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Polyvinylpyrrolidone (PVP) and Na-Alginate Addition Enhances the Survival and Agronomic Performances of a Liquid Inoculant of *Bradyrhizobium japonicum* for Soybean (*Glycine max* (L.) *Merr.*)

Pulak Maitra [1,2,*], Jubair Al-Rashid [2,*], Dipa Mandal [3], Md. Shofiul Azam [3] and Noorain Munim Rasul [2]

[1] Solid-State Fermentation Resource Utilization Key Laboratory of Sichuan Province, Faculty of Agriculture, Forestry and Food Engineering, Yibin University, Yibin 644000, China
[2] Apex Biotechnology Laboratory, Apex Holdings Ltd., East Chandora, Shafipur, Kaliakoir, Gazipur 1751, Bangladesh; noorain@apexbiofertilizer.com
[3] School of International Education, University of Chinese Academy of Sciences, Beijing 100049, China; dipaiuanft112@mails.ucas.edu.cn (D.M.); shofiul.sust@gmail.com (M.S.A.)
* Correspondence: pulak@yibinu.edu.cn (P.M.); jubair@apexbiofertilizer.com (J.A.-R.)

Abstract: Nontoxic polymers PVP and Na-alginate may provide a favorable environment for the survival of bacteria. Therefore, PVP and Na-alginate were added to a growth medium to develop a liquid inoculant of *Bradyrhizobium japonicum* strain. The strain was identified by 16S rDNA sequencing. The addition of PVP (1.8%) and Na-alginate (0.2%) in the medium promoted a better survival (1.93×10^9 cells mL^{-1}) of *B. japonicum* strain compared to the control (3.50×10^2 cells mL^{-1}) after 6 months of storage. The combination of PVP and Na-alginate ensured 1.53×10^7 cells mL^{-1} up to 12 months of storage under ambient temperature (28 ± 2 °C), whereas PVP (1.8%) or Na-alginate (0.2%) alone produced similar cell counts only up to 8 months and 6 months, respectively. Consecutive field experiments proved the efficacy of the liquid inoculant on nodulation and yield of soybean. The combination of PVP and Na-alginate-based inoculation of *B. japonicum* strain significantly increased the nodule number per plant, number of pods per plant, number of seeds per pod, seed yield, and yield per hectare ($p \leq 0.05$). Thus, the combination of PVP- and Na-alginate-based inoculation of *B. japonicum* has great potential to popularize the organic cultivation of soybean.

Keywords: *Bradyrhizobium japonicum*; biofertilizers; soybean; nodulation; crop yield

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1. Introduction

Soybean, one of the essential legume crop plants and a source of the major plant-based protein and oil, is widely grown around the world [1,2]. *Rhizobium* bacteria can fix atmospheric nitrogen by symbiotic association with legume plants in the agro ecosystem and increase the growth and yield of crop plants [3]. Previous study demonstrates that half of the nitrogen in the agricultural ecosystem was fixed by legume–*Rhizobium* symbiosis [4]. Soybean–*Rhizobium* symbiosis is one of the promising legume–*Rhizobium* interactions in the agriculture ecosystem and may fix up to 50% N via its symbiosis with *Bradyrhizobum japonicum* [4–6]. *B. japonicum* recognizes soybean as the host plant when present in its vicinity in the soil. Specialized structures known as nodules form on soybean roots following successful colonization by *B. japonicum* [7]. The bacteria are housed inside the nodules, where they produce ammonia (NH_3) from atmospheric nitrogen (N_2) and deliver it to the soybean plant. In large-scale soybean cultivation, it is necessary to inoculate soybean seeds with high-quality *Rhizobium* to get the maximum yields. Furthermore, the application of inorganic fertilizer adversely affects the soil environment and increases product cost. Therefore, eco-friendly and cost-effective agro-technologies such as legume–Rhizobium technologies are required to increase soybean production.

Previous studies reported that abiotic factors such as drought and high temperature negatively affected the nodule formation because of the low survival of inoculated bacteria in the soil and root system [8,9]. Previously, several carrier substrates were used for the development of carrier-based formulation of *Rhizobium* to increase the survival and nodulation performance in the agro ecosystem [10–12]. However, the major constraints of these formulations were the shorter shelf-life of the substrates used and low survival of *Rhizobium* after seed coating because of high temperature in different climate regions [13]. A previous study also reported that low water availability decreased the survival of peat-based *Rhizobia* inoculants and nodulation performance [14]. Furthermore, *Rhizobium* in the peat carrier is not very practicable for wide adaptation by farmers. The bulk peat soil is more difficult to transport and store compared to liquid inoculants. Furthermore, it is difficult to sterilize peat soil with gamma radiation as the facilities are limited and heat sterilization is not only expensive but may also produce toxic substances. Hence, the selection of liquid carriers suitable for *Rhizobium* bacterial inoculants should consider some essential factors such as cost-effective transport, nontoxic property, and bacterial survival ability [15]. Substantial innovation and improvement in inoculant technology are required to develop commercially available biofertilizer products for farmers in the world. These products need to be highly effective in the field and should possess a good shelf-life at room temperature [16]. Previously, different polymers such as PVP, PEG, gum arabic and Na-alginate have been used for liquid inoculum production to increase the survival of inoculated bacterial as well as shelf-life of the formulated inoculum [17,18]. PVP and Na-alginate may provide a favorable environment for the survival of *Rhizobium* bacteria because of their high water-binding capacity and heat-resistant ability, respectively [17,19]. Furthermore, PVP can protect the bacteria from toxic factors because of its colloidal property [20]. In this study, we developed a combination of PVP- and Na-alginate-based liquid inoculant of *B. japonicum*, evaluate nodulation, and subsequent impact of the soybean growth and yield compared to chemical fertilizers. Therefore, this study has particular significance for the development of a suitable liquid-based inoculant of *B. japonicum* for the increasing demand of soybean production in Bangladesh as well as in the rest of the world. The aim of this study was to (i) evaluate different carriers for *B. japonicum* inoculum preparation, (ii) test the survival and electiveness of PVP- and Na-alginate-based inoculation *B. japonicum*, and (iii) test the agronomic performances of liquid inoculants of *B. japonicum* compared to the inorganic fertilizer. We hypothesized that the PVP and Na-alginate addition would increase the survival of *B. japonicum* in liquid inoculum and enhance the nodulation, growth and yield of soybean.

2. Materials and Methods

2.1. Isolation, Characterization, and Identification of B. japonicum

Nodules were collected by trapping the soil sample from the root collected from Boroibari, Gazipur, Bangladesh from soybean plants. These were surface-sterilized with 70% ethanol for 30 s and then 2% Chloramine T ($C_7H_7ClNO_2SNa$) for 30 s. The surface-sterilized nodules were then crushed with a glass rod. These nodule-crushed extracts were streaked on CRYEMA media (HiMedia Laboratories LLC, PA, USA) containing Congo red. The composition of the media was mannitol (10 g/L), K_2HPO_4 (0.5 g/L), $MgSO_47H_2O$ (0.2 g/L), NaCl (0.1 g/L), yeast extracts (1 g/L), agar (20 g/L), and Congo red solution (0.025 g/L). The pH of the medium was adjusted to 6.8 and sterilized at 15 psi (15 min). The plates were incubated at 28 ± 2 °C for 24–48 h. Most Rhizobia, including *Bradyrhizobium*, lack the ability to absorb Congo red from the YEMA medium [21]. *Rhizobium* colonies remained white, transparent, eminent, and mucilaginous after 24–72 h, whereas other bacteria turned red [22]. Single colonies were then selected and re-streaked on YEMA media (HiMedia Laboratories LLC, PA, USA) to get pure cultures (Figure 1A). Soybean seedlings were grown with an isolate pure culture of *Bradyrhizobium* with N-free Hoagland nutrient solution in a pot under controlled conditions. The composition of N free nutrient solution is the same as Hoagland nutrient solution [23], except without N supplement

element. The soybean seeds were sterilized with 70% ethanol and allowed to germinate on water-soaked filter paper in a sterile chamber. Sterilized germinated seedling of soybean was co-inoculated with isolated pure culture broth in a pot containing sterilized soil supplemented with N free Hoagland nutrient solution and incubated in a growth chamber. Inoculated seedlings grew and produced nodules, while the control seedlings failed to form nodules (Figure 1B).

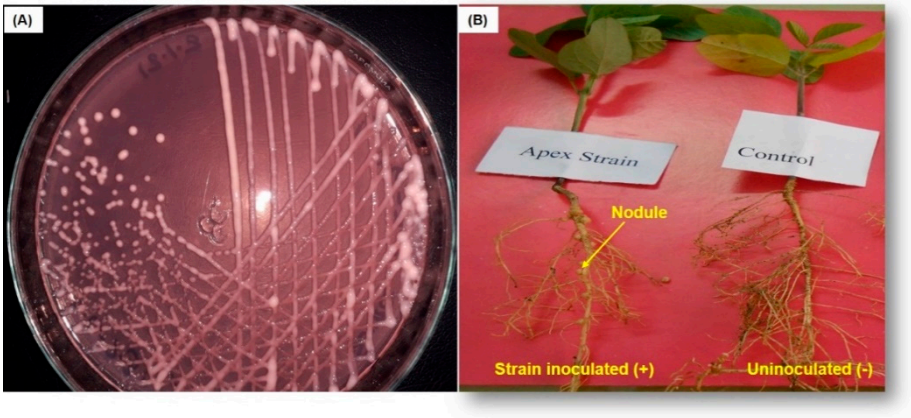

Figure 1. (**A**) Pure culture of *B. japonicum* strain (APEXBJ2); (**B**) Nodule formation of *B. japonicum* strain (APEXBJ2) in soybean seedling grown in pot supplemented with N_2 free nutrients.

B. japonicum was identified by sequencing the first 500 bases of 16S rDNA. Briefly, Genomic DNA was isolated from a single colony from a fresh streak plate isolate of *B. japonicum* using PrepMan®Ultra Sample Preparation Reagent Kit (Applied Biosystems, Foster City, CA, USA). PCR was performed in a MyGene™ Series Peltier Thermal Cycler (LongGene Scientific Instruments Co., Ltd, Hangzhou, China) by using isolated genomic DNA from of *B. japonicum* as a template DNA and 2X PCR Master Mix of Fast MicroSeq®500 16S rDNA PCR Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. We also used sterile deionized water as negative controls during the PCR. PCR products were checked in 1.2% agarose gel in 1 X TAE buffer and purified with an E.Z.N.A Gel Extraction Kit (Omega Bio-Tek, Norcross, GA, USA). No DNA bands were observed in negative controls. The sequencing of the purified PCR product was done at ICDDRB Mohakhali, Dhaka, Bangladesh using Genetic Analyzer 3130 (Applied Biosystems, Foster City, CA, USA). The sequence of the first 500 bases of 16S rDNA was analyzed using ChromasPro v2.0 and blast in NCBI to determine similar sequences. A phylogenetic tree was constructed based on the Neighbor-Joining [NJ] method by using the p-distance model with 1000 replicates to produce bootstrap values in MEGA 6, including the representative sequence obtained during the current study and the reference sequences downloaded from the gene bank [24]. The representative sequence of *B. japonicum* 16S rDNA sequence has been deposited in the NCBI gene bank database and was assigned the accession number KF279677.

2.2. Development of Liquid Inoculant

2.2.1. Microbial Production Media and Cell Culture in Fermenter

A synthetic medium (BJ production media) containing glycerol (12 g), monosodium glutamate (1.0 g), yeast extract (1.0 g), K_2HPO_4 (0.5 g), NaCl (0.1 g), KNO_3 (0.9 g), $(NH_4)_2HPO_4$ (0.4 g), $MnSO_4$ (0.01 g), and $FeSO_4.7H_2O$ (0.05 g) was used as a basal production medium for the liquid inoculant formulation. *B. japonicum* was cultivated to a late exponential/early stationary growth phase at pH 6.8 to 7.0 and 30 °C constant temperature. High cell counts (CFU 5.0×10^9 to 1.2×10^{10}) were produced after culturing in BJ medium for 57–62 h in a fermenter using 2% pure seed culture.

2.2.2. Liquid Inoculant Preparation

Nontoxic polymers such as PVP (1.8%) and Na-alginate (0.2%) were then mixed with the harvested culture and finally packaged into sterile HDPE bottles. The survival of *B. japonicum* was evaluated at monthly intervals up to 1 year by serial dilution and plate-count techniques [25]. The peat-based carrier was sterilized by gamma-irradiation and formulated according to Roughley et al. [26].

2.3. Field Evaluation

2.3.1. Field Trial with Different Carrier Based Inoculation

Geographically the experiment was conducted in January 2014 at the research field of Apex Bio-fertilizer & Bio-pesticides Ltd., Gobindaganj, Gaibandha, Bangladesh (25°11′ N, 89°28′) to study the effect of liquid biofertilizer on the yield of soybean (*Glycine max* L. *Merr.*). The soybean seeds were collected from Bangladesh Agriculture Research Institute (BARI). The soil of the experimental field was comparatively neutral in reaction, having soil pH 6.85 and soil organic matter 1.82%. The fore crop was paddy in this experimental field.

The experiment was laid out in a randomized complete block design (RCBD) with five replicates. The size of each plot was 3 m × 2 m. The distance between any blocks was 1 m. The distance between plots was 1.5 m within each block. Seven treatments were used as experimental variables and assigned randomly in each with five replication. The schematic of the treatment plots is illustrated in Figure 2A. Applied chemical fertilizers were calculated for soybean using the fertilizer recommendation guide 2012 [27]. The 100% application doses of chemical fertilizer for each block were: urea (N): 30.26 g; triple super phosphate (TSP): 148.29 g; muriate of potash (MOP) (K): 101.33 g; gypsum (S): 33.48 g; boric acid (B): 0.80 g. Among the seven treatments (T1–T7), uninoculated fresh seeds were used in two treatments (T1 and T2), and for the other five treatments (T3–T7) seeds were coated with the same strain of *B. japonicum* in different formulations (liquid-based or peat-based). The treatments T1 and T2 were 100% inorganic fertilizers and 25% urea + 100% other inorganic fertilizers, respectively. The other treatments, T3–T7 were T2 + Peat with *B. japonicum*, T2 + Na-Alginate with *B. japonicum*, T2 + PVP with *B. japonicum*, T2 + Na-Alginate + PVP with *B. japonicum*, T2 + Cell culture of *B. japonicum,* respectively. Seeds were inoculated in the field using 3-month-old inoculant. The distance between seed-shown rows was 30 cm and between plants was 7.62 cm for proper development and growth of the plants. The seeds were shown on 10 January in furrows at an approximate depth of 2 cm. The crop was harvested at the maturity stage. Ten comparative plants were selected randomly from each unit plot for recording all data of morpho–physiological traits and yield attributes. Data of nodule number and nodule dry weight were recorded at mid-flowering stage (20 days after first flowering). Grain yield of soybean was determined by harvesting an area of 1 m × 1 m and converting the grain yield value to ton ha^{-1} at 12% moisture content.

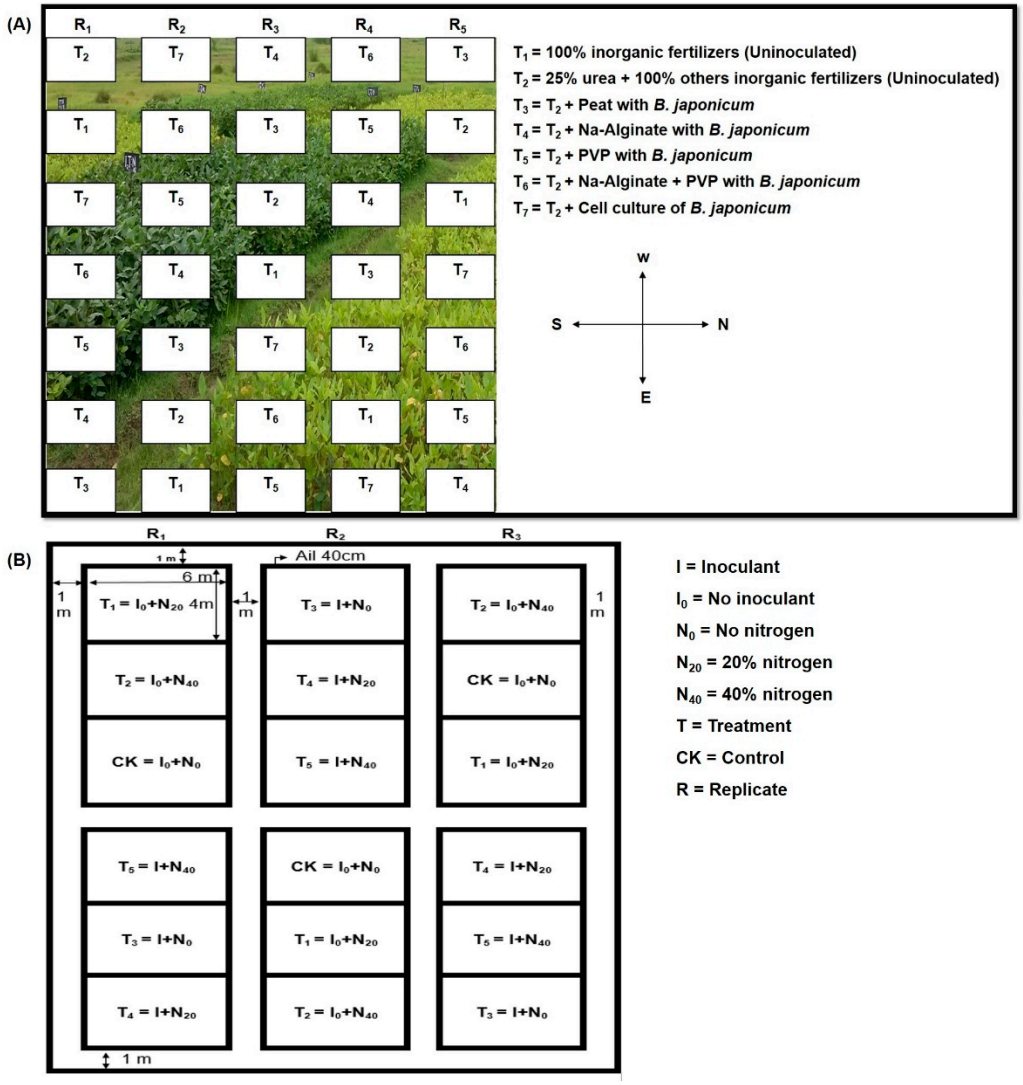

Figure 2. Schematic of field experiments. (**A**) Field trial with different carrier-based inoculation of *B. japonicun*; (**B**) field trial of liquid inoculant with different nitrogen doses on soybean plants.

2.3.2. Field Trial with Different Nitrogen Doses

The formulation selected from the above experiment (*B. japonicum* liquid inoculant) was then evaluated in December 2014 by conducting two field trials at Bangladesh Institute of Nuclear Agriculture (BINA) substations at Noakhali and Rangpur on growth, nodulation, and yield of soybean. The experiments were laid out in a split-plot design, each with three replicates. The size of each plot was 6 m × 4 m. The detailed layout of the experimental plots with treatments is illustrated in Figure 2B. Treatments comprise control (no inoculant (I_0) and no nitrogen (N_0)) and five treatments (T1–T5) with (I) or without *B. japonicum* liquid inoculant (I_0), and three levels of nitrogen viz. without nitrogen (N_0), 20 kg Nitrogen ha^{-1} (N_{20}), and 40 kg nitrogen ha^{-1} (N_{40}) were used in the study. The treatments T1–T5 were $I_0 + N_{20}$, $I_0 + N_{40}$, $I + N_0$, $I + N_{20}$, and $I + N_{40}$, respectively. TSP, MOP, gypsum, zinc sulfate, and boron were applied as a basal application at the rate of 25, 60, 15, 2, and 1 kg/ha P, K, S, Zn, and B, respectively, at final land preparation. Seeds were shown in mid-December 2014. The seeds-showing line distance in each plot was 30 cm, and plant-to-plant distance was 5–7 cm. All fertilizers except urea were applied as a basal dose during final land preparation. Different nitrogen doses (urea) were used to determine the amount of nitrogen fertilizer that the inoculant can replace. We wanted to find out whether the inoculant can replace 100% of the recommended dose of nitrogen fertilizer or whether there is a synergistic effect if the inoculum is used with a low dose of nitrogenous

fertilizer, to determine what should be recommended to the farmers about the use of the inoculum. Data on nodule number and dry weight were recorded at the mid-flowering stage. Yield-contributing parameters and yield were recorded at harvest.

2.4. Statistical Analysis

All statistical analyses were conducted in R program, version 2.15.1 [28]. Tukey's honestly significant difference (HSD) test was used to examine the significant difference of soybean plant growth parameters, nodulation, and yield parameters among different treatments with different carriers added to the liquid inoculum. Two-way analysis of variance (ANOVA) was used to explore the influence of fertilization, liquid inoculant and their interactions on the soybean plant growth parameters, nodulation, and yield parameters, if the data satisfied the normality of distribution and homogeneity of variance among treatments before and after logarithm or root square transformation. Significant differences among different treatments were further tested using Tukey's HSD test at $p < 0.05$ level. For the data that did not satisfy the normality of distribution or homogeneity of variance after transformation, a nonparametric Kruskal–Wallis test was used to examine the effect of fertilization and liquid inoculant, followed by Conover's test using the 'post-hoc.kruskal.conover.test' function in the PMCMR package [29].

3. Results

3.1. Identification of the B. japonicum (APEXBJ2) Strain

The isolated *B. japonicum* strain was identified by sequencing the first 500 bases of 16S rDNA. The sequence obtained showed 94% query cover and 98% identity with *B. japonicum* reference sequences after blasting in NCBI database. The phylogenetic tree illustrated that the representative sequence of *B. japonicum* strain in the current study shared the common clade with the sequences of *B. japonicum* from the gene bank, thus confirming the strain identity (Figure 3).

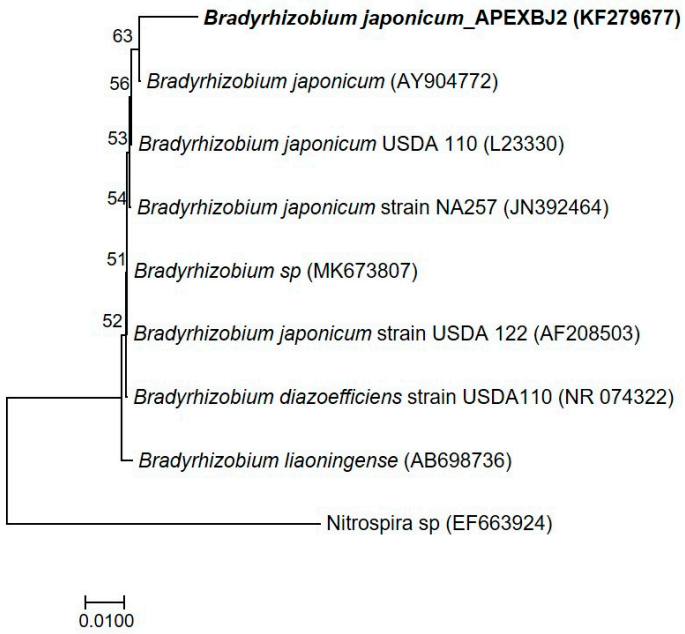

Figure 3. Neighbor-joining tree constructed on the basis 16s rDNA region of *B. japonicum*. The reference sequences were downloaded from GenBank. Bootstrap values were calculated on the basis of resampling the data 1000 times (>50% of the values are shown). *Nitrospira* sp. was used as an out-group. The corresponding accession numbers are indicated in parentheses. Scale bar represents 1% sequence divergence.

3.2. Survival of B. japonicum (APEXBJ2) Strain in Liquid Formulation

The survival of the *B. japonicum* strain in liquid formulation stored at 28 ± 2 °C in the dark was studied. The results showed that the formulation containing PVP and Na-alginate supported more than 1.93×10^9 bacteria mL^{-1} after 180 days of storage. The die-off of *B. japonicum* strain slowly occurred in the harvested culture in the control medium. No viable cells were detected after 240 days in this medium. The liquid inoculum formulated with the combination of PVP and Na-alginate supported an acceptable survival of *B. japonicum*, and provided more than 1.53×10^7 bacteria mL^{-1} at the end of the assay. The formulation that contained PVP only had the highest number of viable cells for the first 120 days, but at the end of the assay (in 360 days), the formulation containing both the additives (PVP and Na-alginate) was the best maintenance of high populations/survival of this strain (Table 1).

Table 1. Survival of *B. japonicum* in liquid formulation at ambient temperature 28 ± 2 °C.

Liquid Carrier	Colony Forming Units (CFU)/mL					
	Initial CFU	**120 Days**	**180 Days**	**240 Days**	**300 Days**	**360 Days**
Control	5.67×10^9	4.53×10^5	3.50×10^2	-	-	-
Na-Alginate (0.2%) + PVP (1.8%)	5.30×10^9	2.53×10^9	1.93×10^9	6.10×10^8	5.10×10^7	1.53×10^7
PVP (1.8%)	5.47×10^9	4.91×10^9	8.40×10^8	1.63×10^7	1.44×10^6	7.25×10^5
Na-Alginate (0.2%)	5.38×10^9	3.70×10^8	6.80×10^7	4.60×10^6	3.90×10^5	1.96×10^5

3.3. Field Experiments

3.3.1. Evaluation of Carrier-Based Formulations

Results showed the effects of different carrier-based formulations of *B. japonicum* strain on nodulation, yield contributing characters, and yields of soybean (Figures 4 and 5). The inoculants had no or a diminutive effect on field stand (Figure 4A), plant height (Figure 4B), and seed size (100 seed weight, Figure 5B), but significantly increased the total number of nodules per plants (Figure 4D) and nodules on taproot over uninoculated treatments (Figure 4E). Seed inoculations promoted root nodulation that subsequently influenced the growth and yield of soybean. The highest number of branches was recorded when seeds were coated with liquid carrier Na-Alginate 0.2% and PVP 1.8% (Figure 4C). Liquid carrier (Na-Alginate 0.2% + PVP 1.8%) inoculated treatment gave the significantly higher number of pods $plant^{-1}$, seeds pod^{-1}, seed weight $plant^{-1}$, and finally the yield of soybean compared to the uninoculated treatments (Figure 5). However, the values were not significantly different from those of the peat-carrier-inoculated treatment. The lowest yield (1.20 ton ha^{-1}) was observed in the treatment with 25% urea fertilizer, and the highest yield (2.45 ton ha^{-1}) was observed in the treatment where the seeds were inoculated with Liquid Carrier (Na-Alginate 0.2% and PVP 1.8%) (Figure 5).

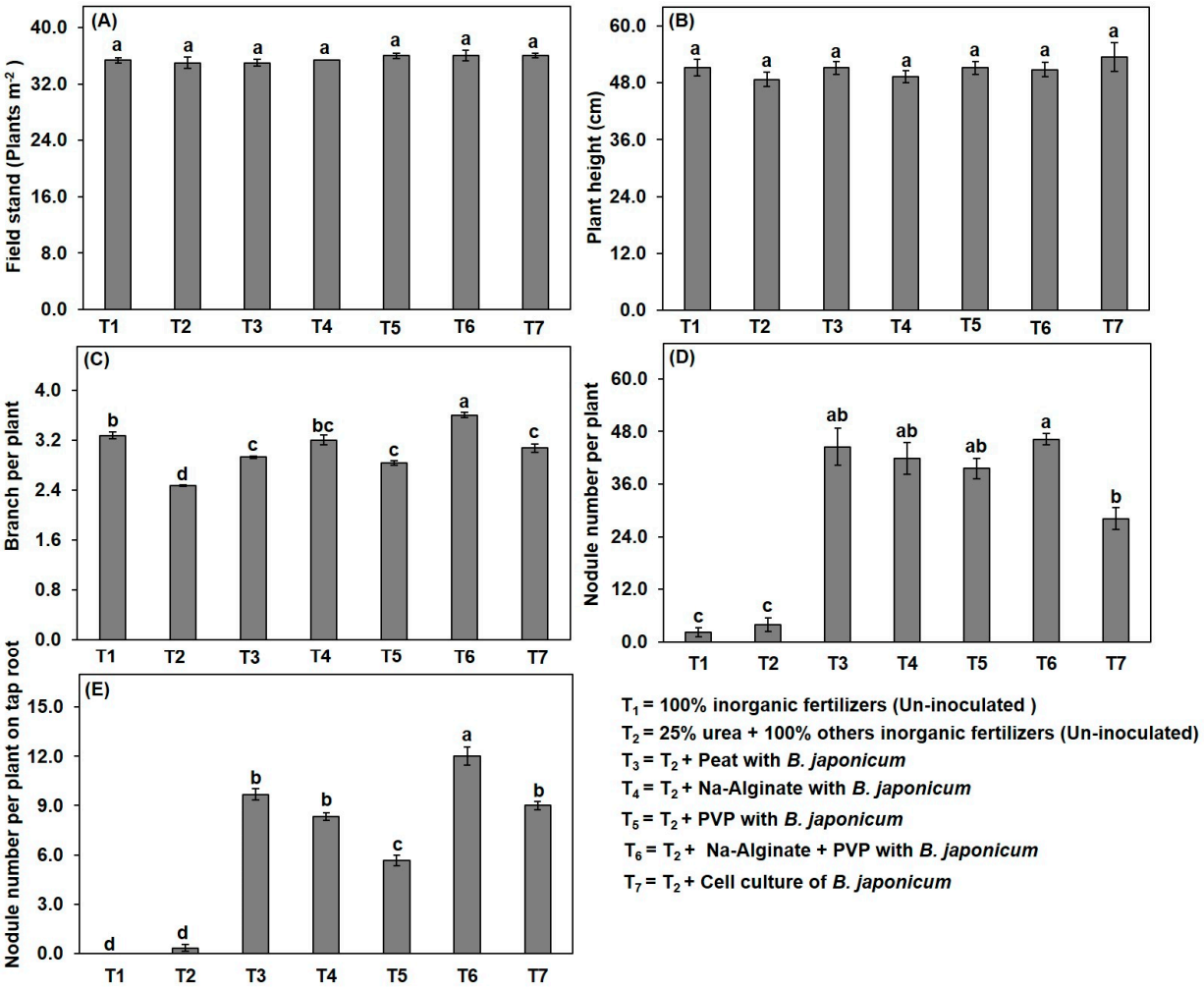

Figure 4. Effect of different carrier-based formulations of *B. japonicum* strain on soybean plant growth parameters and nodulation. (**A**) Field stand; (**B**) plant height; (**C**) branch per plant; (**D**) nodule number per plant; (**E**) nodule number per plant on tap root. Data are means ± SE (n = 5). Bars without shared letters indicate significant differences in treatments at $p < 0.05$.

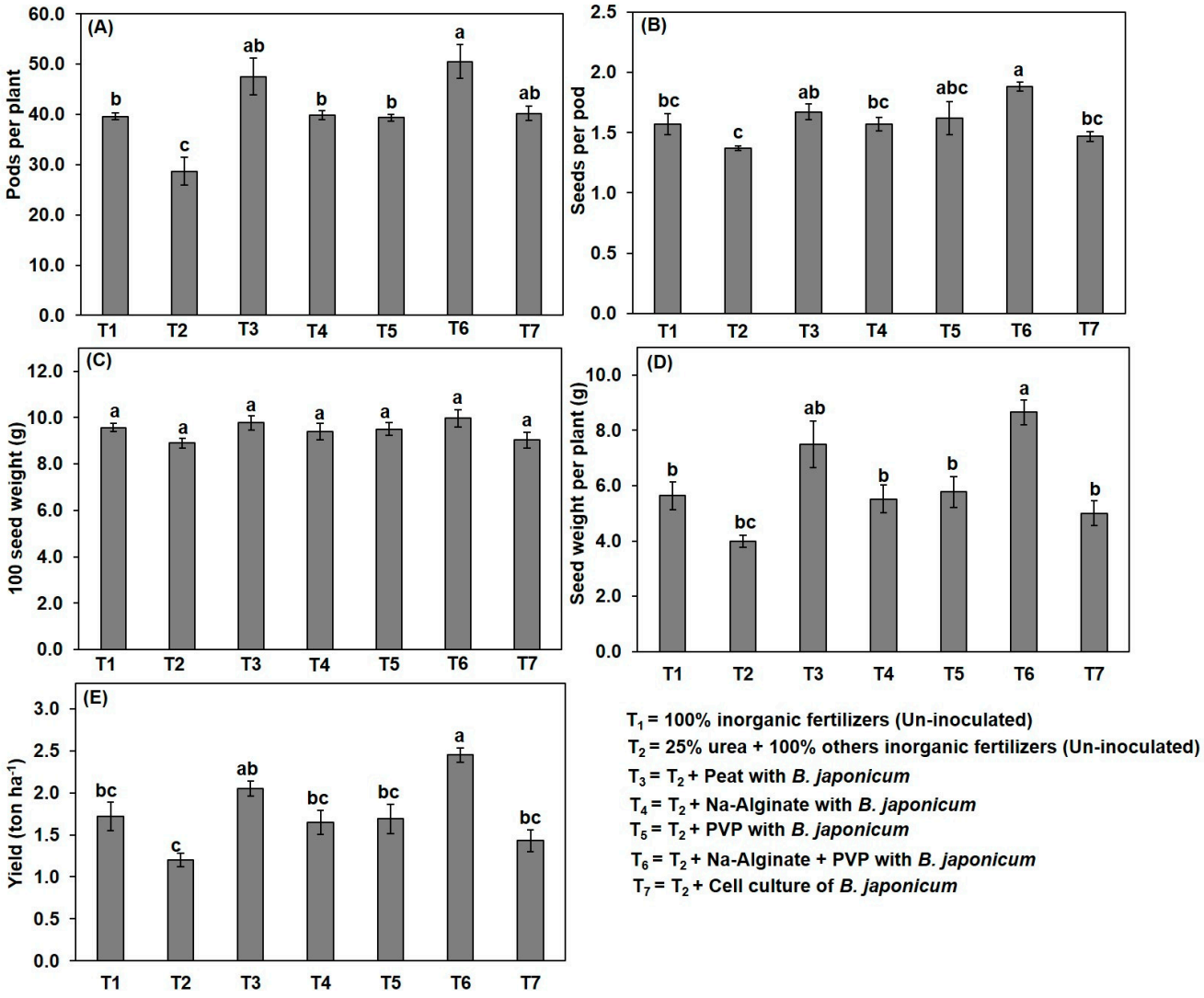

Figure 5. Effect of different carrier-based formulation of *B. japonicum* strain on soybean yield parameters. (**A**) Pods per plant; (**B**) seeds per pod (**C**) 100 seed weight; (**D**) seed weight per plant; (**E**) yield. Data are means ± SE (n = 5). Bars without shared letters indicate significant differences in treatments at $p < 0.05$.

3.3.2. Evaluation of Liquid Formulation with Different Nitrogen Doses

Results of the trials of liquid formulation with different nitrogen doses showed that the liquid inoculant of *B. japonicum* (PVP + Na-alginate based) significantly increased the plant height, nodule number, nodule dry weight, grain yield, and straw yield of soybean compared to the uninoculated control (Figure 6). Nitrogen application showed significant effect on grain yield and straw yield but no significant effect on nodule number and dry weight. Furthermore, the higher dose of nitrogen with inoculant showed a negative effect on nodule number and dry weight (Figure 6B–E). For instance, grain yield and straw yield increased significantly due to nitrogen application. A dose of 40 Kg N ha^{-1} application showed the highest grain and straw yield of soybean (Figure 6D,E). Inoculation without nitrogen recorded similar grain and straw yield as with 40 kg N application without inoculation (Figure 6D,E). Significant interaction effect of fertilization and liquid inoculant was observed in plant height, grain, and straw yield at Rangpur and the Noakhali location (Figure 6A,D,E).

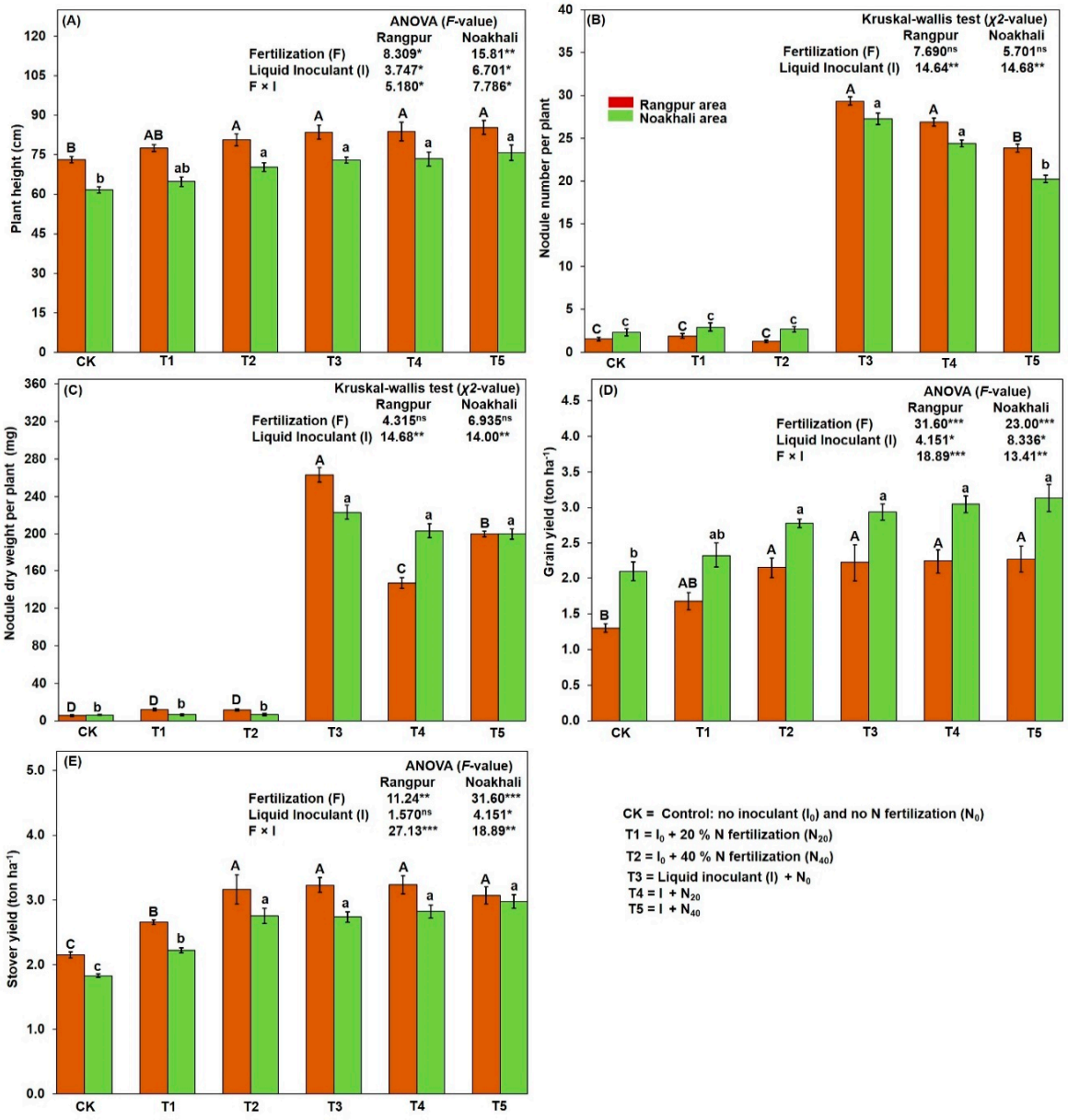

Figure 6. Effect of liquid inoculant (Na-alginate + PVP based) of *B. japonicum* and nitogen application on soybean growth and yield. Two-way ANOVA and Kruskal–Wallis tests illustrate the effect of fertilization (F) and liquid inoculum (I) on (**A**) plant height; (**B**) nodule number per plant; (**C**) nodule dry weight per plant; (**D**) grain yield; (**E**) stover yield of soybean (^{ns} $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Data are means ± SE ($n = 3$). Bars without shared uppercase and lowercase letters indicate significant differences in different treatments in the Rangpur and Noakhali area at $p < 0.05$, respectively.

4. Discussion

The liquid inoculum of *B. japonicum* formulated with the additives PVP (1.8%) and Na-alginate (0.2%) in the culture medium promoted long-term survival of *B. japonicum*. It had a better shelf-life (1.93×10^9 cells/mL) compared to control (3.50×10^2 cells/mL) after 6 months of storage. It also maintained 1.53×10^7 cells/mL after 12 months of storage under ambient temperature (28 ± 2 °C). A safe storage period of 6 months at room temperature is desirable in a commercial inoculant [30]. These results are similar to those of many investigators [17,18,25,31,32], who also reported better survival of microorganisms in liquid formulations amended with polymeric substances. Use of *B. japonicum* liquid formulation, with 5.30×10^9 cells/mL initial counts, may be highly efficient as it is effective

in lower volumes per unit seed weight. Liquid formulations with similar cell counts were also reported in a previous study [33].

The formulations containing single polymeric material of either PVP (1.8%) or Na-alginate (0.2%) recorded 7.25×10^5 and 1.96×10^5 cells/mL, respectively, up to 12 months, but no viable cells were detected in the negative control after storage at 28 ± 2 °C for 6 months. Rapid cell death in negative control may be the result of continued cell growth, which may occur if the temperature exceeds 30 °C, depleting nutrients and accumulating toxic metabolites [17]. PVP is a synthetic vinyl polymer that helps *B. japonicum* to survive [34] while alginate allows cells to be stable at higher temperatures [35,36]. PVP also has adhesive properties, possibly due to its high water-binding capacity, which may maintain water around the cells aiding their metabolism [34,36,37]. PVP has been reported to protect cells against toxic seed coat factors [37]. Furthermore, the high water-holding capacity of PVP slows down the drying rate of media, thus maintaining the moisture level in the media. PVP also has an ability to bind bacterial toxins that were constantly released into the media, when bacterial cells were in stationary phase [37]. Alginate formulations are nontoxic, biodegradable and able to limit heat transfer, and possess high water activities [38,39].

High cell counts of the strain at ambient temperature for the desired period of 6 months was due to multiple factors such as the selected strain, the developed BJ production media containing glycerol as a carbon source [31,34,40], and the use of the two additives. The indigenous strain had better survival capacity in prevalent subtropical temperatures. The BJ production medium was optimized for high cell counts and also played a role in improving the long-term survival of the strain. The use of both PVP (1.8%) and Na-alginate (0.2%) in this liquid formulation in minimal amounts enhanced the survival capacity of the strain over formulations containing either of them. Field studies demonstrated that liquid carrier (Na-Alginate 0.2% + PVP 1.8%) increased yield over the control when applied with 25% urea, and even showed a significant yield increase (42%) over the 100% chemical fertilizer treatment (Figure 5). This result suggests that a low dose of N fertilizer application with *Rhizobium* inoculation stimulates the *Rhizobium*–soybean symbiosis and subsequently increases soybean growth and yield. Grain yield was higher with the liquid inoculum compared to that of peat carrier control, but the difference was not statistically significant. The nodule number in primary root and taproot increased over peat carrier-based treatment. The increased nodule number indicates that the liquid carrier (Na-Alginate 0.2% + PVP 1.8%) inoculant probably supported more viable cells between seed inoculation and root emergence, the time when colonization and infection by the inoculant occur [34]. Several studies done in widely distributed parts of the world have shown that liquid inoculants applied on seed can produce seed yields similar to those obtained with peat-based inoculants [17,34,41,42]. Different liquid formulations of the same strain used in the same location produced different yields in our trials. Soybeans inoculated with liquid formulations containing either PVP (1.8%) or Na-Alginate (0.2%) gave lower seed yields than the peat inoculant, although the reduction was not statistically significant. The performance of a liquid inoculant in the field depends on the survival of the bacteria during storage and after seed coating. Survival can be aided by production media composition and the use of combined additives. The ability of the selected strain to compete with indigenous *Rhizobium*, the efficiency of the strain, tolerance of the strain to fluctuations in soil and climatic conditions are also crucial. Liquid inoculant formulations have been found to perform better for slow-growing strains such as *B. japonicum* [17]. However, many other authors did not obtain as good results with liquid formulations as with the solid ones [43–46]. The difference may be due to the formulations of the liquid inoculants they used.

In our study, similar yield increases were also obtained at the field trials of the liquid inoculant with different nitrogen doses. Nodule number and dry weight increased by 17.9% and 31 times over uninoculated control. Grain yield was 30% higher compared to uninoculated control, whereas straw yield was 19% higher over control at Rangpur, and 27% higher grain yield and 10% higher straw yield was obtained at Noakahali. Similar results

were reported in a previous study [47,48]. Furthermore, our results showed that there is no significant difference between the yields of the 100% nitrogen control and the treatment in which the inoculum was used without any nitrogen. Moreover, the application of higher doses of nitrogen negatively affected the nodule number and size. We have established that this formulated liquid inoculant can maintain a similar or higher count of microorganisms compared to peat during storage and application. It can therefore be used as an alternative to peat for soybean inoculation under field conditions. It has also been found to be able to withstand the subtropical temperatures in Bangladesh as well as other regions of the world. Depending on agronomic practices, and soil, environmental, and climatic factors, the liquid inoculant has been able to replace chemical nitrogen fertilizer completely in our trials.

5. Conclusions

Legume inoculation technology has been available in the agricultural sector for decades. Many constraints limit its dissemination. Combined use of polymeric compounds PVP and Na-alginate enhances the survival of *B. japonicum* in liquid formulation. In our study, a comparatively higher nodulation and yield of soybean was obtained in a field trial of this formulated liquid inoculum over the peat carrier-based treatment. Depending on agronomic practices, and soil, environmental, and climatic factors, the liquid inoculant was able to replace chemical nitrogen fertilizer completely in our study. In the future, this technology can be used to develop *Rhizobium* inoculants as well as formulations of other beneficial microbes, which will be effective in different adverse environmental conditions for sustainable agricultural productivity under global climate change scenarios.

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Abbreviations

PVP	Polyvinylpyrrolidone
PEG	Polyethylene glycol
HDPE	High density polyethylene
BARI	Bangladesh Agriculture Research Institute
BINA	Bangladesh Institute of Nuclear Agriculture
B. japonicum	*Bradyrhizobium japonicum*
NCBI	National Center for Biotechnology Information
USA	United States of America
TSP	Triple super phosphate
MOP	Muriate of potash

References

1. Wilcox, J. World distribution and trade of soybean. In *Soybean: Improvement, Production, and Uses*; Boerma, H.R., Specht, J.E., Eds.; American Society of Agronomy: Madison, WI, USA, 2004; pp. 1–14.
2. Vieira, R.F.; Mendes, I.C.; Reis-Junior, F.B.; Hungria, M. Symbiotic nitrogen fixation in tropical food grain legumes: Current status. In *Microbes for Legume Improvement*; Khan, M.S., Zaidi, A., Musarrat, J., Eds.; Springer: Vienna, Austria, 2010; pp. 427–472.
3. Youseif, S.H.; El-Megeed, F.H.A.; Saleh, S.A. Improvement of faba bean yield using *Rhizobium/Agrobacterium* inoculant in low-fertility soil. *Agronomy* **2017**, *7*, 2. [CrossRef]

4. Hashem, A.; Abd_Allah, E.F.; Alqarawi, A.A.; Al-Huqail, A.A.; Wirth, S.; Egamberdieva, D. Comparing symbiotic performance and physiological responses of two soybean cultivars to arbuscular mycorrhizal fungi under salt stress. *Saudi J. Biol. Sci.* **2016**, *26*, 38–48. [CrossRef] [PubMed]
5. Ma, H.; Egamberdieva, D.; Wirth, S.; Bellingrath-Kimura, S.D. Effect of biochar and irrigation on soybean-*rhizobium* symbiotic performance and soil enzymatic activity in field rhizosphere. *Agronomy* **2019**, *9*, 626. [CrossRef]
6. Rodríguez-Navarro, D.N.; Margaret Oliver, I.; Albareda Contreras, M.; Ruiz-Sainz, J.E. Soybean interactions with soil microbes, agronomical and molecular aspects. *Agron. Sustain. Dev.* **2011**, *31*, 173–190. [CrossRef]
7. Madrzak, C.J.; Golinska, B.; Kroliczak, J.; Pudelko, K.; Lazewska, D.; Lampka, B.; Sadowsky, M.J. Diversity among field populations of *Bradyrhizobium japonicum* in Poland. *Appl. Environ. Microbiol.* **1995**, *61*, 1194–1200. [CrossRef] [PubMed]
8. Egamberdieva, D.; Davranov, K.; Wirth, S.; Hashem, A.; Abd_Allah, E.F. Impact of soil salinity on the plant-growth–promoting and biological control abilities of root associated bacteria. *Saudi J. Biol. Sci.* **2017**, *24*, 1601–1608. [CrossRef]
9. Egamberdieva, D.; Ma, H.; Alimov, J.; Reckling, M.; Wirth, S.; Bellingrath-Kimura, S.D. Response of Soybean to hydrochar-based *Rhizobium* inoculation in loamy sandy soil. *Microorganisms* **2020**, *8*, 1674. [CrossRef]
10. Trivedi, P.; Pandey, A.; Palni, L.M. Carrier-based preparations of plant growth-promoting bacterial inoculants suitable for use in cooler regions. *World J. Microb. Biotechnol.* **2005**, *21*, 941–945. [CrossRef]
11. Abd El-Fattah, D.A.; Eweda, W.E.; Zayed, M.S.; Hassanein, M.K. Effect of carrier materials, sterilization method, and storage temperature on survival and biological activities of *Azotobacter chroococcum* inoculant. *Ann. Agric. Sci.* **2013**, *58*, 111–118. [CrossRef]
12. Htwe, A.Z.; Moh, S.M.; Soe, K.M.; Moe, K.; Yamakawa, T. Effect of biofertilizer produced from *Bradyrhizobium* and Streptomyces griseoflavus on plant growth, nodulation, nitrogen fixation, nutrient uptake, and seed yield of mung bean, cowpea, and soybean. *Agronomy* **2019**, *9*, 77. [CrossRef]
13. Bashan, Y.; de-Bashan, L.E.; Prabhu, S.R.; Hernandez, J.P. Advances in plant growth-promoting bacterial inoculant technology: Formulations and practical perspectives (1998–2013). *Plant Soil* **2014**, *378*, 1–33. [CrossRef]
14. Ardakani, S.S.; Hedari, A.; Tayebi, L.; Mohammadi, M. Promotion of cotton seedlings growth characteristics by development and use of new bioformulations. *Int. J. Bot.* **2010**, *6*, 95–100. [CrossRef]
15. Berninger, T.; Lopez, O.G.; Bejarano, A.; Preininger, C. Sessitsch, A. Maintenance and assessment of cell viability in formulation of non-sporulating bacterial inoculants. *Microb. Biotechnol.* **2018**, *11*, 277–301. [CrossRef] [PubMed]
16. Arora, N.K.; Khare, E.; Maheshwari, D.K. Plant growth promoting rhizobacteria: Constraints in bioformulation, commercialization, and future strategies. In *Plant Growth and Health Promoting Bacteria*; Maheshwari, D.K., Ed.; Springer: Berlin/Heidelberg, Germany, 2011; Volume 18, pp. 97–116.
17. Tittabutr, P.; Payakapong, W.; Teaumroong, N.; Singleton, P.W.; Boonkerda, N. Growth, survival and field performance of *Bradyrhizobial* liquid inoculant formulations with polymeric additives. *Sci. Asia* **2007**, *33*, 69–77. [CrossRef]
18. Girisha, H.C.; Brahmaprakash, G.P.; Mallesha, B.C. Effect of osmoprotectant (PVP-40) on survival of *Rhizobium* in different inoculants formulation and nitrogen fixation in cowpea. *Geobios-Jodhpur-* **2006**, *33*, 151–156.
19. Sukhovitskaia, L.A.; Safronova, G.V.; Klyshko, G.M.; Korolenok, N.V. Survival of *Rhizobium* in monoculture and binary population with rhizosphere bacteria. *Prikl. Biokhim. Mikrobiol.* **2002**, *38*, 73–78. (In Russian) [PubMed]
20. Hubálek, Z. Protectant used in the cryopreservation of microorganisms. *Cryobiology* **2003**, *46*, 205–229. [CrossRef]
21. Ondieki, D.K.; Nyaboga, E.N.; Wagacha, J.M.; Mwaura, F.B. Morphological and genetic diversity of Rhizobian nodulating cowpea (*Vigna unguiculata* L.) from agricultural soils of lower eastern Kenya. *Int. J. Microbiol.* **2017**, *2017*, 8684921. [CrossRef]
22. Somasegaran, P.H.; Hoben, H. *Handbook for Rhizobia: Methods in Legume-Rhizobium Technology*; Springer: Berlin/Heidelberg, Germany, 1994; Volume Xvi, p. 450.
23. Hoagland, D.R. Optimum nutrient solutions for plants. *Science* **1920**, *52*, 562–564. [CrossRef]
24. Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A.; Kumar, S. MEGA 6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* **2013**, *30*, 2725–2729. [CrossRef]
25. Sehrawat, A.; Suneja, S.; Yadav, A.; Anand, R.C. Influence of different additives on shelf life of Rhizobial inoculants for mungbean (*Vigna radiata* L.). *Int. J. Recent Sci. Res.* **2015**, *6*, 4338–4342.
26. Roughley, R.J. The preparation and use of legume seed inoculants. *Plant Soil* **1970**, *32*, 675–701. [CrossRef]
27. FRG. *Fertilizer Recommendation Guide*; Bangladesh Agriculture Research Council (BARC): Farmgate, Dhaka, 2012; 274p.
28. R Development Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2014; Available online: https://www.R-project.org (accessed on 14 April 2021).
29. Pohlert, T. The Pairwise Multiple Comparison of Mean Ranks Package (PCMR). R Package Version 4.1. 2014. Available online: http://cran.r-project/package=PCMR (accessed on 14 April 2021).
30. Roughley, R.J.; Vincent, J.M. Growth and survival of *Rhizobium* spp. in peat culture. *J. Appl. Bacteriol* **1967**, *30*, 362–376. [CrossRef]
31. Taurian, T.; Anzuay, M.S.; Angelini, J.G.; Tonelli, M.L.; Ludueña, L.; Pena, D.; Ibáñez, F.; Fabraet, A. Phos-phate-solubilizing peanut associated bacteria: Screening for plant growth promoting activities. *Plant Soil* **2010**, *329*, 421–431. [CrossRef]
32. Amalraj, E.L.D.; Venkateswarlu, B.; Desai, S.; Kumar, G.P.; Ahmed, S.K.M.H.; Meenakshi, T.; Sultana, U.; Pinisetty, S.; Mangamoori, L.N. Effect of polymeric additives, adjuvants, surfactants on survival, stability and plant growth promoting ability of liquid bioinoculants. *J. Plant. Physiol. Pathol.* **2013**, *1*, 1–5. [CrossRef]

33. Schulz, T.J.; Thelen, K.D. Soybean seed inoculant and fungicidal seed treatment effects on soybean. *Crop Sci.* **2008**, *48*, 1975–1983. [CrossRef]
34. Singleton, P.; Keyser, H.; Sande, E. Development and evaluation of liquid Inoculants. In *Inoculants and Nitrogen Fixation of Legumes in Vietnam*; Herridge, D., Ed.; ACIAR: Canberra, Austrilia, 2002; Volume 109, pp. 52–66.
35. Viveganandan, G.; Jauhri, K.S. Growth and survival of phosphate-solubilizing bacteria in calcium alginate. *Microbiol. Res.* **2000**, *155*, 205–207. [CrossRef]
36. Biradar, B.J.P.; Santhosh, G.P. Role of polymeric additives in formulation, shelf-life and bioefficacy of liquid inoculant of *Pseudomonas fluoresens*. *Int. J. Pure Appl. Biosci.* **2018**, *6*, 123–133. [CrossRef]
37. Deaker, R.; Roughley, R.J.; Kennedy, I.R. Legume seed inoculation technology—A review. *Soil Biol. Biochem.* **2004**, *36*, 1275–1288. [CrossRef]
38. Bashan, Y.; Hernandez, J.P.; Leyva, L.A.; Bacilio, M. Alginate microbeads as inoculant carriers for plant growth-promoting bacteria. *Biol. Fertil. Soils* **2002**, *35*, 359–368. [CrossRef]
39. Zohar-Perez, C.; Ritte, E.; Chernin, L.; Chet, I.; Nussinovitch, A. Preservation of chitinolytic *Pantoae agglomerans* in a viable form by cellular dried alginate-based carriers. *Biotechnol. Prog.* **2002**, *18*, 1133–1140. [CrossRef]
40. Manikandan, R.; Saravanakumar, D.; Rajendran, L.; Raguchander, T.; Samiyappan, R. Standardization of liquid formulation of *Pseudomonas fluorescens* Pf1 for its efficacy against Fusarium wilt of tomato. *Biol. Control* **2010**, *54*, 83–89. [CrossRef]
41. Thao, T.Y.; Singleton, P.W.; Herridge, D. Inoculation responses of soybean and liquid inoculants as an alternative to peat-based inoculants. In *Inoculants and Nitrogenffixation of Legumes in Vietnam*; Herridge, D., Ed.; ACIAR: Canberra, Austrilia, 2002; Volume 109, pp. 67–74.
42. Albareda, M.; Rodriguez-Navarro, D.N.; Camacho, M.; Temprano, F.J. Alternatives to peat as a carrier for rhizobia inoculant: Solid and liquid formulations. *Soil Biol. Biochem.* **2008**, *40*, 2771–2779. [CrossRef]
43. Rice, W.A.; Clayton, G.W.; Olsen, P.E.; Lupwayi, N.Z. Rhizobial inoculant formulations and soil pH influence pea nodulation and nitrogen fixation. *Can. J. Soil Sci.* **2000**, *80*, 395–400. [CrossRef]
44. Kyei-Boahen, S.; Slinkard, A.E.; Walley, F.L. Evaluation of rhizobial inoculation methods for chickpea. *Agron. J.* **2002**, *94*, 851–859. [CrossRef]
45. Clayton, G.W.; Rice, W.A.; Lupwayi, N.Z.; Johnston, A.M.; Lafond, G.P.; Grant, C.A.; Walley, F. Inoculant formula-tion and fertilizer nitrogen effects on field pea: Nodulation, N_2 fixation and nitrogen partitioning. *Can. J. Plant Sci.* **2004**, *84*, 79–88. [CrossRef]
46. Clayton, G.W.; Rice, W.A.; Lupwayi, N.Z.; Johnston, A.M.; Lafond, G.P.; Grant, C.A.; Walley, F. Inoculant formulation and fertilizer nitrogen effects on field pea: Crop yield and seed quality. *Can. J. Plant Sci.* **2004**, *84*, 89–96. [CrossRef]
47. Egamberdiyeva, D.; Qarshieva, D.; Davranov, K. The use of *Bradyrhizobium* to enhance growth and yield of soybean in calcareous soil in Uzbekistan. *J. Plant Growth Regul.* **2004**, *1*, 54–57. [CrossRef]
48. Sibponkrung, S.; Kondo, T.; Tanaka, K.; Tittabutr, P.; Boonkerd, N.; Yoshida, K.-i.; Teaumroong, N. Co-Inoculation of *Bacillus velezensis* Strain S141 and *Bradyrhizobium* strains promotes nodule growth and nitrogen fixation. *Microorganisms* **2020**, *8*, 678. [CrossRef]