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Assessment of some polymers for the formulation of rhizobial liquid inoculants

Mohammed A. Hassan*, Somaya S. Mohamed, Migdam E. Abdelgani

Environment and Natural Resources and Desertification Research Institute, National Centre for Research, Khartoum, Sudan

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Abstract

The present study was conducted to screen and evaluate four polymeric additives, polyvinylpyrrolidone (PVP), polyvinyl alcohol (PVA), polyethylene glycol (PEG) and Gum Arabic at different concentrations for their ability to maintain and support bacterial viability of rhizobial strains TAL 380, USDA 110 and local isolates ENRRI 12 and ENRRI 1 during the initial period of growth compared with growth in YEM medium without additives. The comparison of the different concentrations for each polymer was carried out after 8 days of incubation and Data were expressed as log number of Colony Forming Units (CFU) ml^{-1}. The results of the study clearly indicate that most of the tested liquid formulations supported an adequate survival of rhizobial strains providing more than 10^8 bacteria ml^{-1} at the end of the experiment. Liquid inoculants formulated with Gum Arabic at 0.1%, 0.5% and 0.8% promoted long term survival of ENRRI 1, ENRRI 12 and TAL 380 while PVP at 0.1% supported the growth of strain USDA 110. The selected appropriate concentrations of the polymers for each rhizobial strain and isolate were then recommended for further studies concerning their uses as liquid inoculants. Few concentrations tended to slightly reduce cell density when compared to growth in YEM media. The study confirms the better survival of rhizobial cells in liquid inoculants amended with different concentrations of polymeric additives compared to liquid broth medium during initial growth. In addition, a degree of interaction between strains of rhizobia and polymeric additives was also noticed.

* **Corresponding Author:** Mohammed A. Hassan ✉ mohabdal@hotmail.com

Introduction

Leguminous plants are able to convert atmospheric nitrogen through symbiotic relationship with symbiotic nitrogen fixing bacteria that are naturally found in the soil. The supply of soil nitrogen will mostly be insufficient to satisfy the nitrogen demand of the growing leguminous crops and that creats a need for inoculation with efficient rhizobial strain.

The quality of the carrier, the concentration of the rhizobial cells, tolerance of the strain to temperature, low moisture, aeration and storage conditions, are factors that control the survival of rhizobia in legume inoculants (Denardin and Freire, 2000).

Peat is the most common carriers for rhizobia in legume inoculants. However, peat has some disadvantages *viz.,* variable physical and chemical composition, short shelf life and high cost of preparation. In addition, peat based inoculants had been rejected by large scale farmer due mainly to increased complexity in inoculation (Denardin and Freire, 2000). Therefore, due to the limitations concerning the lack of availability and accessibility of peat deposits in some countries, alternative carriers for inoculant production have been investigated (Albareda *et al*., 2008).

In Sudan, due to unavailability of peat, several studies were carried out to assess the suitability of locally available materials as carriers for rhizobia, also, sterilization of the carriers and shelf life of inoculants were considered (ELsalahi *et al*., 2016). Charcoal was found to be superior in terms of availability and abundance besides its high water holding capacity, its least contamination liability and storage ability of 60 days at room temperature (Elshafie and Elhussein, 1991). Several studies revealed that Rhizobium inoculation improved productivity of leguminous crops and soil fertility (Gadalla *et al.,* 2010). In accord, annual production of *Rhizobium* biofertilizer is increasing but still not satisfactory to meet the actual demand. Recently, expansion in large mechanized fodder production schemes brought about a very large demand on liquid rhizobial inocula (Elsalahi *et al*., 2016).

Commercial rhizobial inoculants have been used over a century and their market is growing steadily to obtain higher crop yields. Rhizobial inoculants are available in several formulations. The most common is granular inoculant which is distributed over the soil after sowing, and hence, in this case high quantities are necessary for an efficient inoculation. Liquid inoculants are commonly used in large areas, since they are better suited for mechanical sowing (Fernandes Junior *et al.,* 2009). The development of carrier materials for enhancing survival of rhizobial inoculant is important for ensuring the maintenance of inoculants quality during storage and transport to the field (Bashan *et al.,* 2013). Therefore, in order to increase the inoculants quality, efficiency, and to reduce costs and environmental impacts, alternative carrier materials including single and composite polymer formulations have been studied (Sarr *et al.,* 2005).

Liquid inoculant formulations contain not only the desired microorganisms and their nutrients but also special cell protectants or additives that promote for longer shelf life and tolerance to adverse conditions (Leo Daniel Amalraj *et al.,* 2012). These polymers, such as sodium alginate, Gum Arabic, polyvinylpyrrolidone (PVP), polyvinyl alcohol (PVA) and polyethylene glycol (PEG) are normally used as adhesive when applied to seed (Leo Daniel Amalraj *et al.,* 2012). Since there is a degree of interaction between strains of rhizobia and additives (Tittabutr *et al.,* 2007), liquid inoculants additives may have to be selected for individual species and strains to maximize their performance. In addition, the maintenance of rhizobial cell concentrations throughout the beginning of the storage period, or in the maturation period, is important because both are adaptative periods of bacterial cells to the new environment (Fernandes Junior *et al.,* 2009).

Therefore, the aim of this research is to evaluate the suitability of some concentrations of Gum Arabic, PVP, PVA and PEG to sustain the bacterial viability during the initial growth period of selected rhizobial strains and isolates that are commonly used as charcoal based inoculants for popular and cash leguminous crops in Sudan.

Materials and methods

Microorganisms

Rhizobial strains TAL 380 and USDA 110 and rhizobial isolates, ENRRI 1 and ENRRI 12 were obtained from Biopesticides and Biofertilizers Department, Environment and Natural Resources and Desertification Research Institute, National Centre for Research, Khartoum, Sudan. TAL 380 strain was previously obtained from NiFTAL project and USDA110 was obtained from U.S. Department of Agriculture, both of them proved to be efficient under Sudan conditions. ENRRI 1 and ENRRI 12 were locally isolated, tested and efficiently used as solid based inoculants.

Rhizobium culture medium

Yeast Extract Manitol Broth (YEMB) composed of (g/l) mannitol, 10g; K_2HPO_4, 0.5g, $MgSO_4.7H_2O$, 0.2g, NaCl 0.1g and yeast extract 0.5g (Somasegaran and Hoben, 1994) was used as a culture basal medium of rhizobia for liquid inoculants formulation. The medium was solidified by adding 16g/l agar when needed.

Liguid inoculant formulation

YEM media were blended with different concentrations of additives: Polyvinylpyrrolidone (PVP) (K40; Sigma) at 1.0, 2.0, 3.0, 5.0% (w/v); Polyethylene glycol (PEG) (3000; Sigma) at 0.1, 0.5, 1.0, 5.0% (w/v); Polyvinyl alcohol (PVA) (Sigma) at 0.1, 0.5, 1.0, 3.0% (w/v) and Gum Arabic at 0.1, 0.3, 0.5, 0.8% (w/v).

Effect of different concentrations of polymers on bacterial growth

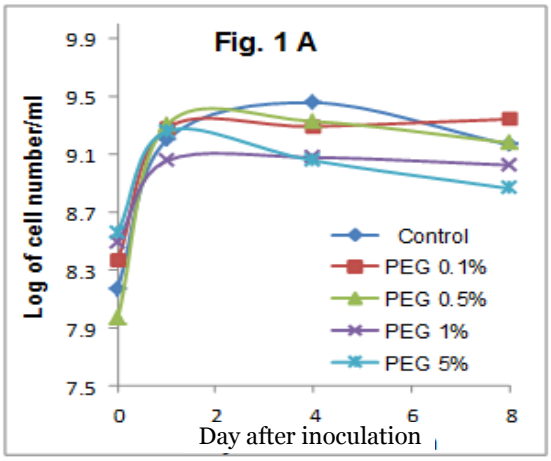
Laboratory experiments were carried out in 250ml Erlenmeyer flasks containing 100 ml of amended medium. 0.1% (v/v) late log phase cultures of the tested rhizobia were inoculated into formulated media, and grown in orbital shaker at room temperature (25-30°C) and 120rpm for 8 days. Liquid inoculants were sampled at day 0, 2, 4 and 8 to determine the total viable rhizobial cells by plate count method (Somasegaran and Hoben, 1994) compared to growth in YEM without additives as a control. Plates were incubated under aerobic conditions at 28±2°C for 48 hrs. Data were expressed as log number of CFU ml^{-1}.

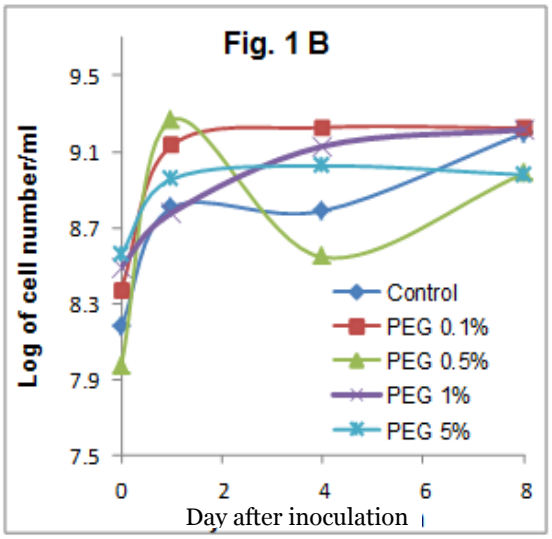
Results and discussion

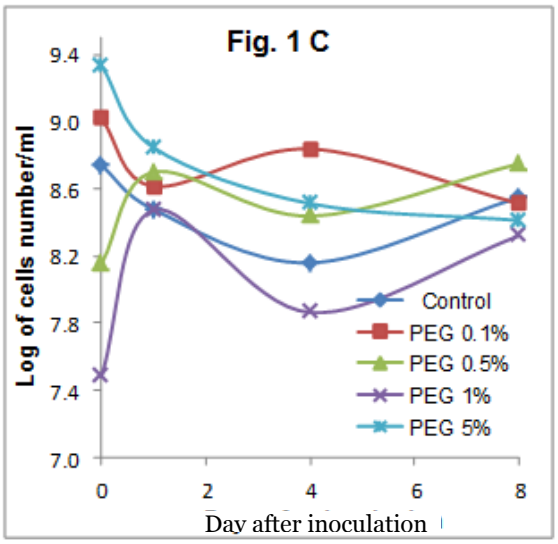
The performance of liquid inoculants containing different concentrations of PVP, PVA, PEG and Gum Arabic on growth of the selected rhizobial strains and isolates compared to growth in YEM without polymeric additives were studied. The results of the study are shown in Fig.s 1- 4.

PEG

The results have shown the ability of all tested concentrations of PEG to sustain more than 10^7 CFU $mL^{-1}$for TAL 380, ENRRI 1, ENRRI 12 and USDA 110 (Fig. 1). 2.2×10^9 CFU mL^{-1} was the highest number of cells for TAL 380 recorded when YEM was amended with 0.1 % PEG followed by 1.5×10^9 CFU mL^{-1} which was obtained by 0.5 % PEG (Fig. 1A) .Also, 0.1% and 1% of PEG recorded the highest population of ENRRI 12 (Fig. 1B) after 8 days of incubation giving $1.6x10^9$ for both concentrations. However, USDA 110 (Fig.1C) showed a loss of viability after 8 days of incubation when the YEM was amended with the different concentrations of PEG compared to control with the exception of 0.5% which gave 5.6×10^9 CFU mL^{-1}. After 8 days of incubation, the population densities of ENRRI 1 at 0.5% and 0.1% PEG exceeded that of control and the highest population number was recorded at 0.1%. (Fig.1D). PEG blended in YEM has also been reported to promote the growth of cells in most of studied rhizobial strains by Tittabutr *et al* (2007). Similarly, highest population densities of *Azotobacter sp, Azospirillum* sp, *Bacillus* sp and *Pseudomonas* sp were recorded in media supplemented with PEG, PVP and glycerol (Dayamani and Brahmaprakash, 2014).







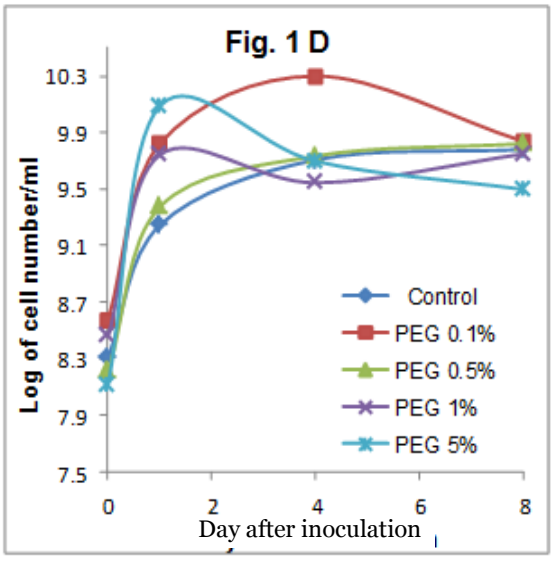
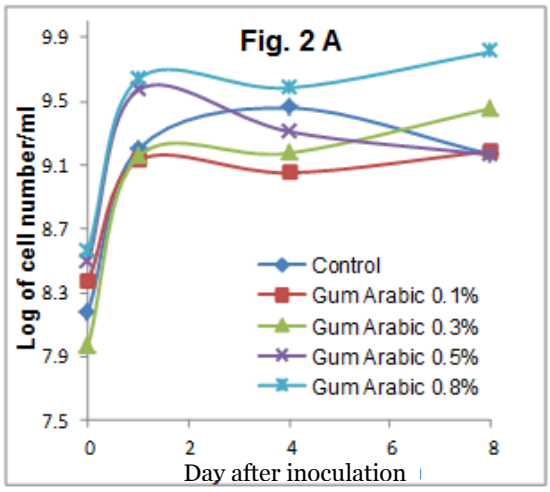
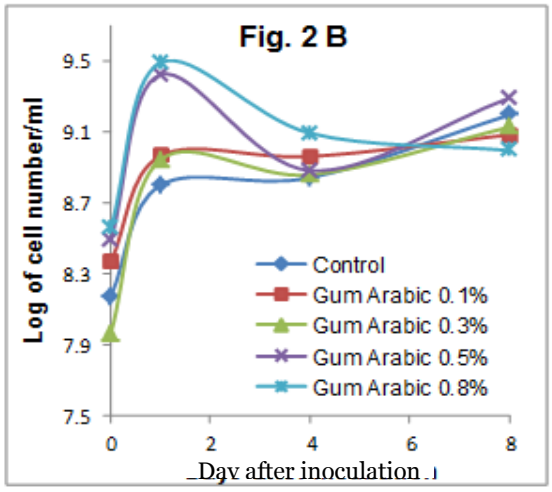


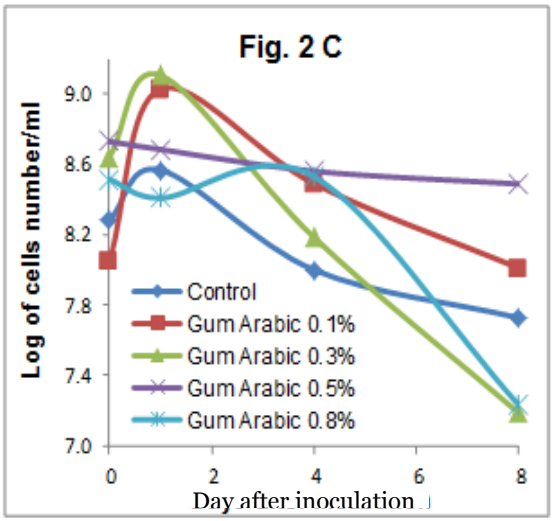
Fig. 1. Effect of different concentrations of PEG on the population density of *Rhizobium* strains TAL 380 (A), ENRRI 12 (B), USDA 110 (C) and ENRRI 1(D).

Gum arabic

The media supplemented with different concentrations of Gum Arabic showed different capacities to maintain adequate survival of the studied rhizobial strains and isolates (Fig. 2). Media supplemebted with 0.3% and 0.8% Gum Arabic, were able to sustain the growth of TAL 380 (Fig. 2A) compared to control, giving 2.8×10^9 and 6.5×10^9 CFU mL^{-1} , respectively. 0.5% was found to be the only concentration that promotes the cell density of ENRRI 12 (Fig. 2B). Highest survival of USDA 110 (Fig. 2C) and ENRRI1 (Fig. 2D) were recorded in liquid inoculants that supplemented with 0.5% and 0.3% Gum Arabic, respectively. Valetti *et al.* (2016) evaluated different polymers and they found that, the greatest bacterial viability of two bradyrhizobial strains were obtained when Gum Arabic was used as stabilizing solution. Previously, Tittabutr *et al.* (2007) reported that Gum Arabic supported the growth of most studied rhizobial strains at 10^8 CFU ml^{-1}







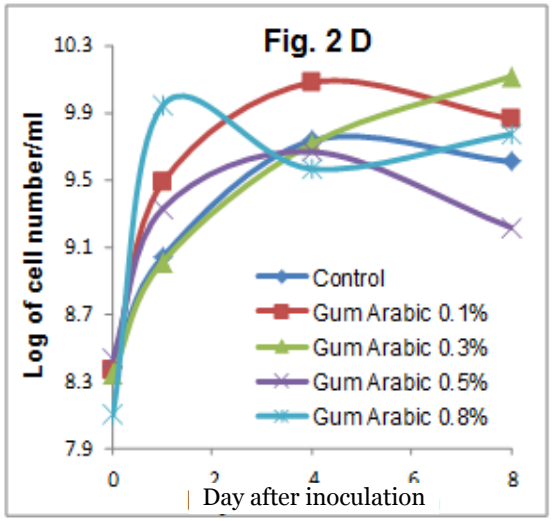


Fig. 2. Effect of different concentrations of Gum Arabic on the population density of *Rhizobium* strains TAL 380 (A), ENRRI 12 (B), USDA 110 (C) and ENRRI 1(D).

PVA

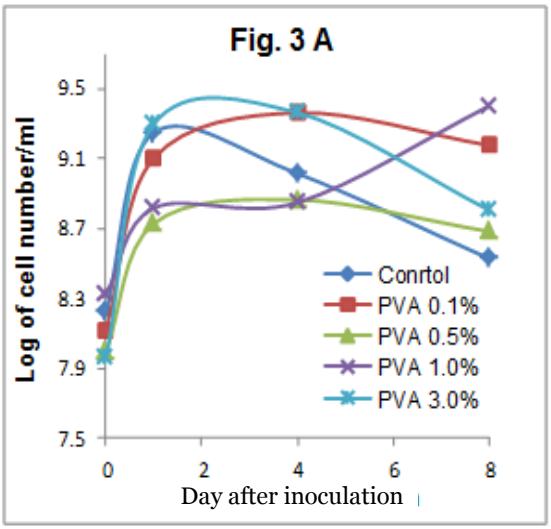
The effect of the different liquid inoculants amended with four concentrations of PVA on the growth of the tested rhizobial strains and isolates are shown in (Fig. 3.) All the concentrations supported an adequate survival of the tested strains and isolates, providing more than 10^8 CFU mL^{-1} at the end of the experiment. The highest numbers of TAL 380 (Fig. 3A) and ENRRI 1 (Fig. 3D) were maintained by the highest concentration (1.0%) of PVA, while the lowest concentration (0.1%) was able to give the highest bacterial density of ENRRI 12 (Fig. 3B) and USDA 110 (Fig. 3C).

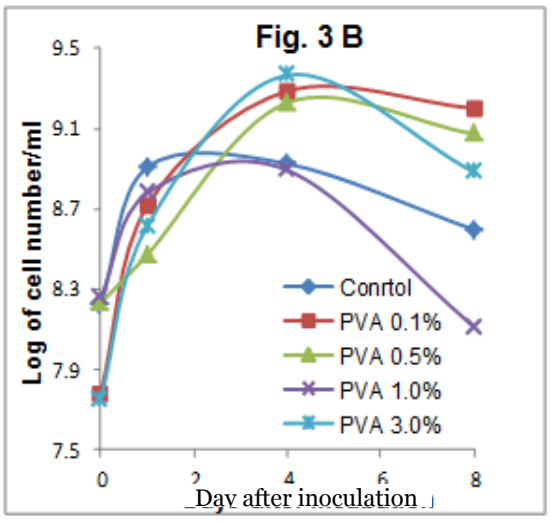
Tittabutr *et al.* (2007) found that PVA could support the growth of *Rhizobium phaseoli* and *Synorhizobium fredii* but some concentrations were not appropriate for *Bradyrhizobium jabonicum*. Leo Daniel Amalraj *et al.* (2012) stated that PVP at 2%.

Concentration

Concentrations showed excellent retention trait of *Bacillus, Azospirillum* and *Azotobacter* and that could be due to their ability to limit heat transfer, their good rheological properties and high water activities. Similarly, Singleton *et al.,* (2002) reported that PVP blended with bradyrhizobium medium showed no adverse affect on growth. Among different additives tested by Kavi Karunya and Reetha (2014),

PVP at 1% supported more population of *Azospirillum brasilense*, *Bacilus subtilis* and *Pseudomonas fluorescens*.





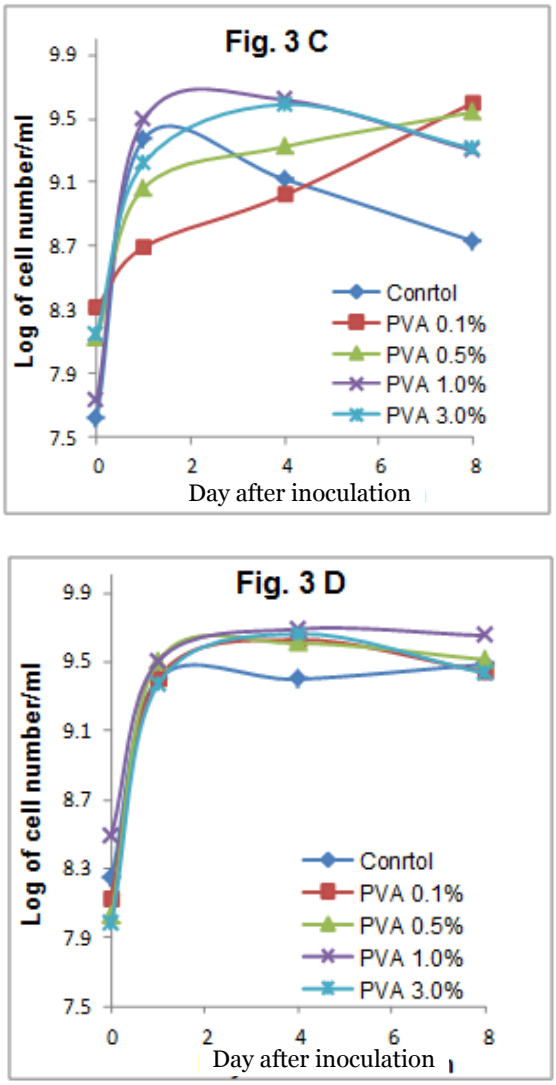
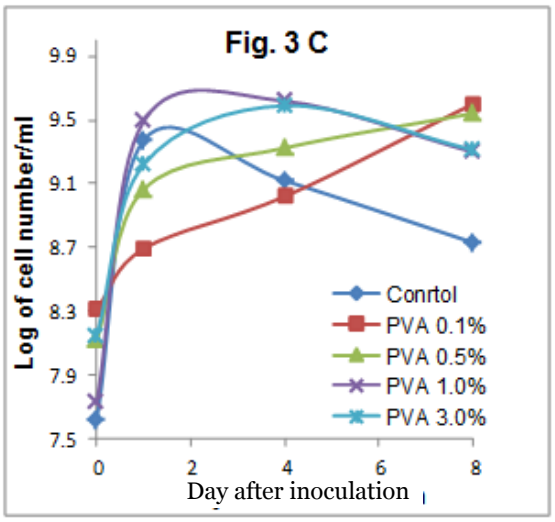


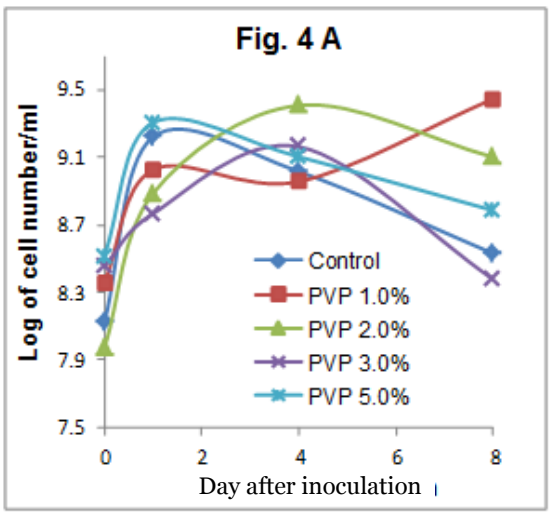
Fig. 3. Effect of different concentrations of PVA on the population density of *Rhizobium* strains TAL 380 (A), ENRRI 12 (B), USDA 110 (C) and ENRRI 1(D).

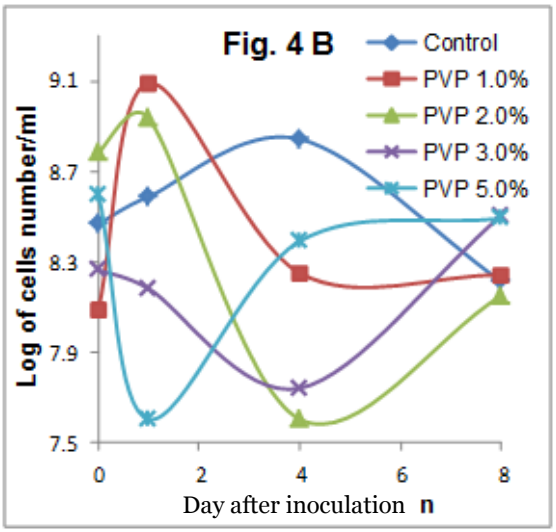
PVP

Survival of the rhizobial strains and isolates were also tested in liquid media supplemented with different concentrations of PVP (Fig. 4). Among the four tested concentrations, 1.0% of PVP showed the highest population of TAL 380 (Fig. 3A) while 5% PVP gave the highest bacterial counts of ENRRI 1 (Fig. 3D) as compared to the other concentrations. Medium that was supplemented with 3.0% PVP was found to be the best formulation for maintaining highest population of ENRRI 12 (Fig. 4B) compared to the other formulations. Maximum bacterial population of USDA 110 was recorded in medium supplemented with 2% PVP (Fig. 4C).

However, although 5% of PVP was not able to support the growth of USDA 110, it gave the highest viable number (6.5×10^9 CFU mL^{-1}) of ENRRI 1.







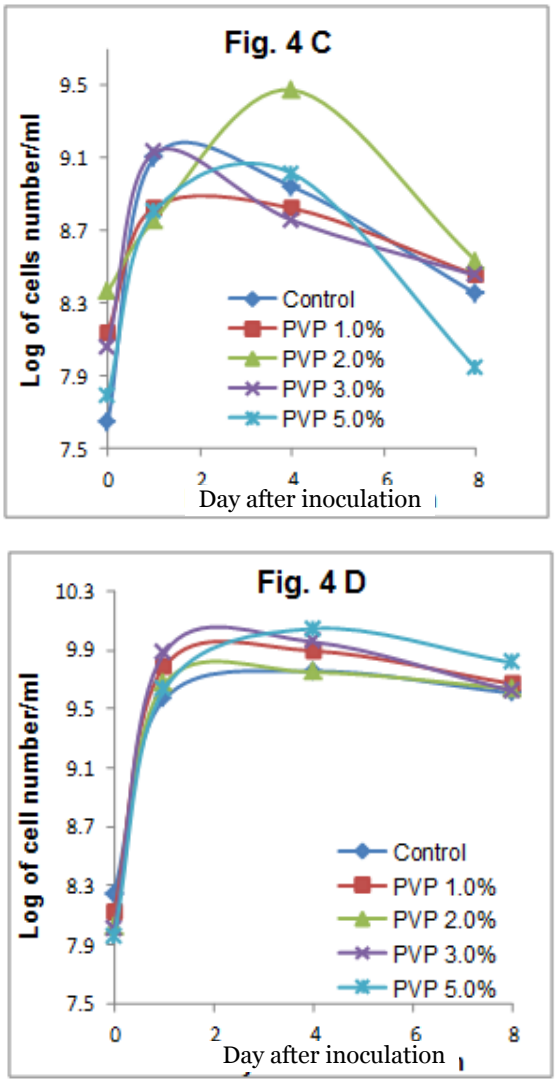


Fig. 4. Effect of different concentrations of PVP on the population density of *Rhizobium* strains TAL 380 (A), ENRRI 12 (B), USDA 110 (C) and ENRRI 1(D).

The results show that, highest population of rhizobial strains ENRRI 1, ENRRI 12 and TAL 380 were recorded in media supplemented with 0.1%, 0.5% and 0.8% Gum Arabic, respectively while medium supplemented with 0.1% PVA promoted the growth of USDA 110 .Therefore, some polymeric additives supported cell growth among all strains and isolates at higher level than YEM and some polymeric additives performed better than others within the studied rhizobial strains and isolates while some concentrations tended to slightly reduce cell density when compared to YEM.

Our results confirmed the previous findings of Tittabutr *et al.* (2007) who stated that there is a degree of interaction between strains of rhizobia and additives that may benefit liquid inoculants performance. Hence, additives may need to be selected for individual species and strains to maximize the performance of individual inoculants as previously stated by Valetti *et al.* (2016).

Liquid inoculant formulation is a solution to the problems associated with processed solid carriers, since they can easily be sprayed on the seeds. (Valetti *et al.,* 2016). This formulation may use broth cultures amended with various additives that promote cell adhesion to seed and enhance rhizobial survival (Deaker *et al.,* 2004). Hence, to produce inoculants of good quality, suitable additives substances for the target rhizobia should be selected (Tittabutr *et al.,* 2007). Therefore, different concentrations of polymer which were appropriate for each rhizobial strain were selected based on their ability to promote cell growth .These selected concentrations should further be used for testing their effects on shelf life of the inoculants.

Polymers have a sticky consistency, which may enhance cell adherence to seeds, and their viscous nature may slow the drying process of the inoculant after application to seed (Temprano *et al.,* 2002). In addition, each polymer has its own special characteristics, PVP has a capacity to bind bacterial toxin that were constantly released into the media, when bacterial cells were in stationary phase (Errington *et al.,* 2002). Gum Arabic is a biopolymer with large molecular weight, adhesive emulsifier and stabilization properties which limits heat transfer and has a high water activity (Mugnier and Jung, 1985). PEG is a water soluble compound with adhesive and sticky consistency. The viscous nature of PEG could slow the drying process of the inoculants (Temperano *et al.,* 2002). PVA is a biomaterial that has been used worldwide in the industrial sector because of its high favourable properties such as biocompatibility and bioadhesiveness (Demerlis and Schoneker, 2003). Those properties most properly were found to support the growth and survival of rhizobial strains and isolates.

Conclusions

The polymeric additives have positive effect on maintaining rhizobial viability during initial growth period. There is a degree of interaction between the different rhizobial strains and different polymers and their concentrations. Results indicate the suitability of using Gum Arabic at 0.1%, 0.5% and 0.8% and PVA at 0.1% as polymeric additives for liquid inoculants production for ENRRI 1, ENRRI 12, TAL 380 and USDA 110, respectively. Furthur studies regarding the use of these concenations for testing the effect of polymeric additives on shelf life of the liquid inoculant and their performance in glass house experiments should be carried out.

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