Journal of Research High School

Vol. 2023 (2) 48 – 56
ISSN 2688-3651 [online]

Effect of Coating Seeds with Micronutrients and Bacterial Consortia on Stomatal Conductance and Yield of Cluster Bean

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Received October 22, 2022; Revised June 20, 2023; Accepted, July 11, 2023

Abstract

The study aims to evaluate the effect of coating seeds with micronutrients in combination with bacterial consortia on stomatal conductance and yields of cluster bean. Greenhouse experiments were carried out during August – December 2021 at Prayoga Institute of Education Research, Bangalore, India. The experiment consisted of 8 different treatments laid out in a randomized block design with five replications. Plant growth-promoting rhizobacteria (PGPR) are known to influence plant growth by various direct or indirect mechanisms. In search of efficient PGPR strains with multiple activities, a total of 10 bacterial strains were isolated from different rhizosphere soil and plant root nodules in the vicinity of Prayoga campus. These test isolates were screened in vitro for their plant growth-promoting traits like the production of *Indoleacetic acid,* production of *Ammonia* and *Phosphate solubilization* and three isolates were selected to develop consortia in different combinations. The micronutrients and bacterial consortia were applied to the seeds in combination as per the studied treatments and drought stress was created during growth stage. The study result revealed improved yields with a decrease in stomatal conductance due to plant adaptation to the stress induced by drought. There was a significant difference in the seed yield of treated treatments with that of the untreated control. Stomatal conductance (mmol m−2 s−1) of five randomly tagged plants was recorded by using a leaf porometer after 40 days and seed yield was recorded. Hence, coating seeds with bacterial consortia and micronutrients had a significant influence on the stomatal conductance and seed yield of cluster bean and this approach can serve as an effective strategy to enhance cluster bean crop performance.

Keywords: Cluster bean, Micronutrients, Rhizobacteria, Seed coating, Stomatal conductance, Yield

1. Introduction

Agriculture provides the principal means of livelihood for the majority of the Indian population. Modern agriculture is not sustainable in long run; Modern agriculture, characterized by intensive practices and heavy reliance on synthetic inputs, has been instrumental in meeting the global demand for food. However, mounting evidence suggests that this system is not sustainable in the long run. Hence the concept of sustainable agriculture has emerged in recent years with an emphasis more on the conservation of natural resources and the environment. Cluster bean (*Cyamopsis tetragonoloba L. Taub*.) or Guar is a major vegetable and industrial crop grown for its tender pods and endospermic gum (30-35 %). Tender pods are rich in nutrients: they contain 16 Kcal of energy, 3.2 grams of protein, 1.4 grams of fat, 10.8 grams of carbohydrate, 65.3 IU of Vitamin A, 49 mg of Vitamin C, 57 mg of calcium and 4.5 mg of iron for every100g of an edible portion (Ashraf & Iram, 2005). Due to their hardiness and tolerance for poor soil and moisture stress conditions, cluster bean is mostly grown in rainfed conditions in arid and semi-arid parts of tropical India. India produces over 80% of the world's cluster bean production (Punia, *et al*., 2009).

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Cluster bean seeds are primarily used for extracting endospermic gum, which has excellent binding characteristics and is in high demand in the culinary industry as an ingredient in sauces and ice creams, among other things. Guar gum is also utilized as a sizing agent, thickener, stabilizer, protective colloid, absorbent, flocculating agent in various industries (Sabahelkheir, *et al*., 2012; Srivastava, *et al*., 2011). Cluster bean pods are utilized as a treatment for diabetes patients, in addition to having a high caloric and nutritional content. For monogastric animals, cluster bean meal (high protein content) produced from the seed coat and germ cell is a good feed. For export, cultivars with a high gum content (>32 %) and viscosity (4000-5000 cps) are desirable (Kumar & Rodge, 2012; Meena & Asrey, 2018). In addition, cluster bean is grown as a green manuring crop in different parts of the world. The husk of cluster bean is used for cattle feed because it contains high protein content (Goudar, *et al*., 2016).

Despite their nutritional importance, cluster bean production has remained low even during the green revolution era. One of the many factors contributing to the cluster bean's low production is a lack of micronutrients in addition to macronutrients. Exogenous sources of these micronutrients are badly needed in different cropping systems due to excessive removal by high-yielding varieties. Micronutrients are extremely effective in little doses to generate optimal results. Micronutrients are important for increasing agricultural productivity. Zn insufficiency is a common micronutrient deficiency in Indian soils. Molybdenum deficiency, by and large, is associated with acid soils. Micronutrient insufficiency was caused by intensification of agriculture with high-yielding crop types, continual application of high-analysis chemical fertilisers, limited supply of organic manures, and little crop residue return to the soil. Micronutrients are required in lesser amounts than macronutrients, yet they are just as vital. Growth suppression or even complete inhibition can occur if any of these components are deficient in the soil or are not properly balanced with other nutrients. In addition to serving as cofactors in enzyme systems and participating in redox reactions, micronutrients play a number of other important roles in plants. Most notably, micronutrients are involved in the fundamental physiological processes of photosynthesis and respiration, and their lack can obstruct these activities thus limiting yield gain in many crops (Mengel, *et al*., 2001). In most crops, seed treatment with micronutrients improves stand establishment, accelerates phenological events, boosts yield, and increases micronutrient content in grain. In some cases, seed treatment with micronutrients outperformed comparable to soil application and foliar spray techniques (Singh and Gandhi, 2015; Quddus, *et al*., 2020). Seed treatment with polymer coating is a simple and cost-effective strategy for resource-poor farmers because of its pronounced influence during the early stages of seedling establishment (Johnson, *et al*., 2005). These treatments can also alter the stomatal response which can play a key role in plant adaptation.

Stomata consist of pores scattered over the relatively waterproof and CO2-tight cuticle covering the leaf surface. Stomata play a key role in plant adaptation to changing environmental conditions as they control both water losses and CO_2 uptake. Stomatal conductance (gl) is a measure of the degree of stomatal opening and can be used as an indicator of plant water status. Stomatal conductance (mmol m−2 s−1) measured by a porometer is the rate of CO2 entering, or water vapour exiting through stomata (Damour, *et al*., 2010), which is dictated by the degree of stomatal opening. In irrigated trials, the handheld porometer allows for quick measurement of leaf stomatal conductance and is suggested for use under water stress (unless extremely slight) because the stomata are normally closed (Shinde, *et al*., 2016).The rhizosphere is a complex ecology in which plant impacts on soil microbes and microorganism effects on plants interact and are interdependent (Mukerji, *et al*., 2006).

Plant-growth promoting rhizobacteria (PGPR) are a type of bacteria that colonizes the roots of plants and promotes their growth (Ghadamgahi, *et al.,* 2022). Sitepu (2008) proved that a variety of bacteria from the genera *Azospirillum, Alcaligenes, Acinetobacter, Bacillus, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Pseudomonas, Rhizobium,* and *Serratia* are associated with the plant rhizosphere and can benefit plant growth. The key processes through which PGPR contributes directly to plant phytohormone production, such as auxins, cytokinin's, and gibberellins are through mineral solubilization, siderophores, and enzyme production (Bhattacharyya, *et al*., 2012).

Undoubtedly, one of the most significant environmental factors affecting crop plant productivity worldwide is drought. And this was reported to greatly affect the yield of cluster bean cultivation. Drought stress decreases the rate of photosynthesis (Kawamitsu, *et al*., 2000). To conserve water, plants cultivated in drought conditions have a decreased stomatal conductance. Keeping in mind the above-mentioned beneficial properties and importance of PGPR

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and micronutrients in promoting plant growth and their adaptation, we hypothesized that coating the seeds with them would increase the PGPR in the rhizosphere and hence regulate the stomatal conductance of cluster bean. Also, we hypothesized that these results would contribute to a better understanding of the responses of cluster bean plants to drought stress.

2. Materials and Methods

2.1 Study Area and Sample Collection

The rhizosphere soils of plants were collected from Prayoga Campus (Ravugodlu, Bengaluru 560082, Karnataka, India) from Prayoga campus (Fig. 1 and 2). The collected soil samples were transported to the laboratory and refrigerated (4 ºC) for further processing. Bacteria were isolated using the serial dilution technique and spread plate technique on nutrient agar (NA). 1gm of rhizosphere soil sample was suspended in 100ml autoclaved 0.85% saline. After sedimentation of solid particles, dilution was made upto10^6. 0.1ml of each dilution was spread by L- shaped rod on NA. The selected isolates were tested for their plant growth-promoting properties, such as *IAA* production, *P* solubilization, and *ammonia* production, were evaluated for some isolates.

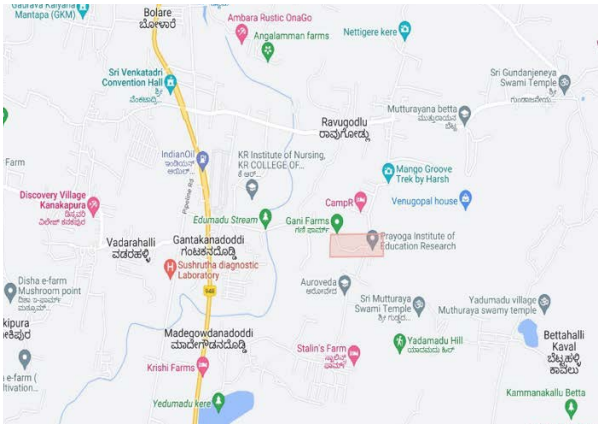

Figure 1. Location of Sampling Site in the Main Campus of Prayoga, Ravugodlu where the Rhizosphere Soil Samples were Collected.

Figure 2. Collection of Rhizosphere Samples from Prayoga Campus, Ravugodlu.

2.2 Test for Plant Growth Promoting Properties

Indole Acetic Acid (IAA) Production

Indole acetic acid production was detected as described by Bric, *et al.,* (1991). Bacterial isolates were inoculated in NA amended with L-Tryptophan and incubated at 37 ºC for 48 h. Fully grown cultures were centrifuged at 3000 rpm for 30 m, the supernatant (2ml) was mixed with two drops of orthophosphoric acid and 4ml of the Salkowaski reagent (50 ml 35% of perchloric acid, 1ml of 0.5mFeCl3 solution). The development of the pink color was indicative of IAA production.

Phosphates Solubilization

Phosphate solubilizing ability of the isolate was checked on Pikovskaya (PVK) medium, incorporated with tricalcium phosphate ($Ca_3 (PO_4)_2$). The isolates were spot inoculated on PVK medium. The formation of a transparent halo zone around the developing colonies indicated *phosphate* solubilizing ability.

Materials required to make Pikovskaya (PVK) medium:

PVK medium contained per litre: glucose,10 g; $Ca_3(PO_4)_2$, 5 g; $(NH_4)_2SO_4$, 0.5 g; NaCl, 0.2g; $MgSO_4W7H_2O$, 0.1 g; KCl, 0.2 g; Yeast extract, 0.5g; $MnSO_4WH_2O$, 0.002 g; and $FeSO_4W7H_2O$, 0.002 g (Surange, *et al.,* 1997).

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Assay for Ammonia Production

The rhizobacterial isolates were tested for the production of *ammonia* in peptone water after incubating at 30 ºC For 48 h. Nessler's reagent (0.5 ml) was added to each tube and observed for the development of a brown to yellow color (Cappucino, *et al*., 1992).

2.3 Gram Staining

The Gram stain technique was carried out using a Gram stain kit. The kit includes 4 bottles of 1X solutions of safranin, acetone-alcohol, Gram's iodine, and crystal violet. The rhizosphere bacterial isolates were maintained and cultivated for 24 hours at 37°C on NA to prepare the microscope slides for use in this study. Each bacterium was smeared separately onto a slide and heat-fixed just before staining. The Gram stain method was carried out in accordance with the instructions provided in a typical microbiology laboratory manual (Michael & Burton, 2002).

2.4 Bacterial Consortia

The isolated bacterial strains were grown on NA for routine use and maintained in nutrient broth with 20% glycerol at - 80 °C for long-term storage. A single colony of bacteria was transferred to 500 ml flasks containing 250 ml of NB and were grown on a rotary shaker (150 rpm) for 48 h at 30 °C. An equal volume of the bacterial suspension was used to make the consortia.

2.5 Coating the Seeds

The micronutrients (like potassium molybdate, $ZnSO_4$, boron etc.,) are applied to the seeds of cluster bean either individually or in combination and grown in a greenhouse facility (Fig. 3 and 4). Micronutrients (like potassium molybdate, $ZnSO_4$, boron etc.,) and/or bacterial consortia are applied to the seeds of cluster bean either individually or in combination (Table 1). The biometric observations on stomatal conductance and growth and yields are recorded. For recording such observations, five plants at random from net plot area were selected from each plot.

Drought stress was created during the vegetative stage by withholding re-watering at and after flowering. Then, around once every week, plants were irrigated with tap water. Hand weeding was used to keep the plots free of weeds. The effects of the drought treatment and covering the seeds with bacterial consortia and micronutrients on seed yield were evaluated at the conclusion of the crop cycle.

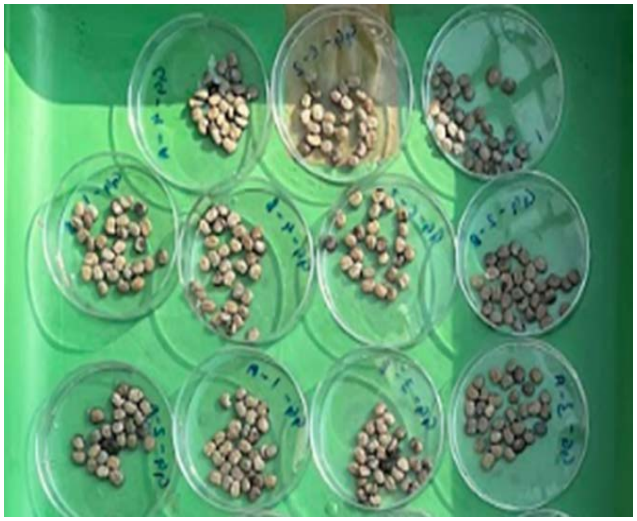

Figure 3. Cluster Bean Seeds Coated with Bacterial Consortia and Micronutrients

Table 1. List of treatments showing respective experimental design

Set Code	Treatment
1a	1+2+MN
1b	1+2
2a	1+2+3+MN
2b	1+2+3
3a	1+3+MN
3b	1+3
4a	2+3+MN
4b	2+3
Control 1	No coating
Control 2	Only starch
Control 3	Only micronutrients

MN - Micronutrients
1, 2, 3 – Isolates

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3. Results and Discussion

Plant growth-promoting rhizobacteria are usually applied to a wide range of agricultural crops for the purpose of growth enhancement, including increased seed germination, plant weight, and harvest yields. PGPR colonization triggers plant growth by bacterial synthesis of plant hormones including *indole-3-acetic acid*, *cytokinin*, and *gibberellins* as well as by increased mineral and nitrogen availability in the soil. Some of them were also known to protect their host plant from pathogenic microorganisms (Handiganoor, *et al*., 2018). In our investigation, four rhizobacteria were isolated from four healthy selected plants from Prayoga.

Figure 4. Planting Seeds in a Small Green House

3.1 Isolation of PGPR

The morphological characteristics of isolates were examined to evaluate colony diversity. It was thought that the isolates' physiological traits varied considerably between the various colonies (Fig. 5). Therefore, 10 bacteria were chosen and used in the following experiments based on the morphological characteristics of isolates, such as the form (circular, filamentous, and irregular), color (white, whitish, yellow, yellowish, creamy, and transplant), elevation (convex, flat, raised, crateriform, and umbonate), and margins (entire, filiform, and undulate) of colonies (Fig. 5 and 6). The isolates were sub-cultured and preserved on NA slants and used for further experiments.

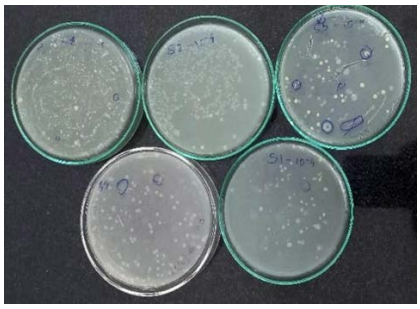

Figure 5. Isolation of Plant Growth Promoting Rhizobacteria by Spread Plate Technique

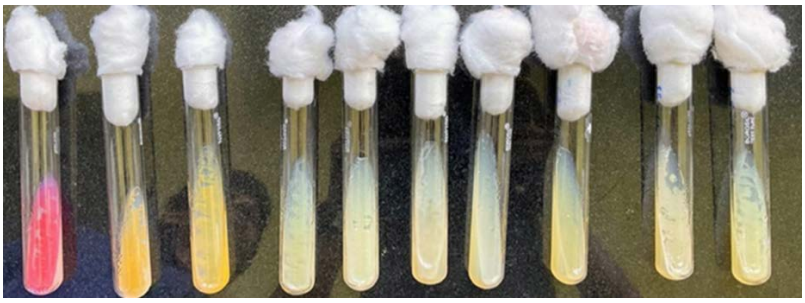

Figure 6. Selected Isolates Maintained on NA Slants

3.2 Physiological properties of isolates

Indole Acetic Acid (IAA) production

A total of 10 selected isolates of PGPB were tested for the production of IAA in the presence of tryptophan. With no addition of tryptophan, production of IAA was not observed. Out of 10 isolates tested, 9 showed positive. The production of IAA was highest in isolates of S4-C3 and S3-C and no IAA was observed in S5-C2 (Fig. 7).

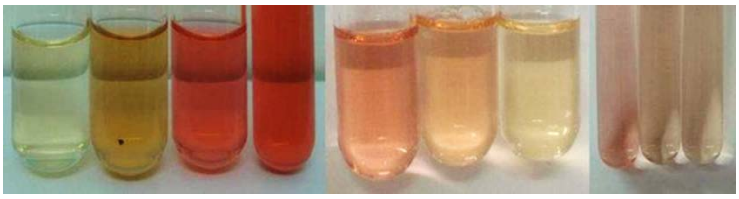

Figure 7. Result of *Indole Acetic Acid* (IAA) production test

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Figure 8. Result of *Phosphate* Solubilization Test

Phosphates Solubilization

A total of 10 selected isolates of PGPB were tested for the production of *phosphate* solubilization. Out of 10 isolates tested, 6 showed positive results. The solubilization of *phosphate* was highest in isolates of S4-C1, S5-C2, S5-C4 and S3-C2 (Fig. 8).

Assay for Ammonia production

A total of 10 selected isolates of PGPB were tested for the production of *ammonia*. Out of 10 isolates tested, 9 showed positive. The production of IAA was highest in isolates of S4-C1 and S5-C2 (Fig. 9). The growth promoting activity of rhizobacterial isolates is summarized in Table 2.

Table 2. Growth promoting activity of Rhizobacterial isolates

Rhizobacterial Isolates	IAA production	PO_4 solubilization	NH_3 production
S4-C3	++	++	++
S4-C1	+	++	+++
S5-C2	-	++	-
S5-C4	+	+	++
S3-C2	++	++	+
S5-C5	+	+	+
S3-C3	+	-	++
S3-C1	+	-	++
S5-C3	+	-	+
S5-C6	+	-	+

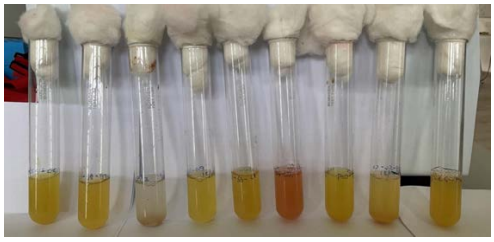

Figure 9. Result of *Ammonia* Production Test

3.3 Gram staining

The results obtained from the assay of the physiological properties of isolates were used to select some isolates for further study. These isolates were tested for gram staining to understand the gram staining result and their morphology (Fig. 10, 11 and 12). Three isolates were selected (S4-C3 – Gram-negative bacilli; S5-C4 – Gram-positive cocci; S3-C2 – Gram-positive bacilli) after gram staining and these isolates were used to make the bacterial consortia using all possible combinations.

Figure 10. Selected Isolates Streaked on NA

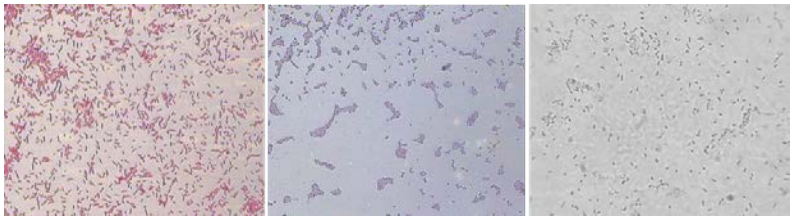

Figure 11. Gram's Staining Result of the Selected PGPR (From left to right: S4-C3 – Gram-negative bacilli; S5-C4 – Gram-positive cocci; S3-C2 – Gram-positive bacilli)

Figure 12. Selected PGPR isolates maintained on NA slants

3.4 Measurement of Stomatal Conductance and Growth

The rhizosphere is a complex ecology in which plant impacts on soil microbes and microorganism effects on plants interact and are interdependent. The key processes through which PGPR contributes directly to plant phytohormone production, such as auxins, cytokinins, and gibberellins, as well as boosting plant nutrition through mineral solubilization, siderophores, and enzyme production (Taiz *et al*., 1991). The turgor pressure and osmotic potential of guard cells are directly related to stomatal conductance (Buckley *et al*., 2013). Stomatal conductance is a function of stomatal

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density, stomatal aperture, and stomatal size (Ziegler *et al.*, 1987). Reductions in stomatal conductance prevent further decreases in water potential by reducing transpiration; also, reductions in water potential can induce stomatal closure, resulting in lowered stomatal conductance. Coating the seeds with PGPR consortia and micronutrients showed beneficial effects on plants adaptation to drought stress and increased seed yield (Fig. 13) (Table 3).

Figure 13. Leaf Porometer (AP4 leaf porometer, Delta-T Devices, UK) used during the experiment (measurement of stomatal conductance)

Stomatal conductance decreased in some of the treatments when they were imposed to drought stress. One of the first responses of plants to drought is stomatal closure, restricting gas exchange between the atmosphere and the inside of the leaf. 'Treatment 2a' showed the lowest stomatal conductance and a better seed yield under drought conditions.

Table 3. Influence of Coating Seeds with Micronutrients and Bacterial Consortia on Stomatal Conductance (mmol m−2 s−1) of Cluster Bean: List of Treatments Showing Respective Experimental Design. The values are the mean and standard deviation of three replications.

Set Code	Treatment	Length (cm)	Stomatal Conductance (mmol m−2 s−1)	Cluster bean yield in g (per plant)
1a	1+2+MN	36 ± 1.14	107 ± 2.61	82.5 ± 2.56
1b	1+2	29 ± 0.74	106 ± 3.44	62.6 ±2.27
2a	1+2+3+MN	49 ± 1.87	77 ± 1.87	118.5 ± 3.65
2b	1+2+3	40 ± 1.47	144 ± 4.81	102.6 ± 2.07
3a	1+3+MN	32 ± 0.98	128 ± 3.65	71.2 ± 1.42
3b	1+3	34 ± 0.87	160 ± 4.03	59.4 ± 0.98
4a	2+3+MN	39 ± 1.04	166 ± 3.75	48.3 ± 1.36
4b	2+3	28 ± 1.01	176 ± 2.93	39.6 ± 1.87
Controls				
C1	No coating	32 ± 0.73	180 ± 4.97	43.6 ± 0.57
C2	Starch	31 ± 0.81	175 ± 4.71	32.4 ± 0.71
C3	Micronutrients	29 ± 0.42	178 ± 3.49	51.4 ± 1.33

MN - Micronutrients
1, 2, 3 – Bacterial isolates

4. Conclusion

Micronutrients viz., zinc, boron and potassium molybdate in combination with standardized PGPR consortia significantly influenced the stomatal conductance of leaf, helping in the better establishment of seedlings and higher yield. Seed coating of cluster bean with the combination of micronutrients with PGPR consortia was shown to be effective in increasing the yields. Stomatal conductance decreased in several of the treatments when they were applied to drought stress. Under drought conditions, "Treatment 2a" displayed the lowest stomatal conductance and the best seed yield. These micronutrients along with the PGPR consortia may be supplied to the plants through seed treatment to improve the stand establishment, better seed yield under drought conditions, micronutrient contents in grain in the

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cluster bean crop. Being an easy and cost-effective method, seed treatment by polymer coating offers an attractive option for resource-poor farmers through its pronounced effect during the early stage of seedling establishment. This positive impact on stomatal conductance was attributed to the synergistic effects of micronutrients and the beneficial bacterial consortia, which likely enhanced nutrient availability, facilitated nutrient uptake, and promoted plant growth. This approach can serve as an effective strategy to enhance crop performance, optimize resource utilization, and contribute to sustainable agriculture. Further studies are warranted to elucidate the underlying mechanisms involved in the observed improvements and to evaluate the long-term effects of seed coating on soil health and crop resilience.

Acknowledgments

We would like to thank Prayoga Institute of Education Research for funding and material support and for giving us the opportunity to work in a research laboratory as high school student. Many thanks to our supervisor, Dr. Venkata Krishna B for guiding us throughout the duration of the research study. This report and the research behind it would not have been possible without his exceptional support. Thanks to Dr. Nagabhushana K S, Research Director, Prayoga for his constant cooperation, encouragement, and facilities. We also thank Mr. Raju and Mr. Narayan, who helped us procure all the milk samples. We would like to extend our gratitude towards the Samvida management for introducing us to this opportunity.

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