ATLANTIS PRESS

Advances in Biological Sciences Research, volume 7

1st International Symposium Innovations in Life Sciences (ISILS 2019)

Rhizospheric Bacteria, Destructors of Toxic Aromatic Compounds

Tatiana Anokhina
Laboratory of plasmid biology
G.K. Scryabin Institute of Biochemistry and Physiology of Microorganisms, FIC Pushchino Scientific Center for Biological Research, RAS
Pushchino, Moscow Region, Russia
to_anohina@rambler.ru

Tatiana Esikova
Laboratory of plasmid biology
G.K. Scryabin Institute of Biochemistry and Physiology of Microorganisms, FIC Pushchino Scientific Center for Biological Research, RAS
Pushchino, Moscow Region, Russia
das3534@rambler.ru

Valetina Polivtseva
Laboratory of cytology
G.K. Scryabin Institute of Biochemistry and Physiology of Microorganisms, FIC Pushchino Scientific Center for Biological Research, RAS
Pushchino, Moscow Region, Russia
kaistia@gmail.com

Leila Iminova
Pushchino State Institute of Natural Sciences
Pushchino, Moscow Region, Russia
uzdleila90@gmail.com

Inna Solyanikova*
Laboratory of microbial enzymology
G.K. Scryabin Institute of Biochemistry and Physiology of Microorganisms, FIC Pushchino Scientific Center for Biological Research, RAS ; Pushchino State Institute of Natural Sciences,
Pushchino, Moscow Region, Russia
innas@ibpm.pushchino.ru

***Abstract*— Bacteria capable of degradation of a number of toxic organic compounds (aromatic hydrocarbons, *n*-alkanes, phenol and its chlorinated derivatives) were isolated from the rhizosphere of plants growing on "clean" and oil-contaminated soil. The most active strain, *Lysinibacillus* sp. Fg1 was able to utilize more than 15 individual substrates. Six bacterial strains of the genus *Pseudomonas* decomposed polycyclic aromatic hydrocarbons, suppressed the growth of phytopathogenic fungi and bacteria, and synthesized phytohormones auxins.**

Keywords—bacteria-destructors, rhizospheric microorganisms, polycyclic aromatic hydrocarbons, phenol, phytoremediation

I. INTRODUCTION

Currently, a huge number of toxic compounds of natural and technogenic origin are circulating in the biosphere. Mono- and polycyclic aromatic hydrocarbons and their derivatives (eg phenol, toluene, chlorinated phenols) are dangerous pollutants due to their widespread distribution and negative impact on living organisms. The most important role in cleaning the environment belongs to microorganisms of different taxonomic groups. Self-recovering of polluted ecosystems is associated with the adaptation of microbial populations to new conditions due to the propagation of strains resistant to pollutants and able to use them as a source of carbon and energy.

Recently, interest in bioremediation technologies for soil cleaning has increased significantly. One such approach is phytoremediation - the combined use of plants and microorganisms associated with them [1, 2]. Microorganisms, decomposing pollutants, reduce the toxic effect on plants, in turn, plants allow microorganisms-destructors to maintain their higher numbers. Obtaining experimental data on the interaction of plants and rhizospheric bacteria on contaminated soils is the basis for increasing the efficiency of phytoremediation technologies.

It was shown that the death and inhibition of plant growth on contaminated soil can occur not only due to the toxic effect of the pollutant, but also due to severe damage of plants by phytopathogenic fungi and the accumulation of fungal metabolites in the soil [3, 4]. Thus, to clean contaminated soils, it is necessary to use strains that can degrade organic pollutants and inhibit the growth of phytopathogenic fungi.

The aim of this work was to isolate and characterize rhizospheric bacterial strains to utilize toxic organic pollutants.

II. EXPERIMENTAL

A. Bacterial strains and cultivation conditions

Bacterial strains were isolated from the rhizosphere of plants (wild grasses) grown on soil contaminated with oil products, as well as from "clean" soils. To isolate strains-destructors, the selected samples were resuspended in saline solution and plated on mineral solid medium M9 [5] with naphthalene / benzoate as the sole sources of carbon and energy. Bacteria were grown at 24–30 °C in plates or in liquid medium at 150 rpm.

B. Molecular biological methods

DNA isolation, amplification, sequencing of the 16S rRNA gene and phylogenetic analysis were carried out by conventional methods [5]. The polymerase chain reaction (PCR) of the genes responsible for the synthesis of bacterial antibiotics phenazine-1-carboxylic acid, 2,4-diacetylphloroglucin, pyoluteorin and pyrrolnitrin was carried out using the primers described previously [6, 7].

C. Determination of the ability of bacteria to utilize pollutants

Selected bacterial cultures were tested for their ability to utilize various aromatic, aliphatic, and chlorinated compounds that were added to the M9 mineral medium as

the sole source of carbon and energy. Growth substrates were used in the following concentrations: naphthalene, phenanthrene, anthracene, fluorene, acenaphthene, phenol, caprolactam - 0.2–1.0 g/l; salicylate, gentisate, protocatechuate, *o*-phthalate, 2-hydroxycinnamic acid, catechol, phenol, benzoate, chlorobenzoates (2-, 3-, 4-chlorobenzoate, 3,5-dichlorobenzoate), 2,4,5-trichlorophenoxyacetic acid – 0.2 g/l; chlorophenols (2-, 3-chlorophenol, 2,3-, 2,4-, 2,5-, 2,6-, 3,4-dichlorophenol, 2,3,4-, 2,4,5-, 2,4,6-trichlorophenol, pentachlorophenol) - 0.1 g/l.

During cultivation of strains on an agar medium, volatile aromatic and aliphatic compounds: naphthalene, benzene, toluene, ethylbenzene, phenol, hexane, octane, nonane, decane, undecane, dodecane, hexadecane, diesel fuel, camphor, coumarin were introduced onto the cover of an inverted Petri dish.

The ability of bacteria to decompose phenol in a liquid medium was tested as follows: all cultures were transferred into agarized Luria-Bertani media (LB). The cells grown on LB were transferred in to the mineral medium of the following composition (g/l): Na_2HPO_4 0.7; KH_2PO_4 0.5; NH_4NO_3 0.75; $MgSO_4$ x $7H_2O$ 0.2; $MnSO_4$ 0.001; $FeSO_4$ 0.02, $NaHCO_3$ 0.25, containing phenol as a source of carbon and energy in various concentrations (100, 300 and 500 mg/l). The growth of cultures was monitored by determining the optical density (OD) at 560 nm, the presence of phenol was determined spectrophotometrically on the device UV-1800 (Shimadzu, Japan) at 220–350 nm.

The pH of the medium was maintained in the range of 7.0–7.2 by adding NaOH. The cultures utilizing phenol at a concentration of 100 mg/l were transferred in fresh mineral medium, the phenol concentration was increased to 300 mg/l. The cultures decomposing phenol at a concentration of 300 mg/l were reseeded in fresh medium with phenol at a concentration of 500 mg/l.

D. The determination of antagonistic activity

The determination of antagonistic activity was carried out by co-cultivation the isolated strains and phytopathogens according to [8]. As indicator phytopathogens, the fungi *Gaeumannomyces graminis* var. *tritici* (strain Ggt 1818), *Rhizoctonia solani*, *Fusarium oxysporum*, *F. graminearum* and bacteria *Erwinia carotovora* B15 were used.

E. The auxin production level

The auxin production level was determined by the colorimetric method with the addition of Salkovsky reagent [9].

III. RESULTS AND DISCUSSION

The ability of rhizospheric strains to survive and effectively utilize toxic pollutants is a prerequisite for their use in phytoremediation [10]. Many organic pollutants negatively affect the phytoremediation process, inhibiting plant growth due to metabolic disorders and a decrease in bacterial degradation as a result of bactericidal exposure [11–13].

In this work, more than 30 strains of bacteria, capable of utilizing various pollutants (aromatic, aliphatic and chlorine-containing compounds) as the sole source of carbon and energy, were isolated from rhizosphere samples. Screening of the collection showed that substrates such as benzoate, phenol, naphthalene, and *n*-alkanes (the number of C6 – C16 carbon atoms) were most easily utilized by isolated strains.

More than half of the studied strains grew on these compounds. The most stable for conversion by the strains were benzene and its derivatives - toluene and ethylbenzene, as well as chlorine-containing phenols and benzoates. Most strains did not use these substrates. Only in some cases, an insignificant growth was observed on an agar medium supplemented with chlorophenols or chlorobenzoates. Identification of the most active strains-destructors based on 16S rRNA gene sequencing showed that they belong to different taxonomic groups of bacteria, both gram-positive (*Lysinibacillus*) and gram-negative (*Pseudomonas*, *Stenotrophomonas*) (Table I).

Since phenol belongs to the pollutants of priority, having a negative effect on the metabolism of plants already at a concentration of 500 mg/l, isolated strains were tested for the ability to utilize this compound. The phenol degrading strains were isolated from the rhizosphere of plants growing on both “clean” and contaminated soils.

TABLE I. CHARACTERIZATION OF THE MOST ACTIVE STRAINS-DESTRUCTORS

Strain	Source of isolated strain	Growth substrate
Stenotrophomonas sp. Fch7	Rhizosphere of plants from uncontaminated soil (Kazakhstan)	Phenol, 2-chlorophenol
Lysinibacillus sp. Fg1	Rhizosphere of plants from contaminated soil (Kazakhstan)	Caprolactam, phenol, benzoate, octane, nonane, decane, hexadecane, dodecane, undecane, 2-chlorophenol, 3-chlorophenol, pentachlorophenol, 2,4,5-trichlorophenoxyacetic acid, 2,5-dichlorophenol, 2,6-dichlorophenol 2,4,6-trichlorophenol, protocatechuate
P. fluorescens IC7, IID5, VB1, OV29	Rhizosphere of wild cereals, oil-contaminated soil (Western Siberia)	Phenanthrene, anthracene, fluorene, acenaphthene, naphthalene, salicylate, protocatechuate
P. fluorescens A1	Wild cereal rhizosphere, gas station territory (Pushchino, Moscow region)	Phenanthrene, anthracene, fluorene, acenaphthene, naphthalene, salicylate, protocatechuate
P. chlororaphis OV17	Rhizosphere of wild cereals, oil-contaminated soil (Western Siberia)	Phenanthrene, anthracene, fluorene, acenaphthene, naphthalene, 2-methylnaphthalene, salicylate, benzoate, protocatechuate
P. chlororaphis IG1	Rhizosphere of wild cereals, oil-contaminated soil (Republic of Tatarstan)	Salicylate, benzoate
Pseudomonas sp. OV9, OV25	Rhizosphere of wild cereals, oil-contaminated soil (Western Siberia)	Phenanthrene, anthracene, fluorene, acenaphthene, naphthalene, 2-methylnaphthalene, salicylate, protocatechuate

Two strains (*Lysinibacillus* sp. Fg1 and *Stenotrophomonas* sp. Fch7) actively grew and decomposed phenol in high, up to 500 mg/l, concentration. Strain *Lysinibacillus* sp. Fg1 isolated from the contaminated area showed destructive activity towards to more than 15 individual compounds (Table I).

Lysinibacteria are considered to be very promising agents for bioremediation. Bacteria of this genus can not only decompose stable pollutants, but also belong to the group a plant growth-promoting bacterium [14]. *Stenotrophomonas* sp. Fch7 isolated from clean soil did not have significant biodegradable activity, however, it was also able to utilize the more toxic 2-chlorophenol. Fig. 1 shows a growth graph of a strain of *Stenotrophomonas* sp. Fch7 in mineral medium supplemented with 500 mg/l of phenol.

It is known that some representatives of rhizospheric bacteria are able to improve plant growth due to various mechanisms [15, 16]. The most striking example of their positive effect is the synthesis of phytohormones and the protection of plants from phytopathogens.

The use of multifunctional strains that have a complex of useful features, in particular phytostimulating properties and the ability to biodegradation of organic pollutants, in phytoremediation technologies seems very promising. In this regard, the antagonistic activity of strains-destructors towards phytopathogenic fungi *Gaeumannomyces graminis* var. *tritici* (strain Ggt 1818), *Rhizoctonia solani*, *Fusarium oxysporum*, *F. graminearum* and *Erwinia carotovora* B15 bacteria was studied.

Strains *P. chlororaphis* OV17 and IG1 showed antagonistic activity against fungal and bacterial phytopathogens, and the zones of inhibition of growth of phytopathogens were comparable with the zones in the positive control (*P. chlororaphis* strain BS1393 from the IBPM RAS collection). *P. fluorescens* strains IC7, IID5, VB1, A1, OV29 had less antagonistic activity and suppressed only the growth of fungi (Fig. 2, Table II).

Antifungal phenazine metabolites were detected in extracts of the culture fluid of the investigated pseudomonads using thin layer chromatography (TLC). Strains *P. chlororaphis* OV17 and IG1, releasing yellow-orange pigments into the culture medium, synthesized phenazine-1-carboxylic acid (PCA) (Rf 0.8) and 2-hydroxyphenazine (Rf 0.3). Only phenazine-1-carboxylic acid was detected in *P. fluorescens* IC7, IID5, VB1 and A1 culture fluid. The structure of these phenazine derivatives was confirmed by mass spectrometry. In the strain *P. fluorescens* OV29, which showed weak antagonistic activity against phytopathogenic fungi, under these cultivation conditions, no major spots were detected during TLC. Perhaps its effect on phytopathogens is associated with the production of other metabolites, for example, siderophores.

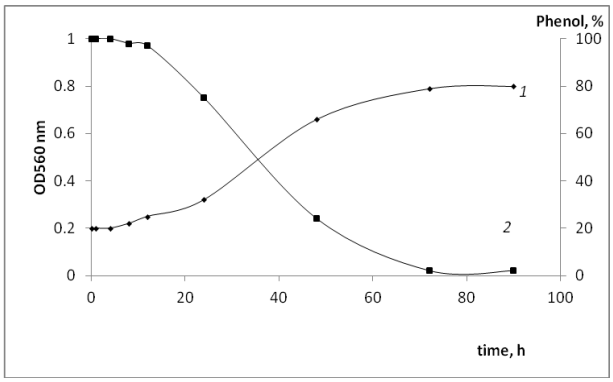

Fig. 1. The growth of strain *Stenotrophomonas* sp. Fch7 in a liquid mineral medium with phenol as the sole source of carbon and energy. Graph growth (*1*) and phenol decreasing (*2*).

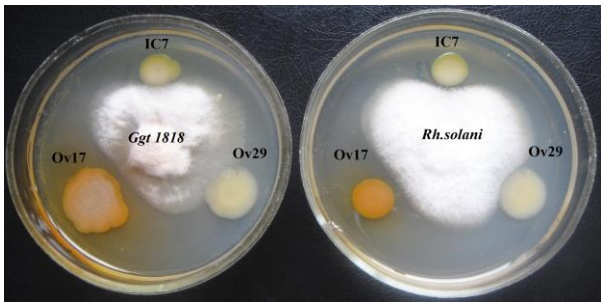

Fig. 2. Inhibition of growth of phytopathogenic fungi *G. graminis* var. *tritici* (Ggt 1818) and *R. solani* by strains of rhizospheric bacteria when their co-grown.

TABLE II. CHARACTERIZATION OF THE ANTAGONISTIC ACTIVITY OF THE STRAINS OF THE GENUS *PSEUDOMONAS*

Strain	Phytopathogen suppression zone, mm				Produced antibiotics	Auxins, µg/ml
	R. solani	*Ggt 1818*	*F. oxysporum*	*E. carotovora*		
P. chlororaphis OV17	4 ± 1	4 ± 1	5 ± 1	3 ± 1	PCA/2-hydroxyphenazine	14.0 ± 1.6
P. chlororaphis IG1	7 ± 2	4 ± 1	4 ± 1	3 ± 1	PCA/2-hydroxyphenazine	13.4 ± 1.2
P. fluorescens IC7	< 2	< 2	3 ± 1	–	PCA	3.2 ± 0.7
P. fluorescens IID5	< 2	< 2	< 2	–	PCA	3.5 ± 1.0
P. fluorescens VB1	< 2	< 2	3 ± 1	–	PCA	3.5 ± 1.0
P. fluorescens A1	7 ± 2	< 2	8 ± 2	–	PCA	8.4 ± 1.1
P. fluorescens OV29	2 ± 1	–	< 2	–	–	3.6 ± 0.7

Pseudomonas strains that showed antagonistic activity were selected for analysis for the presence of genes involved in the biosynthesis of various antibiotics. The strains were screened by PCR using specific primers designed to detect the genes involved in the biosynthesis of 2,4-diacetylphloroglucinol (*phlD*), pyrrolnitrin (*prnC*), pyoluteorin (*pltB*) and phenazine-1-carboxylic acid (*phzCD*) which showed antibiotic activity and are fungicidal and bactericidal compounds. The amplification results are presented in Fig. 3.

Using primers PCA2a and PCA3b, complementary to the *phzCD* phenazine operon sequence, identical DNA fragments were amplified in five strains and the control strain *P. fluorescens* 2-79 (Fig. 3, A). A positive PCR response was also obtained for strains OV17 and IG1 for the *prnC* gene, which is necessary for the biosynthesis of pyrrolnitrin (Fig. 3, B). The genes necessary for the synthesis of pyoluteorin and 2,4-diacetylphlorogluinol were not amplified.

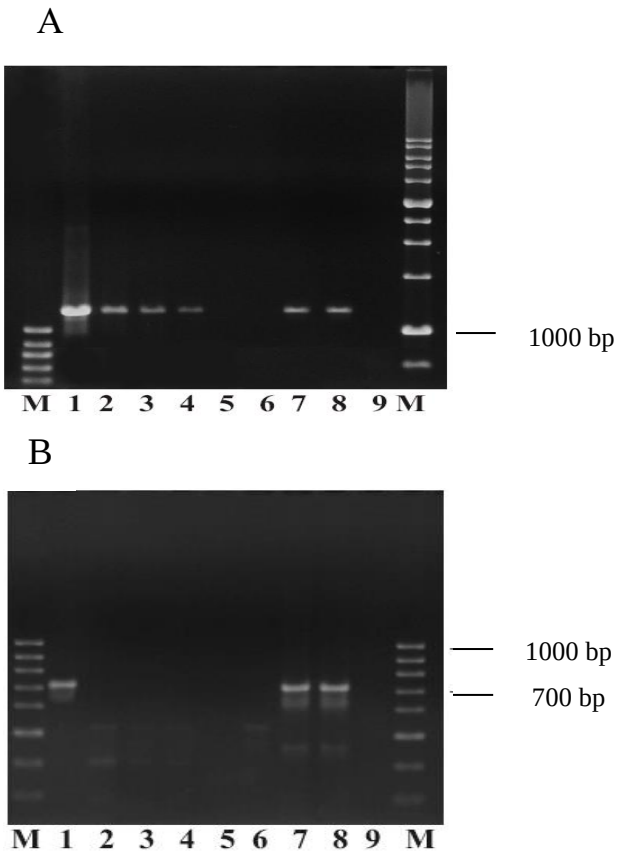

Fig. 3. Electrophoregram of amplification products of the phenazine operon fragment *phzCD* (A) and *prnC* gene necessary for the synthesis of pyrrolnitrin (B) in rhizospheric PAH destructive bacteria. M - DNA marker (SibEnzyme, Russia). A: 1 - *P. fluorescens* 2-79 (phenazine-1-carboxylic acid producer, positive control), 2 - IC7, 3 - IID5, 4 - VB1, 5 - OV9, 6 - OV29, 7 - OV17, 8 - IG1, 9 - *P. fluorescens* Q2-87 (producer of 2,4-diacetylphloroglucinol, negative control); B: 1 - *P. fluorescens* Pf-5 (producer of pyrrolnitrin, pyoluteorin and 2,4-diacetylphloroglucinol, positive control), 2 - IC7, 3 - IID5, 4 - VB1, 5 - OV9, 6 - OV29, 7 - OV17, 8 - IG1, 9 - *P. fluorescens* 2-79 (phenazine-1-carboxylic acid producer, negative control).

It is important to note that the efficiency of phytoremediation is significantly affected by the viability of plants under pollution conditions and their accumulation of vegetative mass. Exogenous auxins produced by rhizosphericbacteria are known to stimulate the growth and development of bacteria [17]. All the rhizosphere strains-destructors studied in this work synthesized auxins in an amount of about 3–14 μg/ml. The highest concentration of auxins was found in the culture medium of *P. chlororaphis* OV17 and IG1 strains - 14.0 ± 1.6 and 13.4 ± 1.2 μg/ml, respectively.

IV. Conclusion

The bacteria-destructors of toxic organic compounds, which can also inhibit the growth of phytopathogenic bacteria and fungi, have been isolated and characterized. These strains are promising candidates for the creation of biological products based on them for cleaning polluted territories. The introduction of rhizospheric bacteria-destructors capable of colonizing plant roots into contaminated soil can help increase the stability of plant-microbial associations and intensify phytoremediation processes. The expected positive effect of such bacteria is associated not only with a decrease in the level of toxic load on the soil, but also with the release of biologically active substances that have a positive effect on plants.

Acknowledgment

The reported study was partially funded by RFBR according to the research project № 18-34-00964.

References

[1] X. Zhuang, J. Chen, H. Shim, Z. Bai, “New advances in plant growth-promoting rhizobacteria for bioremediation”, Environ Int., vol. 33(3), pp. 406–413, 2007.

[2] K.E. Gerhardt, X.-D. Huang, B.R. Glick, B.M. Greenberg, “Phytoremediation and rhizoremediation of organic soil contaminants: Potential and challenges”, Plant Sci., vol. 176, pp. 20–30, 2009

[3] N.A. Kireeva, N.F. Galimzyanova, A.M. Miftakhova, “Micromycetes from oil-contamineted soils and their phytotoxicity”, Mycology and phytopathology (in Russian), vol. 34(1), pp. 36–41, 2000.

[4] N.A. Kireeva, M.D. Bakaeva, A.M. Miftakhova, “Litic activity of micromycetes in oil-contaminated soils as a factor of phytotoxicity”, Agricultural chemistry (in Russian), vol. 9, pp. 75–81, 2006.

[5] J. Sambrook, E.F. Fritsch, T. Maniatis, “Molecular cloning: A Laboratory Manual”, Cold Spring Habor Laboratory press, 479 p. 1989.

[6] J. Raaijmakers, D.M. Weller, L.S. Thomashow, “Frequency of antibiotic-producing *Pseudomonas* spp. in natural environments”, Appl. Environ. Microbiol., vol. 63, pp. 881–887, 1997.

[7] O.V. Mavrodi, B.B. McSpadden Gardener, D.V. Mavrodi, R.F. Bonsall, D.M. Weller, L.S. Thomashow, “Genetic diversity of *pltD* from 2,4-diacetylphloroglucinol-producing fluorescent *Pseudomonas* spp”, Phytopathology, vol. 91(1), pp. 35–43, 2001.

[8] H. Hamdan, D.M. Weller, L.S. Thomashow, “Relative importance of fluorescent siderophores and other factors in biological control of *Gaeumannomyces graminis* var. *tritici* by *Pseudomonas fluorescens* 2-79 and M4-80R”, Appl. Environ. Microbiol., vol. 57(11), pp. 3270–3277, 1991.

[9] S.A. Gordon, R.P. Weber, “Colorimetric estimation of indole-acetic acid”, Plant Physiol., vol. 26, pp. 192–195, 1951.

[10] H. Saleem, M. Arslan, K. Rehman, R. Tahseen, M.Afzal, “*Phragmites australis* – a helophytic grass – can establish successful

partnership with phenol-degrading bacteria in a floating treatment wetland", Saudi J. Biol. Sci., vol. 26(6), pp. 1179–1186, 2019.

[11] A.S. Ucisik, S. Trapp, "Uptake, removal, accumulation, and phytotoxicity of phenol in willow trees (*Salix viminalis*)", Environm. Toxicol. Chem., vol. 25, pp. 2455–2460, 2006.

[12] S. Kottuparambil, Y.-J. Kim, H. Choi, M.-S. Kim, A. Park, J. Park, W. Shin, T. Han, "A rapid phenol toxicity test based on photosynthesis and movement of the freshwater flagellate, *Euglena agilis Carter*", Aquatic Toxicol., vol. 155, pp. 9–14, 2014.

[13] T. Phenrat, P. Teeratitayangkul, I. Prasertsung, R. Parichatprecha, P. Jitsangiam, N. Chomchalow, S. Wichai, "Vetiver plantlets in aerated system degrade phenol in illegally dumped industrial wastewater by phytochemical and rhizomicrobial degradation", Environ. Sci. Pollut. Res., vol. 24(15), pp. 13235–13246, 2017.

[14] S.A. Martínez, J. Dussán, "*Lysinibacillus sphaericus* plant growth promoter bacteria and lead phytoremediation enhancer with *Canavalia ensiformis*", Environ. Prog. Sustain. Energy, vol. 37, pp. 276–282, 2018.

[15] B.J. Lugtenberg, F. Kamilova, "Plant-growth-promoting rhizobacteria", Annu. Rev. Microbiol., vol. 63, pp. 541–556, 2009.

[16] B.R. Glick, "Plant growth-promoting bacteria: mechanisms and applications", Scientifica (Cairo), Article ID 963401, 2012.

[17] S. Spaepen, J. Vanderleyden, R. Remans, "Indole-3-acetic acid in microbial and microorganism-plant signaling", FEMS Microbiol. Rev., vol. 31(4), pp. 425–448, 2007.