Inoculation technology for legumes based on alginate encapsulation

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Abstract. The main purpose of seeds inoculation is to provide the sufficient number of viable efficient bacteria that are able to actively colonize the plant roots immediately after germination. One of the promising forms of bacterial preparations is cells encapsulation in the polymer gel. Advantages of using alginate microspheres are slow, controlled release of bacteria, biodegradation in the soil and an increased shelf life. As a result of this study the effectiveness of using capsulated biopreparation was established to increase the nitrogen-fixing potential of legumes. The advantage in colonization activity is shown in comparison with other forms of the biopreparations due to the slow release of rhizobium from the capsules. The optimal composition for formulation is established which ensures the storage of biopreparation for more than 1 year. The prospect of using encapsulated biopreparations under adverse environmental conditions and for joint application with chemical pesticides and agrochemicals is analyzed.

Key words: biopreparations, encapsulation, sodium alginate, legume crops, inoculation.

INTRODUCTION

Immobilized preparative forms of microorganisms in biofertilizers are perspective for use in sustaintable agriculture and attracting the attention of researchers and farmers in present time. In this form bacterial cells more resistance to aggressive environmental factors (Digat, 1991; Amiet-Charpentier et al., 1999; Lebsky et al., 2001; Bashan, 2014) as well, a number of researchers (Bashan & Gonzalez, 1999) point to the possibility of increasing the shelf life of bacteria in alginate granules up to 14 years.

Especially this technology can be relevant in creation and use of biopreparations based on nitrogen-fixing microorganisms, as the most effective method for productivity increasing of crops, yield quality and effectiveness of inoculants for legume plants (Burton, 1976; Jung, 1982; Chen & Huang, 1988; Carrillo-Gracia et al., 2000).

The main criteria for choosing of carrier for nodule bacteria is the cheapness, ease for use, moisture capacity and non-toxicity (Lewis & Papaviz, 1985; DeLucca et al.,

1990; Smith, 1992; Cassidy, 1996; Fenice et al., 2000). Peat has been used for a long time for these purposes but the main drawback of peat is the high variability of the samples depending on their origin. The organic polymers (carrageenan, agar, gelatin, chitosan and alginate) can be used as an alternative to peat, organic waste, inert materials.

The technology of inclusion in alginate gels refers to the soft methods of immobilization – cells remain alive and can carry out polyenzyme processes. The positive property of gel is the cells ability to multiply, as well as its ability to dissolve when the pH and temperature changes. The polymer can be sterilized by autoclaving and, in addition, the immobilization process is reversible, and processed by addition of a Ca^{2+} binding agent (eg EDTA, citric acid, monovalent cations or complex anions – citrates, phosphates, lactates). This makes it possible to isolate viable cells and facilitate to study of their properties (Thompson, 1980; Stormo & Crawford, 1992; Trevors et al., 1992; Carrillo & Bashan, 1997; Amiet-Charpentier, 1998). The advantages of alginate using are slow, controlled release of bacteria, biodegradation in the soil, and an increased shelf life.

MATERIALS AND METHODS

Materials
Sodium alginate was purchased from Sigma Aldrich (Alginic acid sodium salt, low viscosity) as 2 wt % solution. Other chemicals were reagent-grade products (Fluka) used without further purification. *Rhizobium* cells were cultured in YMB medium (Yeast Mannitol Broth). The standard medium includes mannitol, sucrose or glycerol as the carbon source, yeast extract as a source of nitrogen, growth factors, and mineral salts.

Bacterial strains
Mesorhizobium ciceri ST-282 and *Bradyrhizobium japonicum* M8 strains received from work collection of laboratory were used in this work.

Immobilization procedures
For the formation of alginate granules various compositions are used, as indicated in Table 1. In all experiments sodium alginate was used in an amount of 2.0% from total volume. Clays of kaolin, bentonite, gelatin and pectin were used as modifiers on the basis of literature data. (Ajayi et al., 2012; Devi & Kakat., 2013; Belscak-Cvitanovic et al., 2015; Batista et al., 2017) The composition also included glycerin (as osmoprotector) and sucrose for better dissolution of alginate and as additional source of nutrition. All ingredients were dissolved in distilled water and sterilized at 121 °C for 20 minutes. After sterilization centrifuged suspension rhizobial bacteria in amount of 20% of total volume of preparation was added to the mixture and mixed on shaker for 30 minutes. To determine the viscosity of the alginate matrix viscometric method was used, to determine the acidity the pH was measured using pH-meter. Then granules were formed with a mechanical dispenser in a 1.0–3.0% sterile calcium chloride solution and held for 30–40 minutes with slow stirring. Water-soluble sodium alginate was converted to water-insoluble calcium alginate. The resulting granules were washed with sterile 0.85% NaCl solution and stored in sealed bags. Diameter of granules was determined using microscope with occlusal ruler.

Table 1. Labeling of samples and main analytical data

Sample	Encapsulation mix	Mean bead Diameter, mm	Bacteria cell Density, 10^8CFU g^{-1}	Cell leakage after 24 h
1	Na alginate + gelatine	3.8 ± 0.2	82	39%
2	Na alginate + pectin	4.9 ± 0.4	78	42%
3	Na alginate + kaolin	3.5 ± 0.6	86	10%
4	Na alginate + bentonite	3.7 ± 0.4	83	28%

Microorganism viability evaluation during immobilization treatments

1 g of microspheres loaded with microbial cells was destruction by adding EDTA 5% and homogenized with 10 cm^3 of distilled water to obtain complete and homogeneous dispersion of cells. The cell density of *Mesorhizobium ciceri ST-282* and *Bradyrhizobium japonicum* M8 was evaluated by plate count on yeast mannitol agar. Plates incubated for 7 days at 28 °C. The viability was determined for both free and entrapped cells, in alginate beads. The results were expressed as cfu living cell per 1 g of microspheres.

Cell leakage and colonization activity

Seeds of chickpea Volgogradskyi-10 cultivar selected in N.I. Vavilov Research Institute of Plant Industry, Russia were used. Chickpea seeds were surface-sterilized in a mercuric chloride solution for 7 min, washed in sterile water to remove mercuric chloride and germinated for 2 days in Petri dishes with moist vermiculite at 28 °C under sterile conditions. After that, chickpea seeds were placed in specially prepared gnotobiotic conditions (Simons et al., 1996). Sprouted seeds were sown on stainless steel grids placed into sterile glass vessels containing 100 mL of deionized water, mineral salts and 6.5 g of microspheres whiz bacteria *M. ciceri ST-282*. Incubated in a growth chamber at 16–18 h day light/darkness cycle and a temperature of 23–18 °C for 7 days.

Microbial cells leak from the microspheres was determined by plating on YMA media. The amount of bacterial cells was determined every 24 hours, for 19 days in the medium where chickpea plants were grown.

Fluorescent *in situ* Hybridization (FISH) and Confocal Laser Scanning Microscopy (CLSM)

Samples were fixed in 4% paraformaldehyde solution mixed with phosphate buffer in the volumetric proportion of 3:1 (Sambrook, 2001). For further treatment separate microsections of chickpea roots were placed in sterile 1.5 mL tubes. Fixed samples were hybridized with rRNA specific oligonucleotide probes following (Shcherbakov et al., 2013). We used for hybridization an equimolar mixture of universal bacterial samples EUB338, EUBII338, EUBIII338 (Amann et al., 1990; Daims et al., 1999). In the end the samples were mounted on an object slide and covered with a ProLong Gold Antifade reagent (Invitrogen, Germany).The preparations were studied on the Leica TCS SPE confocal microscope (Leica Mycrosystems, Germany). To detect oligonucleotide probes labeled with 6FAM, Cy3 and Cy5 fluorochromes, lasers with awave length of 488, 532 and 635 nm were used. Fluorescence was registered in the range of 508–566 nm, 665–607 nm and 657–709 nm, respectively.

Biodegradation of alginate microspheres

Microspheres with immobilized bacteria were placed in glass vessels containing moist soil for growing plants, 30 microspheres per vessel and were buried 5 cm below the soil surface in the natural soil described below. The soil was aged at 30 °C for 15 days and kept slightly below saturation with water, adding distilled water if necessary. Every 3 days, they were pulled out of the soil, and each of the beads was examined under stereoscopic microscope (Stemi 508, Zeiss, Germany). The diameter of all microspheres was measured immediately, the capsules were placed back into the soil after the measurements.

Inoculation of chickpea and soybean plants with microbead inoculant containing *Mesorhizobium ciceri* ST-282 and *Bradyrhizobium japonicum* M8

Field tests of 2016 were carried out on the experimental field of ARRIAM Research Institute, St. Petersburg, Pushkin. The area of experimental plots of 25 m², four-time repetition of experiments. For field experiment we used chickpea and soybean seeds var. Krasnokutsky and var. Lidiya. The seeding rate was 500,000 seeds ha⁻¹.

Biopreparation granules mixed with the seed at the rate of 1% by weight of the seeds. Options which used liquid preparation was treated as previously written. The seeds were sown when the soil temperature 7–8 °C, to the depth of 6–7 cm.

Statistical analysis

Results for nodulation, growth parameters, cell number in biopreparations were subjected to variance analysis. All measurements in each experiment were performed three times independently, producing similar results. Three independent experiments were conducted for each variant. They were considered as biological replications.

The means were compared by the least significant difference (LSD) test at $P = 0.05$ with the Diana Software (ARRIAM, St Petersburg, Russia).

RESULTS AND DISCUSSION

Development of biopreparation composition for immobilized bacteria

Immobilization by adsorption and incorporation into the spatial structure of alginates is the most mild and preferred method for fixation living bacterial cells. The polysaccharide chains are first joined together by hydrogen bridges when the alginate gel is formed, and then these chains form the cellular structure by binding to calcium ions. In the middle of each cell is the calcium ion (Idris & Suzana, 2006; Cruz et al., 2013).

On Fig. 1 shown the forms of preparations containing bacteria *Mesorhizobium ciceri* ST 282. The diameter of alginate granules was 3–4 mm.

As a result of preliminary studies it was found that the survival of bacterial cells in a liquid culture is extremely low, the titer drops significantly during 1−1.5 months by 3–6 orders. The composition No.1 with gelatin and No.2 with pectin, had a lower viscosity compared with compositions containing clays. The high acidity of pectin made it difficult to form the polymer matrix, so in the No.2 variant the granules did not have a spherical shape. Granules from composition No.3 with kaolin and No. 4 with bentonite

had a spherical shape. According to literature date bentonite and kaolin use as structure-forming agent by increasing the viscosity of alginate to maintain the spherical shape of the granules, while the spherical shape is maintained even at lower concentrations of alginate. The addition of clays increased the viscosity and improved the stability of granules and their mechanical strength, in addition, the clays possess sorption properties. Variation of bentonite content in biopreparations does not effect on effectiveness of immobilization and the number of 'trapped' cells does not change (Bashan, 1986b; Paul et al., 1993; Gonzalez & Bashan, 2000). Clays have a lower cost compared to pectin and gelatin.
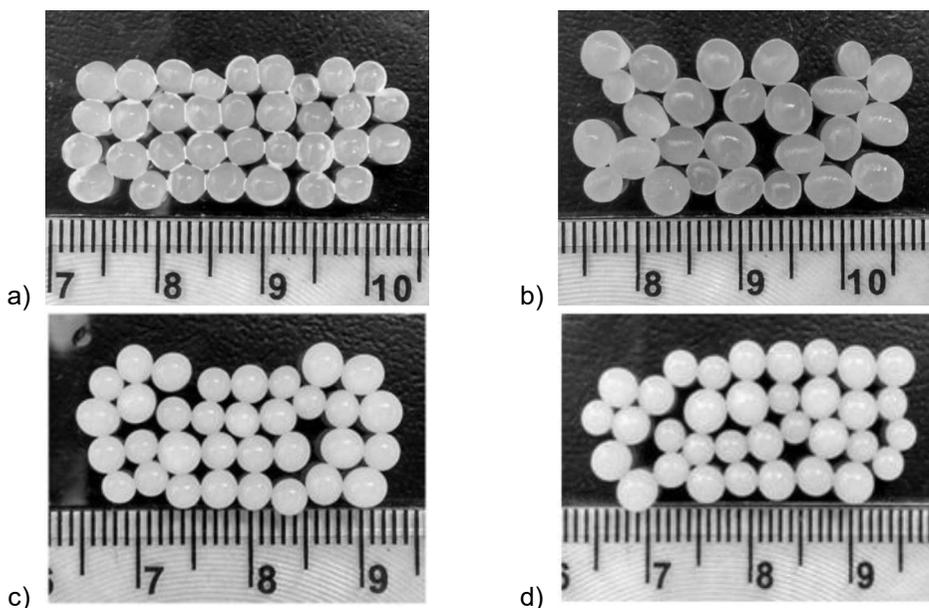

Figure 1. The granullar form of biopreparation with immobilized cells *Mesorhizobium ciceri ST-282*: a – alginate + gelatin; b – alginate + pectin; c – alginate + kaolin; d – alginate + bentonite.

For further studies the composition containing 2% kaolin as a modifier of biopreparation was chosen (Table 1). The samples of biopreparation with immobilized microorganisms *Mesorhizobium ciceri* ST-282 and *Bradyrhizobium japonicum* M8 was stored in sterile polyethylene bags with airtight clasps at a temperature of 4 °C. Each month during the year the number of cells in Ca-alginate granules was determined.

Survival of bacterial cells in alginate granules

The alginate granules were used with initial concentration of bacterial cells *Mesorhizobium ciceri* ST-282 $(8.6 \pm 0.3) \cdot 10^9$ CFU g^{-1} and *Bradyrhizobium japonicum* M8 $(10 \pm 0.4) \cdot 10^9$ CFU g^{-1}. The Fig. 2 shows the changes in the cell number of bacteria in biopreparation during storage period (1 year).

As the result of studies it was determinate the survival of bacteria in alginate granules, it was found that the titer of immobilized cells decreased smoothly during the first 3 months of storage (Fig. 2). There was a slight decrease in the titer of living cells at the subsequent period of storage from 4 to 12 months, the number of bacteria was

maintained stably within the error of measurement. The cell number of *Mesorhizobium ciceri* ST-282 became $19.5 \cdot 10^8$ CFU g^{-1}, and *Bradyrhizobium japonicum* M8 – $29.5 \cdot 10^8$ CFU g^{-1} by the end of the year of storage. Thus, the developed formulation containing nodule bacteria immobilized into the granules of the alginate gel is able to stably preserve the bacteria during long-term storage which create more opportunities for its use in agriculture.
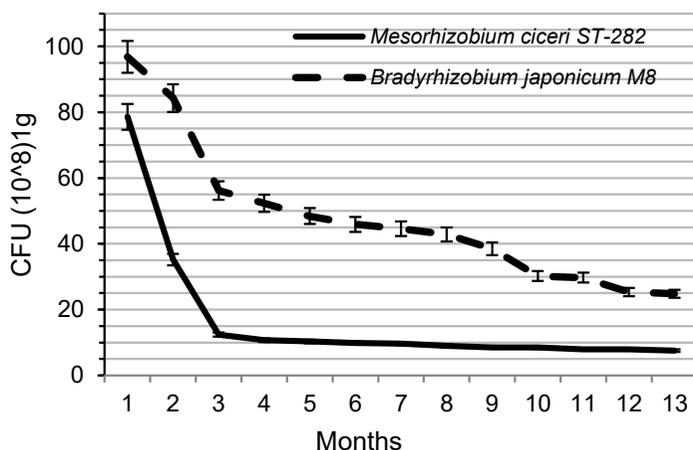

Figure 2. Cell number of bacteria *Mesorhizobium ciceri* ST-282 and *Bradyrhizobium japonicum* M8 in alginate granules depending on the storage time.

Degradation of Ca-alginate granules in soil

The microbiological and fermentative activity of the soil is active factor of hydrolytic and degradation processes. It is known that many soil microorganisms degrade natural polymers using them as a substrate for growth (Vassilev et al., 1997; Wan et al., 1992). There is the gradual degradation of the alginate gel under the action of the soil microorganisms and also due to substances that bind Ca^{2+} ions (Fravel et al., 1985; Sadasivan & Neyra, 1985; Kenney, 1997; Roger et al., 2006) in the soil. Study the biodegradation process noted that the presence of capsules in the soil (as additional substrate) activated the microorganisms while the processes of mineralization of organic matter were more active in experimental soil samples than in the control (soil without polymer). The investigated microbiological soil background is generally characteristic of rich chernozem soils. The total number of microorganisms from experimental soil samples was on the average 1.5 and 1.3 times higher than in control. The number of microorganisms of hydrolytes and oligotrophs significantly increased in the presence of alginate capsules what indicates more intensive processes of destruction of organic substances in experimental soil variants compared to the control. Thus, microorganisms actively reacted to the introduction of the polymer, as an additional nutrient substrate, into their habitat by its decomposition and utilization.

Changes in the shape and structure of alginate beads were noticeable after 7 days. The size of beads was significantly reduced in comparison with control (Fig. 3) after 14 days from beginning what indicate the biodegradation process of alginate capsules.
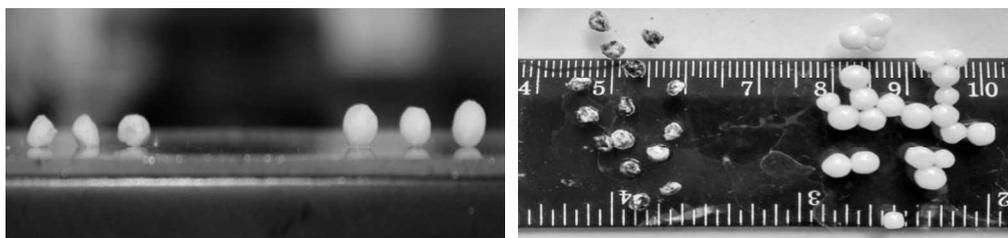
Figure 3. Visual assessment of biodegradation: A – after 7 days of biodegradation process, B – after 14 days of biodegradation process (on the left – alginate capsules, from vessels with soil, on the right – capsules that were kept in sterile Petri dishes).

The release dynamics of bacterial cells from granules into environment

Dynamics of bacterial release into environment has been slow at initial period (Fig. 4). During the first 10 days the release of bacteria into external environment was low at the level of 5–8% from initial content. After 20 days of experiment the content of nodule bacteria in media increased up $19.5 \cdot 10^8$ CFU g^{-1} for *Mesorhizobium ciceri* ST-282 and $29.5 \cdot 10^8$ CFU g^{-1} for *Bradyrhizobium japonicum* M8.
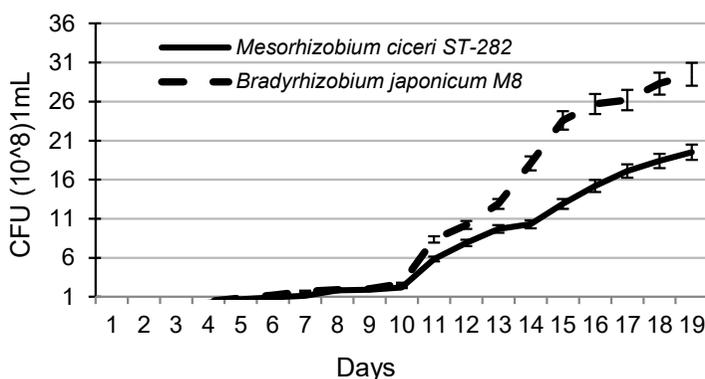

Figure 4. The release dynamics of bacteria *Mesorhizobium ciceri* ST-282 and *Bradyrhizobium japonicum* M8 from alginate granules.

The increased exudation of plant roots with organic acids, which stimulates the multiplication of rhizobium cells and accelerates the destruction of the polymer matrix, thereby facilitating the release of bacteria into the environment from alginate granules. The sharp increase in the number of bacteria coincides with period of active growth and development of root system which promotes active bacterial colonization of plant roots. As is known, a significant part of nodule bacteria is killed before the germination of plant as adverse conditions and lack of nutrition adversely affect them. The introduction of nodule bacteria in the alginate gel provides a sustained release of bacteria and retains them for successful colonization (Shcherbakova et al., 2017).

Colonization activity and spatial localization of bacteria on the roots

The colonization potential of nodule bacteria from alginate granules was rather high, bacteria are well established on the surface of chickpea roots. The maximum values

of root survival were recorded for the variant using alginate granules and amounted to $(8.3 \pm 0.7) \cdot 10^5$ CFU g^{-1}, in the control variant the number of bacterial cells was an order of magnitude lower $(6.4 \pm 0.4) \cdot 10^4$ CFU g^{-1}. It should be noted that the growth-stimulating effect on the variant where the alginate granules were used was the best.

The cells of *Mesorhizobium ciceri* ST-282 was localized on the surface and inside the root using specific oligonucleotide probes allowed the visualization. The roots for hybridization were selected on the 14th day of experiment. The Fig. 5 illustrates the location of bacterial microcolonies which were found both on the rod and on the lateral roots.
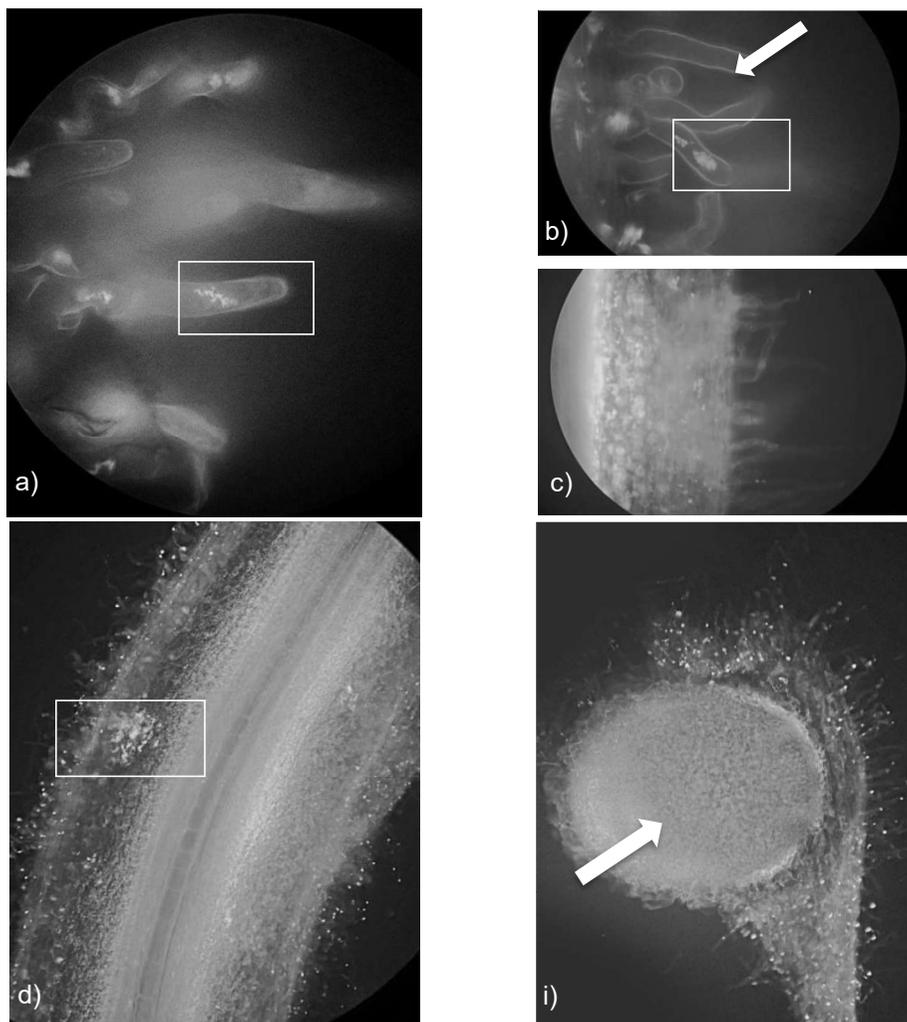

Figure 5. Localization of *Mesorhizobium ciceri* ST-282 on the surface and inside the root of chickpea plants using fluorescent *in situ* hybridization and epiluminescence microscopy: a and b – the root hairs with introduced bacteria, 400X; c – the general view of the root part with bacteria colonized its surface, 200X; d – formation of nodule, bacterial cells and initiation bacteroides development, 40X; i – longitudinal section of nodule, 40X.

The highest density of microbial populations was found on the lateral roots of the plant in the places of so-called 'feeding points' where the roots exudate nutrients. As is known, infection of legume plants with nodule bacteria begins with the reaction of bacteria to the appearance of signaling and nutrients released by host roots. The cells are attach to the root surface. The twisting and deformation of root hairs induced by bacteria (Fages, 1990; Elsas et al., 1992; Hernández-Carmona et al., 1999) can be seen on Figs 5, a and b (root hairs with introduced bacteria, the places of penetration of bacteria into the root hair are visible). Fig. 5, c shows the general view of the root part of the root with bacteria colonized on its surface.

Nodule bacteria penetrate the twisted root hairs at the point of greatest bending and introduced into them in the form of tubular structure called infectious filament. These tubes carry the rhizobium cells usually in the single chain to the base of the root hair (to the basal cell) (Bashan & Holguin, 1994; Grube et al., 2009). Immediately after release bacteria encapsulated by the cytoplasmic membranes of the host cells and never come into direct contact with the cytoplasm of host cells (Bashan & Levanony, 1989; Puente et al., 1999).

On Fig. 5, d present the beginning of formation the future nodule and localization of bacterial cells, initiation of bacteroides formation. Fig. 5, i shows the longitudinal section of nodule, the compaction nodule part adjacent to the root hair is seen, from the side of outer wall of nodule the localization of bacteroids and more loose structure is observed what indicates the further growth of nodule.

Field experiments with capsulated biopreparations for chickpea and soybean
The inoculation experiments were carried out on the experimental sites to assess the effectiveness of the microspheres modifier (with chickpea and soybean). Microspheres by themselves are not demonstrate any improvement in plant growth compared to non-inoculated control agents (Table 2). However, the inoculation of both chickpea and soybean plants with *Mesorhizobium ciceri* ST-282 and *Bradyrhizobium japonicum* M8 encapsulated in the alginate microspheres described in this study significantly increased the number and weight of nodules (Fig. 6, a and b).

Table 2. Influence of encapsulated biopreparations on nodules number and weight of chickpea and soybean plants in field experiments

Crop	Sample	The number of nodules in one plant	Node weight in one plant, g in one plant
Chickpea	Control (non inoculation)	6.4 ± 0.43	1.23 ± 0.08
	Inoculation *Mesorhizobium ciceri* ST 282	18.8 ± 0.28	3.61 ± 0.11
	Inoculation *Mesorhizobium ciceri* ST 282 encapsulated in the alginate microspheres	27.5 ± 0.32	6.64 ± 0.16
Soybean	Control (non inoculation)	11.8 ± 0.43	1.13 ± 0.08
	Inoculation *Bradyrhizobium japonicum M8*	24.6 ± 3.13	4.52 ± 2.7
	Inoculation *Bradyrhizobium japonicum M8*encapsulated in the alginate microspheres	43.5 ± 0.32	8.64 ± 4.19

The main advantages of alginate preparations are their non-toxic nature, soil degradation, slow release of microorganisms into the soil (van Elsas & Heijnen, 1990; Bashan 1998) and almost unlimited shelf life (Bashan & Gonzalez, 1999; Bashan & Davis, 2000). The preparation of microalginate granules of 1–3 mm in diameter containing bacteria is quite easily and is a mufti step (Bashan, 1986a; Kobayashi et al., 1997). Several preparations based on alginate were is evaluated for agricultural purposes, including encapsulation of rhizobial bacteria, able to enter into symbiosis with leguminous plants. This technology was also used to encapsulate *Mesorhizobium ciceri* and *Bradyrhizobium japonicum*, which were successfully used to inoculate the plants of chickpea and soybean in field conditions. Encapsulated rhizobia, showed significantly improved survival rates on non-encapsulated cells. What influenced the formation of nodules on the roots of plants. So when using bacteria encapsulation the number of nodules increased by 46 and 76% and the weight of nodules by 80 and 90% in comparison with non-encapsulated rhizobia.

CONCLUSIONS

This work defines some aspects of safe and general immobilization technology of symbiotic microorganisms for agricultural use. In particular, the study confirms the efficiency of the capture of bacteria in the polymer matrix. The method of production of sodium alginate beads with the use of a coal concentrate makes it possible to obtain granules with a narrow distribution of diameters and gradual release of trapped cells. The viability of immobilized cells is shown, the leakage is gradual and biological activity is retained. These positive effects are the ability to extend the stability of rhizobial bacteria and the range of their use. Encapsulation of rhizobia also allows effective protection of bacteria against ultraviolet and other adverse effects. As a result of the studies for encapsulation, a combination of alginate + kaolin was chosen as the most physically stable combination for obtaining clusters, supporting the maximum number of bacterial cells. Microspheres with a diameter of 3–5 mm can contain $> 10^8$ bead CFU g^{-1}, therefore even one ball of this diameter will be sufficient to inoculate the family. Wet microspheres are poorly suited for agricultural use, since very few farmers have the technical capacity to cover in the field, but future developments may allow the use of these wet biopreparations, such as fertilizers in the drip irrigation system.

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