TÜBİTAK

Turk J Biol
36 (2012) 487-492

doi:10.3906/biy-1012-4

Colonization of *Mycobacterium phlei* in the rhizosphere of wheat grown under saline conditions

Dilfuza EGAMBERDIEVA

Department of Biotechnology and Microbiology, Faculty of Biology and Soil Sciences, National University of Uzbekistan, Vuzgorodok, Tashkent 100174 - UZBEKISTAN

Received: 13.12.2010 ● Accepted: 24.08.2011

Abstract: One of the natural reservoirs of potentially human-pathogenic bacteria is believed to be the rhizosphere. The aim of the present work was to test nontuberculous mycobacterium *Mycobacterium phlei* MbP18 for its ability to colonize the rhizosphere of wheat and to evaluate its effect on plant growth under saline conditions. In competitive wheat root tip colonization assays, *M. phlei* MbP18 showed poor competitive colonization of the wheat rhizosphere compared to the reference strain. The strain produced lipase, amylase, cellulase, and pectinase and grew well in the presence of high salt (up to 4% NaCl) and at high temperatures (up to 40 °C). It was also able to utilize a wide range of carbohydrates for growth. The strain produced indole-3-acetic acid and proved to be very efficient in promoting a significant increase in the shoot and root of wheat under saline conditions. In conclusion, the results of this study indicate that *M. phlei* MbP18 has beneficial effects on plant growth under saline conditions through its ability to produce different biologically active compounds such as cell wall-degrading enzymes and the phytohormone auxin. However, its competitive colonization abilities in the rhizosphere are poor. In light of this observation, attempts should be made to manage the rhizosphere in order to prevent colonization of the rhizosphere by pathogens. This will help remove mycobacteria from habitats where humans or animals can be exposed.

Key words: *Mycobacterium* phlei, colonization, enzymes, indole-3-acetic acid, plant growth stimulation

Introduction

Human infections associated with plants have increased in many countries in the world (1). According to Gagliardi and Karns (2), animal manures that are often applied to land as fertilizer are not specifically treated to reduce their bacterial content. As a result, large numbers of bacteria, including potential pathogens, are applied to soil along with the manure, and these pathogens can reach the rhizosphere of plants; their survival and interaction with plants is greater than previously thought (3).

A group of nontuberculous mycobacteria (NTM) have increased in prevalence and are causing disease worldwide in both immunocompromised and immunocompetent people (4,5). Many NTM have been found in a variety of environments including natural and municipal water, soil, plants, animals, and humans (6,7). Moreover, several studies support the view that environmental strains are indistinguishable from clinical isolates in terms of genotypic, taxonomic, or metabolic properties (8-10).

Mycobacterium phlei is considered to be an opportunistic NTM and is usually saprophytic, but has been reported to cause septic arthritis in humans (11) as well as infections in cats (12) and cattle (13). As these bacterial species do not transmit from person to person, their source is believed to be environmental.

They have been recovered from boreal forest soil (14), water ecosystems (15), and drinking water (16). However, their reservoirs are not yet well understood. The plant root is believed to be a natural reservoir of opportunistic pathogens, an alternative host for human pathogens to survive in the environment, and a vehicle to recognize the animal or human host once the pathogen is ingested (17). Assuming that the selected bacteria are indeed of warm-blooded origin, the question of how they successfully compete with the local indigenous rhizosphere microflora arises. The aim of this study was to evaluate the colonization of *M. phlei* strain MbP18 in the rhizosphere of wheat and to investigate its interactions with plants.

Material and methods

Microorganisms

The bacterial strain *M. phlei* MbP18 was taken from the culture collection of the National University of Uzbekistan. The strain was previously isolated from the rhizosphere of wheat (18). *Pseudomonas fluorescens* PCL1285 (a Tn*5*luxAB derivative of WCS365) was acquired from the culture collection of Leiden University, the Netherlands. Strain WCS365 is an excellent colonizer of various plant root systems (19).

Traits involved in plant growth promotion

Hydrogen Cyanide (HCN) production by the bacterial strain was determined according to the method described by Castric (20), lipase activity was determined according to the method described by Howe and Ward (21), protease activity was determined according to the method described by Brown and Foster (22), glucanase activity was determined according to the method described by Walsh et al. (23), and cellulase activity was detected using the substrate carboxymethylcellulose in top-agar plates (24). Pectinase was performed according to the method of Smibert and Krieg (25).

The production of acid from carbohydrates was tested in peptone water broths containing carbohydrates such as glucose, arabinose, xylose, glycose, galactose, saccharose, lactose, maltose, glycerol, mannitol, and Andrade's indicator at 1% (26).

The production of indole-3-acetic-acid (IAA) was determined according to the method of Bano and Musarrat (27). Tested bacterial strains were grown in LC medium, a modification of Luria broth base, Miller (Difco), amended with 1%-4% NaCl with and without tryptophan (100 µg/mL) and incubated at 28 °C. After 4 days of cultivation, 2-mL aliquots of bacterial culture were removed from each tube and centrifuged at 13,000 × *g* for 10 min. Supernatant fluid (1 mL) was transferred to a fresh tube, to which 100 µL of 10 mM orthophosphoric acid and 2 mL of reagent (1 mL of 0.5 M $FeCl_3$ in 50 mL of 35% $HClO_4$) were added. After 25 min, the absorbance of the developed pink color was read at 530 nm. The IAA concentration in the culture was determined by using a calibration curve of pure IAA as a standard.

In order to determine the optimum salt concentration for growth, the bacterial strain was cultured in LC medium supplemented with 1%, 2%, 3%, and 4% NaCl (w/v). The growth rate of bacteria isolates was determined by spectrophotometer after 2, 4, 6, 8, 10, 12, and 14 h. The bacterial strain growth was measured as optical density (OD) at 600 nm. The growth at different temperatures was observed in LC medium after incubation at 10, 30, and 40 °C.

The bacterial strain was tested in vitro against *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Forl), *Gaeumannomyces graminis* pv. *tritici* (Ggt), *Pythium ultimum*, *Alternaria alternate*, and *Botrytis cinerea* using a plate bioassay with potato dextrose agar.

Fungal strains grown in agar plates at 28 °C for 5 days and disks of fresh culture of the fungus (5 mm in diameter) were cut out and placed in the center of a 9-cm petri dish. Bacteria were streaked onto the test plates perpendicular to the fungi. Plates were incubated at 30 °C for 7 days until the fungi had grown over the control plates without bacteria. Antifungal activity was recorded as the width of the zone of growth inhibition between the fungus and the test bacterium.

Competition assay for colonization of wheat root tip

For the competitive colonization of wheat root tip assay the strain *M. phlei* MbP18 was grown overnight in LC medium. One milliliter of an overnight culture

was sedimented by centrifugation (13,000 × g) and the supernatant was discarded. The cells were resuspended in 1 mL of phosphate buffered saline (PBS). Cell suspensions were adjusted to OD_{620} = 0.1, corresponding to a cell density of about 10^8 cells/mL. Cell suspensions of competing strains were mixed equally, and the virtual ratio in the mixture was determined by plating 10^{-5} and 10^{-6} dilutions on LC agar and LC agar supplemented with kanamycin (Km). In the case of competition of the isolate, PCL1285 was used. Sterile wheat seedlings were dipped into the mixed cell suspensions and incubated for 10 min. Inoculated seedlings were sown in sterile glass tubes with sand, as described by Simons et al. (28). The inoculation treatments were set up in a randomized design with 10 replications. Plants were grown for 7 days in plant growth chambers with 16 h of daylight at 24 °C, and then 1 cm of root tip was collected from the plantlets. Bacterial cells were removed from the root tip by vortexing in PBS and plated with a spiral plater on LC medium containing X-gal (40 μg/mL) to distinguish between the strains set in competition.

Plant growth promotion under gnotobiotic condition

The effects of inoculation with *M. phlei* MbP18 on wheat seedlings grown under saline conditions were studied under gnotobiotic conditions. Gnotobiotic experiments were carried out in test tubes (25 mm in diameter, 200 mm in length) containing 60 g of sterilized sand, as described by Simons et al. (28). Saline conditions were obtained by adding 100 mM NaCl to the plant nutrition solution (PNS). Seeds were germinated on 1% water agar in the dark at 28 °C. Bacteria were grown in LC medium for 24 h, and bacterial suspensions were adjusted to an OD at 620 nm of 0.1 (OD_{620} = 0.1), corresponding to a cell density of about 10^8 cells/mL. Germinated seeds were placed in the bacterial suspension with sterile forceps and shaken gently for a few seconds. After standing for 10 min, inoculated seedlings were planted in sterile glass tubes with 1 seed per tube. The seedlings were grown in a growth cabinet with a 16-h light period at 22 °C and an 8-h dark period at 16 °C. After 10 days of growth, the length of shoots and roots and the dry matter of plants were measured.

Statistical analysis

Data were tested for statistical significance using the analysis of variance package included in Microsoft Excel 98, and comparison was done using Student's t-test. Mean comparisons were conducted using a least significant difference (LSD) test (P = 0.05). Standard error and LSD results were calculated.

Results

The strain *M. phlei* MbP18 was tested for competitive wheat root tip colonization against *P. fluorescens* PCL1285, the best-known competitive root tip colonizer (28). *M. phlei* MbP18 showed poor competitive colonization of the wheat rhizosphere (0.8×10^3 CFU/cm root tip) when compared with the reference strain *P. fluorescens* PCL1285 (12.3×10^3 CFU/cm root tip).

The strain produced lipase, amylase, cellulase, and pectinase, and it grew well in the presence of high salt (up to 4%) and at high temperatures (up to 40 °C) (Table; Figure 1). The strain showed antagonistic

Table. Characteristics of *Mycobacterium phlei* MbP18 isolated from wheat grown in salinated soil.

Properties	*M. phlei* MbP18
Production of enzymes	
Lipase	+
Glucanase	-
Protease	-
Pectinase	+
Cellulase	+
Amylase	+
Utilization of	
Glucose	+
Arabinose	+
Xylose	+
Galactose	+
Saccharose	+
Lactose	+
Maltose	+
Mannitol	+
Plant growth promotion	
Control shoot (8.5 cm)	11.2 ± 0.9
Root (12.4 cm)	13.2 ± 0.6
Dry weight (0.017 g)	0.027 ± 0.08
Growth at 40 °C	+

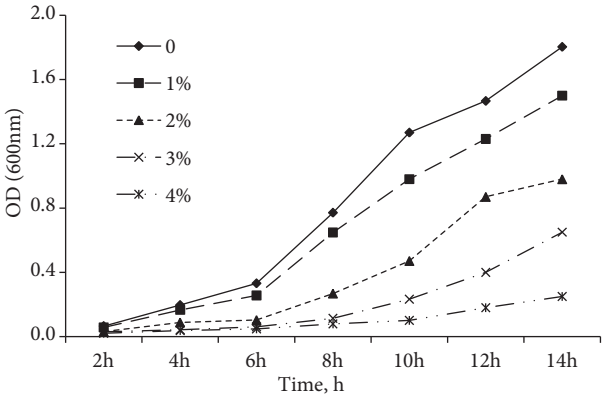

Figure 1. Effects of NaCl (0%, 1%, 2%, 3%, and 4%) on the growth of *Mycobacterium phlei* MbP18 after 2, 4, 6, 8, 10, 12, and 14 h. Growth was measured as OD at 600 nm.

activity toward the phytopathogenic fungi *F. oxysporum* f. sp. *radicis-lycopersici*, *G. graminis* pv. *tritici*, *A. alternate*, and *B. cinerea*.

The patterns of acid production from carbohydrates produced by *M. phlei* MbP18 showed that it produced acid from glucose, arabinose, xylose, glycose, galactose, saccharose, lactose, maltose, and mannitol (Table).

IAA production was tested in the absence and presence of 100 µg/mL of the auxin precursor tryptophan, and results obtained from 4-day-old cultures showed that *M. phlei* MbP18 produced IAA. The presence of tryptophan strongly stimulated IAA production by the strain. The salinity did not strongly inhibit the IAA-producing ability of the bacterial strain (Figure 2).

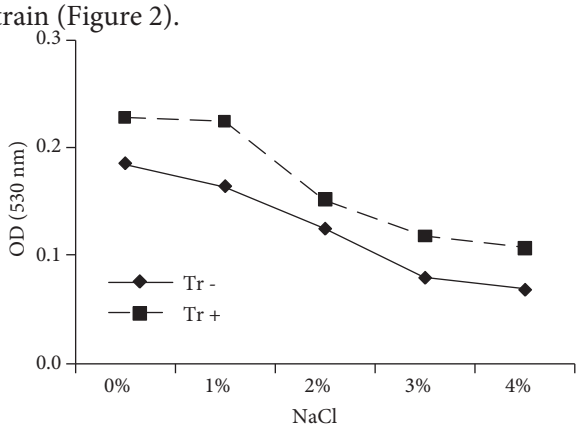

Figure 2. The production of IAA by *Mycobacterium phlei* MbP18 (bacteria were grown for 4 days at 28 °C in the absence and presence of 100 µg/mL of the auxin precursor tryptophan (Tr).

As for the plant growth-promoting properties of *M. phlei* MbP18, the strain significantly stimulated shoot growth (up to 32%), root growth (up to 6%), and dry matter contents (up to 52%) in wheat grown under saline conditions (Table).

Discussion

Manure is directly used to fertilize nutrient-poor soil, and it is an obvious point source of pathogens. The use of manure may expose humans to enteric pathogens through food, water, or direct contact with crops; it also perpetuates an on-farm cycle of farm animal infection with human pathogenic enteric bacteria (29). Potentially pathogenic species are assumed to colonize and establish in the roots of crops and become enriched through utilization of carbon sources present in the root exudates (30). According to Roberts et al. (31), the rhizosphere attracts not only beneficial bacteria to colonize the roots, but also human pathogens that have evolved to respond to the same signals.

In this study, *M. phlei* MbP18 was able to colonize wheat root. However, the competitive colonization ability of the strain was poor compared with the reference strain.

Similar results were found in a previous study, in which potential human pathogenic strains such as *P. aeruginosa*, *B. cereus*, *Staphylococcus* sp., and *Acinetobacter* sp. showed poor colonization of wheat root (32). In a study published by Morales et al. (33), it was also shown that the survival and colonization of potentially pathogenic human-associated bacteria in the rhizosphere of plants is poor and that their persistence in and colonization of plants may decrease with coinoculation of pathogens with naturally occurring bacteria (34,35).

The biochemical characterization of *M. phlei* MbP18 showed that it produces several enzymes such as lipase, cellulase, pectinase, and amylase. Caballero et al. (36) reported that protease syntheses by bacterial strains have been implicated in their pathogenicity. It is also known that secretion of these enzymes by microbes can sometimes result in the suppression of plant pathogen activities (37). *M. phlei* showed antagonistic activity against pathogenic fungi such as *F. oxysporum* f. sp. *radicis-lycopersici*, *G. graminis* pv. *tritici*, *A. alternate*, and *B. cinerea*.

M. phlei MbP18 was salt-tolerant (up to 4% NaCl) and temperature-resistant (up to 40 °C), and thus the strain was able to survive in the rhizosphere of the plant due to its competitiveness and persistence under saline, arid soil conditions. The high temperature of the soil in arid regions creates conditions that are favorable for bacteria that originate from warm-blooded animals. In earlier reports, *M. phlei* and *M. thermoresistibile* were the only 2 mycobacteria that could grow at temperatures of up to 52 °C (38). *M. phlei* MbP18 was able to utilize a wide range of carbohydrates for growth. This ability is well suited to the rhizosphere, because the strain is able to use a wide variety of carbon sources as nutrients.

The results showed that *M. phlei* MbP18 establishes beneficial interactions with the plant, which lead to stimulation of plant growth. In previous studies, the strain also increased maize, pea, and cotton growth, as well as nutrient uptake in arid soils (39). Dell'Amico et al. (40) reported that inoculation of canola (*Brassica napus*) with *Mycobacterium* sp. ACC14 promoted plant growth. The bacterial strain *M. phlei* MbP18 was able to produce the IAA that has been implicated in the stimulation of plant growth (41).

In conclusion, the results of this study indicated that *M. phlei* has the potential to produce different biological active compounds, such as cell wall-degrading enzymes and IAA. In addition, the strain utilizes a wide range of carbohydrates as carbon and energy sources. The strain may positively affect plant growth and has the ability to survive under ecologically stressed conditions, including hot summer temperatures and saline and nitrogen-deficient soils. However, its competitive colonization abilities in the wheat rhizosphere are poor. In light of this observation, efforts should be made to manage the rhizosphere in such a way that it becomes more resistant to colonization by pathogens. This will help remove mycobacteria from habitats in which humans or animals can be exposed.

Acknowledgments

This study was supported by the UNESCO/L'Oreal Fellowship for Women in Science. The author would like to thank Zulfiya Kucharova for technical assistance.

Corresponding author:

Dilfuza EGAMBERDIEVA

Department of Biotechnology and Microbiology

Faculty of Biology and Soil Sciences

National University of Uzbekistan

100174 Vuzgorodok, Tashkent - UZBEKISTAN

E-mail: egamberdieva@yahoo.com

References

1. Anderson GL, Kenney SJ, Millner PD et al. Shedding of foodborne pathogens by *Caenorhabditis elegans* in compost-amended and unamended soil. Food Microb 23: 146-53, 2006.
2. Gagliardi JV, Karns JS. Persistence of *Escherichia coli* O157:H7 in soil and on plant roots. Env Microb 4: 89-96, 2002.
3. Warriner K, Ibrahim F, Dickinson M et al. Interaction of *Escherichia coli* with growing salad spinach plants. J Food Prot 66: 1790-7, 2003.
4. Falkinham JO. Epidemiology of infection by nontuberculous mycobacteria. Clin Microb Rev 9: 177-215, 1996.
5. Primm TP, Luceron CA, Falkinham JO 3rd. Health impacts of environmental mycobacteria. Clin Microb Rev 17: 96-108, 2004.
6. Kirschner RAJ, Parker BC, Falkinham JO. Epidemiology of infection by nontuberculous mycobacteria. *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum* in acid, brown-water swamps of the southeastern United States and their association with environmental variables. Amer Rev Resp Dis 145: 271-5, 1992.
7. Laukkanen H, Soini H, Kontunen-Soppela S et al. A mycobacterium isolated from tissue cultures of mature *Pinus sylvestris* interferes with growth of Scots pine seedlings. Tree Physiology 20: 915-20, 2000.
8. Kiewitz C, Tuemmler B. Sequence diversity of *Pseudomonas aeruginosa*: impact on population structure and genome evolution. J Bact 182: 3125-35, 2000.

9. Finnan S, Morrissey JP, O'Gara F et al. Genome diversity of *Pseudomonas aeruginosa* isolates from cystic fibrosis patients and the hospital environment. J Clin Microb 42: 5783-92, 2004.
10. Madhaiyan M, Poonguzhali S, Lee JS et al. *Enterobacter arachidis* sp. nov., a plant-growth-promoting diazotrophic bacterium isolated from rhizosphere soil of groundnut. Int J Sys Evol Microb 60: 1559-64, 2010.
11. Aguilar JL, Sanchez EE, Carrillo C et al. Septic arthritis due to *Mycobacterium phlei* presenting as infantile Reiter's syndrome. J Rheumatol 16: 1377-8, 1989.
12. Jang SS, Hirsh DC. Rapidly growing members of the genus *Mycobacterium* affecting dogs and cats. J Amer Anim Hosp Ass 3: 217-20, 2002.
13. Pavlik I, Dvorska L, Matlova L et al. Mycobacterial infections in cattle in the Czech Republic during 1990-1999. Veterinarni Medicina 47: 241-50, 2002.
14. Niva M, Harnesmaa A, Haahtela K et al. Actinobacterial communities of boreal forest soil and lake water are rich in mycobacteria. Bor Env Res, 11: 45-53, 2006.
15. Covert TC, Rodgers MR, Reyes AL et al. Occurrence of nontuberculous mycobacteria in environmental samples. App Envir Microb 65: 2492-6, 1999.
16. Kubalek I, Mysak J. The prevalence of environmental mycobacteria in drinking water supply systems in a demarcated region in Czech Republic, in the period 1984-1989. Eur J Epid 12: 471-4, 1996.
17. Tyler HL, Triplett EW. Plants as a habitat for beneficial and/or human pathogenic bacteria. Ann Rev Phyt 46: 53-73, 2008.
18. Egamberdiyeva D, Hoflich G. Effect of plant growth-promoting bacteria on growth and nutrient uptake of cotton and pea in a semi-arid region of Uzbekistan. J Arid Envir 56: 293-301, 2004.
19. de Weert S, Dekkers LC, Kuiper I. Generation of enhanced competitive root-tip-colonizing *Pseudomonas* bacteria through accelerated evolution. J Bacter 186: 3153-9, 2004.
20. Castric PA. Hydrogen cyanide, a secondary metabolite of *Pseudomonas aeruginosa*. Can J Microb 21: 613-8, 1975.
21. Howe TG, Ward JM. The utilization of Tween 80 as carbon source by *Pseudomonas*. J Gen Microb 92: 234-5, 1976.
22. Brown MR, Foster JH. A simple diagnostic milk medium for *Pseudomonas aeruginosa*. J Clin Path 23: 172-7, 1970.
23. Walsh GA, Murphy RA, Killeen GF et al. Technical note: detection and quantification of supplemental fungal β-gluconase activity in animal feed. J Anim Sci 73: 1074-6, 1995.
24. Hankin L, Anagnostakis SL. Solid media containing carboxymethylcellulose to detect C_x cellulase activity of microorganisms. J Gen Microb 98: 109-15, 1977.
25. Smibert RM, Krieg NR. Phenotypic characterization. In: Gerharelt P, Murray RGE, Wood WA et al. eds. Methods for General and Molecular Bacteriology. American Society of Microbiology; 1994: pp. 607-54.
26. Cowan ST. Cowan and Steel's Manual for the Identification of Medical Bacteria, 2nd ed. Cambridge University Press. Cambridge; 1974.
27. Bano N, Musarrat J. Characterization of a new *Pseudomonas aeruginosa* strain NJ-15 as a potential biocontrol agent. Cur Microb 46: 324-8, 2003.
28. Simons M, van der Bij AJ, Brand I et al. Gnotobiotic system for studying rhizosphere colonization by plant growth-promoting *Pseudomonas* bacteria. Mol Plant Microbe Int 9: 600-7, 1996.
29. Lugtenberg BJJ, Dekkers L, Bloemberg GV. Molecular determinants of rhizosphere colonization by *Pseudomanas*. An Rev Phyt 39: 461-90, 2001.
30. Gilbert GS, Parke JL, Clayton MK et al. Effects of an introduced bacterium on bacterial communities on roots. Ecol 74: 840-54, 1993.
31. Roberts DP, Dery PD, Yucel I et al. Importance of *pfkA* for rapid growth of *Enterobacter cloacae* during colonization of crop seed. Appl Env Microb 66: 87-91, 2000.
32. Egamberdieva D, Kucharova Z. Selection for root colonising bacteria stimulating wheat growth in saline soils. Biol Fertil Soils 45: 561-73, 2009.
33. Morales A, Garland JL, Lim DV. Survival of potentially pathogenic human-associated bacteria in the rhizosphere of hydroponically grown wheat. FEMS Microb Ecol 20: 155-62, 1996.
34. Cooley MB, Pathirana S, Wu HJ et al. Members of the *Arabidopsis HRT/RPP8* family of resistance genes confer resistance to both viral and oomycete pathogens. Plant Cell 12: 663-76, 2003.
35. Jablasone J, Warriner K, Griffiths M. Interactions of *Escherichia coli* O157:H7, *Salmonella typhimurium* and *Listeria monocytogenes* plants cultivated in a gnotobiotic system. Int J Food Microb 99: 7-18, 2005.
36. Caballero AR, Moreau JM, Engel LS et al. *Pseudomonas aeruginosa* protease IV enzyme assays and comparison to other *Pseudomonas* proteases. Anal Bioch 290: 330-37, 2001.
37. Bull CT, Shetty KG, Subbarao KV. Interactions between Myxobacteria, plant pathogenic fungi, and biocontrol agents. Plant Dis 86: 889-96, 2002.
38. Ratledge C. Nutrition, growth and metabolism. In: Ratledge C, Stanford L. eds. Biology of the Mycobacteria. Academic Press; 1982: pp. 186-212.
39. Egamberdiyeva D, Hoflich G. Influence of growth promoting bacteria on the growth of wheat at different soils and temperatures. Soil Biol Biochem 35: 973-8, 2003.
40. Dell'Amico E, Cavalca L, Andreoni V. Improvement of *Brassica napus* growth under cadmium stress by cadmium-resistant rhizobacteria. Soil Biol Biochem 40: 74-84, 2008.
41. Frankenberger JWT, Arshad M. Microbial synthesis of auxins. In: Frankenberger WT, Arshad M. eds. Phytohormones in Soils. Marcel Dekker; 1995: pp. 35-71.