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Analysis of populations and physiological characterization of microorganisms in rhizospheres of plants with antagonistic properties to phytopathogenic nematodes

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Abstract

Populations of rhizosphere microflora of plants which have demonstrated an antagonism toward phytopathogenic nematodes, including velvet bean (*Mucuna deeringiana*), castor bean (*Ricinus communis*), sword bean (*Cannavalia ensiformis*), and Abruzzi rye (*Secale cereale*), were compared to the rhizosphere microflora of soybean. Population densities of total bacteria were significantly lower for young Abruzzi rye, mature velvet bean, and mature castor bean, and fungi from mature velvet bean than for soybean. Population densities of spore-forming bacilli were significantly higher for Abruzzi rye than for soybean. Population densities of coryneform bacteria for mature sword bean and velvet bean were significantly higher than for soybean. All seedling test plants supported significantly higher population densities of chitinolytic fungi than soybean. On mature plants, chitinolytic bacteria were significantly higher on all test plants except velvet bean. Populations of endophytic root bacteria for three of the four test plants were significantly higher than for soybean. Fifty randomly selected bacterial strains from seedlings and mature plants of soybean and each test plant were characterized for various physiological traits associated with rhizosphere competence, including chitinolytic activity, gelatin hydrolysis, production of hydrogen cyanide, starch hydrolysis, phenol oxidation, siderophore production, and production of antifungal compounds (inhibition of *Pythium ultimum* and/or *Rhizoctonia solani*). There was a strong trend to increased frequency in each of the physiological tests with bacteria from test plants in comparison to those from soybean. The frequency of starch hydrolysis was up to 24 times greater for strains from test plants than for soybean strains, and siderophore production was up to 22 times more frequent for test plants. These results demonstrate that, compared to soybean, plants with properties antagonistic to phytopathogenic nematodes have a distinct rhizosphere microflora.

Introduction

Several nematicides have been banned or are currently under review because of environmental concerns including toxicity and groundwater contamination, leaving gaps in control strategies for phytopathogenic nematodes. This situation has increased interest in developing alternatives to chemical nematicides, i.e. biological control.

One biological control strategy which is currently the subject of intensive research efforts with phytopathogenic fungi is the use of root-associated bacteria as seed treatments (Kloepper, 1991; Weller, 1988). Recent work suggests

that some rhizosphere bacteria may also provide protection from phytopathogenic nematodes. Sikora (1988) isolated rhizosphere bacteria from crop roots and tested them for control of nematodes in field soils; 7.2% of strains from sugarbeet reduced infection by *Heterodera schachtii*, 9% from potato reduced infection by *Globodera pallida*, 9.8% from cotton reduced infection by *Meloidogyne incognita*, and 8.3% from peanut reduced infection by *M. arenaria*. In a subsequent study (Oostendorp and Sikora, 1989), *Pseudomonas fluorescens* strain A-59, which was effective against *H. schachtii* in greenhouse assays, induced a significant 75% reduction in infection and resulted in a significant yield increase. McInnis et al. (1990) reported that a strain of *P. aureofaciens* isolated from suppressive soil inhibited infection of peach seedlings by the ring nematode, *Criconemella xenoplax*.

The starting point for studies of biological control with rhizosphere bacteria is often the isolation and characterization of bacteria from root environments where disease is lacking, such as suppressive soils, or from healthy plants in diseased fields (Cook and Baker, 1983). One possible source of biological control agents for phytopathogenic nematodes is the rhizosphere of plants which demonstrate antagonistic properties to nematodes (Mashkoor et al., 1990). Castor bean (*Ricinus communis*) exudates are inhibitory to nematodes (Lear, 1959), and in a previous study in Alabama (Rodríguez-Kábana et al., 1988), cropping of castor bean significantly reduced the population of *Meloidogyne arenaria* juveniles on a subsequent peanut crop. Velvet bean (*Mucuna deeringiana*) plants and extracts reduced the gall index and infection of tomato by *M. incognita* (Vincente and Acosta, 1987). In a separate study (Rodríguez–Kábana et al., 1990), jack bean (*Cannavalia ensiformis*), velvet bean, and showy crotalaria (*Crotalaria spectabilis*) failed to support development of *M. arenaria* and *Heterodera glycines*. Abruzzi rye (Pedersen and Rodríguez–Kábana, 1991) and sword bean (Grandados Alvarez, 1989) have also been implicated in antagonism toward phytopathogenic nematodes. The precise mechanisms by which these plants are antagonistic to nematodes are unknown. It is possible that part of the mechanism includes stimulation of specific components of the indigenous rhizosphere microflora which are in themselves antagonistic to nematodes. Some plants which are antagonistic to nematodes produce toxins, such as ricin produced by castor bean (Rich et al., 1989). It is likely that these toxins are present in the root zone and that the indigenous rhizosphere microflora of toxin-producing plants is physiologically distinct from the indigenous microflora of nontoxin-producing plants.

The purpose of this study was to gain information on what types of microorganisms inhabit the rhizospheres of plants with antagonistic properties to nematodes. We initiated this project as part of an ongoing effort to select root-colonizing bacteria as biological control agents for phytopathogenic nematodes. Data are presented on total rhizosphere population densities of various microorganisms and the physiological characterization of representative bacteria from each antagonistic plant in comparison to a nonantagonistic soybean control.

Materials and methods

Preparation of treatments for sampling

Two randomized block design experiments were used to grow plants for sampling. Each experiment consisted of the following six treatments, replicated eight times: soybean (cv. 'Davis'), velvet bean, castor bean, sword bean, Abruzzi rye, and a nonplanted soil control. Each replicate consisted of one container with four plants per container. Containers were 2-L plastic bottles with the bottom removed. The bottles were inverted, and glass wool was placed in the opening of the screw-cap end. These containers were wrapped with opaque black plastic and inverted into a polyvinyl chloride support tube. The containers were filled with a 2:1 mixture of field soil and sand. The field soil was a Pacolet fine sandy loam (clayey, kaolinitic, thermic, typic, kahapludults) from the agricultural research farm on the Auburn University campus. The containers were left unplanted or planted with the appropriate seeds, watered, and placed in the greenhouse.

Sampling of roots and soil

One experiment was sampled when seedlings were at the first true leaf stage (8–14 days after planting), and the other was sampled when plants were near maturity (3 months after planting). Sampling for rhizosphere populations was done by removing soil from the containers and taking a total of 0.5–2.0 g fresh weight of roots plus tightly adhering soil, which corresponded to a volume which, when not compressed, would fit into a 40-mL beaker. Roots from each treatment and each of 8 replicates were weighed and placed into 250-mL erlenmeyer flasks eontaining 50 mL 0.02 *M* sterile phosphate buffer (pH = 6.8). Flasks were shaken on an orbital shaker at 150 rpm for 1 hr., and serial 10-fold dilutions were prepared to 10^{-3}. The 10^{-1} and 10^{-3} dilutions were plated onto test media using a spiral plater (Spiral System Instruments, Bethesda, MD).

In the experiment involving mature plants, endophytic populations of bacteria were estimated from the same root samples used to calculate the rhizosphere samples. After preparing the dilutions described above, roots were removed and surface-sterilized by agitating in 1.5% NaClO (30% household bleach) for 10 min. Roots were rinsed six times in sterile water, and 0.1 mL of the final rinse was placed into 5-mL tryptic soy broth (Difco, Detroit, MI) as a check for sterility. Roots were ground in 5 mL sterile phosphate buffer using autoclaved mortars and pestles. Dilutions were prepared to 10^{-2}, and the 10^{0} and 10^{-2} dilutions were spiral-plated onto the test media. Mean populations were calculated only from plants for which the sterility check lacked microbial growth.

Media and microbial enumeration

In the experiment involving seedlings, rhizosphere populations of spore-forming bacilli were determined using the same root samples. After plating the general rhizosphere samples, tubes containing the 10^{0}, 10^{-2}, and 10^{-4} dilutions were placed in a water bath at 80°C for 20 min to kill asporogenous bacteria (Norris et al., 1981). Tubes were cooled to room temperature and spiral-plated onto tryptic soy agar (TSA) (Difco).

Several different media were used to estimate rhizosphere and endophytic microbial populations. Soil extract agar (SEA) (Johnson and Curl, 1972) and 5% tryptic soy agar (5% TSA) (Difco, Detroit, MI) were used for enumerating bacteria which grow better under low-nutrient conditions. Tryptic soy agar (TSA) (Difco) was used as a general-purpose bacterial medium. CNS was used to enumerate coryneform bacteria (Gross and Vidaver, 1978). Ohio agar was used for enumeration of total fungi (Johnson and Curl, 1972). Chitinolytic microorganisms were plated on colloidal chitin agar (Rodríguez-Kábana et al., 1983). Chitinolytic bacteria were enumerated after incubation for 36 h and fungi after 96 h. The experiment with mature plants used TSA, 5% TSA, CNS, SEA, chitin agar, and Ohio agar for external rhizosphere microbial populations and TSA and 5% TSA for endophytic bacterial populations. In the experiment with seedlings 5% TSA, TSA after heat treatment (as described above), CNS, Ohio agar, and chitin agar were used for external rhizosphere microbial populations. Endophytic bacterial populations were not determined for seedlings.

Plates were incubated at 28°C for 24–96 h, and colonies were enumerated using a laser colony counter with Bacterial Enumeration software (Spiral System Instruments). Treatment means were calculated by averaging the Log cfu g^{-1} (colony-forming units/g fresh root weight) for each replicate, since rhizosphere bacteria are distributed in a lognormal pattern (Loper et al., 1984). Treatment means for velvet bean, castor bean, sword bean and Abruzzi rye on each medium were statistically compared to means for soybean by conducting a two-way analysis of variance using SAS software (SAS Institute, Inc., Cary, NC) with the Log cfu g^{-1} values of each replicate.

Physiological characterization of rhizosphere bacteria

Fifty rhizosphere bacterial isolates from each plant in each experiment were transferred at random from 5% TSA plates and were purified on TSA. Isolates were characterized for various

physiological traits which may confer enhanced survival or antagonism toward nematodes. These traits included production of chitinases, gelatin hydrolysis, hydrogen cyanide production (experiment with seedlings only), starch hydrolysis, oxidation of phenol, production of antifungal compounds, and siderophore production.

Chitinase production was determined by the presence of clear zones surrounding 72-h-old colonies on collodial chitin agar (Rodríguez-Kábana et al., 1983).

Gelatin hydrolysis was determined by inoculating 3.0 mL gelatin medium (Difco) in tubes with bacteria, incubating 10 days at 28°C, and placing tubes at 4°C for 1 h. Liquefaction of the semisolid gelatin medium was a positive reaction for gelatin hydrolysis.

Production of HCN was determined using a modification of the procedure of Millar and Higgins (1970) by growing bacteria on TSA supplemented with 4.4 g L^{-1} glycine and placing filter paper strips soaked in picric acid solution (2.5 g picric acid, 12.5 g Na_2CO_3, 1 L water) in the lid of the petri dish. Dishes were sealed with parafilm and incubated for 2–4 days. HCN production was indicated by a change in the color of the filter paper from yellow to reddish brown.

Starch hydrolysis was determined according to the methods in the American Phytopathological Society Guide for Identifying Phytopathogenic Bacteria (Schaad, 1988). Inoculated starch agar plates were flooded with Lugol's iodine after 48 hr incubation at 28°C, and starch hydrolysis was indicated by the presence of clear zones surrounding the bacteria.

Oxidation of phenol was determined by testing growth on gallic acid agar (per liter water: 15 g agar, 2.0 g $NaNO_3$, 0.5 g $MgSO_4$, $7H_2O$, 1.0 g KH_2PO_4, 2.0 mg thiamine HCl, and 425 mg gallic acid). Presence of growth was indicated by formation of a visible lawn within 7 days of inoculation.

Production of antifungal compounds was determined by streaking bacteria onto the outer region of TSA plates inoculated in the center with plugs of 48-h-old cultures of *Pythium ultimum* and *Rhizoctonia solani*. Plates were examined after incubation at 25°C for 4 days for zones of fungal growth inhibition around bacteria.

Siderophore production was determined using CAS agar plates acording to Schwyn and Neilands (1987). Production of siderophores was indicated by the presence of a yellow-orange halo around bacteria after 2–4 days incubation.

Results

Population densities of microorganisms isolated from roots

Total bacterial population densities, as isolated on 5% TSA, were lower for each test plant than for soybean in the seedling experiment (Table 1). The difference was significant for Abruzzi rye. Similarly, in the experiment with mature plants (Table 2), each test plant yielded lower populations of total bacteria isolated on 5% TSA than soybean, and the difference was significant for velvet bean. A comparison of mean population densities from young plants (Table 1) and mature plants (Table 2) indicates that bacteria continued to grow as plants matured and reached final values of 8.6 to 9.3 Log cfu g^{-1} root.

The mean populations estimated for total bacteria were similar using 5% TSA and full-strength TSA (Table 2). Soil extract agar (SEA) yielded mean population densities 0.5 to 1.0 Log units lower than 5% TSA (Table 2). The results with SEA showed the same trend as with 5% TSA, i.e. all test plants had lower mean population densities than soybean, and the difference was significant with velvet bean and castor bean.

Mean population densities of coryneform bacteria were generally 2 to 3.5 Log units lower than the total population detected on 5% TSA (Tables 1 and 2). With the exception of mature velvet bean, the mean population densities of test plants detected on CNS were not lower than for soybean. Mature sword bean had a significantly higher mean population of coryneforms than soybean, i.e. Log 6.58 vs Log 5.87 cfu g^{-1} root (Table 2). Coryneform populations did not increase at the same rate as the total bacteria as the plants matured, and increases from young to mature plants were generally less than 1.0 Log unit. In the case of velvet bean, there was a 0.5-Log decrease.

Populations of spore-forming *Bacillus* spp.

Table 1. Microbial populations in soil and rhizospheres of young plants

Treatment	Mean log cfu g^{-1} root on various media[a]					
	5% TSA	TSA-heated	CNS	Ohio agar	Chitin agar	
					Bacteria	Fungi
Soybean	7.65	5.46	5.50	4.14	3.40	3.06
Velvet bean	7.31	5.50	5.69	3.89	3.21	3.83*
Castor bean	7.54	5.76	5.48	4.36	4.33*	4.78*
Sword bean	7.57	5.50	5.66	4.06	1.88*	3.93*
Abruzzi rye	7.13*	6.21*	5.70	4.10	4.54*	5.07*
Soil control	6.07	4.38	4.56	3.87	3.17	2.41
LSD ($p = 0.05$) =	0.36	0.36	NSD	NSD	0.54	0.43

* Indicates significant difference from mean population in soybean rhizosphere at $p = 0.05$.
[a] The media selected for specific groups of microorganisms: 5% TSA for total bacteria, TSA following heat treatment for bacilli, CNS for coryneform bacteria, Ohio agar for total fungi, and chitin agar for chitinolytic fungi and bacteria.

were slightly higher on all test plants than on soybean (Table 1). The difference was significant for Abruzzi rye which had a mean of Log 6.21 compared to soybean with Log 5.46 cfu g^{-1} root.

Total fungal population densities were generally lower on test plants than on soybean at both plant ages sampled (Tables 1 and 2). Mature velvet bean yielded significantly fewer fungi than soybean, while fungal population densities of the other test plants were not significantly different from that of soybean (Table 2). A comparison of mean fungal populations for young (Table 1) and mature (Table 2) plants indicates that fungi generally increased about 1.5 Log units as plants matured.

Population densities of chitinolytic microorganisms showed more variation than the other microbial groups. With young plants (Table 1), castor bean and Abruzzi rye supported significantly higher populations of chitinolytic bacteria than did soybean, while sword bean had significantly lower populations than soybean. The population of chitinolytic bacteria on young soybean was Log 3.4 cfu g^{-1} root (Table 1) which declined to Log 1.4 cfu g^{-1} root on mature plants (Table 2). In contrast, with all test plants except velvet bean, population densities of chitinolytic bacteria increased as plants matured. Mean chitinolytic bacterial populations were significantly higher than soybean (Log 1.4 cfu g^{-1} root)

Table 2. Microbial populations in soil, rhizospheres, and endorhizospheres of mature plants

Treatment	Mean log cfu g^{-1} root on various media[a]								
	External							Internal	
	TSA	5% TSA	CNS	SEA	Chitin agar		Ohio agar	TSA	5% TSA
					Bacteria	Fungi			
Soybean	9.08	9.33	5.87	8.18	1.41	3.75	5.59	6.27	5.99
Velvet bean	8.66*	8.63*	5.18*	7.50*	2.20	3.90	4.62*	6.10	6.07
Castor bean	8.80*	8.92	5.63	7.71*	4.70*	2.31	5.29	6.88	7.03*
Sword bean	9.19	9.24	6.58*	7.94	5.30*	4.30	5.15	7.46*	7.59*
Abruzzi rye	8.93	9.16	6.20	8.04	6.29*	0.60*	5.64	7.68*	7.54*
Soil control	8.18	7.46	3.99	6.76	3.23	4.23	4.70	–	–
LSD ($p = 0.05$) =	0.27	0.49	0.64	0.42	1.67	1.95	0.97	0.71	0.75

* Indicates significant difference from mean population in soybean rhizosphere at $p = 0.05$.
[a] The media selected for specific groups of microorganisms: 5% TSA, TSA, and SEA for total bacteria, CNS for coryneform bacteria, Ohio agar for total fungi, and chitin agar for chitinolytic fungi and bacteria.

for mature castor bean (Log 4.7 cfu g^{-1}), sword bean (Log 5.3 cfu g^{-1}), and Abruzzi rye (Log 6.3 cfu g^{-1}) (Table 2). With chitinolytic fungi, population densities were significantly higher on all young test plants than on soybean (Table 1). As plants matured, chitinolytic fungal populations increased less than 1 Log unit on soybean, velvet bean, and sword bean, decreased 2.4 Log units on castor bean, and decreased 3.9 Log units on Abruzzi rye.

Populations of endophytic bacteria in mature plants (Table 2) were 1.6 to 3.3 Log units lower than the mean population densities of external bacteria on 5% TSA; however, sizeable endophytic population densities were detected. Three of the four test plants (castor bean, sword bean, and Abruzzi rye) had significantly more internal bacteria than soybean.

Physiological characterization of rhizosphere bacteria

Fifty randomly selected bacterial isolates from 5% TSA for each test plant and soybean using both young and mature plants were characterized for various physiological traits as previously described. Chitinolytic activity was generally infrequent with only one chitinolytic isolate identified from young soybean (Table 3) and none from mature soybean (Table 4). Chitinolytic activity was generally more frequent with all test plants, with the highest frequencies noted for strains from sword bean.

Gelatin hydrolysis was noted with nine strains from young soybean. Strains from young castor bean and Abruzzi rye had more than twice the frequency of gelatin hydrolysis than the soybean strains (Table 3). The number of strains demonstrating hydrolysis of gelatin decreased with all plants at maturity (Table 4).

No strains from soybean produced HCN (Table 3). HCN production was noted for one strain from castor bean, two from velvet bean, and three from sword bean and Abruzzi rye.

Only one strain from young and mature soybean hydrolysed starch. Strains from all test plants had higher frequencies of starch hydrolysis for both young (Table 3) and mature (Table 4) plants. Seventeen strains from young castor bean and 24 from Abruzzi rye hydrolysed starch.

Phenol oxidation, as determined by growth on gallic acid agar, was evidenced by three strains

Table 3. Physiological characterization of bacteria from rhizospheres of young plants[a]

Test plant	Chitinolytic activity	Gelatin hydrolysis	HCN production	Starch hydrolysis	Phenol oxidation	Antibiosis on TSA Toward		Siderophore production
						Pythium	Rhizoctonia	
Soybean	1	9	0	1	3	4	5	6
Velvet bean	2	10	2	6	23	10	22	19
Castor bean	3	19	1	17	20	12	16	26
Sword bean	4	14	3	2	20	14	22	20
Abruzzi rye	2	22	3	24	8	2	6	17

[a] Based on evaluation of 50 bacterial strains from each test plant; see text for details.

Table 4. Physiological characterization of bacteria from rhizospheres of mature plants[a]

Test plant	Chitinolytic activity	Gelatin hydrolysis	Starch hydrolysis	Phenol oxidation	Antibiosis on TSA Toward		Siderophore production
					Pythium	Rhizoctonia	
Soybean	0	7	1	2	9	12	1
Velvet bean	1	7	13	8	18	33	22
Castor bean	2	8	8	22	7	15	19
Sword bean	3	11	13	24	12	13	13
Abruzzi rye	0	10	11	29	17	16	13

[a] Based on evaluation of 50 bacterial strains from each test plant; see text for details.

from young soybean and two strains from mature soybean. Strains from all test plants had substantially higher frequencies of phenol oxidation than strains from soybean. On mature plants, over 20 strains from castor bean, sword bean and Abruzzi rye oxidized phenol.

Production of antifungal compounds was determined by testing for antibiosis toward Pythium and Rhizoctonia. With the exception of strains from young Abruzzi rye, strains from the test plants had a higher frequency of antibiosis than strains from soybean. Strains from young velvet bean, castor bean, and sword bean exhibited antibiosis at frequencies two to three times higher than strains from soybean (Table 3). More strains from all plants were antagonistic to Rhizoctonia than to Pythium.

Siderophore production was exhibited by six strains from young soybean and one strain from mature soybean (Tables 3 and 4). In contrast, strains from young test plants produced siderophores at frequencies three to four times higher than strains from soybean (Table 3), while strains from mature test plants had frequencies 13 to 22 times higher than strains from soybean (Table 4).

Discussion

The results indicate that the test plants which exhibit antagonism to phytopathogenic nematodes support a distinct rhizosphere microflora in comparison to soybean. While the total external rhizosphere bacterial population densities on test plants were generally not significantly different from soybean (Tables 1 and 2), there were substantial differences in the physiological profiles of the strains. The physiological tests used for evaluation of strains (Tables 3 and 4) are those which have been suggested to aid in bacterial competition in the rhizosphere. In general, the lowest frequency of activity in these physiological tests occurred with strains from soybean. Levels of activity for strains from test plauts were highest for siderophore production, phenol oxidation, starch hydrolysis, and production of antifungal compounds. The frequency of activity for strains from test plants was often many times higher than for strains from soybean. For example, starch hydrolysis for strains from young Abruzzi rye (Table 3) was 24 times the rate for strains from soybean.

The results from estimations of population densities of specific types of rhizosphere microorganisms support the conclusion that the microflora of plants antagonistic to nematodes is distinct from that of soybean, even if the total bacterial population densities show little change. All seedling test plants had higher populations of bacilli than soybean, suggesting that *Bacillus* spp. are favored relative to the total bacterial microflora. The same trend was observed for coryneform bacteria (Tables 1 and 2) from young and mature test plants. Another strong suggestion that plants with antagonism toward nematodes contain distinct specific groups of bacteria is indicated by the data on populations of chitinolytic bacteria on mature plants (Table 2). All test plants supported higher populations than soybean, and three of the test plants had increases of over 3.3 Log units compared to soybean, i.e. over 3,000 times the population. All seedling test plants supported significantly higher populations of chitinolytic fungi than soybean (Table 1), while the populations on all mature test plants were less than soybean. Therefore, it appears that chitinolytic fungi operate in rhizospheres of plants with antagonism to nematodes early in the season, and that chitinolytic bacteria predominate as plants mature. With Abruzzi rye, populations of chitinolytic fungi decreased 4.5 log units from seedlings to mature plants for unknown reasons.

That three of the four test plants supported significantly higher population densities of endophytic bacteria compared to soybean (Table 2) was unexpected. Given that one mechanism of antagonism toward nematodes by the test plants is thought to be production of toxins (Mashkoor et al., 1990), one might have expected lower internal populations of bacteria in the test plants. The internal bacteria of the test plants are likely adapted to growth in the presence of toxins and may encounter less total competition from general bacteria.

A debate exists among rhizosphere microbiologists concerning which media to use for estimation of total bacterial population densities. It is recognized that many rhizosphere bacteria

will not grow on standard bacteriological media, partly because the media are nutritionally richer than the root environment. In this study, three different media were used for estimating total bacteria (Table 2): tryptic soy agar (TSA), 5% TSA, and soil extract agar (SEA). With four of the five plant species used, the recoverable bacterial populations were highest on 5% TSA. This medium is considerably more convenient to prepare than SEA. In addition, the batch-to-batch consistency of nutrient status is higher with 5% TSA than with SEA.

The results suggest that specific groups of microorganisms have become adapted to rhizospheres of plants which have antgonistic properties to phytopathogenic nematodes. Hence, these rhizospheres should be examined as sources of microbial antagonists to nematodes. Further work should be done to identify the bacterial strains which were physiologically characterized to see if specific taxa correlate with physiological activity and/or predominate in rhizospheres of the test plants.

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