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Development of Efficient Suspension Formulation of Starch Industry Wastewater Grown *Sinorhizobium Meliloti* for Agricultural Use

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Abstract: **Liquid and alfalfa seed coated formulations of Sinorhizobium meliloti were developed as potential bio-inoculant. S. meliloti used in the present study was successfully cultivated using starch industry wastewater as the feedstock. The additives, such as sucrose, sorbitol, polyvinylpyrrolidone (PVP), polyethylene glycol (PEG), sodium alginates, and different mixtures of these compounds were evaluated for their capacity to maintain the viability of S. meliloti during 13 weeks of refrigerated storage. All formulations were found to be capable of maintaining a viable cell count greater than 10^9 CFU/mL. Sucrose (10% w/v) was found to be the best additive to extend the half-life of the formulation to as long as 83 weeks. During 4 months of seed storage at ambient temperature, fresh liquid formulation of PEG and PEG-sorbitol applied to alfalfa seeds maintained the cell viability of 103 CFU/seed, which is equivalent to Canadian standards for commercial formulations. Micrographs showed that S. meliloti cells were distributed differently on the seed surface (as aggregates or uniformly distributed) according to the additives used. 13 week old PVP-sorbitol formulation applied on alfalfa seed was the most efficient formulation to facilitated highest shoot yield on dry biomass basis.**

Keywords: Sinorhizobium Meliloti, **Formulation, Storage, Viability, Plant Yield**

I. Introduction

Application of bio-fertilizers has been showing an increasing trend for the last few decades as an alternative to soil amendments by chemical fertilizers. For legumes, inoculation of Rhizobium in soils/ on seeds was demonstrated to be an efficient agricultural practice as bio-fertilizer [1]. The challenge of bio-fertilizer formulations is to provide a suitable microenvironment for the bacteria to survive in soil conditions when inoculated in the soil and guarantee its efficiency under field conditions. Selection of suitable additives, concentration of different additives and industrial formulation processes are considered as key factors of microbial formulations [2]. Alfalfa nodulating fast growing Sinorhizobium meliloti is usually formulated in solid carriers, while liquid inoculants are the most suitable formulations especially for using in planting machines [1].

Solid inoculant carrier requires series of industrial processing steps, such as mixing, drying, sterilizing, milling, neutralization, packaging and uses large amount of costly additives; which can affect commercial competitiveness of the inoculant [3]. In addition, the problem of suitable carrier availability leads to an additional point to be considered. An overview of studies carried out on bio-inoculants has shown that preparation of liquid formulation of selected microorganism followed by pre-inoculation of seeds using such formulation is a potential alternative of solid carriers [3]. Moreover, a high cost of industrial production of inoculum has been attributed to the culture media; therefore, development of formulation based on industrial wastewater is particularly an interesting field of research. In previous work, it was demonstrated that starch industry wastewater (SIW) can be used to grow Sinorhizobium meliloti, which resulted in almost similar cell count (>10^9 CFU/mL) to that of standard synthetic medium [4]. In this study, the conditions for stable and efficient liquid and seed coated formulations of SIW grown *Sinorhizobium meliloti* have been optimized by using several classes of additives (polymers and sugars) and their mixtures.

II. Material and Methods

A. *Microorganisms and inoculum preparation*

Sinorhizobium meliloti A2 (Agriculture and Agri-food Canada, Sainte-Foy, Québec, Canada) was used in the current study. Inoculum was produced in yeast mannitol broth (YMB) medium composed of K2HPO4 (0.5 g/L), MgSO4.7H2O (0.2 g/L), NaCl (0.1g/L), yeast extract (1.0 g/L) and mannitol (10.0 g/L) [5]. This medium contained the following constituents. Cells were grown at 30±1 °C for 24 h on an Excella E25 rotary shaker (NewBrunswick Scientific, Edi son, US) at 180 rpm. 3% (v/v) inoculum containing 10^9 CFU/mL was used throughout the investigation.

Starch industry wastewater (1.4% w/v total solids, ADM Ogilvie; Candiac, Québec, Canada) was used for for the cultivation Sinorhizobium meliloti [6]. 0.1% (w/v) yeast extract was added and medium pH was first adjusted to 7 [4]. The medium was sterilized at 121 ± 1°C for 15 min

which was followed by fermentation for 72 h at 30°C under similar incubation conditions as inoculum production.

Cell count was estimated by plating on YMA plate, supplemented with Congo red (0.25% w/v) after a serial dilution using saline solution (NaCl, 0.85% w/v). Incubation of plates was carried out at 30 ± 1°C for 72 h.

B. Liquid formulations

Cells were grown until 4.5 x 10^9 CFU/mL which was followed by development of suspension formulations using fermented SIW. Additives that were tested for formulations comprised: sorbitol at concentrations of 1, 2 and 5% (w/v); sucrose at 2, 5 and 10 % (w/v); sodium alginate at 0.2, 0.3 and 0.5 % (w/v); polyvinyl pyrrolidine (PVP) (Mw=100000) at 1, 2 and 5 % (w/v) and polyethylene glycol (PEG) (Mw=8000) at 1, 2 and 5% (w/v). A combination of additives tested in the present study was composed of 1% (w/v) of PVP with 5% (w/v) of sucrose, 2% (w/v) of sorbitol and 0.3% (w/v) of sodium alginate. Similarly, in another combination 1% (w/v) of PEG was mixed with 5% (w/v) of sucrose. All additives were sterilized prior to their application and all formulations were kept at 4 °C. The efficiency of the formulations was measured based on the survival of bacteria which was determined every 3 weeks of storage.

C. Seed coated formulations

The selected chemicals additives used for seed coating were (w/v): PVP, 1%; polyethylene glycol, 1%; sucrose, 5%; sorbitol, 2%; and sodium alginate, 0.3%. Likewise, a combination of 1% PVP either with 5% of sucrose or 2% of sorbitol or 0.3% of sodium alginate was studied. Formulations based on 1% polyethylene glycol either with sucrose (5%) or sorbitol (2%) and sodium alginate (0.3%) were also studied. In these experiments, fresh fermented broth with viable cell count of 4.5x109 CFU/mL served as control.

Alfalfa (Medicago sativa L) seeds were coated with fresh liquid formulations (FLF) as well as 13 week old liquid formulations (OLF). In order to coat the cells, 4 g of seeds were vigorously mixed for 2 min with 2 mL of each formulation (at ratio 2:1) and kept in contact for 2 h at ambient temperature. Drying was achieved by placing the samples in laminar flow for 12 h. Coated seeds (initial viable cells ≈ 10^6CFU/seed) were stored at ambient temperature (23 ±1°C) in petri-dishes until viable cells concentration decreased below 103CFU/seed.

Viability of the coated *S. meliloti* was determined at various storage times. In order to determine the cells viability on the coated seeds, about 45 coated seeds were mixed with 4.5 mL of saline solution (NaCl, 0.85% w/v, supplemented with Tween 80 at 0.1% w/v) for 2 min; serial dilutions were used for cell count. YMA plate supplemented with Congo red (0.25% w/v) was used for cell plating and incubation of plates was carried out at 30±1 ºC for 72 h.

D. Plant yield and nodulation index

Alfalfa seeds pre-inoculated with FLF (PEG 1% and PEG1%-Sorbitol 2%) and with OLF (sucrose 5% and PVP1%-sucrose 5%) were tested for their symbiotic efficiency. For each formulation, 10 alfalfa coated seeds were sown in sterilized plastic growth pouches (Mega international of Minneapolis, Mexico) fed with nutrient N-free solution [5]. Fifteen pouches were used for each formulation. After germination, plants were kept in growth room (at 20°C with a photoperiod of 16 h and at 15°C during nights). Plants were irrigated regularly with deionized water. Plant yield was determined based on dry matter (plants were dried at 70°C for 48h) after 6 weeks of growth. To calculate the nodulation index, visual estimation of nodule number (3 for several, 2 for many, and 1 for few) was multiplied by the color (2 for pink and 1 for white) by the size of root nodules (3 for large, 2 for medium and 1 for small); maximum nodulation index is18 [7].

These tests were carried after 24h of coating, and after 12 and 6 weeks of seed storage for FLF and OLF, respectively (which corresponded to viable cells per seed of ≈ 3x10^3CFU/seed).

E. Microscopic observation

Distribution of Sinorhizobium meliloti and additives used on seeds were examined using a scanning electron microscope (Carl Zeiss SMT, Cambridge, England). Coated seeds were first fixed to metal stud, coated with mild gold at a thickness of 0.01 mm and then exposed to an accelerate voltage of 10KV.

F. Statistical analyses

Statistical analyses were carried out using R version 2.10.1 (The R Foundation for Statistical Computing ISBN 3-900051-07-0). Analysis of variance (ANOVA) was used to evaluate significance of the difference between the responses. Difference was considered significant at $p < 0.05$ (α was fixed to 0.05). The error bars on the Figures indicate the mean standard deviations for three replicates.

III. Results and Discussion

A. Survival of cells in liquid formulation during refrigerated storage

A1. Sucrose formulations

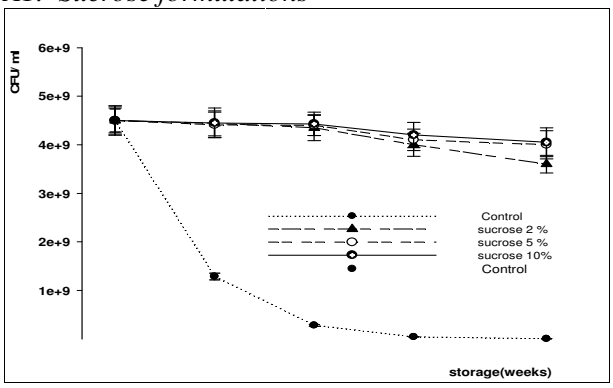

Fig 1(a)

Figure 1a shows the cell viability during 13 weeks of storage at 4°C for sucrose at concentrations of 2, 5 and 10% (w/v). These formulations maintained higher cell

viability than the control (fermented broth). Viable population was similar at all concentrations studied, i.e., 2, 5 and 10% (w/v) until 9 weeks of storage (≈ 4.4 ×10^9 CFU/mL). At 13th week, for the formulation involving 2% of sucrose, viable cell count was found to be 3.6×10^9 CFU/mL. Compared to this, formulations with 5 and 10% sucrose exhibited higher stability which was close to the initial cell count (4.1 and 4.2×10^9 CFU/mL, respectively). Viability of cells in the control decreased steeply by approximately one log cycle after 6 weeks of storage and reached 2.8 ×10^8 CFU/mL (Figure 1a). At the end of storage period of 13 weeks, viable cell count was 4.17×10^5 CFU/mL. Major decrease was only in the first six weeks; afterwards it was slow.

Sucrose has been rarely investigated as suspending agent and it has been commonly used as growth substrate and non-ionic osmo-regulatory agent. It was proved that addition of sucrose helps to maintain the stability of proteins and cell structure during osmotic stress [8]. Mechanisms involved in the stability of formulation are mainly related to the hydrogen bonding between sucrose and proteins, which may be responsible for cell stabilizing effect [9, 10]. Gouffi et al. [11] demonstrated that sucrose is an effective osmo-protectant for *S. meliloti*, *R. leguminosarum* bv. *trifolii* and *phaseoli.* Sucrose has been showed to be a superior suspending agent in comparison to glucose, xylose, tryptophane, salicin, saline and water for the protection of *Streptococcus pyogenes* C203 and *Escherichia coli* communion during desiccation [9] and was able to enhance survival of *R. japonicum* USDA 138 in biopolymer gels formulations [12]. In the present study, efficiency of sucrose as suspending and protecting agent for refrigerated storage of fermented broth containing *S. meliloti* has been determined.

A2. Sorbitol formulations

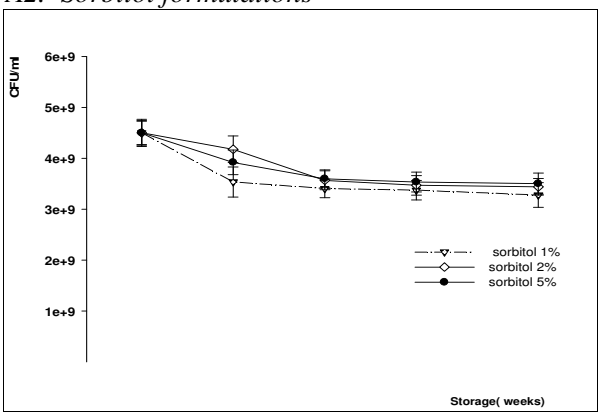

fig 1(b)

One of the major microbiological applications of sorbitol is cell protection during freeze drying; [13, 14]. Annear [15] pointed out the efficiency of sorbitol-peptone mixture in preserving several vacuum dried bacteria on cellulose fibers at room temperature. The mechanisms by which sorbitol can protect living cells could be the stabilization of lipids and proteins of the cell membrane [16]. Sorbitol was also demonstrated to be efficient in stabilization of the structure of collagen [17] and in protection of ovalbumin, lysozyme, conalbumin, and α-chymotrypsinogen against heat denaturation [18]. Sorbitol has been reported to strengthen the bridge interactions between polypeptide chains by replacing the water molecules [19].

The storage profiles of formulations with different concentrations of sorbitol ranging from 1 to 5 % (w/v) are given in Figure 1b. In the first six weeks of storage, viability increased with the concentration of sorbitol added. For instance, at 3rd week, cell count of ≈ 3.5×10^9 CFU/mL was observed for 1% sorbitol; whereas cell count of ≈4×10^9 CFU/mL was obtained for 2 and 5% of sorbitol. From 6th to 13th week of storage, all sorbitol formulations were equally effective and maintained an unchanged *S. meliloti* population with the final cell count of 3.3×10^9 CFU, 3.4 ×10^9 CFU/mL and 3.5×10^9 CFU/mL for 1, 2 and 5% of sorbitol, respectively. Thus, it can be concluded that viable cell count of *S. meliloti* depends on both initial cell count as well as sorbitol concentration.

A3. Polyethylene glycol (PEG) formulations

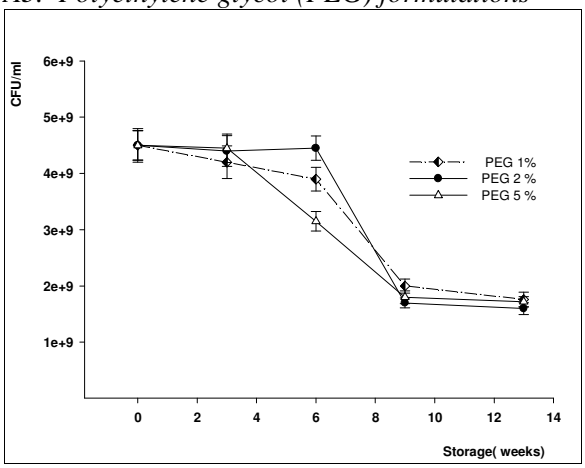

fig 1(c)

PEG at different concentrations such as 1, 2 and 5% (w/v) was evaluated as an additive to preserve the viability of *S. meliloti*. Figure 1c shows the viability profile during storage of these formulations. *S. meliloti* viability varied among the concentrations studied in the first nine weeks of storage. A steady phase in cell count was observed for first 3 and 6 weeks for the formulation comprising of 5 % and 1 and 2 % of PEG, respectively. Later, there was a sharp decline in viable cell count of all formulations until 9th week. The slope indicating decline in viable cells count was more pronounced in the case of 1% than that of 2 and 5% of PEG. From the 9th week to the end of storage period, a stable phase in cell viability was observed for all PEG concentrations studied, and the final cell count ranged from 1.6 X 10^9 to 1.7 X 10^9 CFU/mL.

It has been demonstrated that polyethylene glycol is an effective cryoprotectant for proteins [20] and microorganisms subjected to freeze-drying [21]. From literature reports it has been observed that addition of PEG 6000 to soil provided protection for fast growing strains [22]; [9]. Priming soybean seeds in PEG 8000 solution improved germination and seedling establishment by

stimulating physiological and biochemical activities [23]. PEG 3000 was studied by Tittubtr et al. (2007) in liquid formulation and they observed that addition of this polymer led to the development of stable formulations of *Azorhizobium caulinodans* IRBG23 and *Bradyrhizobium japonicum* USDA110 during 6 months of storage. Thus, present finding and literature data suggest that PEG could be an effective agent for the preparation of stable formulation of *S. meliloti.*

A4. Polyvinyl pyrrolidine (PVP) formulations

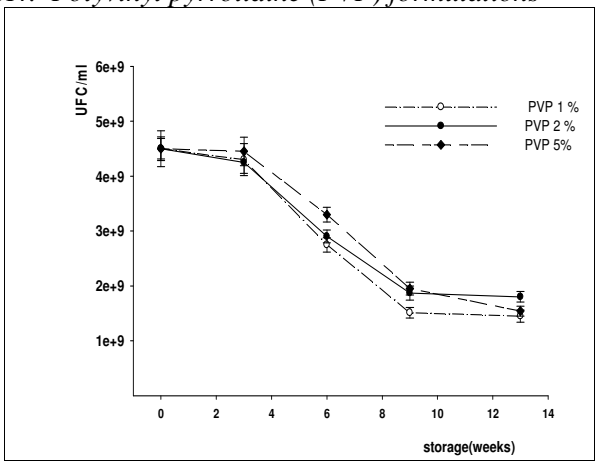

fig 1(d)

PVP with average molecular weight of 10000 was also tested as an additive for the preparation of *S. meliloti* formulation. 1, 2 and 5 % (w/v) of PVP was used for the assessment and the results have been presented in Figure 1d. Among these concentrations, 2% of PVP maintained the highest cell viability with a final viable cell count of ≈1.8×10^9 CFU/mL. For 1 and 5% of PVP, final cell counts were found to be 1.45×10^9 CFU/mL and 1.54×10^9 CFU/mL, respectively. The result suggests that relatively high concentration of PVP may be unfavourable for preserving the viability of *S. meliloti.* The viscosity of the formulation prepared using high concentration of PVP may be the factor behind observed difference in cell viability.

PVP has been described as a polymer with high water-binding capacity, largely used as seed coating adhesive for bacteria. Some researchers observed that the insoluble PVP was able to bind seed exudates, naturally mobilized during inoculation and seed germination (Deaker et al.2007). PVP improved viability of *B. japonicum* SEMIA 5019 when mixed with the culture broth and peat and applied on soybean seed [3, 24]. Generally, under the storage conditions studied, PVP formulations maintained comparable cell viability to that of PEG. Tittubtr et al [25] proposed an adsorption mechanism by which the polymer formed a thin molecular layer on the surface of the individual particles, which resulted in a stabilized suspension that prevents cell membrane damage, consequent reduction of O_2 and nutrient diffusion from media to cells, leading to a stable cell count during storage.

A5. Sodium alginate formulations

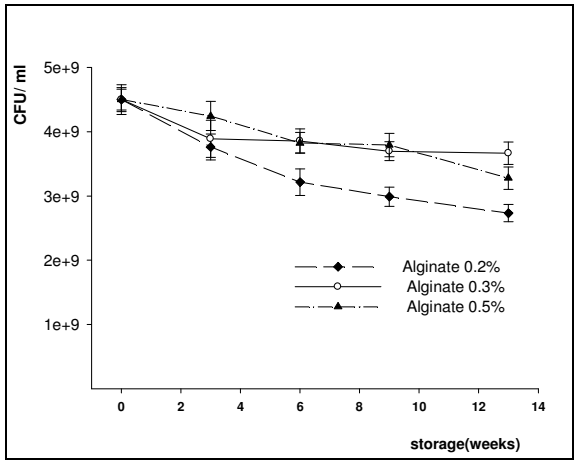

fig 1(e)

Figure 1 e shows the storage profile of sodium alginate formulations. For all sodium alginate concentrations studied during 13 weeks of storage period, i.e., 0.2, 0.3 and 0.5% (w/v), the formulations showed a viability of cells more than $3X10^9$ CFU/mL. The formulation containing 0.3 % of sodium alginate was the most effective in promoting rhizobial survival with a cell count of ≈3.7×10^9 CFU/mL. On the contrary, for 0.2 and 0.5% of sodium alginate it was only $2.7x10^9$ CFU/mL and 3.3×10^9 CFU/mL, respectively.

Efficiency of sodium alginate has been already demonstrated for cell encapsulation and it is the most common biopolymer used for industrial purposes [26-29]. As a constituent of seed coating formulation, main advantages of alginate are biocompatibility and biodegradability in the soil [2]. [25] observed that 0.1% (w/v) of sodium alginate added to liquid inoculants improved the survival of *Bradyrhizobium japonicum* USDA110, and *Mesorhizobium ciceri* USDA 2429 cultivated in standard media, which remained at cell concentrations of 10^7-10^8 cells/mL and *Sinorhizobium fredii* HH103 from 10^5 to 10^6 cells/mL after 6 months of storage at 28-30ºC. [12] attributed the protective nature of alginate and biopolymer in general to its high water activity and stabilizing effect of cell membrane [30, 31].

A6. Formulation with combination of additives

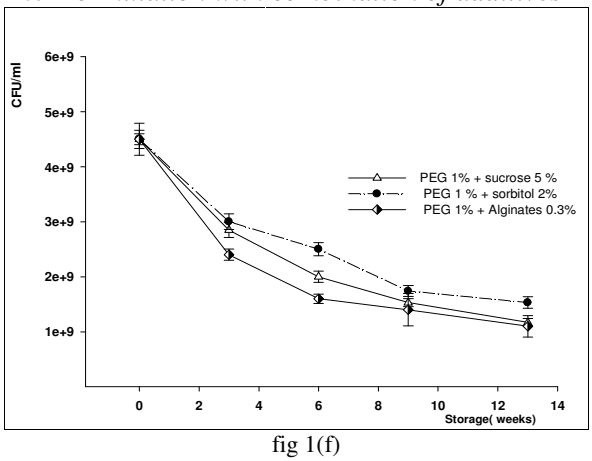

fig 1(f)

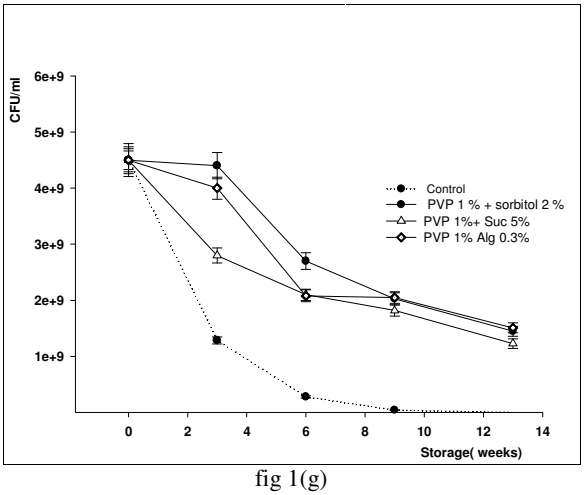

fig 1(g)

Figure 1. Survival of *Sinorhizobium meliloti* in liquid formulations at 4°C (a) sucrose; (b)sorbitol; (c) PEG; (d) PVP; (e) alginate; (f) PEG + sucrose, PEG+ sorbitol, PEG + alginates; (g) PVP + sucrose, PVP+ sorbitol, PVP+ alginates

Mixture formulations were developed in order to study the effect of combination properties of sorbitol, alginates, sucrose, PEG and PVP during refrigerated storage. Results of mixture of PEG-sorbitol, PEG -sucrose, PEG-alginates and PVP-sorbitol, PVP-sucrose, and PVP-alginates are shown in Figure 1f and 1g. The survival of bacteria was slightly higher in the formulations of (PEG 1% w/v + sorbitol at 2% w/v) and (PVP 1% w/v + sorbitol at 2% w/v) during storage at 4ºC. After 12 weeks, the cell count was 1.53 and 1.45 x10^9 CFU/mL for PEG (1% w/v) + sorbitol (2% w/v) and PVP (1% w/v) + sorbitol (2% w/v), respectively.

B. Comparison between formulations

Comparison was carried out between formulations to select the most effective additives and concentrations that can be used to promote the *Sinorhizobium meliloti* survival in liquid formulation, during storage at 4ºC. The comparison was based on calculation of half-life ($t_{1/2}$) according to the following Equation:

Viable cell count = a*exp (-b*t)), t: storage period in weeks, a and b are constants

$t_{(1/2)}$=-[log((initial viable cell count) /2*a)]/b (1)

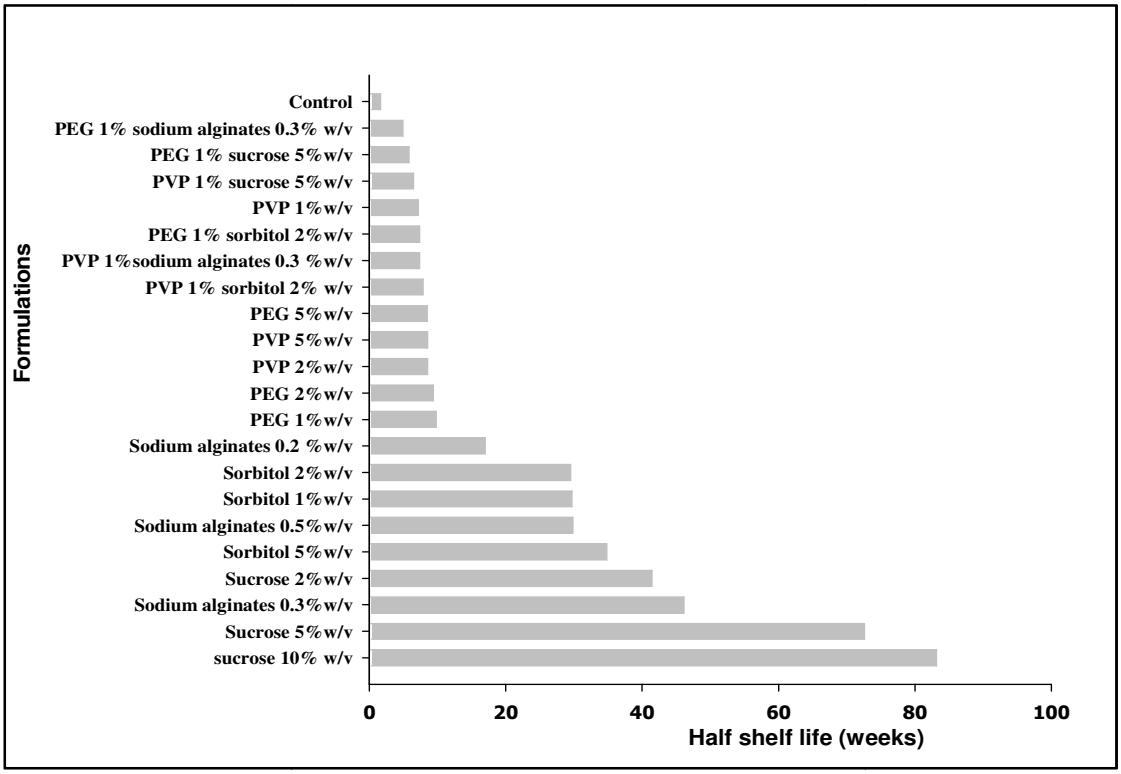

Figure 2. Half-life of different liquid formulations involving *S. meliloti*

The half-life results for the different formulations are shown in Figure 2. All probability values *p* related to the models used were < 0.05. The most efficient formulations were those containing sucrose followed by alginates, sorbitol, PEG, PVP and finally combination formulations. The best sucrose concentration was 10% w/v; with a half-life of approximately 83 weeks, while half-life of control was 1.6 weeks. Half-life of sucrose at 5 and 2% w/v were 72.5 and 42 weeks, respectively. Alginates at 0.3 % w/v was the second best formulation, this concentration guaranteed a half-life of 44 weeks. Half-life of *Sinorhizobium meliloti* at all sorbitol concentrations were similar (around 29 weeks). Sucrose formulation (5% w/v) showed a half-life of 72.5 weeks, whereas, half-life for PEG and PVP 1% (w/v) each were found to be 9.8 and 7.2 weeks, respectively. When 5% (w/v) of sucrose was mixed with 1% (w/v) of PEG or PVP, the half-life decreased to 6.4 and 5.8 weeks, respectively. A similar

reduction in half-life has been observed in the case of formulations comprising sorbitol (1% w/v) and PEG and PVP 1% (w/v) each where the half-life was decreased from 29.5 weeks to 7.4 and 7.9 weeks, respectively. Exact mechanism of these dramatic reductions in half-life of formulations is a subject of further research.

C. Effect of storage and additives on Sinorhizobium meliloti viability on coated seeds

C1. After 24h of storage

Old liquid formulation (OLF) and fresh liquid formulation (FLF) were compared for their ability to support survival of *Sinorhizobium meliloti* when applied to alfalfa seeds and stored for 24h. In order to simulate real storage conditions, coted seeds were stored at ambient temperature without humidity control. For this investigation, inoculant efficiency (%) was calculated as:

(%) cells viability on seeds = viable cell count per seed after 24h/ initial cell count applied per seed (2)

Initial cell count applied per seed= cell count in formulation (CFU/mL) / Number of seeds mixed with 1 mL of formulation (3)

Table 1. Percent cell viability on seeds after 24h of coating

Formulations	PVP 1%	PVP1%-Sucrose 5%	PVP1%-Sorbitol 2%	PVP1%-Alginates 0.3%	PEG 1%	PEG1%-Sucrose 5%	PEG1%-Sorbitol 2%	PEG1%-Alginates 0.3%	Sucrose 5%	Sorbitol 2%	Alginates 0.3%
Fresh Liquid Formulation (FLF)	14.4	18.2	13	12.8	14.9	22.3	17.2	19.2	21.7	13.8	3.1
Old Liquid Formulation (OLF)	8	8.6	11	10	11.6	15.9	ND*	13	1.5	0.4	2

* ND: Not Determined

There was a substantial decrease of *Sinorhizobium meliloti* viability (%) on alfalfa seeds after 24h in all formulations (Table1). The most efficient seed formulations were PEG 1% -sucrose 5% and PEG 1%-alginates 0.3% for both FLF and OLF. These formulations maintained the viability of 22.3 and 19.2 % of cells added per seed for FLF; and 15.9% and 13% for OLF, respectively. Addition of 0.3% (w/v) of alginates, 2% (w/v) of sorbitol or 5% (w/v) of sucrose to PEG and PVP formulation (1% w/v each) improved inoculant survival.

Compared to seeds coated with fresh liquid formulation (FLF), cell viability was found to decrease for all additive types when old/stored liquid formulations (OLF) were used for the purpose. This was mainly observed for sucrose (5%), where cell viability was found to be 21.7% for FLF and it decreased to 1.5% for OLF. Likewise, in the case of sorbitol (2%), when FLF was replaced by OLF, viable cell count was decreased from 13.8% to 0.4%. During seed coating, the first important formulation property was adhesiveness and protection against desiccation [25]: weak adhesion resulted in low immobilized cell count on seed surface. This could possibly explain the low inoculant efficiency observed in the case of alginates formulation at 0.3 % w/v, where quantity of polymer added was the lowest used in this study. It was demonstrated that survival of rhizobia on seed depends on kinetics of water loss and/or moisture sorption characteristics of the polymeric adhesives and anti-desiccant used [8]. Studying the survival of *R. leguminosarum* bv. *trifolii* coated to glass beads under dry conditions, [32] described two distinct phases of death in relation to loss of water; the first: between 0 and 24 h, characterized by a rapid loss in cell numbers, the second phase was characterized by a decline of the death rate and continued loss of water.

Efficient formulation should protect cells after coating, especially against desiccation, the phenomenon which induces changes in cells membrane permeability during storage. When polymers are combined with sucrose and sorbitol, cell membrane is protected against osmotic stress conditions, increasing oxygen permeability and toxic effect of seed exudates, by complementary properties of additives used. PVP and PEG have a high water binding capacity which could maintain sufficient water around cells, reduced permeability and death and explain these efficiencies [25]. Sorbitol and sucrose stabilized cell membrane during osmotic stress conditions and improved survival of rhizobia on seeds and beads [9, 33]. Therefore, formulation of these components will combat against desiccation and osmotic pressure.

When liquid formulations were stored for 13 weeks (OLF) prior to seed coating, a general decrease was observed in the percentage of cells viability in comparison to FLF. This could be mainly due to the altering effect of long time storage in cell membranes [34], resulting in increase of cell sensitivity to desiccation and higher percentage of death.

C2. During storage

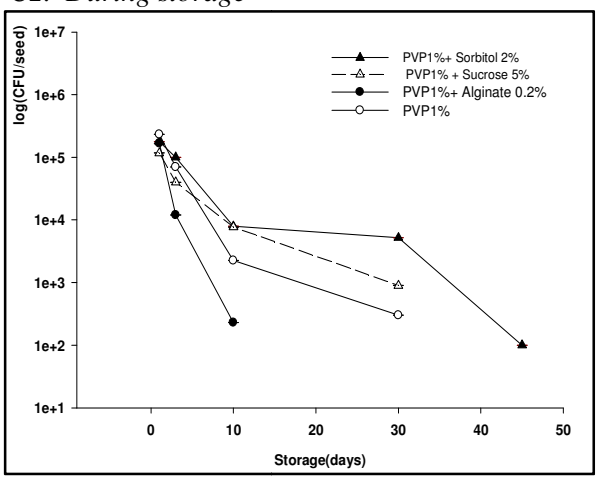

fig3(a)

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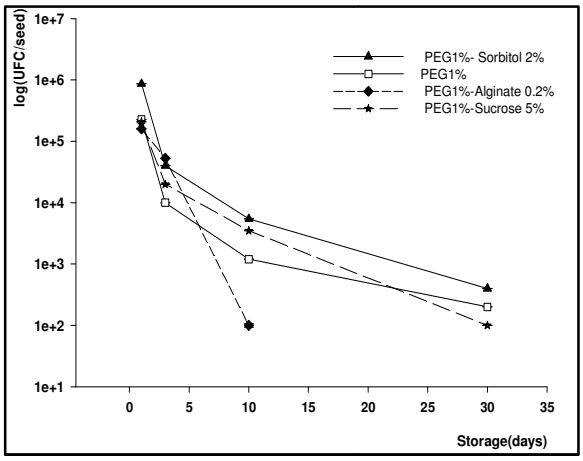

fig 3(b)

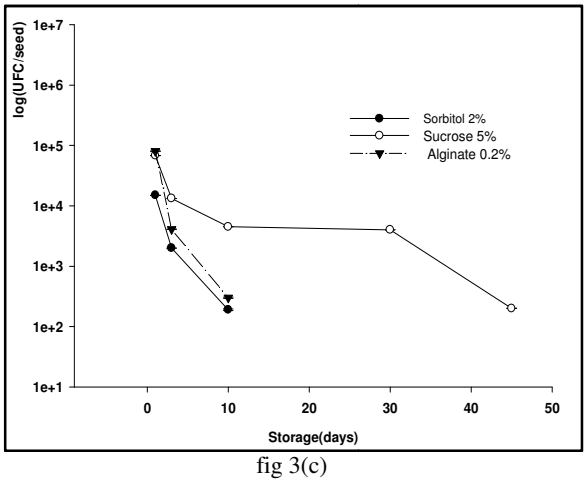

fig 3(c)

Figure 3. Survival of *S. meliloti* cells on alfalfa seeds coated with OLF supplemented with: (a) PVP, (b) PEG, (c) sucrose, sorbitol and alginates

Results of cells viability on coated seeds are shown in Figures 3 and 4 for FLF and OLF, respectively. During storage, the effect of desiccation continued following drying. To select the most efficient additives, a comparative study was carried out between seeds coated with (FLF) and those coated with (OLF). Seeds were stored at ambient temperature for definite periods as specified later. The number of live rhizobial cells is one of the most important criteria of inoculant quality determination. Improving survival of the cells on seeds would directly affect nodulation and subsequent yield of the plant [9]. According to Canadian Standards (CFIA) for commercial products, minimum 10^3 *Sinorhizobium meliloti* cells should be delivered per seed [7].

All the formulations tested were equally effective in maintaining the initial rhizobial population higher than 10^4 CFU/seed after 24h of coating. Best protection during storage was achieved by application of fresh formulations. Survival of cells on seeds coated with PEG (1%) and combination of PEG (1%) with sorbitol (2%) was in conformity with Canadian standards over 130 days of storage as seen in Figure 4 b. The protective nature of these formulations was possibly due to PEG properties. In general, polymers have the ability to limit water transfer (evaporation) by acting as a barrier, which could protect cells during long time storage [12].

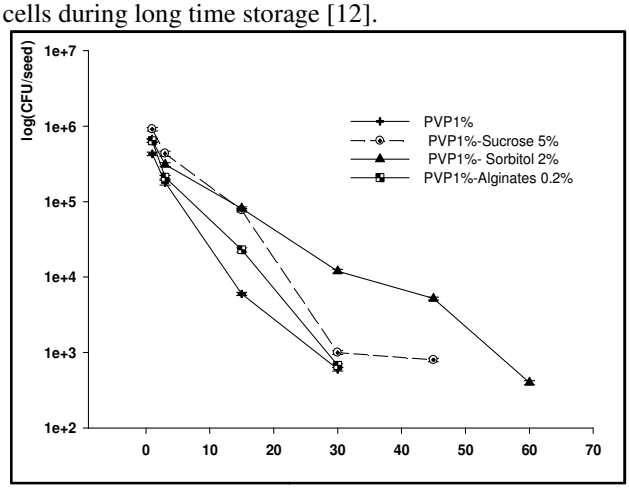

fig 4(a)

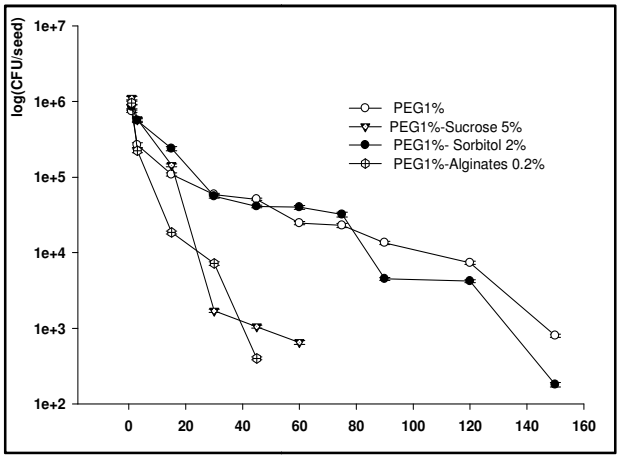

fig 4(b)

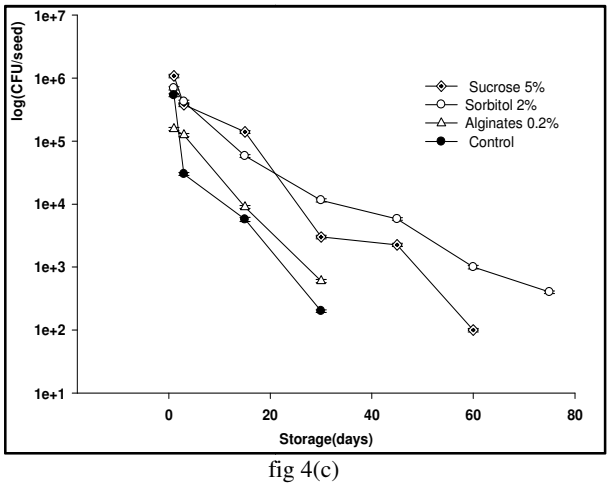

fig 4(c)

Figure 4: Survival of *S. meliloti* cells on alfalfa seeds coated with fresh liquid formulation (FLF) supplemented with: (a) PVP, (b) PEG, (c) sucrose, sorbitol and alginates. Control is fermented SIW.

In the present investigation, when seeds were coated with stored formulations, best cell viability was obtained

for sucrose (5%) based formulation, followed by combination of PVP (1%) with sorbitol (2%). In all cases, during 35 days of storage cell viability was always found to be more than $3.5x10^3$ CFU/seed, which is higher than Canada standards of minimum 3 $x10^3$ CFU/seed.

These results suggested an existing interaction between period of storage of liquid inoculant before application to seeds and the additives. In fact, physiology of cells seems to be negatively affected by prolonged liquid inoculant storage due to morphological changes [35]. When applied to seeds, *S. meliloti* cells are directly exposed to oxygen which acts as an additional factor affecting viability, damaging proteins, membranes and nucleic acids [9]. Sorbitol and sucrose were demonstrated to be efficient in maintaining macromolecular structure which may improve biological integrity, and consequently survival of cells. The protective function was related to the ability of these additives to replace the water lost during desiccation and thus preventing formation of unfavourable conformations in labile structures [36].

D. Inoculant evaluation for plant yield and nodulation index

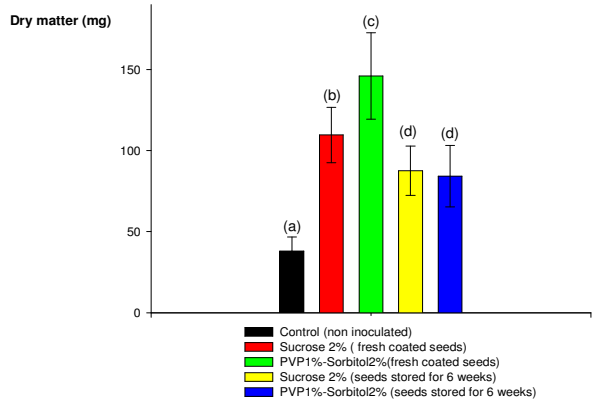

fig5(a)

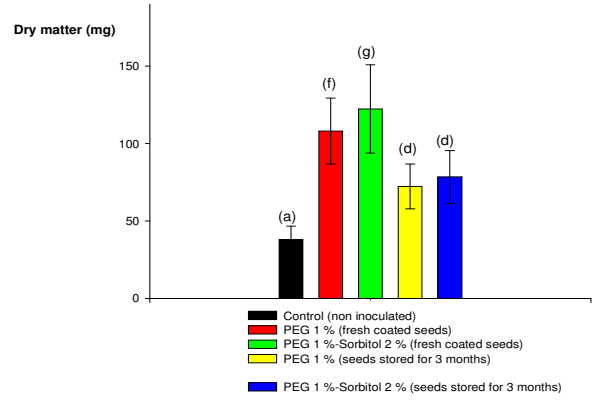

fig 5 (b)

Figure 5. Shoot dry matter yield of alfalfa inoculated with: (A) Seed coated by 13 weeks old stored liquid formulation (OLF); (B) Seed coated by fresh liquid formulation (FLF). Responses indicated by the same letter in the Figure are not significantly different at P <0.05.

Shoot dry plant matter yield of seeds coated with OLF and FLF are shown in Figures 5a and 5b, respectively. For fresh coated seeds, best results were obtained for seeds coated with OLF of PVP (1%)-sorbitol (2%), followed by seeds coated with FLF of PEG (1%)-sorbitol (2%). Plant yields for these two combined formulations and control (uncoated seeds) were 145 mg and 122 mg, and 38 mg, respectively. When the sown seeds were coated with formulations of sucrose (5%) and PEG (1%), dry matter content was almost similar, i.e., around 105 mg.

Stored coated seed formulations were sown when viable cell count was between 3 and 3.5 10^3 CFU/seed, which corresponded to a seed storage period of 6 and 12 weeks for OLF and FLF, respectively. Plant yields were found to be almost similar for all the cases.

As determined by nodulation index, all formulations attributed similar alfalfa nodulation index, which was 18, the maximum possible index value. This similar observed nodulation could be due to the fact that all formulations were applied at very high number of *Sinorhizobium meliloti* cells (higher than 10^5 CFU/seed). The same tendency was observed for stored coated seed formulations with a nodulation index of 12.

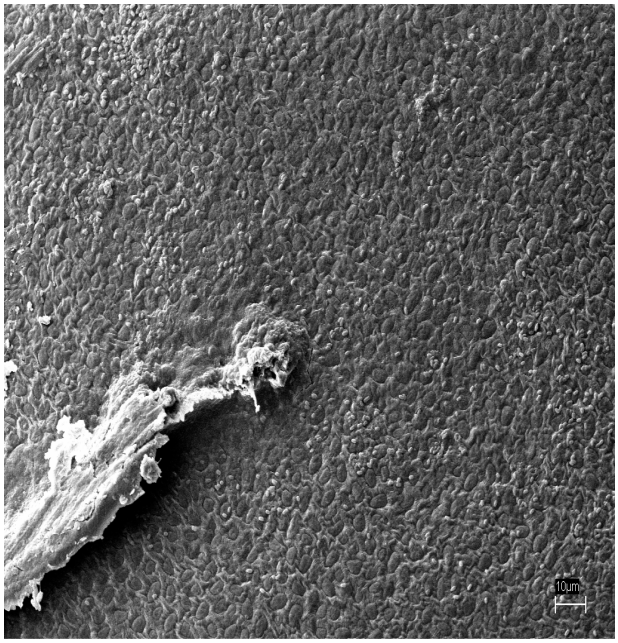

fig 6(a)

The effect of formulations on plant yield was not dependent only on the number of viable cells per seed at the time of sowing, but also on the additives. For example, plant yield was 122 mg for the formulation of PEG-sorbitol and the same was 147 mg for the formulation of PVP-sorbitol although, the later had 8 times lesser number of rhizobial cells than PEG-sorbitol. The same trend was observed when the results for OLF of sucrose (5%) and FLF of PEG (1%) were compared. In both the cases, the plant yields were almost the same, although viable cell count was 10 times higher in the case of PEG (8.5 x 10^5 CFU/seed) based formulation.

Relatively better performance of some formulations containing lower number of viable cells could be due to specific chemical and physical properties that may protect cells from dehydration damage on coated seeds. This hypothesis is supported by microscopic observations of coated seeds (Figure 6 and 7) which showed that cells are

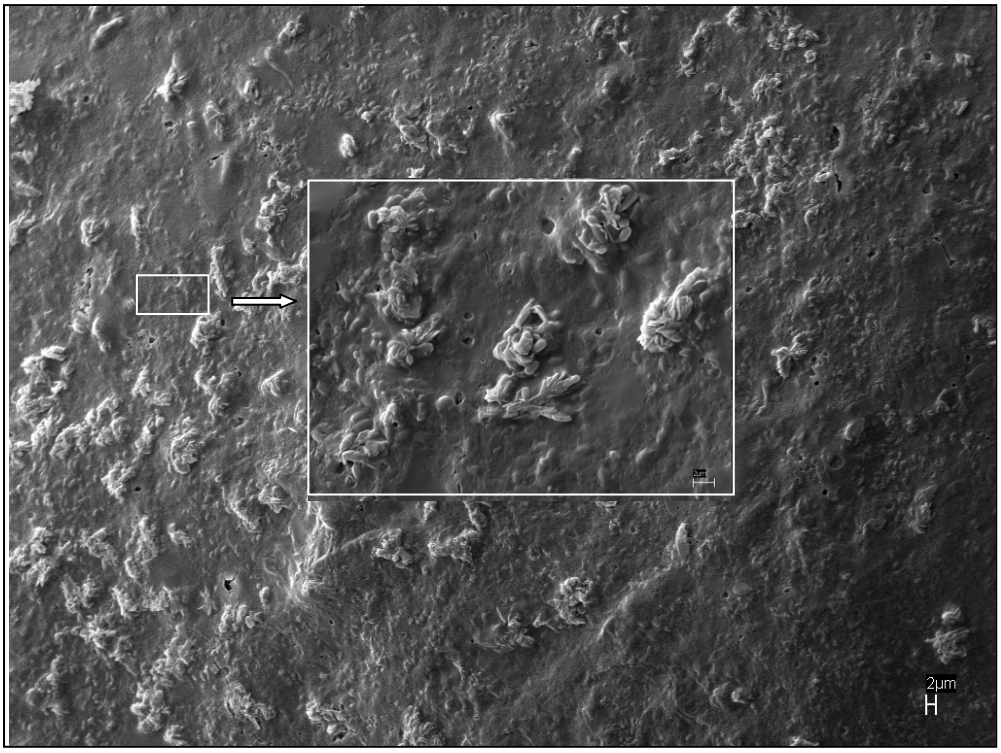

fig 6(b)

Figure 6. Micrographs of *S. meliloti* on alfalfa seeds coated with sucrose (5%) (A), and PVP (1%)-sorbitol (2%) (B)

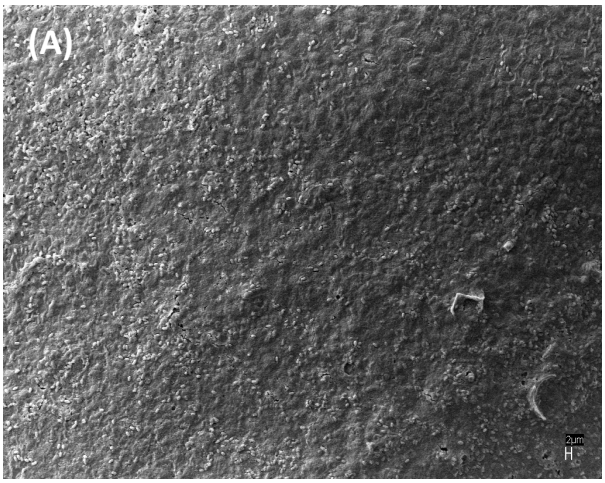

fig 7(a)

distributed on seed surface in a varied manner depending on the additives. For PVP-sorbitol OLF, cells were entrapped in a matrix and bound together on the cluster; seed surface was rough due to the higher repartition of these aggregates with approximately 50 cells per cluster. Such kind of arrangement allowed better protection of cells, binding more water which improved cellular metabolic activity, the initial step of nodulation process, and would consequently increase nitrogen fixation and plant yield. On the contrary, for seeds coated with PEG and PEG-sorbitol formulations, individual cells were uniformly distributed in multiple layers. The response of sucrose coating was similar to PEG and PEG-sorbitol, but with less number of layer of cells on the seed surface. Thus, these non-aggregated cells are not likely protected against the damage caused by water uptake during seed imbibition. This distribution may slowdown the rhizobium growth by delaying the recovery of optimal cellular activity and consequently delaying nodulation process. In our experiment, although we did not see differences in the nodulation index after 6 weeks, but appearance of nodules could be delayed with these formulations. Thus, formulation efficiency is not only related to the initial cell count applied and the period of storage; other factors concerning cell distribution on the seed surface do play a major role during nodulation.

This study showed the complexity of criteria for determining bioinoculant efficiency. Many questions remain regarding the physiological response of cells to the liquid formulation from storage phase to the final application phase of sowing. In fact, maintaining high level of viable cell count in liquid or seed coated formulations cannot be considered as the only criterion to select best formulation. During all storage phases and subsequent final application, bioinoculant formulation should guarantee cell performance until seed germination and plant growth.

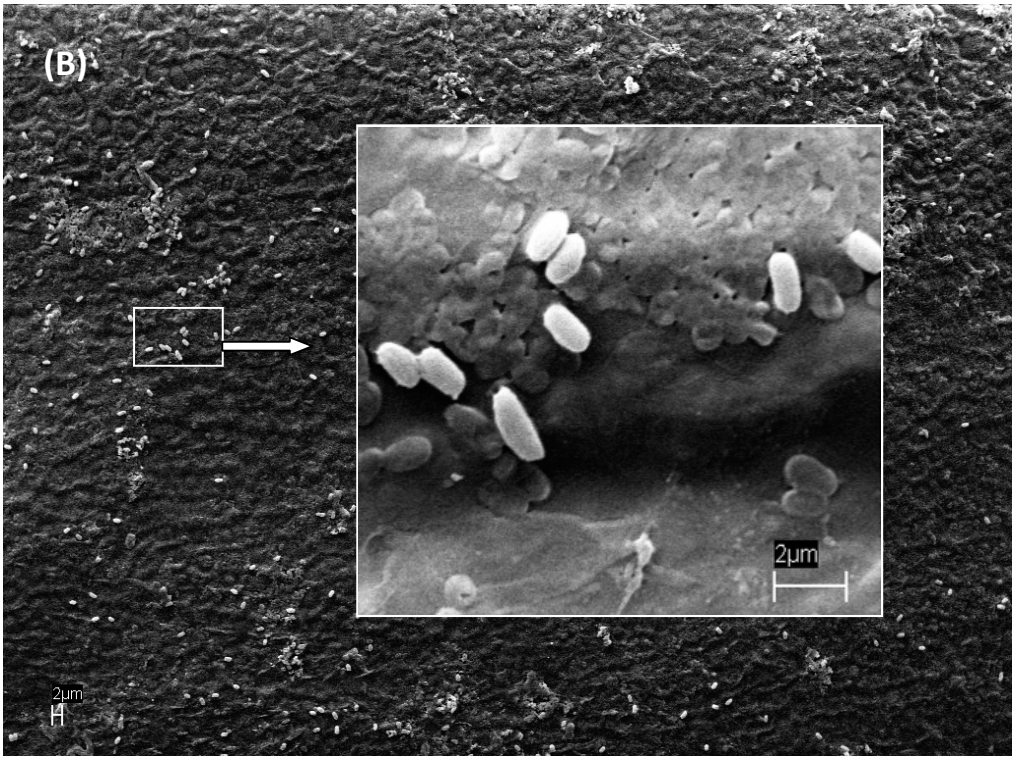

fig 7(b)

Figure 7. Micrographs of *S. meliloti* on alfalfa seeds coated with PEG (A), and PEG-sorbitol (B)

IV. Conclusions

Biofertilizer formulation can be developed by growing *Sinorhizobium meliloti* in starch industry wastewater which can increase the economic feasibility of the process by replacing expensive synthetic media components. All the liquid formulations developed in the present investigation were found to maintain *S. meliloti* cell viability higher than 10^9 CFU/mL. With the longest half-life of nearly 83 weeks, sucrose (10 %) was found to be best additive to preserve the viability of *S. meliloti* during storage in liquid formulation. For seed coated formulations, fresh liquid formulation (FLF) supplemented with PEG and PEG-sorbitol guaranteed a viable cell count of more than 10^3 CFU/seed during 4 months of storage. The effectiveness of formulations was tested on alfalfa and the highest shoot dry matter was obtained with multi additive formulations, i.e., PVP-sorbitol and PEG-sorbitol for OLF and FLF, respectively. Thus, these formulations have been recommended for the development of *Sinorhizobium meliloti* liquid biofertlizer. Following seed coating, highly variable cell distribution on the seed surface was observed for different additives used. Further studies are needed to understand the mechanisms to determine the viability of microbial cell coated on the seed surface.

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