DE GRUYTER OPEN

Material Science and Applied Chemistry doi: 10.1515/msac-2015-0013

2015/32

Immobilization and Survival of Root Nodule Bacterium *Rhizobium leguminosarum* Biovar *viciae*

Signe Žvagiņa[1], Zaiga Petriņa[2], Vizma Nikolajeva[3], Anita Lielpētere[4],
[1–3]*University of Latvia*, [4]*Bioefekts Ltd.*

***Abstract* — *Rhizobium leguminosarum* biovar *viciae* is a bacterium, which can establish nodules on roots of legumes. Rhizobial inoculants are used in agriculture as bio-fertilizers. Peat, clay powder and three kinds of expanded clay were tested for the immobilization of bacteria. The results showed that carrier material influences the success of immobilization and that storage temperature influences the survival. The best results were achieved with maintenance of bacteria in a suspension and immobilization on peat. We recommend storage of rhizobial products at a temperature of −18 °C or 4 °C.**

***Keywords* — Ceramic, clay, immobilization, peat, *Rhizobium leguminosarum*.**

I. Introduction

Rhizobium leguminosarum biovar *viciae* is a soil bacterium that can establish nodules on roots of field pea (*Pisum sativum*), faba bean (*Vicia faba*), lentil (*Lens culinaris*), and vetch (*Vicia sativa*) and fixes atmospheric nitrogen (N_2) in symbiosis with host plants [1]. This often improves the productivity of the crop.

These bacteria are Gram-negative heterotrophs with great metabolic plasticity that allows survival in many different environments, for review see [2]. Before competitive infection of the root and nodule formation, rhizobia should grow in soil without host plant and should be able to colonize the plant rhizosphere. The responses of autochthonous rhizobia to environmental factors influence their competence and nodule formation [3]. Selected rhizobial strains are used as plant growth promoting bio-fertilizers.

The first artificial inoculants were liquid bacterial cultures added to seed or directly to the soil [4]. Today commercial rhizobia inoculants can be purchased by farmers in solid, liquid or freeze-dried form. However, there is evidence of a marked decrease in viable cell numbers after freeze-drying of rhizobia [5]. Solid peat-based inoculants are commonly used [6]. Comparisons of liquid cultures with peat inoculants confirm the superiority of the later. Liquid cultures seem to lack the protection given by peat [4]. The carrier is the delivery vehicle of live bacteria. A sustainable carrier should have good water holding and aeration characteristics, provide support for bacteria, be non-toxic, easily sterilized and produced, simply handled and environmentally friendly [7]. A major role of inoculant formulation is to provide more suitable microenvironment, even temporarily, to bacteria introduced in the soil [8]. The inoculant should have sufficiently long shelf life. Most commercial carrier materials are cheap and naturally abundant, for example, peat and soil fractions. Inoculants come in four basic dispersal forms: powders, slurries, granules and liquids, for review see [8]. It is believed that peat formulations have been developed into effective and acceptable carriers, but their development has almost reached its limits. Thus, Albareda et al. [9] evaluated bagasse, cork compost, attapulgite, sepiolite, perlite and amorphous silica as alternatives to peat and found compost and perlite to be superior to peat in maintenance of different rhizospheric bacteria. But soybean inoculated with compost, perlite and liquid formulations produced seed yields that were not significantly different from those produced by peat-based inoculants. Fraser [10] began to develop a granular type inoculant already several decades ago. Granules with counts of $200 \cdot 10^6$–$500 \cdot 10^6$ g^{-1} are produced. It is possible that the granular form is better than powder inoculants for rhizobia under stressful planting conditions [11].

The objective of this study was to determine the survival of *R. leguminosarum* biovar *viciae* in liquid and on different carrier materials with the goal of developing improved formulations for rhizobia.

II. Materials and Methods

R. leguminosarum biovar *viciae* strain MSCL 802 was used in this study. The strain was cultivated in R2A agar (*SIFIN*, Germany) for 3 days at 20 °C temperature.

We tested five materials as carriers: peat (from *Bioefekts Ltd.*, Latvia); clay powder (*Ceplis Ltd.*, Latvia); oval aggregates of expanded clay (*Fibo S* from *Maxit*, Germany, and *Kano-p* from *Kurzemes Sēklas Ltd.*, Latvia); and cylindrical (on average 5×10 mm) ceramic granules with apparent porosity 17.8 %, specific surface area 4.30 $m^2 \cdot g^{-1}$ and bulk density 1.58 $g \cdot cm^{-3}$, made from Devonian clay (Plančі deposit), sintered at 1200 °C. Ceramic granules were characterized at the Institute of Silicate Materials, Riga Technical University.

All carriers were sterilized by autoclaving at 121 °C for 20 min. 15 g of sterile carrier material in a Petri dish was watered with 50 ml of suspension of *R. leguminosarum* (Fig. 1) with concentration of 10^{10} CFU ml^{-1}. Materials were stored at 20 °C and the excess liquid was decanted after 2.5 h. Carrier materials with inoculants were stored for 16 days at 20 °C temperature and periodically sampled. Experiments were conducted in duplicate. Peat, powder and granules were scrubbed and ground in a sterile mortar with a pestle in sterile water to recover the bacteria adhered to granules. The number of rhizobial CFU in the initial suspension and in the final liquid of detached bacteria was determined by plating tenfold serial dilutions on R2A agar plates using the spread plate method. Results were expressed as CFU ml^{-1} for suspension or as CFU g^{-1} for dry carrier. The respective detection limits were 10 CFU ml^{-1} or 2 CFU g^{-1}. Materials were weighted and

the moisture content of solid inoculants was calculated at sampling.

1 g of peat and cylindrical ceramic granules in 10 ml vials with screw caps was inoculated with 3 ml of *R. leguminosarum* suspension as previously and inoculants together with bacterial suspension were stored at four temperatures, i.e., 30 °C, 20 °C, 4 °C, and −18 °C. The number of CFU per gram or ml was determined as previously after 8, 20, 42 and 50 days. Experiments were conducted in duplicate.

The proportion of live and dead bacteria cells was determined using epifluorescence kit *LIVE/DEAD® BacLight™ Bacterial Viability Kit L7007* (*Life Technologies, Molecular Probes®*) after 42 days of storage.

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.

A B C D E F

Fig. 1. Suspension of *R. leguminosarum* without (A) and with carrier materials: B — *Kano-p*; C — cylindrical ceramic granules; D — *Fibo S*; E — peat; F — clay powder.

III. Results and Discussion

The suspension contained living *R. leguminosarum* cells. According to our data, rhizobia were successfully immobilized in all tested carrier materials — clay, peat, *Fibo-S*, *Kano-p* and cylindrical ceramic granules. *Kano-p* carrier contained from $10^{9.4}$ CFU g^{-1} and peat contained $10^{9.8}$ CFU g^{-1} of rhizobia (Fig. 2). However, these materials had different effects on bacterial viability during storage. The number of live bacteria had decreased by $10^{1.3}$ CFU g^{-1}, $10^{4.1}$ CFU g^{-1}, $10^{4.7}$ CFU g^{-1}, $10^{5.6}$ CFU g^{-1} and $10^{9.5}$ CFU g^{-1} in peat, clay, *Fibo-S*, *Kano-p* and cylindrical ceramic granules, respectively, after 16 days at 20 °C temperature.

The wet mass of carriers significantly increased after watering with bacterial suspension (Fig. 3). The mass increased by 19 %, 23 %, 44 %, 81 % and 91 % in *Kano-p*, cylindrical ceramic granules, *Fibo-S*, peat and clay, respectively. The amount of absorbed fluid did not correlate significantly ($r = 0.1332$, $p > 0.05$) with the amount of immobilized cells. The absorbed fluid was lost during three days of storage at 20 °C. Data showed that the tested peat initially contained about 66 % of moisture which was lost after about seven days of storage. Other materials initially contained from 0 % (*Fibo-S*) to 7 % (clay) moisture.

The decrease or loss of survival is frequently confounded with effects of moisture loss [12]. Data obtained according to our materials was not in agreement with such statements. The tested peat can be considered to be the best material for immobilization of *Rhizobium*. The greatest survival was demonstrated by suspension with loss of $10^{0.5}$ CFU g^{-1} after 16 days of storage at 20 °C (Fig. 2). After 16 days, peat had dried out and lost 65 % of the initially contained moisture and bacteria — $10^{1.3}$ CFU g^{-1}. All tested ceramic materials — cylindrical ceramic granules and oval aggregates of expanded clay, *Kano-p* and *Fibo-S*, were less suitable for the immobilization of rhizobia than peat due to a greater loss of viability. However, other studies have shown usefulness of specific ceramic materials for immobilization of several microorganisms [13]–[15]. Several factors that improve survival regarding desiccation are known. Thus, accumulation of trehalose may result in better survival. The accumulation of exopolysaccharide may also acts as a barrier reducing excessive water loss [16]. This opens opportunities for further research.

Liquid inoculants can simplify the production process. However, it is shown that bacterial survival in liquid and on inoculated seeds is worse because bacteria lack a carrier [9]. Peats from different areas of the same bog as well as peats from diverse areas differ greatly in their suitability as carriers for bacteria [17]. Therefore, it is necessary to examine the potential of particular commercial products. Final peat moisture content of 40–50 % after addition of rhizobia appeared favourable for most peats according to literature. Moisture contents below 30 % or above 60 % are generally unfavourable for survival [4]. However, 60 % moisture content was recommended for sterile peat [18]. Although our sterile peat initially contained 66 % moisture, further its content increased to 81 % during watering and then was lost after storage, it demonstrated a good survival of immobilized bacteria. We must agree with Singleton *et al.* [19] that other materials have not demonstrated performance characteristics equivalent to peat-based inoculant products.

Population declined over time, even under proper storage conditions. The study showed the influence of storage temperature on the survival of rhizobia in suspension and immobilized in peat and cylindrical ceramic granules (Fig. 4). Most bacteria remained alive in suspension and in peat but the tested ceramic granules did not support long-term survival above 0 °C (4 °C, 20 °C or 30 °C). Survival was also reduced in ceramic granules at −18 °C in comparison to suspension and peat (Table I). Results obtained using plate count technique were in agreement with the fluorescence LIVE/DEAD cell viability assay which distinguishes live bacteria with intact plasma membranes from dead bacteria with compromised membranes (Fig. 5). Better survival was observed when bacteria were maintained at −18 °C compared to 4 °C or above. Kaljeet *et al.* [20] also stored carrier materials (peat, rice husk and rice husk plus kaolin) which were inoculated with rhizobia at 4 °C and 28 °C for eight weeks and found that reduction of number of viable cell count is over a time less for peat compared to other tested materials. In experiments provided by Meade *et al.* [5], viable cell numbers of bacteria stored in peat decreased steadily from 10^{11}–10^{12} CFU g^{-1} to 10^{9} CFU g^{-1} or less during 26 weeks of storage at room temperature or at 4 °C. Absolutely optimum storage conditions for rhizobia were under refrigeration.

The performance of different formulations of rhizobia will be assayed under field conditions in future experiments.

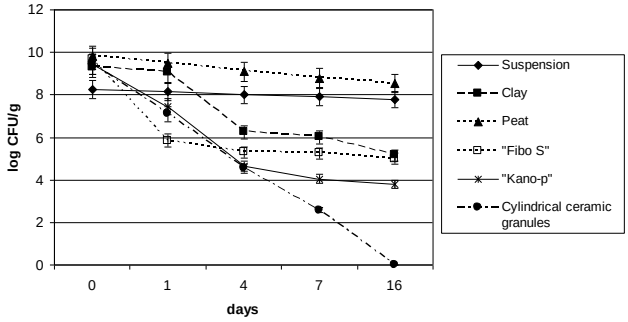

Fig. 2. Survival of *R. leguminosarum* in suspension and five carrier materials stored at 20 °C. The experiment was conducted in duplicate.

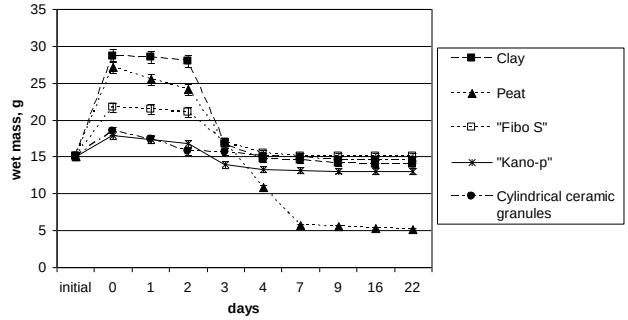

Fig. 3. Changes of wet mass of carriers with immobilized bacteria during storage at 20 °C. 50 ml of bacterial suspension were applied to 15 g of each carrier on day 0. The experiment was conducted in duplicate.

TABLE I

LOSS OF VIABILITY OF *R. LEGUMINOSARUM* AFTER 50 DAYS OF STORAGE AT DIFFERENT TEMPERATURES, LOG CFU G^{-1}

Location of bacteria	Temperature, °C			
	−18	4	20	30
Suspension	0.15	0.90	0.99	2.81
Peat	0.41	1.10	2.34	3.04
Cylindrical ceramic granules	0.80	9.00	9.00	9.00

IV. CONCLUSION

Studies have shown that carrier material influences the success of immobilization and maintenance temperature influences the survival of *R. leguminosarum*. The best results were achieved with maintenance of bacteria in suspension and immobilization on peat. We recommend storage of *R. leguminosarum* products (suspension or peat) at −18 °C or 4 °C temperature.

ACKNOWLEDGEMENT

Authors are grateful to R. Švinka and V. Švinka for providing and characterization of ceramic granules. This study was supported by the National Research Programme of Latvia "Sustainable Use of Forests and Mineral Deposits — New Products and Technologies (ResProd)", as well as EU FP7 Research Project EUROLEGUME, No. 613781.

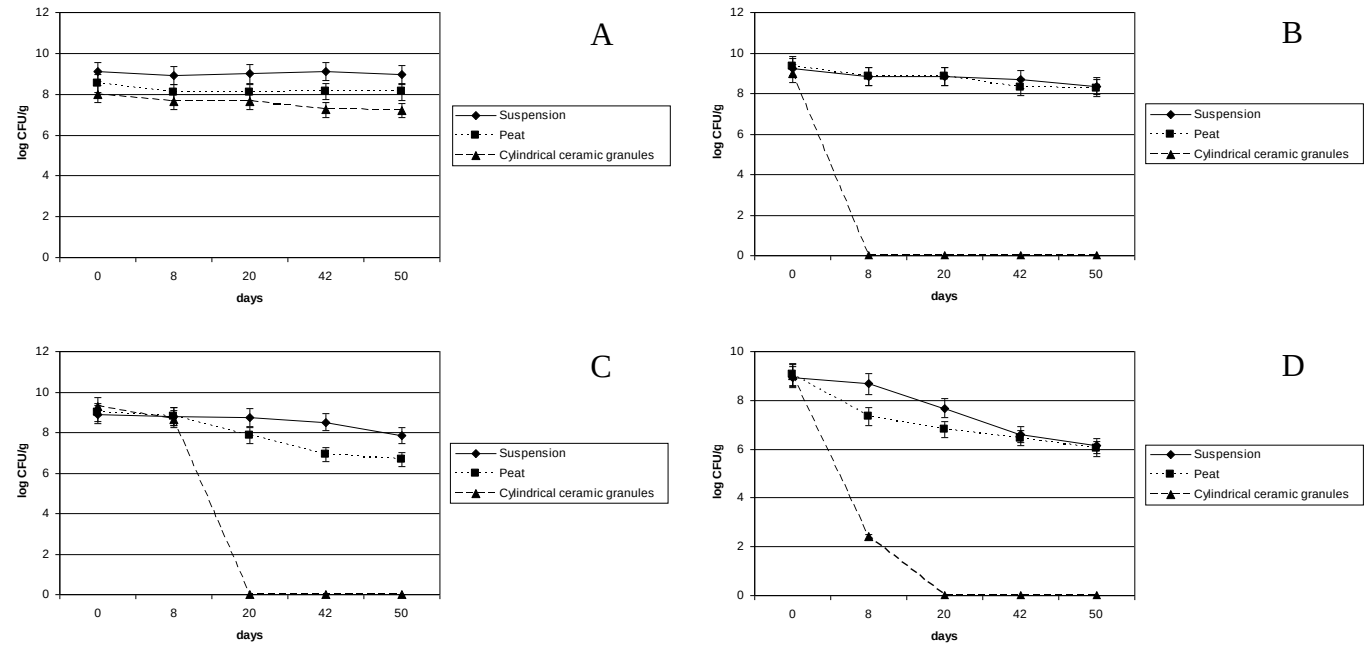

Fig. 4. Survival of *R. leguminosarum* in the suspension and carrier materials stored at −18 °C (A), 4 °C (B), 20 °C (C) and 30 °C (D). 1 g of peat and cylindrical ceramic granules was inoculated with 3 ml of *R. leguminosarum* suspension. The experiment was conducted in duplicate.

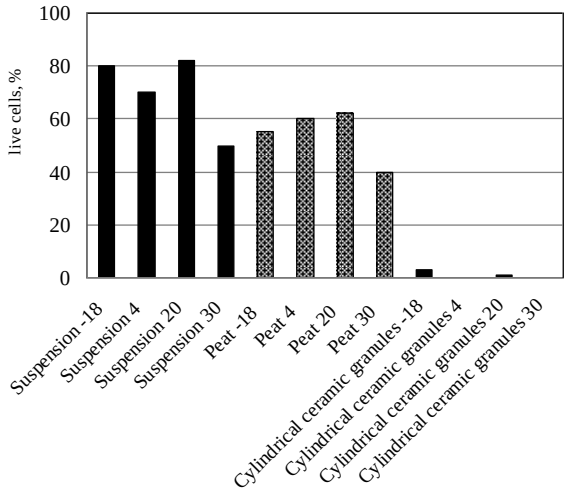

Fig. 5. Proportion of live bacteria in suspension and carrier materials after 42 days of storage at −18 °C, 4 °C, 20 °C or 30 °C, determined by LIVE/DEAD cell viability assay.

REFERENCES

1. **Kuykendall, L.D., Young, J.M., Martinez-Romero, E.,** et al. Genus I. *Rhizobium* Frank 1889, 338[AL]. In: Bergey's Manual of Systematic Bacteriology, 2nd ed., Vol. 2, Part C. Ed. G.M. Garrity. Springer, New York, 2005, pp. 325–340. http://dx.doi.org/10.1007/0-387-29298-5_82
2. **Rivas, R., Garcia-Fraile, P., Velazquez, E.** Taxonomy of bacteria nodulating legumes. *Microbiology Insights*, 2009, vol. 2, pp. 51–69.
3. **Vlassak, K.M., Vanderleyden, J.** Factors influencing nodule occupancy by inoculant rhizobia. *Crit. Rev. Plant Sci*., 1997, vol. 16, pp. 163–229. http://dx.doi.org/10.1080/07352689709701948
4. **Hamdi, Y.A.** Application of nitrogen-fixing systems in soil improvement and management. Rome: Food and Agriculture Organization of United Nations, 1982, 188 p.
5. **Meade, J., Higgins, P., O`Gara, F.** Production and storage of *Rhizobium leguminosarum* cell concentrates for use as inoculants. *J. Appl. Microbiol*., 2008, vol. 58, pp. 517–524.
6. **Laranjo, M., Alexandre, A., Oliveira, S.** Legume growth-promoting rhizobia: An overview on the *Mesorhizobium* genus. *Microbiological Research*, 2014, vol. 169, pp. 2–17. http://dx.doi.org/10.1016/j.micres.2013.09.012
7. **Khavazi, K., Rejali, F., Seguin, P., Miransari, M.** Effect of carrier, sterilisation method, and incubation on survival of *Bradyrhizobium japonicum* in soybean (*Glycine max* L.) inoculants. *Enzyme and Microbial Technology*, 2007, vol. 41, pp. 780–784. http://dx.doi.org/10.1016/j.enzmictec.2007.06.011
8. **Bashan, Y**. Inoculants of plant growth-promoting bacteria for use in agriculture. *Biotechnology Advances*, 1998, vol. 16, pp. 729–770. http://dx.doi.org/10.1016/S0734-9750(98)00003-2
9. **Albareda, M., Rodriguez-Navarro, D.N., Camacho, M., Temprano, F.J.** Alternatives to peat as a carrier for rhizobia inoculants: Solid and liquid formulations. *Soil Biology & Biochemistry*, 2008, vol. 40, pp. 2771–2779. http://dx.doi.org/10.1016/j.soilbio.2008.07.021
10. **Fraser, M.E.** A method of culturing *Rhizobium meliloti* on porous granules to form a pre-inoculant for lucerne seed. *J. Appl. Bact.*, 1975, vol. 39, pp. 345–351. http://dx.doi.org/10.1111/j.1365-2672.1975.tb00581.x
11. **Smith, R.S.** Legume inoculant formulation and application. *Can. J. Microbiol.*, 1992, vol. 38, pp. 485–492. http://dx.doi.org/10.1139/m92-080
12. **Herridge, D.F., Hartley, E., Gemell, L.G**. Rhizobial counts in peat inoculants vary amongst legume inoculant groups at manufacture and with storage: Implications for quality standards. *Plant and Soil*, 2014, vol. 380, pp. 327–336. http://dx.doi.org/10.1007/s11104-014-2087-8
13. **Muter, O., Potapova, K., Nikolajeva, V., Petriņa, Z., Griba, T., Patmalnieks, A., Švinka, R., Švinka, V.** Comparative study on bacteria colonization onto ceramic beads originated from two Devonian clay deposits in Latvia. *Scientific Journal of RTU: Material Science and Applied Chemistry*, 2012, vol. 26, pp. 134–139.
14. **Muter, O., Berzins, A., Potapova, K., Strikauska, S., Stelmahere, S.** Bacteria immobilization on ceramic beads for soil remediation technologies. *Journal of International Scientific Publications: Ecology & Safety* (ISP: EB), 2011, vol. 5, pp. 152–162.
15. **Nikolajeva, V., Griba, T., Petriņa, Z.** Factors influencing adhesion of *Pseudomonas putida* on porous clay ceramic granules. *Environmental and Experimental Biology*, 2012, vol. 10, pp. 77–80.
16. **Deaker, R., Roughley, R.J., Kennedy, I.R.** Legume seed inoculation technology – a review. *Soil Biology & Biochemistry*, 2004, vol. 36, pp. 1275–1288. http://dx.doi.org/10.1016/j.soilbio.2004.04.009
17. **Burton, J.C.** *Rhizobium* species. In: Microbial technology. Vol. 1. Microbial processes. Ed. H.J. Peppler, D. Perlma. New York: Academic Press, 1979, pp. 29–58. http://dx.doi.org/10.1016/b978-0-12-551501-6.50007-3
18. **Roughley, R.J**. 1970. The preparation and use of legume seed inoculants. *Plant and Soil*, 1970, vol. 32, pp. 675–701. http://dx.doi.org/10.1007/BF01372900
19. **Singleton, P., Keyser, H., Sande, E.** Development and evaluation of liquid inoculants. In: Inoculants and nitrogen fixation of legumes in Vietnam. Ed. D. Herridge. ACIAR Proceedings, 2002, No. 109, pp. 52–66.
20. **Kaljeet, S., Keyeo, F., Amir, H.G.** Influence of carrier materials and storage temperature on survivability of rhizobial inoculant. *Asian J. Plant Sci.*, 2011, vol. 10, pp. 331–337. http://dx.doi.org/10.3923/ajps.2011.331.337

Signe Žvagiņa, *Bc. biol.* (2015), is a master level student of biology in University of Latvia with specialization in microbiology. She is a laboratory assistant in Institute of Microbiology and Biotechnology, University of Latvia.
E-mail: sz11051@lu.lv

Zaiga Petriņa has a master's degree in biology, University of Latvia. She is a research assistant in Institute of Microbiology and Biotechnology and in Faculty of Biology, University of Latvia. She has worked in various scientific projects related to microbiology. Z. Petriņa is a member of the Microbiological Society of Latvia.
E-mail: zaiga.petrina@lu.lv

Vizma Nikolajeva, *Dr. biol.*, docent and senior researcher at the Department of Microbiology and Biotechnology, Faculty of Biology, University of Latvia, has participated in various projects related to microbiology. V. Nikolajeva obtained a diploma in biology from the University of Latvia. Her *Dr. biol.* thesis was "Catalytic properties and biological activity of adenylate deaminase produced by microscopic fungus *Penicillium lanoso-viride*"(1983, Lomonosov Moscow State University, Russia; 1992, A. Kirchenstein Institute of Microbiology and Virology, Latvia). Her fields of specialization are general microbiology, environmental microbiology and food microbiology. V. Nikolajeva is a member of the Microbiological Society of Latvia.
E-mail: vizma.nikolajeva@lu.lv

Anita Lielpētere, *Dr. biol.*, is a microbiologist. She obtained a diploma in biology from the University of Latvia and has participated in several scientific projects in the A. Kirchenstein Institute of Microbiology and Virology and in the University of Latvia. In 1993, she created a company *Bioefekts Ltd.* that already more than 20 years produces microbiological preparations for agriculture.
E-mail: info@bioefekts.lv

Signe Žvagiņa, Zaiga Petriņa, Vizma Nikolajeva, Anita Lielpētere. Gumiņbaktērijas *Rhizobium leguminosarum* biovar *viciae* imobilizācija un dzīvotspēja.

Rhizobium leguminosarum biovar *viciae* ir augsnes baktērija, kas spēj veidot gumiņus uz zirņu, pupu, lēcu un vīķu saknēm un saistīt atmosfēras slāpekli simbiozē ar augiem. Gumiņbaktērijas izmanto lauksaimniecībā kā bioloģiskus mēslošanas līdzekļus augu augšanas veicināšanai. Komerciālos gumiņbaktēriju preparātus var iegādāties sausā, šķidrā vai liofilizētā formā. Parasti izmanto sausos sagatavotos mēslošanas līdzekļus uz kūdras bāzes. Šī pētījuma mērķis bija imobilizēt *R. leguminosarum* un noteikt tās dzīvotspēju šķidrumā un dažādos nesējmateriālos, lai izstrādātu uzlabotus gumiņbaktēriju preparātus. Imobilizēšanai izmantoja piecus sterilizētus materiālus: kūdru, māla pulveri, divu veidu ovālus šūnainās keramikas agregātus un cilindriskas keramikas granulas, kas izgatavotas no Planču depozīta Devona perioda māla. Imobilizāciju veica 2,5 stundu laikā 20 °C temperatūrā. Vēlāk kūdru, pulveri un granulas noberza un sasmalcināja ūdenī ar sterilu piestiņu, lai atdalītu pielipušās baktērijas. Baktēriju kolonijas veidojošo vienību skaitu noteica, izsējot iegūto suspensiju atšķaidījumus Petri traukos ar agarizētu barotni. Dzīvotspēju noteica arī ar LIVE/DEAD epifluorescences metodi. Iegūtie rezultāti parādīja, ka nesējmateriāls ietekmē imobilizācijas sekmes un ka uzglabāšanas temperatūra ietekmē *R. leguminosarum* dzīvotspēju. Vislabākos rezultātus ieguva, uzglabājot baktērijas suspensijā vai imobilizējot uz kūdras. Rekomendējam uzglabāt *R. leguminosarum* produktus −18 °C vai 4 °C temperatūrā.