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Full Length Research Paper

Effect of high temperature on rhizobia survival on different leguminous seeds inoculated with liquid formulations

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Liquid inoculants formulated with different polymeric additives viz., polyvinylpyrrolidone PVP, polyethylene glycol (PEG), polyvinyl alcohol PVA and Gum Arabic were evaluated for their ability to support *Rhizobium* survival on coated seeds. Inoculated faba bean, alfalfa, chickpea, mung bean, guar and soybean seeds were incubated at high temperatures (40°C and 45°C) for 24 and 48 hour. The number of rhizobia per seed of each treatment was determined using the plate count method. Liquid inoculants containing 1% PEG was found to maintain the highest number of cells surviving on faba bean seeds giving 12.4% increase compared to charcoal-based inoculants. Liquid inoculants amended with 0.8% Gum Arabic and 0.1% PEG recorded the maximum population of about 2.4×10^6 and 2×10^6 cells/ alfalfa seed, respectively. Highest population 8×10^5 cells/ chickpea seed was recorded in liquid inoculant formulated with 0.5% Gum Arabic. For mung bean, liquid inoculants amended with 0.1% Gum Arabic was found to record a maximum population of about 1×10^6 and 9×10^5 cells/seed after 24 and 48 h of storage at 45°C, respectively. Liquid inoculants amended with 0.1% and 0.5% PVA were found to be better in supporting *Rhizobium* population on guar seeds than charcoal based inoculants after 48 h. Liquid inoculants containing 0.5 % PVA and 0.1 % Gum Arabic could support the survival of rhizobial cell to 9.6% and 8.1% over charcoal based inoculant after 24 and 48 h from inoculation of soybean seeds at 45°C. The study concluded that liquid formulations could either promote rhizobial population or sustain rhizobial number equivalent to charcoal based inoculants. Moreover, Survivability of liquid formulations varied with the strain, seed and polymeric additives

Keywords: Polymers, Rhizobia, Temperature, Inoculation rate, seeds.

INTRODUCTION

Biofertilizers are important components of integrated nutrients management which plays a key role in productivity and sustainability of soil. They are cost-effective, ecofriendly and renewable sources of plant

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nutrients. Legume inoculants should provide a high number of viable and effective rhizobia to the rhizosphere in order to allow rapid colonization, nodulation and nitrogen fixation so as to maximize legume yield potential (Deaker et al*.,* 2006). Seed inoculation is a more economical way of introducing rhizobia to the rhizosphere than soil application since the latter requires large volumes of inoculants to ensure adequate distribution of rhizobia.

Survival of rhizobia during stress like high temperatures and desiccation is affected by the carrier material used, the type of culture medium in which the cells were produced and by the incubation or storage period of inoculant after carrier impregnation (Bashan et al., 2014).The quality of legume inoculant is determined by how many viable cells are in the inoculant and how well they survive after application to the seeds (Singleton et al*.,* 2002). Besides that, inoculants carrier material should have properties such as dissolve well in water so that the bacteria can be released and able to tolerate harsh conditions (Kaljeet et al*.,* 2011). In order to maintain survival of rhizobia, especially in tropical and subtropical regions of the world where inoculants are often exposed to extremely hot and dry conditions, many polymers have been used as additives for inoculant production due to their ability to limit heat transfer, good rheological properties and high water activities (Sandra and Rebeca, 2015).

Inoculated seeds are normally sown into a furrow, which may expose the inoculant to temperatures at or above 40°C (Hafeez et al., 1991). Temperature is known as one of the environmental factors affecting survival and nitrogen fixation of rhizobial inoculant (Hungria and Vargas, 2000).Using an approach of varying the inoculants rates to achieve a range of rhizobial number on the seeds, Roughley et al.,(1993) found that increasing the number of rhizobia applied to the seed increased the number of nodules and grain yield and the responses were almost linear. Thus, it is important to determine the duration of bacterial survivability in the coated seeds and the rate of application of the inoculants to ensure the desired and effective level of bacterial population. Therefore, in this study, the role of liquid inoculants formulated with the appropriate polymeric additives on survivability of rhizobial cells on coated seeds at high temperature was investigated compared to coating with charcoal based inoculant.

MATERIALS AND METHODS

Rhizobia Culture Medium

Yeast Extract Mannitol 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 medium for rhizobia, basal medium for liquid inoculants formulation and charcoal-based inoculants preparation. The medium was solidified by adding 16g agar when needed.

Bacterial Strains Preservation

Rhizobial strains and isolates 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 NifTAL project, USDA 3386 and USDA 3100 were obtained from U.S.A. Department of Agriculture and ENRRI 1 was locally isolated. They are efficiently used as solid based inoculants. Rhizobial strains were preserved by streaking on YEMA slants and they were kept in the refrigerator at 4°C during the study.

Preparation of Charcoal-based Inoculants

A volume of 400 ml Yeast Extract Mannitol (YEMB) in 500 ml conical flasks was autoclaved for 15 minutes at 15 ib/in² and 121°C. A loopful of each strain was transferred aseptically to a flask containing YEMB and left in an orbital shaker for 24-48 hour. Cultures were serially diluted in distilled water and plate count method (Somasegaran and Hoben, 1994) was used to check for inoculum quality (should contain at least 1×10^9 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 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 polyethylene 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.

Liquid Inoculant Formulation

YEMB media were amended with different concentrations of additives: polyvinylpyrrolidone PVP (K40; Sigma) at 1.0, 2.0, 3.0 and 5.0% (w/v); polyethylene glycol (PEG) (3000; Sigma) at 0.1, 0.5, 1.0 and 5.0% (w/v);); polyvinyl alcohol PVA (Sigma) at 0.1, 0.5, 1.0 and 3.0% (w/v) and Gum Arabic at 0.1, 0.3, 0.5 and 0.8% (w/v).The formulated media were autoclaved for 15 minutes at 15 ib/in² and 121°C.A loopful of each strain was transferred aseptically to a flask containing the formulated YEMB and left in an orbital shaker for 24-48 hour.

Inoculant Survival on Seeds at High Temperature

The formulated rhizobial liquid inoculants (2 ml) were used to inoculate 50 g of faba bean, alfalfa, chickpea, mung

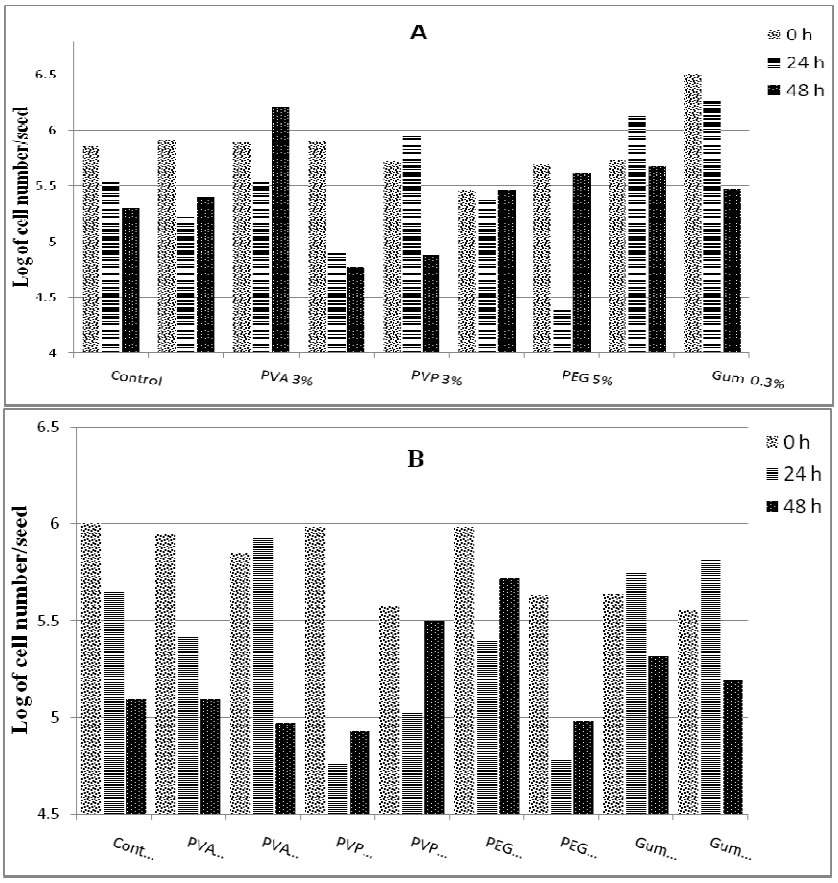

Figure 1: Survival of rhizobial strain TAL 1399 on faba bean seeds coated with liquid formulations at (A) 40°C and (B) 45°C.

bean, guar and soybean seeds. Seeds were shaken for few minutes until all seeds were uniformly wetted with the liquid inoculant. For inoculation with charcoal-based inoculants, a small amount of 40% (w/v) Gum Arabic was added to the seeds and mixed thoroughly to wet all seeds then charcoal- based inoculants were added and mixed until all seeds were totally coated. Then inoculated seeds were left to air dry for five minutes.Seeds from each treatment were divided into two groups and placed in Petri dishes. Each group was divided to three lots for each formulation. One group of Petri dishes was incubated at 40°C and the other one was incubated at 45°C. The number of rhizobia per seed of each treatment was determined at 0, 24, and 48 h from inoculation using the plate count method as described by Somasegaran and Hoben (1994). Samples were replicated and duplicate plate counts were made from each dilution. The average number of the colony forming unit was then calculated.

RESULTS AND DISCUSSION

Survival of Liquid Formulations on inoculated Seeds After Storage at High Temperature

Faba Bean Seeds

Different *Rhizobium* inoculant formulations showed different capacity to maintain an adequate survival of rhizobial cells on seeds coated with strain TAL1399 and incubated at 40°C and 45°C for 24 and 48 h (Figure1A and B). Liquid inoculants amended with 0.5 and 0.3% Gum Arabic were found to be more effective in maintaining high population of rhizobia at about 1×10^6 cells/seed and 2×10^6cells/seed at 40°C after 24 h from inoculation, respectively. Moreover, 6×10^5cells/seed was obtained by liquid inoculants amended with each concentration of Gum Arabic at 45°C after 24 h. PVA at 3% maintained the

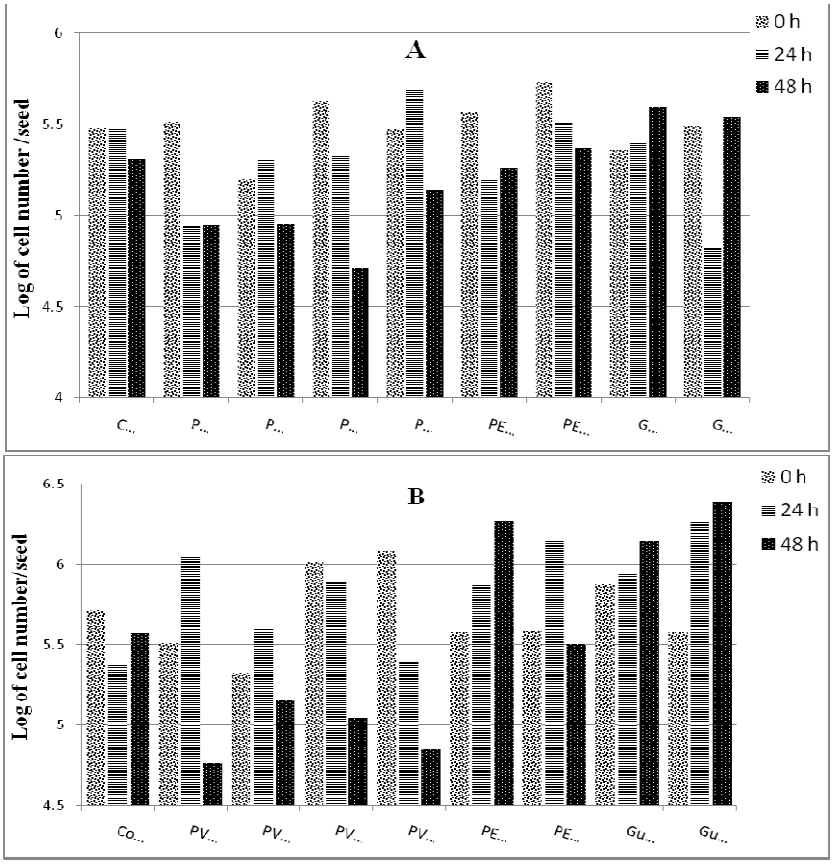

Figure 2: Survival of rhizobial strain TAL 380 on alfalfa seeds coated with liquid formulations at (A) 40°C and (B) 45°C.

highest population at about 2×10^6 cells/seed compared to the other treatments after 48 h of storage at 40°C. However, at 45°C, the viable counts of the inoculant formulated with 3% PVA sharply declined after 48 h of storage. Liquid inoculants containing 1% PEG maintained the highest number of cells surviving on seeds giving 12.4% increase compared to charcoal-based inoculants. These findings are in agreement with the result of Valetti et al., (2016) that liquid inoculant formulated with Gum Arabic had optimal bacterial growth and the highest bacterial concentration and viability during 6 months at room temperature (25 °C). At zero time, the population count of all tested liquid formulation and charcoal based inoculants exceeded the standard number at both temperatures 40°C and 45°C. However, different trends were followed by each formulation during the storage period.

Alfalfa Seeds

The number of viable cells on the alfalfa seeds was determined at 0, 24, and 48 h after inoculation with TAL 380 and incubated at 40°C and 45°C. (Figure2 A and B).Charcoal-based inoculants and liquid inoculants containing PVA, PVP, PEG and Gum Arabic could maintain the number of the cells at 40°C to the standard number. Liquid inoculant formulated with 2% PVP gave 5×10^5cells/seed which was the highest viable count at 40°C after 24 h, followed by 0.5% PEG and charcoal based inoculant. 0.3% Gum Arabic gave 4×10^5 cells/seed followed by PEG at about 2.3×10^5 cells/seed and charcoal based inoculants at about 2×10^5 cells/seed after 48 h. Liquid inoculants containing 0.8% Gum Arabic and 0.5% PEG could maintain the number of surviving cells on seed at about 2×10^6 and 1.3×10^6 cells/seed respectively, at 45°C after 24 h, while charcoal based inoculant maintained about 2×10^5 cells/seed. Similar trends were followed after 48 h of incubation, liquid inoculants amended with 0.8% Gum Arabic, 0.1% PEG recorded the maximum population of about 2.4×10^6 and 2×10^6 cells/seed, respectively.

A sharp reduction in population counts was recorded by liquid inoculant amended with 1% PVP, where the initial count was about 4×10^5 cells/seed decreased to 0.5×10^5 cells/seed after 48 h of storage at 40°C. However, charcoal based inoculants maintained the population count throughout the storage period with more than 10^5 cells/seed. At 45°C, liquid inoculants amended with 0.8%

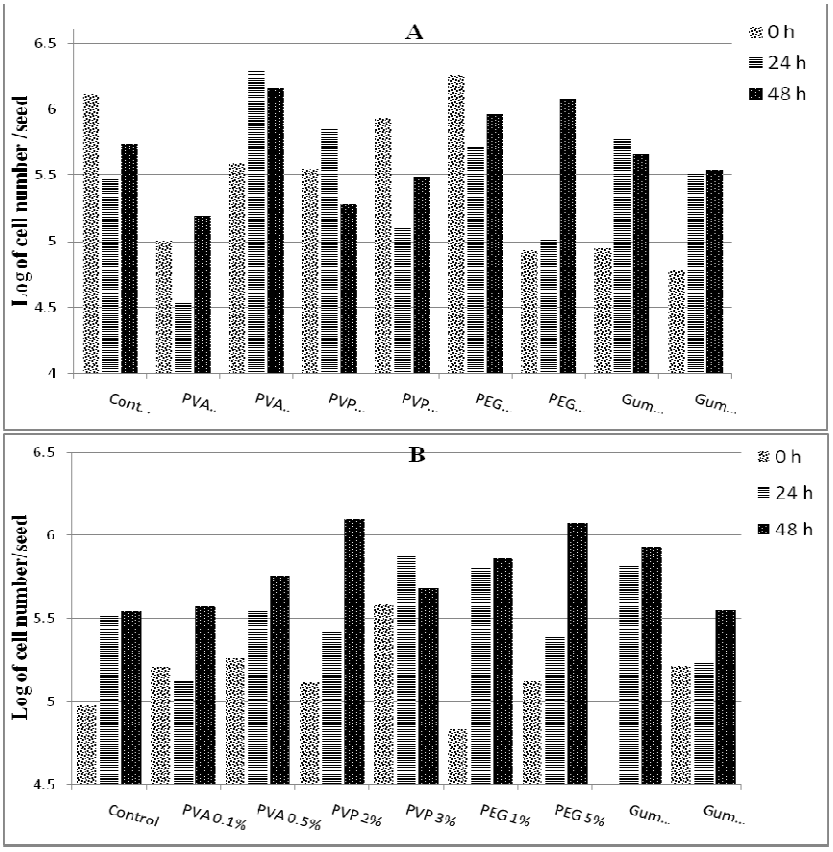

Figure 3: Survival of rhizobial strain USDA 3100 on chickpea seeds coated with liquid formulations at (A) 40°C and (B) 45°C.

Gum Arabic recorded a sharp increase in viable counts from the initial time at about 3.6×10^5 cells/seed to 4×10^6 cells/seed after 48 h from inoculation.

Chickpea Seeds

The results (Figure 3 A and B) revealed that all liquid formulations and charcoal-based inoculant of strain USDA 3100 were able to sustain *Rhizobium* number to the standard level at both 40°C and 45°C. Most of the liquid formulations followed the trend of charcoal based inoculants; the viable counts or population density started to increase from the initial count at zero time up to 48 h from inoculation. Among all tested additives, PVA at 0.5% was found to record high population of about 2×10^6 and 1×10^6 cells/seed at 40°C after 24 and 48 h of inoculation, respectively. Liquid inoculant amended with 3% PVP was found to record a maximum population of about 7×10^5 cells/seed, followed by 1% PEG and 0.5% Gum Arabic which were both found to sustain rhizobial growth at about 6×10^5 cells/seed at 45°C and 24 h from inoculation. Among the different formulations, the maximum number of rhizobial cells per seed were observed with liquid inoculants amended with 2% PVP and 5% PEG at 45°C after 48 h. Also, liquid inoculant formulated with 0.5% Gum Arabic recorded 8×10^5 cells/seed which was considered as a high population, whereas, charcoal based inoculant recorded 3×10^5 cells/seed after storage at 45°C at for 24 and 48 h.

Mung bean Seeds

The results demonstrated that different additives had different abilities to protect rhizobial cells on mung bean seeds at 40°C and 45°C (Figure 4 A and B). All liquid formulations and charcoal based inoculant could sustain the viability of rhizobial cells. At 40°C, liquid inoculants formulated with 3% PVP supported the maximum population at about 2.4×10^6 and 2×10^6 cells/seed after 24 and 48 h from inoculation, respectively. Among the different formulations, liquid inoculants amended with 0.1% Gum Arabic was found to record a maximum population at about 1×10^6 and 9×10^5 cells/seed after 24 and 48 h of storage at 45°C, respectively. Minimum populations were observed in liquid inoculant formulated with 3% PVA after 24 h and 0.5% PEG after 48 h from inoculation. Increase of

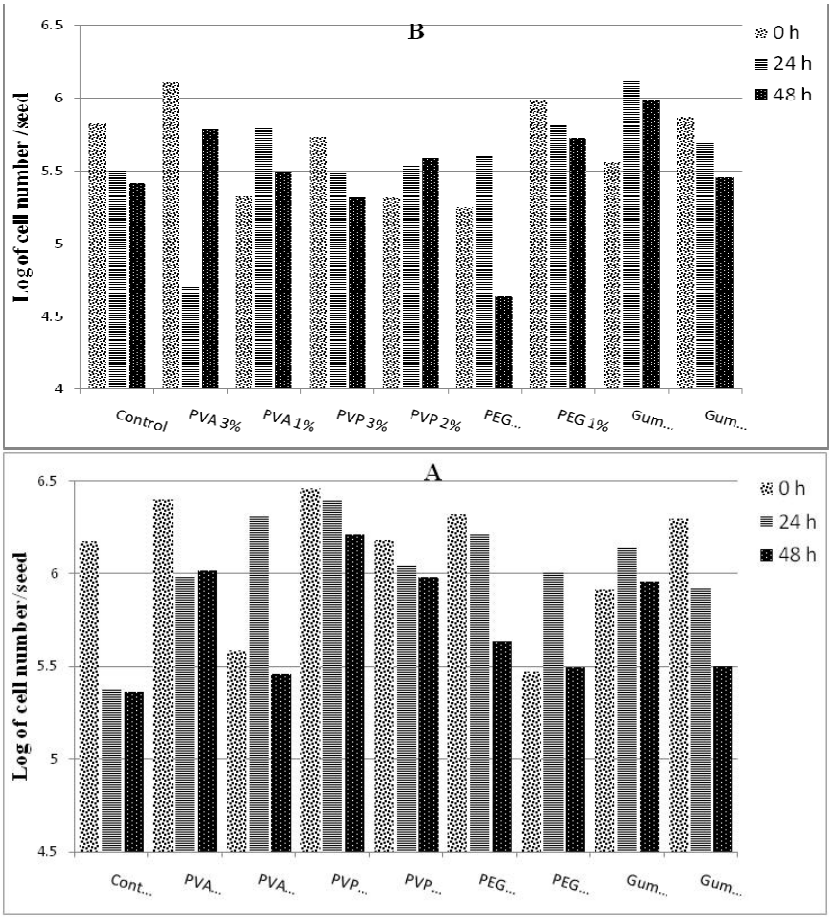

Figure 4: Survival of rhizobial strain TAL 209 on mung bean seeds coated with liquid formulations at (A) 40°C and (B) 45°C.

temperature was found to reduce the viable number/seed for each formula, hence, better survival of *Rhizobium* strain TAL 209 was observed at 40°C rather than 45°C. Dayamani and Brahmaprakash(2014) found that liquid inoculants containing PEG 4000 at the 2 % level increase the population density of Acinetobacter sp. significantly to the extent of 9 times compared to control, and further amending media with PEG 400 at all concentrations, PEG 600 and PEG 6000 at the 2 % level increase population density substantially.

Guar Seeds

The effects of polymeric additives on the population density of *Rhizobium* strain USDA 3385 are shown in (Figure 5A and B). Charcoal based inoculant, liquid inoculants amended with 0.5% PEG, 0.5% Gum Arabic could maintain the maximum number of surviving cells on seeds at about more than 10^6 cells/seed at 40°C after 24 h of storage. The population of rhizobia was found to be supported at a higher level at about 4×10^5 cells/seed in liquid inoculant formulated with 0.5% PEG at 40°C after 48 h from inoculation.

At 45°C, four liquid inoculants amended with polymers PVA (0.5%), PVP (5%), PEG (1%) and PEG (0.5%) showed sharp increases in cell density compared to their initial number at zero time and to charcoal based inoculant after 24 h from inoculation. Moreover, after 48 h, liquid inoculants amended with 0.1% and 0.5% PVA were found to be better in supporting *Rhizobium* population on seeds than charcoal based inoculants. These results are in accord with the results reported by Dayamani and Brahmaprakash (2014) that The population density of Pseudomonas sp. significantly increased by the addition of PVP K-15 at all concentrations and the extent of increase was almost seven times by the addition of PVP K-15 at the 2 % level

Soybean Seeds

The population of rhizobia was found to be supported to a higher level in liquid inoculants formulated with PVP, PVA

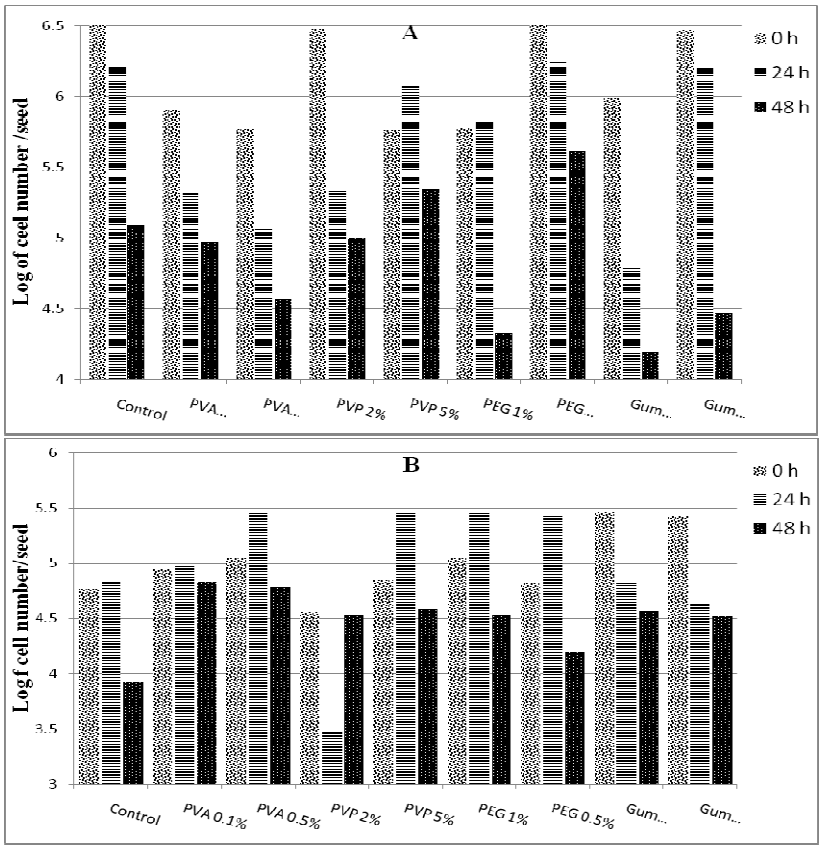

Figure 5: Survival of rhizobial strain USDA 3385 on guar seeds coated with liquid formulations at (A) 40°C and (B) 45°C.

and Gum Arabic throughout the storage period at both temperatures 40°C and 45°C (Figure 6A and B) The maximum surviving population of ENRRI 1 at 40°C was 1.4×10^6 cells/seed obtained by 1% PVP after 24 h from inoculation and 1×10^6 cells/seed obtained by 0.1% Gum Arabic after 48 h from inoculation. Liquid inoculants containing 0.5 % PVA and 0.1 % Gum Arabic could support the survival of rhizobial cell to 9.6% and 8.1% over charcoal based inoculant after 24 and 48 h from inoculation at 45°C. Previous studies found that liquid inoculant containing Gum Arabic could maintain the number of rhizobial cell on seed about 10^4-10^5 cells/seed or remain 74% of cell survival after 48 h (Tittabutr, 2005).

DISCUSSION

High temperatures usually affect the survival of cells on seeds, especially in tropics where soil temperature can be greater than 40°C. Additives to the broth will improve inoculants quality such as better adhesion to seed, binding or inactivating soluble seed coat toxins and enhance rhizobial survival during storage and after exposure to extreme environmental conditions after inoculation and seed planting.

The first step to assure a successful seed inoculation with rhizobial inoculant is the number of viable cells on seed which should be high enough to colonize the root hair and start the interaction between rhizobia and leguminous seeds after seed germination. Factors such as temperature, humidity, and toxic substances all affect the survival of rhizobia in the seed-coating agent.

Girisha et al. (2006) stated that a number of cells surviving on groundnut seeds are higher when *Rhizobium* liquid inoculant was used compared to carrier-based inoculant. The chemical constituents of liquid formulation permitted more number of bacteria to survive on seeds, possibly by offering protection against many biotic and abiotic factors.

Catroux et al. (2001) reported that nodulation and yield increase when the numbers of viable rhizobia per inoculated seed increase as accomplished by having a greater number of viable rhizobia in inoculant itself or by using higher than normal rates of inoculation, or by minimizing the death of rhizobia after inoculation.

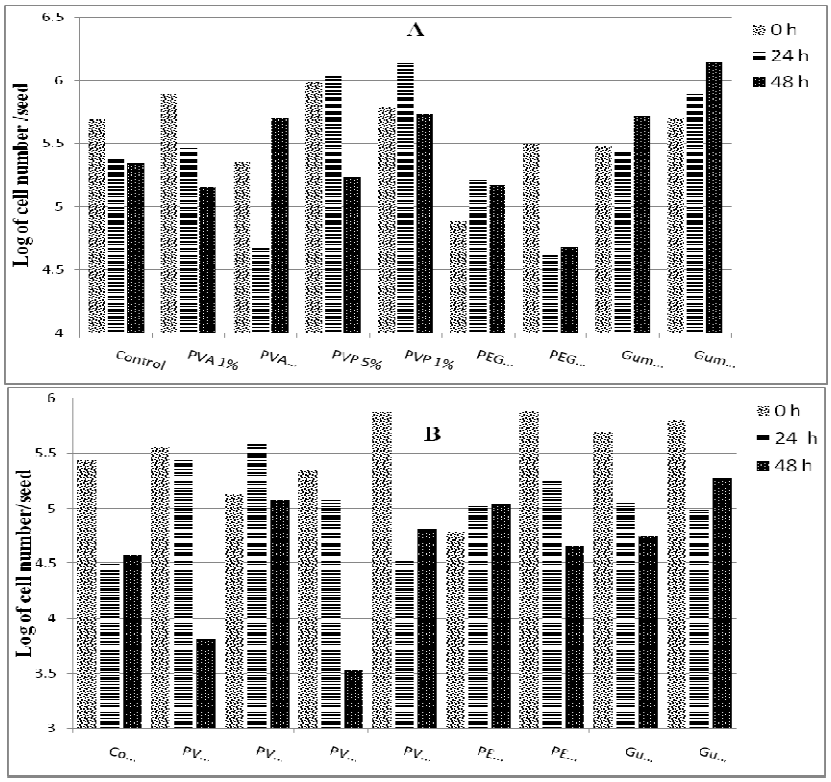

Figure 6: Survival of rhizobial isolate ENRRI 1 on soybean seeds coated with liquid formulations at (A) 40°C and (B) 45°C.

Inoculated seeds with the different formulations were incubated for 24 and 48 h at 40°C and 45°C without controlling the humidity. Our results confirmed the previous findings by Tittabutr et al. (2007) that polymers have the ability to protect cells from temperature fluctuation after inoculation. However, different additives showed different abilities to protect rhizobial cells on seeds at high temperature after inoculation. However, different additives showed different abilities to protect rhizobial cells on seeds at high temperature. The most widely accepted standard for a number of rhizobia delivered per seed are 10^3, 10^4 and 10^5 rhizobia for small, medium and large seeds, respectively (Lupwayi et al*.,* 2000). Most of the polymeric additives and their different concentrations were found to maintain the cell viability more than 10^5 cells/seed. However, Gum Arabic and PVA additives showed better performance han others on a wide range of rhizobial strains and leguminous seeds. Gum Arabic was found to support the maximum survival of TAL 1399, ENRRI 1, TAL 380 and TAL 209 on seeds after inoculation, PVA could maintain a high population of rhizobial cells on seed at about 10^4-10^6 cells/seed of strains USDA 3100, USDA 3385 and ENRRI 1. Moreover, the number of viable cells on seeds decreased as temperature increased for most formulations. Polymers such as PVP, PEG and Gum Arabic and PVA have adhesive properties, sticky consistency which may enhance cell adherence to seed and their viscous nature may slow the drying processes of the inoculants after application to seed (Leo Daniel et al*.,* 2013).The viability of *Bacillus megaterium* liquid formulation inoculated onto black gram, maize and soybean starts to decline after 36 h of incubation (Gomathy et al*.,* 2008).

CONCLUSION

Liquid inoculants amended with polymeric additives *viz*., polyvinylpyrrolidone, polyethylene glycol, polyvinyl alcohol and Gum Arabiccan improve the survival of rhizobial cells on the seed at high temperature. Therefore, liquid formulations could overcome many problems associated with seed exposure to high temperature. The performances of liquid formulations were found to be better than or equivalent to charcoal based inoculants. Among the tested additives, Gum Arabic was found to support maximum survival for most rhizobial strains at high temperature.

However, different additives had different abilities to protect rhizobial cells on seed at high temperature

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