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MICROBIAL COMMUNITY IN A PRECURSORY SCENARIO OF GROWING TAGETESPATULA IN A LUNAR GREENHOUSE

A confined prototype plant-microbial system is elaborated for demonstration of growing pioneer plants in a lunar greenhouse. A precursory scenario of growing *Tagetes patula* L. in a substrate anorthosite which is similar mineralogically and chemically to lunar silicate rocks includes the use of a microbial community. Microorganisms served for preventive substrate colonization to avoid infection by deleterious microorganisms as well as for bioleaching and delivering of nutritional elements from anorthosite to plants. A model consortium of a siliceous bacterium, biocontrol agents, and arbuscular mycorrhizal fungi provided an acceptable growth and blossoming of *Targetes patula* L. under growth limiting factors in terrestrial conditions.

INTRODUCTION

The plants will be a practical necessity for humans living in permanently manned lunar bases, and they will provide fresh food, oxygen, and clean water for lunar explorers. The ornamental plants are needed for psychological purpose. Combinating esthetic impression with more practical traits such as medicinal, nutritional, biopesticidal, some ornamental plants could be considered as a valuable element of regenerative life support system in lunar habitats. It is anticipated that lunar regolith will be used as a substrate for plant growing at the early beginning of missions. Some petrogenic elements that compose lunar minerals (Fe, K, Na, Ca, etc.) may serve as an essential source of plant nutrition ions. Potentially, nearly a half of them the plant could get from a lunar rock, and such ions could be leached from lunar-sourced rocks by microorganisms. This would reduce the cost of a plant nutrition program. It is well established that some species of bacteria of genus Bacillus are able to dissolve alumino-silicates and to liberate elements like Fe, Si, K, etc., from rocks, making them available for the plant [1, 5, 11, 19]. Bacteria belonging to the genera Thiobacillus and Pseudomonas as well as fungi belonging to the genus Cladosporium were also shown to destroy alumino-silicates [14, 17]. Siliceous and some other bacteria as well as fungi could be used for bioleaching the lunar regolith.

Except bioleaching, microbial communities may serve as means to optimize plant growth regime via provision of substances for nutrition, growth regulation, plant protection against pathogens, via removing plant 'wastes' and improving the plant environment [3, 8]. The use of microbial communities in lunar garden will allow one to avoid imbalance in microbial cenosis because the microorganism-free lunar rocks (used as a substrate) might be colonized with accidentally delivered from the Earth deleterious micro-astronauts. Colonization of the plant/substrate system by bacteria-antagonists would be the preventive means that could protect the plant from occupancy of phytopathogenic microorganisms and therefore stabilize a microbial community in artificial ecosystems.

A water movement within the plant would be a paramount issue at low atmospheric pressure on the Moon. The means by which plants can avoid desiccation may be arbuscular-mycorrhizal (AM) fungi that improve water regime in plants [2]. In addition, AM fungi are known as transporters of cations of metals to the plant [6, 18], and they could be used for inoculations of soil sources of low bioavailability.

Especially they are active in company with mycorrhiza helper rhizobacteria [20]. In such soils AM could increase absorption and translocation to the plant of ions released from rocks by bacteria. The purpose of this research is experimental examination of the use of rationally assembled defined microbial community in a precursory scenario of cultivating *Tagetes patula* on silicate substrate in the controlled environment.

MATERIALS AND METHODS

Prototype plant growth system includes French marigold (*Tagetes patula* L. cv. Carmen) that has been chosen due to a set of beneficial traits. Wheat (*Triticum aestivum* L. cv. Katyusha) was used for determination of phytotoxic effect of model bacteria. *Silicate rocks* were used in this study: anorthosite (deposits Penizevichi and Turchynka, both from Korosten Pluton, Zhytomyr region) and silica rock (Khmelnytskyi region) in fractions of 5.0 mm fragments and as dust (less than 0.5 mm). Rocks were fragmented and milled when needed and heated during 2 h at 600 °C.

Microorganisms were used in experiments: *Pseudomonas* sp. IMBG163, *Pseudomonas aureofaciens* IMBG164, *Xanthomonas maltophilia* IMBG147, *Paenibacillus* sp. IMBG156, *Klebsiella oxytoca* IMBG26, and *Pantoea agglomerans* IMV56 (kindly provided by Prof. R. Gvozdyak, Institute of Microbiology and Virology of NASU). *Glomus* sp. isolated from the zinc violet in FRG and kindly provided by H. Bothe (Cologne University).

Cultivation and identification of microorganisms. Bacteria were cultivated overnight in the following liquid media: *Paenibacillus* sp. — in MZ [15], *Pseudomonas* sp. IMBG163 — in KB [7], and rest of species — in LB [12] and used at a density of 10^6 CFU/ml for seed or substrate inoculation. To estimate external root colonization, root sections were vortexed in 0.9 % NaCl, and serial dilutions were plated in LB to discriminate between *P. aureofaciens* and *P.agglomerans* (on orange and yellow colony color); *Pseudomonas* sp., and *Paenibacillus* sp. — in KB and MZ, respectively. To isolate *K. oxytoca* and *X. maltophilia*, chloramphenicol and rifampicin (both of 50 mu g/ml) were added to LB agar, respectively. Microscopic analysis of bacteria was performed by standard staining the sample after fixation under microscope ML-2 (LOMO, Russian Federation). Visualization of fungi was conducted with standard method of incubation of roots with lactic acid [16].

Culture-independent method T-RFLP was used for the study of bacterial community composition. The communities harvested from fresh plant material (both a stem and a root), and isolated whole-community DNA was analyzed, using T-RFLP (Terminally Labeled Restriction Fragment Length Polymorphism) in ALF System (Pharmacia). Total DNA isolation from plant material was performed as a manufacturer of the kit, the MoBio (USA), specifies. Fragments of the *rrn* gene (16S rDNA) of the bacteria associated with plant were amplified with the eubacterial primers F507 (labeled at the 5'end with Cy5) and R1353 and subjected to a T-RFLP analysis. The primers were synthesized in Syntol (Russian Federation). In preliminary experiments characteristic sizes of T-RFs (*Hha*I and *Hae*III, Fermentas, Lithuania) for either bacterium of consortium have been determined.

The evolution of bacterial population of Paenibacillus. Batch experiments were performed in a 50 ml stationary culture where a rock or a dust material (0.5 g) were used. The evolution of bacterial population of *Paenibacillus* was monitored within a period of two months. Samples of whole population [unattached and attached bacteria; the latter were washed off with Tween 20 (0.02 %) on shaker during 30 min] were in parallel spread on selective MZ agar and examined microscopically to enumerate periodically their presence in the system. Corrosion of rocks was determined visually and microscopically.

Absorption of Paenibacillus exometabolites on the surface of anorthosite particles was examined by method of microelectrophoresis [4]. Bacterial exopolysaccharide (EPS) was extracted as Zakharova recommended [24].

Plant growth conditions. Plastic transparent containers of (160 by 90 mm) or growth chambers of a locker-based facility with an experiment hardware and facility infrastructure, the Plantkord, were used to produce the substrate-plant microcosms. Portions (260 g) of either substrate (anorthosite or zeolite) were added to the microcosms. The sterile phosphate solution was pumped by a peristaltic pump located outside the containers through a valve at the bottom of each chamber below the surface of the substrate in the first case, and automatically in the second case. The inoculum of *Glomus* sp. has been added to inorganic substrates (10 %) when needed. The microcosms were planted with ten seeds or left unplanted. The growth containers were accommodated within the controlled environment room under a 16/8 day (light/dark) photoperiod with light supplied at an intensity of 55 $\mu molm^{-2}s^{-1}$ and 22 °C. Disinfections of seeds when needed was performed with 1.0 % sodium hypochlorite supplemented with 0.02 % Tween-20 for 1 min, followed by incubation in 70 % ethanol

(1 min) and washing three times (1 min each time). At the end of the experiment all plants were harvested and morphometric analyses were performed.

Statistical tests of the significance of differences between means were based on Student's T-test.

RESULTS AND DISCUSSION

Growth of silicate bacterium Paenibacillus sp. on silicate substrates. Two types of anorthosite served us for demonstration of its availability to be used as both a substrate and a source of macro- and microelements for plant growing. Anorthosites of Turchynka type are composed of olivine, orthopyroxen, plagioclase minerals (mainly, labradorite) and are most similar to lunar anorthosite chemically and mineralogically [10]. Anorthosite of Penizevichi deposit is plagioclase of 73—86 %, and accessory minerals compose a group of olivin, ilmenite, orthoclase, biotite [13].

The Penizevichi type of anorthosite as lunar rock analog and silica rock as additional specimen have been used to study the pattern of colonization and survival of *Paenibacillus* sp. on fragments of rocks and on dust. The siliceous bacterium *Paenibacillus* sp., natural resident of the earthly silica, has been isolated and shown its friendly relationships with a variety of gramnegative bacteria [15]. Attempts were made to determine the proportions of bacteria attached to solid particles or freely suspended in the medium using a combination of serial dilutions and a microscopic technique. Results represented in Table 1 display change in the population size of *Paenibacillus* sp. culturable cells in different microcosms. The attached cells looked like long vegetative slime cells without spores and covered with a huge amount of exopolysaccharide, on contrast to freely suspended in the medium cells, middle-sized, mainly spore-conferring. Microscopic observation revealed that the attached bacteria were more physiologically active, and that might be connected with their putative capability of

Table 1. Average Populations of the Paenibacillus Sp. Culturable Cells in Presence of Different Silicate Substrates

Variant of a substrate	Unattached, CFU/ml		Attached*, CFU/g	
	7 days	28 days	7 days	28 days
Anorthosite, rocks (Penizevichi)	2000	82	11	2.3E+05
Anorthosite, dust	2100	1.0E+07	—	—
Silica, rocks	130	12	62	1.7E+05
Silica, dust	10	3.6E+07	—	—

* Bacterial cell were washed off from silicate rocks

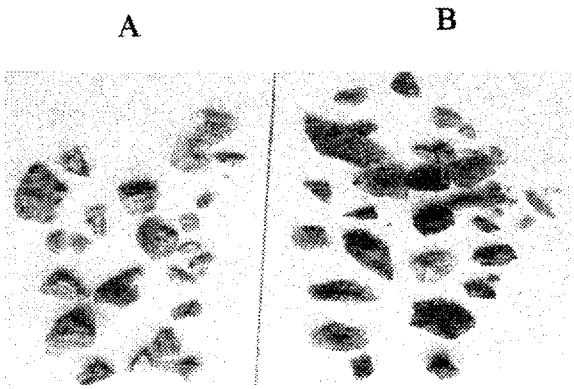

Fig. 1. Images of anorthosite rocks: A — after a contact with a nutrient medium (without bacteria) within a month; B — with the culture of *Paenibacillus* sp.

biodegradation/bioleaching of anorthosite [5, 17]. The same difference in a single population has been demonstrated earlier between attached and unattached cells with respect to traits such as cell size, reproductive rate, and exopolymer production [9, 22, 23]. Our data support the hypothesis of Vandevivere and Kirchman (1993) that solid surfaces may stimulate attached bacteria to produce exopolymers [21].

Corrosion on the Penizevichi anorthosite surface (as well as on silica rock) was observed by the naked eye after 28 days of incubation under optimal conditions (Fig. 1). It was expressed in a formation of iron oxide. The same changes we observed on surface of the Turchynka type anorthosite which served as a substrate for marigold growing at the end of the plant vegetation. No signs of changes were observed in both unplanted and planted chambers without bacteria.

Adhesion of bacteria on silicate particles. The initial stage of bioleaching/biodegradation is bioadhesion of microorganisms on mineral surface. A diffusion layer of exometabolites (that is different in different microbes) plays a crucial role in bioadhesion/hetercoagulation [4]. Water-soluble exometabolites, including EPS, being absorbed on surface of certain mineral, change the charge of the target surface (the ζ-potential) and thus a power of electrostatic repulsion. Absorption of both small quantities of EPS and total exometabolites produced by *Paenibacillus* sp. resulted in a change of the ζ-potential of anothosite particles, on contrast to control specimen of mineral (in absence of biomaterial) (Fig. 2). Estimation showed that *Paenibacillus* needed at least 200 times less EPS for attachment than it was able to produce. Anxiety about possible destroying EPS by bacterial community which are able to consume it as carbon source was not sound.

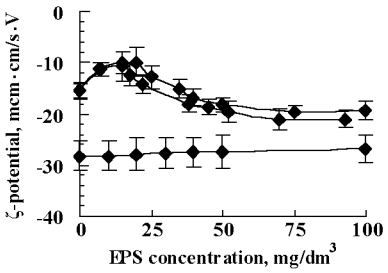

Fig. 2. Dependence of the electrokinetic ζ-potential of anorthosite particles (lines 1 and 2) and silica rock (line 3) on concentration of exopolysaccharide (line 1) and total exometabolites (containing the same quality of EPS produced by *Paenibacillus* sp. (line 2), and EPS synthesised by *Chlorella vulgaris* (line 3)

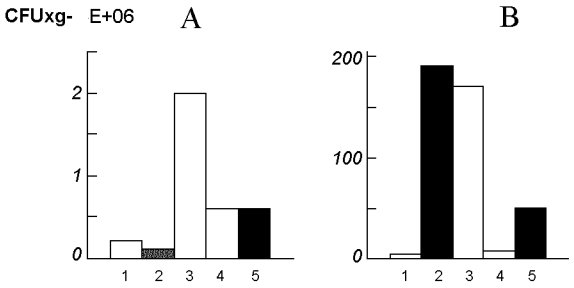

Fig. 3. Dynamics of populations of bacterial assemblage in the marigold rhizosphere: A — two weeks after a planting; B — six weeks later (*1 — Klebsiella oxytoca, 2 — Paenibacillus sp., 3 — Pseudomonas sp., 4 — Pantoea agglomerans, 5 — Pseudomonas aureofaciens*)

Table 2. Impact of Bacterial Inoculations on Growth of Wheat in Inorganic Substrate

Variant	Average dry weight of par a plant, g	% to control	Average height of a stem, mm	% to control	Average length of a root, mm	% to control
Control	0.027a	100.0	386a	100.0	110a	100.0
Pseudomonas sp. IMBG163	0.031b	115.7	404a	104.7	125b	113.9
Paenibacillus sp. IMBG156	0.032b	117.0	465b	120.5	1255b	114.1
Pseudomonas sp. + *Paenibacillus* sp.	0.029b	107.0	353a	94.0	145b	123.0

Note: Values followed by the same letter in a column are not significantly different (p = 0.05) by Student's T-test.

Impact of Paenibacillus sp. *on development of plant growth in zeolite.* According to the cumulative data gained, *Paenibacillus* sp. had a positive impact on wheat growth (Table 2). Average dry weight of inoculated wheat was higher up to 117 % as compared to control, and affected both development of shoots and root system positively.

A study of the marigold rhizosphere bacterial composition of consortium was performed after two and six weeks of plant growth. Temporal changes in community composition resulting from species replacement could be expected in microcosms just as they do in natural systems. There were no shifts in community composition in the rhizosphere of marigold within six weeks. Analysis of bacterial community by culture-independent method showed that bacteria were able to colonize plant roots and survive within plant vegetative period (data not shown). In parallel, it was exhibited by microbiological method that there were differences in the number of CFU of either strain among different sampling times within a 45-day period. Number of bacteria isolated from 1 g of fresh root increased ten-fold in populations of *K. oxytoca* and *P. agglomerans*, and hundred-fold in populations of both species of pseudomonads. Exception was *Paenibacillus* sp. that at the beginning of period of examination had a small-size population and later, after two weeks, was getting to develop rapidly. Finally, it had outgrown the partners in the consortium (Fig. 3). The growth of this bacterium seems to be favored by both bacterial partners and plant-host. It has to be noted that the *Paenibacillus* rhizosphere population had originated from a substrate where it was applied initially, on contrast to other partners that were used for seed inoculation.

Tagetes patula have been colonized well by *Glomus* sp. which formed arbuscules inside the plant roots.

Marigold seed germination in anorthosite substrate was stimulated by bacterial consortium and approached to 100 %. Control seeds (without bacteria) germinated 50—80 % worse as compared to inoculated ones. After a period of 54 days cocultivation of French marigold with the consortium of microorganisms the plant began to blossom. The control plants died after 3—4 weeks of growth.

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ВИКОРИСТАННЯ МІКРОБНОЇ СПІЛЬНОТИ У ПОПЕРЕДНЬОМУ СЦЕНАРІЇ ВИРОЩУВАННЯ TAGETES PATULA У МІСЯЧНІЙ ОРАНЖЕРЕЇ

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Розроблено прототип замкненої системи рослина-мікроорганізми для демонстрації можливості вирощування перших рослин у місячній оранжереї. Попередній сценарій вирощування *Tagetes patula* L. у субстраті анортозиту, який подібний до місячної породи як за складом мінералів, так і за хімічною будовою, передбачає використання мікробної спільноти. Мікроорганізми слугували для запобіжного заселення субстрату з метою уникнення інфекції шкідливих мікроорганізмів, а також для видобування та доставки поживних елементів з анортозиту до рослин. Модельний консорціум силікатної бактерії, бактерій-антагоністів та мікоризних грибів забезпечував задовільний розвиток та цвітіння *Tagetes patula* L. у наземних умовах під впливом факторів, що обмежують розвиток рослини.