suspension, 250 µL was inoculated into conical flasks (250 mL) containing 50 mL of sterile deionized water. Subsequently, 25 rice seedlings (grown aseptically for 2 weeks) were transferred to each of these conical flasks, cotton-plugged, covered with foil at the bottom, and kept on an orbital shaker (50 rpm) in the plant-growth chamber for 48 h (maintained at 25 ± 2 °C with 16 h light/8 h dark photoperiod). The experiment was repeated thrice, with uninoculated flasks serving as control (Rekha et al. 2018a).

Collection of exudates

After 48 h of bacterial treatment, the root exudates accumulated in the deionized water were collected (Rekha et al. 2018a). Subsequently, the same volume of fresh deionized water (sterile) was replaced and the rice plants were incubated in the plant-growth chamber for another 48 h. Immediately after sampling (at 48 and 96-h post-inoculation, hpi), the exudate samples were filtered to remove the root sheathings and root-border-like cells using 0.4 µm syringe filters (Millex-HV, Merck, MA, USA). The exudate samples were then frozen using liquid nitrogen and lyophilized in a freeze dryer at –37°C under vacuum. The obtained crude root exudate (~2 mg g^{-1} fresh weight of roots) was then resuspended in methanol (1 mL) and filter-sterilized using 0.22 µm syringe filters (Millex-GV, Merck, MA, USA) for high-performance liquid chromatography (HPLC) analysis.

Further, to determine the effect of rice root exudates on the growth of RR4, an aliquot of the samples was taken in parallel and spread-plated on the nutrient agar (NA) medium with appropriate dilutions. On overnight incubation at 37°C, the plates were observed for colonies and the colony-forming units (CFU) were recorded. As the strain RR4 was tagged with rifampicin

resistance, spread-plating in rifampicin-amended medium enabled colony counts of RR4 alone and avoided the possible growth of endophytes.

HPLC analysis of root exudates for MA and SA

For analysis of malic acid, root exudate samples were injected into Phenomenex-RP C18 column (250 × 4.6 mm, 5 µm, Phenomenex, CA, USA). The mobile phase used was 5 mM sulfuric acid and 100% methanol (75:25, v v^{-1}). The flow rate was set at 0.5 mL min^{-1} and the compounds eluted were detected at 254 nm of photodiode array (PDA) detector (Rekha et al. 2018a). The presence of malic acid in the samples was identified by comparing the chromatograms with the chromatogram of the standard L-malic acid (Sigma-Aldrich, MO, USA).

To detect SA in the root exudates, HPLC analysis was carried out with a mobile phase of methanol and 0.1% formic acid (68:32, v v^{-1}) using the Phenomenex-RP C18 column (250 × 4.6 mm, 5 µm, Phenomenex, CA, USA). The flow rate was set at 0.8 mL min^{-1} and the elution of compounds was detected using a PDA detector at 280 nm. The presence of SA in the samples was analyzed by comparing their retention times with that of the standard (Salicylic acid, HiMedia Laboratories, Mumbai, India). The significance of the data obtained from three independent experiments was statistically analyzed through analysis of variance (SPSS software, ver. 16.0, SPSS Inc., Chicago, USA).

SA test

Based on HPLC results, SA test was performed using a spectroscopy method (Warrier, Paul, and Vineetha 2013). Being a potent antimicrobial, SA test was performed to determine the toxicity concentrations of SA for RR4 and to determine the capacity of RR4 to utilize SA as a carbon source. The culture suspension of RR4 was prepared with an optical density (OD_{600}) of 0.4. One percent from this pre-inoculum was inoculated into culture tubes containing 10 mL of sterile nutrient broths, supplemented with varying SA concentrations (0, 0.05, 0.2, 1, 5, and 10 µg mL^{-1}). On 24 h incubation, 1 mL from each of the culture broths was taken and centrifuged to obtain cell-free supernatant. To the supernatant (1 mL), 1 mL of ferric chloride reagent (1 g ferric chloride in 100 mL 1% hydrochloric acid) was added and the volume was made to 10 mL using sterile distilled water. In parallel with the samples, the reaction was set with the standard SA (with concentrations of 5, 10, 15, 20, 25, and 30 µg mL^{-1}). After 10 min, the absorbance of the reaction mixtures (samples and standards) was measured at 525 nm. A standard graph was plotted using the absorbance values to estimate the concentration of SA in the samples. The experiment was performed in triplicate. The same procedure was repeated for the culture supernatants collected at 48 h.

In parallel, the viability of RR4 in the presence of SA was estimated by spread-plating the SA-amended culture broths (sampled at 24 h and 48 h incubation time), with appropriate dilutions, on the nutrient medium. After overnight incubation, the colonies were counted to calculate colony-forming units. The experiments were carried out in triplicate and analysis of variance was performed.

Results

Effect of B. subtilis *RR4 on the root exudation of MA*

Analysis of HPLC data showed that RR4 gradually enhanced the levels of MA in the root exudates of rice plants. There was an increase in MA levels at 48 hpi (Figure 1); however, on normalization with the plant and the bacterial controls, the increase was found to be non-significant (0.8-fold, p-value = 0.14); whereas, at 96 hpi, a significant increase in MA levels (after normalization with the controls) was observed in RR4-treated rice root exudates (12.3 ± 0.2 µg g^{-1} of root fresh weight, constituting a 3-fold increase, p-value <0.05) (Figure 2).

Impact of B. subtilis *RR4 on root exudation of SA*

A similar analysis for SA through HPLC showed the levels of SA were enhanced in the exudates of RR4-treated plants. As observed for MA, on

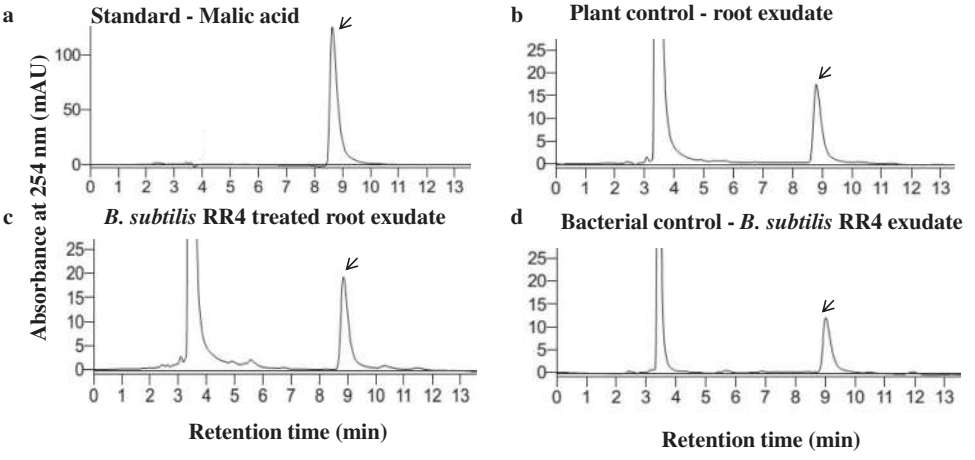

Figure 1. Chromatographic illustration of *B. subtilis* RR4-induced changes in the levels of malic acid (MA) in rice root exudates sampled at 48 hpi. The figure illustrates the levels of MA in the *B. subtilis* RR4-treated root exudate sample (C) in comparison with that of the standard – malic acid (A), the control plant root exudate (B) and the bacterial control (*B. subtilis* RR4) exudate (D). The peak for MA is indicated by arrows. The data are significant with a p-value <0.05, $n = 3$ (ANOVA).

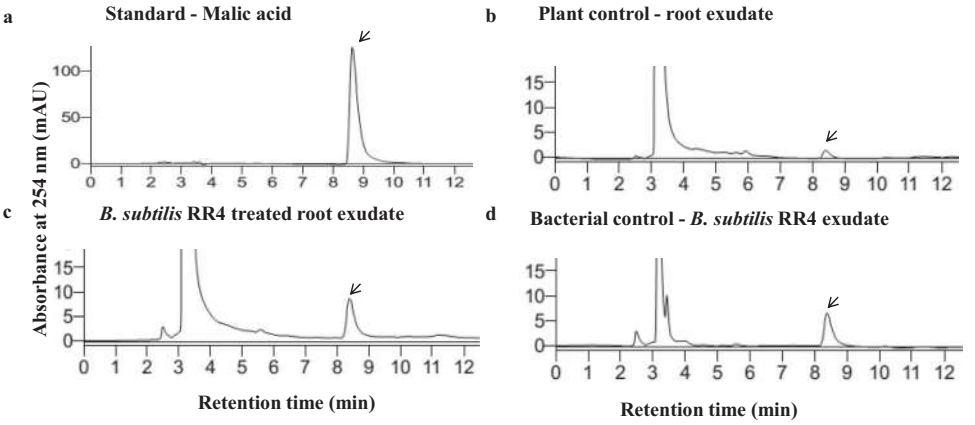

Figure 2. Chromatographic illustration of the *B. subtilis* RR4-induced changes in the levels of malic acid (MA) in rice root exudates sampled at 96 hpi. The figure illustrates the levels of MA in the *B. subtilis* RR4-treated root exudate samples (C) in comparison with that of the standard – malic acid (A), the control plant root exudates (B) and the bacterial control (*B. subtilis* RR4) exudate (D). The peak for MA is indicated by arrows. The data are significant with a p-value <0.05, $n = 3$ (ANOVA).

normalization with the plant and the bacterial controls, RR4-induced root exudation of SA was found to be non-significant (0.31-fold) at 48 hpi (p-value = 0.1) (Figure 3). However, at 96 hpi, there was a significant increase in SA levels (0.228 ± 0.007 μg g^{-1} of root fresh weight, constituting a 7-fold increase, p-value <0.05) in the exudates of RR4-treated plants, as compared with the control (Figure 4).

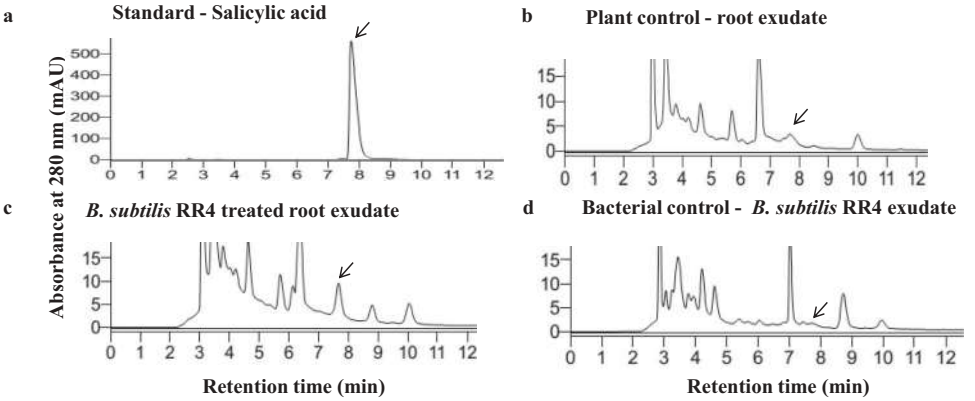

Figure 3. Chromatographic illustration of the RR4-induced changes in the levels of salicylic acid (SA) in rice root exudates sampled at 48 hpi. Chromatograms illustrate the levels of SA in the *B. subtilis* RR4-treated root exudate samples (C) in comparison with that of the standard – salicylic acid (A), the control plant root exudates (B) and the bacterial control (*B. subtilis* RR4) exudate (D). The presence of peak for SA (retention time – 7.6 min) is indicated by arrows. The data are significant with a p-value <0.05, $n = 3$ (ANOVA).

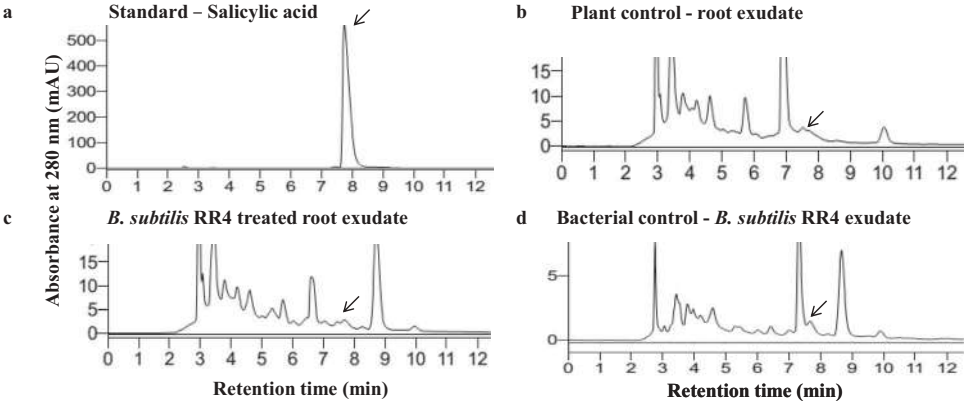

Figure 4. Chromatographic illustration of the RR4-induced changes in the levels of salicylic acid (SA) in rice root exudates sampled at 96 hpi. Chromatograms illustrate the levels of SA in the *B. subtilis* RR4-treated root exudate samples (C) in comparison with that of the standard – salicylic acid (A), the control plant root exudates (B) and the bacterial control (*B. subtilis* RR4) exudate (D). The presence of peak for SA (retention time – 7.6 min) is indicated by arrows. The data are significant with a p-value <0.05, $n = 3$ (ANOVA)

Effect of SA on growth and metabolism of RR4

SA being an antimicrobial, SA test was performed to assess the ability of RR4 to breakdown/utilize SA. The results showed an increase in levels of SA in the culture tubes amended with low SA concentrations (0.05 and 0.2 μg mL^{-1}), whereas, at higher concentrations, the levels of total SA were decreased (p-value <0.05) (Figure 5). This indicated that low concentrations did not affect the proliferation and metabolism of bacteria. Moreover, higher levels of total SA than the amended concentrations indicated the ability of RR4 to secrete SA in the culture broth. Higher concentrations of SA seemed to have a toxic effect on the bacterial growth, which could possibly have led to its rapid breakdown (97–99%) by the bacteria in the culture broths containing higher SA concentrations.

Similarly, the test performed through spread-plating of SA-amended culture broths and further calculation of CFU showed a decrease in CFU at SA concentrations of 1 μg mL^{-1} (Tables 3 and 4). At lower concentrations (up to 5 μg mL^{-1}), there was a significant increase in CFU of RR4 cells, relative to the control. These results corroborate those obtained through the SA test. The bacterial cell densities in SA-amended media were found to be in the range of 10^8–10^9 CFU mL^{-1} at 24 hpi and increased at 48 hpi.

Discussion

Under the natural conditions, microbes present in the soil help the plant with growth promotion through secretion of phytohormones and siderophores;

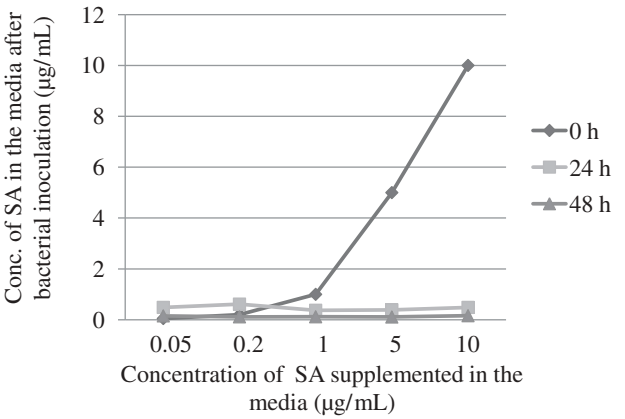

Figure 5. Illustration of the impact of *B. subtilis* RR4 on externally supplied salicylic acid (SA). The figure shows the SA profile of RR4 in SA amended nutrient broths at 24 and 48 h. The data are mean of the biological triplicate. Standard deviation among the replicates ranged from 0.005 to 0.02 µg mL^{-1}. Analysis of variance showed the data to be significant (p-value <0.05).

solubilization of essential minerals like phosphate and biocontrol of phytopathogens. In turn, root exudation of compounds, such as organic acids, amino acids, and sugars, helps the bacteria in their growth and metabolism. This metabolite trafficking in the rhizosphere, particularly root exudation, plays a critical role in influencing the microbiota of the soil. The components in the root exudates can either be beneficial or detrimental to the microbes in the soil. Though the plants are in continuous contact with the bacterial and fungal pathogens in the rhizosphere, only very few are successful in causing diseases. This self-defense is attributed to the exudation of compounds with antimicrobial activity (for example, phenolics). On the other hand, compounds, such as organic acids, enhance the potential of rhizobacteria to form biofilm onto plant roots (Rudrappa et al. 2008). Meharg and Killham (1995) have demonstrated the role of rhizobacteria in inducing root exudation of organic acids. This study aimed to analyze the impact of a PGPR on the exudation of specific metabolites (malic acid and salicylic acid) in rice.

The isolate from the rice rhizosphere, *B. subtilis* RR4, was reported to possess plant growth-promoting ability and induce MA biosynthesis in rice roots (Rekha et al. 2018a). The study also showed RR4 to have positive chemotaxis toward increasing MA concentrations. Here, investigation on the impact of RR4 in root exudation of MA demonstrated positive results. There was a 3-fold increase in root exudation of MA in RR4-treated rice plants, indicating the ability of RR4 to induce MA exudation; this correlates with the chemotaxis results obtained by Rekha et al. (2018a). This suggests that preference of RR4 to MA as a carbon source could be a possible reason for the induction of MA exudation by rice roots. Positive chemotaxis by the PGPR also points to the fact that MA could be utilized for their growth and

metabolism. Earlier studies have also demonstrated a similar rhizobacteria's preference for MA (Zhulin and Armitage 1993; Ling et al. 2011; Chen et al. 2013; Yuan et al. 2015). Bacterial catabolic pathways for MA were also shown to be enhanced in the presence of MA (Meyer et al. 2011). As discussed earlier, other than chemotaxis and recruitment of beneficial bacteria, root-exuded MA plays a role in mitigation of biotic and abiotic stresses (Rudrappa et al. 2008; Chen et al. 2013). With diverse growth-regulatory roles, MA promotes the growth of plants (Fernie and Martinoia 2009). This clearly suggests that RR4 influences the root exudation of metabolites for its growth, which in turn, helps the plant indirectly in growth promotion through the accumulated MA in the rhizosphere. This indicates that a possible mechanism of plant-growth promotion by species of *Bacillus* is its capacity to induce root exudation of MA.

HPLC analysis for SA showed that progressive colonization by RR4 significantly enhanced the root exudation of SA. Root colonization by a PGPR has been shown to induce, directly or indirectly, SA biosynthesis in plants (Solano et al. 2008). Consequently, the increased SA levels enhance the expression of several defense-response genes, such as those encoding β-1,3-glucanases, chitinases, and pathogenesis-related proteins; ultimately the plant attains immunity to pathogen attacks (Pieterse et al. 2009). Our previous study reported that RR4 induced a defense response in rice plants through SA-mediated signaling and enhanced the root biosynthesis of SA (Rekha et al. 2018b). Stimulation of plant defense responses being a major function of SA, our results (for SA) suggested that RR4 induced defense response in rice plants gradually and through different modes – systematically through enhanced accumulation of SA in roots (Rekha et al. 2018b) and externally through enhanced exudation of SA through roots, as observed in this study.

SA being a potent antimicrobial, SA test was performed to demonstrate if RR4 could protect itself from the deleterious effect of the exuded SA. The test showed that the concentrations of SA observed in the treated root exudates promoted bacterial growth and metabolism. However, at higher concentrations (1 µg), there was almost a complete breakdown of SA (97–99%) by RR4. This is in accordance with the previous studies, which have reported an induction of genes associated with the aromatic compound catabolism and energy generation in response to root exudates (Lugtenberg and Dekkers 1999; Mark et al. 2005). Moreover, the concentrations of SA induced by RR4 in the root exudates are much less than the toxicity concentrations reported for plants (Nazar et al. 2011; Rivas-San Vicente and Plasencia 2011; Bastam, Baninasab, and Ghobadi 2013). This suggests that RR4-induced root exudation of SA is non-detrimental for both the symbiotic partners involved (rice and RR4). These results showed the impact of PGPR-induced root exudation

on the native micro-flora under field conditions, which needs to be explored further.

As discussed above, SA can serve as either a nutrient source or an antimicrobial chemical. Hence, the effect of SA on the proliferation of RR4 was investigated. Preliminary plating experiments investigating the effect of root exudates on the growth of RR4 showed a gradual increase in the bacterial population (increase in CFU) in the presence of root exudates relative to bacterial control (Tables 1 and 2). Moreover, the cell densities remained in the range of 10^6–10^9 CFU mL^{-1}, which is necessary to exert a beneficial effect on plant growth (DeFlaun and Gerba 1993; Raaijmakers et al. 1995; Raaijmakers, Vlami, and De Souza 2002; Pieterse et al. 1996; NandeeshKumar et al. 2008; Nihorimbere et al. 2012). However, to

Table 1. Effect of root exudates on the population of *Bacillus subtilis* RR4 in a hydroponic set-up (with deionized water).

		CFU† (mL^{-1})	
S.No	Treatment	48 h	96 h
1.	Bacterial control (RR4)	$(3.9 \pm 0.03) \times 10^6$	$(1 \pm 0.06) \times 10^6$
2.	Treated (RR4 + rice plant)	$(9.4 \pm 0.06) \times 10^6$	$(1.8 \pm 0.06) \times 10^9$

†Colony-forming units (CFU) are represented as mean values ± standard deviation. At 0 h, CFU = $(1.8 \pm 0.1) \times 10^7$ mL^{-1}.

Table 2. Analysis of variance for colony-forming units of *B. subtilis* RR4 representing its population in a hydroponic set-up with deionized water with/without rice plants.

Analysis of variance for Colony-forming units of *B. subtilis* RR4					
Source of variation df	SS	MS	F	P-Value	F crit
Between groups 4	8.99E+14	2.25E+14	6.026543	0.002	2.776289
Within groups 24	8.96E+14	3.73E+13			

Table 3. Effect of salicylic acid on the viability of *Bacillus subtilis* RR4.

		CFU† (mL^{-1})	
S. No.	Salicylic acid (µg mL^{-1})	24 h	48 h
1.	0	$(3.6 \pm 0.06) \times 10^6$	$(4.7 \pm 0.1) \times 10^8$
2.	0.05	$(6 \pm 0.2) \times 10^6$	$(7.2 \pm 0.2) \times 10^8$
3.	0.2	$(19.2 \pm 0.25) \times 10^6$	$(15.8 \pm 0.6) \times 10^8$
4.	1	$(14.1 \pm 0.6) \times 10^6$	$(12.5 \pm 0.6) \times 10^8$
5.	5	$(13.7 \pm 0.63) \times 10^6$	$(14.4 \pm 0.9) \times 10^8$
6.	10	$(9.6 \pm 0.23) \times 10^6$	$(8.3 \pm 0.6) \times 10^7$

†Colony-forming units (CFU) are represented as mean values ± standard deviation. ANOVA analysis (p-value <0.05, $n = 3$).

Table 4. Analysis of variance for colony-forming units of *B. subtilis* RR4 representing its viability in salicylic acid-amended media (with varying concentrations).

Analysis of variance for colony-forming units of *B. subtilis* RR4						
Source of variation	df	SS	MS	F	P-Value	F crit
Between groups	4	45.86E+14	1.46E+14	8.236775863	0.000204	2.689628
Within groups	30	5.48E+14	1.83E+13			

demonstrate the sole effect of SA, samples from SA-amended culture broths were spread-plated to determine the CFU. The results showed that at concentrations of up to 5 μg mL^{-1}, there was a significant increase in the CFU of RR4 relative to control, whereas at higher concentrations, a reduction in the viability of RR4 was observed (Tables 3 and 4). These results corroborate the results obtained through SA test and confirm that the bacteria use the exuded SA for their growth and metabolism. Moreover, a study demonstrating the effect of root exudates on soil microbiome showed lower SA concentrations (15–62 nmol) to be beneficial for enriching the bacterial community in the rhizosphere (Badri et al. 2013). Although SA is well known as an antimicrobial chemical, this study provides evidence on its beneficial effect on bacterial growth and metabolism.

Conclusion

Overall, we have demonstrated the effect of *B. subtilis* RR4 on root exudation of MA and SA in rice plants. Further, the impact of SA on the metabolism and proliferation of RR4 was demonstrated. HPLC analysis showed the potential of RR4 in enhancing root exudation of MA and SA from rice roots. This is an early study demonstrating the effect of a PGPR on root exudation of important metabolites, such as MA and SA. We have also shown that the levels of SA exuded from the RR4-treated roots do not affect the survival of RR4 in the rhizosphere. Similar studies with different PGPRs should help in identifying the potential of microorganisms in inducing root exudation of important phytochemicals, which should be useful in preparing bio-formulations with an improved effect (enhanced PGPR viability) to ultimately enhance crop productivity and stress tolerance in plants.

Disclosure statement

The authors declare no competing financial interests.

References

Asaka, O., and M. Shoda. 1996. "Biocontrol of *Rhizoctonia Solani* Damping-off of Tomato with *Bacillus Subtilis* RB14." *Applied and Environmental Microbiology* 62: 4081–4085.

Bacilio-Jiménez, M., S. Aguilar-Flores, E. Ventura-Zapata, E. Pérez-Campos, S. Bouquelet, and E. Zenteno. 2003. "Chemical Characterization of Root Exudates from Rice (*Oryza Sativa*) and Their Effects on the Chemotactic Response of Endophytic Bacteria." *Plant and Soil* 249: 271–277.

Badri, D. V., J. M. Chaparro, R. Zhang, Q. Shen, and J. M. Vivanco. 2013. "Application of Natural Blends of Phytochemicals Derived from the Root Exudates of Arabidopsis to the Soil Reveal that Phenolic-related Compounds Predominantly Modulate the Soil Microbiome." *Journal of Biological Chemistry* 288 (7): 4502–4512.

Badri, D. V., and J. M. Vivanco. 2009. "Regulation and Function of Root Exudates." *Plant, Cell and Environment* 32: 666–681.

Bashan, Y., and L. E. De-Bashan. 2002. "Protection of Tomato Seedlings against Infection by *Pseudomonas Syringae* Pv. Tomato by Using the Plant Growth-promoting Bacterium *Azospirillum Brasilense*." *Applied and Environmental Microbiology* 68 (6): 2637–2643.

Bastam, N., B. Baninasab, and C. Ghobadi. 2013. "Improving Salt Tolerance by Exogenous Application of Salicylic Acid in Seedlings of Pistachio." *Plant Growth Regulation* 69: 275–284.

Chandran, D., J. Rickert, Y. Huang, M. A. Steinwand, S. K. Marr, and M. C. Wildermuth. 2014. "Atypical E2F Transcriptional Repressor DEL1 Acts at the Intersection of Plant Growth and Immunity by Controlling the Hormone Salicylic Acid." *Cell Host and Microbe* 15: 506–513.

Chen, X. H., J. Vater, J. Piel, P. Franke, R. Scholz, K. Schneider, A. Koumoutsi, et al. 2006. "Structural and Functional Characterization of Three Polyketide Synthase Gene Clusters in *Bacillus Amyloliquefaciens* FZB42." *Journal of Bacteriology* 188: 4024–4036.

Chen, Y., F. Yan, Y. Chai, H. Liu, R. Kolter, R. Losick, and J. H. Guo. 2013. "Biocontrol of Tomato Wilt Disease by *Bacillus Subtilis* Isolates from Natural Environments Depends on Conserved Genes Mediating Biofilm Formation." *Environmental Microbiology* 15 (3): 848–864.

Curzi, M. J., C. M. Ribaudo, G. D. Trinchero, J. A. Curá, and E. A. Pagano. 2008. "Changes in the Content of Organic and Amino Acids and Ethylene Production of Rice Plants in Response to the Inoculation with *Herbaspirillum Seropedicae*." *Journal of Plant Interaction* 3 (3): 163–173.

DeFlaun, M. F., and C. P. Gerba. 1993. "Monitoring Recombinant DNA Microorganisms and Viruses in Soil." In *Soil Microbial Ecology: Application in Agricultural and Environmental Management*, edited by F. B. J. Metting, 131–150. Washington: Marcel Dekker.

Fariduddin, Q., S. Hayat, and A. Ahmad. 2003. "Salicylic Acid Influences Net Photosynthetic Rate, Carboxylation Efficiency, Nitrate Reductase Activity and Seed Yield *Brassica Juncea*." *Photosynthetica* 41: 281–284.

Fernie, A. R., and E. Martinoia. 2009. "Malate. Jack of All Trades or Master of a Few?" *Phytochemistry* 70: 828–832.

Gao, X., F. Zhang, and E. Hoffland. 2009. "Malate Exudation by Six Aerobic Rice Genotypes Varying in Zinc Uptake Efficiency." *Journal of Environmental Quality* 38: 2315–2321.

Harwood, C. S., M. Rivelli, and L. N. Ornston. 1984. "Aromatic Acids are Chemoattractants for *Pseudomonas Putida*." *Journal of Bacteriology* 160: 622–628.

Ishihara, T., K. T. Sekine, S. Hase, Y. Kanayama, S. Seo, Y. Ohashi, T. Kusano, et al. 2008. "Overexpression of the *Arabidopsis Thaliana EDS5* Gene Enhances Resistance to Viruses." *Plant Biology* 10: 51–461.

Kamilova, F., L. V. Kravchenko, A. I. Shaposhnikov, T. Azarova, N. Makarova, and B. Lugtenberg. 2006. "Organic Acids, Sugars and L-tryptophane in Exudates of Vegetables Growing on Stonewool and Their Effects on Activities of Rhizosphere Bacteria." *Molecular Plant-Microbe Interactions* 9: 250–256.

Klessig, D. F., and J. Malamy. 1994. "The Salicylic Acid Signal in Plants." *Plant Molecular Biology* 26 (5): 1439–1458.

Krebs, B., B. HöDing, S. M. KüBart, A. Workie, H. Junge, G. Schmiedeknecht, R. Grosch, et al. 1998. "Use of *Bacillus Subtilis* as Biocontrol Agent. Activities and Characterization of *Bacillus Subtilis* Strains." *Journal of Plant Disease and Protection* 105: 181–197.

Lebeis, S. L., S. H. Paredes, D. S. Lundberg, N. Breakfield, J. Gehring, M. Mcdonald, S. Malfatti, et al. 2015. "Plant Microbiome. Salicylic Acid Modulates Colonization of the Root Microbiome by Specific Bacterial Taxa." *Science* 349 (6250): 860–864.

Ling, N., W. Raza, J. Ma, Q. Huang, and Q. Shen. 2011. "Identification and Role of Organic Acids in Watermelon Root Exudates for Recruiting *Paenibacillus Polymyxa* SQR-21 in the Rhizosphere." *European Journal of Soil Biology* 47 (6): 374–379.

Lipton, D. S., R. W. Blanchar, and D. G. Blevins. 1987. "Citrate, Malate, and Succinate Concentration in Exudates from P-sufficient and P-stressed *Medicago Sativa* L. Seedlings." *Plant Physiology* 85: 315–317.

Lugtenberg, B. J., and L. C. Dekkers. 1999. "What Makes Pseudomonas Bacteria Rhizosphere Competent?" *Environmental Microbiology* 1 (1): 9–13.

Lynch, J. M., and J. M. Whipps. 1990. "Substrate Flow in the Rhizosphere." *Plant and Soil* 129: 1–10.

Malamy, J., J. Henning, and D. F. Klessig. 1992. "Temperature-dependent Induction of Salicylic Acid and Its Conjugates during the Resistance Response to Tobacco Mosaic Virus Infection." *Plant and Cell* 4: 359–366.

Mark, G. L., J. M. Dow, P. D. Kiely, H. Higgins, J. Haynes, C. Baysse, A. Abbas, et al. 2005. "Transcriptome Profiling of Bacterial Responses to Root Exudates Identifies Genes Involved in Microbe-plant Interactions." *Proceedings of the National Academy of Sciences of the United States of America* 102 (48): 17454–17459.

Medvedev, S. S., and I. V. Markova. 1991. "Participation of Salicylic Acid in Gravitropism in Plants." *Doklady Akademii Nauk SSSR* 316: 1014–1016.

Meharg, A. A., and K. Killham. 1995. "Loss of Exudates from the Roots of Perennial Ryegrass Inoculated with a Range of Micro-organisms." *Plant and Soil* 170: 345–349.

Meyer, F. M., M. Jules, F. M. P. Mehne, D. Le Coq, J. J. Landmann, B. Görke, S. Aymerich, et al. 2011. "Malate-mediated Carbon Catabolite Repression in *Bacillus Subtilis* Involves the Hprk/Ccpa Pathway." *Journal of Bacteriology* 193 (24): 6939–6949.

NandeeshKumar, P., K. RamachandraKini, H. S. Prakash, S. R. Niranjana, and H. ShekarShetty. 2008. "Induction of Systemic Resistance against Downy Mildew on Sunflower by Rhizobacteria." *Journal of Plant Interaction* 3 (4): 255–262.

Nazar, R., N. Iqbal, S. Syeed, and N. A. Khan. 2011. "Salicylic Acid Alleviates Decreases in Photosynthesis under Salt Stress by Enhancing Nitrogen and Sulfur Assimilation and Antioxidant Metabolism Differentially in Two Mungbean Cultivars." *Journal Plant Physiology* 168: 807–815.

Neal, A. L., S. Ahmad, R. Gordon-Weeks, and J. Ton. 2012. "Benzoxazinoids in Root Exudates of Maize Attract *Pseudomonas Putida* to the Rhizosphere." *PloS One* 7 (4): e35498.

Nihorimbere, V., H. Cawoy, A. Seyer, A. Brunelle, P. Thonart, and M. Ongena. 2012. "Impact of Rhizosphere Factors on Cyclic Lipopeptide Signature from the Plant Beneficial Strain *Bacillus Amyloliquefaciens* S499." *FEMS Microbiology and Ecology* 79: 176–191.

Pajerowska-Mukhtar, K. M., W. Wang, Y. Tada, N. Oka, C. L. Tucker, J. P. Fonseca, and X. Dong. 2012. "The HSF-like Transcription Factor TBF1 Is a Major Molecular Switch for Plant Growth-to-defense Transition." *Current Biology* 22: 103–112.

Parmer, N., and K. R. Dadarwal. 1999. "Stimulation of Nitrogen Fixation and Induction of Flavonoid like Compounds by Rhizobacteria." *Journal of Applied Microbiology* 86: 36–44.

Peters, N. K., J. W. Frost, and S. R. Long. 1986. "A Plant Flavone, Luteolin, Induces Expression of *Rhizobium Meliloti* Nodulation Genes." *Science* 233: 977–980.

Pieterse, C. M., S. C. Van Wees, E. Hoffland, J. A. Van Pelt, and L. C. Van Loon. 1996. "Systemic Resistance in Arabidopsis Induced by Biocontrol Bacteria Is Independent of Salicylic Acid Accumulation and Pathogenesis-related Gene Expression." *The Plant Cell* 8 (8): 1225–1237.

Pieterse, C. M. J., D. Van Der Does, C. Zamioudis, A. Leon-Reyes, and S. C. M. Van Wees. 2012. "Hormonal Modulation of Plant Immunity." *Annual Review of Cell and Developmental Biology* 28: 489–521.

Pieterse, C. M. J. L., A. Leon-Reyes, S. Van Der Ent, and S. C. M. Van Wees. 2009. "Networking by Small-molecule Hormones in Plant Immunity." *Nature Chemical Biology* 5: 308–316.

Raaijmakers, J. M., M. Leeman, M. M. P. Van Oorschot, I. Van der Sluis, B. Schippers, and P. A. H. M. Bakker. 1995. "Dose-response Relationships in Biological Control of Fusarium Wilt of Radish by *Pseudomonas* Spp." *Phytopathology* 85: 1075–1081.

Raaijmakers, J. M., M. Vlami, and J. T. De Souza. 2002. "Antibiotic Production by Bacterial Biocontrol Agents." *Antonie Van Leeuwenhoek* 81: 537–547.

Ramarathnam, R., S. Bo, Y. Chen, W. G. Fernando, G. Xuewen, and T. De Kievit. 2007. "Molecular and Biochemical Detection of Fengycin- and Bacillomycin D Producing *Bacillus* Spp., Antagonistic to Fungal Pathogens of Canola and Wheat." *Canadian Journal of Microbiology* 53 (7): 901–911.

Rekha, K., B. Baskar, S. Srinath, and B. Usha. 2018a. "Plant Growth Promoting Rhizobacteria *Bacillus Subtilis* RR4 Isolated from Rice Rhizosphere Induces Malic Acid Biosynthesis in Rice Roots." *Canadian Journal of Microbiology* 1: 20–27.

Rekha, K., R. MohanKumar, K. Ilango, A. Rex, and B. Usha. 2018b. "Transcriptome Profiling of Rice Roots in Early Response to *Bacillus Subtilis* (RR4) Colonization." *Botany* 96: 749–765.

Richardson, A. E., J. M. Baréa, A. M. McNeill, and C. Prigent-Combaret. 2009. "Acquisition of Phosphorus and Nitrogen in the Rhizosphere and Plant Growth Promotion by Microorganisms." *Plant and Soil* 321: 305–339.

Rivas-San Vicente, M., and J. Plasencia. 2011. "Salicylic Acid beyond Defence: Its Role in Plant Growth and Development." *Journal of Experimental Botany* 62 (10): 3321–3338.

Robert-Seilaniantz, A., M. Grant, and J. D. Jones. 2011. "Hormone Crosstalk in Plant Disease and Defense: More than Just Jasmonate-salicylate Antagonism." *Annual Review of Phytopathology* 49: 317–334.

Rudrappa, T., K. J. Czymmek, P. W. Paré, and H. P. Bais. 2008. "Root-secreted Malic Acid Recruits Beneficial Soil Bacteria." *Plant Physiology* 148: 1547–1556.

Ryu, C. M., M. A. Farag, C. H. Hu, M. S. Reddy, H. X. Wei, P. W. Paré, and J. W. Kloepper. 2003. "Bacterial Volatiles Promote Growth in Arabidopsis." *Proceedings of the National Academy of Sciences of the United States of America* 100: 4927–4932.

Schilirò, E., M. Ferrara, F. Nigro, and J. Mercado-Blanco. 2012. "Genetic Responses Induced in Olive Roots upon Colonization by the Biocontrol Endophytic Bacterium *Pseudomonas Fluorescens* PICF7." *PloS One* 7: e48646.

Seyfferth, C., and K. Tsuda. 2014. "Salicylic Acid Signal Transduction: The Initiation of Biosynthesis, Perception and Transcriptional Reprogramming." *Frontiers in Plant Science* 5: 697.

Solano, R. B., B. J. Maicas, P. M. T. De La Iglesia, J. Domenech, and G. F. J. Mañero. 2008. "Systemic Disease Protection Elicited by Plant Growth Promoting Rhizobacteria Strains: Relationship between Metabolic Responses, Systemic Disease Protection, and Biotic Elicitors." *Phytopathology* 98 (4): 451–457.

Toro, M., R. Azcon, and J. M. Barea. 1997. "Improvement of Arbuscular Mycorrhiza Development by Inoculation of Soil with Phosphate Solubilizing Rhizobacteria to Improve Rock Phosphate Bioavailability (^{32}P) and Nutrient Cycling." *Applied and Environmental Microbiology* 63: 4408–4412.

Uzunova, A. N., and L. P. Popova. 2000. "Effect of Salicylic Acid on Leaf Anatomy and Chloroplast Ultrastructure of Barley Plants." *Photosynthetica* 38: 243–250.

Vacheron, J., G. Desbrosses, M. L. Bouffaud, B. Touraine, Y. Moënne-Loccoz, D. Muller, L. Legendre, et al. 2013. "Plant Growth-promoting Rhizobacteria and Root System Functioning." *Frontiers in Plant Science* 4: 356.

Vidhyasekaran, P. 2015. "Plant hormone signaling systems in plantinnate immunity." In: *Baluška, František (ed) Salicylic Acid Signaling in Plant Innate Immunity, Springer Dordrecht*, pp 27–122.

Vlot, A. C., D. A. Dempsey, and D. F. Klessig. 2009. "Salicylic Acid, a Multifaceted Hormone to Combat Disease." *Annual Review of Phytopathology* 47: 177–206.

Warrier, R. R., M. Paul, and M. V. Vineetha. 2013. "Estimation of Salicylic Acid in Eucalytpus Leaves Using Spectrophotometric Methods." *Genetics and Plant Physiology* 3 (1): 90–97.

Wu, Y. Y., and K. Zhao. 2013. "Root-exuded Malic Acid versus Chlorophyll Florescence Parameters in Four Plant Species under Different Phosphorus Levels." *Journal of Soil Science and Plant Nutrition* 13 (3): 604–610.

Yuan, J., N. Zhang, Q. Huang, W. Raza, R. Li, J. M. Vivanco, and Q. Shen. 2015. "Organic Acids from Root Exudates of Banana Help Root Colonization of PGPR Strain *Bacillus Amyloliquefaciens* NJN-6." *Scientific Reports* 5: 13438.

Zhulin, I. B., and J. P. Armitage. 1993. "Motility, Chemokinesis, and Methylation-independent Chemotaxis in *Azospirillum Brasilense*." *Journal of Bacteriology* 175 (4): 952–958.