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SURVIVAL AND VIABILITY OF *BRADYRHIZOBIUM JAPONICUM* IN DIFFERENT LIQUID MEDIUM

PREŽIVLJAVANJE I ODRŽIVOST *BRADYRHIZOBIUM JAPONICUM* U RAZLIČITIM TEKUĆIM MEDIJIMA

Gabriella Kanižai Šarić, K. Prtenjača, Ivana Majić

ABSTRACT

The microbiological inoculants present on the market come in various formulations and forms. Inoculants used in pre-sowing bacterization of legumes in our country are traditionally prepared on peat as the highest quality carrier of bacteria. However, the requirements of the manufacturer are focused on liquid forms of inoculants. Therefore, the aim of this paper is to determine the optimal composition of the liquid medium that will support the growth of *Bradyrhizobium japonicum*, the soybean symbiont. Three liquid nutrient medium formulations were included in the study: mannitol-yeast medium, modified mannitol-yeast medium, and glycerol medium stored at two temperatures (4 and 25 °C), and *B. japonicum* cell viability was determined over 6 months. The results showed that the largest number of rhizobia (on average $4x10^9$ zo $9x10^8$) was obtained on yeast-mannitol medium at 4 °C as well as on modified yeast mannitol medium where their number remained constant throughout the storage time and was $5x10^7$ cfu ml^{-1} at 25 °C. Further research should include testing other rhizobial protectors in order to increase the number of viable cells in longer time periods.

Key words: soybean, liquid inoculants, protective compounds, temperature, storage time

SAŽETAK

Mikrobiološki preparati prisutni na tržištu dolaze u različitim formulacijama i oblicima. Inokulanti koji se primjenjuju u predsjetvenoj bakterizaciji leguminoza u našoj zemlji se tradicionalno pripravljaju na tresetnom kao najkvalitetnijem nosaču bakterija, međutim zahtjevi proizvođača su usmjereni na njihove tekuće forme. Stoga je cilj ovog rada utvrditi optimalan sastav tekuće podloge koja će podupirati rast *Bradyrhizobium japonicum*, simbionta

soje. U istraživanje su uključene tri formulacije tekuće hranjive podloge: manitol-kvasac podloga, modificirana YM (yeast-mannitol) podloga i podloga s glicerolom koje su pohranjene na dvije temperature (4 i 25 °C) te je vijabilnost stanica *B. japonicum* utvrđena kroz 6 mjeseci. Rezultati pokazuju da je najveći broj rizobija (u prosjeku od $4x10^9$ do $9x10^8$) dobiven na kvasac-manitolovoj podlozi na 4 °C te na modificiranoj kvasac manitol podlozi gdje je njihov broj ostao konstantan tijekom cijelog vremena skladištenja i iznosio je $5x10^7$ cfu ml^{-1} na 25 °C. Daljnja istraživanja bi trebala biti usmjerena na ispitivanje i drugih protektora rizobija u cilju povećanja vijabilnosti stanica i u dužem vremenskom periodu.

Ključne riječi: soja, tekući inokulanti, protektivne tvari, temperatura, vrijeme skladištenja

INTRODUCTION

Soybean (*Glycine max* L. Merr.) is the most commonly sown legume worldwide and represents a nutritionally essential part of the humans and domestic animals diets due to high grain protein content (Hungria and Mendes, 2015). Pre-sowing bacterization of soybean seeds with inoculants present on the market ensures successful infection and nodulation of soybean roots with effective strains of *Bradyrhizobium japonicum*. This measure is a standard agricultural practice because it allows the fixation of 0-450 kg of atmospheric N ha^{-1} per year (Ormeño-Orrillo et al., 2012). The quality of all inoculants is determined by a number of factors and the most important is to ensure a large number of living cells of rhizobium bacteria (greater than $2x10^9$ per g^{-1}) without or with minimal contamination (Lupwayi et al., 2000). In addition, *B. japonicum* strains incorporated into the inoculant must be highly effective, viable, and retain their properties during storage but must also be tolerant to stressful conditions such as acidity, drought, high temperature and chemical pesticides (Lupwayi et al., 2000.; Ben Rebah et al., 2007.). Furthermore, the quality of the inoculant is determined by they efficiency and easy application, appropriate product shelf life, proper packaging and clear instructions for use on each package (Lupwayi et al., 2000). Inoculants carrier must satisfy certain criteria, they must be: available, uniform in composition, cheap in price, nontoxic to the bacteria, with high water retaining capacity and with good nutrient content to allow bacterial growth (Hungria et al.. 2005). Peat are the most commonly used carrier of solid rhizobial inoculants because it

satisfies all the above mentioned properties. However, peat stocks are limited and this technology requires grinding, drying, neutralization and sterilization (Hungria et al., 2005). Researchers are investigating the properties of other potential carriers of inorganic (perlite, vermiculite) or organic (compost) origin (Temprano et al.; 2002, Albareda et al., 2008; Blažinkov et al., 2015). Liquid inoculants are easier to handle but bacterial survival is lower (Singleton et al., 2002.; Albareda et al., 2008). This type of inoculant include a various broth formulations. The application of protective materials such as: polyvinylpyrrolidone (PVP), polyethylene glycol (PEG), gum arabic glycerol, glucose, mannitol, trehalos or FeEDTA improve the quality of liquid inoculants, protect bacterial cells and allow better adhesion to seeds (Temprano i sur., 2002.; Singleton et al., 2002.; Tittabutr et al., 2007). The aim of study was to determine efficacy of yeast-mannitol medium as standard medium for rhizobial growth and two modified medium with protective compounds on viability of *B. japonicum* cell incubated on two temperatures (4 and 25 °C) over a period of 6 months.

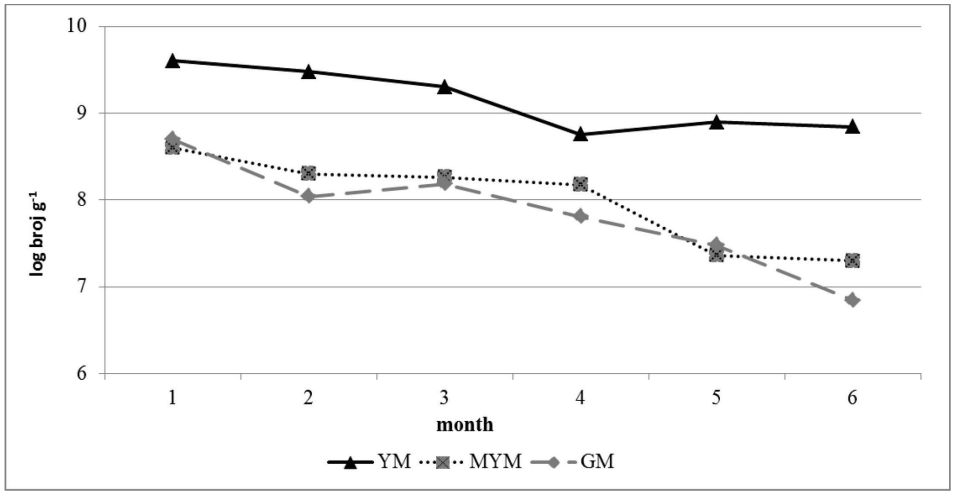
MATERIALS AND METHODS

An in vitro experiment was performed which included *Bradyrhizobium japonicum* DSM 1755. Pure cultures of the tested strain were inoculated on solid yeast-mannitol medium (Vincent, 1970), and incubated for 7 days at 28 °C. The grown colonies were transferred to a (I.) liqiud yeast-mannitol medium (YM) (Vincent, 1970), (II.) modified yeast-mannitol medium (mannitol 1 g, K_2HPO_4 0.5 g, $MgSO_4x7H_20$ 0.2 g, NaCl 0.2 g, yeast 0.1 g, PVP 20g (MYM) and (III.) glycerol medium (glycerolum 12 ml, mannitol 1 g, K_2HPO_4 0.8 g, $MgSO_4x7H_20$ 0.5, NaCl 0.1 g, yeast 2 g, PVP 20g (GM) aerated medium (Singleton et al., 2002.). After 5 days on incubation on optimal temperarures inoculants were stored at two temperatures: 4 and 25 °C over a period of 6 months. Once a month, microbiological analysis was performed to determine the number of viable *B. japonicum* cells according to the dilution method while the average cell number was expressed as cfu per ml.

RESULTS AND DISCUSSION

Microbiological analysis showed that in YM medium at 4 °C, the number of viable cells was $4x10^9$ at the beginning of experiment. During the months the number decreased slightly and remained at $9x10^8$ cells at the end of the 6th

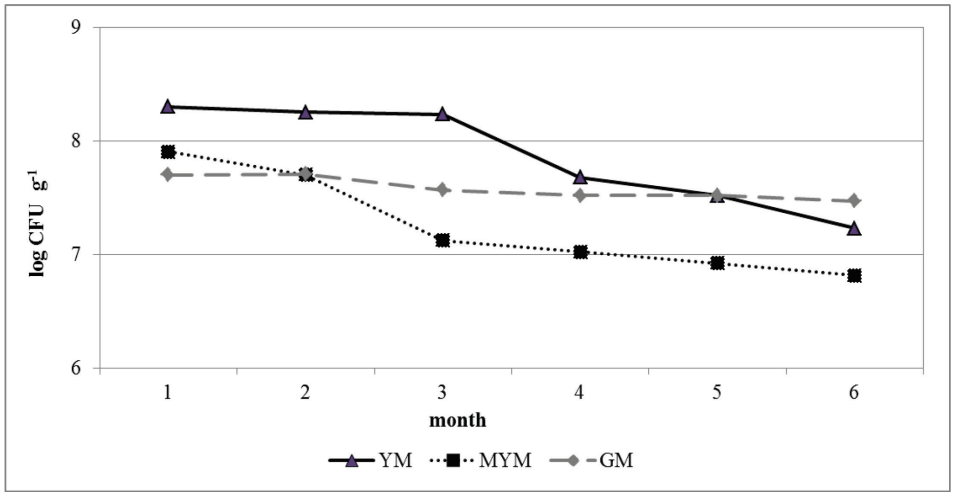
month. MYM and GM at the beginning of the experiment had the same initial number of cells of $7x10^8$, in the modified medium cell number remained at $2x10^7$ until the end of the 6th month, while in the glycerol medium *Bradyrhizobium* cell number dropped to $8x10^6$ (Graph 1.).



Graph 1 Viability of B. japonicum in different liquid medium at 4 °C
Grafikon 1. Vijabilnost B. japonicum u različitim tekućim podlogama na 4 °C

Minimum quality standards for the number of rhizobial cells varies in different countries in the range from $5x10^7$ to $1x10^9$ cells per gram (or ml) of freshly prepared inoculant (Lupwayi et al., 2000), although many countries, as well as the Republic of Croatia, do not have a prescribed legislative framework and quality control is carried by the producers themselves. Storage temperature is a significant factor for the survival of rhizobia and it is higher at 4-10 °C compared to 28 °C (Herridge i sur., 2002). Also, long-term storage of rhizobial inoculants is not recommended due to the occurrence of physiological changes in rhizobial cells that may prolong the time to nodulation (Tittabutr et al., 2007). In research of Menendez et al. (2014) liquid inoculant was stable at 4 °C for at least 8 months. They increased yest extract in media and additional nitrogen salts further contributed to incraese cell density to $5x10^9$ ml^{-1}. The authors also noted that exopolysaccharides are primary components of the biofilm that protects bacteria from desiccation and function as reserve energy source.

A smaller number of viable cells was found at a storage temperature of 25 °C. In YM medium at 25 °C, the initial number of viable cells was $2x10^8$ and at the end of the 6th months it remained at $2x10^7$. In GM the cell number was kept constant throughout the storage time at $5x10^7$**.** In MYM $8x10^7$ viable cells were found at the beggining of experiment which droped to $7x10^6$ at the end of the experiment (Graph 2).



Graph 2 Viability of B. japonicum in different liquid medium at 25 °C
Grafikon 2. Vijabilnost B. japonicum u različitim tekućim podlogama na 25 °C

In the study of Singleton et al. (2002) after 6 months of storage the number of viable cells remained nearly constant in modified Vincent medium, glycerol and Vincent medium at 25 °C (10^9, 10^9, 10^8). The authors propose that C and N starvation at the beginning of the stationary growth phase creates stress resistance in rhizobial cell. Tittabutr et al. (2007) investigated survival of rhizobia in peat and liquid inoculant (modified yaest manitol medium + PVP, cassava, alginate, PEG, arabic gum, polyvinylalcohol at 28 °C. Peat based inoculants supported cell survival above 10^8 cells g^{-1} after six months of storage and liquid inoculants modified with sodium alginate supported cell survival from 10^5 to 10^8 cells g^{-1} depending on cultivated rhizobial strain after six months of storage. Authors concluded that for commercial purpose a safe storage period of 6 months is desirable. Maurice at al. (2001) investigated

survival of *B. japonicum* in commercial liquid inoculants over a period of 8 years at 20 °C. Results showed that cells remained viable and culturable at 10^8 cell ml^{-1} with cell physiological changes. More than 85% of the bacteria were unable to propagate but remained viable.

Further research is needed that will include other protective substances that provide high viability of rhizobial cells in order to increase the quality of these products. It is also necessary to investigate the effectiveness of such formulations of liquid inoculants in field conditions.

CONCLUSIONS

All tested broths stored at 4 °C showed higher viability compared to those stored at 25 °C. Yeast manitol broth showed satisfactory cell viability in the 6 months of storage. Further reserach is needed to identify new, more appropriate broth medium and to identify new additives in order to improve inoculants quality with optimal number of rhizobial cells.

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Author's addresses – Adresa autora:
izv. prof. dr. sc. Gabriella Kanižai Šarić,
e-mail: gkanizai@fazos.hr,
izv. prof. dr. sc. Ivana Majić,
Faculty of Agrobiotechnical Sciences Osijek,
Josip Juraj Strossmayer University in Osijek,
Vladimira Preloga 1, Osijek, Croatia

Krešimir Prtenjača, student,
Faculty of Agrobiotechnical Sciences Osijek,
Josip Juraj Strossmayer University in Osijek,
Vladimira Preloga 1, Osijek, Croatia

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