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COST-EFFECTIVE FORMULATION OF BIO-FERTILIZER USING AGRICULTURAL RESIDUES AS CARRIERS AND DETERMINATION OF SHELFLIFE OF BIO-FERTILIZER INOCULANTS

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Key words: Bio-fertilizer, *Rhizobium* sp., *Azotobacter* sp., *Trichoderma* sp., Rice husk ash, Alluvial soil.

Abstract

Traditionally, inorganic chemical-based fertilizers is used for soil management strategies, which can cause public health and environmental threats. Alternatively, bio-fertilizer can be used to increase the productivity and sustainability of soil without causing environmental pollution. The present study aimed to cost-effectively produce bio-fertilizer using agricultural residues and determine the shelflife and efficacy of the bioinoculants. We used sterilized rice husk ash and alluvial soil (1:2) to prepare cost-effective carriers. *Rhizobium* sp., *Azotobacter* sp., and *Trichoderma* sp. were grown in a newly designed culture medium for economic production as bio-inoculants. The efficacy of the formulated bio-fertilizer was tested on a small scale, where it significantly improved the growth of the sponge gourd (*Luffa aegyptiaca*) plant ($p<0.01$). The formulated bio-fertilizers were stored at room temperature for one year. Initially, the total viable count of microorganisms was 8.0×10^7 CFU/g in the formulated bio-fertilizer. The total viable count of the bio-inoculants increased significantly after one month (2.2×10^8 CFU/g) and one year (2.2×10^9 CFU/g). Rice husk ash might have supported the growth and survival of the bioinoculants under room temperature (25°C) because of its nutrient retention capacity, adsorptive capability, and high content of silica. Therefore, this study suggests that sterile rice husk ash combined with alluvial soil can be used as a carrier for bio-fertilizers formulation with *Rhizobium* sp., *Azotobacter* sp., and *Trichoderma* sp. bioinoculants.

Introduction

Soil infertility leads to decreased crop yield and therefore is a burning issue in developing countries. Fertilization plays a vital role in agriculture by increasing crop yield[1,2]. Farmers generally use chemical fertilizers for higher production of crops[3]. However, several researchers reported that the long-term use of chemical fertilizers would decrease soil fertility and water holding capacity[4,5]. The continuous application of

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chemical fertilizer causes decay of soil quality and fertility. Additionally, chemical fertilizers also cause air pollution as well as groundwater pollution through eutrophication of water.

However, agriculture has undergone various scientific innovations to make it more efficient over the years. Researchers suggest to replace chemical fertilizers with bio-fertilizers. The application of bio-fertilizers provides many benefits over chemical fertilizers[6]. Bio-fertilizers contain selected strains of microorganisms grown in the laboratory and mixed with appropriate carriers, that colonize the rhizosphere and enhance plant growth by making nutrients available. Bio-fertilizers enhance soil fertility and carbon content[7] and enrich soil nutrients by nitrogen fixation and solubilization of potassium and phosphate[8]. It was also reported that bio-fertilizer application increased crop production by increasing nitrogen fixation and contents of essential amino acids [9]. Microorganisms in bio-fertilizers play a significant role in compact nutrient regulation systems[10]. Moreover, N_2-fixing microorganisms convert atmospheric N_2 into NH_3 through the biological nitrogen fixation process[11]. Several plant growth-promoting microorganisms such as *Azotobacter*, *Azospirillum, Bacillus*, *Pseudomonas, Rhizobium, Trichoderma,* and Yeast are being used as bio-inoculants in bio-fertilizer production[12,13].

Prolonged shelflife without any harmful effects on the environment is another advantage of bio-fertilizer[8]. Many approaches are used for bio-fertilizer formulation such as solid carrier-based, polymer-based, metabolite, and liquid formulations[14]. A low-cost carrier-based bio-fertilizer formulation would be economical and, therefore, highly acceptable to farmers of developing countries. This study aimed to produce and formulate bio-fertilizer in a cost-effective manner using *Rhizobium* sp., *Azotobacter* sp., and *Trichoderma* sp. as bio-inoculants, and rice husk ash and alluvial soil as carriers. This investigation also determined bio-inoculants'shelflife and evaluated the efficacy of the bio-fertilizer in improving plant growth.

Materials and Methods

Sample collection and determination of antibiotic sensitivity of the used organisms: Stock cultures of *Rhizobium* sp., *Azotobacter* sp., and *Trichoderma* sp. were collected from the Food, Nutrition, and Agriculture Research Laboratory, Centre for Advanced Research in Sciences, University of Dhaka, Dhaka-1000. Cultures of *Rhizobium* sp. and *Azotobacter* sp. were resuscitated into 5 ml Tryptone Soy Broth (TSB) in a test tube and incubuted at 37°C and 150 rpm for 24h. *Trichoderma* sp. was resuscitated into Sabouraud Dextrose Broth (SDB) and incubated at 30°C and 150 rpm for 24 h. *Rhizobium* sp. and *Azotobacter* sp. were tested for antibiotic susceptibility on the Mueller-Hinton agar medium by Kirby-Bauer disc diffusion technique against 26 antibiotics following the standard protocol [15]. Bacterial lawns were prepared by introducing suspensions of *Rhizobium* sp. and *Azotobacter* sp. on Muller Hinton agar. The antibiotic discs were introduced correctly on the lawn of bacteria on Muller Hinton agar. The plates were then incubated for 12-18 h at

37°C and observed if any inhibition zone (mm) was formed. The sensitivity or the resistance of *Rhizobium* sp. and *Azotobacter* sp. to antibiotics were determined by observing the absence or presence of growth around the discs [16]. The organisms which showed growth around the antibiotic disc are resistant, and the isolates whose growth is inhibited by the antibiotic are sensitive to that antibiotic.

Determination of growth curve of the organisms: *Rhizobium* sp. and *Azotobacter* sp. were inoculated into 100 ml Luria Bertani (LB) broth and incubated in an orbital shaker incubator (New Brunswick Scientific, Excella E25, USA) at 37°C and 150 rpm for 48h. The density of the organisms was measured in an absorbance spectrophotometer (SpectronicGENESYS 5,USA) at 600 nm with an interval of 3h for 48h to find out the duration of the exponential growth phase of these organisms.

Media preparation for economic production of the bio-fertilizer: Different molasses concentrations and pH conditions were assessed to identify the best economic culture medium. *Rhizobium* sp., *Azotobacter* sp., and *Trichoderma* sp. were grown in media containing varying molasses concentrations (0.3% and 0.4%) and at different pH (6, 7, and 8). To achieve that, we designed two culture media composed of molasses (0.3 or 0.4 g), yeast extract (0.1g), $MgSO_4$ (0.02g), K_2HPO_4 (0.05g), and potassium phosphate buffer (0.1M) for 100 ml.

For these organisms, two sets of 9 flasks of molasses media were prepared (three media of pH-6, 7, 8 for each organism). The organisms were inoculated into each flask of prepared media and incubated at 37°C for *Rhizobium* sp. and *Azotobacter* sp. and at 30°C for *Trichoderma* sp. in an orbital shaker at 150 rpm for 24h. After incubation, 100 µl of appropriately diluted culture was spread on TSA and PDA media, and the plates were then incubated at 37°C for 24h and 30°C for 48h, respectively. After incubation, CFU/ml was enumerated for all different media combinations.

Formulation of bio-fertilizer: Rhizobium sp. and *Azotobacter* sp. were inoculated in molasses medium which showed maximum biomass production, and incubated in an orbital shaker at 37°C for two days at 150 rpm. *Trichoderma* sp. was also inoculated in the best molasses medium and incubated at 30°C for five days at 150 rpm. Finally, suspensions of 100 ml *Rhizobium* sp. (5×10^7 CFU/ml), 100 ml *Azotobacter* sp. (5×10^7 CFU/ml), and 100 ml *Trichoderma* sp. (6×10^7 CFU/ml) were prepared. In this experiment, sterilized alluvial soil and rice husk ash were used as carrier material. The 0-30 cm depth layer of soil was collected from Mirpur area and rice husk ash was collected from a local market in Dhaka, Bangladesh. The soil and ash were air-dried, sieved (5 cm sieve), and then autoclaved to sterilize them. A sterile beaker was filled with 1.0 kg sterilized soil, 0.5 kg sterilized ash, and 300 ml suspensions of *Rhizobium* sp. (100 ml), *Azotobacter* sp. (100 ml), and *Trichoderma* sp. (100 ml). Plastic zip lock bags were sterilized for 30 minutes under UV light to ensure aseptic conditions. The prepared bio-fertilizer was placed in a plastic ziplock bag, sealed, and stored in a dry environment at 25-30°C.

Determination of shelflife of bio-inoculants in bio-fertilizer: Viable counts were taken to determine the shelflife of the bio-inoculants. The viability of the organisms was tested at 0, 30, and 365 days. Standard spread plate procedures using TSA media were used to determine the total viable microbial count in the stored bio-fertilizer.

Evaluation of the effectiveness of the bio-fertilizer: The formulated bio-fertilizer was applied on sponge gourd (*Luffa aegyptiaca*) plants to investigate the effects on the growth of the plants. The natural unsterile alluvial soil was used for this purpose. The planting soil was prepared by combining alluvial soil and sand in a 3:1 ratio. Sponge gourd seeds were collected from a local market in Dhaka. To investigate the growth promoting activity, two pots (Pot A and B) were prepared. Pot A contained 3000 g regular planting soil as the negative control, and Pot B contained 600 g bio-fertilizer and 2400 g regular soil (25 g bio-fertilizer/100 g soil). The seeds were then sowed in both pots. Regular watering and monitoring were carried out while maintaining the same environmental conditions. Shoot length of each sponge gourd plant was measured after 7, 15, 30, and 45 days of sowing the plant.

Statistical analysis: Analysis of Variance (ANOVA) for different groups was accomplished using the SPSS vs. 24.0. The Tukey HSD test was performed to detect the significant differences ($p< 0.05$) between means. All graphs were prepared using GraphPad Prism vs. 8.0.

Results and Discussion

Antimicrobial susceptibility of the bio-inoculants: Antibiotic susceptibility pattern of the candidate bacteria was tested, which showed resistance to some antibiotics and sensitivity to many other antibiotics. The antibiotic susceptibility pattern of *Rhizobium* sp. and *Azotobacter* sp. was done against 26 antibiotics. *Rhizobium* sp. showed resistance against five antibiotics (amoxicillin, aztreonam, ampicillin, cephalexin, and oxacillin), and *Azotobacter* sp. showed resistance against four antibiotics (amoxicillin, ampicillin, cephalexin, and oxacillin). Genetic mutation is a grave fact that can increase the resistance by changing the genetic code of DNA due to chemical agents and the transfer of the resistant gene from a virulent to a susceptible pathogen[17]. However, bio-fertilizers organism's resistance to antibiotics has some advantages. If the field soils contain some sort of antibiotics, the bio-inoculants of the bio-fertilizer would be inhibited. But in this case, the used organisms would not be inhibited efficiently as these organisms possess resistance to some antibiotics. A previous study showed that the resistance of *Rhizobium* to antibiotics increases its survivability in the soil[18]. Therefore, these candidate antibiotic-resistant *Rhizobium* sp. and *Azotobacter* sp. would endure in an antibiotic stressed state and help increase soil productivity.

Growth curve of Rhizobium sp. and Azotobacter sp.: The growth curves were obtained to determine the duration of log phases of both candidates (Fig. 1). *Rhizobium* sp. and *Azotobacter* sp. entered into the log phase of growth within 6 and 3 h of inoculation,

respectively. Both inoculants entered into the stationary phase of growth after around 18 h of inoculation into media.

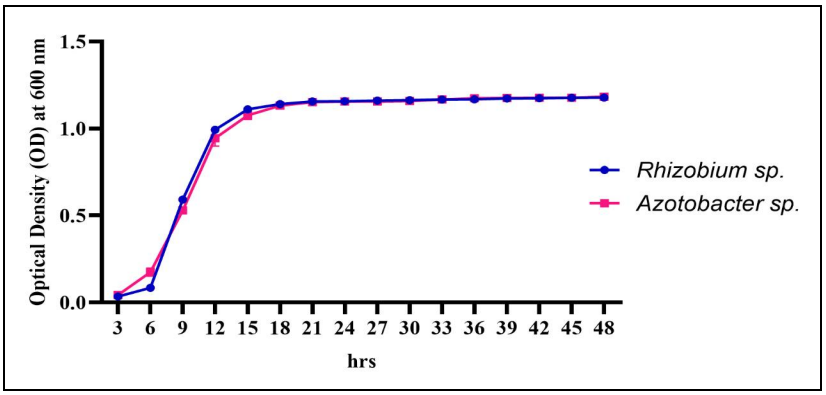

Fig. 1. Growth curve of *Rhizobium* sp. and *Azotobacter* sp.

Optimized conditions for maximum production of the biomass: In this study, the growth parameters of *Rhizobium* sp., *Azotobacter* sp., and *Trichoderma* sp. were optimized for cheaper production of the maximum biomass. These candidate organisms were grown in a molasses-containing medium to reduce the cost of biomass production. Molasses is one of the byproducts of the sugar industry in our country, which is readily available and a very economical source for growing these organisms. All of these organisms showed significant growth in the molasses-containing medium. Both *Rhizobium* sp. and *Azotobacter* sp. showed maximum growth (7.78 CFU/ml and 7.78 CFU/ml, respectively) in 0.4% molasses media with pH 8.0 (Fig. 2a and Fig. 2b). On the other hand, *Trichoderma* sp. showed maximum growth (7.85 CFU/ml) in 0.4% molasses containing media at pH 7.0 (Fig. 2c).

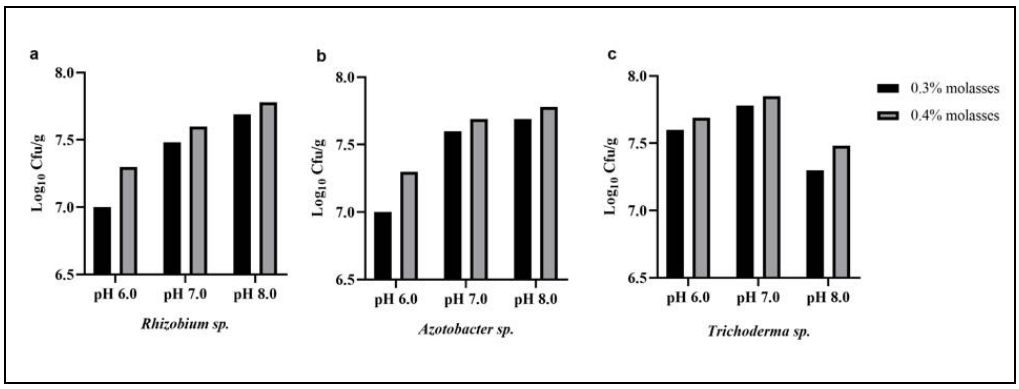

Fig. 2. Determination of media composition for best economic production of bio-fertilizer. a) Optimized molasses concentration (0.4%) and best pH condition (8.0) for *Rhizobium* sp., b) Optimized molasses concentration (0.4%) and best pH condition (8.0) for *Azotobacter* sp., c) Optimized molasses concentration (0.4%) and best pH condition (7.0) for *Trichoderma* sp.

Shelflife of bio-fertilizer: A good bio-formulation should have a good shelflife and be non-polluting, effective, and readily biodegradable. Hence, the shelflife estimation is an essential step of bio-fertilizer formulation [19,20]. Storage conditions, formulation strategy, carrier, and packaging material affect the shelf life of inoculants in bio-fertilizer[21]. The stability and viability of bio-fertilizers were determined for 12 months under room temperature storage (25 ± 2°C). *Rhizobium* sp., and *Azotobacter* sp., were grown in media containing 0.4% molasses with pH 8.0. *Trichoderma* sp. was grown in media containing 0.4% molasses with pH 7.0. Before mixing with the carrier materials, the total viable count of these organisms was determined. The total viable count of *Rhizobium* sp., and *Azotobacter* sp., and *Trichoderma* sp., was 5×10^7 CFU/ml, 5×10^7 CFU/ml, and 6×10^7 CFU/ml, respectively. After mixing with the sterile carrier materials, the total viable count of microorganisms in the bio-fertilizer was 8×10^7 CFU/g. After 30 days of storage, the total viable count of microorganisms in the stored bio-fertilizer was slightly increased to 2.2×10^8 CFU/g. After one year, the total viable count of microorganisms in the formulated bio-fertilizer was also increased to 2.2×10^9 CFU/g, demonstrating carrier materials' ability to support the growth of the inoculants. In this present study, the shelflife of bio-fertilizer was improved by allowing the microorganism to grow in a non-competitive micro-environment through pre-sterilization of the carrier materials (rice husk ash and soil), as previously reported by Yardin *et al*.[22]. The organisms might be propagated in the bio-fertilizer during storage at room temperature due to the nutrient content of molasses, rice husk ash, and soil[23].

Cost-effectiveness of the formulated bio-fertilizer: The market price of alluvial soil was 25 BDT\kg, rice husk ash 20 BDT\kg, and molasses 45 BDT/kg. In the experiment, one kg soil (25 BDT), half kg rice husk ash (10 BDT), and 1.6g molasses (to grow microorganisms) were used to make bio-fertilizer. In total, it costs about 36 BDT to make 1.5 kg of bio-fertilizer. In the market, the price of available bio-fertilizer is 150 BDT\kg, but in this study, it costs only about 24 BDT/kg of bio-fertilizer. However, the commercialization of bio-fertilizers depends on the survivability of candidate organisms in the formulations for a more extended period [24]. For industrial application, the carrier materials must support the viability of the organisms for at least 2 to 3 months[20,25]. The rice husk ash and soil formulation proved to be a cheaper and suitable carrier material for the bio-fertilizer formulation, and after 12^{th} month of storage, the total viable count indicates its suitability for commercial purposes.

Evaluation of bio-fertilizer on the growth of sponge gourd plants: Bio-fertilizer treatment improved the growth of sponge gourd plants compared with those of control. The shoot growth of sponge gourd plants was excellent and high in bio-fertilizer mixed soil compared to that grown in regular soil. The growth of sponge gourd plants after 7, 15, 30, and 45 days of sowing in regular soil and bio-fertilizer treated soils are presented in Figs 3-4.

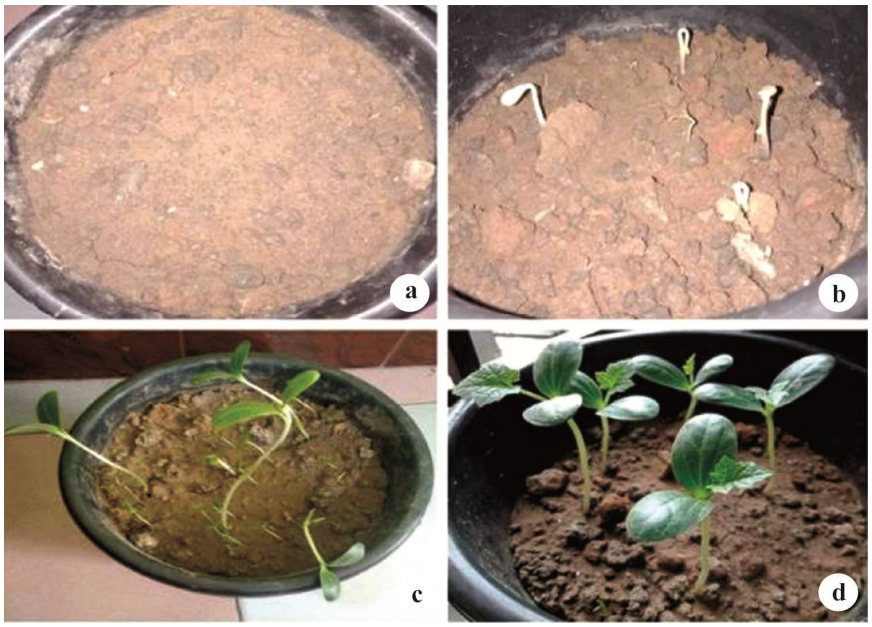

Fig. 3. Growth of sponge gourd after 7 days (a and b) and 15 days (c and d) of sowing. Here, a) Pot A (regular soil) after 7 days, b) Pot B (25 g bio-fertilizer/100 g of soil) after 7 days, c) Pot A after 15 days, and d) Pot B after 15 days.

Fig. 4. Growth of sponge gourd after 30 days (a and b) and 45 days (c and d) of sowing. Here, a) Pot A (regular soil) after 30 days, b) Pot B (25 g bio-fertilizer/100 g of soil) after 30 days, c) Pot A after 45 days, and d) Pot B after 45 days.

The effects of bio-fertilizer (25 g bio-fertilizer/100 g soil) treatments on the shoot length of sponge gourd plants for 45 days from sowing are presented in Fig. 5. The shoot length of sponge gourd plants grown in regular soil (Pot A) had the lowest value ($p<0.01$) of shoot length on each successive day of interval compared to that grown in bio-fertilizer mixed soil (Pot B). The highest difference of shoot length ($p<0.01$) of sponge gourd plant was observed after 45 days.

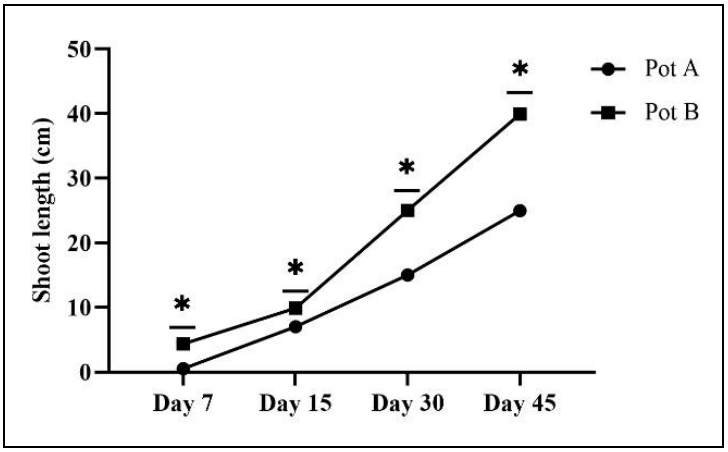

Fig. 5. Effects of bio-fertilizer treatments on shoot length of sponge gourd plants for 45 days from sowing. Pot A: Regular soil, Pot B: Bio-fertilizer mixed soil (25 g bio-fertilizer/100 g soil). * indicates significant differences between Pot A and Pot B ($p<0.01$).

A similar elevation of crop yield (around 10-40 %) through bio-fertilizer was reported by Bhardwaj *et al*.[9]. Bio-fertilizer usually increase soil fertility by fixing nitrogen and solubilization of phosphates[26]. *Rhizobium* sp. is well known to increase plant growth by producing phytohormones such as IAA, gibberellic acid and cytokinins[27]. Gholami *et al* reported that *Azotobacter* increases plant growth by enhancing seed germination[28]. The present study found that the germination of sponge gourd seeds in bio-fertilizer mixed soil was faster than in regular soil. Hossain and Sumon reported that N contents, N uptake by seed and shoot growth of plant was significantly influenced through dual effect of *Azotobacter* and *Rhizobium*[29]. *Rhizobium* increased grain yield compared to control as reported by Hossain[30]. Plant growth stimulation by *Trichoderma* sp. has been reported in pea and tomato[31]. *Trichoderma* has the ability to solubilize phosphate and micronutrients that could be made available to plant[32]. The candidate organisms in bio-fertilizer might increase plant height by improving the nutrient status of the soil.

As the agricultural waste residues (rice husk ash) are readily available and cost-effective, their use as carriers for the formulation of bio-fertilizer is a commonly analyzed choice [33]. In this study, rice husk ash and soil were used as carrier materials for bio-fertilizer formulation. Rice husk ash can increase the nutrient content of the soil, the yield

of crops, sequestration of carbon, retention of water, decrease the leaching of nitrogen and reduce the toxicity in soils[34]. Rice husk ash and soil are readily available in our country and are the cheapest substrate compared to peat, powder, and granule, which are also used as the carrier for bio-fertilizer formulation. The shelf life of bio-inoculants in bio-fertilizer was also found satisfactory. Any carrier material should maintain microorganisms in a viable state. In this study, the viable count of bio-inoculant was increased during the entire storage period. Rice husk ash enables the non-competitive multiplication of candidate organisms and maintenance of the candidates in a nutrient-rich environment.

However, this study comes with some limitations. The bio-fertilizer application was carried out on a small scale. It is necessary to carry out large-scale field applications to support the findings.

Conclusion

The production of crops is dependent primarily on chemical fertilizers, which has a dangerous impact on human health and the environment. The study's objective was the cost-effective production and formulation of bio-fertilizer, which was achieved through using molasses as a nutrient source for bio-inoculants and rice husk ash and soil as carrier materials. The formulated bio-fertilizer improved plants' growth and production. Shelflife of the bio-fertilizer was also found quite impressive. The large-scale production of inoculum using the by products or wastes of the industry as components of culture media and formulations is a way to decrease the costs of agricultural technologies.

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