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Growth, Survival and Field Performance of Bradyrhizobial Liquid Inoculant Formulations with Polymeric Additives

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ABSTRACT: Liquid inoculant has become a preferred method for inoculating legumes with bradyrhizobia. Finely ground peat has been the standard of quality for inoculants for many years. Suitable peat for an inoculant carrier is difficult to find and limited in supply in many locations. We evaluated six different polymeric additives (polyvinyl pyrrolidone (PVP), polyethylene glycol (PEG), polyvinyl alcohol (PVA), gum arabic, cassava starch, and sodium alginate) for their ability to support growth and promote survival of several strains of bradyrhizobia and rhizobia during storage. Some concentrations of various additives to yeast extract mannitol (YEM) media promoted higher cell density compared to cells cultured in YEM media alone. There was a large interaction between strains of rhizobia and the additives in relation to cell survival. Shelf-life of liquid inoculant formulations depended on the strain of rhizobia and additives, when stored at room temperature. Liquid inoculants formulated with sodium alginate promoted long-term survival of all rhizobial strains, but its effect on cell survival was not as great as peat. Peat also provided the greatest protection to cells of bradyrhizobia after application to the seed surface followed by incubation at 40°C. Survival of bradyrhizobia was maintained at 10^5 cells/seed after 48 hours of incubation. Liquid inoculant formulated with gum arabic, sodium alginate, PVP, or cassava starch supported survival at only 10^4-10^5 cells/seed, while PEG and PVA additives performed poorly and cell numbers fell to 10^3 cells/seed at 48 hours after inoculation. One-week old liquid inoculants with bradyrhizobia were tested for their ability to nodulate and fix nitrogen under field conditions. We found that liquid inoculant performance was as good as that of peat based inoculant.

KEYWORDS: additive, bradyrhizobia, liquid inoculant, polymeric substance, survival.

INTRODUCTION

Bacterial inoculant is a formulation that contains one or more beneficial bacterial strains or species in an easy-to-use and economical carrier material. Inoculant is the means to transport living bacteria from the factory and introduce them onto living plants, so they may produce the desired effects on plant growth.[1] Peat is the most frequently used carrier for the rhizobial inoculant industry because it has characteristics such as high water holding capacity and high surface area that support rhizobial growth and survival in large numbers. However, peat is not available in many countries, especially in the tropics, and will be depleted in many areas in the future.[2] Moreover, peat-based inoculant carrier requires a significant amount of processing, such as mining, drying, milling and neutralizing, before its use in a commercial production system. Processing peat requires costly investments in equipment and for small production operations is usually not feasible.[3] Peat-based carriers are also difficult to process to consistent characteristics, and are not easily used with precision planting equipment.[4]

Liquid inoculant formulations are one solution to the problems associated with processing solid carriers. Liquid inoculant formulations may use various broth cultures amended with agents that promote cell survival in the package and after application to seed or soil. Liquid inoculant is easily adapted to advanced seeding equipment, since it can be sprayed onto the seed as it passes through the seed auger and dries before it travels into the seed bin on the planter.[5] Various liquid media have been used to culture rhizobia. These media normally consist of carbon, nitrogen and vitamin sources, which promote the growth of rhizobia. However, additives to the broth can be made that will improve inoculant quality, such as including better adhesion to seed, stabilizing the product, binding or inactivating soluble seed coat toxins, and enhancing rhizobial survival during storage and after exposure to

extreme environmental conditions after inoculation and seed planting. The later issue is perhaps, the most important. Inoculated legume seed are sometimes sown into soil with temperatures reaching 40°C.[6] High temperature is an important environmental factor that affects rhizobia survival and nitrogen fixation.[7]

Additives to liquid inoculant formulations should have a role in protecting rhizobial cells on seed at high temperature and during desiccation. Many kinds of polymers have been used for inoculant production because of their ability to limit heat transfer, their good rheological properties and high water activities.[8] These polymers, such as methyl cellulose, gum arabic, polyvinyl pyrrolidone (PVP) and alginate are normally used as adhesive compounds with solid based carriers when they are applied to seed. Polymers are also used to entrap rhizobia in microbeads.[9,10,11] Polymers that are soluble in liquid inoculant formulations make for convenient batch processing of inoculant and make seed application a simple process for farmers. Polymers used in this study were selected based on their properties, such as solubility in water, non-toxicity, and complex chemical nature, which prevents microorganisms in the soil from rapidly degrade the polymeric coating.[9] Our objective is to evaluate the performance of polymeric substances that are suitable for producing quality liquid inoculant that is equivalent to peat-based inoculant under field conditions

Materials and Methods

Microorganisms

Rhizobia from several genera were used in this experiment. *Bradyrhizobium japonicum* USDA110 and *Azorhizobium caulinodans* IRBG23 were obtained from Thailand Department of Agriculture, Bangkok, Thailand. *Rhizobium phaseoli* TAL1383, *Sinorhizobium fredii* HH103, and *Mesorhizobium ciceri* USDA2429 were received from the University of Minnesota, USA.

Medium and liquid inoculant formulation

Yeast extract mannitol media[3] (YEM) containing (g/l) 10.0 mannitol; 0.5 K_2HPO_4; 0.2 $MgSO_4.7H_2O$; 0.1 NaCl and 0.5 yeast extract, was the basal medium used to evaluate the effect of additives on cell growth (details of each additive are listed in Table 1). A modified YEM medium (referred to here as G5 medium[4]), which is composed of (g/l) 1.0 mannitol; 0.5 K_2HPO_4; 0.2 $MgSO_4.7H_2O$; 0.1 NaCl; 1.0 yeast extract; 1.0 glucose; 0.5 arabinose; 200 mM Fe-EDTA and 4 ml glycerol, was used as a basal medium for liquid inoculant formulation with selected appropriate concentrations of additives.

Preparation of peat-based inoculant

Raw peat material was received from Thailand Department of Agriculture, Bangkok, Thailand (total nitrogen, 0.8-1.3%; organic matter, 55-75%; water holding capacity, 70-140%; pH 4.2-5.0; particle size, 80-100 mesh). Peat was neutralized with 10% (w/w) of $CaCO_3$ to obtain the final pH at 7.0. Each 100 g of peat was packed in a low density polypropylene bag, heat sealed and sterilized by autoclaving at 121°C for 1 hour. Rhizobia were grown in YEM liquid medium until late log phase (>10^8 cells/ml), and were injected into sterilized peat to obtain the final moisture at 40%. Peat inoculants were well mixed and incubated at room temperature (28-30°C) for one week before use. The cell population in peat inoculant was determined by adding 10 g of inoculant to 90 ml of sterile distilled water and making a 10-fold dilution series. Then, 0.1 ml aliquots of the appropriate dilutions were spread on YEM agar medium. Colonies were counted after incubation at 28°C for 7 days.

Testing the effect of additives on cell growth

YEM medium was blended with different concentrations of additives as follows: PVP at 1.0, 2.0, 3.0 and 5.0% (w/v); PEG at 0.1, 0.5, 1.0 and 5.0% (w/v); PVA at 0.1, 0.5, 1.0, 3.0% (w/v); Gum Arabic at 0.1, 0.3, 0.5, 0.8% (w/v); Sodium alginate at 0.1, 0.2, 0.3 and 0.5% (w/v); and cassava starch at 0.1, 0.5, 1.0 and 3.0% (w/v). The experiments were carried out in 125 ml Erlenmeyer flasks containing 50 ml of amended medium. Late log phase cultures of rhizobia were inoculated into test media (0.10% v/v), and grown in an incubator at 28°C, shaking at 200 rpm for 6 and 9 days for fast- and slow-growing species, respectively. The viable cell populations in YEM medium and YEM with additives were determined by plate counts on YEM agar media. Based upon the effect of the additives on cell growth, the appropriate concentration of each additive for different species of rhizobia were selected (Table 2) for further experiments.

Testing the effect of additives on seed germination

Liquid and peat-based inoculants of *B. japonicum* USDA110 were used to evaluate their effect on seed germination. Twenty milliliters of liquid inoculant formulated with appropriate concentrations of additives, and slurrys of peat inoculant[12] (5 g of peat with 15 ml of water) were inoculated onto 100 g of soybean (SJ2) seeds and gently mixed until the seeds were uniformly wet. The three replicates of inoculated seeds were placed in moist vermiculite, and placed under light control for 3 days with a light intensity of 400 µmole.m^{-2}.s^{-1} and temperature ranging from 28-30°C. The proportion of germinated seeds for each liquid treatment were determined and compared to germination of seed inoculated with peat.

Table 1. List of additives used in this study

Additives	Company	*Viscosity (cP)	Characteristic	References
Polyvinyl pyrrolidone PVP-40T (PVP)	Sigma	25.0 (at 2.0 % (w/v))	large molecular weight (Av. Mol. Wt. 40000), water soluble compound with stabilization and adhesive properties, high water binding capacity, high phenolic compound binding capacity, useful in reducing toxic substances released from seed coat	4, 9, 16, 19
Polyethylene glycol (PEG)	Sigma	18.4 (at 1.0 % (w/v))	small molecular weight (Av. Mol. Wt. 3000), water soluble compound with adhesive properties	18, 31
Polyvinyl alcohol(PVA)	Sigma	18.0 (at 0.5 % (w/v))	large molecular weight (Av. Mol. Wt. 70,000-100,000), fully hydrolyzed (98-99.5 %), hot water soluble compound, with stabilizing properties, useful in reducing the extent of protein precipitation or the coagulation of cells	9, 14, 32, 33
Gum arabic	Carlo	17.4 (at 0.3 % (w/v))	biopolymer, large molecular weight compound with adhesive properties, emulsification and stabilization, limit heat transfer, high water activity	8, 16, 19
Sodium alginate	Carlo	38.2 (at 0.1 % (w/v))	large molecular weight, nontoxic compound with adhesive property, limit heat transfer, high water activity, useful in supporting long term survival of inoculant	8, 23, 34
Tapioca Flour (Cassava starch)	Thai Better Food Co. Ltd.	35.2 (at 1.0 % (w/v))	large molecular weight biopolymer compound with stabilizing properties, limit heat transfer, high water activity, used as thickener, and also as a binder	8, 32

*Viscosity measurements were carried out with a viscometer (Brookfield DV-III Ultra, Brookfield Engineering Laboratories, Inc., USA) and using the spindle no. 2 at 25°C with a speed of 200 rpm.

Table 2. Additive concentrations selected for formulation of liquid inoculant based on maximum growth of rhizobia

	Concentration of additives (%) used with each strain of rhizobia				
Carriers	*Bradyrhizobium*	*Azorhizobium*	*Mesorhizobium*	*Rhizobium*	*Sinorhizobium*
PVP	2.0	2.0	2.0	5.0	2.0
PEG	1.0	0.5	0.5	1.0	1.0
PVA	0.5	0.1	0.5	0.5	0.5
Arabic gum	0.3	0.3	0.1	0.1	0.3
Cassava	1.0	0.5	1.0	1.0	0.1
Alginate	0.1	0.1	0.1	0.1	0.1

Testing the survival of inoculated bradyrhizobia on seed at high temperature

Peat-based inoculant and liquid inoculant of *B. japonicum* USDA110 containing appropriate concentrations of additives, were used to inoculate three replicate batches of SJ2 soybean seed (100 g seed/replicate) with 2 ml of liquid inoculant or 0.5 g of peat with 1.5 ml of water as sticker.[3] Seed was coated in sterile 1.0 liter flasks and shaken for approximately 1 min until all seeds were uniformly wet. Immediately following inoculation, seeds were incubated at 40°C. The number of viable cells remaining on the seed was determined at 0, 24, and 48 h after inoculation. Ten seeds were removed from each replicate and transferred to test tubes containing 10 ml of sterile 0.85% NaCl. Test tubes were shaken vigorously for 5 min to wash the inoculum off the seeds, and then 1 ml of resulting suspension representing cells derived from 1 seed was removed to a sterile tube. Ten-fold dilutions were made from this sample and 100 µl of each dilution was spread on YEM-congo red agar medium[3] containing 5 µg/µl of tetracycline as a selective marker for this strain of *Bradyrhizobium*. Plates were incubated at 28°C for 7 days, and the colonies occurring on each plate were counted.

Field experiment

The efficiency of *B. japonicum* USDA110 liquid

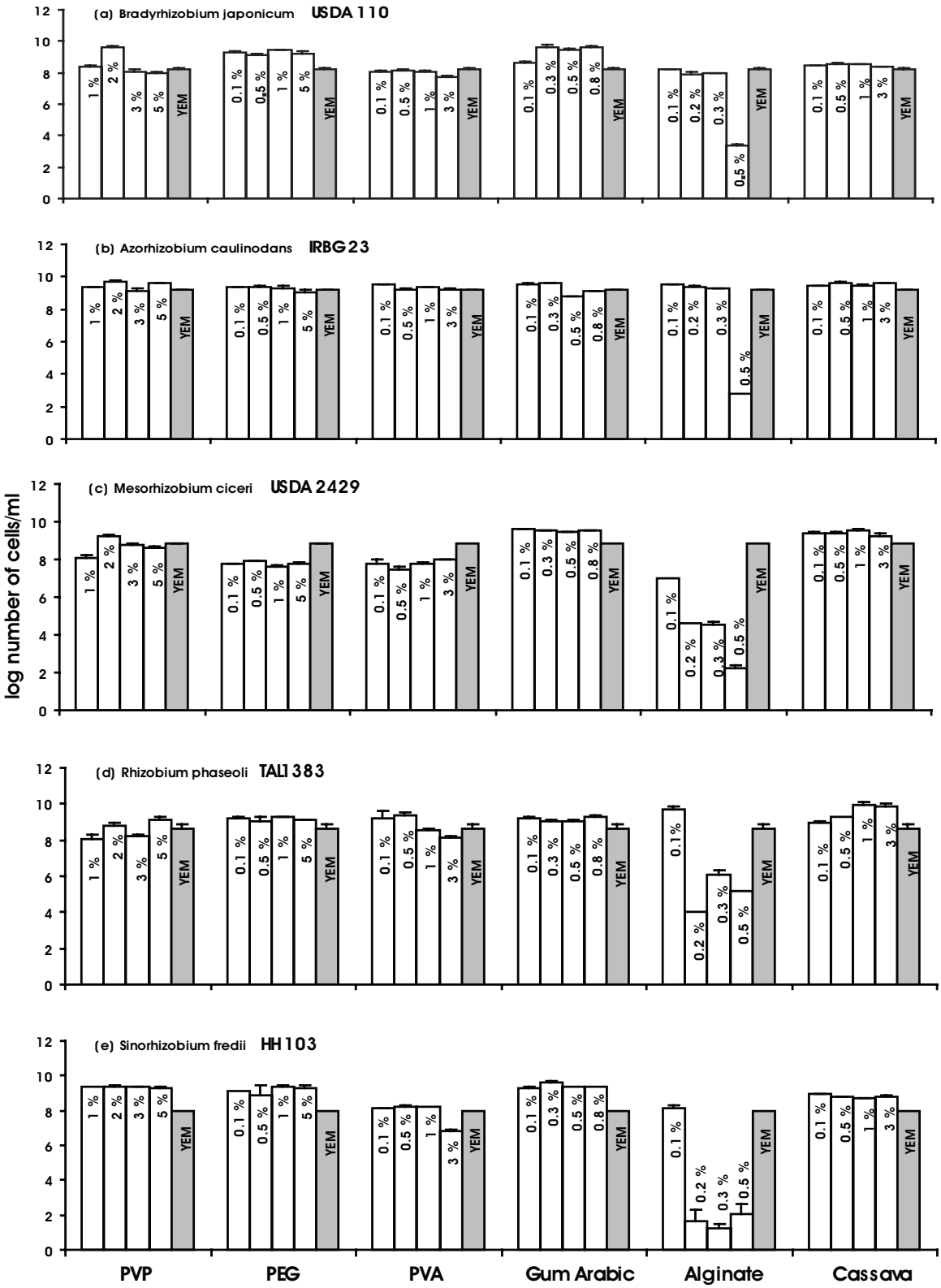

Fig 1. The amount of rhizobial cells after culturing in YEM liquid medium and YEM liquid medium amended with different concentrations of additives (% w/v). Cassava refers to cassava starch. Additives are described in Table 1.

inoculant formulations were tested with soybean variety SJ2 in a field experiment located on the Suranaree University of Technology farm. The field soil was characterized as sandy loam soil, which has pH 6.48, and contained 0.92, 36.0, 74.5, 1146.0, 160.0, 55.5, 37.8 and 0.2 ppm organic matter, P_2O_5, K_2O, Ca, Mg, Fe, Mn and Zn, respectively. Different liquid inoculant formulations were tested in the field and compared to peat-based inoculant. Plots were arranged in a Randomized Complete Block Design with four replications. Plots were 1.0 m × 4.0 m, and seeds were planted in two rows (60 plants per plot). Seeds were inoculated in the field using one-week-old liquid inoculant. Sixty days after planting, nodule number, plant dry weight and seed weight were determined. The data were analyzed statistically using the Statistical Analysis System.[13] Analysis of Variance (ANOVA) and means comparison were made using Duncan's Multiple Range Test (DMRT).

Liquid inoculant production and survival during prolonged storage

Cells of *B. japonicum* USDA110 and other species of rhizobia were grown in 2 liter Erlenmeyer flasks containing 1.4 liters of modified G5 medium with appropriate concentrations of additive (Table 2). Air was continuously pumped through a 0.45 µm filter into the medium until reach maximum cell concentrations of 10^9 cells/ml were reached. A 20 ml aliquot of each cell culture was inoculated into sterile polypropylene bags, which were then heat sealed. These liquid inoculants were stored at room temperature (28-30°C) and the viability of cells was determined every month by plate count.

Results

Effect of additives on cell growth

The effects of six additives to YEM medium on the final cell concentration of rhizobia and bradyrhizobia are shown in Figure 1. There were few adverse effects of any additive on final cell concentration; some polymeric additives, such as PVP, cassava starch and gum arabic supported cell growth among all species at higher levels than in YEM. Some concentrations of PEG, PVA, and sodium alginate tended to slightly reduce cell density when compared to YEM media. PEG blended in YEM could promote the growth of cells in all strains of rhizobia except *M. ciceri* USDA2429. Also, PVA could support the growth of *R. phaseoli* TAL1383, *S. fredii* HH103 and *A. caulinodans* IRBG23, but some concentrations were not appropriate for *M. ciceri* USDA2429 and *B. japonicum* USDA110. Alginate had the largest effect on cell growth, depending on the concentration; alginate affected the growth of all species tested. Increasing the concentration of alginate in YEM medium above 0.1 % (w/v) reduced the number of *R. phaseoli* TAL1383 and *S. fredii* HH103 cells in the culture, but there was no effect on *B. japonicum* USDA110 and *A. caulinodans* IRBG23 until the concentration of alginate increased to 0.5 % (w/v). The selected concentration of additive for each species was based on the concentration that supported maximum growth of the species (Table 2). The designated concentrations were used for all further experiments.

Survival of bradyrhizobia on soybean seed stored in elevated temperatures after inoculation

The role that polymeric additives can play in protecting rhizobial cells on seed incubated at high temperature was investigated and compared with traditional peat-based application of rhizobia to seed (Fig. 2). The results demonstrated that different additives had different abilities to protect rhizobial cells on seed at high temperature. The number of viable cells on seed decreased with time from inoculation for all formulations. Peat-based carrier maintained the highest number of bradyrhizobial cells on seed, with 5.1×10^5 cells/seed surviving at 48 h. Liquid inoculant containing gum arabic, sodium alginate, PVP, and cassava starch could maintain the number of surviving cells on seed at about 3.7×10^5, 2.0×10^5, 8.8×10^4, and 1.8×10^4 cells/seed at 48 h, respectively. Liquid inoculant containing PEG and PVA maintained only 2.0×10^3 and 2.4×10^3 cells/seed after 48 h.

Effect of bradyrhizobial liquid inoculant formulation with polymeric additives on soybean seed germination and field performance

Polymeric additives were inoculated on soybean seed, and their effects on germination were determined. Neither peat-based inoculant nor liquid inoculant

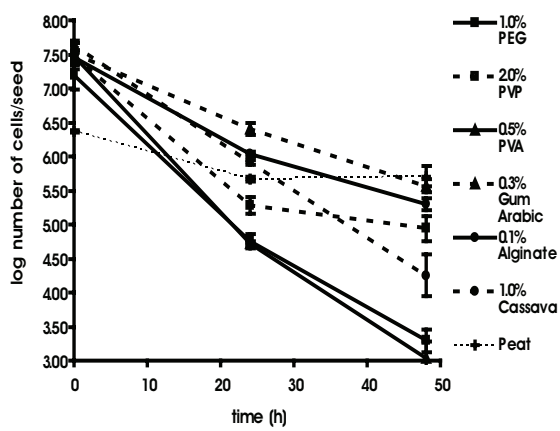

Fig 2. Survival of *B. japonicum* USDA110 on SJ2 soybean seed after peat and different liquid inoculants were inoculated onto seed and incubated at 40°C for 48 h

containing polymeric additives had an effect on the germination of soybean seeds (data not shown).

One-week old liquid inoculants of bradyrhizobia containing different additives were used for a field experiment to evaluate their effects on the number of nodules, plant dry weight and seed weight compared to peat-based inoculant (Table 3). The results revealed that liquid inoculant formulation with all polymeric additives produced nodulation and yields of soybean equivalent to those produced with the peat inoculant.

Long-term survival of rhizobia in liquid inoculant formulation with different polymeric additives

Survival of bradyrhizobia and rhizobia from other species in different liquid inoculant formulations were shown in Figure 3. Peat-based inoculant supported cell survival of every strain above 10^8 cells/g and 10^7 cells/g for slow-growing and fast-growing species, respectively, after six months of storage (Fig. 3a). Liquid inoculant containing sodium alginate supported survival of every strain, especially *B. japonicum* USDA110, *A. caulinodans* IRBG23, and *M. ciceri* USDA2429, which remained at cell concentrations from 10^7-10^8 cells/ml after 6 months. This carrier could also support the survival of *R. phaseoli* TAL1383 and *S. fredii* HH103 at 10^5 cells/ml for 6 months of storage (Fig. 3b). Liquid inoculant containing PVP could support the survival of *B. japonicum* USDA110 and *A. caulinodans* IRBG23 up to 6 months at a cell concentration higher than 10^8 cells/ml. While the survival of *M. ciceri* USDA2429 and *R. phaseoli* TAL1383 was maintained at 10^6 cells/ml after 6 months, *S. fredii* HH103 did not survive in this formulation (Fig. 3c). Liquid inoculant containing PEG could support the survival of *B. japonicum* USDA110 and *A. caulinodans* IRBG23 at cell concentration higher than 10^8 cells/ml only 5 months, but PEG could not support the survival of *M. ciceri* USDA2429, *R. phaseoli* TAL1383 and *S. fredii* HH103 (Fig. 3d). Inoculant containing PVA was generally less effective, except for its excellent performance with *A. caulinodans* IRBG23, which had a cell concentration of more than 10^8 cells/ml after 6 months of storage (Fig. 3e). Gum arabic supported most strains at 10^8 cells/ml, but *R. phaseoli* TAL1383 and *S. fredii* HH103 did not survive with this additive (Fig. 3f). Inoculant containing cassava starch, maintained the survival of *A. caulinodans* IRBG23 and *B. japonicum* USDA110 at cell concentrations of more than 10^8 cells/ml for 6 months, respectively. Cassava starch had only a moderate positive effect on the survival of *R. phaseoli* TAL1383, but *M. ciceri* USDA2429 and *S. fredii* HH103 failed to survive (Fig. 3g).

Table 3. Effectiveness test of *B. japonicum* USDA110 liquid inoculant formulation with polymeric additives on field performance with soybean SJ2

Carriers	Nodule number/ plant	Plant dry weight (kg/hectare)	Seed weight (kg/hectare)
Control	2 c	993.3 c	1429.8 b
Peat	23 ab	1398.0 ab	1883.0 a
PVP (2% w/v)	29 ab	1424.0 ab	1936.0 a
PEG (1% w/v)	36 ab	1556.0 ab	2014.3 a
PVA (0.5% w/v)	22 ab	1384.8 ab	1803.0 a
Arabic gum (0.3% w/v)	30 ab	1533.5 ab	1942.0 a
Cassava starch (1% w/v)	40 a	1785.0 a	2122.8 a
Alginate (0.1% w/v)	33 ab	1572.5 ab	2051.8 a

Means with different letters are significantly different at $p<0.05$

Discussion

The results of this research show that there is a degree of interaction between strains of rhizobia and additives that may benefit liquid inoculant performance. Our results indicate that, although some additives can be identified that generally perform better than others with a wide range of rhizobia and bradyrhizobia, to maximize performance of liquid inoculants additives may have to be selected for individual species and strains. Some polymeric substances, such as PVP have been blended with medium for normal culturing conditions of bradyrhizobia with no adverse affect on growth.[4] Since cells cannot use these polymers as an energy source, they have other properties supporting the growth and survival of cells. PVP is used as commercial product to increase the yield of yeast in fermentation.[14] PVP is believed to detoxify the fermentation media by complexing with the phenolic-type, shelf-limiting toxins in the media.[4] However, for other polymers, there are no reports about how they support cell growth in mass culture conditions.

Desiccation and high temperature also influence the survival of cells on seed.[15,16] In our experiment, the inoculated seeds were incubated for 48 hours at 40°C under atmospheric pressure without controlling the humidity. In practice, the temperature of the soil can be greater than 40°C during the midday in tropical regions.[6] However, the soil temperature is not constant at 40°C, especially during the night. Cell death under real field conditions may actually be lower than the results we obtained in this experiment. These results confirmed the ability of polymers to protect cells from extreme changes in environment after inoculation onto seed. Although some of the polymers maintained the cell viability at only 10^3 cells/seed, it has been reported that in soils devoid of specific rhizobia, concentrations of even 100-1,000 cells per seed could produce

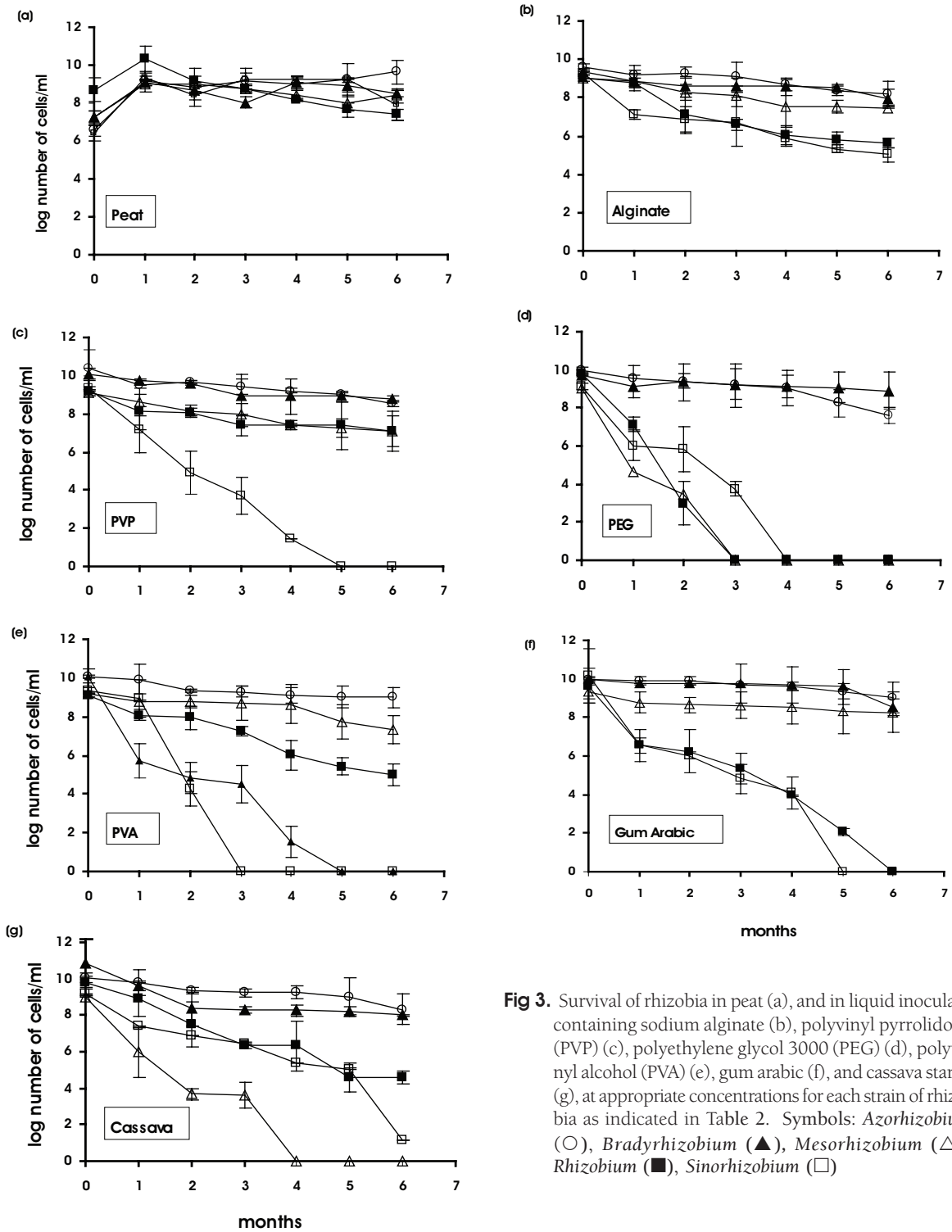

Fig 3. Survival of rhizobia in peat (a), and in liquid inoculant containing sodium alginate (b), polyvinyl pyrrolidone (PVP) (c), polyethylene glycol 3000 (PEG) (d), polyvinyl alcohol (PVA) (e), gum arabic (f), and cassava starch (g), at appropriate concentrations for each strain of rhizobia as indicated in Table 2. Symbols: *Azorhizobium* (○), *Bradyrhizobium* (▲), *Mesorhizobium* (△), *Rhizobium* (■), *Sinorhizobium* (□)

satisfactory nodulation.[17] These reports have been confirmed by the results of our field test (Table 3), which demonstrated that there were no statistical differences among liquid inoculant polymers and peat-based inoculant in soybean nodulation and yield.

Various polymers, such as PVP, PEG and gum arabic, have adhesive properties. They have a sticky consistency, which may enhance cell adherence to seed, and their

viscous nature may slow the drying process of the inoculant after application to seed.[18] PVP also has a high water binding capacity, which could maintain water around the cells for their metabolism.[4,9] PVP and gum arabic have been reported to protect cells against toxic seed coat factors.[16,19] In addition, as concentrations of salts increase in the cell environment as the liquid inoculant dries, stabilizing polymers such as PVA may be useful in reducing the extent of protein precipitation or coagulation of cells. Maintenance of macromolecular structure may improve biological integrity, thus leading to improved survival.[9] Biopolymers such as cassava starch, alginate and gum arabic have the ability to limit heat transfer and also have high water activities.[8] These might be the mechanisms that improve the survival of rhizobial cells on the seed and gives rise to nodulation and nitrogen fixation in the field that is equivalent to peat-based inoculant.

For commercial purposes a safe storage period of 6 months is desirable. Peat is widely used in the final stage of the preparation of legume inoculants, and generally constitutes a suitable carrier for this purpose.[20] It is a common perception that rhizobia do not survive well in liquid inoculant, especially when stored without refrigeration.[4] In our experiment, the storage temperature was 28-30°C, which could reduce cell survival in all carriers. Because of the cost of building large cold storage at the manufacturing plant and the inconvenience to farmers to keep inoculant refrigerated, we used a relatively high range of temperature for assessing cell survival during storage.

In liquid inoculant, rhizobial cells encounter starvation stress or nutrient depletion[21]. After cells enter into the stationary phase prior to packaging, nutrient depletion may be severe and may cause the higher reduction of viable cell in liquid inoculant compared to peat. The rate of decline in viable cells differed between strains and formulation of liquid inoculant. We showed that the survival of cells was dependent on both the additive types and strain of rhizobia.

The reason these polymers maintain viable cells at a high concentration is not exactly known. Alginate is the most common polymeric material for encapsulation of microorganisms for commercial use.[22] The main advantages of alginate inoculants are their nontoxic nature, degradability and slow release of the entrapped microorganisms into the soil.[1] It has been reported that plant-growth-promoting bacteria (PGPB) could survived for 14 years in dry alginate beads at ambient temperature.[23] Furthermore, alginate may be amended with nutrients to improve short-term survival upon inoculation.[24] Moreover, some polymeric additives such as PVP, PVA and starch have stabilization property. This protective property is known as colloidal stabilization. The improvement of survival is analogous to the protective colloid effect where bacteria represent one colloid and the suspension the other.[9] The polymer is absorbed in a thin molecular layer on the surface of the individual colloidal particles resulting in a stabilized suspension that prevents coalescence of cells,[14] which might block the O_2 and nutrient diffusion from media to cells.

The survival of cells was also dependent on the strain of rhizobia. Slow-growing rhizobia have been reported to survive longer than fast-growing rhizobia in liquid and peat-based inoculants and in the soil.[25,26] In liquid inoculant, rhizobia must encounter nutrient starvation and O_2 depletion during storage. These stress conditions may induce changes in the cell such as changes in cell morphologies, and reductions in cell division, cell protein content, and DNA and RNA synthesis.[27] Therefore, the ability to establish a low rate of endogenous metabolism and the ability to utilize endogenous energy reserves are mechanisms by which bacteria maintain viability under stress conditions.[25] This might be the reason why the production of liquid inoculant appears more appropriate for slow-growing rhizobia than fast-growing rhizobia.[28]

Rhizobial inoculant should not be stored for very long periods since induced physiological changes of rhizobial cells may increase the time to nodulation. Therefore, the efficiency of rhizobial inoculant may decrease over the time of storage.[29] In one reported field test, inoculant stored for one year produced a lower percentage of nodule occupancy than recently produced inoculant.[30] Therefore, proper storage and management of rhizobial inoculant in the distribution network is important to ensure proper product performance.

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