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Developing Liquid Rhizobium Inoculants with Enhanced Long-Term Survival, Storage Stability, and Plant Growth Promotion Using Ectoine Additive

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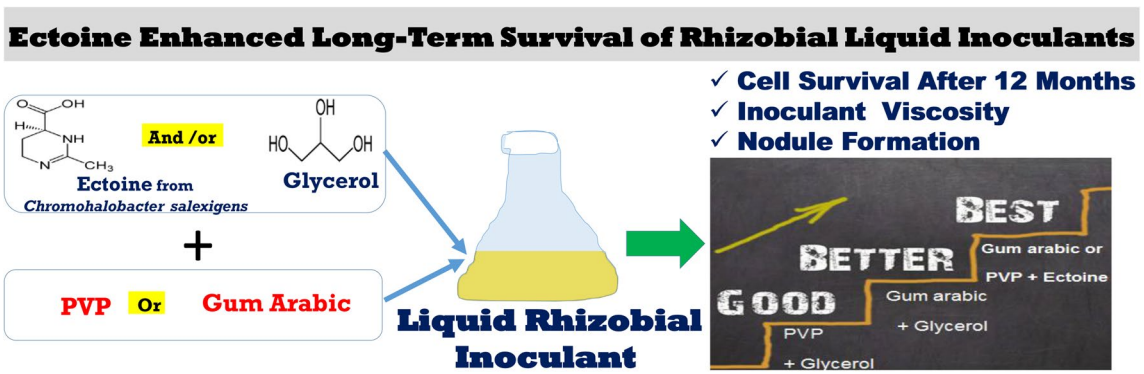
Abstract

Liquid microbial inoculants have recently received great attention due to their vital roles for sustainable agricultural practices. However, long-term conservation under ambient temperature conditions and deleterious environmental factors might negatively impact microbial cell survival and limit their efficacy in the field. Thus, developing efficient liquid formulation providing prolonged survival of rhizobia in the final product and after an application is crucial. Therefore, this study investigates the effect of various additives on the long-term survival of rhizobia stored in liquid cultures at room temperature (25 °C) for 12 months. Various yeast sucrose media amended with polyvinylpyrrolidone (PVP) or gum arabic as colloidal agents in combination with ectoine (as a compatible solute) and/or glycerol were evaluated. A dramatic decline in viable cell count was obtained in formulas amended only with PVP from Log 8.5 to Log 5 in the first six months and then to Log 1.5 after 12 months. In contrast, rhizobia stored at PVP-based formulas amended with 10 mg L^{-1} ectoine exhibited almost constant survival level till the end of the storage period. The same trend was obtained using formulas based on gum arabic as a colloidal dispersing agent; however, less decline in cell count using a formula containing gum arabic alone as compared to using PVP. On the other hand, PVP based formulas exhibited higher viscosity compared with another formula. Increased viscosity till the 8th month of storage was achieved in the presence of ectoine indicating the increase of exopolymeric substances production. Electrophoretic protein pattern of rhizobial cells (stored for 12 months) exhibited several low molecular weight protein bands in cells stored in PVP based formula with ectoine as compared to the other treatments. Thus, the amendment of the liquid formulation of rhizobia bioinoculant with PVP plus ectoine not only improved cell survival but also enhanced the culture viscosity and consequently ameliorate the colonization and performance of rhizobial inoculants.

Graphic Abstract



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Introduction

Microbial inoculant formulation is a promising technology that has been designed to safely improve the productivity of economic crops in the long-run application. It was previously reported that the major part of bio-fertilizers is produced in the form of carrier-based inoculants. However, solid carrier-based technology is considered as an energy-consuming process and involving milling, as well as sieving of the solid carrier in addition to pH adjustment are required [1, 2]. On the other hand, liquid bio-inoculant formulation has become the ideal substitution of carrier-based inoculants for many reasons [3]. For example, in liquid bio-inoculants, rhizobia broth cultures are supplemented with substances that maintain cell viability during storage and after application for seeds or soil. These additives protect rhizobial cells from drastic conditions such as high temperature and desiccation. The microbial cells of liquid bio-inoculant can tolerate high temperatures up to 55 °C [4]. The average shelf-life of most solid carrier-based bio-fertilizers is about 180 days; however, the shelf-life period could be increased up to two years when the liquid formulation is used.

Liquid inoculant formulations have been observed to serve as effective inoculants for rhizobia. Liquid formulations are typically aqueous, oil, or polymer-based products that are generally consisted of certain compounds serving as cell protectants in addition to specific nutrient media components used for the growth of plant growth-promoting rhizobacteria (PGPR) [5]. Polysaccharides such as gums, carboxymethylcellulose, and polyalcohol derivatives are commonly used to alter the fluid properties of these formulations [6]. Liquid formulations offer several advantages including stabilization of the microorganisms during production, packaging, distribution, and storage, and ease mixing with the seeds. Other applicable features are the protection of microbes from unfavorable environmental field conditions and enhancement of microbial activity at the site of application through increasing its viability, generation, adherence, and interaction with the target crops [7].

Ectoines (1,4,5,6-Tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) are low-molecular weight compounds produced by a wide range of halophilic microbial species. They are highly soluble in water (about 4 M at 20 °C) and can act as a compatible solute [8–10]. Ectoines have been classified as an excellent universal osmoprotectant [11–13]. Ectoine and hydroxyl ectoine are also reported to protect living cells against abiotic stress factors such as heating, desiccation, and freezing [14–16]. These criteria introduce the appropriate application of ectoine in different fields related to agriculture, molecular biology, food industries, biotechnology, and pharmacy [17]. Louis et al. investigated the protective function of hydroxyectoine and ectoine on *Escherichia coli* K12 and *E. coli* NISSLE 1917 during drying and subsequent storage. They found that those tetrahydropyrimidines molecules could introduce protection to freeze-dried *E. coli* NISSLE 1917 [18].

Besides, several compounds have been also studied for their potential as cell protectors during rhizobia production and storage. For example, polyvinylpyrrolidone (PVP) has been used as a polymer that protects the microbial cell from toxic seed defeated releasing from seed coat during germination. Moreover, it has high water binding capacity which alleviates the drying of the inoculant at the field. Fe-EDTA and glycerol had been also added to the liquid formula of rhizobia as iron and carbon source, respectively. Glycerol can also protect cells from the effects of desiccation [19]. Moreover, polysaccharides such as gum arabic, carboxymethyl cellulose (CMC), and polyalcohol derivatives are widely used to adjust the fluid properties of liquid formulations [6]. Tittabutr et al. studied the potential of bradyrhizobial liquid formulations amended with gum arabic, sodium alginate, polyethylene glycol (PEG), polyvinyl pyrrolidone (PVP), polyvinyl alcohol (PVA), and cassava starch compared with peat-based inoculant under field conditions and they concluded that liquid inoculants based on sodium alginate enhanced long-term survival of tested rhizobial strains in culture media while peat provided the highest protection to microbial cells after application to the seeds and incubation at 40 °C. Gum arabic based formulas maintained the bradyrhizobia number at 10^5 cells/seed after 48 h of incubation. Also, liquid inoculant amended with sodium alginate, PVP, or cassava starch supported survival at only 10^4–10^5 cells/seed [20].

The unavailability of suitable formula in which bacteria are allowed to multiply and survive for long period due to the short shelf-life of bio-fertilizers is a major constraint. Therefore, developing effective liquid rhizobial inoculants with enhanced long-term survival and storage stability is a critical challenge. Consequently, this study aimed to assess the effect of PVP or gum arabic in combination with ectoine and/or glycerol on the survival of rhizobia in liquid formulation along 12 months of storage.

Material and Methods

Bacterial Strain and Growth Conditions

Rhizobium sp. SARS 81 was provided from Bacteriology Laboratory, Sakha Agricultural Research Station, Kafr El-Sheikh, Egypt. Pure cultures were routinely maintained on Yeast Extract Mannitol Agar (YEMA) medium.

Ectoine Source

Ectoine was extracted from 48 h old culture of *Chromohalobacter salexigens* strain TA1 growing in Sehgal and Gibbons complex broth medium (SGCb medium) [21]. Briefly, cell pellets of *C. salexigens* were collected by centrifugation at 5000 rpm undercooling and were washed by phosphate buffer (pH 7.0) supplemented with NaCl (200 g L^{-1}). Washed cells were resuspended overnight in 80% (*v/v*) ethanol then centrifuged at 5000 rpm undercooling. The supernatant was used as a source of ectoine. The concentration and purity of ectoine in the supernatant was determined by HPLC with a TSK-GEL reversed-phase column (Tosoh, Japan) as described by Zhang et al. [22]. Ethanolic solution of ectoine was concentrated in rotary-evaporator at 60 °C and the resulted ectoine were dissolved in distilled water to obtain 1000 mg L^{-1} concentration (stock solution), filtered through 0.22 µ syringe filter and amended to the Yeast- Sucrose Media as a solution.

Experimental Design

Eleven formulations were prepared as shown in Table 1 to investigate the performance of compatible solutes (ectoine and/or glycerole) in combination with dispersant agents (PVP or gum arabic) for improvement of rhizobia survival in liquid inoculants and its effect on nodulation efficiency at the end of 12 months storage.

Shelf Life of Liquid Inoculant and Storage Condition

Cells were grown in 500 mL Erlenmeyer flasks containing 200 mL of yeast sucrose medium and adjusted at pH 6.8 with the appropriate concentrations of additives before sterilization as described earlier. The flasks were incubated at 30 °C for 48 h on a rotary shaker at 150 rpm. Aliquots of 50 mL of culture were distributed into 100 mL capacity sterile screw-capped glass bottles. These bottles were stored at room temperature and the total viable count, culture pH, and culture viscosity were determined every month. Data of viable count obtained by plate count were expressed as Log CFU mL^{-1}.

Viscosity Estimation

The rhizobial culture was centrifuged at 5000 rpm for 20 min, and the viscosity was measured in the supernatant using U shaped Ostwald viscometer using distilled water as a reference liquid according to the following equation:

$$\eta_s = \frac{\eta_w \rho_s t_s}{\rho_w t_w}$$

where ρ_s is the density of a sample, t_s is the time of outflow of the sample, ρ_w is the density of water, t_w is the time of the outflow of water η_w is the viscosity of water. The resulting data were expressed in centipoise unit.

Quantification of Exopolymeric Substances (EPS).

Cells were harvested by centrifugation at 5000 rpm for 20 min, and the supernatant was treated with two volumes of cold ethanol, held overnight at − 18 °C, and then centrifuged undercooling (4 °C) at 10,000 rpm for 20 min using BECKMN J2-MC centrifuge. The polysaccharide pellets were dissolved in warm distilled water, dialyzed against distilled water for several runs, lyophilized, and then weighted. The obtained product were analyzed for total carbohydrate and protein content [23].

Total Carbohydrates Determination

One mL of 5% phenol solution was added to 0.1 mL of 1% EPS solution. Then, 5 mL of concentrated H_2SO_4 was added rapidly to the mixture, shaked, and left aside for 30 min at room temperature. The color density was measured at

Table 1 Composition of the different formulations used in this study

Treatment	Formulation composition
T_1	Yeast-Sucrose media (control)
T_2	Yeast-Sucrose media + 2% PVP
T_3	Yeast-Sucrose media + 2% PVP + 0.5% glycerol
T_4	Yeast-Sucrose media + 2% PVP + 1.0% ectoine solution (10 mg L^{-1})
T_5	Yeast-Sucrose media + 2% PVP + 0.5% glycerol + 1.0% ectoine solution (10 mg L^{-1})
T_6	Yeast-Sucrose media + 0.3% Gum Arabic
T_7	Yeast-Sucrose media + 0.3% Gum arabic + 0.5% glycerol
T_8	Yeast-Sucrose media + 0.3% Gum arabic + 1.0% ectoine solution (10 mg L^{-1})
T_9	Yeast-Sucrose media + 0.3% Gum arabic + 0.5% glycerol + 1.0% ectoine solution (10 mg L^{-1})
T_{10}	Yeast-Sucrose media + 0.5% glycerol
T_{11}	Yeast-Sucrose media + 1.0% ectoine solution (10 mg L^{-1})

490 nm wavelength using JENWY 6705 spectrophotometer. Glucose was used as a standard solution [24].

Protein Patterns

Rhizobia cells of each treatment were harvested by centrifugation at 5000 rpm for 15 min at 4 °C, washed by 50 mmol L^{-1} Tris–HCl (pH 7.5) and resuspended in 200 µL of the same buffer. The cells were sonicated for 5 min at 15 s intervals at 4.0 °C using a sonicator Vibra-Cell 100 W model (Sonic & Materials Inc., Danbury, CT, USA). Lysates were centrifuged at 10,000 rpm for 20 min at 4 °C to remove cell debris. Protein electrophoresis was carried out in 15% SDS–polyacrylamide gels [25]. Gels were fixed and stained with Coomassie-Brilliant Blue as described by Arndt et al. [26].

Pot Experiment

Seeds of cowpea (*Vigna unguiculata* L. Walp.) obtained kindly from Legumes Research Department, Field Crops Research Institute, Agriculture Research Center, Egypt were surface sterilized by soaking in 2.5% sodium hypochlorite for 3 min and then washed 5 times in sterile distilled water and then mixed with liquid inoculant at a rate of 10 mL inoculant for 100 seeds before sowing in pots of 5 kg capacity filled with sterilized clay soil collected from experimental farm of Sakha Agriculture Research Station (SARS) at a depth of 0−20 cm with the following composition pH 8.10, organic matter (OM) 1.54%, total nitrogen 479 mg kg^{-1}, available phosphorous 11 mg kg^{-1}, available potassium 365 mg kg^{-1}, available Zn 0.75 mg kg^{-1}, available Fe 5.35 mg kg^{-1} and available Mn 2.85 mg kg^{-1}. The experiment was conducted in triplicats and each pot received two sterilized cowpea seeds inoculated according each treatment. At 60 days from planting, the plant was pulled gently and roots were cleaned and washed with running tap water then the nodule number, nodules dry weight and plant dry weight were determined.

Statistical Analysis

Data were statistically analyzed using Co Stat-statistical package software version 6.303. One-way analysis of variance (ANOVA) was used to compare the treatments. Multiple comparisons were done using Tukey's range tests at $P < 0.05$. All results are the means of three independent replicates as specified above.

Results and Discussion

Survival of Rhizobia in Different Liquid Formulations

Different liquid formualations were prepared for the rhizobial growth and storage as shown in Table 1. Rhizobia showed well growth and multiplication in yeast sucrose media at all treatments during the incubation period at room temperature with an average viable count reached about log 9 CFU mL^{-1} (Table 2). In general, the average viable count was increased in all treatments reaching about Log 10.2 CFU mL^{-1} after storage for two months. Almost all formulas had conserved viable cells until the fifth month

Table 2 Tracking the changes in total viable count (Log CFU mL^{-1}) of rhizobia under the influence of different amendments during 12 months of preservation at room temperature

Treatments [YSM supplemented with:]	Log CFU mL^{-1} at various storage period (months):											
	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th
— (YSM only)	8.69	10.3	10.0	10.2	10.3	10.3	10.0	9.89	7.88	7.75	5.48	5.46
PVP	8.62	10.2	10.4	10.2	9.94	5.23	2.82	1.93	1.67	1.65	1.63	1.80
PVP + glycerol	8.59	10.3	10.3	8.83	8.49	4.94	2.08	1.65	1.64	1.61	1.59	1.54
PVP + Ectoine	9.03	10.3	10.5	10.1	10.3	10.3	10.3	10.3	10.3	10.3	9.94	9.93
PVP + glycerol + Ectoine	9.27	10.1	10.4	10.1	10.1	10.3	8.63	8.00	8.05	7.54	5.42	5.40
Gum Arabic	8.12	10.2	10.2	9.76	9.76	9.65	9.94	9.75	8.00	6.79	5.61	5.60
Gum Arabic + glycerol	8.40	10.4	10.1	9.83	10.3	10.2	9.98	9.83	7.99	7.13	6.11	6.08
Gum Arabic + Ectoine	8.51	10.3	10.3	9.99	10.2	10.3	10.1	10.0	7.22	6.59	6.24	6.22
Gum Arabic + glycerol + Ectoine	8.44	10.4	10.4	10.1	10.1	10.3	10.3	10.1	9.70	9.63	9.12	9.10
Glycerol	8.78	10.3	9.90	10.2	10.2	9.89	9.94	9.63	6.96	5.86	3.45	3.41
Ectoine	9.32	10.3	10.4	10.2	10.2	10.2	9.94	9.89	9.96	9.89	9.11	9.10
L.S.D. 0.01	0.05**	0.04**	0.11**	0.10**	0.12**	0.08**	0.07**	0.46**	0.11**	0.08**	0.08**	0.42**

YSM yeast-sucrose media, *PVP* polyvinylpyrrolidone at 2%; glycerol at 0.5%; Gum Arabic at 0.3%; Ectoine solution at 1.0% (10 mg L^{-1})

**mean significant

Table 3 Tracking the changes in pH of culture media under influence of different during 12 months of preservation at room temperature

Treatments [YSM supplemented with:]	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th
— (YSM only)	5.48	5.40	6.03	6.30	6.33	5.82	5.73	5.63	5.40	5.20	5.05	4.96
PVP	5.60	6.07	6.00	5.73	4.94	4.83	4.82	4.76	4.66	4.52	4.32	4.09
PVP + glycerol	5.03	5.87	4.70	4.77	4.83	4.71	4.70	4.60	4.50	4.40	4.23	3.97
PVP + Ectoine	5.00	5.44	4.60	4.67	4.57	4.40	4.31	4.21	4.12	4.08	3.95	3.75
PVP + glycerol + Ectoine	5.43	5.81	4.50	4.62	4.53	4.29	4.39	4.29	4.07	4.27	4.17	4.10
Gum Arabic	5.20	5.87	5.03	4.97	4.80	4.73	4.57	4.47	4.17	4.03	3.87	3.70
Gum Arabic + glycerol	5.57	5.60	5.00	5.55	5.61	4.52	4.60	4.50	4.27	4.10	4.00	3.93
Gum Arabic + Ectoine	5.70	5.92	4.82	4.71	4.70	4.19	4.21	4.14	4.14	4.07	4.01	3.97
Gum Arabic + glycerol + Ectoine	5.73	6.00	4.93	4.69	4.73	4.55	4.45	4.25	4.05	3.92	3.78	3.72
Glycerol	5.30	5.73	5.87	6.03	6.13	5.20	5.15	5.02	4.75	4.49	4.25	4.18
Ectoine	5.64	6.00	5.75	5.71	6.50	5.58	5.61	5.38	5.19	5.06	4.52	4.40
L.S.D. 0.01	0.41**	0.39**	0.36**	0.38**	0.28**	0.21**	0.22**	0.14**	0.17**	0.42**	0.58**	0.66**

YSM yeast-sucrose media, *PVP* polyvinylpyrrolidone at 2%; glycerol at 0.5%; Gum Arabic at 0.3%; Ectoine solution at 1.0% (10 mg L^{-1})
**mean significant

of storage where some treatments started to decline after that. A sharp decline in the viable count was obtained in the treatment T_2 (YSM + PVP) and T_3 (YSM + PVP + Gly) that reached down to Log 1.8 and Log 1.54, respectively (Table 2). Therefore, the presence of glycerol only as a compatible solute and osmoprotectant did not prevent/recover the decline in viable count in the presence of PVP. While the treatments amended with ectoine were able to survive for a longer time than other treatment even in the presence of PVP or gum arabic as dispersant agents where the best treatment was T_4 (PVP + Ectoine) which recorded viable Log count 9.93 at the end of the storage period.

Sucrose was used as a carbon source due to its protective properties. It was reported to enhance the survival of certain *Rhizobium* spp [27–29], and stimulate the accumulation of compatible solute glycine betaine (betaine) inside the cell [30]. Sucrose is also used by bacterial cells for the synthesis of glycocalyx which play a significant role in the survival of various microorganisms [31, 32]. Polyvinylpyrrolidone (PVP) and gum arabic were used to maintain the bacterial growth suspended in the growth medium along the storage period through what is called colloidal stabilization that prevents protein precipitation or cell coagulation [5].

At the current work, the reduction in cell count of rhizobia was recorded in treatments containing 2% PVP alone or with glycerol after 5 months of storing. This may be attributed to the that PVP has high phenolic content and high binding activity to polar and hydrophobic molecules [33], which may affect the plasma membrane of the microbial cell especially when exposed for a prolonged time. This sharp decline in the viable count at treatments 2, 3 might also be attributed to the consumption of sucrose in the 5th month. So, the medium became free from not only carbon source but also compatible solute (sucrose) which preserves the cell from injury under osmotic pressure caused by PVP. Our results are in agreement with previous research which showed that compounds with high-molecular-weight (C_6 to C_{12}) such as sucrose can improve the survival of bacteria in dried biopolymers [29] or enhance the survival of *Rhizobium* spp. upon desiccation and/or dry formulation [27, 28].

On the other hand, ectoine could retrieve the survival of cells for a longer storage periods. The viable count in

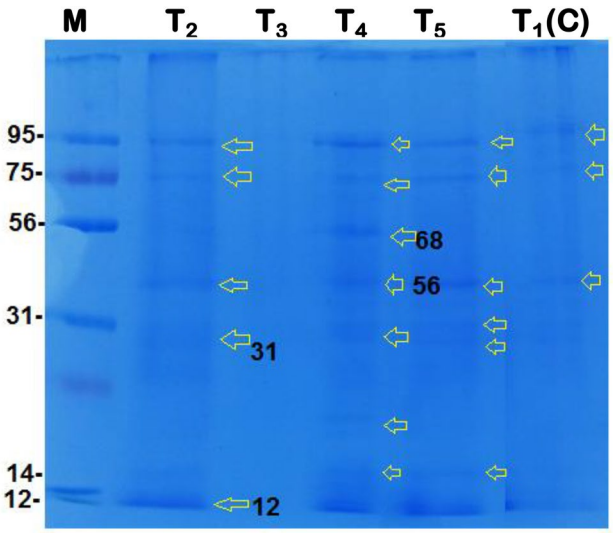

Fig. 1 SDS-PAGE of total soluble protein of *Rhizobium* sp. (vigna) tal169 stored for 12 months at room temperature. Lane M: molecular mass standards (kit Mw- GF-1000- Sigma). Lane T_2, Culture media amended with 2% PVP; lane T_3, Culture media amended with 2% PVP + 0.5% glycerol; lane T_4, Culture media amended with 2% PVP + 1.0% Ectoine [10 mg L^{-1}]; Lane T_5, Culture media amended with 2% PVP + 0.5% glycerol + 1.0% Ectoine [10 mg L^{-1}]; lane T_1 (control). The numbers beside arrows indicate the changes observed as explained in the text

Table 4 Tracking the changes in viscosity (centipoise unit) of culture media under influence of different amendments during 12 months of preservation at room temperature

Treatments [YSM supplemented with:]	Viscosity (centipoise unit) at various storage period (months):											
	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th
— (YSM only)	2.60	4.50	5.30	4.80	7.50	7.20	7.00	7.10	7.70	7.50	7.40	7.50
PVP	5.50	18.6	25.7	34.6	39.5	56.8	59.8	57.0	61.5	61.5	57.3	57.3
PVP + glycerol	5.30	14.3	16.5	17.3	20.8	20.8	25.4	25.3	27.6	27.9	23.5	23.1
PVP + Ectoine	5.80	20.8	64.0	120.2	204.2	339.9	362.6	362.1	355.3	317.2	317.2	317.1
PVP + glycerol + Ectoine	5.30	21.0	61.5	93.5	136.4	136.1	159.0	170.0	170.2	181.4	186.1	181.9
Gum Arabic	1.40	3.20	9.50	16.5	20.7	61.7	68.6	74.7	81.5	86.6	91.2	95.5
Gum Arabic + glycerol	1.70	7.00	12.1	19.5	30.5	57.3	64.1	68.3	72.7	73.4	79.9	79.8
Gum Arabic + Ectoine	1.80	7.90	34.4	50.5	80.0	136.2	136.3	136.1	145.1	147.4	147.5	143.1
Gum Arabic + glycerol + Ectoine	1.50	4.70	18.8	39.1	57.3	113.5	120.5	124.8	134.1	136.3	136.1	140.9
Glycerol	1.60	5.70	5.00	7.00	9.40	9.60	11.8	11.5	12.0	11.9	11.7	11.6
Ectoine	1.30	1.60	3.00	4.10	7.10	7.10	7.50	7.20	7.50	7.30	6.90	7.20
L.S.D. 0.01	0.29**	0.61**	0.89**	0.88**	0.88**	0.76**	1.05**	0.45**	0.58**	0.73**	1.05**	0.89**

YSM yeast-sucrose media, *PVP* polyvinylpyrrolidone at 2%; glycerol at 0.5%; Gum Arabic at 0.3%; Ectoine solution at 1.0% (10 mg L^{-1})

**mean significant

formulas containing ectoine remain constant until the 9th month of storage then a gradual decrease was observed after that except in the case of T_4 (YSM + PVP + Ectoine) whereas the cell viable count remains constant until the end of the storage period.

Compatible solutes as betaine had been long used as additives in liquid formulations of PGPRs. Among several products, ectoine showed a promising ideal compatible solute in preserving the viability of liquid rhizobial culture at the current study. Ectoine can protect and stabilize proteins, nucleic acids, and whole cells against those stresses where they can collapse and proteins tend to be denatured especially in the worm environment. Zhang et al. proposed that ectoine improved cell division and assimilation of glucose, and protected the relative enzymes during ethanol production by *Zymomonas mobilis* [34]. Talibart et al. investigated the role of ectoine in the osmo-adaptation of *Rhizobium meliloti* and they found that ectoine improved the growth of *R. meliloti* under adverse osmotic conditions (0.5 M NaCI) without being accumulated by the cells. It was suggested that the mechanism of action is situated at the gene expression level [13]. This finding explains the difference in protein profiles between the treatments as shown in Fig. 1. Ectoine also proved to osmoprotectant several species of rhizobia such as *Rhizobium leguminosarum, Bradyrhizobium japonicum*, and *Rhizobium* sp. [13].

Changes in pH and Viscosity (Centipoise Unit) of Culture Media

Results shown in Table 3 indicated that, although there are statistical differences between the treatments during the storage period of liquid inoculants in the pH values, however, this change cannot be attributed to the effect of any of the additives used in the experiment. All treatments showed a significant decrease in pH values at the end of the storage period compared to the pH measured at the initial time.

On the other hand, there are great variations in viscosity between different treatments (Table 4). As expected, the formulas containing colloidal agents possess the highest viscosity rate but it continued to increase during the continuos storage period. The increase in rheological properties with the time indicates that the viscosity resulted from the production of exopolymeric substances (EPS). This suggestion is confirmed by the estimation of polysaccharide in all formulations under study. The increase in viscosity was not proportional to the increase in the viable count where the viscosity continued to increase although the bacterial count was constant. The highest increase in viscosity was achieved in treatments containing ectoine that reached ˃300 centipoise units in treatment T_4 and up to 140 centipoise unit in treatments T_5, T_8, and T_9. On the other hand, the increase in viscosity was stopped when the viable cell count started to decline (Table 4). Formulations containing glycerol showed lower viscosity compared with the analogous treatment without glycerol (Table 4). Glycerol based media are used as a carbon source by many rhizobial strains. Lorda and Balatti studied the growth behavior of *B. japonicum* in glycerol-based media under various environmental conditions. They showed a rapid growth rate in a medium supplemented with glycerol instead of mannitol. The current work suggested that the rhizobial strain at glycerol- amended medium used glycerol as a protective agent. Glycerol possesses high water

binding capacity which protects rhizobial cells from desiccation during application to the seeds [35].

Quantification of Exopolymeric Substances (EPS)

The varied viscosities among treatments were attributed to differences in polysaccharide production. The quantification of exopolymeric substances and carbohydrate content of culture media after 12 months was shown in Table 5. This could be a fruitful area of the investigation if the effect of PVP and polysaccharides on cell survival is synergistic. Polysaccharide plays several roles in rhizobia survival as it protects rhizobial cells from desiccation, metallic cations, and/or stress-induced by acid soil.

Treatments with high viscosity gave a higher value for EPS that reached to 6.8 g L^{-1} at treatment 9 containing ectoine. The carbohydrate content was higher than 80% in all treatments. Although PVP containing formulas possess a higher viscosity compared to other formulas containing gum arabic; however, the EPS content in the last was higher due to the chemical nature of gum arabic which is composed mainly of carbohydrate (Treatments T7, T8, and T9).

Nodule Formation of Rhizobia Stored in Different Formulations

Generally, the viscosity and the ability of liquid inoculant to easily adhesion to plant parts is a preferable and limiting character for microbial liquid formulation. It helps microorganisms to colonizes the target surfaces and increase the bacterial population on the seeds. In the current study, the high viscosity of treatments especially those containing ectoine would be advantageous that can play an effective role in the nodulation process.

At the end of the storage period, the nodulation efficiency of stored cultures was examined to evaluate the viability of rhizobia and its validity to form nodules. In this experiment, only equal volumes of stored cultures were added to equal quantity of seeds to investigate the actual activity of stored inoculates, thus, the number of viable cells was not equal between stored treatments. It was clear that the application of treatments with high rhizobial number achieved the highest nodulation nodule formation as shown in Table 6. This was reflected on plant growth parameters as plant dry weight and nitrogen content were increased. Formulation containing ectoine and PVP (T4 and T5) showed the highest nodule number (23, 17 nodule $^{-1}$), with plant dry weight (3.64 and 3.45 g) and N-content (1.61, 1.32%), respectively. The application of other treatments exhibited lower values as indicated in Table 6.

Liquid inoculant formulation of cowpea rhizobia prepared with PVP as an osmoprotectant had been observed to have higher shelf life than those without the PVP amendment [36]. In contrast, some researchers reported a negative effect on cell viable count by the addition of PVP. Dayamani and Brahmaprakash reported that the viable count of *Azospirellium* was decreased with PVP at a 0.5% level [7].

Electrophoretic Protein Pattern

The electrophoretic protein patterns of cells stored for 12 months in liquid formulations containing PVP are shown in Fig. 1. As indicated, formulations amended with ectoine affected the synthesis of cell proteins. Comparative analysis of the lanes showed that the protein bands are similar in

Table 5 Quantification of exopolymeric substances extracted from rhizobia cultures after preservation for 12 months and their carbohydrate content

Treatments [YSM supplemented with:]	Exopolymeric substances (g/L)	Carbohydrates content (%)
— (YSM only)	1.33	92.0
PVP	2.10	92.1
PVP + glycerol	2.30	92.3
PVP + Ectoine	5.90	92.0
PVP + glycerol + Ectoine	4.73	89.3
Gum Arabic	3.10	83.3
Gum Arabic + glycerol	6.41	81.2
Gum Arabic + Ectoine	6.03	82.0
Gum Arabic + glycerol + Ectoine	6.83	85.0
Glycerol	1.13	95.0
Ectoine	2.35	94.1
L.S.D. 0.01	0.39**	0.23**

YSM yeast-sucrose media, *PVP* polyvinylpyrrolidone at 2%; glycerol at 0.5%; Gum Arabic at 0.3%; Ectoine solution at 1.0% (10 mg L^{-1})

**mean significant

Table 6 Evaluating the nodulation efficiency of rhizobia stored in different formulations on Cowpea plant in pot experiment

Treatments [YSM supplemented with:]	No. nodules/plant	Nodules dry weight (mg/plant)	Plant dry weight (g)	N% in plant
— (YSM only)	7.20	43.4	2.77	1.21
PVP	2.10	9.80	2.08	1.21
PVP + glycerol	0.90	7.70	1.87	1.20
PVP + Ectoine	23.1	100.1	3.64	1.75
PVP + glycerol + Ectoine	17.0	34.8	3.45	1.61
Gum Arabic	8.90	49.9	2.86	1.32
Gum Arabic + glycerol	11.7	65.6	3.17	1.43
Gum Arabic + Ectoine	10.5	68.1	3.14	1.47
Gum Arabic + glycerol + Ectoine	15.4	72.8	3.24	1.52
Glycerol	7.20	30.3	2.56	1.28
Ectoine	12.6	72.3	3.08	1.49
L.S.D. 0.01	2.64**	45.9**	0.08**	0.05**

-*YSM* yeast-Sucrose media, *PVP* polyvinylpyrrolidone at 2%; glycerol at 0.5%; Gum Arabic at 0.3%; Ectoine solution at 1.0% (10 mg L^{-1})

**mean significant

case of treatment amended with glycerol and control (lane 2, 5). Also, these bands were common in all PVP treatments. Two low molecular weight protein bands (31 and 12 kDa) appeared in the treatment containing PVP alone (lane 1) which were not produced under control condition. Furthermore, two protein bands of molecular weights 68, and 56 kDa were appeared at the treatment containing ectoine in its formulation.

Fetyan and Mansour previously reported that a halotolerant strain *R. meliloti*-n11 showed two new protein bands (48 and 32 kDa) under salt stress that were not produced under normal condition. Also, they found protein bands of molecular weights 108, 68, and 53 kda were intensified under stress conditions [37]. The presence of ectoine regulates the genes responsible for the alleviation of osmotic stress.[13]. Further studies to characterize the chaperons produced under different stress conditions pending necessary to give more clear mechanism of the inoculant additives.

Conclusion

In this study, various liquid formulations containing PVP or gum arabic in combination with ectoine and/or glycerol were evaluated for rhizobia (*Rhizobium* sp. SARS 81) growth and storage. The liquid formulation containing ectoine plus PVP was considered the best for developing efficient rhizobial liquid bio-inoculants with enhanced long-term survival and storage stability. Amongst all additives, ectoine at 10 mg L^{-1} exhibited the best performance of microbial cells during the prolonged storage of up to 12 months at room temperature. It improved rhizobial survival under storage and enhanced production of exopolymeric substances that lead to increased viscosity and consequently potential for better application as a stronger adhesive to the plant surfaces and colonization performance. Besides this, formulations containing ectoine showed better nodules formation with *Vigna unguiculata.* Also, some stress protein bands with molecular weights 68, and 56 kDa appeared in the cells stored in the formulations containing ectoine. Further efforts should be done to determine the possible mechanism of ectoine and the maximum preservation period.

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Compliane with Ethical Standards

Conflict of interest The authors declare that they have no competing interests.

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