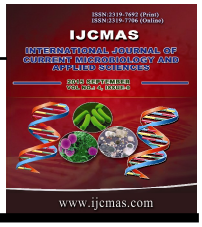
Int.J.Curr.Microbiol.App.Sci (2015) 4(9): 501-510

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 9 (2015) pp. 501-510**
http://www.ijcmas.com



Original Research Article

Antagonistic Activity of Siderophore Producing Rhizobacteria Isolated from the Semi-Arid Regions of Southern India

Neha Singh and Ajit Varma*

Amity Institute of Microbial Technology, E-3 Block, Sector-125, Amity University, Noida, UP-201303, India
**Corresponding author*

A B S T R A C T

The present study aims at assessing the antagonistic activity of previously reported six rhizobacteria, *viz*. SJPB (*Acinetobacter baumannii*), SJPB-2a (*Aeromonas hydrophila*), SJPB-2b (*Acinetobacter* sp.), BJJ-4 (*Pseudomonas alcaliphila*), BJJ-5a (*Klebsiella pneumoniae*) and BJJ-7 (*Pseudomonas brassicacearum*), isolated from the semi arid regions of Southern India (Singh *et al.*, 2015). These isolated rhizobacteria were qualitatively assessed for cell wall degrading enzymes activity. Additionally, in comparison to control, isolated rhizobacteria demonstrated varying potential for antifungal activity against the phytopathogen *Fusarium oxysporium*. The results of the present study, thus, indicate the potential of harnessing the benefit of plant growth promoting rhizobacteria as biocontrol agents.

Keywords

Rhizobacteria, PGPR, Antagonistic activity, *Fusarium oxysporium*, Biochemical assays

Introduction

Rhizobacteria that wield advantageous effect on plant growth and development and colonize the roots of the plants or rhizosphere are termed plant growth promoting rhizobacteria (PGPR) (Vacheron *et al.*, 2013). PGPRs are a part of rhizobacterial community and occupy as much as 2 to 5% of total rhizobacterial population constituting a cardinal part of rhizosphere biota (Kevin-Vessey, 2003). Interestingly the mechanism responsible for imparting plant growth promoting properties to the plant growth promoting rhizobacteria is not fully comprehended (Cattelan *et al.*, 1999). It may be attributed to the ability to produce plant hormones indoleacetic acid (IAA), gibberellic acid, cytokinins, and ethylene; fix nitrogen; suppress the growth of phytopathogenic microorganisms by production of siderophore, β-1,3-glucanse, chitinases, antibiotics, and cyanide; and solubilise phosphate and other nutrients. PGPRs effectively institute themselves in soil ecosystem due to their high compliance in a wide variety of environments, faster growth rate and biochemical adaptability to metabolize a wide range of natural and xenobiotic compounds (Bhattacharyya and Jha, 2012).

Biocontrol of plant pathogen is a cardinal PGPR trait. They produce siderophores and synthesize antibiotics and thus defending the plant from phyto-pathogens (Neilands and Leong, 1986). They also synthesize extracellular enzymes like chitinase, protease or lipase and β-1,3-glucanase which break fungal cells. They also produce hydrogen cyanide which exhibits antifungal activities and pose competition with plant pathogen (Dowling and O'Gara, 1994; Loper *et al.,* 1997). PGPR play significant role in inhibiting fungal pathogen and also considerable enhancement of plant growth. Various researchers have documented that rhizobacteria display rapid growth and compete for carbon and energy source against fungal pathogen and thus acting as biological control (Neilands and Leong, 1986; O'Sullivan and O'Gara, 1992; Dowling and O'Gara, 1994). Reports have also claimed that the entire rhizobacterial populations have the potential of causing fungistasis in rhizosphere (Handelsman and Stabb, 1996). Recent studies have also point out induced systemic resistance, antibiosis and pathogen- antagonist interaction as three important mechanisms for biocontrol of phytopathogens (van Loon *et al.,* 1998; van Loon and Bakker, 2003). Production of antibiotics has been reported from a large number of Pseudomonas strains. These antibiotics have the ability to inhibit pathogenic bacteria, fungi, pathogenicity of higher organisms and in some cases higher organisms (Raaijmakers *et al.,* 2002).

PGPR stimulate the plant protection against pathogens and arrest the pathogenic activity of rhizobacteria through Induced Systemic Resistance mechanisms (ISR) (Van Loon and Bakker, 2006a,b). Induced Systemic Resistance mechanisms have been documented in nearly 15 plant species (van Loon and Bakker, 2006a,b). PGPR also increase the plant growth by Induced Systemic Resistance (Kloepper *et al.,* 2004). The inducible defense mechanisms include production of pathogenesis proteins, production of antimicrobial phytoalexins and re-construction of plant cell wall (Hammond-Kosack and Jones, 1996; Conrath *et al.,* 2006).

In the present study, we aimed at assessing the antagonistic activity effect of previously reported six rhizobacteria, *viz*. SJPB (*Acinetobacter baumannii*), SJPB-2a (*Aeromonas hydrophila*), SJPB-2b (*Acinetobacter* sp.), BJJ-4 (*Pseudomonas alcaliphila*), BJJ-5a (*Klebsiella pneumoniae*) and BJJ-7 (*Pseudomonas brassicacearum*), isolated from the semi arid regions of Southern India (Singh *et al.,* 2015).

Materials and Methods

Isolation and maintenance of rhizobacteria

The rhizosphere dwelling bacteria beneficial for plant growth were isolated from the rhizospheric soil samples employing the serial dilution plate method. Suitable amount of dilution was spread on nutrient, Jensen's, King's B and TSA agar plates. These spread plates were then incubated at 28 ± 2°C for 24-48 h. Post incubation morphologically distinct colonies were picked from the plate and were maintained as pure cultures with regular transfer to fresh media plates and stored for future use. The rhizobacterial isolates were maintained at -20°C in equal volumes of nutrient broth and 30% (v/v) glycerol.

Catalase activity

The catalase test was carried out by standard method. 3–4 drops of hydrogen peroxide (H_2O_2) was added to rhizobacterial colony which was grown on nutrient agar medium. The effervescence on the glass slide demonstrates catalase activity.

Hydrogen cyanide (HCN) production

Selection of rhizobacterial isolates for hydrogen cyanide (HCN) production was done in accordance to the protocol described by Bakker and Schipperes (Bakker and Schippers, 1987). Rhizobacterial cultures were inoculated on king's B plate supplemented with 4.4 g/lit of glycine. Further Whatman filter paper No. 1 absorbed in 0.5% picric acid solution (in 2% sodium carbonate) was put inside the lid of the agar plate. The agar plates were preserved with parafilm and incubated at 30±0.1°C for 4 days. Appearance of orange to dark brown color demonstrated HCN production.

Antifungal activity

The rhizobacterial isolates were studied for their intrinsic ability to inhibit phytopathogenic fungus with help of dual culture method on PDA plates. Independently a loopful of 48 hrs old rhizobacterial culture grown on nutrient agar media was inoculated on one side leaving 1 cm from the margin, and then 6 mm disc of fungal phytopathogen culture was localized on the other side. Plates without rhizobacteria were used as control. The plates were inoculated at 25±2 ºC for 4-5 days. Antifungal activity was determined from the inhibition of mycelial growth of the phytopathogen in the route of vigorously growing bacteria. The percentage of inhibition was calculated by subtracting the distance (mm) of fungal growth in the track of an antagonist from the phytopathogenic fungal radius.

The percent inhibition was calculated using the formula:

Percent inhibition= (R-r)/R ×100

Where 'r' is radial growth of the fungal growth conflicting the bacterial colony and, R is the radial growth of the phytopathogenic fungus in control plate.

Cell wall degrading enzymes

Protease activity

The qualitative assay for determination of protease activity (casein degradation) was evaluated from clear zone formation in skimmed milk agar media plate. The agar plates were used and dot inoculated with test rhizobacteria and incubated at 30º C for 3 days. Appearance of halo zone formation around the colony is indicative for cell wall degrading enzyme synthesis.

Lipase activity

Assay for detection of lipase producing rhizobacteria makes use of acridine orange-olive oil-agar media. The growth medium constituents are (g/L): nutrient broth, 8.0; NaCl & agar-agar. The pH 7.0 of the medium was adjused, autoclaved & cooled to about 60ºC. Then olive oil 10g/L was mixed with constant and forceful stirring. The media was ready to be poured into petri plates under aseptic conditions. The rhizobacteria culture was inoculated in the solidified media plates. Lipase producing bacteria were spotted on spread plates post 48 h of incubation at 27ºC. The hydrolysis of substrate is credited to the formation of orange fluorescent halos around rhizobacterial colonies visible upon exposure to U.V. radiation.

Amylase activity

Starch agar media was made, autoclaved and poured on petriplates. The rhizobacteria were inoculated and the plates were incubated for 24 hours at 27ºC. In presence of starch, iodine changes color from a yellow-brown to blue-black. The plates were

flushed with iodine and it wass allowed to stand for 10 minutes. The starch agar plate transforms into blue-black in presence of starch which is a negative response for the hydrolysis of starch. In fact appearance of clear zone surrounding the growth is a positive response and asserts the fact that the starch has been hydrolysed in the area surrounding the rhizobacteria. If in case rhizobacteria secretes and liberates amylase, starch hydrolysis in the agar will not take place.

Statistical analysis

All the above reported data are represented as mean ± SD for a minimum of three replicates. Student's T-test was performed to assess the significance of the results derived from the experiments (**indicates $p<0.005$).

Results and Discussion

Six rhizobacteria were isolated from the rhizospheric soil samples from semiarid regions of Secunderabad (Southern India), which demonstrated significant plant growth promoting activities, including siderophore production (Singh *et al.*, 2015). Siderophore is a biocontrol mechanisms belonging to PGPRs groups under iron limiting conditions. PGPR produces a range of siderophore which have kinship towards iron. Therefore, the availability of iron would overpower the growth of pathogen organisms including plant pathogenic fungi. Iron is a limiting bioactive metal in soil and essential for growth of soil microorganisms. The iron concentration in the soil is exceptionally low (10^{-7}M) enough to hinder the growth of soil microorganism (10^{-8}-10^{-6}M) (Guerinot, 1994). Rhizobacteria are equipped with some strategies to acquire iron. An important tactic is the production of siderophores. The rhizobacteria that can produce siderophores compete for iron with soil borne pathogens. Siderophore producing bacteria are great in plant growth promotion.

All the six rhizobacterial isolates were previously found to be siderophore producing (Singh *et al.*, 2015). In the present study, these six isolates were characterized for their antagonistic activities *viz*., screening for their HCN Production, Catalase-, Antifungal-, Lipase-, Amylase-, and Protease-activity.

Catalase activity

Rhizobacterial isolates exhibiting catalase activity are highly resistant to environmental, mechanical and chemical stress. Excluding BJJ-4, all the bacterial isolates in the present study exhibited catalase activity (Table 1A). BJJ-5a and SJPB-2a exhibited maximum effervescence

HCN production

HCN production by rhizobacteria has been claimed to play a vital role in the biological control of pathogens. In the present study, only BJJ-7 demonstrated positive activity for HCN production, which may act as an inducer of plant resistance (Table 1B).

Antifungal activity

Antagonistic activities of the bacterial isolates were assessed in terms of inhibition zone diameter as a pointer of the reduction in growth of phytopathogenic fungus *F. oxysporum*. BJJ-5a showed antagonism against fungal phytopathogens *F.oxysporum* (Fig. 1A). Calculation of percentage inhibition of radial growth indicated that BJJ-5a showed maximum inhibition of 64.2% and BJJ-4 showing least inhibition of 15% (Table 2, Fig. 1).

Table.1 Qualitative analysis of (a) catalase activity and (c) HCN production by rhizobacteria

(a)

Isolates	Results
SJPB	+ Weak
SJPB-2a	++++ Excellent
SJPB-2b	+ Weak
BJJ-4	- (No effervescence)
BJJ-5a	++++ Excellent
BJJ-7	+++ Good

(b)

Isolates	Results
SJPB	- (No activity)
SJPB-2a	- (No activity)
SJPB-2b	- (No activity)
BJJ-4	- (No activity)
BJJ-5a	- (No activity)
BJJ-7	++++ Excellent

Table.2 Quantitative determination (Percentage inhibition of radial growth-PIRG) of the antifungal activity of rhizobacteria against fungal pathogen *F. oxysporum*

Isolates	Radial Growth (mm)			Mean (mm)	PIRG %
	Replicate 1	Replicate 2	Replicate 3		
Control	40	42	38	40.0	0.0
SJPB	28	24	27	26.3	34.2
SJPB-2a	26	25	24	25.0	37.5
SJPB-2b	29	29	27	28.3	29.2
BJJ-4	37	32	33	34.0	15.0
BJJ-5a	15	12	16	14.3	64.2
BJJ-7	22	24	25	23.7	40.8

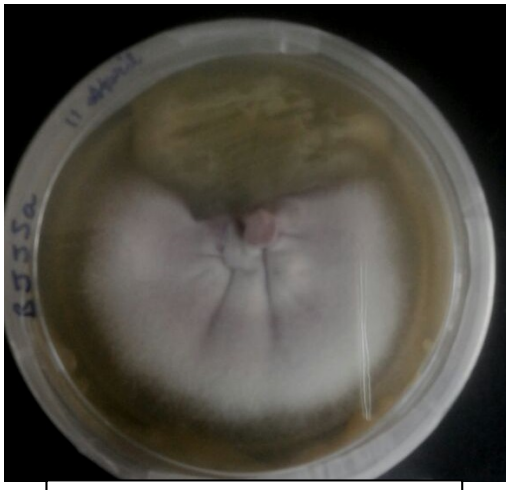
Table.3 Summarized table of various plant growth properties of different rhizobacteria

Isolates	Catalase activity	HCN production	Antifungal activity	Cell wall degrading enzymes		
				Lipase activity	Amylase activity	Protease activity
SJPB	+	-	+++	++	+++	-
SJPB-2a	++++	-	+++	+++	++++	-
SJPB-2b	+	-	++	++++	+++	-
BJJ-4	-	-	+	+++	++	-
BJJ-5a	++++	-	++++	++++	++++	++
BJJ-7	+++	++++	+++	-	++++	++++

++++ Excellent, +++ Good, ++ Average, + Weak, - No effervescence/No activity

Fig.1 a) Representative photograph of antagonism activity of BJJ-5a against fungal phytopathogen *Fusarium oxysporium* b) Antifungal activity of rhizobacteria against phytopathogenic fungus *Fusarium oxysporium*

Control



BJJ-5a

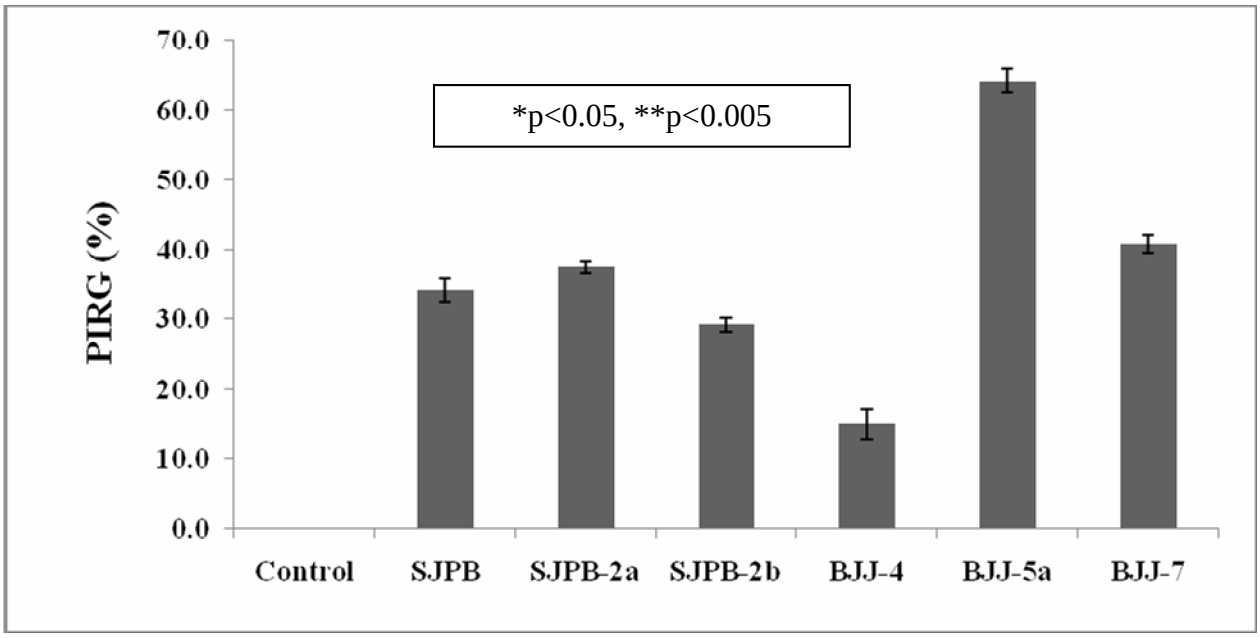
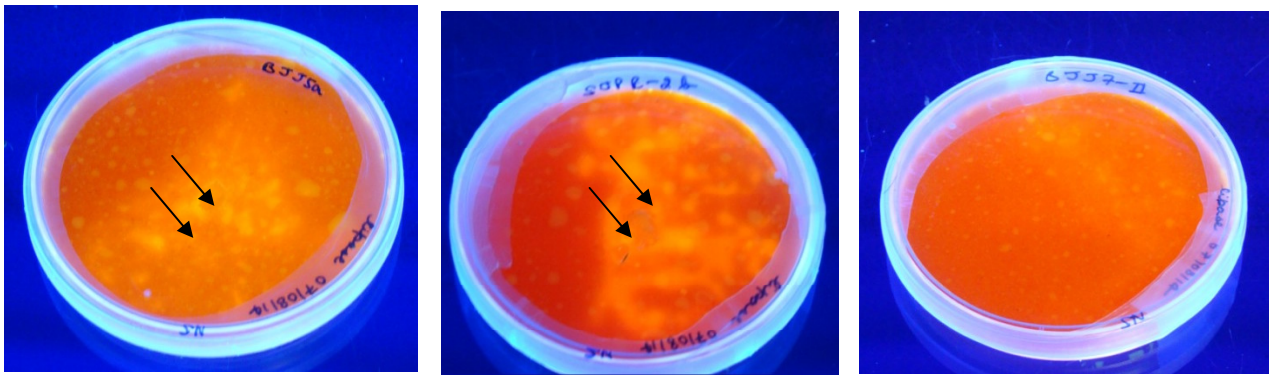


Fig.2 Representative photographs of rhizobacteria demonstrating lipase activity



BJJ-5a | SJPB-2b | BJJ-7

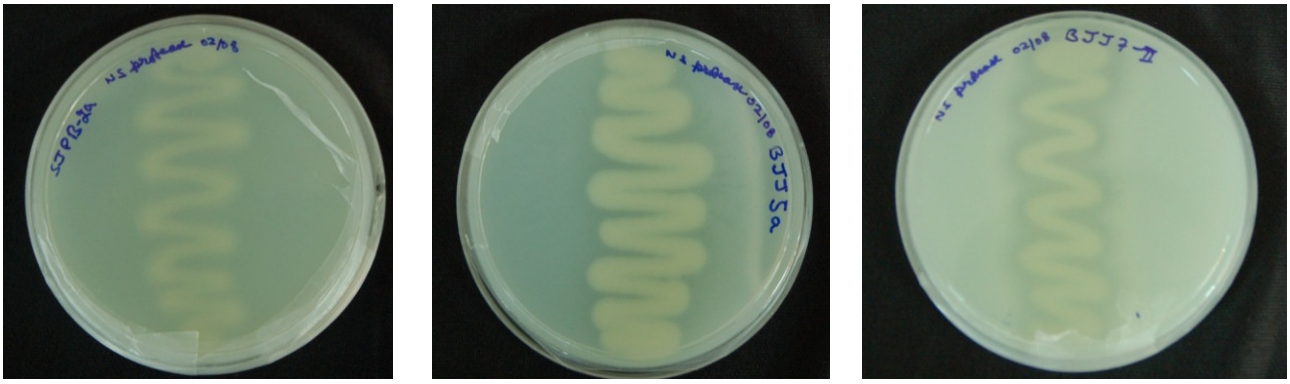
SJPB	SJPB-2a	SJPB-2b	BJJ-4	BJJ-5a	BJJ-7
++ Average	+++Good	++++ Excellent	+++ Good	++++Excellent	- (No activity)

Fig.3 Representative photographs of rhizobacteria demonstrating amylase activity

SJPB-2a | BJJ-5a | BJJ-7

SJPB	SJPB-2a	SJPB-2b	BJJ-4	BJJ-5a	BJJ-7
+++ Good	++++ Excellent	+++ Good	++ Average	++++ Excellent	++++ Excellent

Fig.4 Representative photographs of rhizobacteria demonstrating Protease activity



SJPB-2a | BJJ-5a | BJJ-7

SJPB	SJPB-2a	SJPB-2b	BJJ-4	BJJ-5a	BJJ-7
- (No activity)	**- (No activity)**	**- (No activity)**	**- (No activity)**	**++ Average**	**++++ Excellent**

Lipase activity

Detection of lipase producing rhizobacteria was screened on acridine orange- olive oil-agar media. The rhizobacteria culture was inoculated in the solidified media plates. Lipase producing bacteria were spotted on spread plates post 48 h of incubation at

27ºC. The hydrolysis of substrate is attributed to the formation of orange fluorescent halos around rhizobacterial colonies visible upon exposure to U.V. radiation BJJ-5a again and SJPB-2b showed excellent lipase activity (qualitative) and BJJ-7 exhibited no lipase activity (Fig. 2).

Amylase activity

Amylase activity can be observed by clear zone on starch agar medium. After 2-3 days of incubation the plates were flooded with Iodine solution. Iodine solution is allowed to stand for 1 min, the development of clear zone around the colony indicative of positive result for starch hydrolysis test showed Amylase activity. All the six rhizobacterial isolates showed amylase activity. BJJ-5a, BJJ-7, SJPB-2a showed strong amylase activity (Fig. 3).

Protease activity

Proteolytic enzyme production was determined by the development of a clear zone around the colony on skim milk agar medium. Two rhizobacteria showed proteolytic activity. BJJ-7 showed excellent protease activity, BJJ-5a showed average protease activity whereas the rest of the four isolates showed no protease activity (Fig. 4).

PGPRs inhabit the roots of plant and the area adjoining the root and augment plant growth and development through myriad of mechanisms. Nevertheless, the precise mechanism by which PGPR kindle plant growth is not clearly understood, although several processes like production of phytohormones, suppression of phytopathogenic fungi, activation of phosphate solubilization and promotion of the mineral nutrient uptake have so far been thought to be the reasons behind plant growth promotion (Lalande *et al.,* 1989; Glick, 1995).

In present study, previously reported plant-beneficial bacteria isolated from rhizosphere of Jatropha plant, were screened for different antagonistic activity *viz.* HCN production, Antifungal-, Catalase- Amylase-, Protease-activity. Cell wall degrading enzymes were qualitatively assayed (Table 3) to understand the mechanisms behind the biocontrol of pathogens. Isolated PGPRs demonstrated unique ability to secrete enzymes that may help to degrade the cell wall of pathogens. Furthermore, rhizobacteria BJJ-5a demonstrated maximal antagonism against fungal phytopathogens *F. oxysporum*, which suggests potential for antagonistic activity against phytopathogens. Present study, thus reflects the application of Plant Growth Promoting Rhizobacteria (PGPR), having significant antagonistic effect that may prove beneficial to the plant, additional to rhizobacteria's plant growth promoting properties.

Acknowledgement

This work was supported by Defence Institute of Bioenergy Resources, Pithoragarh, India and Defence Research & Development Organisation, Secunderabad, India. We are thankful to Dr. K. Annapurna from Indian Agriculture Research Institute, Delhi, India for her instrumental support.

References

Bakker, A. W., B. Schippers. 1987. Microbial cyanide production in the rhizosphere in relation to potato yield reduction and Pseudomonas SPP-mediated plant growth-stimulation. *Soil Biol. Biochem.,* 19(4): 451–457.

Bhattacharyya, P. N., D. K. Jha. 2012. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World J. Microbiol. Biotechnol.,* 28(4): 1327–1350.

Cattelan, A. J., P. G. Hartel, J. J. Fuhrmann.

1999. Screening for Plant growth–promoting rhizobacteria to promote early soybean growth. *Soil Sci. Soc. Am. J.*, 63(6): 1670–1680.

Conrath, U., G. J. Beckers, V. Flors, P. Garcia-Agustin, G. Jakab, F. Mauch, M. A. Newman, C. M. Pieterse, B. Poinssot, M. J. Pozo, A. Pugin, U. Schaffrath, J. Ton, D. Wendehenne, L. Zimmerli, B. Mauch-Mani. 2006. Priming: getting ready for battle. *Mol. Plant Microbe Interact.*, 19(10): 1062–1071.

Dowling, D. N., F. O'Gara. 1994. Metabolites of Pseudomonas involved in the biocontrol of plant disease. *Trends Biotechnol.*, 12(4): 133–141.

Glick, B. R. 1995. The enhancement of plant growth by free-living bacteria. *Can. J. Microbiol.*, 41(2): 109–117.

Guerinot, M. L. 1994. Microbial iron transport. *Annu. Rev. Microbiol.*, 48: 743–772.

Hammond-Kosack, K. E., J. D. Jones, 1996. Resistance gene-dependent plant defense responses. *Plant Cell*, 8(10): 1773–1791.

Handelsman, J., E. V. Stabb. 1996. Biocontrol of Soilborne Plant Pathogens. *Plant Cell*, 8(10): 1855–1869.

Kevin-Vessey, J. 2003. Plant growth promoting rhizobacteria as biofertilizers. *Plant Soil*, 255(2): 571–586.

Kloepper, J. W., C. M. Ryu, S. Zhang, 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Phytopathology*, 94(11): 1259–1266.

Lalande, R., N. Bissonnette, D. Coutlée, H. Antoun, 1989. Identification of rhizobacteria from maize and determination of their plant-growth promoting potential. *Plant Soil*, 115(1): 7–11.

Loper, J., B. Nowak-Thompson, C. Whistler, M. Hagen, N. Corbell, M. Henkels, V. Stockwell, 1997. Biological control mediated by antifungal metabolite production and resource competition: an overview. International Plant Growth Promoting Rhizobacteria Workshop.

Neilands, J. B., S. A. Leong, 1986. Siderophores in relation to plant growth and disease. *Ann. Rev. Plant Physiol.*, 37(1): 187–208.

O'Sullivan, D. J., F. O'Gara. 1992. Traits of fluorescent Pseudomonas spp. involved in suppression of plant root pathogens. *Microbiol. Rev.*, 56(4): 662–676.

Raaijmakers, J. M., M. Vlami, J. T. de Souza. 2002. Antibiotic production by bacterial biocontrol agents. *Antonie Van Leeuwenhoek*, 81(1-4): 537–547.

Singh, N., K. Mishra, A. Varma. 2015. Isolation, screening and characterization of PGPRs from the semi-arid rhizospheric soil of *Jatropha curcas*. *J. Endocytobiosis Cell Res.*, 26: 13–20.

Vacheron, J., G. Desbrosses, M. L. Bouffaud, B. Touraine, Y. Moenne-Loccoz, D. Muller, L. Legendre, F. Wisniewski-Dye, and C. Prigent-Combaret. 2013. Plant growth-promoting rhizobacteria and root system functioning. *Front Plant Sci.*, 4: 356–375.

van Loon, L. C., P. A. Bakker, C. M. Pieterse, 1998. Systemic resistance induced by rhizosphere bacteria. *Ann. Rev. Phytopathol.*, 36: 453–483.

Van Loon, L. C., P. A. H. M. Bakker, 2006a. Induced systemic resistance as a mechanism of disease suppression by rhizobacteria. In: Z. Siddiqui (Ed.), PGPR: Biocontrol and Biofertilization. Springer Netherlands. Pp. 39–66.

van Loon, L. C., P. A. H. M. Bakker, 2006b. Root-associated bacteria inducing systemic resistance. *Plant-Associated Bacteria*. S. Gnanamanickam, Springer Netherlands. Pp. 269–316.

van Loon, L. C., P. A. H. M. Bakker. 2003. Signalling in Rhizobacteria-Plant Interactions. In: H. de KroonE. W. Visser (Eds), Root ecology. Springer Berlin Heidelberg. 168: 297–330.