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# The shelf life of Rhizobial liquid inoculants amended with different polymeric additives

**Somaya S. Mohamed[1], Mohammed A. Hassan[2], Migdam E. Abdelgani[3]**

[1]Environment, Natural Resources and Desertification Research Institute. National Centre for Research, Sudan
[2]Environment, Natural Resources and Desertification Research Institute. National Centre for Research, Sudan
[3]Environment, Natural Resources and Desertification Research Institute. National Centre for Research, Sudan

**Correspondence Author:** Somaya S Mohamed, Associate Professor Environment, Natural Resources and Desertification Research Institute. National Centre for Research. P.O. Box 6096. Khartoum, Sudan.
E-mail:- somasirmo@gmail.com

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**Abstract**

**One of the main problems in rhizobial inoculant technology is the poor survival of microorganisms during storage. Liquid inoculants amended with polymers has considered as a solution to the problems associated with shorter shelf life and poor quality. This study was conducted to improve the shelf life of previously selected liquid formulations of rhizobial strains TAL 380, TAL 209, TAL 1399, ENRRI 1, USDA 3385 and USDA 3100. The polymeric additives used were polyvinylpyrrolidone (PVP), polyethylene glycol (PEG), polyvinyl alcohol (PVA) and Gum Arabic. Yeast Extract Mannitol (YEM) broth without the addition of polymers and charcoal based inoculant were used as check treatments. The formulated liquid inoculants were stored at room temperature (25-35°C) and under refrigeration at 4°C for a period of two months. Spread plate method was used to determine the colony forming units (CFU) per ml at two weeks intervals. Liquid inoculants formulated with PEG (0.1%),PVP (1% and 2%), PVA (1% and 3%), and Gum Arabic (0.3%) supported the growth of ENRRI 1, TAL 380, USDA 3100, TAL 1399, TAL 209, and USDA 3385, respectively. Charcoal based inoculant harboured lowest cell counts when compared to liquid inoculants amended with the polymeric additive. The results revealed that all tested formulations were able to support cell growth as high as $10^9$ CFU/ml and liquid *Rhizobium* inoculants amended with polymeric additives can be stored without loss of viability for two months at both room temperature and at 4°C. In conclusion, the study suggests the efficiency of these polymeric additives in improving the shelf life of rhizobial liquid inoculants.**

**Keywords:** Rhizobia, Liquid inoculant, Shelf life, Additives

## INTRODUCTION

Nitrogen is still the most limiting nutrient in agricultural production that plays a role in overcoming low productivity in Sub-Saharan African (Balume et al., 2015). Biological nitrogen fixation (BNF) is mediated in nature by microorganisms that convert inert $N_2$ to biologically useful ammonium salt. The association between the rhizobia and leguminous plants is the most studied and well-characterized symbiosis (Xavier et al., 2010). Rhizobia-legume association is exploited in agriculture by rhizobial inoculants production and the use of improved and high-quality inoculants is a strategy to increase the BNF benefits in agroecosystems (Fernandes Júnior et al., 2012).

Rhizobia has to face extremes of low moisture regime in soil and should have the capacity to withstand extremes of soil and weather condition, better interaction ability, nodule-forming and nitrogen-fixing capacity and long shelf life in a carrier (Deshmukh et al., 2014). One of the main problems in inoculants technology is the survival of micro-organisms during storage. Several parameters such as culture medium, physiological state of microorganisms when harvested, rates of drying, storage temperature and water activity of the inoculants influence their shelf life (Goudar et al., 2017). Under the tropical condition, high temperature may adversely affect the survival of rhizobia in the packaged inoculants and also in the inoculated seeds in the field (Deshmukh et al., 2014). Therefore, the effect of temperature on the survivability of inoculants should be detected during storage and distribution since adequate low-temperature facilities are not readily available.

The liquid formulation may provide a solution to some of the problems associated with carrier-based inoculants. Additives to liquid inoculants formulations should have a role in protecting rhizobial cells on seeds at high temperature and during desiccation. Many kinds of polymers *viz.*, Polyethylene glycol PEG, polyvinylpyrrolidone PVP, Polyvinyl alcohol PVA and Gum Arabic have been used for inoculants production because of their ability to limit heat transfer, their excellent survival properties and high water

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activity (Deshmukh et al., 2014). In the tropics, the liquid formulation may have advantages for a small scale local inoculants manufacturers and that is due to unavailability of quality peat deposits. Even when they do mining, drying and grinding are expensive and requires a considerable capital investment in equipment (Singleton et al., 2002). In Sudan, much research has been done on *Rhizobium* strains selection and inoculation response. Researchers were prompted to evaluate several local materials to be used as microbial carriers. Charcoal was found to be superior in terms of availability and abundance besides its high water-holding capacity and its least contamination liability (Elsalahi et al., 2016). Partially sterilized charcoal is used for inoculants production in the belief that inoculants will be used shortly two months of storage at room temperature after manufacture (Elshafie and Elhussein, 1991). Since Sudan has no legislation for inoculants standards yet, the Biopesticides and Biofertilizers Department which is the only *Rhizobium* inoculants producer in Sudan follows the international standards that the manufactured inoculants should have more than $10^8$ CFU/g carrier inoculants (Elsalahi et al., 2016). However, research conducted on the inoculants production and formulation technology is limited. Liquid biofertilizer formulation could be considered as one potential strategy for improving the shelf-life of biofertilizer. Unlike solid carrier-based formulations, liquid formulations allow the manufacturer to include a sufficient amount of nutrients, cell protectants, and inducers responsible for cell formation to ensure prolonged shelf-life. The objective of this study is to assess the survival of rhizobia in liquid inoculants amended with different polymeric additives as means to improve their shelf life during three months of storage at room temperature (25-35˚C) and in the refrigerator (4˚C).

## 2. MATERIALS AND METHODS

### Microorganisms

Rhizobial strains and isolates for this study were obtained from The Biopesticides and Biofertilizers Department, Environment, Natural Resources and Desertification Research Institute, National Centre for Research, Khartoum, Sudan. TAL 380, TAL 1399 and TAL 209 strain were previously obtained from a Nif TAL project, USDA 3386 and USDA 3100 were obtained from the U.S.A. Department of Agriculture and ENRRI 1 was locally isolated. They are efficiently used as solidly based inoculants.

### *Rhizobium* culture medium

Yeast Extract Mannitol (YEM) broth medium was used as a basal medium for liquid inoculant formulations and for preparation of charcoal inoculants. Broth medium without additives was used as a control. A loopful of each rhizobial strain and isolate was inoculated into YEM broth and incubated in a rotary shaker at 120 rpm for 24 h at room temperature (25-35°C).

### Formulation of liquid inoculants

According to the findings of a previous study concerning the assessment and selection of the appropriate polymeric additives for each strain, liquid inoculants formulated with 0.3% Gum Arabic and 0.1% PEG were used for ENRRI 1, 1% PVP and 0.8% Gum Arabic for TAL 380, 2% PVP and 0.5% Gum Arabic for USDA 3100, 3% PVA and 3% PVP for TAL209, 0.1% PVA and 0.3% Gum Arabic for USDA 3385 and 0.5% PVA and 5% PVP for TAL 1399, were prepared and packed in sterilized transparent glass bottles of 100 ml capacity.

### Preparation of charcoal-based inoculant

A volume of 400 ml Yeast Extract Mannitol (YEMB) in 500 ml conical flasks were autoclaved for 15 minutes at 15 ib/in² and 121°C. The broth was inoculated by the chosen strain, a loopful of each strain was transferred aseptically to flasks containing YEMB and left in an orbital shaker for 24-48 hour. Cultures were serially diluted in distilled water to check for inoculum quality (should include at least $1\times10^9$ Colony-forming units CFU/ml). Charcoal fragments were collected from the local market, milled and sieved to pass through a 0.5 mm mesh screen, and packed in thin-walled polypropylene bags and then oven sterilized at 100° C for 3 h. A volume of 400 ml of inoculated YEMB was then added separately to 1kg of ground charcoal and mixed by hand until it became uniform and friable in texture. Each 500 g of inoculated charcoal were then packed in a polythene bag, with a pore size of 0.05 mm, sealed and left in the laboratory at room temperature (25-35°C) to gain a maximum number of rhizobial cells till use.

### The shelf life of liquid and charcoal inoculants

Two batches of each treatment (charcoal and liquid inoculants) were prepared, one batch was stored at room temperature (25-35°C) and the other batch was stored at 4°C. Shelf life for each inoculant was assessed by colony count using spread plate method (Somasegaran and Hoben, 1994) at 15 days intervals starting from zero-day and up to 60 days. Serial dilution was prepared and 0.1 ml from each diluent of liquid *Rhizobium* culture was evenly distributed on yeast extract mannitol agar (YEMA) in Petri dishes. Two replicates were used for the last three dilutions 7, 8 and 9 of each treatment. Dishes were incubated at 28°C for 3 days. Colonies were counted according to plate count methods. Data were expressed as log CFU $ml^{-1}$.

## 3. RESULTS AND DISCUSSION

### 3.1 ENRRI 1

The results showed that the population of Rhizobia isolate ENRRI 1 was maintained in all selected formulations up to 60 days of storage. At room temperature (Fig. 1 A), the maximum number of colonies was observed in liquid inoculant amended with 0.1% PEG after 60 days of storage followed by liquid inoculant formulated with 0.3% Gum Arabic then charcoal-based inoculant.

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At 4°C (Fig. 1 B), the maximum number of colonies was observed in liquid inoculants amended with 0.1% PEG followed by liquid inoculants amended with 0.3% Gum Arabic, then YEMB medium. The minimum number of colonies was observed in charcoal based inoculant. During the storage period at both room temperature and in the refrigerator, different patterns of growth were followed by the different liquid formulations and the number of colonies mostly exceeded $10^8$ cells/ml.

**3.2 TAL 380**

Liquid inoculants with the different formulations were able to maintain the viability of *Rhizobium* strain TAL 380 throughout the two months and the highest numbers of colonies were maintained in liquid inoculants amended with 1% PVP after 60 days of storage at room temperature (Fig. 2 A). Although minimum reduction of the population was observed throughout the storage period in liquid inoculant amended with 0.8% Gum Arabic but the number of colonies remained within the standard level. The number of colonies of TAL 380 in the different liquid formulations at 4°C started to decline from the initial count up to the end of storage period except in liquid inoculants amended with 1% PVP where the population started to increase from the initial count and recorded the highest population at about $1.2\times10^9$ cells/ml after 60 days of storage compared to the other treatments, (Fig. 2 B).

**3.3 USDA 3100**

Results displayed in Figure 3 A revealed that control, charcoal-based inoculant and liquid inoculant formulated with 0.5% Gum Arabic supported the survival of USDA 3100 at cell concentration higher than $10^8$ cells/ml till 60 days at room temperature. Moreover, liquid inoculant formulated with 2% PVP was found to record bacterial colonies higher than$10^9$ cells/ml after 60 days of storage at room temperature. The effects of storage for 60 days at 4°C on the cell population of USDA 3100 in the different formulations are shown in (Fig. 3 B). Liquid inoculants were found to support cell growth at levels higher than the population number in YEM and charcoal-based inoculant. The highest number of colonies ($1.1\times10^9$) was maintained by liquid inoculants amended with 2% PVP till 60 days of storage, followed by 0.5% Gum Arabic and then charcoal-based inoculants.

**3.4 TAL 209**

The effect of storage at room temperatures on the survival of TAL 209 is presented in Figure (4 A). The results showed maximum populations were recorded in liquid inoculants amended with 3% PVA ($3.1\times10^8$ cells/ml). The other treatments gave population densities that were very close to each other, control ($1.8\times10^8$ cells/ml) followed by charcoal-based inoculant ($1.7\times10^8$ cells/ml) and then liquid inoculant formulated with 3% PVP ($1.6\times10^8$ cells/ml) after 60 days of storage at room temperature. Survivability of TAL 209 during 60 days of storage at 4°C is explained in Figure (4 B). At 60 days of storage, liquid inoculant formulated with 3% PVA showed the highest number of colonies $9.3\times10^8$ cells/ml whereas the minimum population at about $2.9\times10^8$ cells/ml were recorded in charcoal based inoculant. Liquid inoculants amended with PVA at concentration 3% could retain most of its viability till 60 days compared to the other treatments at the two storage conditions. PVP also supported the growth and no reduction in population was recorded after two months of storage compare to the initial number of population at 4°C.

**3.5 USDA 3385**

Liquid inoculant formulated with 0.3% Gum Arabic was able to sustain rhizobia cells from the initial count up to 60 days of storage at room temperature and recorded the highest population at about $1.5\times10^9$ after 60 days of storage (Fig. 5 A). The number of cells in liquid inoculants amended with 0.1% PVA supported the population of rhizobia cells in the initial 30 days of storage giving more than $10^9$ cells/ml, then started to drop till the 60th day of storage and ended with $2.6\times10^8$ cells/ml. The highest initial population of cells ($1.5\times10^{10}$) at zero-day of storage was recorded by charcoal inoculant and then dropped to $2.5\times10^8$ after 60 days of storage. Similarly, the population growth in YEM broth declined from $6.5\times10^9$ cells/ml to $1.8\times10^8$ after 60 days of storage. Results displayed in (Fig. 5 B) revealed that all the tested formulations were able to support rhizobial growth up to 60 days at 4°C. Maximum rhizobial counts were determined in liquid inoculant amended with 0.3% Gum Arabic, followed by liquid inoculant formulated with 0.1% PVA.

**3.6 TAL 1399**

The population dynamics of TAL 1399 in the growth media amended with the appropriate polymeric additives and charcoal-based inoculants that were stored at room temperature and at 4°C for 60 days are presented in Figures (6 A) and (6 B) respectively. All the treatments could support the survival of TAL 1399 more than $10^8$ cells/ml throughout the storage period at room temperature. Liquid inoculants containing 0.5 % PVA could highly support the growth of TAL 1399 and had a cell concentration of about $2.1\times10^9$ cells/ml after two months of storage at room temperature. Also, all the treatments supported the bacterial growth more than $10^8$ cells/ml during the storage period at 4°C (Fig. 5 B). After 60 days of storage at 4°C, the highest number of colonies ($3\times10^9$ cells/ml) were observed in liquid inoculants formulated with 0.5% PVA followed by PVP at concentration 5% ($2.8\times10^9$ cells/ml) and charcoal-based inoculant ($2\times10^9$ cells/ml). The lowest number of colonies was retained in control at about $2.2\times10^8$ cells/ml and $1\times10^9$ cells/ml at room temperature and at refrigeration, respectively.

The results revealed that all tested formulations were able to support cell growth as high as $10^8$ CFU/ml and increase the surviving population at the higher level of growth than YEM medium without any additive during 60 days of storage at both room temperature and refrigeration. Charcoal based inoculant harboured lowest cell counts when compared to liquid formulations containing polymeric additives. Moreover, marginal differences were observed between population densities supported by the different inoculants formulations within each rhizobial strain. PEG at concentration 0.1% could promote the growth of ENRRI 1

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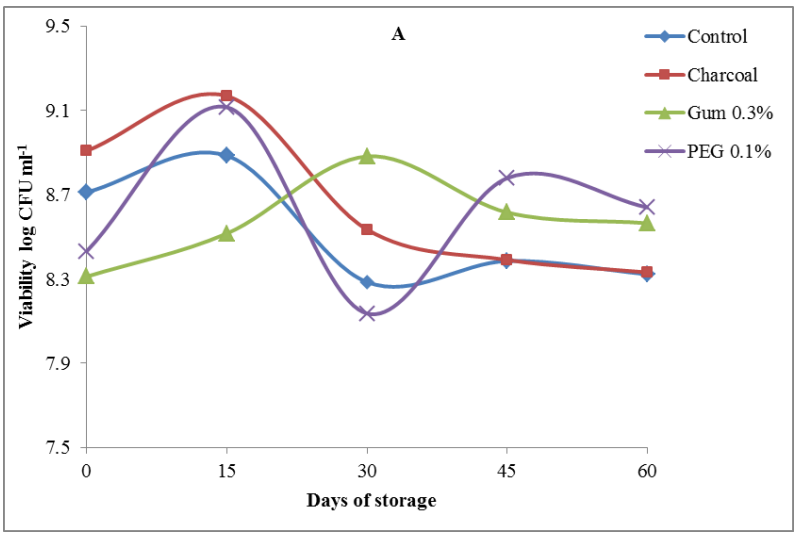
while Gum Arabic could support the growth of USDA 3385. Also, PVP at 1% and 2% concentrations could promote the growth of TAL 380 and USDA 3100, respectively. Moreover, PVA could support the growth of TAL 1399 and TAL 209 at concentrations 0.5% and 3%, respectively. Hence, all polymeric additives proved to be a suitable supplement for enhancing the shelf life of *Rhizobium* with mostly $10^8$ cells/ml which are the quality standard for liquid inoculants even in the absence of adequate cooled storage condition.

Our results are in concurrence with earlier studies where survival of *Rhizobium* cells in the liquid formulation has been enhanced due to the action of chemical amendments added to the medium. Singleton et al. (2002) developed liquid formulations of *Rhizobium* by adding various additives in the yeast extract mannitol media and claimed a cell number of $1\times10^{10}$ cells/ml in the liquid inoculants. Similarly, liquid inoculants formulated with PVP and Gum Arabic were found to support good survival of *Bradyrhizobium japonicum* up to 240 days (Deshmukh et al., 2014). Also, Kumaresan and Reetha, 2011, found higher population of *Azospitillum brasilense* due to addition of 0.3% Gum Arabic and 0.2% PVP up to 11 month of storage under ambient temperature (28°C to 32°C), whereas PEG (1%), PVP (0.5%) and lignite carrier recorded the same population up to 8 months, 6 months and 5 months, respectively.

Liquid inoculants formulated with 4% PVP for *Azotobacter vinelandii* have been observed to have the highest cell population than other additives (Mounik et al, 2016). Also, Vendan and Thangaraju (2006) reported $10^8$ cells/ml up to 10 months storage at room temperature for a liquid formulation of *Azospirillim brasilence* amended with PVP. Liquid biofertilizer of *Rhizobium*, *Azotobacter*, *Azospirillum* and *Bacillus megaterium* formulated with PVP in addition to glycerol at the rate of 0.5% each retained maximum number of colonies, followed by PEG and Gum Arabic for a period of 180 days at 28+2˚C (Deshmukh et al., 2014). The shelf life of *Azospirillum* sp. and PSB could be enhanced up to 9 months at room temperature in a liquid inoculant formulated with PVP and trehalose, respectively (Surendra and Akhila, 2016).

Liquid formulations unlike solid carrier-based inoculants, allow the manufacturer to induce sufficient amount of nutrients and cell protectants to ensure prolonged shelf life, tolerance to temperature (Amalraj et al., 2013), good survival properties and high water activity (Deshmukh et al., 2014). PVP is a synthetic polymer with high water holding capacity that appears to slow down the drying rate of media, thus maintaining the moisture level in the media around the cells for their metabolism (Deaker et al., 2007). PVP also has the capacity to bind bacterial toxin that was constantly released into the media when bacterial cells were in a stationary phase (Santosh, 2015). PEG is water soluble with an adhesive and sticky consistency. Its viscous nature slows the drying process of the inoculants (Santosh, 2015). Gum Arabic is adhesive, emulsifier and has a stabilization property which limits heat transfer, has water activity and reported to protect cells against toxic factors (Tittabutr, 2005). The sticky consistency of these polymers may enhance cell adherence to seeds and their vicious nature may slow the drying process (Temprano et al., 2002).

Similar to the findings of Balume et al. (2015), storage conditions did not affect the quality of inoculants. Survival at room temperature is an important characteristic of inoculants formulation since building large cold storage rooms at the production plant is expensive (Tittabutr, 2005). However, rhizobial inoculants should not be stored for a prolonged time that may exert changes in the physiological activity leading to decrease in nodules number (Maurice et al., 2001). Moreover, Girisha et al. (2006) observed that *Rhizobium* inoculants can be stored without loss of viability for more than one year and stated that liquid formulations are better than carrier-based materials. Therefore, as the performance of rhizobial inoculants decreases with the increase of time the inoculant is stored (Catroux et al., 2001), proper inoculants production management and distribution of inoculants should keep the time between manufacture and application to a minimum especially when cooling facilities are not available.



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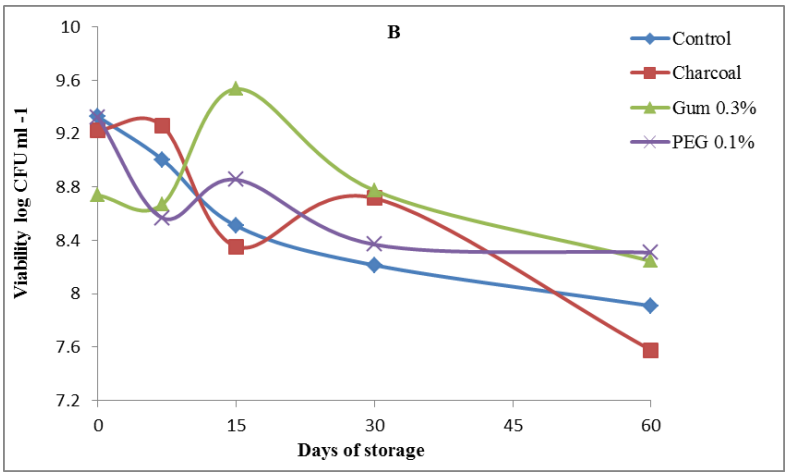
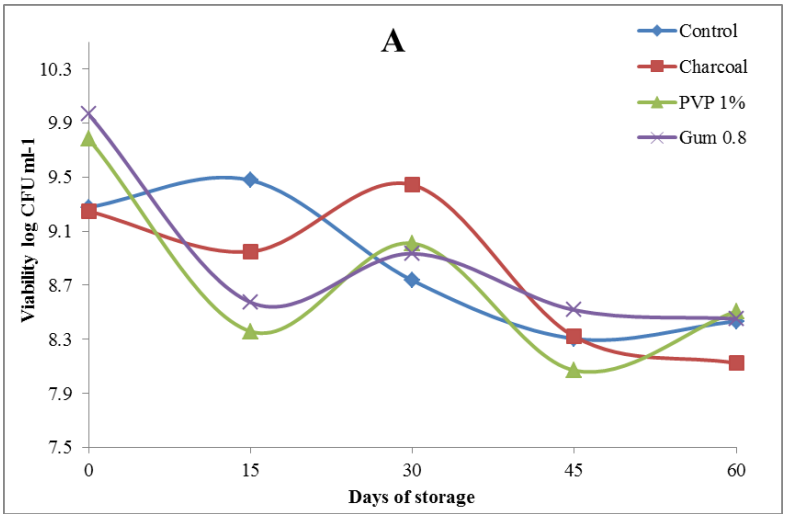



**Figure 1:** Shelf life of ENRRI 1 in different formulations at two storage conditions (A) room temperature (25-35˚C) and (B) refrigerator (4˚C).



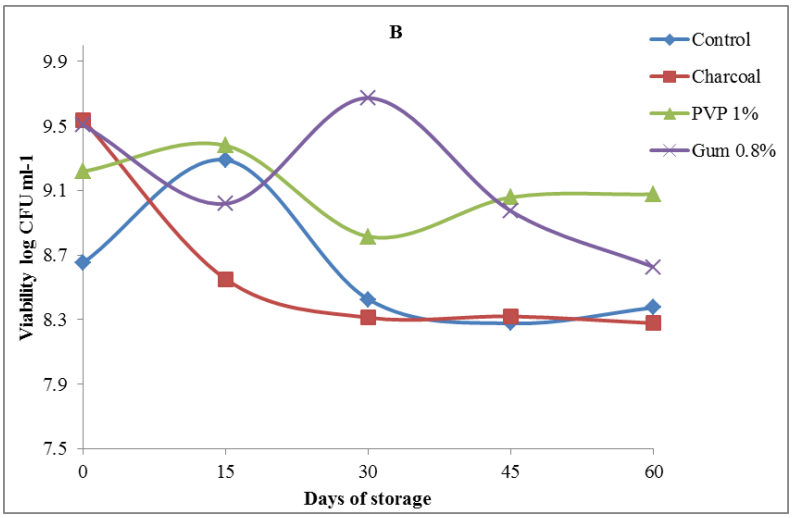




**Figure 2:** Shelf life of TAL 380 in different formulations at two storage conditions (A) room temperature (25-35˚C) and (B) refrigerator (4˚C).

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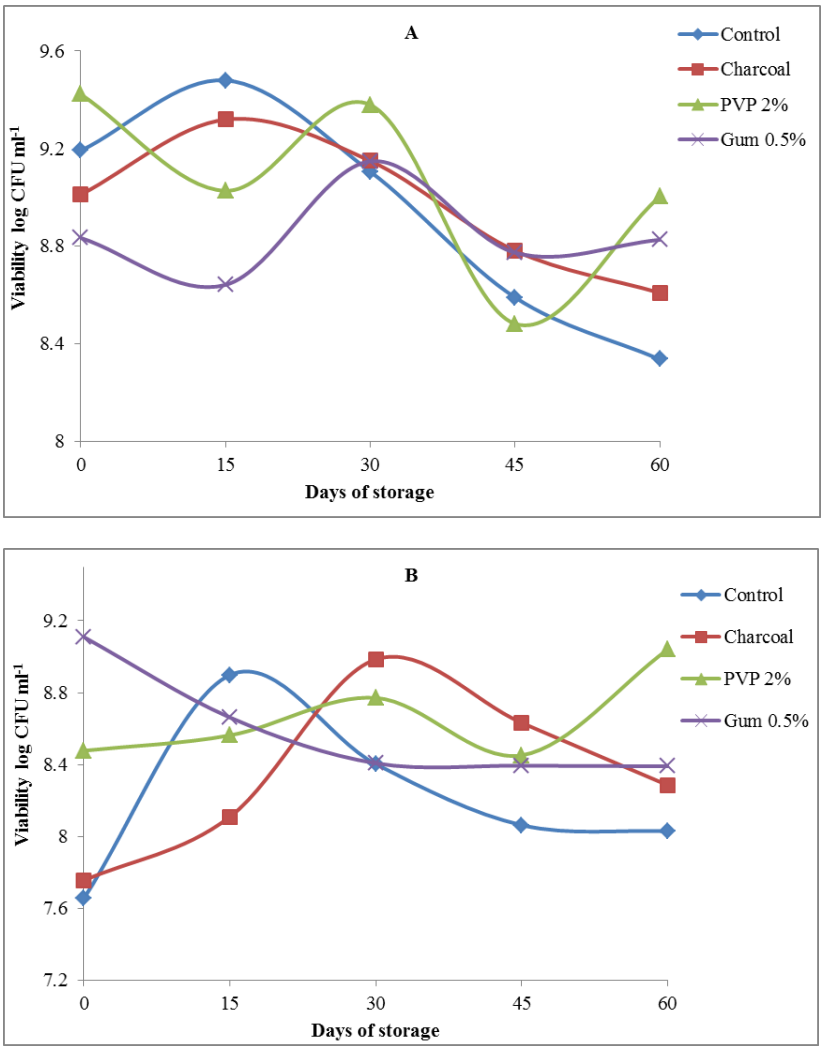
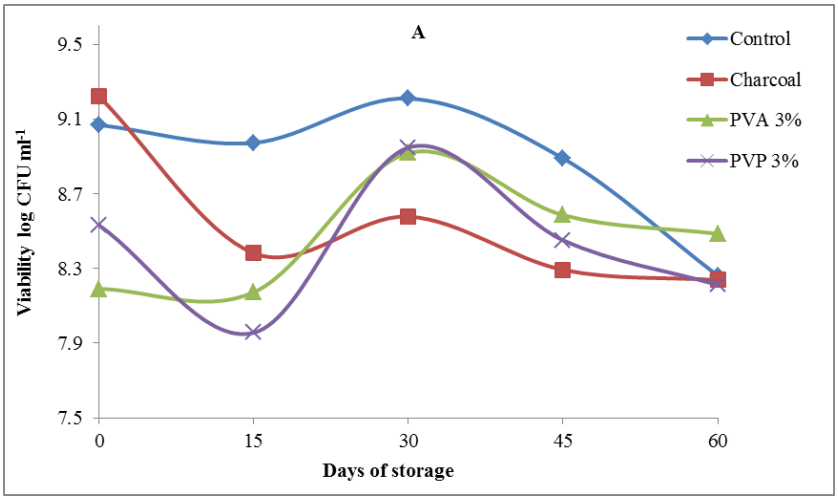



**Figure 3:** Shelf life of USDA 3100 in different formulations at two storage conditions (A) room temperature (25-35 °C) and (B) refrigerator (4°C).



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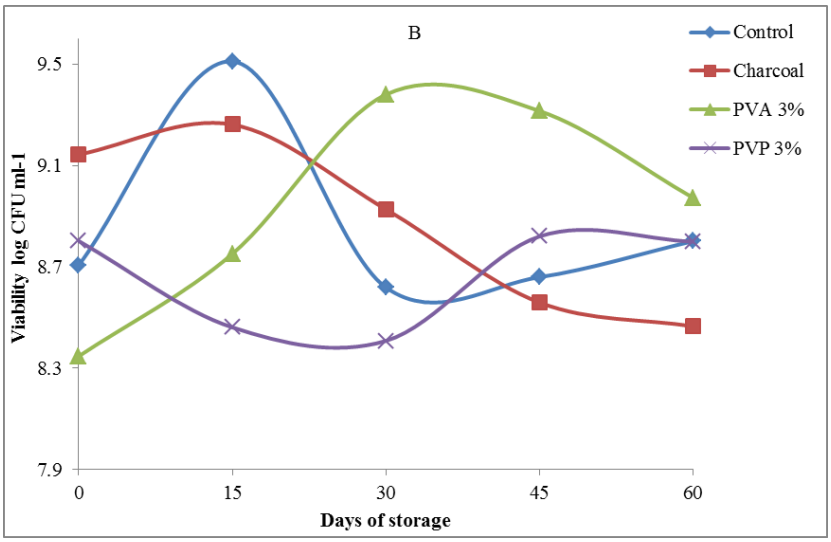
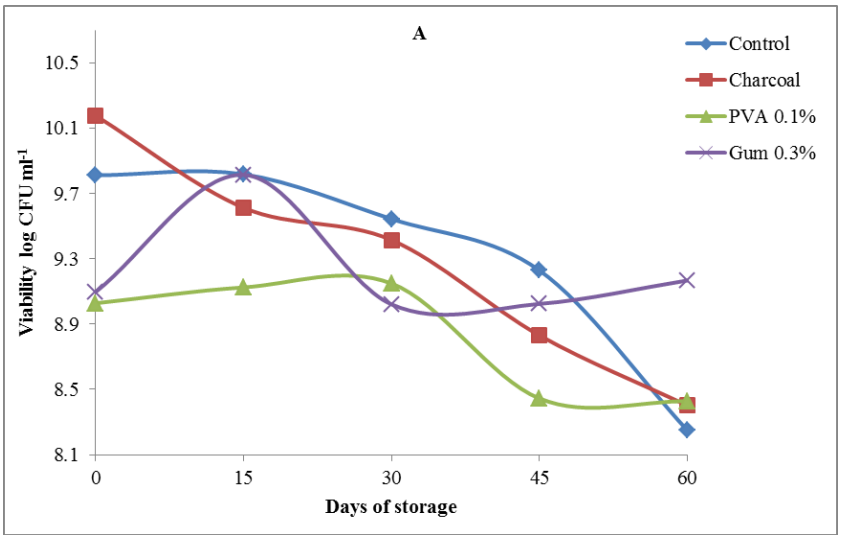



**Figure 4:** Shelf life of TAL 209 in different formulations at two storage conditions (A) room temperature (25-35˚C) and (B) refrigerator (4˚C).



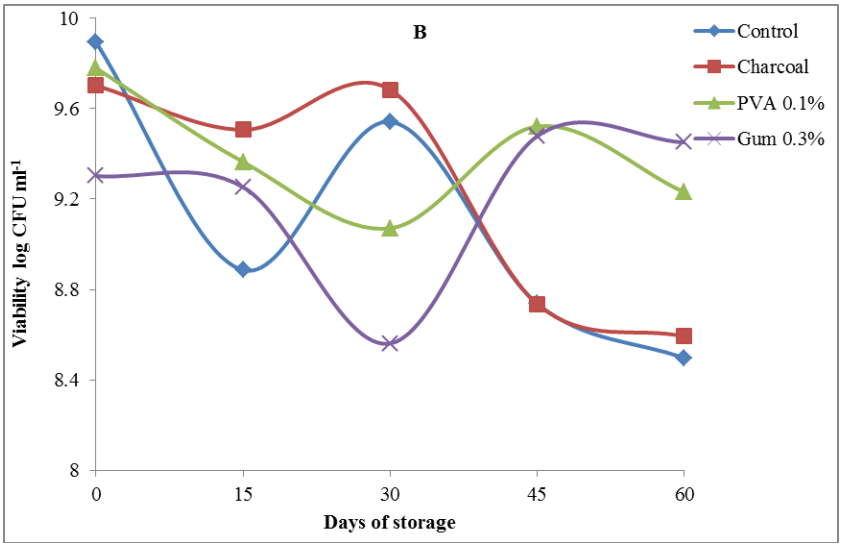




**Figure 5:** Shelf life of USDA 3385 in different formulations at two storage conditions (A) room temperature (25-35˚C) and (B) refrigerator (4˚C).

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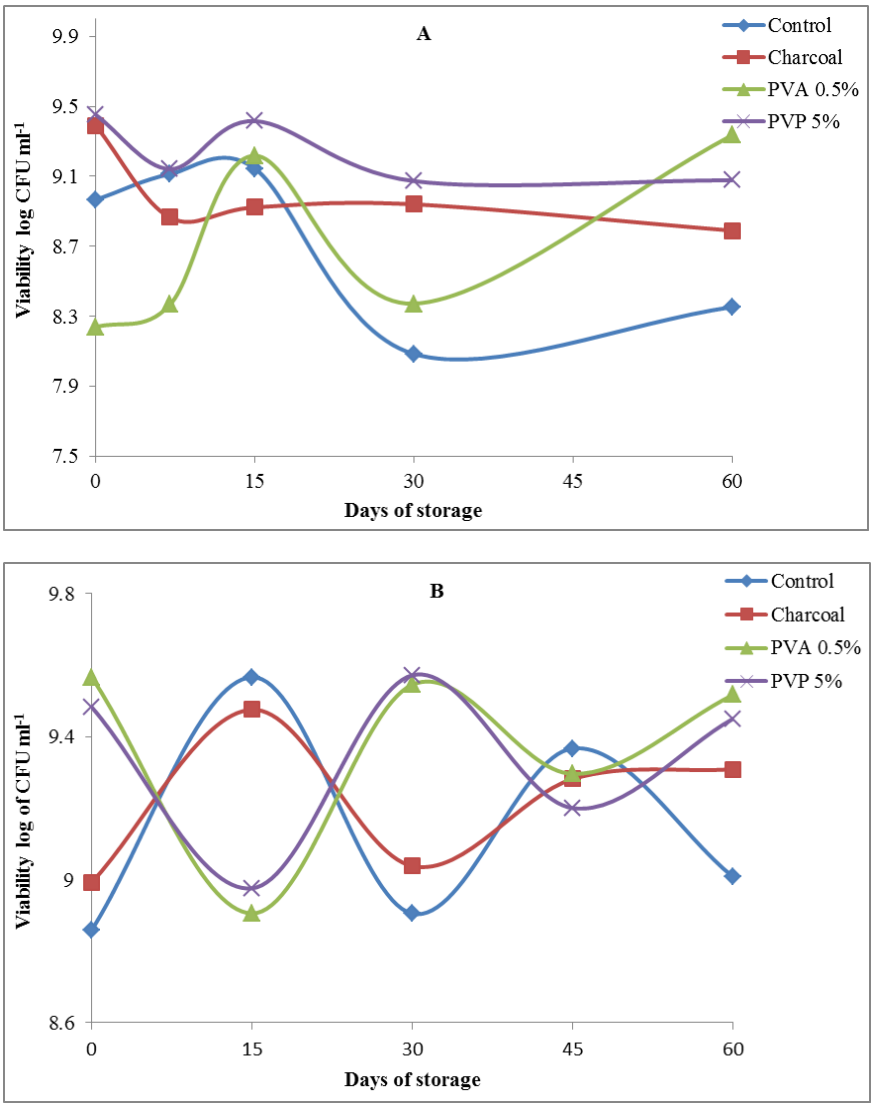


**Figure 6:** Shelf life of TAL 1399 in different formulations at two storage conditions (A) room temperature (25-35˚C) and (B) refrigerator (4˚C).

## 4. CONCLUSIONS

From this study, it has been concluded that liquid inoculants amended with polymeric additives supported the survival of *Rhizobium* inoculants up to 60 days at both room temperature and at 4°C. Moreover, their population density was found to exceed that of charcoal- based inoculants which are usually used for inoculant production. The results also showed that the survival of cell was dependent on both the type of additive and the strain of rhizobia.

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