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Synergism and Compatibility Study of Micro-Encapsulated Formulation of Nitrogen-Fixing Bacteria with Improved Viability and Functionality for Tropical Regions

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Abstract

This study is aimed at evaluating the impact of spray drying on survival and retention of functional properties of nitrogen–fixing bacteria (*Bradyrhizobium japonicum*). The impact of spray drying on physiochemical properties of encapsulated material and nitrogen fixing ability of bacteria under greenhouse conditions were investigated. The results showed 90% survivability of microencapsulated *Bradyrhizobium japonicum* with natural polymer after spray drying. The co-inoculation of microencapsulated *B. japonicum* and Mycorrhizae showed synergistic interactions. It was found to be effective for enhancing the growth of soybean plant. Our results confirmed that micro-encapsulation of *B. japonicum* is an effective approach to develop more viable and stable biological formulations.

Keywords: *Bradyrhizobium japonicum,* Microencapsulation, Mycorrhizae, Spray drying, Seed coating

Introduction

Soybean is considered as a rich source of protein for human and animal nutrition. Also, it is one of the most economically relevant crops in agriculture across the globe. Unfortunately, the excessive use of chemical fertilizers especially nitrogen fertilizers to increase the production of soybean has raised various environmental concerns related to soil fertility and soil biodiversity. Therefore, in order to reduce the dependency of farmers on chemical fertilizers, novel strategies are needed. In this context, nitrogen fixing bacteria (NFB) have been suggested as an attractive alternative to reduce the application of N- based fertilizers on soils by farmers [1].

Currently, biofertilizers based on NFB are widely investigated and commercialized as an alternative to traditional nitrogen based fertilizers. Most of the NFB could not produce spores like *Bacillus* sp. against adverse external environment conditions and different storage conditions resulting low shelf life of the biofertilizer. Hence, an optimized microencapsulation process for such NFB requires stabilizing cells, potentially enhancing their viability and stability in the production, storage and handling. Encapsulated cell formulations have several advantages over free cell formulations for example, encapsulation of cells protects them from biotic stresses [2], and abiotic stresses (the inhibitory effect of toxic compounds) [3], improves physiological activity [4], provides enhanced survival and supply of encapsulated nutritional additives [5], increases cell densities and preferential cell growth in various internal aerobic and anaerobic zones of encapsulated material.

Reports on the spray drying of bacteria are scarce but [6] some studies showed promising results on the dehydration of microorganisms by thermal process indicating that the highest survival is between less than 1% and 100% dependent on the different growth media, drying media, drying systems and microorganisms. Similarly, Campos et al. [1], have done microencapsulation of bacterial cells by spray drying with 79% of survival rate. The present study was undertaken to characterize and compare the effects of spray dried *B. japonicum* on survival, viability and retention of nitrogen-fixing property under greenhouse conditions with other different bioformulation.

Materials and Methods

Collection of the Bradyrhizobium culture

The bacterial strain (*Bradyrhizobium japonicum*) was collected from the culture collection bank of The Energy and Resources Institute (TERI), New Delhi, India. Culture was revived by streaking it on yeast extract mannitol agar supplemented with 0.25% Congo red dye (CRYEMA). The YMA plates were incubated at 28±2°C for 4 days and colonies showing a slightly raised, colorless and opaque phenotype were selected. The primary identification of the strain was based on gram stain and morphology [7].

Feed preparation for bacterial cell microencapsulation

Feed (100 ml) was prepared by centrifuging 1000 ml of the *B. japonicum* culture broth at 8,000 g for 10 minutes at 4°C. After this, the supernatant was discarded and the pellet was kept at 4°C. Gum acacia and maltodextrin are selected for immobilization of living cells. They provide good mechanical stability and easy release of the encapsulated cells. Different combinations were developed for preparing feed (Table 1), by dissolving cell biomass into sterilized 30 ml of distilled water. Followed by addition of this mixture into phosphate buffer and final volume of every combination was made upto 100 ml. The feed suspension was kept in an orbital shaker at 30°C at 150 rpm for 30 minutes so that the cells get adapted to the suspension medium and used subsequently for drying.

	Composition 1	Composition 2	Composition 3
Gum acacia	6%	3%	1%
Maltodextrin	30%	13%	15%
KCl	0.1%	0.3%	0.1%
Sucrose	2%	1%	0.5%
Cell cake	4%	4%	4%

Table1. Formulation of the feed for spray drying

Spray drying conditions

In order to synthesize microspheres loaded with microbes, encapsulation of *B. japonicum* utilizing the optimized procedure was performed. The optimized parameters such as inlet temperature (Ti), outlet temperature (To), aspirator rate, pump percent efficiency were used in the Buchi Mini Spray dryer (B-290). The aspirator was set to 100%, and compressed air from zero air cylinder at 5 bar with a flow rate of 40 units was passed into the nozzle through air inlet. The feed was passed through the feed-inlet. The inlet temperature was set as 65°-70°C. The outlet air temperature was maintained around 30-32°C by controlling feed pump rate in the range 20-23%. After the completion of each experimental run, the powder was collected from the cyclone and cylindrical parts of the dryer chamber by lightly sweeping the chamber wall as described by Bhandari et al. [8]. The powders were then packed in sterilized glass vials and stored in desiccator for further analysis.

Solid powder recovery and moisture content

The percentage of solid powder recovery was calculated based on following equation: Percentage (%) solids powder recovery = Sp/Sf x100. Where Sf is the estimated dry weight of solids in feed, and Sp is the dry weight of solids collected in product collector. The main objective of calculating the solid powder recovery was to know the loss of solid powder due to its deposition in drying chamber and cyclone separator. Moisture content was determined using standard laboratory protocol.

Viable cell count

The viability in microencapsulated product was estimated. Known weight (about 1 g) of microencapsulated product was reconstituted in 10 ml of buffer media (0.1mM PBS. pH 7.2) and kept under mild stirring condition at low temperature (4°C) over night. Serial dilutions (10^{-1} –10^{-7}) were made in sterile saline solution (NaCl 0.85 %) and then 0.1 mL of each dilution was spread onto CRYMA plates. Colony forming units (CFU) were enumerated after incubation at 30°C till 72 h by using standard protocol.

Morphology of microcapsule

The microcapsule morphology was visualized by using scanning electronic microscopy (SEM). The dried powders of microcapsules were first fixed on aluminum stubs. These stubs were then coated with carbon tape and metalized with palladium using a sputter Coater. After this, the observations were taken in a scanning electronic microscope (Zeiss EVO MA10, London, UK) at 10kv voltage.

Effect of encapsulated formulation on growth parameters under greenhouse conditions

Efficacy evaluation of encapsulated formulation on growth parameters such as plant height, total shoot biomass, root fresh and dry biomass, number of root nodules, nodule fresh and dry biomass per plant was performed with six replicates with the following treatments: + N: Inorganic nitrogen; -N: Non treated control; T1: seed coating with lyophilized inoculum; T2: liquid inoculation; T3: seed coating with encapsulated inoculum. T4: liquid inoculum of *B. japonicum* and TERI-AMF (*Glomus intraradices*) at the rate 10 spore/seed, T5: encapsulated *B. japonicum* and TERI-AMF (*Glomus intraradices*) at the rate 10 spore /seed, %N: nitrogen content. They were analyzed by a multivariate analysis using principal components (PCA) using five replicates per treatment and performed with XLSTAT software.

Nitrogen uptake

Total nitrogen in shoot was estimated by Kjeldahl digestion method [9] using the following equation:

% Nitrogen = (Sample titer value – blank titer value) × 0.1N H_2SO_4 × 14 × 100

Mycorrhizal colonization in plant roots

The percentage of root colonization was estimated by the modified method as described by Giovanneti and Mosse [10]. Data were recorded as frequency distributions from samples containing 25, 50, and 100 segments.

Statistical analysis

All data was analyzed using a commercial software package (SPSS statistics 21, IBM). One way analysis variance (ANOVA) was used to determine nitrogen content in all the treatments. Statistical significance was determined at the $P \leq 0.05$ level using Duncan post hoc test. All the results were presented as average value ± standard deviation of three replicates.

Results and Discussion

Microencapsulated bacterial cells

The three different compositions of the feed mixtures had shown variance in cell survivability depending on the inlet and outlet temperatures. The maximum 58% solid encapsulated powder was recovered by using the composition 3 with viable cell $1x10^8$cfu g-1 and with 12% moisture content (Table 2). The results from this study showed that it is possible to produce a spray dried bacteria powder using a less expensive process than freeze drying with a high level of cells ($>10^{10}$cfu g-1) and viability during the process. Improvements in the bacteria (type, resistance to stress, temperature, etc.), and in the process, could improve the effectiveness of the drying of non-sporulating bacteria [6].

Morphology of microencapsule

Morphology study of microencapsule was performed by using scanning electron microscope (SEM). It provided information on the degree of integrity and porosity of the microcapsules [11]. The diameter of the spherical microcapsules obtained in this study ranged from 10-100 µm as shown in Figure.1. The outer surfaces of the microcapsules were characterized by the presence of indentation. No cracks were also found.

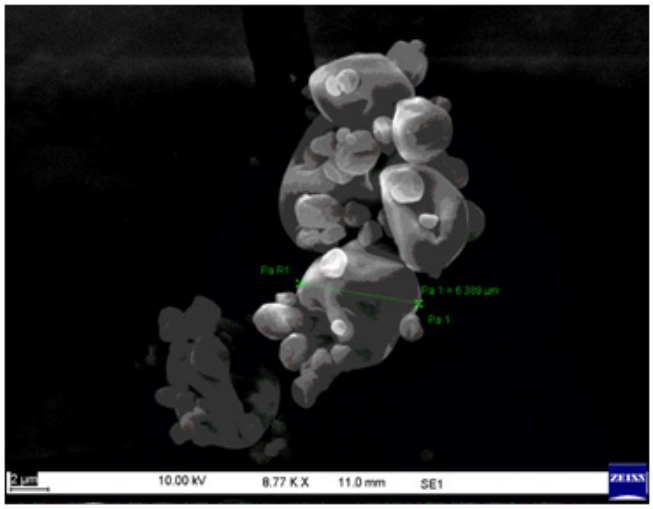

Figure 1: Results of the synthesized microcapsule containing *Bradyrhizobium japonicum.*

Greenhouse experiments for the assessment of plant growth promotion

Inoculation with encapsulated *B. japonicum* + *G. intraradices* showed higher response for all the growth parameter such as shoot height, fresh weights of roots and shoots, dry weight of roots and shoot when compared with other treatments including the controls. However, inoculation with micro-encapsulated *Bradyrhizobium japonicum* alone also recorded higher performance in all the measured parameters over the other treatments (Table 3). Root development was also positively responded to the bacterial inoculation. There was 2% increase in root biomass of the plants of T3 treatment (seed coating with encapsulated inoculum) when compared to other treatments. Co-inoculation of G. intaradices and encapsulated *Bradyrhizobium japonicum* led to higher nodulation and nodule biomass (Table 4). A significant increase in nodule number as well as nitrogen uptake was observed in treatments with seed coating with encapsulated inoculum (T3) and encapsulated *Bradyrhizobium japonicum* and TERI-AMF (T5). Whereas, there was no significant difference between (liquid inoculation) T2 and seed coating with encapsulated inoculum (T3).Remarkable outcome of this in vivo study was that the encapsulated inoculum outperformed the T1 treatment (lyophilized *Bradyrhizobium japonicum*) and was almost equal to positive control (with nitrogen).

Table 2: Detail observations for each parameter with specific feed input

Composition No.	Rh (%)	RT(°C)	Inlet(°C)	Outlet (°C)	Feed flow rate (%)	Soil powder recovery (%)	CFU per gram powder	Moisture content (%)	Survivability (%)
1	41.9	28.0	70	32	23	55	$5.0x10^7$	12.8	86
2	37.0	28.0	70	30	22	56	$8.0x10^7$	12.6	88
3	37.0	28.0	65	30	20	58	$1.0 x10^8$	12.0	90

Table 3: Effect of *Bradyrhizobium japonicum* on shoot and root biomass of soybean (height, fresh and dry weight) grown under controlled conditions in greenhouse at TERI GRAM, Gurgaon, India*.

Treatments	Shoot height (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	Root fresh weight(g)	Root dry weight(g)	%N
+N	30.56 ± 0.54bc	3.20 ± 0.05bc	0.61 ± 0.008bc	0.37 ± 0.007b	0.04 ± 0.0011a	3.20 ± 0.06a
-N	26.30 ± 0.47c	1.40 ± 0.03e	0.18 ± 0.003d	0.09 ± 0.002d	0.01 ± 0.0008d	0.59 ± 0.02c
T1	31.68± 0.62b	2.32 ± 0.14d	0.35 ± 0.016c	0.20 ± 0.023c	0.02 ± 0.0018c	2.41 ± 0.09b
T2	33.5167 ± 1.19ab	3.22 ± 0.07b	0.64 ± 0.019ab	0.39 ± 0.014ab	0.04 ± 0.0012a	3.15 ± 0.06ab
T3	33.75 ± 0.45ab	3.46 ± 0.18ab	0.65 ± 0.020ab	0.39 ± 0.016ab	0.03 ± 0.0017b	3.14 ± 0.10ab
T4	34.50 ± 1.19a	3.18 ± 0.07c	0.63 ± 0.019b	0.401 ± 0.014a	0.04 ± 0.0012a	3.20 ± 0.06a
T5	34.64 ± 0.45a	3.52 ± 0.18a	0.73 ± 0.020a	0.42 ± 0.016a	0.04 ± 0.0017a	3.21 ± 0.10a

+ N: Inorganic nitrogen; -N: Non treated control; T1: seed coating with lyophilized inoculum; T2: liquid inoculation; T3: seed coating with encapsulated inoculum. T4: liquid inoculum of *Bradyrhizobium japonicum* and TERI-AMF (Glomus intraradices) at the rate 10 spore/seed, T5: encapsulated B. japonicum and TERI-AMF (*Glomus intraradices*) at the rate 10 spore /seed, %N: nitrogen content. Values with different letters(s) in a column are significantly different ($p \leq 0.05$). Data reported as mean ±SE for 6 samples. *All values are mean of three replications (five plants from each treatment). Values in the column followed by different letters indicate significant difference to Duncan's post hoc analysis at 90 days after sowing.

Table 4: Effect of B. japonicuminoculation on nodule number, fresh and dry weight of soybean plant.

Treatments	Number of Nodules	Nodule fresh weight	Nodule dry weight
T1	5.3 ± 0.33c	0.13 ± 0.01c	0.037 ± 0.06e
T2	8.2 ± 0.33b	0.29 ± 0.04bc	0.07 ± 0.05d
T3	8.3 ± 0.33b	0.31 ± 0.01b	0.08 ± 0.03c
T4	10.8 ± 0.54ab	0.38 ± 0.06a	0.09 ± 0.08b
T5	11.5 ± 0.5a	0.40 ± 0.03a	0.12 ± 0.06a

*All values are mean of three replications (five plants from each treatment). Values in the column followed by different letters indicate significant difference to Duncan's post hoc analysis at 90 days after sowing.

These positive effects of nitrogen fixing strain on plant growth are correlated with changes in root morphology, normally increasing the lateral root and root hair number and length [12]. In addition, this study also confirms that encapsulated process is effective and enhances the viability of cell numbers. An increase in nodule number showed that the encapsulated B. japonicum carried higher population of cells and the slow release property of Maltodextrin and gum acacia could represent an attractive approach to be used as wall materials in encapsulation of non-sporulating bacterial cells.

Multifactorial comparison of the greenhouse experiments using PCA

Correlation analysis clearly indicates relationship between the measured parameters and their co-ordinates (treatments). The principal component analysis (PCA) and their correlation are shown in Figure.2. The first two factorial axes represent 97.46% of the variance of the data. Parameters such as root fresh weight, shoot dry weight, shoot fresh weight and N uptake clustered together at positive side of both factors while root dry weight was located at strong positive side of F1. Parameters such as shoot height were located at slightly negative side of factor F2. In terms of treatments, the PCA revealed that five treatments were clustered into four groups. The first group has strong positive correlation with both the factors (F1 & F2) and this group included treatments like inorganic nitrogen (+N), liquid inoculation (T2): liquid inoculum of *Bradyrhizobium japonicum* and TERI-AMF (T4), and encapsulated *Bradyrhizobium japonicum*and TERI-AMF (T5), while second group containing treatment (T3) was more correlated with factor F1 and negatively correlated with factor F2. Third group was negatively correlated with the both the factors and contained seed coating with lyophilized inoculum (T1) treatment. Fourth group contained only negative control (-N) which was strongly correlated with factor F1 and less positive correlated with factor F2.

Mycorrhizal colonization

To better understand the effect of AMF (*Glomus intraradices*) inoculation on the growth of soybean, mycorrhizal colonization was assessed and the results are shown in Figure.3. The colonization in root samples was higher when the plants inoculated only with *Glomus intraradices* and was lower in plants when inoculation with B. japonicum and *Glomus intraradices* was performed. The highest value was 92% in T6 followed by 85% in T5 in the 130-day samples.Co- inoculation of encapsulated *Bradyrhizobium japonicum* and *Glomus intraradices* enhanced nitrogen uptake and nodule formation when compared with inoculation of encapsulated *Bradyrhizobium japonicum* alone (Table 4).This can be attributed to balanced nutrition received by plants and due to improved absorption of minerals like N and P or a general improvement in the development of roots [13-14].

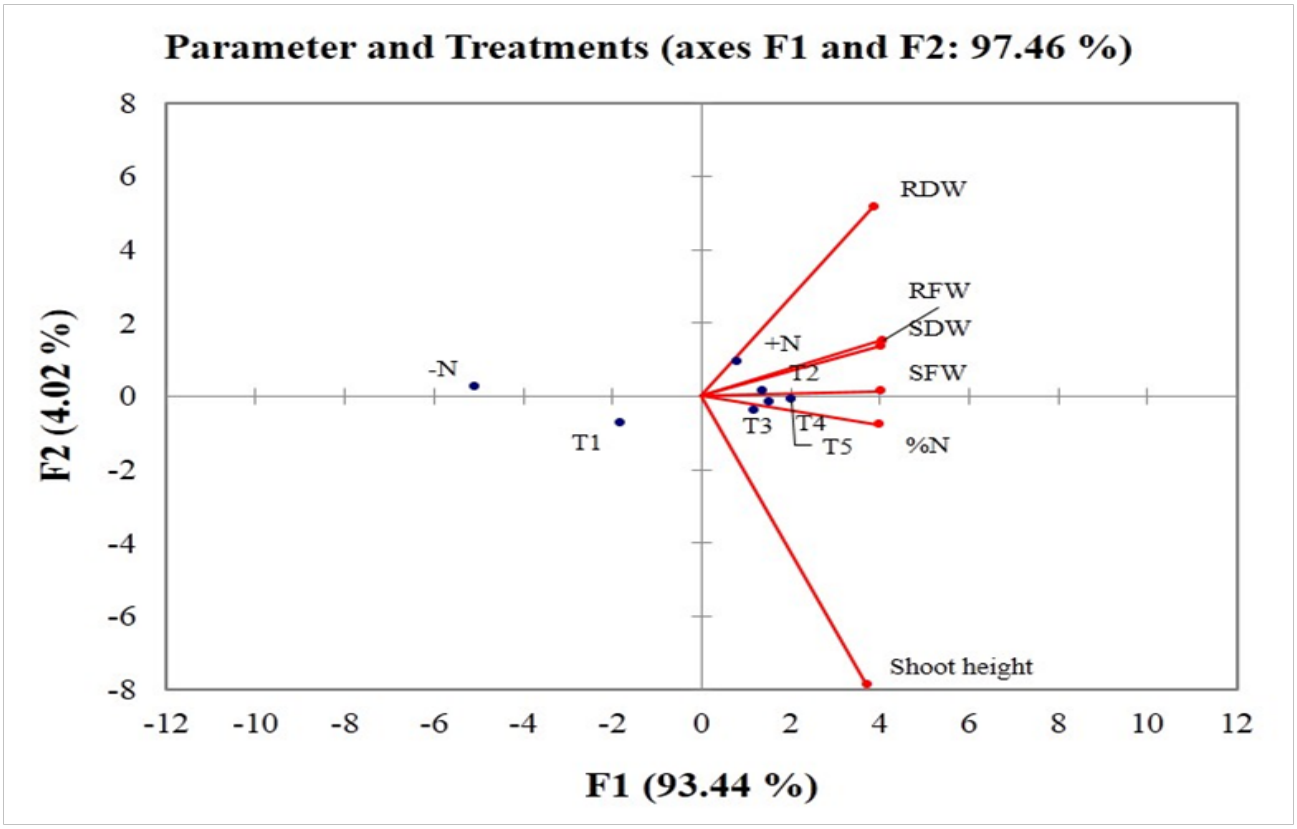

Figure2: Multifactorial comparison of the greenhouse experiments using PCA. Correlation between six measured parameters. Parameters codes RDW root dry weight, SDW shoot dry weight, SFW shoot fresh weight, RFW root fresh weight, % N nitrogen uptake. Treatments codes + N: Inorganic nitrogen; -N: Non treated control; T1: seed coating with lyophilized inoculum; T2: liquid inoculation; T3: seed coating with encapsulated inoculum. T4: liquid inoculum of *Bradyrhizobium japonicum* and TERI-AMF at the rate 10 spore/seed, T5: encapsulated *Bradyrhizobium japonicum* and TERI-AMF at the rate 10 spore /seed,

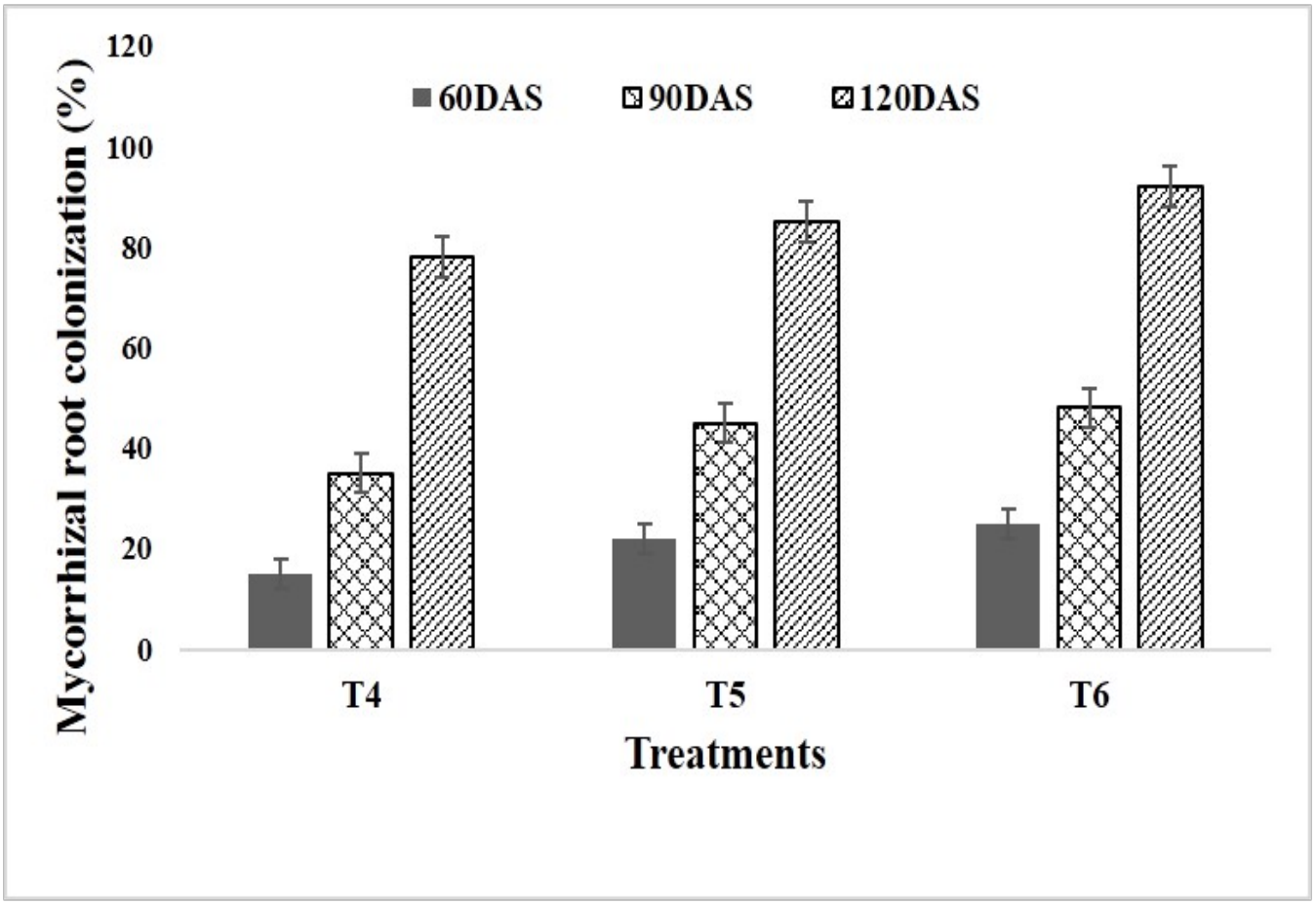

Figure 3: Mycorrhizal (AMF) root colonization of soybean roots at 60, 90 and 120 day after sowing (DAS). A total of 400 root samples were analyzed over the course of this experiment. Treatment codes T4: liquid inoculum of *Bradyrhizobium japonicum* and TERI-AMF at the rate 10 spore/seed, T5: encapsulated *Bradyrhizobium japonicum* and TERI-AMF at the rate 10 spore /seed, T6: only AMF at the rate 10 spore /seed.

Conclusions

In conclusion, this study provides valuable information regarding the practical application of microencapsulation of microbes to be used by farmers in fields as bio-inoculants with improved shelf-life. Furthermore, by incorporating bio-inoculant in seed coating process, seed coating companies can provide farmers with convenient ready-to-use product.

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