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INFLUENCE OF DIEBACK DISEASE ON SOIL CHEMISTRY AND BACTERIAL DIVERSITY IN THE RHIZOSPHERE OF MANGO PLANTS

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Abstract

Influence of dieback disease on the soil chemistry and the morphological, biochemical, and molecular characteristics of bacteria isolated from the rhizosphere of mango plants was studied. The soil samples collected from the rhizosphere of the healthy and dieback-affected mango plants were analyzed for their physicochemical parameters and biochemical characteristics of bacterial isolates. The dieback disease resulted in a significant decrease in pH, water holding capacity, organic matter, total nitrogen content, available phosphorus content, and available potassium content. The number of bacterial isolates showing oxidase production and lactose and sucrose fermentation was increased while the number of those showing urease and hydrogen sulphide production was decreased in the rhizosphere of diseased plants. The genus *Bacillus* showed maximum prevalence (97%) among the identified genera of rhizobacteria. Eight bacterial species were found to be absent and eight new species appeared in the rhizosphere of diseased plants. The study would be a significant contribution to the literature regarding the dieback mango disease and its relationship with soil composition and its microflora.

Key words: bacterial population, dieback mango disease, phylogenetic analysis, rhizobacteria, soil parameters, 16S ribosomal RNA gene

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Introduction. The rhizosphere of plants provides a diverse atmosphere for the interaction and growth of microbial communities [1]. The plant root exudates and nutritional composition of the soil significantly affect rhizospheric soil composition and microbial growth and reproduction. The interactions between the plants, microorganisms, and the prevailing environmental factors may be beneficial or harmful to the plants [2]. The nutrient deficiency and some other factors such as drought, lack of shade, drying winds, and microbial infections make plants more vulnerable to the attack of fungal pathogens resulting in a quick decline of plants also known as dieback disease [3].

Mango, botanically known as *Mangifera indica* L., is under serious threat of quick decline or dieback disease in Pakistan and other mango producing countries. This disease is known to be caused by the fungal pathogens *Botryosphaeria ribis, Lasiodiplodia theobromae,* and *Ceratocystis fimbriata* [4]. Previously, studies have been reported on the detection of dieback disease in mango, its effects on the biochemical and phytochemical composition of mango stem bark [5].

However, limited information was found on the impact of dieback disease on bacterial population and soil parameters of mango plants. Therefore, the present study was designed to evaluate the effect of dieback disease on the physicochemical parameters and bacterial population in the rhizosphere of mango plants in Southern Punjab, Pakistan.

Materials and methods. Soil samples were collected from the rhizosphere of healthy ($n = 8$) and dieback-affected mango plants ($n = 8$) at a depth of 20 cm followed by analysis of soil parameters, isolation of rhizobacteria. The pH of the soil-water suspension (1:1) and electrical conductivity (EC) of the aqueous extracts were determined by the previously reported methods using a pH meter (Jenway 3540, Fisher Scientific, Leicestershire, UK) and EC meter (Chemtrix 70, Chemtrix Inc., Hillsboro, USA), respectively [6]. The water holding capacity (WHC), organic matter (OM) total nitrogen content (TNC), available phosphorus content (APC), and available potassium content (AKC) of the soil were determined by standard protocols [7–9].

The suspension of rhizosphere soil sample (10 g) in sterile ringer solution (90 ml) was homogenized at 300 × g for 10 min using a shaker-incubator (Labtech LSI-3016A, Namyangju-City, Kyonggi-Do, Korea). The rhizobacteria were isolated from the solution by serial dilution (10^{-1}–10^{-6}) technique, cultured, and purified using the method described earlier [10]. The purified bacterial colonies were preserved in 50:50 w/w nutrient-glycerol broths at −70 °C. The morphological features of different colonies were examined under a light microscope (Olympus CH-2, Microscope Central, Feasterville, Pennsylvania) while the biochemical tests were performed following the previously reported standard protocols [11].

The DNA was extracted from the bacterial isolates grown in LB broth in the shaker incubator at 300 × g and 37 °C, for 18–24 h using a DNA extraction kit (Vivantis Technologies, Selangor Darul Ehsan, Malaysia). The amplification of

bacterial-specific 16S ribosomal RNA gene was performed by polymerase chain reaction (PCR) in a thermal cycler (Sure Cycler 8800, Agilent Technologies, Inc., Santa Clara, USA) using universal primers derived from the *Escherichia coli* 16S rRNA sequence using the protocols described earlier [12]. The sequence analysis of the purified PCR products was performed by Macrogen Inc. (Korea) using Sanger sequencing techniques. The sequences of the 16S rRNA gene were aligned manually and compared with the new homologous 16S ribosomal RNA sequences available on the basic local alignment search tool (BLAST) in the National Center for Biotechnology Information (NCBI) database [13]. The 16S rRNA gene sequence was deposited at the Gene bank and is accessible under the accession numbers MK467575 to MK467574S.

The evolutionary history of the bacterial isolates was determined by the Neighbour-Joining method [14]. The computational analysis of the evolutionary distances was performed as the number of base substitutions per site using the Maximum Composite Likelihood method [15]. The evolutionary analyses were conducted in molecular evolutionary genetic analysis (MEGA X) software.

Statistical analysis. The results of the physicochemical parameters and bacterial population of rhizosphere soil were presented as mean ± SD of ten replicates and the means were separated by Tukey's multiple range tests using one-way analysis of variance (ANOVA) in SPSS statistical software.

Results. The texture of soil samples collected from the rhizosphere of both healthy and dieback-affected mango plants was found to be silty clay loam. The pH, EC, and WHC of rhizosphere soil obtained from the healthy and dieback-affected mango plants were found to be 6.12 ± 0.74 and 7.72 ± 0.71, 1.51 ± 1.14 and 2.12 ± 0.36 µS cm^{-1}, and 21.41 ± 4.20 and 41.3 ± 4.72%, respectively. Organic matter, TNC, APC, and AKC of the rhizosphere of the healthy and dieback-affected mango plants were found to be 1.19 ± 0.37 and 2.02 ± 0.24%, 0.11 ± 0.09 and 0.23 ± 0.08 mg kg^{-1}, 0.28 ± 0.08 and 0.07 ± 0.02 mg kg^{-1}, and 4.50 ± 2.20 and 13.4 ± 2.72 mg kg^{-1}, respectively (Table 1). The bacterial population in the rhizosphere of the healthy and dieback-affected mango plants was found to be 29.98 ± 6.59 and 33.10 ± 3.80 10^{-4}×CFU/g soil, respectively. The analyzed physicochemical parameters except for EC of the rhizosphere of healthy and dieback-affected mango plants were statistically different ($p < 0.05$) with relatively higher values of pH, WHC, OM, TNC, APC, AKC in the rhizosphere of healthy plants.

Morphological and biochemical characteristics of bacteria. The majority of the bacterial colonies in the rhizosphere of healthy and dieback-affected plants were observed as broad (46–47%) and opaque (100%) with irregular colony shape (26–56%), smooth texture, and entire margins (83–100%) and *Bacilli* cell shape (78–97%) and cream colour (44–46%) (Table 2). The number of bacterial isolates with orange-brown colour and those possessing gram-negative staining and spore-forming abilities was increased in the rhizosphere of dieback-affected

Table 1

Physiochemical parameters and bacterial population in the rhizosphere of healthy and dieback mango plants

Parameters	Healthy mango plants*	Dieback-affected mango plants	Sum of Squares	Mean Square	F-value	p-value
pH	7.72 ± 0.71	6.12 ± 0.74	10.24	10.24	19.50	0.001**
Electrical conductivity (µS cm^{-1})	2.12 ± 0.36	1.51 ± 1.14	1.50	1.50	2.09	0.170
Water holding capacity (%)	41.3 ± 4.72	21.41 ± 4.20	1586.03	1586.03	79.27	0.000
Organic matter (%)	2.02 ± 0.24	1.19 ± 0.37	2.73	2.73	28.2	0.000
Total nitrogen content (%)	0.23 ± 0.08	0.11 ± 0.05	0.048	0.048	6.45	0.024
Available phosphorus content (mg kg^{-1})	0.28 ± 0.088	0.07 ± 0.02	0.170	0.170	26.11	0.000
Available potassium content (mg kg^{-1})	13.4 ± 2.72	4.50 ± 2.20	315.06	315.06	51.36	0.000
Bacterial population (10^{-4} CFU*/g)	33.1 ± 3.80	29.98 ± 6.59	39.69	39.69	1.37	0.261

*CFU: Colony forming units, **The values are significantly different at a 95% confidence level ($p \leq 0.05$) using Duncan's multiple range tests

plants. The rhizosphere of healthy mango plants also showed the presence of *Klebsiella, Paraclostridium, Enterococcus*,and *Acinetobacter* morphologies while that of dieback-affected plants showed *Pseudomonas, Staphylococcus, Enterobacter,* and *Pelagirhhobacter* morphologies.

The majority of the bacterial isolates obtained from the rhizosphere of healthy and dieback-affected mango plants showed the production of catalase (97 and 100%), oxidase (40 and 60%), urease (31 and 26%), lactose (83 and 89%), sucrose (20 and 31%), hydrogen disulphide (9 and 3%) and indole (40 and 43%) and fermentation of glucose (94 and 97%), citrate utilization (86 and 89%) and nitrate reduction (94 and 95%), respectively. The number of oxidase producing and lactose and sucrose fermenting bacteria was significantly increased while that of urease and hydrogen sulphide producing bacteria was decreased in the rhizosphere of dieback-affected plants (Fig. 1a–c).

Bacterial identification by 16S rRNA gene sequences. The 16S rRNA sequences showed 36 species of bacteria isolated from the rhizosphere of healthy plants and 35 species from that of dieback-affected plants. The sequences of the 16S rRNA gene of the bacteria isolated from the rhizosphere of healthy plants were split into four monophyletic groups (MPG1-MPG4) along with the presence of *Klebsiella pneumoniae.* The MPG4 was found to be the largest clade consist-

T a b l e 2

Morphological characteristics of bacterial isolates obtained from rhizosphere of healthy and dieback mango plants

		Healthy plants* ($n = 36$)		Dieback-affected plants ($n = 35$)	
Tested parameter	Characteristics	Number of isolates (n)	Percentage	Number of isolates (n)	Percentage
Colony size	Large	17	47	16	46
	Medium	12	33	12	33
	Small	17	47	7	20
Colony shape	Circular	16	44	14	40
	Irregular	20	56	9	26
Colour	Cream	16	44	16	46
	Blue green	5	14	3	9
	Light yellow	6	17	6	17
	Pink	3	8	3	9
	Orange brown	2	6	4	11
	Yellowish	4	11	3	9
Opacity	Opaque	36	100	35	100
Texture	Smooth	36	100	29	83
Margin	Entire	36	100	35	100
Gram staining	Gram +ive	29	81	26	74
	Gram –ive	7	19	9	26
Cell morphology	Bacilli	28	78	29	97
	Klebsiella	3	8		
	Microbacterium	1	2	1	3
	Paraclostridium	1	2		
	Enterococcus	1	2		
	Acinetobacter	1	2		
	Pseudomonas			1	3
	Enterobacter			1	3
	Staphylococcus			1	3
	Pelagirhhobacter			1	3
Spore formation	+ive	26	72	29	97

ing of 12 species including three strains of *Bacillus haynesii,* two strains of each of *Bacillus mojevinsis, Bacillus amyloliquefaciens,* and *Bacillus subtilis,* and one strain of each of *Bacillus velezensis, Bacillus tequilensis,* and *Bacillus australimeris.* MPG3 was the second largest group consisting of 10 species including five strains of *Bacillus paramycoides,* three strains of *Bacillus tropicus,* and one strain of each of *Staphylococcus haemolyticus* and *Bacillus cereus.* MPG2 showed the presence of two strains of *Bacillus tropicus* and one strain of each of *Enterococcus faecalis, Bacillus oceandisediminis,* and *Paraclostridium benzoelyticum.* MPG1 consisted of three strains of *Klebsiella aerogenes* and one strain of each of *Microbacterium esteraromaticum, Acinetobacter pittii,* and *Enterobacter ludwigii* (Fig. 2a).

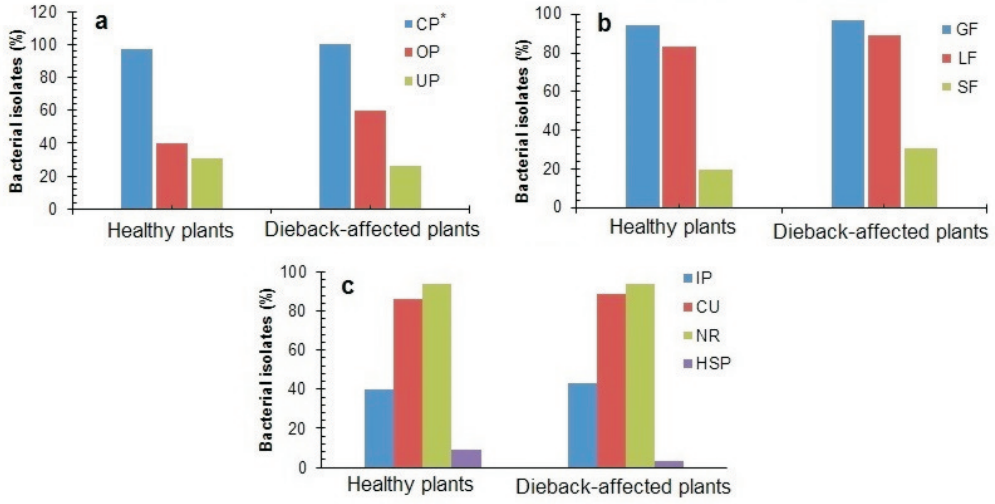

Fig. 1. Biochemical characteristics of the bacteria isolated from the rhizosphere of healthy and dieback-affected mango plants: a) Enzyme production; b) Sugar fermentation; c) Other metabolic activities. *CP: Catalase production, OP: Oxidase production, UP: Urease production, GF: Glucose fermentation, LF: Lactose fermentation, SF: Sucrose fermentation, IP: Indole production, CU: Citrate utilization, NR: Nitrate reduction, and HSP: Hydrogen sulphide production

The bacterial isolates obtained from the rhizosphere of dieback-affected plants were split into five monophyletic groups (MPG1-MPG5) along with the presence of *Bacillus pseudomycoides, Staphylococcus hominis, Pelagirhabdus alkalitolerans,* and *Microbacterium esteraromaticum* (Fig. 2b). MPG1 clade consisted of *Pseudomonas plecoglossicida, Pseudomonas aeruginosa*, and *Enterobacter ausburie.* MPG2 was the largest group consisting of 13 *Bacillus* strains including three strains of each of *Bacillus haynesii* and *Bacillus subtilis,* two strains of each of *Bacillus safensis* and *Bacillus amyloliquefaciens,* and one strain of each of *Bacillus firmus, Bacillus oceanisediminis,* and *Bacillus mojavensis.* MPG3 clade consisted of one strain *of each of Bacillus paramycoides, Bacillus tropicus,* and *Bacillus bingmayongensis* while MPG4 clade consisted of one strain of *Bacillus tropicus* and two strains of *Bacillus paramycoides.* MPG5 was the second largest clade consisting of eight species including four strains of each of *Bacillus tropicus* and *Bacillus paramycoides.*

The phylogenetic analysis identified the genus *Bacillus* as the most prevalent genus along with some other genera of bacteria with a relatively higher percentage in the rhizosphere of dieback-affected plants (97%) than that of healthy plants (78%). The Bacillus clade of the bacterial isolates obtained from the rhizosphere of healthy plants was subdivided into *Bacillus Paramycoides* (13.8%), *Bacillus tropicus* (13.8%), *Bacillus haynesii* (8.3%), *Bacillus subtilis* (5.55%), *Bacillus mojaven-*

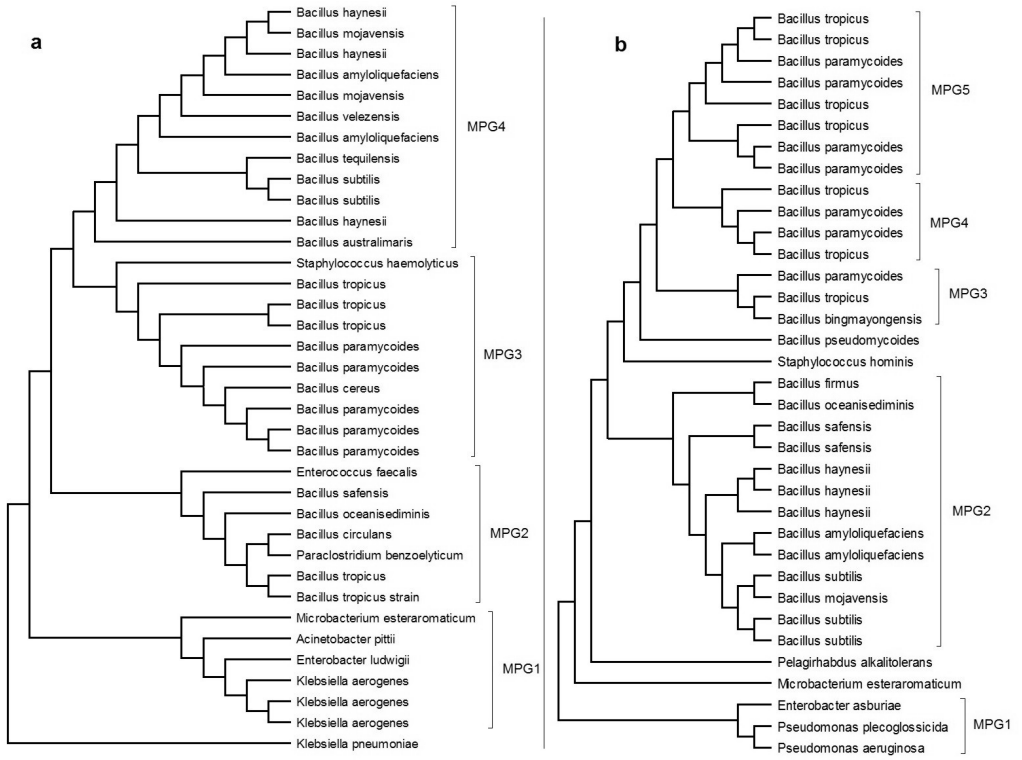

Fig. 2. Phylogenetic tree of 16S rRNA gene extracted from the bacteria isolated from the rhizosphere of a) Healthy and b) Dieback affected mango plants MPG: Monophyletic groups

sis (5.55%), *Bacillus amyloliquefaciens* (5.55%), and *Klebsiella aerogenes* (8.3%) clades. The *Bacillus* clade of the bacterial isolates obtained from the rhizosphere of dieback-affected plants was also subdivided into *Bacillus tropicus* (20%), *Bacillus paramycoides* (20%), *Bacillus haynesii* (8.57%), *Bacillus mojavensis* (5.55%), *Bacillus subtilis* (8.57%), *Bacillus amyloliquefaciens* (5.71%), *Bacillus safensis* (5.71%), and *Klebsiella aerogenes* (8.3%) clades.

Discussion. The observed variation in physicochemical parameters of the rhizosphere of healthy and dieback-affected plants indicated that dieback disease has significantly affected the physical properties and nutritional composition of the soil. However, the variance in physical properties and nutritional composition of soil did not affect the overall population of bacteria in the rhizosphere of healthy and dieback-affected plants. The decrease in the pH, WHC, OM TNC, APC, and AKC in the rhizosphere of dieback-affected plants as compared to that of healthy plants may be attributed to the altered root exudation affecting the physical properties and solubility and availability of minerals and nutrients [16].

The observed variation in the morphology and biochemical parameters of the bacteria isolated from healthy and dieback-affected plant rhizospheres may be as-

sociated with the potential effects of dieback disease on the microbial population present in the rhizosphere of mango plants. The presence of different bacterial strains in the same clade suggested a close evolutionary association among these strains. The long-term survival of *Bacillus* species in the soil may be attributed to the formation of resistant-endospore and the production of antibiotics [17]. The disappearance of *Bacillus velezensis, Staphylococcus haemolyticus, Enterococcus faecalis, Paraclostridium benzoelyticum, Acinetobacter pittii, Enterobacter ludwigii, Klebsiella aerogenes,* and *Klebsiella pneumoniae*, and the appearance of some new species including *Bacillus bingmayongensis, Bacillus pseudomycoides, Staphylococcus hominis, Bacillus firmus, Pelagirhabdus alkalitolerans, Enterobacter ausburie, Pseudomonas plecoglossicida,* and *Pseudomonas aeruginosa* in the rhizosphere of dieback affected plants may be attributed to the potential effect of dieback disease in the rhizoflora of mango plants. The disappearance of the native bacterial species and the appearance of new species in the rhizosphere of dieback-affected mango plants may be correlated with the change in their root exudates and soil chemistry [18].

In light of the above discussion, it has been concluded that the dieback disease of mango plants significantly affects the soil chemistry and bacterial population. The dieback-induced emergence of new species of rhizoflora may in turn affect the susceptibility of the mango plant. The dieback disease-induced changes in soil chemistry and bacterial diversity may also have ecological effects on the growth and susceptibility of nearby plants. The study would be a valuable contribution to the literature regarding the consequences of dieback disease on the soil chemistry and bacterial diversity in the rhizosphere of mango plants throughout the world.

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REFERENCES

[1] Van Veen J. A., J. A. W. Morgan, J. M. Whipps (2000) Methodological approaches to the study of rhizosphere carbon flow and microbial population dynamics. The Rhizosphere: Biochemistry and Organic Substance at the Soil-Plant Interface: Biochemistry and Organic Substance at the Soil-Plant Interface, CRC Press, 373 pp.

[2] Bais H. P., T. L. Weir, L. G. Perry, S. Gilroy, J. M. Vivanco (2006) The role of root exudates in rhizosphere interactions with plants and other organisms, Annual Reviews in Plant Biology, **57**, 233–266.

[3] Agrios G. N. (2005) Plant pathology, 5th edn, San Diego, Elsevier Academic Press.

[4] Masood A., S. Saeed, S. F. Silveira, C. N. Akem, N. Hussain, M. Farooq (2011) Quick decline of mango in Pakistan: survey and pathogenicity of fungi isolated from mango tree and bark beetle, Pakistan Journal of Botany, **43**, 1793–1798.

[5] Saeed A., M. A. Shad, H. Nawaz, M. N. Shafqat, Z. Muneer et al. (2016) Quick decline disease disturbs the levels of important phytochemicals and minerals in mango trees (*Mangifera indica*), International Journal of Agronomy, 2016, 1–7.
[6] Mekeague J. A. (1978) Manual of soil sampling and methods of analysis, 2nd edn, Canadian Society of Soil Science, 907 pp.
[7] Buresh R. J., E. R. Austin, E. T. Craswell (1982) Analytical methods in 15 N research, Fertilizer Research, **3**, 37–62.
[8] Walkley A., I. A. Black (1934) An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method, Soil Science, **37**, 29–38.
[9] Qian P., J. J. Schoenaru, R. E. Karamanos (1994) Simultaneous extraction of available phosphorus and potassium with a new soil test: A modification of Kelowna extraction, Communications in Soil Science and Plant Analysis, **25**, 627–635.
[10] Ibrahim M., F. Ahmad, H. Nawaz, M. Aslam, M. A. Shad (2020) Studies on correlations between soil chemistry and bacterial population in rhizosphere of Bt and non-Bt cotton and characterization of rhizobacteria, Journal of Taibah University for Science, **14**, 1463–1474.
[11] Sule I. O., T. O. Agbabiaka, B. K. Saliu, A. B. Bello, A. B. Adeboye (2017) Bacteriological and physicochemical assessment of selected brands of bottled water in Ilorin, Nigeria, Al-Hikmah Journal of Pure and Applied Sciences, **4**, 15–22.
[12] Porteous L. A., J. L. Armstrong (1993) A simple mini-method to extract DNA directly from soil for use with polymerase chain reaction amplification, Current Microbiology, **27**, 115–118.
[13] Altschul S. F., T. L. Madden, A. A, Schäffer, J. Zhang, Z. Zhang et al. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Research, **25**, 3389–3402.
[14] Saitou N., M. Nei (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees, Molecular Biology and Evolution, **4**, 406–425.
[15] Tamura K., M. Nei, S. Kumar (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method, Proceedings of the National Academy of Sciences, **101**, 11030–11035.
[16] Huang G., G. Guo, S. Yao, N. Zhang, H. Hu (2016) Organic acids, amino acids compositions in the root exudates and Cu-accumulation in castor (*Ricinus communis* L.) under Cu stress, International Journal of Phytoremediation, **18**, 33–40.
[17] Amin M., Z. Rakhisi, A. Z. Ahmady (2015) Isolation and identification of Bacillus species from soil and evaluation of their antibacterial properties, Avicenna Journal of Clinical Microbiology and Infection, **2**, e23233.
[18] Schroth M. N., D. C. Hildebrand (1964) Influence of plant exudates on root-infecting fungi, Annual Review of Phytopathology, **2**, 101–132.

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