Multiplication and Viability of some *Rhizobium* Strains to be used as Inoculants for Agricultural Biomass Production

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Abstract
Rhizobia are well known for their capacity to establish a symbiosis with legumes. They inhabit root nodules, where they reduce atmospheric nitrogen and make it available to the plant. Biological nitrogen fixation is an important component of sustainable agriculture, and rhizobial inoculants have been applied frequently as biofertilizers. In this review we approach the subject of legumes inoculation in order to improve the nitrogen fixing capacity. In the first part of the experiment, the Rhizobium strains were cultivated on media indicated in the literature as optimal for bacterial growing and development in laboratory conditions. Afterwards, the Rhizobium strains that have grown and accumulate biomass were tested in different conditions of pH and salinity. The biomass accumulation was determinate by spectrophotometer. The obtained values shown that the Rhizobium strains tested can be used to inoculate the legumes cultivated on acid, basic and alkaline soils. Finally, the stability in real time of two strains of Rhizobium (Rhizobium meliloti and Rhizobium japonicum) mixed with different supports was evaluated during a 6-months period. The supports studied were: peat, peat and calcium carbonate, zeolite, and ceramic. 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).

Keywords: extreme pH, inoculation, Rhizobium, salinity, support, zeolite

1. Introduction

Rhizobia encompass a range of bacterial genera, including *Rhizobium*, *Bradyrhizobium, Sinorhizobium, Mesorhizobium, Allorhizobium,* and *Azorhizobium*, which are able to establish a symbiosis with leguminous plants [1]. They induce the formation of specialized organs, called nodules, on roots or stems of their hosts, in which they reduce atmospheric nitrogen and make it available to the plant for his own protein synthesis. The importance of legumes in establishing and keeping soil fertility it was been recognized long time ago. Symbiotic nitrogen fixation is an important source of nitrogen, and the various legume crops and pasture species often fix as much as 200 to 300 kg nitrogen per hectare. Inoculation of legumes with rhizobial strains selected for high N_2-fixing capacity can improve nitrogen fixation in agriculture, particularly when local rhizobial strains are absent from soils or ineffective [2]. The inoculation process consists in administration of viable bacteria from *Rhizobium* genus on legumes seeds, before seeding, to coat seeds with a sufficiently high number of viable N-fixing bacteria of the correct strain to provide early and effective nodulation of that legume in the field, to facilitate subsequent nitrogen fixation. Initially have been used pure cultures cultivated in nutrient agar in 1896 by Nobe and Hiltne. Later it been used sterile soil impregnated with *Rhizobium* bacteria and afterwards it been used turba as a support for the bacteria used at inoculation [3].
The primary aim of this work is to test the capacity of the *Rhizobium* strains to grow in extreme conditions of pH and salinity, in order to

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inoculate the legumes cultivated on acid, saline or alkaline soils. Secondary aim is: to achieve a practical methodology and a specific material for use as support (support) in obtaining commercial microbial products containing *Rhyzobium* cells viable in stable population for a long period of time.

2. Materials and methods

Bacterial strains:

1. *Rhizobium meliloti* CMIT10,
2. *Rhizobium meliloti* CMIT11,
3. *Rhizobium japonicum* CMIT12,
4. *Rhizobium trifolii* CMIT13,
5. *Rhizobium leguminosarum* CMIT14.

Culture media:

- *Liquid YM medium* (Medium A): K_2HPO_4 0,5g; MgSO 0,2g; NaCl 0,1g; Yeast extract 0,4g, Mannitol 2g; Distillate water 1000 ml
- *Solid YMA medium (*Medium B): at medium A formula 15 g agar was added.
- *Liquid sucrose medium (*Medium C): K_2HPO_4 0,75g; $MgSO_4$ 0,4g; $CaCO_3$ 0,4g; Yeast extract 3g; Sucrose 10g; distillate water 1000 ml

Establishing the multiplication rate of *Rhizobium* strains:

In order to obtain inoculants of high concentration it is necessary to cultivate the *Rhizobium* strains in vitro and obtain high density biomass of living bacteria. For this purpose it has been used the *Rhizobium* strains from Collection of Industrial Microorganisms from Faculty of Animal Science and Biotechnology, Timisoara (CMIT). The *Rhizobium* strains were cultivated on specific media as Medium A and Medium B. Their evolution was determined by spectrophotometer. The accumulation of bacteria biomass in the culture medium determines an increase of medium turbidity, which can be determined by photometry. The specific wavelength with maximum absorbance for *Rhizobium* bacteria is 660 nm.

Growing capacity of *Rhizobium* strains in saline condition

Rhizobiums are telluric bacteria that develop symbiotic interactions whit legumes. Many of these bacteria are sensitive to water deficit from soil, pH and salinity, which affect negatively the nitrogen fixation capacity and therefore decrease the productivity of legumes. It has been estimated that 23% of agricultural soils are affected by salinity problems [4].

Most crops are sensitive to high levels of salinity and in the case of legumes there is an additional issue, because not just the plant is affected, but microsymbiont as well [5]. To determine the growing capacity of *Rhizobium* strains in condition of extreme salinity, the strains from the CMIT have been cultivated on mediums whit different salt concentration: 0,2 M; 0,15 M; 0,1 M; 0,05 M; 0,025 M; 0,0125 M; 0,00625 M; 0 M.

After 48 h of cultivation on 30^{o}C the cultures were washed from the surface of solid medium and the DO of these cells suspensions was determined.

Growing capacity of *Rhizobium meliloti* CMIT11 in different pH conditions

The *Rhizobium meliloti* CMIT11 strain was used to evaluate its capacity to accumulate biomass in different pH values (4, 5, 6, 7, 8, 9). In the first part of this experiment has been shown that de *Rhizobium meliloti* CMIT11 strain is the most vigorous and has capacity to accumulate biomass in both culture media.

In order to determine the capacity to grow in different pH condition, cultures in liquid medium (medium C) were carried out with pH values between 4-9. These cultures were made in Erlenmeyer flasks, using a temperature – controlled water bath with shaking device.

Obtaining seeds inoculants containing *Rhyzobium* cells

- 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 microorganism with symbiotic specificity for small-seeds species of *Medicago, Melilotus and Trigonella*; and *Rhizobium japonicum,* a microorganism with symbiotic specificity for large-seeds species as *Glycine max.*

- Supports for cells immobilization: *peat*, *zeolite,* c*eramics*, c*alcium carbonate*,

- Obtaining cells-support mixtures:

Stock-cultures of *Rhyzobium* were multiplied in agar YMA media in 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°C at 200 rpm. The number of viable cells was determined in fresh cultures, and the necessary number of cells / g support 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 supports, and the necessary number of rhyzobia to be inoculated / 1 seed (Date and Roughley, 1977). The fresh cultures of *Rhyzobium* were mixed with supports mentioned above and eight types of products were obtained:

► 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°C in a dark ventilated chamber. Probes of 1 gram from each mixture were taken and the number of CFU on YMA-agar plates was determined. Each dry mixture were introduced in plastic bags, air-tight sealed and preserved in a refrigerator at +4°C.

► 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°C in a dark ventilated chamber. Probes of 1 gram from each mixture were taken and the number of CFU on YMA-agar plates was determined. Each dry mixture were introduced in plastic bags, air-tight sealed and preserved in a refrigerator at +4°C.

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

3. Results and discussion

After determination of DO to display the cells growth, and graphic representation of *Rhizobium* biomass accumulation on media A and C, it has been observed that strains from medium A were less developed comparatively with strains from medium C. The evolution of microorganisms growing on both media is represented in Figures 1 and 2.

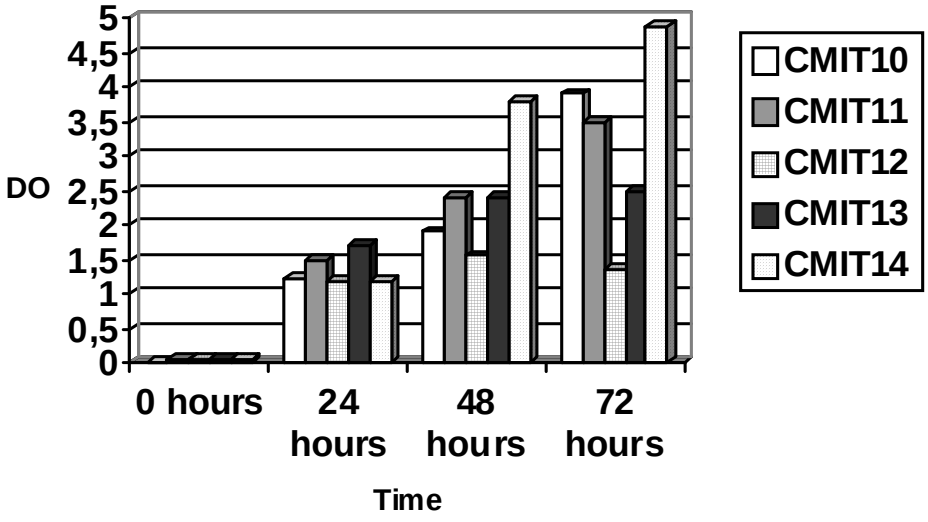

Figure1. Evolution of *Rhizobium* biomass accumulation on medium C (the values of optic density represented on y axis are obtained after dilution corrections)

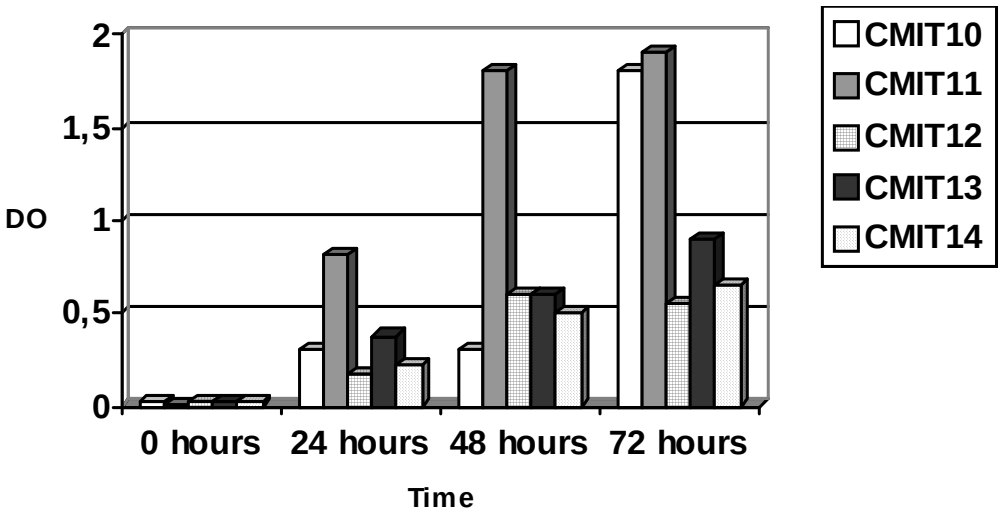

Fig. 2 Evolution of *Rhizobium* biomass accumulation on medium A (the values of optic density represented on y axis are obtained after dilution corrections)

The *Rhizobium trifolii* CMIT13 strain was cultivated on medium B at different salt concentration. After 48 h of culture bacteria scows a good developed well in salinity condition. The results can be seen in Figures 3 and 4. At concentration of 0.1 M NaCl in culture medium, bacteria growith was improved.

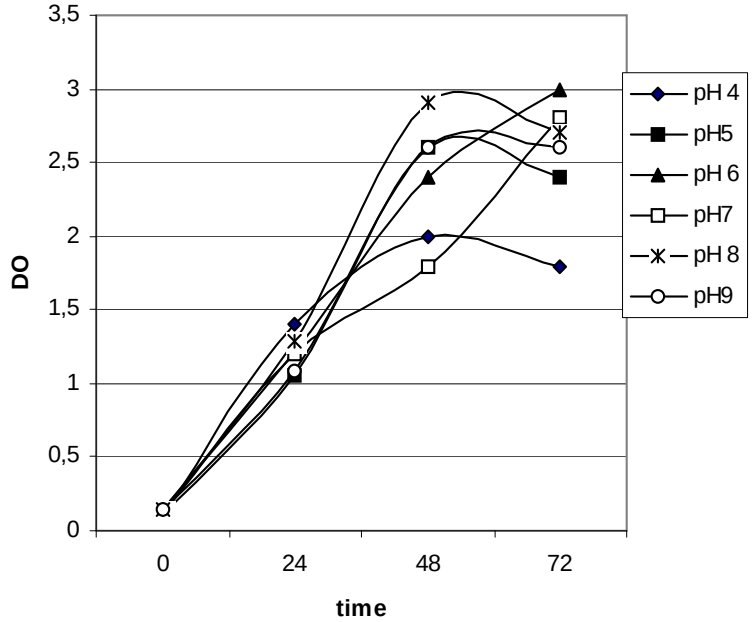

Fig. 3 Representation of biomass accumulation *Rhizobium trifolii* CMIT13 cultivated on medium B formulas with different pH

The optimal development and maximal biomass accumulation of *Rhizobium trifolii* CMIT13 strain were obtained at pH 6-7. At both acid and basic pH, after 24 hours bacteria reach the decline phase.

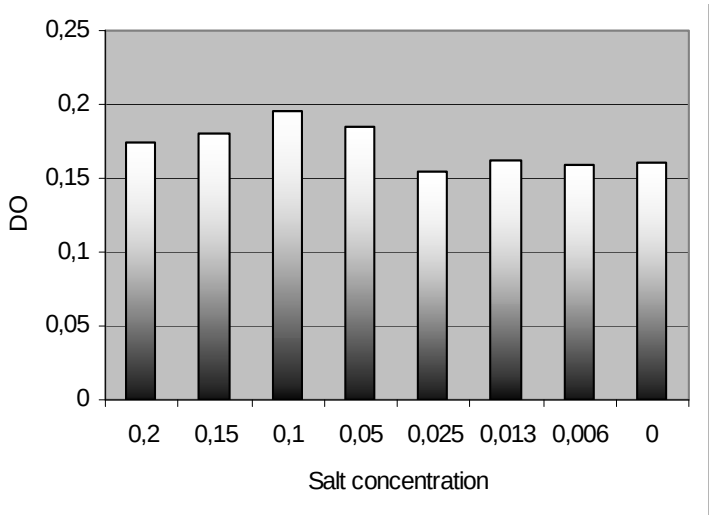

Figure 4. Development of *Rhizobium trifolii* CMIT13 at different salt concentrations

Inoculants preparation:
The necessary number of cells/g support 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 supports, 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 inoculants conditioning was made for 1.3 x 10^8 bacteria / g product.
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 inoculant.
The dry products of *Rhyzobium* mixed with supports and preserved at +4°C were tested periodically by mixing 1 gram in a test tube with 10 ml of 0.9% saline solution. After a vigorous vortex, dilutions in 0.9% saline solution were carried out and inoculated in YMA agar plates. The obtained results regarding viability in time of *Rhyzobia* in dry products are illustrated in Figures 5 and 6.

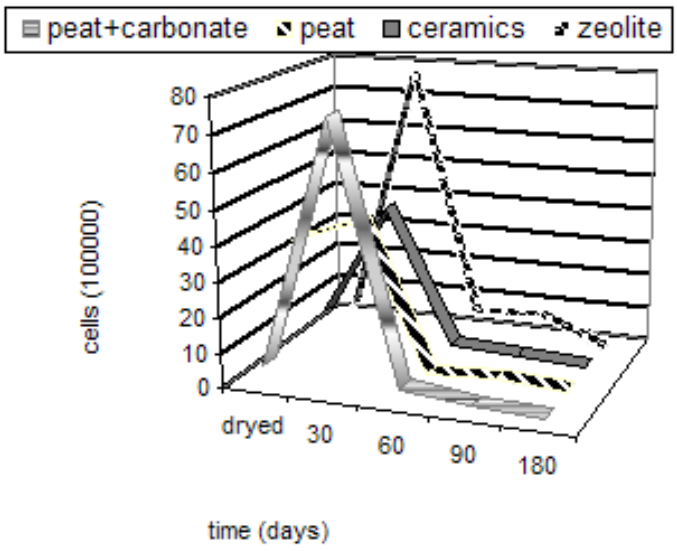

Figure 5. Viability of *Rh. meliloti* in 1 gram of dry products containing peat, peat+calcium carbonate, ceramics and zeolite, stored at +4°C for 6 months.

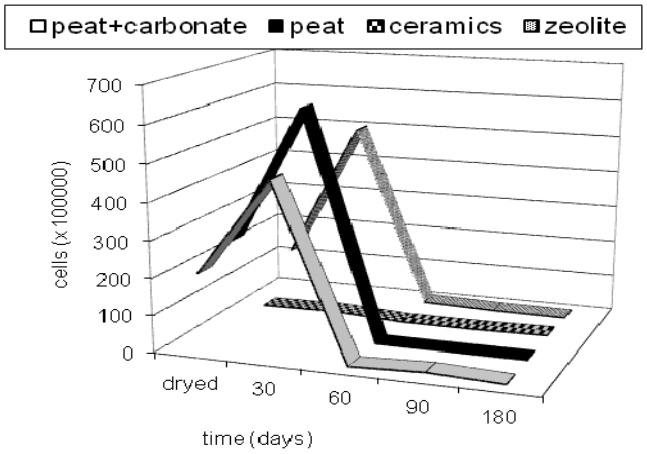

Figure 6. Viability of *Rh. japonicum* in 1 gram of dry products containing peat, peat+calcium carbonate, ceramics and zeolite, stored at +4°C for 6 months.

The results presented in figures 5 and 6 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 support 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 support 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°C, the *Rhyzobium* cells continued the multiplication in the most supports, 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 seems that this is the tendency of the optimum cell density, indifferent of the initial density of the cells mixed with the support.

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 supports to obtain dry inoculants of nitrogen fixing bacteria for use in legumes seed inoculation.

4. Conclusions

The *Rhizobium* CMIT can be used to obtain an inoculant for legumes seeds.

The *Rhizobium* strains can be grown using cheep and accessible culture medium (medium C).

The *Rhizobium* strains can grow in conditions of extreme pH and salinity and therefore can be used as inoculant for legumes cultivated on acid, saline and alkaline soils.

All products tested (peat, zeolite, ceramics, calcium carbonate) can be used as supports for conditioning *Rhyzobium* cells and maintain the viability of these microorganisms in the period of time studied in this work.

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