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Quality Attributes of Co-inoculants Based on Rhizobia and Phosphate Solubilizing Bacteria under Different Storage Conditions

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Abstract: *The present study was carried out to assess the survivability of two different rhizobial strains, two rhizobial isolates and one isolate of phosphorus solubilizing bacteria(PSB) as co–cultured inoculants which were constructed by two methods either mixing equal weights of charcoal based inoculants of each bacterial candidate to stand as C1 co-inoculant, or mixing equal volumes of bacterial candidates' broth cultures prior to impregnation into partially sterilized charcoal to stand as C2 co-inoculant. Co-cultured inoculants were stored either at room temperature (25-30° C) or under refrigeration (4° C) for three months. Results revealed that bacterial viability in C1 and C2 co-inoculants were more than 10^9 cfu/g and 10^7 cfu/g for Rhizobium and PSB, respectively in all studied packets. Although storage at room temperature recorded fewer numbers of viable cells than under refrigeration, both storage conditions recorded the standard viable counts. The study provides the possibility of construction of Rhizobium and PSB co- inoculants in partially sterilized charcoal. The effect of some factors such as the ratios of Rhizobium and PSB inoculants, nutrient availability and soil microbial activity on the viability and activity of Rhizobium and PSB should be considered in further studies.*
Keywords*: Rhizobium, PSB, viable count, storage, mixed inoculant.*

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I. Introduction

Biofertilizers of plant growth promoting bacteria, especially *Rhizobium* and phosphorus solubilizing bacteria (PSB) are considered to be an alternative method for increasing crop yield and quality that can reduce the pollution hazards of N and P chemical fertilizers.

Rhizobia are the only nitrogen fixing bacteria living in a symbiotic relationship with legumes[1]. Biological nitrogen fixation by rhizobia is one of the effective methods that improve plant growth and productivity[2]. Phosphorus is the second essential nutrient, after nitrogen, required for plant growth. Soil phosphorus is often abundant either as insoluble organic or inorganic form. A large portion of inorganic phosphate applied to soil as chemical fertilizer is rapidly immobilized and becomes unavailable to plants. Thus, the release of insoluble and fixed forms of phosphorus is an important aspect of increasing soil phosphorus availability[3]. A diverse group of soil microflora is reported to be involved in solubilizing insoluble phosphate complexes and supplying plants with available phosphorus especially in soils with limited phosphorus [4,5].

The construction of an inoculant with a consortium of microbes with multiple beneficial functions such as N_2 fixation and phosphate solubilization in addition to other plant growth promoting properties is a positive new development that a single co-inoculant can be used effectively for increasing the productivity of crops[6]. During this interaction, nitrogen-fixing microorganisms provide nitrogen to the plants and consequently improve the nitrogen status of the soil, while PSB enhance plant growth by supplying phosphates. Besides their role in atmospheric nitrogen fixation and phosphorous solubilization, they also play a rule in producing plant growth hormones providing better nutrient uptake and increasing tolerance towards drought and moisture stress.

The positive effects of microbial co-inoculation on plant yield, soil physiochemical properties, soil enzymes activity and soil microbial community had been extensively investigated [7]. Some reports of the effect of combined inoculation with nitrogen fixing and phosphate solubilizing bacteria (PSB) on plants growth and yield are available worldwide. In Sudan, *Rhizobium* inoculants have been produced by the National Center for Research since 1992 as mono cultured inoculant and proved to be an effective technology[8]. Moreover, application of mono culture inoculants of *Rhizobium* or PSB and their interaction for improvement of crop yield and seed quality is well documented [9].Therefore, the present study was conducted to assess the survivability of

Rhizobium and PSB as co-cultured inoculants at room temperature (25-30° C) and under refrigeration (4º C) during three months.

II. Materials and Methods

Bacterial strains:

Rhizobial strains and isolates in this study were obtained from Biopesticides and Biofertilizers Department, Environment, Natural Resources and Desertification Research Institute, National Centre for Research, Sudan. *Rhizobium* strains TAL1399 and TAL 380 were originally obtained from NIFTAL project, U.S.A, while ENRRI 12 and ENRRI 9 were locally isolated and were also deposited in the Department. The selected rhizobial strains and isolates for this study, were tested under Sudan conditions and proved to be efficient and are routinely used in commercial inoculants production. The Phosphate solubilizing bacteria was also locally isolated, deposited in the Department of Biopesticides and Biofertilizers and proved to be efficient.

Rhizobium **broth cultures:**

Yeast Extract Mannitol Agar (YEMA) composed of (g/l) mannitol, 10: K_2HPO_4, 0.5: $MgSO_4.7H_2O$, 0.2: NaCL, 0.1; yeast extract, 0.5: agar, 15 [10] was used as culture medium for rhizobia, and sucrose (10g) was used as the carbon source instead of mannitol. The medium was solidified by adding agar when needed. The pH was adjusted to 6.8 -7.0. The medium was dispensed into 100 ml conical flasks, wrapped with aluminum foil and sterilized by autoclaving at 15 lb/in^2 and 12° C for 15-20 minutes. A loopful of each strain/isolate was transferred aseptically to flasks containing YEMB once the media had cooled to below 30° C and then left on an orbital shaker for 24-48 hours. Cultures were serially diluted and viable colonies were counted (should contain at least 1×10^9 cfu/ml).

Phosphate solubilizing bacteria (PSB) broth cultures:

Phosphate solubilizing bacteria was cultured on meat peptone broth which contains (g/ l) peptone, 7.5; NaCL, 5; meat extract, 5 and autoclaved for 15 minutes at 15 lb/in^2 and 121° C. A loopful of phosphate solubilizing bacteria was transferred aseptically to flasks containing the broth media. Cultures were left on a shaker for 3 days at room temperature (25-30° C).Colony count was determined using spread plate method.

Mixed Inoculants and shelf life:

Charcoal was collected, ground using hammer mill and sieved to pass through a 0.5 mm mesh screen. Charcoal was partially sterilized with hot air oven at 100° C for two hours, and then after cooling, mixed manually with the broth cultures. The volume of inoculants added was to make for 40% of the water holding capacity of the carrier material. Co-inoculuants were constructed by two different methods, (i) Mixing equal volumes of *Rhizobium* and PSB broth cultures, then mixed with partially sterilized charcoal [5] to construct C1 co-inoculant, (ii) each culture (*Rhizobium* or PSB) was separately added to partially sterilized charcoal then mixed with each other, afterwards, on equal weight basis to construct C2 co-inoculant. Carriers impregnated with broth cultures were mixed well, packed in polyethylene bags and immediately sealed to maintain the required moisture content. Packets were stored at room temperature (25-30º C) or refrigerator (4º C). A random sample was picked from each packet of the inoculants, and the viable number of colony forming units per gram (cfu/g) [11]was determined for *Rhizobium* and PSB during three months of storage at two weeks intervals.

III. Results

The growth inhibition or promotion of *Rhizobium* strains and isolates and PSB was studied in co-cultured charcoal based inoculants as presented in figures (1-4). No antagonistic interaction between the two types of bacteria was observed.

Interaction of *Rhizobium* **isolate ENRRI 9 and PSB co-inoculant:**

Charcoal based inoculants supported the standard population levels of *Rhizobium* isolate ENRRI 9 and phosphorus solubilizing bacteria (PSB) up to 3 months. The shelf life of both types of bacteria was satisfactory at the two constructions (C1&C2) at room temperature (25-30° C) (fig.1A), and refrigeration (4° C) (fig.1B). The initial populations at room temperature for *Rhizobium* (isolate ENRRI 9) and PSB were 9×10^8 cfu/g and 6×10^8 cfu/g, respectively in C1 and 9×10^8 cfu/g and 5×10^8, respectively in C2 (fig.1A). The corresponding initial populations under refrigeration (fig.1B) were 9×10^8 cfu/g and 6×10^8 cfu/g for ENRRI 9 and PSB, respectively, in C1, and 9×10^8 cfu/g and 5×10^8 cfu/g, respectively in C2.

The bacterial populations in partially sterilized charcoal inoculants stored at room temperature, at the end of the storage period were 3×10^7 cfu/g and 9×10^8 of ENRRI 9, 3×10^8 cfu/g and 6×10^8 cfu/g of PSB, in C1 and C2 respectively. At refrigeration, the surviving populations of ENRRI 9 and PSB were 3×10^7 cfu/g, 1×10^7 cfu/g in C1 and 2×10^8 cfu/g and 4×10^8 cfu/g in C2, respectively.

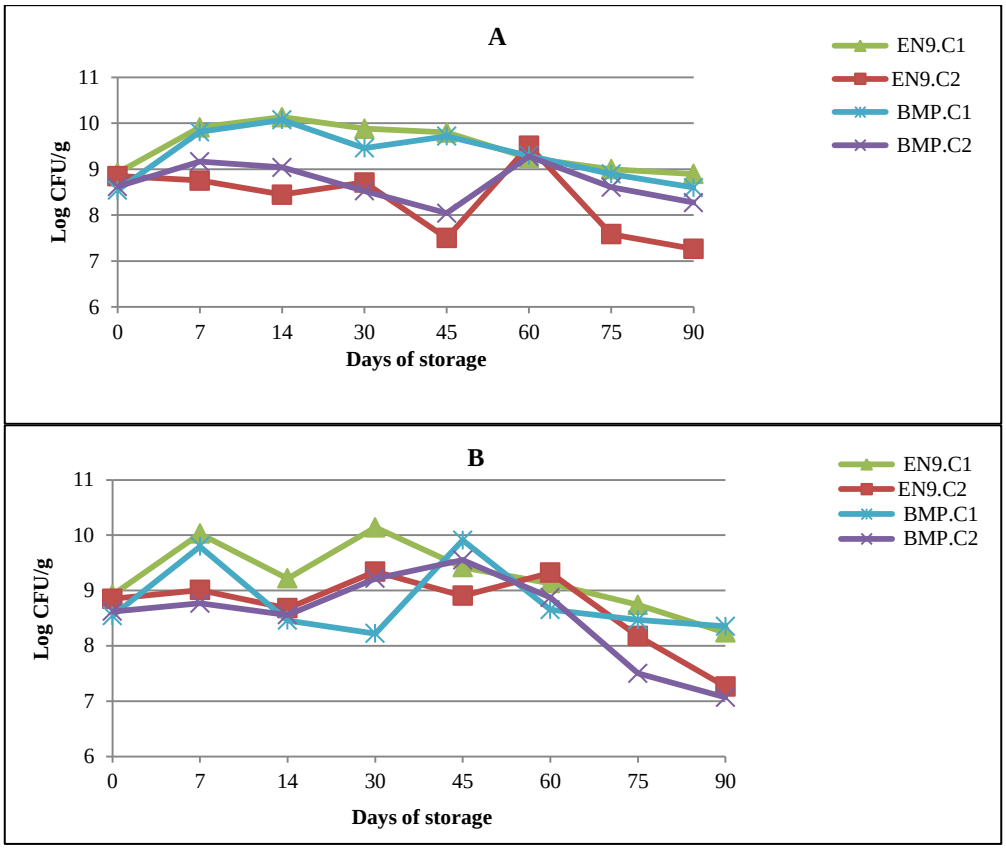
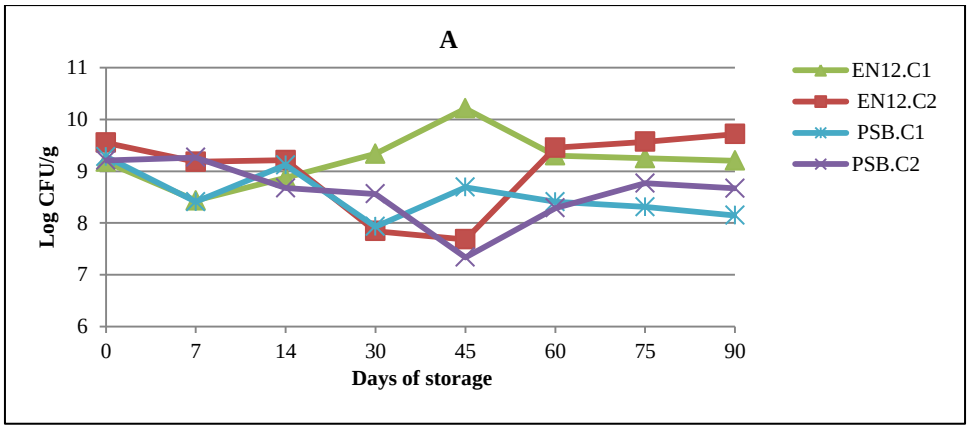


Fig. (1): Shelf life of ENRRI 9 and PSB in C1 and C2 at two storage conditions (A) room temperature (25-30°C) and (B) refrigeration (4°C).

Interaction of *Rhizobium* isolate ENRRI 12 and PSB co-inoculant:

The initial populations of ENRRI 12 and PSB in C1at room temperature (fig.2A) were 6×10^9 cfu/g and 2×10^9 cfu/g, respectively. The corresponding populations in C2 were 2×10^9 cfu/g and 3×10^9 cfu/g, respectively. Under refrigeration (fig.2B), the initial surviving populations were estimated as 5×10^9 cfu/g of ENRRI 12 and 2×10^9 cfu/g of PSB in C1, 2×10^9 cfu/g of ENRRI 12 and 3×10^9 cfu/g of PSB in C2.

After 3 months of storage, the colony forming units of ENRRI 12 per g were 7×10^9 and 9×10^9 inC1, 2×10^9 and 3×10^9 in C2at room temperature and refrigeration**,** respectively. The population counts of PSB were 7×10^8 and 4×10^8 cfu/g inC1, and 1×10^8 cfu/g and 6×10^8 cfu/g in C2 at room temperature and refrigeration, respectively. The results showed that the shelf life of *Rhizobium* isolate and PSB were satisfactory up to 3 months in both constructions (C1&C2).



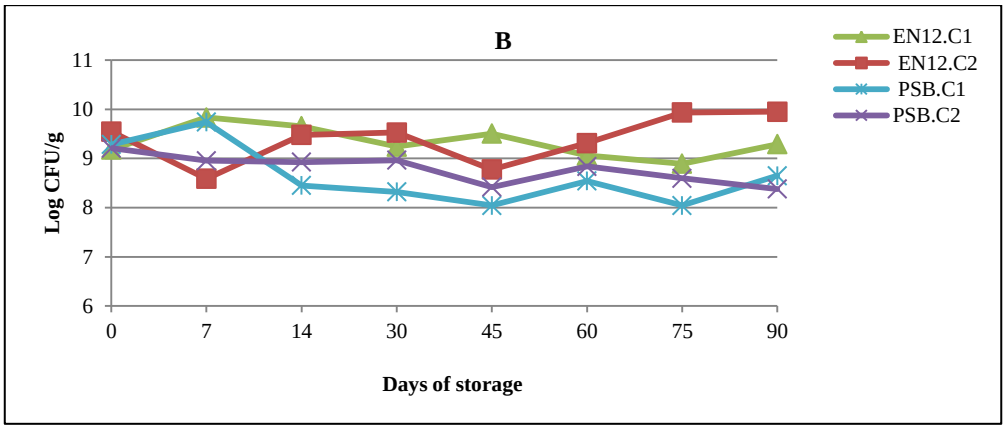


Fig. (2): Shelf life of ENRRI 12 and PSB in C1&C2 at two storage conditions (A) room temperature (25-30°C) and (B) refrigeration (4°C).

Interaction of *Rhizobium* strain TAL 380 and PSB co-inoculant:

The maximum population density of *Rhizobium* strain TAL 380 was above 6×10^9 cfu/g in both constructions. At room temperature (fig.3A), 6×10^9 cfu/g and 6×10^8 cfu/g were the maximum populations counted for PSB in C1 and C2, respectively. After 90 days of storage, 1×10^9 cfu of *Rhizobium*/g was recorded in both constructions at room temperature and under refrigeration. However, PSB recorded 6×10^9 cfu/g in C1 and 8×10^7 cfu/g in C2.Under refrigeration, the population densities of *Rhizobium* strain TAL 380 recovered were (8×10^8) cfu/g in C1 and (1×10^9) cfu/g in C2 after 3 months of storage, while, PSB recorded 2×10^8 and 3×10^8 cfu/g inC1 and C2, respectively (fig.3B). The population densities for both *Rhizobium* strain TAL 380 and PSB in the two constructions and at the two storage temperatures were found to be within the permissible limit.

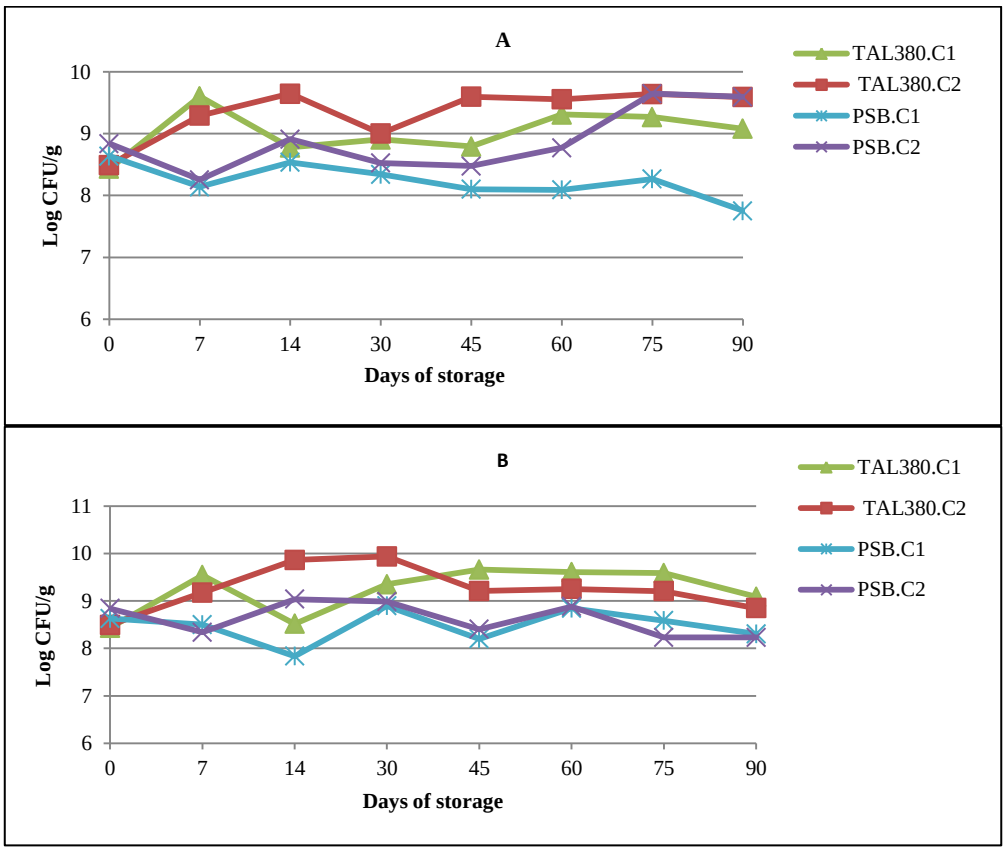


Fig. (3): Shelf life of TAL 380 and PSB C1 and C2 at two storage conditions (A) room temperature (25-30° C) and (B) refrigeration (4° C).

Interaction of *Rhizobium* strain TAL 1399 and PSB co-inoculant:

Maximum population (8×10^8 cfu/g) of *Rhizobium* strain TAL1399 was supported by C1 compared to (5×10^7 cfu/g) in C2 at room temperature after 3 months of storage. The results of PSB count showed population densities of 3×10^8 cfu/g and 5×10^7 cfu/g in C1 and C2 co-inoculants, respectively, after 3 months of storage at room temperature (fig.4A). A consortium of TAL 1399 and PSB maintained population densities to 3×10^9 cfu/g and 9×10^8 cfu/g in inoculants based on broth mixtures and 1×10^9 cfu/g and 6×10^8 cfu/g in C2 co-inoculants, respectively, after 3 months of storage under refrigeration (fig.4B). Results showed that storage under refrigeration increased cell counts over storage at room temperature. However, colony counts for the two types of bacteria were found to be within the standard limit at both room temperatures (25-30°C) and refrigeration (4°C).

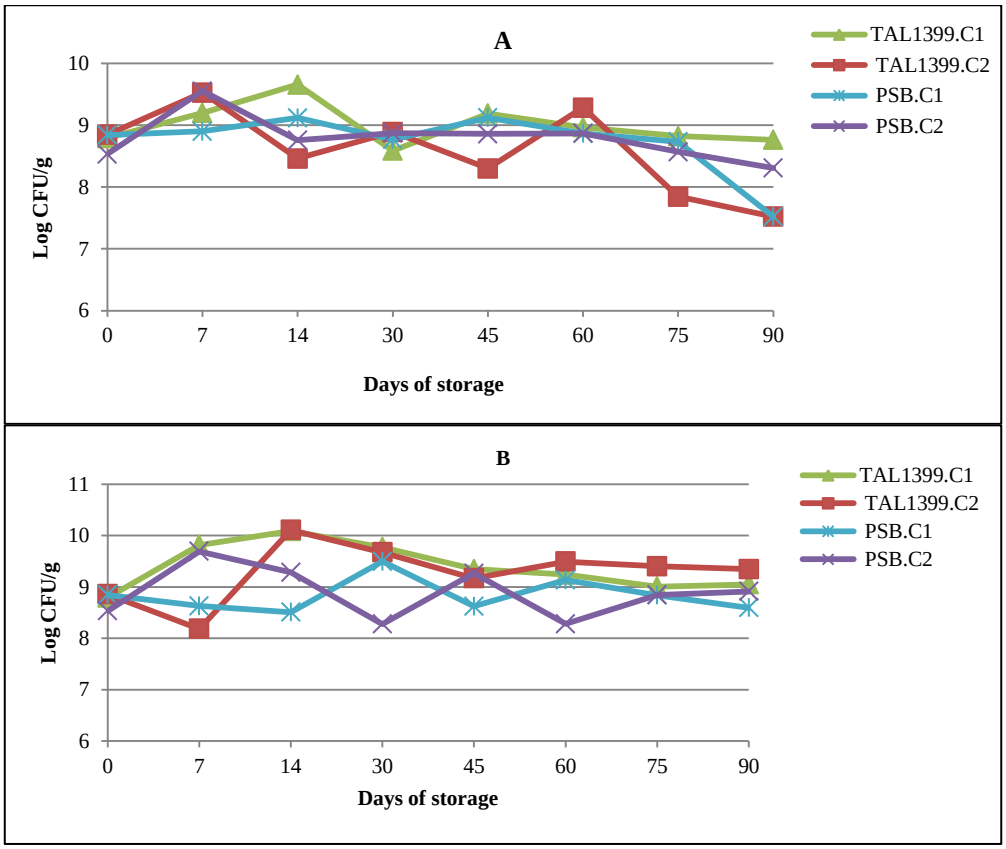


Fig. (4): Shelf life of TAL 1399 and PSB in C1 and C2 at two storage conditions (A) room temperature (25-30°C) and (B) refrigeration (4°C).

IV. Discussion

Application of selected carrier materials for bacterial inoculants production, have long been practiced and proved to be beneficial to protect and support bacteria. In Sudan, charcoal has been considered as the most suitable carrier material[8].Oven sterilization of charcoal at 100º C for two hours is now adopted for commercial production of *Rhizobium* biofertilizer as it proved to be efficient , much convenient and can safely be stored for 6–10 weeks under ambient temperature (up to 37° C) [12]. Earlier reports suggested the usefulness of co-inoculation over mono inoculation, especially of microorganisms that are synergistic to each other [5]. The application of rhizobia in combination with phosphate solubilizing bacteria proved to be a suitable approach to promote plant growth ,enhance nodulation , nitrogen fixation[13] and increase grain yield [14].

Many researchers proved the efficiency of co-inoculation by different *Rhizobium* and phosphate solubilizing bacterial strains and their interaction on yield and seed quality of some leguminous crops viz., lablab bean[15], faba bean [16]and groundnut[17].However, the biological activity of rhizobia and PSB, may decline rapidly if the handling and storage of the co-inoculants are not done in discipline way. In this study, the effect of impregnation of partially sterilized charcoal with *Rhizobium* and PSB to form co-inoculants by either mixing the broth cultures of the two types of bacteria before adding charcoal powder, or mixing together the charcoal based inoculants of the two types of bacteria, on the survivability of *Rhizobium* and PSB were recorded in figures (1-

4). Results showed that, for most strains, mixing broth cultures (C1) recorded higher survival values for both *Rhizobium* and PSB over consortia based on mixing solid inoculants (C2), although the two formulations maintained viable counts within the standard limit. The shelf life of a consortium of *Azospirillum*, *Azotobacter*, *Bacillus megatherium* and *Pseudomonas fluorescens* was found to be satisfactory up to six months as reported by [18].Also,[19] observed no antagonistic effect between *Azospirillum*, *Bacillus* and *Pseudomonas* and used to be compatible and hence used as a consortium. The results also confirmed pervious finding by [20] that a co-culture of multiple inoculants in sterilized fine wood charcoal powder containing more than 2×10^8 cells/g of carrier was possible.

Temperature also plays an important role in the survival of *Rhizobium* and PSB co-inoculants or consortia. In this study, when the co-inoculants were stored at 4° C, the dynamics of the bacterial population were similar to those kept at room temperature, although the final population being higher for co-inoculants kept at 4°C than at room temperature. However, the viable counts for the tested bacteria in the two constructions were found to be within or above the standard values. The use of co-inoculants based on mixing broth cultures of *Rhizobium* and PSB and storage at room temperature (25-30° C) for 3 months could be a useful satisfactory formulation, especially when cold storage facilities are not available. The result of a study conducted by [18] indicated that the storage temperatures of 25° C as well as 30° C were optimum for the survival of co-cultured inoculants. Also, [21] showed that after 8 weeks of storage, different carrier materials were able to sustain higher viable counts of rhizobia and *Pseudomonas* co-inoculants at room temperature (22-28° C) than at 4° C. The feasibility of procuring the inoculants, application and the cost factor are the major constraints for the farmers to harvest the benefits of using these inoculants [20]. Therefore, formulation of bacterial co-inoculants by mixing broth cultures is found to be easier and practicable than mixing solid inoculants. It is also more feasible that only one mechanical mixer is required for mixing broth cultures, while in case of consortia based on solid mixtures, at least two mechanical mixers are required that may encourage contamination.

V. Conclusion

The study revealed that it is possible to develop charcoal based co-cultured inoculants containing *Rhizobium* and PSB to promote plant growth and provide N and P. A consortium of rhizobia and PSB constructed by mixing the broth cultures and storage at room temperature, were found to be the best formulation and conditions for production of *Rhizobium* and PSB co-inoculants. Such inoculants acquired a wider acceptance as a convenient feasible application. However, more studies on the effect of multiple inoculants on growth and yield of different crops are required.

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