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Survival of potentially pathogenic human-associated bacteria in the rhizosphere of hydroponically grown wheat

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Abstract

Plants may serve as reservoirs for human-associated bacteria (H-AB) in long-term space missions containing bioregenerative life support systems. The current study examined the abilities of five human-associated potential pathogens, *Pseudomonas aeruginosa*, *Pseudomonas cepacia*, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Escherichia coli*, to colonize and grow in the rhizosphere of hydroponically grown wheat, a candidate crop for life support. All of these bacteria have been recovered from past NASA missions and present potential problems for future missions. The abilities of these organisms to adhere to the roots of axenic five-day-old wheat (*Triticum aestivum* L. cv. Yecora rojo) were evaluated by enumeration of the attached organisms after a one hour incubation of roots in a suspension (approximately 10^8 cfu ml^{-1}) of the H-AB. Results showed that a greater percentage of *P. aeruginosa* cells adhered to the wheat roots than the other four H-AB. Similarly incubated seedlings were also grown under attempted axenic conditions for seven days to examine the potential of each organism to proliferate in the rhizosphere (root colonization capacity). *P. cepacia* and *P. aeruginosa* showed considerable growth, *E. coli* and *S. aureus* showed no significant growth, and *S. pyogenes* died off in the wheat rhizosphere. Studies examining the effects of competition on the survival of these microorganisms indicated that *P. aeruginosa* was the only organism that survived in the rhizosphere of hydroponically grown wheat in the presence of different levels of microbial competition.

Keywords: Human-associated bacteria (H-AB); Rhizosphere; Adherence; Root colonization capacity; Microbial competition

1. Introduction

The goals of the United States space program include the expansion of human presence and activity beyond Earth's orbit into other parts of the solar system. In order to accomplish these goals the development of life support systems capable of sustaining human life for space missions of long duration is needed. The Controlled Ecological Life Support System (CELSS) program is part of the National Aeronautics and Space Administration (NASA) design to use higher plants as CO_2/O_2 converters and sources of food in bioregenerative life support systems [1].

Human-associated bacteria (H-AB) will undoubtedly be present in a CELSS. Since direct contact between humans and plants in a CELSS is inevitable, the plants could serve as reservoirs for H-AB. Both

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the United States and Russian space programs have reported changes in the human immune system and in microbiological infections during spaceflight [2–4]. Information from these studies indicates that spaceflight can be expected to result in a compromised human immune system and in a possible increase in the numbers and distribution of potentially pathogenic microorganisms.

The rhizosphere, where the continual input of readily assimilable organic substrates stimulates microbial growth, can contain up to 10^{11} cells $gram^{-1}$ of root in plants grown in prototype hydroponic production systems [5,6]. The growth of human pathogens as part of this large microbial population is a major concern due to the potential effects on human health. The competition between H-AB and resident rhizosphere microflora is a very relevant area of CELSS research, particularly since the composition of the resident flora can be manipulated, through inoculation, to a much greater degree in a closed, space-based system relative to an open, Earth-based system.

The aim of this study was to investigate the potential survival of H-AB in the rhizosphere of hydroponically grown wheat by evaluating the abilities of H-AB: (1) to adhere to the roots of axenic five-day-old wheat, (2) to grow and proliferate in the rhizosphere (root colonization capacity) without microbial competition, and 3) to compete with diverse microbial communities for resources critical to survival. *Pseudomonas aeruginosa*, *Pseudomonas cepacia* and *Escherichia coli* are commonly found in the environment and are opportunistic pathogens causing urinary tract infections, septicemia, and pneumonia. Skin and other infections are often caused by *Staphylococcus aureus* and *Streptococcus pyogenes*. All of these bacteria have been recovered from past NASA missions and present potential problems for future missions [7–9].

2. Materials and methods

2.1. Bacterial strains

Five potentially pathogenic H-AB were used in this study: (1) *P. aeruginosa* ATCC 27853, (2) *S. aureus* ATCC 25923, (3) *S. pyogenes* ATCC 49399, (4) *E. coli* ATCC 23590, and (5) a *P. cepacia* strain recovered from the potable water systems of past space shuttle missions, and obtained from the Bionetics Microbiology Group in the Bioastronautics Operation and Support Unit at Kennedy Space Center, Florida.

2.2. Seed sterilization, germination and sterility test

Wheat seeds (*Triticum aestivum* L. cv. Yecora rojo) were sterilized using a combination of mercuric chloride and hydroxylamine hydrochloride [10]. Sterilized seeds were placed on filter paper (Whatman, qualitative, 10 cm) moistened with sterile distilled water in small (100 × 10 mm) glass Petri plates. The seeds were incubated in a bench-top plant growth chamber consisting of a glovebox (model 11000, Labconco, Inc., Kansas City, MO) containing three fluorescent lamps, a fan to extract hot air, and a humidifier. The conditions inside the plant growth chamber were as follows: 20 h/4 h light/dark, photosynthetic photon flux of 113.5 $\mu moles/m^2$ per sec, continuous temperature at 25°C, and 65% relative humidity. After 5 days, wheat seedlings were randomly picked for different experimental treatments. Sterility was checked by randomly selecting six wheat seedlings and placing them in a 50 ml culture tube containing 15 ml 0.55% R2A agar. The sterility test tubes were checked during the course of the experiments for any visible bacterial growth in the rhizosphere.

2.3. Plant culture

Seedlings were transferred to sterile, wide-mouth glass vessels containing 50 ml of 1/4-strength Hoagland's nutrient solution. Seedlings were placed into slits of autoclaved, foam plugs, which were inserted into wide-mouth glass vessels containing 50 ml of 1/4-strength Hoagland's nutrient solution. Nylon strips (Nytex, 63 μm aperture, Wildco, Saginaw, MI) were permeated with nutrient solution and used to wick up the solution to the wheat seedlings until roots reached the solution. The wide-mouth glass vessels were covered with a black plastic cover to prevent algal growth. Plants were grown in the bench-top chamber for 7 days. The vessels containing the wheat seedlings were inserted into a wood

frame holder and placed on top of a shaker (Autoshake R, type 886013, B. Braun, Germany) with agitation at 75 rpm inside the PGC. The agitation maintained dissolved oxygen levels above 6 ppm (188 μmoles) in the jars (Oxygen meter, model 51B, YSI, Inc., Yellow Springs, OH).

2.4. Adherence assay

Bacteria in late logarithmic phase were harvested from the R2A culture broth by centrifugation at $2000 \times g$ (model IEC, HN-SII, Damon/IEC Division, Needham, MA) for 20 min at 25°C [11]. The bacterial pellets were resuspended in 13 ml of filtered-sterilized 1/4-strength Hoagland's solution (pH 5.5) [12]. Cell densities were adjusted turbidimetrically on a Beckman DU-64 spectrophotometer by dilution in 1/4-strength Hoagland's solution to an optical density of 0.1 at 600 nm (approximately 10^8 cfu ml^{-1}).

Five-day-old wheat seedlings were incubated in the bacterial suspensions for one hour with slow agitation (40 rpm) at 25°C, and rinsed by mixing in a vortex-mixer for 10 sec in 20 ml of fresh 1/4-strength Hoagland's solution. Those bacterial cells remaining on the seedlings were considered to be firmly bound [11]. The roots were shaken in 0.1% NaP solution containing glass beads (3 mm) for 3 min, and the suspension was serially diluted on R2A agar. Plates were counted after two days of growth at 37°C. Root dry weights were determined immediately after the roots were dried at 70°C for 48 h.

2.5. Root colonization capacity assay

The roots of sterile, five-day-old wheat seedlings were soaked in 10 ml of bacterial suspensions of the respective H-AB as described above and grown for seven days in the bench-top plant growth chamber under the conditions outlined in Section 2.3. Enumeration of rhizosphere bacteria was performed following the protocol described in Section 2.4.

2.6. Microbial competition studies

2.6.1. Inoculum preparation

Three types of mixed microbial samples were used to evaluate the ability of H-AB to compete for survival in the rhizosphere. The inocula were selected based on potential approaches to the inoculation of plant growth systems in space, ranging from strict decontamination to specific inoculation to enrich for diversity.

A sample of microorganisms from the clean room animal handling facility at the Life Sciences Support Facilities, Hangar L, KSC, was used to simulate the type of inoculum likely to exist when using the current approach to decontamination of spaceflight life science experiments. Surface samples were taken by rolling a sterile cotton-tipped swab across a small surface area (10 cm) that had been recently decontaminated with Quatracide PV (Pharmacal Research Laboratories, Inc., Naugatuck, CT). The swab was placed in 50 ml R2A broth, with agitation (200 rpm) at 25°C. After 55 h, 1-ml samples from this culture were placed into 1.2-ml sterile Nalgene cryogenic tubes and 0.2 ml of sterile glycerol were added. The samples were stored at −70°C.

An intermediate approach of bacterial inoculation to the plant growth system, involving neither strict decontamination nor enrichment, is routinely employed for plant growth experiments at KSC. Microbial inoculum is limited due to the isolation of the chambers, but system components (hardware, distilled water, seeds) are not sterilized. Roots were cut from the rhizosphere of 68-day-old wheat (*Triticum aesticum*) grown in a controlled growth chamber at the KSC CELSS facilities. A section of this root mat was suspended in 20 ml of sterile 0.1% sodium pyrophosphate and glass beads (3 mm, 7.69 g). The solution was hand-shaken for 3 min. A 5-ml sample from this suspension was grown in a 250-ml Erlenmeyer flask containing 100 ml R2A broth (DIFCO, Detroit, MI) with agitation (200 rpm) at 25°C. Samples were fixed and stored as described above.

Estimates of the species richness of soil microbial communities are very high [13]. Inoculation of plant systems with a soil sample, therefore, reflects a strategy of maximizing potential species richness. Soil samples were taken from the top 10 cm of soil from an orange grove in Arcadia, Florida. The pH of the soil in distilled water was 4.5 The soil was mixed well and the soil bacteria were grown in 50-ml Erlenmeyer flasks containing 100 ml R2A broth and 1 g of soil. After 24 h and 96 h, 1-ml samples from this culture were fixed and stored as described above.

The background microbial communities (high-, medium-, and low-level diversity inocula) were grown (20 μl of frozen culture stock in 20 ml R2A broth) for 16 h prior to inoculation into experimental treatments.

2.6.2. Microbial competition experiments

Microbial competition experiments were identical to the root colonization capacity tests except that the mixed microbial inocula described above were added to the nutrient solution of the vessels following introduction of the H-AB to the roots of the plants. Initial tests evaluated the growth of each of the H-AB when the low- and medium-diversity inocula were introduced at a density of 10^6 cfu ml^{-1}. Follow-up tests evaluated the growth of *P. aeruginosa* when all three types of inocula were introduced at a density of 10^1 or 10^6 cfu ml^{-1}. Viable counts of rhizosphere communities after seven days of plant growth were performed as described above. H-AB were distinguished from the background community based on colony morphology. Identification of isolates that were not easily classified by colony morphology was performed using the Vitek Automicrobic system (bioMerieux Vitek, Inc., Hazelwood, MO).

2.7. Experimental design and statistical analyses

The microbial diversity of samples was calculated from analysis of the colony morphology of 25 colonies grown on R2A agar plates. Each colony was given a six-number code based on the following criteria: colony size, pigmentation, form, elevation, margin, and surface. Each unique six-digit code was considered a single type of organism for estimating microbial diversity. The Shannon index was used to calculate diversity [14]. The diversity indexes were used as indicators of potential competition. Specific growth rates (r) per day were calculated as follows: $1/t \times [\ln (N_f/N_0)]$, where t = time, N_f = number of bacteria at day 7, and N_0 = number of bacteria at time zero (day 1) [15].

All experiments were performed in a completely randomized design with four replicates. A replicate consisted of a wide-mouth glass vessel containing one wheat plant. Results from identical experiments were combined for analysis. The bacterial counts were all logarithmically transformed prior to statistical analysis. For determining the mean, the zero values were replaced by the limit of detection value. A zero value did not actually indicate that the introduced bacteria were not present on the root, but rather that the introduced bacteria were below the minimum detectable level. Significant differences among treatments were determined at $P < 0.05$ by using single and double factor analyses of variance (ANOVA).

3. Results and discussion

3.1. Adherence assay

Adherence to the root surface is one of the earliest steps in root colonization by bacteria. The adherence

Table 1
Adherence of human-associated bacteria (H-AB) to the roots of wheat after a one-hour incubation

H-AB	H-AB density in suspension (log_{10} cfu $root^{-1}$)					H-AB density after 1 h incubation (log_{10} cfu $root^{-1}$) [a]					Average % adherence [b]
	Replicate experiments					Replicate experiments					
	1	2	3	4	5	1	2	3	4	5	
P. aeruginosa	9.41	9.46	9.45	9.14	9.43	6.99 ± 0.37	7.52 ± 0.19	7.18 ± 0.51	7.54 ± 0.18	6.83 ± 0.75	1.03 ± 0.70
S. aureus	8.08	8.18	8.53	8.12	8.18	4.79 ± 0.44	5.29 ± 0.44	5.12 ± 0.11	5.04 ± 0.14	4.59 ± 0.2	0.08 ± 0.05
S. pyogenes	7.25	7.49	7.94	8.14	7.98	3.82 ± 0.20	4.30 ± 0.24	4.98 ± 0.15	5.05 ± 0.20	4.79 ± 0.15	0.08 ± 0.03
P. cepacia	8.83	9.31	9.31	9.16	9.25	5.66 ± 0.30	4.94 ± 0.33	5.96 ± 0.36	5.89 ± 0.27	6.06 ± 0.39	0.06 ± 0.03
E. coli	9.12	9.08	9.21	9.10	9.04	5.89 ± 0.18	4.73 ± 0.49	6.42 ± 0.86	5.74 ± 0.19	5.51 ± 0.50	0.04 ± 0.02

[a] Mean ± standard deviation at 95% confidence level for four replicate samples.
[b] Average % adherence = (H-AB cfu $root^{-1}$ adhering to roots after 1 h/H-AB cfu $root^{-1}$ in suspension) × 100. Mean ± standard deviation of five experiments.

assay used in this study focused on the rapid, firm adhesion of each bacterium to the wheat roots. *P. aeruginosa* cells adhered to the roots of wheat in higher numbers than *P. cepacia*, *E. coli*, *S. aureus*, and *S. pyogenes* (Table 1). The number of cells that adhere to the roots in this assay can be affected by the density of microorganisms in the suspensions in which the roots are soaked. Since the cell density in the soaking solutions was slightly different among bacterial types as a result of different optical properties of the microorganisms we also compared the adherence abilities of the different bacteria by examining the percentage of the total bacteria in suspension that adhered to the roots (Table 1). These results also indicated that *P. aeruginosa* displayed significantly greater adherence (average of 1.03%) than all of the other four H-AB (average ranges from 0.04 to 0.08%).

Several factors may have contributed to the greater percent adherence of *P. aeruginosa* to the roots of wheat. Initial adherence is considered to depend mainly on surface properties of bacteria, such as net surface charge [11]. Contact between the bacteria and the root surface can be established at the molecular level by extracellular polymeric substances (EPS) produced by some bacteria. The EPS may form a bridge between a bacterium and the root surface because the EPS are not subject to the same degree of repulsion [16]. Synthesis of exopolysaccharide materials (slime) appears to be the most important factor for irreversible adherence of bacteria [17]. The production of slime has been shown to be important for colonization of both medical devices [18] and cell surfaces [19]. *P. aeruginosa* is known to be a slime-producing pathogen. *P. aeruginosa* strains produce EPS that form a loose capsule of organized linear strands of polysaccharide radiating outwards from the cell surface. Exopolysaccharides, also known as glycocalyx, produced by the bacterial cells glue them firmly to these surfaces. Studies of the interaction of *P. aeruginosa* with respiratory mucosa in vitro reported that when *P. aeruginosa* was seen (using scanning electron microscopy) associated with the mucosal surface including mucus, cilia, unciliated cells and extruded cells, an extracellular matrix material was often seen bridging the gap between the bacteria themselves and between bacteria and the mucosal component with which the bacterium was associated [20]. Other adhesins, such as exoenzyme S [21] and alginate [22] have also been identified in *P. aeruginosa*.

Additional studies may focus on the ability of H-AB to grow on root exudates, chemotaxis, and agglutination of plant exudates. Other studies may also look at H-AB cell wall-associated surface components that promote specific interactions with root surface cells.

3.2. Root colonization capacity

Axenic conditions were difficult to maintain and some contamination was always present in these experiments. The term 'attempted axenic conditions' will be used to more accurately describe the condi-

Table 2
Root colonization capacity of human-associated bacteria (H-AB)

H-AB	Average H-AB density ($\log_{10}$ cfu root^{-1}) [a]				Average r [c]
	Experiment 1		Experiment 2		
	Time 0	Time 7	Time 0	Time 7	
P. cepacia	6.63 ± 0.34	7.31 ± 0.59	5.96 ± 0.36	8.22 ± 0.10	0.50 ± 0.30
E. coli	5.17 ± 0.24	6.31 ± 0.32	6.42 ± 0.86	6.74 ± 0.33	0.30 ± 0.34
P. aeruginosa	7.54 ± 0.26	8.31 ± 0.05	7.18 ± 0.51	8.22 ± 0.33	0.28 ± 0.05
S. aureus	6.10 ± 0.22	6.05 ± 0.4	5.12 ± 0.11	5.43 ± 0.30	0.07 ± 0.07
S. pyogenes	4.76 ± 0.28	(0) [b]	4.98 ± 0.15	(0)	−0.53 ± 0.04

[a] Mean ± standard deviation at 95% confidence level for four replicate samples.
[b] Arithmetic zero, below detection limit of viable counts (2×10^3 cfu root^{-1}).
[c] Mean ± standard deviation at 95% confidence level for two experiments ($n = 8$).
Specific growth rate (r) = $1/t \times [\ln(N_t/N_0)]$. See Section 2.7.

tions maintained in these experiments. The sterility tests (see Section 2.2) done at the beginning of the experiments showed no bacterial growth in the roots of the five-day-old wheat seedlings. These results suggest that the contamination present in the plant growth studies occurred during either the planting of the seeds in the plant growth vessels or in the course of the experiment. The most likely source of contamination during the experiment is the water from the humidifier which, at times, could be seen as condensate on the surface of the plant growth vessels. One contaminant (*Pseudomonas acidovorans* or *Pseudomonas pickettii*) was present in all the treatments, including the controls (sterile wheat plant with no inoculum) at an average density of 6.49 (0.66 $\log_{10}$ cfu root^{-1}).

Under the attempted axenic conditions, *P. aeruginosa*, *P. cepacia*, *E. coli*, and *S. aureus* persisted in the rhizosphere, while *S. pyogenes* decreased to levels below the detection limit of viable counts (2×10^3 cfu root^{-1}). The human-associated bacteria cell densities ($\log_{10}$ cfu root^{-1}) at time zero (after 1 h incubation) and at time 7 (after seven days) are reported in Table 2. There is some variation between the H-AB cell densities obtained in the two experiments, but the results indicate that *P. cepacia* had the highest growth rate (0.50), followed by *E. coli* (0.30), *P. aeruginosa* (0.28), and *S. aureus* (0.07). Under the same conditions, *S. pyogenes* died off (−0.53). It is interesting to note that although *P. aeruginosa* had the highest percent adherence and colonization capacity, it did not have the greatest specific growth rate per day. This suggests that adherence alone is not a good predictor of the ability of a microorganism to proliferate in the presence of low competition.

Motility and the nutrient requirements for growth of a bacterium may be important factors in determining the colonization capacity of these organisms. The rhizosphere may enrich for bacteria that are best suited for rapidly utilizing simple carbon sources [23]. Previous studies have also shown that chemotactic attraction of rhizosphere bacteria by root exudate and root mucilage can play a role in root colonization [24]. The two best colonizers in this study, *P. aeruginosa* and *P. cepacia*, are nutritionally versatile and motile. The two pseudomonads are known to utilize a large number of carbon sources and nitrogen compounds as sole carbon sources, and are motile by one or several polar flagella. The intermediate colonizer, *E. coli*, is motile by peritrichous flagellation, but not as nutritionally versatile as the pseudomonads. The poorest colonizers in this study, *S. aureus* and *S. pyogenes*, are non-motile bacteria with less nutritional diversity. The combinations of these two factors, motility and nutritional versatility, would be important in allowing bacteria to reach and utilize exudates as the roots grow.

3.3. Microbial competition

The capacity of these H-AB to colonize the rhizosphere will also depend on their ability to compete with other potential root colonizers for resources

Table 3
Effects of microbial competition on the survival of *Pseudomonas aeruginosa* in the rhizosphere of wheat

Competition treatments	Diversity index [a]	*P. aeruginosa* cell density at time 7 ($\log_{10}$ cfu root^{-1}) [b]	*P. aeruginosa* specific growth rate per day [c]
High-level microbial diversity:			
High density	3.60	7.36 ± 0.09	0.11
Low density	2.57	8.41 ± 0.13	0.46
Medium-level microbial diversity:			
High density	2.76	7.76 ± 0.21	0.25
Low density	1.56	8.29 ± 0.25	0.43
Low-level microbial diversity:			
High density	1.37	8.52 ± 0.07	0.49
Low density	1.00	8.70 ± 0.21	0.56

[a] Shannon index of microbial diversity. See Section 2.7.
[b] Mean ± standard deviation at 95% confidence level for four replicate samples.
[c] Specific growth rate per day = $1/t \times [\ln(N_t/N_0)] \cdot N_0 = 7.02 \pm 0.09$.

within the rhizosphere. Initial experiments evaluating all five H-AB indicated that *P. aeruginosa* was the only microorganism that persisted at detectable levels in the presence of either the low or medium-level microbial diversity inocula. After seven days growth, the average $\log_{10}$ cfu root^{-1} of *P. aeruginosa* cells in the low- and medium-level microbial diversity competition treatments was 8.65 ± 0.08 and 8.43 ± 0.55, respectively. *P. cepacia*, *E. coli*, *S. pyogenes*, and *S. aureus* were below the detection limit of viable counts (2.0×10^3 cfu root^{-1}).

A follow-up experiment was designed to study the effects of both the diversity and the density of the background microbial community on the survival of *P. aeruginosa*. Growth of *P. aeruginosa* was inversely related to both of these factors (Table 3). A two-way factor analysis of variance (ANOVA) indicated that there is a statistically significant density effect ($P < 0.0001$), as well as a diversity effect ($P < 0.0001$) on the density of *P. aeruginosa* in the rhizosphere after seven days growth with competition. A significant interaction effect (i.e., density–diversity) was also present ($P < 0.0093$). The interaction significance was due to a decrease in the effect of density at lower levels of diversity. The difference in the specific growth rates of *P. aeruginosa* between the high and low density of the low-, medium-, and high-level diversity inocula treatments was 0.35, 0.18, and 0.07, respectively (Table 3). The difference in microbial diversity of the high- and low-density inocula also decreased at lower levels of diversity, as predicted by dilution/extinction phenomena. In essence, less extinction occurs upon dilution of a low-diversity inoculum relative to a high-diversity inoculum since relatively fewer types of microorganisms are present at higher densities.

These results indicate the importance of not only the quantity, but also the type of competing microorganisms on the persistence of *P. aeruginosa* in the rhizosphere. In this experiment, the composition of the community was described by a diversity index, and although persistence was negatively correlated with diversity, it is unclear if diversity has a direct negative effect on the survival of *P. aeruginosa*. A direct effect of diversity would suggest an interactive component of competition. In other words, a more diverse community would contain a greater percentage of specialists with narrow niche width, leaving fewer resources available for the maintenance of a large population of *P. aeruginosa*. An alternative, and indirect, effect of diversity could be the greater probability for the presence of individuals which show direct antagonism against *P. aeruginosa* in a more diverse community. Future experiments that test individual members of the low-, medium-, and high-level diversity inocula for antagonism against *P. aeruginosa* could help identify the relative importance of these alternative explanations for the inhibitory effect of diversity on the survival of *P. aeruginosa*.

3.4. Future studies

The results from the current study provide some insight into the ability of H-AB to survive in the rhizosphere of wheat and how microbial competition may play an important role in planning effective inoculation strategies in a closed, space-based system. We have shown that the root colonization capacity of a bacterium does not predict its survival under microbial competition. The density and composition of the microbial competitors affect the survival of introduced H-AB in the rhizosphere of wheat. Future studies could examine the survival of different strains of these five H-AB under microbial competition to evaluate the consistency of conclusions. These studies could include specific genotypic or phenotypic markers to track the introduced H-AB so that more realistic scenarios of introduction of these bacteria into the rhizosphere may be used.

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