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CONDITIONING MICROBIAL PRODUCTS CONTAINING NITROGEN FIXING BACTERIA WITH DIFFERENT SOLID EXCIPIENTS

CONDIŢIONAREA FOLOSIND DIFERIŢI EXCIPIENŢI SOLIZI A UNOR PRODUSE MICROBIENE CONŢINÂND BACTERII FIXATOARE DE AZOT

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The stability in real time of two strains of Rhizobium (Rhizobium meliloti and Rhizobium japonicum) mixed with different excipients was evaluated during a 6-months period. The excipients studied were: peat, peat and calcium carbonate, zeolite, and ceramic. Liquid cultures and excipients mixtures were dried (12-14% humidity), sealed in plastic bags and preserved at +4^{o}C. The cells were activated periodically by suspending aliquots from dry products in 0.9% saline solution. The viability of Rhizobium cells was evaluated by cultivation of diluted suspensions in YMA plates. The number of viable cells is decreasing during drying in all cases, increase in the first month of storage, and remains constant or decrease very slowly during storage for all obtained dry products containing rhizobia mixed with solid dry excipients. The highest number of viable cells at the end of the experiment was obtained in ceramic with Rhizobium japonicum (8x10^{5} cells/gram), and the lowest number of viable cells was obtained in zeolite with Rhizobium meliloti (1,1x10^{3} cells/gram).
***Key words**: Rhizobium, excipient, peat, calcium carbonate, zeolite, ceramic, inoculants.*

Introduction

There are several methods described for inoculation of legume seeds with rhizobia before seeding. Also, there are several excipients and supports described for microbial products used in pretreatment of seeds before seeding for a beater nodulation of the future plant roots and a higher amount of fixed nitrogen (Bulletin Pedologique de la FAO no.49, Adjei 2006, Date 1977). All mentioned methods of inoculation reach a common important point: the specific amount of inoculant to be mixed with seeds in order to achieve the optimum number of rhizobia fixed on seed tegument. The most types of fresh-culture inoculants (rhizobia in liquid or agar media) consists in a number of viable cells that decrease with a rate depending of a large amount of factors (type of

strain, composition of media, incubation parameters, stage of cells, conditions of storage etc). All these factors lead to an inoculant containing an unpredictable number of viable cells at a certain moment of storage. The purpose of the present study is to achieve a practical methodology and a specific material for use as excipient (support) in obtaining commercial microbial products containing Rhyzobium cells viable in stable population for a long period of time. The proposed methodology and materials may be used in obtaining other microbial products, just by making small modifications in each case.

Materials and Methods

Microorganisms: two strains from the Collection of Industrial Microorganisms from Faculty of Animal Science and Biotechnology Timisoara, Romania were used in this study: *Rhizobium meliloti,* a specie with symbiotic specificity for small-seeds species of *Medicago, Melilotus and Trigonella*; and *Rhizobium japonicum,* a specie with symbiotic specificity for large-seeds species as *Glycine max.*

Excipients:

1. ***Peat***, an accumulation of partially decayed vegetation matter. It forms when plant material, usually in marshy areas, is inhibited from decaying fully by acidic conditions. It is composed mainly of peat moss or sphagnum, but may also include other marshland vegetation: trees, grasses, fungi, as well as other types of organic remains, such as insects, and animal corpses.

Peat material is either fibric, hemic, or sapric. Fibric peats are the least decomposed, they are composed of undecomposed fiber. Hemic peats are somewhat decomposed, and sapric are the most decomposed. The last type was used in this experiment.

2. ***Zeolite,*** a mineral that have a micro-porous structure. Is a hydrated alumino-silicate mineral with an "open" structure that can accommodate a wide variety of cations (positive ions), such as Na^+, K^+, Ca^{2+}, Mg^{2+} and others. These positive ions are rather loosely held and can readily be exchanged for others in a contact solution. We have used a natural zeolite formed from volcanic rocks and ash layers reacted with alkaline groundwater. The rock was ground and sieved with 2 mm sieve. Color: grey-green.

2. ***Ceramics***, produced from argil that has relatively high water content. The ceramic used in this study have the following characteristics: powder product (granulometry < 1mm), porous structure (min 75%), color brick-colored, dry density: 100-150 kg/m^3, water absorption min. 200%, inert to chemicals or biological agents.

3. ***Calcium carbonate***, is a chemical compound, with chemical formula $CaCO_3$. It is commonly used in medicine, agriculture, construction, chemical industry, etc. The

product used in this study is a white powder, with granulometry < 1mm, water absorption min. 200%.

Obtaining cells-excipient mixtures:

Stock-cultures of Rhyzobium were multiplied in agar YMA media in 160/16 test tubes at 28^{o}C for 4 days. These cultures represent the inoculum used to initiate liquid cultures of Rhyzobium in 300 ml Erlenmayer flasks, incubated 4 days at 28^{o}C at 200 rpm. The number of viable cells was determined in fresh cultures, and the necessary number of cells / g excipient was calculated accordingly to the following factors: the number of viable rhizobia/ml of liquid culture, the weight of 1000 seeds, the quantity of seeds that can be properly mixed with the powdery excipients, and the necessary number of rhyzobia to be inoculated / 1 seed (Date and Roughley, 1977). The fresh cultures of Rhyzobium were mixed with excipients mentioned above and eight types of products were obtained:

1. 100 mL of *Rh. japonicum* culture was separately mixed with 100 grams of: peat, peat and calcium carbonate, zeolite, and ceramic. The wet mixtures were dryed at 40^{o}C in a dark ventilated chamber. Probes of 1 gram from each mixture were taken and CFU on YMA-agar plates was done. Each dry mixture were introduced in plastic bags, air-tight sealed and preserved in a refrigerator at +4^{o}C.
2. 100 mL *Rh. meliloti* was separately mixed with 700 grams of: peat, peat and calcium carbonate, zeolite, and ceramic. The wet mixtures were dryed at 40^{o}C in a dark ventilated chamber. Probes of 1 gram from each mixture were taken and CFU on YMA-agar plates was done. Each dry mixture were introduced in plastic bags, air-tight sealed and preserved in a refrigerator at +4^{o}C.

Samples were taken at 30, 60, 90, 180 days of storage in refrigerator for analysis of viability in real time of Rhyzobium cells in dry products, in above mentioned excipients.

Results and Discussions

Conditioning inoculants:

The necessary number of cells / g excipient was calculated accordingly to the following factors: the number of viable rhizobia/ml of liquid culture, the weight of 1000 seeds, the quantity of seeds that can be properly mixed with the powdery excipients, and the necessary number of rhyzobia to be inoculated / 1 seed.

For *Rh. meliloti,* 10 kg of seeds will be inoculated with 70 g of inoculant. The minimum number of bacteria / seed necessary to start a proper nodulation is cca 1600. The conditioning was made for 1.3 x 10^{8} bacteria / g excipient.

For *Rh. japonicum,* 25 kg of seeds will be inoculated with 70 g of inoculant. The minimum number of bacteria / seed necessary to start a proper nodulation is cca 25000. The conditioning was made for 5.5 x 10^{7} bacteria / g excipient.

The dry products of Rhyzobium mixed with excipients and preserved at +4°C were tested periodically by mixing 1 gram in a test tube with 10 ml of 9% saline solution. After a vigorous vortex dilutions in 9% saline solution were carried out and inoculated in YMA agar plates. The obtained results regarding viability in time of Rhyzobium bacteria in dry products are illustrated in figures 1 and 2.

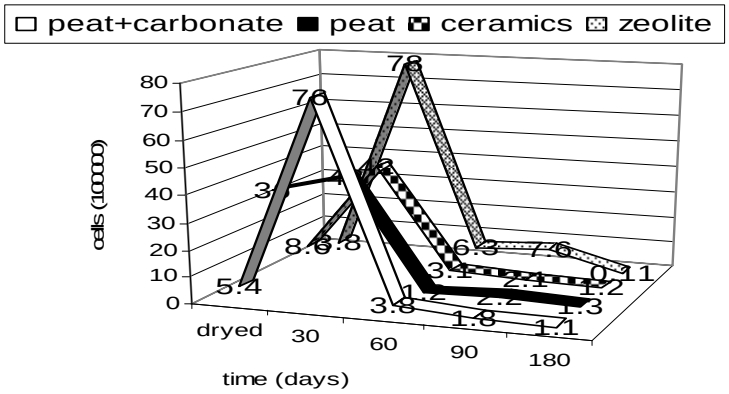

Figure 1. Viability of *Rh. meliloti* in 1 gram of dry products containing peat, peat+calcium carbonate, ceramics and zeolite, stored at +4°C for 6 months.

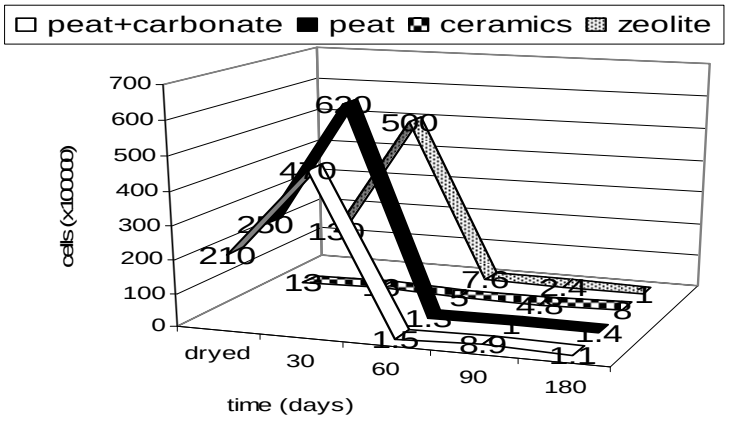

Figure 2. Viability of *Rh. japonicum* in 1 gram of dry products containing peat, peat+calcium carbonate, ceramics and zeolite, stored at +4°C for 6 months.

The results presented in figures 1 and 2 indicate that during drying an important part of the bacteria are inactivated. The number of viable *Rh. meliloti* decrease from 1 x 10^8 cells / g excipient to 3.8 x 10^5 / g in zeolite, and to 3.6 x 10^6 / g in peat. The number of viable *Rh. japonicum* decrease from 5.5 x 10^7 bacteria / g excipient to 2.5 x 10^7 / g in peat, and to 1.3 x 10^6 / g in ceramic. In the first month of storage at +4^{o}C, the *Rhyzobium* cells continued the multiplication in the most excipients, excepting *Rh. japonicum* in ceramics, where the number of cells is constant. After the second month of storage, the number of viable cells reached a constant value, in the range of 10^5. It seams that this is the tendency of the optimum cell density, indifferent of the initial density of the cells mixed with the excipient.

Conclusions

1. All products tested in this experiment can be used as excipients for conditioning Rhyzobium cells and maintain the viability of these microorganisms in the period of time studied in this work.
2. Although the initial density of bacteria / gram of excipient was different for the two strains (1 x 10^8 cells of *Rh. meliloti* / g excipient and 5.5 x 10^7 cells of *Rh. japonicum* / g excipient), during drying the number of viable cells decreased with a high rate in case of *Rh. meliloti* (down to 10^5) and remains in the same range (10^7) in case of *Rh. japonicum*.
3. From the second month of storage to the end of the experiment, the number of cells in the tested products reached a constant value in the range of 1 x 10^5, excepting *Rh. meliloti* in zeolite (1.1 x 10^4), *Rh. japonicum* in ceramic (8 x 10^5) and *Rh. japonicum* in peat (1.4 x 10^4). All tested products that keep the number of viable cells constant for a long time can be recommended as excipients to obtain dry inoculants of nitrogen fixing bacteria for use in legumes seed inoculation.

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În această lucrare a fost evaluată timp de 6 luni stabilitatea a două tulpini de Rhizobium (Rhizobium meliloti şi Rhizobium japonicum) combinate cu diferiţi excipienţi. Excipienţii studiaţi sunt: turbă, turbă cu carbonat de calciu, zeolit şi ceramică. Amestecurile de exciienţi şi culturi lichide au fost uscate (12 – 14 % umiditate), închise în pungi de plastic şi conservate la +4°C. Celulele au fost activate periodic prin prelevarea de probe şi suspendarea acestora înser fiziologic. Viabilitatea celulelor de Rhizobium a fost evaluată prin cultivarea de suspensii diluate în plăci Petri cu mediu YMA. Numărul de celule viabile scade în timpul uscării în toate cazurile, urcă în prima lună de păstrare şi apoi rămâne constant sau descreşte foarte încet în toate produsele obţinute conţinând bacterii fixatoare de azot şi excipient solid uscat. Cel mai mare număr de celule viable a fost obţinut în cazul ceramicii cu Rhizobium japonicum ($8x10^5$ celule/gram), iar cel mai scăzut număr de celule viabile s-a obţinut în cazul zeolit cu Rhizobium meliloti ($1,1x10^3$ celule/gram).

Cuvinte cheie: Rhizobium, excipient, turbă, carbonat de calciu, zeolit, ceramică, inoculanţi.