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Immobilization and Survival of Plant Growth-Promoting Bacteria *Streptomyces griseoviridis* and *Azotobacter* sp.

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Abstract
One of the problems in the production of microbial preparations for agriculture is the maintenance of bacterial viability. One of the methods of improving the viability is an immobilization in the carrier material. Immobilization of plant growth-promoting bacteria *Streptomyces griseoviridis* and *Azotobacter* sp. in the peat and experimental ceramic granules was studied. Bacterial viability in sterile aqueous suspension and in the immobilized state was tested during 10 months of storage at three different temperatures: 20 °C, 4 °C and -18 °C. The peat and ceramic granules bound 49.3% and 47.8% of the added *Azotobacter* sp. and 26.7% and 8.2% of the added *S. griseoviridis* respectively. The number of bacterial colony-forming units (CFU) tended to increase during the first three days of storage at 4 °C and 20 °C in the suspension and in immobilized conditions and a gradual decrease of CFU was observed during storage of both bacteria with the exception of *S. griseoviridis*-containing peat at 20 °C. It is recommended to store immobilized *S. griseoviridis* in the peat at room temperature (20 °C) and *Azotobacter* sp. in the peat at -18 °C. Freezing had a good influence on the viability of *Azotobacter* sp. and is recommended for storage of *Azotobacter* sp. in the suspension and in the peat. In the case of immobilization in the ceramic granules, it is recommended to store both microbial preparations at a low temperature, best of all at -18 °C. Bacterial suspensions in sterile water can be stored at 4 °C.

Keywords: Streptomyces, Azotobacter, Immobilization, Survival, Temperature

1. Introduction
There is a growing interest in the use of plant growth-promoting microorganisms also called biostimulants due to the demand for chemically uncontaminated food. The European Biostimulants Industry Council states that "plant biostimulants contain substance(s) and/or microorganisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality" [1]. Active agents of biostimulants can be Gram-negative (*Azotobacter, Azospirillum, Bradyrhizobium, Pseudomonas, Rhizobium*) and Gram-positive (*Bacillus, Paenibacillus, Streptomyces*) bacteria [reviewed in 2]. The objects of this study were *Streptomyces griseoviridis* and *Azotobacter* sp. *Streptomyces* produce phytohormones including indole-3-acetic acid [3] as well as siderophores for complexing iron and different enzymes (amylase, catalase, lipase) and usually possess cellulolytic, chitinolytic and xylanolytic activity [4]. *Azotobacter* species are free living nitrogen fixing bacteria that also produce indole-3-acetic acid [5] as well as gibberellin and siderophores [6].
One of the biggest problems in the production of microbial preparations is the maintenance of bacterial viability. The period of validity depends on the storage temperature and carrier material of microorganisms. Many microorganisms have the ability to bind to the surface of various organic and inorganic, natural or artificial materials and many immobilization applications are described [7]. To select the most suitable material for

immobilization, bacterial viability should be checked at several storage temperatures [8]. Previously we studied immobilization and survival of symbiotic nitrogen-fixing bacterium *Rhizobium leguminosarum* biovar *viciae* [9].
The aim of this study was to immobilize *Streptomyces griseoviridis* un *Azotobacter* sp. in the peat and ceramic granules and evaluate their viability during storage at 20 °C, 4 °C and -18 °C for 10 months as well as to compare these parameters with bacterial viability in the liquid.

2. Materials and methods

Streptomyces griseoviridis MSCL 369 and *Azotobacter* sp. MSCL 299 were used in this study. The strains were cultivated in R2A agar (SIFIN, Germany) for 5 days at a temperature of 20 °C.
The tested carriers were peat (obtained from "Bioefekts" Ltd., Latvia) and experimental cylindrical (on average 5 x 10 mm) ceramic granules with an apparent porosity of 17.8%, a specific surface area of 4.30 m^2/g and bulk density 1.58 g/cm^3 made from Planci deposit (Latvia) of Devonian clay, sintered at 1200 °C and characterized in the Institute of Silicate Materials Riga Technical University. One gram of each carrier was placed in 20-ml polypropylene test tubes with caps and sterilized by autoclaving at 121 °C for 20 min. Sterile carriers were watered with three milliliters of fresh suspension of *S. griseoviridis* or *Azotobacter* sp. with OD_{540} 0.235 and 0.358 respectively. After 2.5 hours of storage at 20 °C, the excess of bacterial suspension was decanted from the test tubes with ceramic granules and discharged. A series of 1.5 ml eppendorf tubes with 0.4 ml of bacterial suspensions was prepared separately. Each tube was used for bacterial viability testing only once.
The prepared tubes were stored at a temperature of -18 °C, 4 °C and 20 °C and sampled after 1, 4, 7, 14, 42 and 302 days. Experiments were conducted in duplicate. Peat and granules were scrubbed and ground in a sterile mortar with a pestle in sterile water to recover the adhered bacteria. The number of colony-forming units (CFU) in the initial suspension and in the final liquid of detached bacteria was determined by plating 10-fold serial dilutions on R2A agar plates using the spread plate method. The results were expressed as CFU per millilitre of suspension or per gram of dry carrier. The respective detection limits were 10 CFU/ml or 2 CFU/g. Means and standard deviations were calculated. Analysis of variance and the Student *t*-test were used to test differences among groups. $P < 0.05$ was considered statistically significant.

3. Results and discussion

Both *S. griseoviridis* and *Azotobacter* sp. were immobilized in the peat and ceramic granules (Figure 1). The peat and ceramic granules bound 49.3% and 47.8% of the added *Azotobacter* sp. and 26.7% and 8.2% of the added *S. griseoviridis* respectively. The peat absorbed moisture together with the bacteria, therefore, the immobilized cell count was higher than that in the granules. According to our previous experiments with similar immobilization of *Rhizobium leguminosarum*, the amount of absorbed fluid does not correlate significantly with the number of immobilized cells [9]. Furthermore, the peat particles were smaller, with a greater surface than the ceramic granules, so the peat could bind more bacterial cells than the granules could. Nevertheless, immobilization of *Azotobacter* sp. in ceramic granules was more successful than immobilization of *S. griseoviridis* (Figure 1).
The amount of bacterial CFU tended to increase during the first three days of storage at 4 °C and 20 °C in the suspension and in immobilized conditions (Figure 2 and 3) but the differences were not statistically significant ($P>0.05$). Bashan [10] also observed proliferation of inoculated bacteria in the peat substrate after three days. He explained it with the presence of available nutrients in the peat. In the case of *S. griseoviridis*, the effect could also be due to the fragmentation of mycelium [11] during storage resulting in an increased amount of CFU.
Hereafter, a gradual decrease of CFU was observed during storage of both bacteria (Figure 2 and 3) with the exception of *S. griseoviridis*-containing peat at 20 °C (Table 1). The results obtained coincide with Junaid et al. [12] and Sabaratnam and Traquair [13], i.e. survival remains the best at low temperatures because bacterial metabolism slows and the use of nutrients and formation of toxins is reduced. From a practical point of view, we assumed that the storage is good or acceptable if the decrease of CFU did not exceed log 2 after 10 months. It fulfilled all variants with suspensions and peat as well as variants with ceramic granules and *S. griseoviridis* stored at 4 °C or -18 °C. *S. griseoviridis* showed a greater viability than *Azotobacter* sp. at 20 °C and 4 °C but a lesser viability in the frozen state, i.e. at -18 °C. However, freezing had a good influence on the viability of

Azotobacter sp. and freezing is recommended for storage of *Azotobacter* sp. in the suspension and in the peat. This is consistent with evidence of Phiromtan et al. [14] that *A. vinelandii* maintained survival at 5 °C and -16 °C after 90 days of storage.

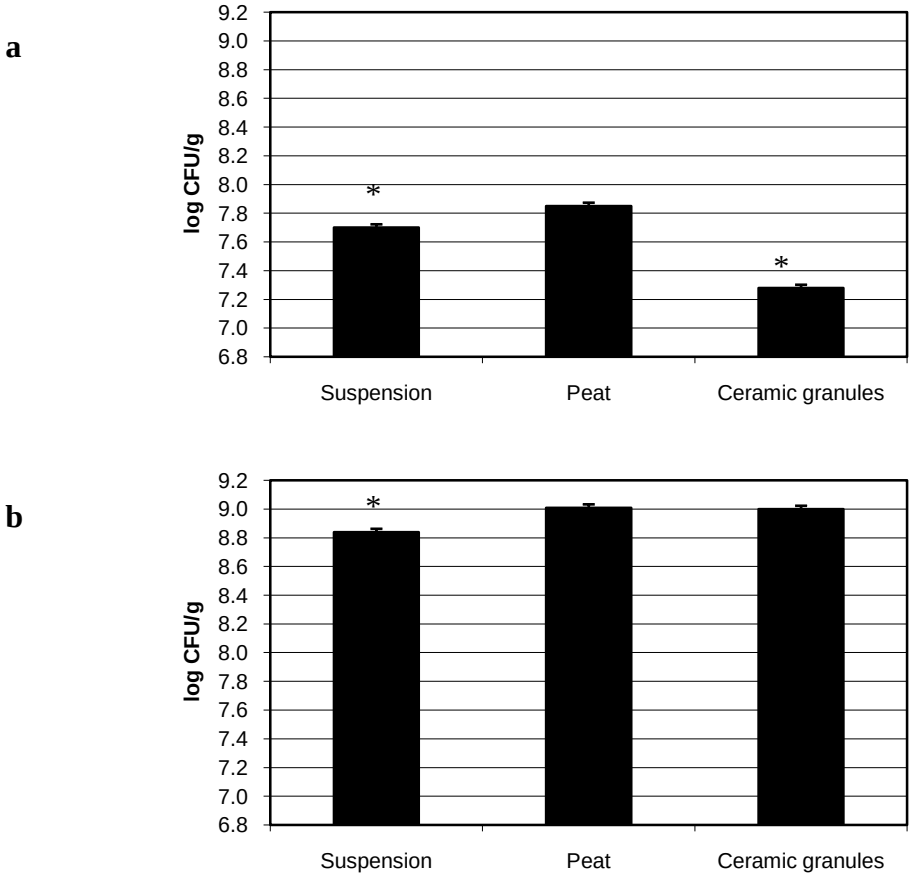

Figure 1: The number of immobilized bacteria in the peat and in the ceramic granules compared to the number of bacteria in the suspension. a – *S. griseoviridis*; b – *Azotobacter* sp. * – significant difference in comparison with peat (P <0.05).

In the case of bacterial immobilization in the ceramic granules, it is strongly recommended to store microbial preparations at a low temperature, best of all at -18 °C. Compared to *Rhizobium leguminosarum* [9], it is evident that *Azotobacter* sp. has the same trends in storage capabilities. Perhaps it is because both are Gram-negative bacteria, i.e. possess similar cell wall structures. It is known that *Azotobacter* [15] and *R. leguminosarum* [16] produce extracellular polysaccharides that have positive effects on the formation of biofilms and support existence in different adverse conditions, including desiccation.

Table 1: Loss of viability of *S. griseoviridis* and *Azotobacter* sp. after 10 months (302 days) of storage at different temperatures, log CFU/g. Data are presented as means of two experiments ± SD.

Location of bacteria	*S. griseoviridis*			*Azotobacter* sp.		
	-18 °C	4 °C	20 °C	-18 °C	4 °C	20 °C
Suspension	1.08±0.05	0.70±0.04	1.06±0.05	0.94±0.02	1.04±0.07	1.12±0.06
Peat	0.77±0.04	0.32±0.02	0.00±0.00	0.38±0.02	0.91±0.05	1.42±0.07
Ceramic granules	1.28±0.06	1.58±0.08	2.20±0.11	2.52±0.13	4.00±0.20	7.00±0.31

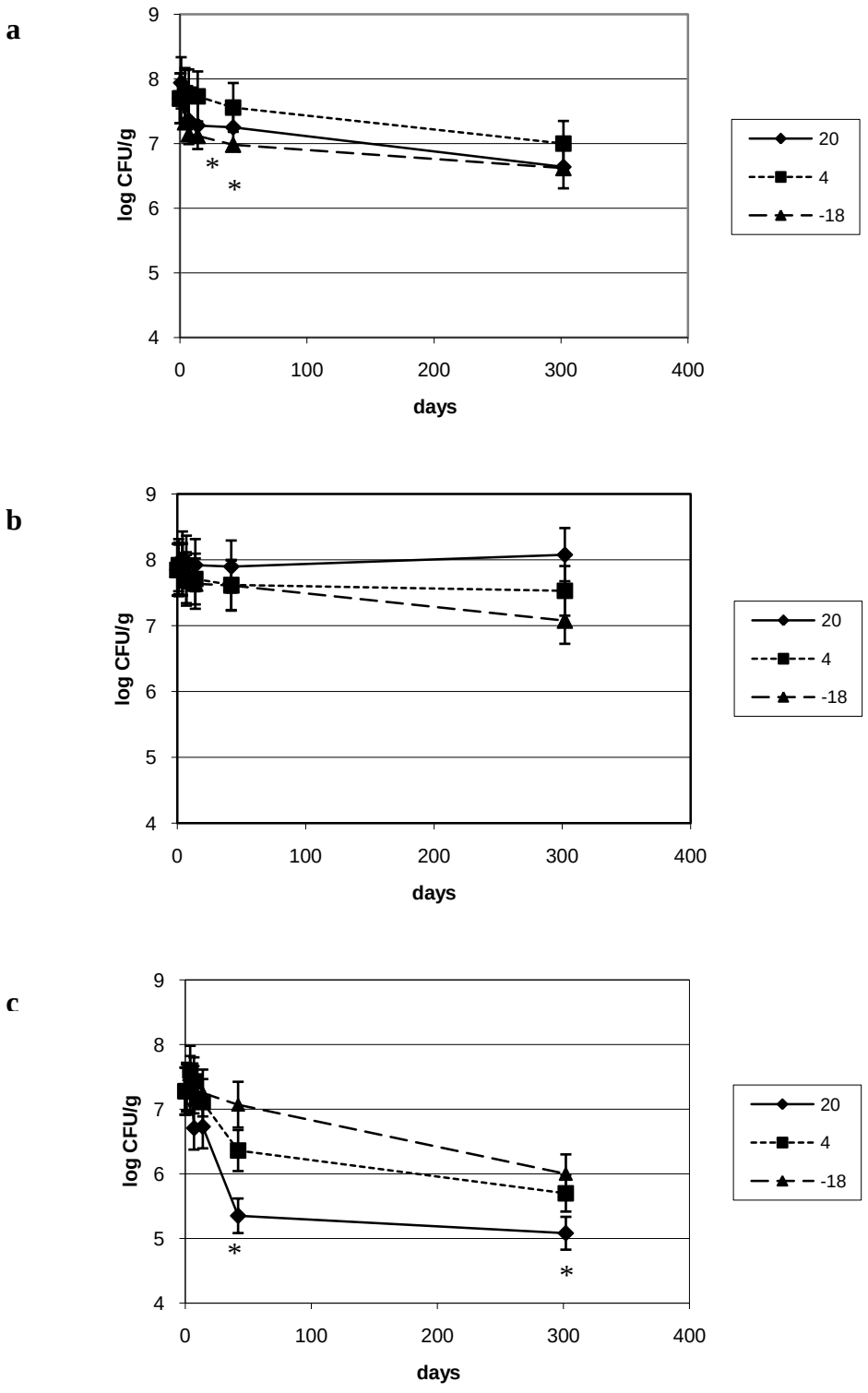

Figure 2: Viability of *Streptomyces griseoviridis* in the suspension (a) and in the peat (b) and ceramic granules (c) at different temperatures (20 °C, 4 °C, -18 °C). * – significant difference in comparison with 4 °C (P <0.05).

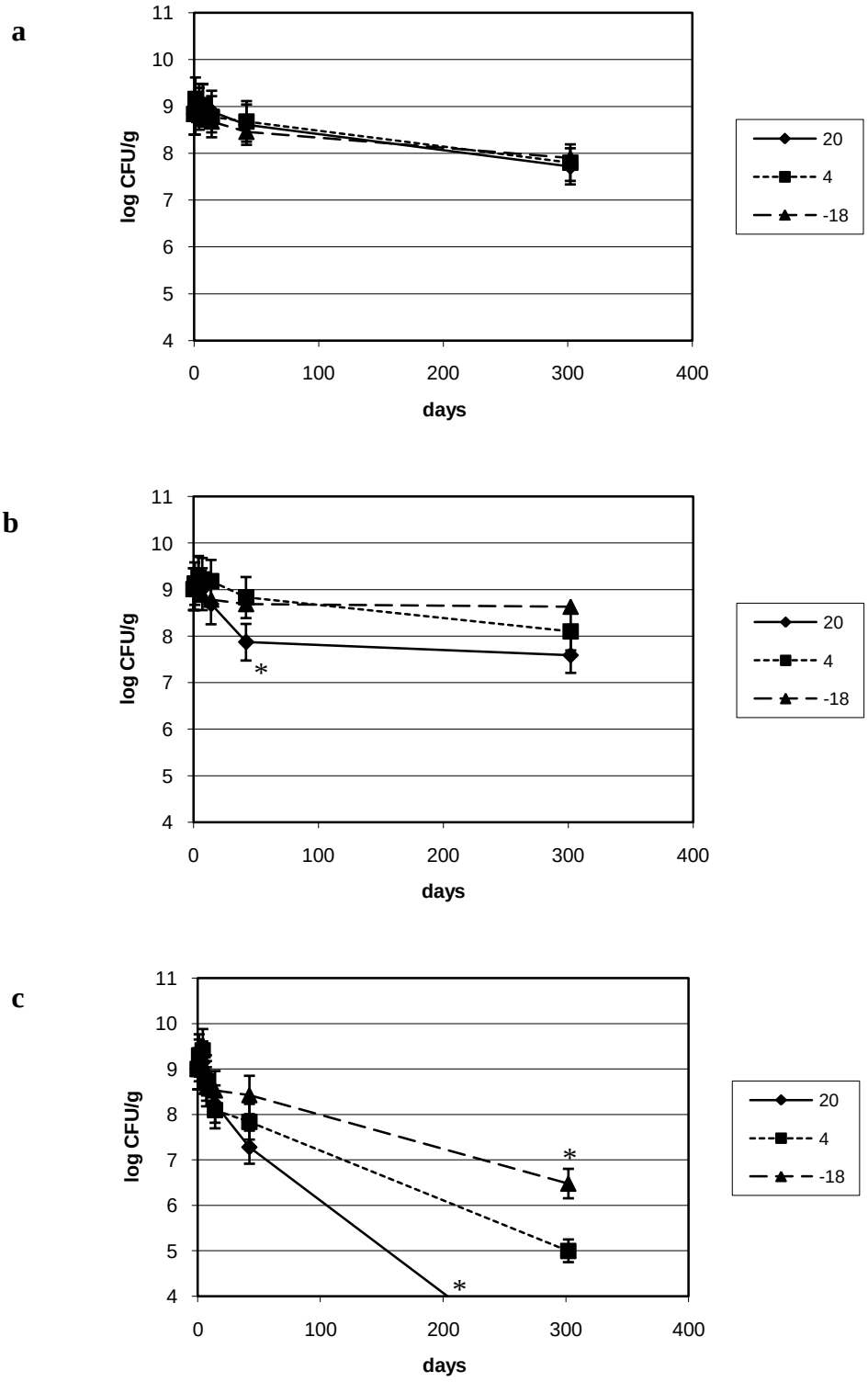

Figure 3: Viability of *Azotobacter* sp. in the suspension (a) and in the peat (b) and ceramic granules (c) at different temperatures (20 °C, 4 °C, -18 °C). * – significant difference in comparison with 4 °C (P <0.05).

Conclusions

Immobilization of *Streptomyces griseoviridis* and *Azotobacter* sp. took place in both the peat and the ceramic granules, but more bacteria bound to the peat. Both the carrier material and storage temperature affected bacterial viability. The number of bacteria increased during the first three days of storage at 4 °C and 20 °C. Afterwards, a gradual decrease of viability was observed in all variants with the exception of *S. griseoviridis*-containing peat at 20 °C. It is recommended to store immobilized *S. griseoviridis* in the peat at room temperature (20 °C) and *Azotobacter* sp. in the peat at -18 °C. In the case of bacterial immobilization in the ceramic granules, it is recommended to store microbial preparations at a low temperature, best of all at -18 °C. Bacterial suspensions in sterile water can be stored at 4 °C for at least 10 months.

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