

A study on enhancing crops growth rate using Biofertilizers made with species of Rhizobium, Azotobacter, Phosphate Solubilizers, and Potassium Solubilizers

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Abstract—India, being a major agricultural country, faces increasing demands for food crops due to its burgeoning population. To meet this demand sustainably, the utilization of fertilizers is crucial. Biofertilizers, derived from living organisms like bacteria and fungi, offer a natural and eco-friendly alternative. In this study, biofertilizers incorporating Rhizobium, Azotobacter, Phosphate solubilizers and Potassium solubilizers were developed and evaluated for efficacy. Through extensive screening and enrichment processes, these beneficial microorganisms were cultivated. The efficacy of the biofertilizers was assessed through controlled experiments on various crops including wheat, chickpea, moog and fenugreek. Results indicated significant improvements in crop growth and yield compared to control groups. This research highlights the potential of biofertilizers in enhancing agricultural productivity while promoting sustainability and environmental health.

Keywords: Biofertilizers, Rhizobium, Azotobacter, Phosphate solubilizers, Potassium solubilizers.

I. INTRODUCTION

By 2050, it is projected that the global population will grow by 9 billion individuals. To adequately nourish this expanding population, there is a necessity to enhance crop production twofold by embracing innovative and environmentally friendly technologies, with a specific emphasis on the microenvironment of the rhizosphere. Creating conducive environmental conditions and ensuring the presence of fertile, well-maintained soils are crucial factors in attaining this objective (Safdar et al., 2022).

To achieve optimal plant growth i.e enhanced crop production, it is crucial to have a well-balanced and adequate supply of nutrients (Mohammadi & Sohrabi, 2012). Chemical fertilizers are popular for their elevated nutrient content, fulfilling the needs of high-yield, nutrient-demanding crops (Kumar et al., 2022). However, prolonged use of chemical fertilizers possess environmental risks such as water pollution and air emissions, degrades soil quality through acidification and nutrient imbalances, and contributes to biodiversity loss by disrupting soil microorganisms. This can lead to reduced soil fertility over time, emphasizing the need for sustainable agricultural practices mitigate these adverse effects. (Safdar et al.2022)

Soil infertility poses a significant challenge to crop yield, particularly in developing nations and among resource-poor farmers. Without addressing soil fertility issues, the benefits of improved crop varieties and more efficient farming practices may be limited. Integrated soil fertility management (ISFM), which incorporates strategies such as natural resource conservation, biological nitrogen fixation (BNF), and enhanced input efficiency, proves effective in restoring soil fertility. Biofertilizers, integral to integrated nutrient management, play a vital role in enhancing soil productivity and sustainability while being environmentally friendly and cost-effective inputs for farmers. These biological fertilizers offer a renewable supplement to chemical fertilizers in creating a sustainable agricultural system (Mohammadi & Sohrabi, 2012)

Various microorganisms and their interactions with crop plants are being harnessed to develop biofertilizers. These microorganisms can be categorized in various ways depending on their characteristics and roles in plant growth (Paul & Dubey, 2014). Organisms commonly utilized in the formulation of biofertilizers include those with nitrogen fixing capabilities (N-fixer), potassium solubilizing traits (K-solubilizers), and phosphorous solubilizing characteristics (P-solubilizers). These can be used individually or in combination with molds or fungi to enhance their effectiveness (Mohammadi & Sohrabi, 2012).

I.I. NEED OF NITROGEN FIXING BACTERIA

Nitrogen is one of important nutrients, essential for growth and development of plants. Nitrogen is a major source that makes upto 78% of the atmosphere.(Mohammadi & Sohrabi, 2012) In USA the first inoculant of *rhizobium* was made and in 1930

private enterprise commercialized it. In 1895, Nobbe and Hiltner applied for patents in England and United States for a legume-inoculants that later marketed as Nitrogen Nitrogen-fixation biologically is a way to convert elemental nitrogen into usable form (Thomas & Singh, 2019). Nitrogen fixing bacteria convert atmospheric N₂ to organic compounds. (Mushtaq et al., 2020) They are free living such as *Azotobacter*, *Azospirillum*; symbiotic such as rhizobium, *Azolla* etc. The N₂-fixing bacteria associated with non-legumes plants are *Achromobacter*, *Arthrobacter*, *Beijerinckia*, etc. (Mohammadi & Sohrabi, 2012) Although many of genera and species of nitrogen fixing bacteria are isolated from rhizosphere of various cereals. *Azolla* biofertilizer is used in rice cultivation. *Azotobacter* reducing the need for synthetic nitrogen fertilizers. Their effectiveness depends on soil conditions and microbial interactions (Aquilanti et al., 2004).

Biofertilizers, like nitrogen-fixing bacteria, enhance soil fertility by converting atmospheric nitrogen into a plant-usable form. Phosphate-solubilising microorganisms (e.g., mycorrhizal fungi) in biofertilizers increase phosphorus availability for plant growth (Mahanty P, Bhattacharjee S, Goswami M, Bhattacharyya P, Das B, Ghosh A, Tribedi P., 2016).

They improve nutrient uptake efficiency and contribute to enhanced soil health by promoting microbial diversity. Biofertilizers support sustainable agriculture, reducing reliance on chemical fertilizers and mitigating environmental impacts. Their environmental friendliness, lower cost-effectiveness in the long run, and adaptability to various cropping systems contribute to resilient agro-ecosystems. Certain biofertilizers, like those with beneficial microorganisms (e.g., *Trichoderma*), offer bio control against soil-borne pathogens and pests. Overall, bio fertilizers play a crucial role in sustainable and environmentally friendly farming practices, aligning with agro-ecological principles (Malusa F, Pinzari F, Canfera L., 2016).

I.II. NEED OF PHOSPHORUS-SOLUBILISING BACTERIA

Phosphorus (P) is an essential nutrient for plant growth and development, playing a crucial role in various physiological processes, such as energy transfer, DNA synthesis, and root development. However, despite its importance, plants face challenges in taking up phosphorus from the soil. Phosphorus naturally tends to form insoluble compounds in the soil, particularly with calcium, iron, and aluminium (Kalayu, 2019). These insoluble forms make it difficult for plants to directly uptake phosphorus. Phosphorus has low mobility in soil, meaning it does not move easily through the soil profile. As a result, even if there is an adequate amount of phosphorus in the soil, plants may struggle to access it if it is not in proximity to their root systems. To overcome these challenges, phosphate fertilizers are used. However, chemical fertilizers, especially phosphorus-based ones, can contribute to water pollution when runoff carries excess nutrients into water bodies whereas Phosphate solubilising bio fertilizers offer a more environmentally friendly alternative by promoting nutrient uptake efficiency and reducing the risk of nutrient leaching (Saxena et al., 2019). By improving the availability of phosphorus in the soil, phosphate-solubilising bio fertilizers can potentially reduce the need for supplemental phosphorus fertilization, leading to cost savings for farmers. This is particularly beneficial in regions where access to commercial fertilizers is limited or expensive (Khan et al., 2014).

Microorganisms, particularly bacteria and fungi referred to as phospho-microorganisms, play a vital role in rendering insoluble phosphorus accessible to plants. Certain soil bacteria and specific fungi possess the ability to convert insoluble phosphate in the soil into soluble forms by releasing organic acids. These acids not only lower the soil pH but also induce the dissolution of bound phosphate forms, enhancing its availability to plants. Examples of P-solubilizers are *Bacillus megaterium*, *Bacillus circulans*, and *Pseudomonas straita*. (Mohammadi & Sohrabi, 2012).

I.III. NEED OF POTASSIUM SOLUBILISING BACTERIA

Relationships Soil is a complex mixture of minerals, water, air, organic materials, and billions of species. It also undergoes bio-geochemical transformations, which alter its makeup. The ability of the soil to provide necessary plant nutrients, such as N, P, K, and micronutrients, which are frequently unavailable in free form or are present in small amounts in the soil, is referred to as soil fertility. Root-associated beneficial microorganisms are crucial allies in this situation (De Zelicourt et al. 2013). It is well known that microbes can supply plants with nutrients through a variety of methods (Zhao et al., 2016). The number and diversity of bacteria are influenced by the soil conditions such as organic carbon, temperature, moisture, electrical conductivity and other chemicals as well as by the number and types of plants found in those soils. Therefore, soil-grown plants are immersed in a sea of microorganisms especially bacteria (Glick, 2012). Recent studies show that most plant. Furthermore plants are able to choose their own root micro flora from the surrounding soil. Put differently, every single plant species has a distinct community of related microorganisms. The ecology is known to suffer from the continuous use of chemical fertilizers, particularly those high in phosphorus, nitrogen, and potassium (Adesemoye and Kloepper, 2009).

Potassium (K), which is essential for plant growth, metabolism, and development, is the most significant nutrient for plants, after nitrogen (N) and phosphorus (P). K is necessary to activate more than 80 distinct enzymes that are crucial for plant defence against a biotic stressors, diseases, and pests (Bashir et al., 2019). The majority of plant species need microbial interactions to survive, according to recent studies (de Zelicourt et al., 2013; Ma et al., 2016). Moreover, plants have the capacity to choose their own root micro flora from the nearby soil. Put differently, a distinct group of microorganisms is associated with each specific plant species. Beneficial plant–microbial interactions need reciprocal recognition and extensive response coordination on the part of the microbial and plant (de Zelicourt et al., 2013). According to Cheng et al. (2010), the relationships that arise between bacteria and plants can be beneficial (e.g., PGPRs, plant growth-promoting rhizobacteria), detrimental (e.g., pathogens), or neutral for the plant. In certain cases, the influence of a bacterium may also shift as the soil does. The following bacteria help plants in some ways: (i) those that grow symbiotic nodules on the roots of their hosts and fix nitrogen; (ii) endophytic bacteria that invade internal plant tissues without harming the host; (iii) bacteria that can colonize the rhizosphere and plant root surfaces in a competitive manner; and (iv) soil-dwelling bacteria (Glick, 2012).

In agriculture, beneficial bacteria are defined as any bacteria that colonize the roots of plants following inoculation onto seed and improve plant growth by increasing seed emergence, plant weight, and crop yields. Despite the limited understanding of soil bacteria-plant interactions, a number of these bacteria are used commercially as adjuncts to agricultural practice (Glick, 2012). These bacteria include *Burkhol deriacepacia*, *Delfitia acidovorans*, *Paenibacillus macerans*, *Pantoea agglomerans*, *Pseudomonas spp.*, *P. aureofaciens*, *P. chlororaphis*, *P. fluorescens*, *P. solanacearum*, *Bacillus spp.*, *B. mucilaginous*, *B. pumilus*, *B. subtilis*, *B. amyloliquefaciens*, *B. fimus*, *B. licheniformis*, *B. megaterium*, *Agrobacterium radiobacter*, *Azospirillum brasilense*, *A. lipoferum*, *Azotobacter chroococcum*, *P. syringae*, *Serratia entomophilia*, *Streptomyces spp.*, *S. griseoviridis*, and *S. lydicus* (Glick, 2012).

II. MATERIALS

Species of Rhizobium, Azotobacter, Phosphate solubilising bacteria and Potassium solubilising bacteria. Culture media of Congo Red Yeast Mannitol Agar media for Rhizobium spp. Ashby's Mannitol Broth for Azotobacter spp. Pikovaskaya's media for Phosphorous solubilising bacteria. Aleksandrov's media for Potassium solubilising bacteria. Samples like root nodules of leguminous plants, soil and compost sample and all the setup requirements.

III. METHODOLOGY

III.I. ISOLATION OF MICROORGANISMS

A) ISOLATION OF RHIZOBIUM SPP.

2-3 root nodules of leguminous plants (here fenugreek) were washed with water and then with a solution of HgCl₂ to get rid of any contamination. In aseptic conditions, the root nodules were crushed between two glass slides until the white liquid was released. The white liquid was then taken and isolated on a CRYEMA (Congo red yeast extract Mannitol Agar) plate. The medium is composed of Mannitol 10 (g/l), calcium carbonate 2.5 (g/l), magnesium sulphate 0.2 (g/l), Dipotassium sulphate 0.5 (g/l), yeast extract 1.0 (g/l), NaCl 0.1 (g/l), 1% Congo red 2.5ml, Agar 20 (g/l), dissolved in 1000 ml of distilled water. The pH of the medium was adjusted to 7.2 before autoclaving at 15 lbs pressure (121°C) for 15 minutes. 1gm of the sample was then inoculated in 9 ml of saline and serial dilutions were conducted in order to get the desired concentration of sample. The sample was then isolated on another CRYEMA plate to ensure presence of any Rhizobium. The plates were incubated at 37°C for 3 days. Pinpoint colourless colonies were obtained on the medium, indicating presence of Rhizobium. A single isolated colony was picked and re-isolated on another CRYEMA plate to obtain a pure culture (Hala et al., 2019).

B) ISOLATION OF AZOTOBACTER SPP.

1gm of the soil sample was inoculated in 9ml of saline. This suspension was vortexed well and allowed to settle. 1ml of the supernatant was then inoculated in Ashby's Mannitol Broth, which is a selective media to isolate *Azotobacter sp.* Ashby's Mannitol Broth contains components like Mannitol 20 (g/l), Dipotassium phosphate 0.2 (g/l), Magnesium sulphate heptahydrate 0.2 (g/l), NaCl 0.2 (g/l), Potassium sulphate 0.1 (g/l), Calcium carbonate 5.0 (g/l) in 1000ml distilled water. The pH of the medium was maintained at 7.0. The broth was incubated at 37°C for a week. On obtaining slimy, brown growth inside the flask, the growth was isolated on Ashby's Mannitol Agar having the same composition as the broth, but with an addition of 20g agar. This medium was again incubated at 37°C for a week. Clear and distinct Azotobacter colonies were obtained on the medium. One colony was picked and reisolated on Ashby's Mannitol Agar in order to obtain a pure culture.

C) ISOLATION OF PHOSPHOROUS SOLUBILISING BACTERIA.

One gram of the sample was mixed with 9 ml of autoclaved saline solution. The mixture was then vigorously vortexed and left undisturbed for some time, allowing the soil particles to settle at the bottom of the container. Afterward, one ml of the clear liquid supernatant was transferred and added to Pikovskaya's agar, which contained tricalcium phosphate (TCP) as the source of phosphate. The Pikovskaya's medium consisted of yeast extract 0.10 (g/l), dextrose 2.00 (g/l), calcium phosphate 1.00 (g/l), ammonium sulphate 0.10 (g/l), potassium chloride 0.04 (g/l), magnesium sulphate 0.02 (g/l), manganese sulphate 0.0001 (g/l), ferrous sulphate 0.0001 (g/l), and dissolved in 200 ml of distilled water. The pH of the medium was adjusted to 7.2 before autoclaving at 15 lbs pressure (121°C) for 15 minutes. After the initial inoculation, the first enrichment flask was placed on a rotary shaker and incubated for four days at room temperature, ranging from 27 to 30 degrees Celsius. During this time, the broth was closely monitored for turbidity and any signs of contamination, with particular attention paid to the absence of any fungal growth. To confirm the presence of phosphate-solubilising bacteria, a small sample of the first enrichment broth was isolated and spread onto sterile PVK agar plates. These plates were then incubated at room temperature for 48 hours, and colonies with a clear halo around them were identified as PSB. Finally, these colonies were transferred onto PVK agar slants to obtain pure culture slants.

D) ISOLATION OF POTASSIUM SOLUBILISING BACTERIA.

1gm of soil sample was inoculated in 9 ml of saline. After vigorous vortexing, the suspension was allowed to settle and the supernatant was used for further inoculation. The sample was inoculated in four different medias with Aleksandrov media as reference, since it's a selective media for potassium solubilizers. Banana peel extract, cucumber extract, potassium sulphate and potassium chloride medias were used respectively for inoculation. The cucumber extract broth is composed of Magnesium sulphate 0.005 (g/100ml), calcium carbonate 0.01 (g/100ml), cucumber extract 1ml, 0.5 (g/100ml), ferric chloride 0.005 (g/100ml), calcium phosphate 0.2 (g/100ml) dissolved in 100 ml of distilled water. Ingredients of the banana extract broth include magnesium sulphate 0.005 (g/100ml), calcium carbonate 0.01 (g/100ml), banana extract 1ml, glucose 0.1 (g/100ml), ferric chloride 0.001 (g/100ml), calcium phosphate 0.2 (g/100ml) dissolved in 100ml distilled water. Potassium chloride broth is made up of all the above ingredients, only the banana extract was replaced by potassium chloride. The same applies for potassium sulphate broth, the potato extract was replaced by potassium sulphate. The media broths were incubated at 37°C for 1 week. The cucumber extract broth supported the growth of organisms, the best. Hence this medium was chosen for further procedures. A loopful of suspension was picked from the cucumber extract broth and isolated on a cucumber extract agar. This medium was isolated at 37°C for 1 week to obtain isolated colonies of potassium solubilising bacteria. A clear halo was observed around the colonies, confirming that the colonies were indeed of KSB.

III.II. MORPHOLOGICAL CHARACTERIZATIONS

The structure of the isolated bacterial colonies was inspected by applying gram staining according to the standard protocol. All the cultures were studied under 100X oil immersion lens of the compound microscope.

III.III. CULTURE MAINTENANCE

Sub culturing of isolated strains on appropriate media and maintenance of pure culture at suitable temperatures.

III.IV. INOCULUM PREPARATIONS

Cultivation of microorganisms in liquid media until reaching the desired cell density. Harvesting and washing cells for subsequent inoculation.

III.V. TESTING SYNERGISTIC AND ANTAGONISTIC ACTIVITY

To enhance the efficacy of the isolated bacterial strains as biofertilizers, they needed to be used in a consortium. In order to that, all the isolates needed to be compatible with each other and not inhibit growth of others. To check this, cross-streak method was used for each isolate. Nutrient agar was used as the medium as it supports the growth of all organisms. The plates were left to incubate at room temperature for 24 hours. If a zone of inhibition was observed, this indicated antagonistic activity against the isolated bacteria.

III.VI. BIO FERTILIZER FORMULATION

Mixing appropriate ratios of Rhizobium, Azotobacter, Phosphorous solubilising bacteria and Potassium solubilising bacteria separately and preparing Consortium of all four cultures.

III.VII. FIELD APPLICATION

Application of formulated bio fertilizers to crop soil. Incorporation of bio fertilizer into the soil by tillage or irrigation.

III.VIII. CROP GROWTH MONITORING

Regular monitoring of crop and checking the parameters of crop (e.g., Height and No. of leaves). Assessment and nutrient uptake of plants.

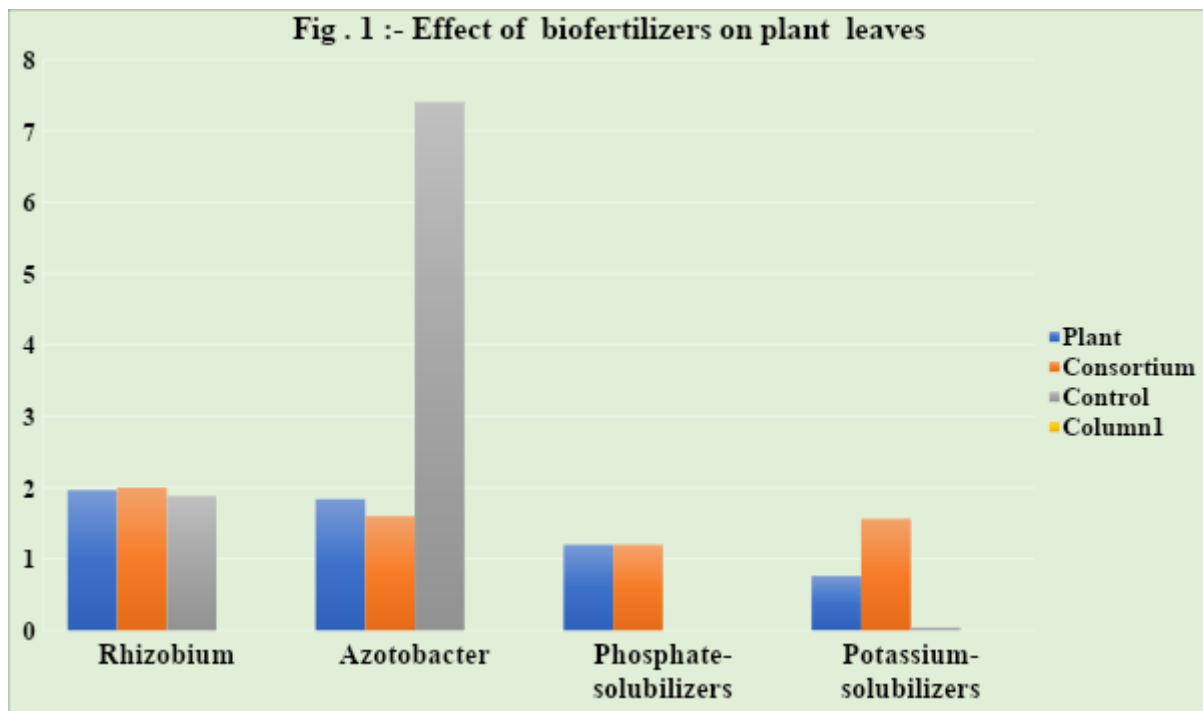
III.IX. DATA ANALYSIS

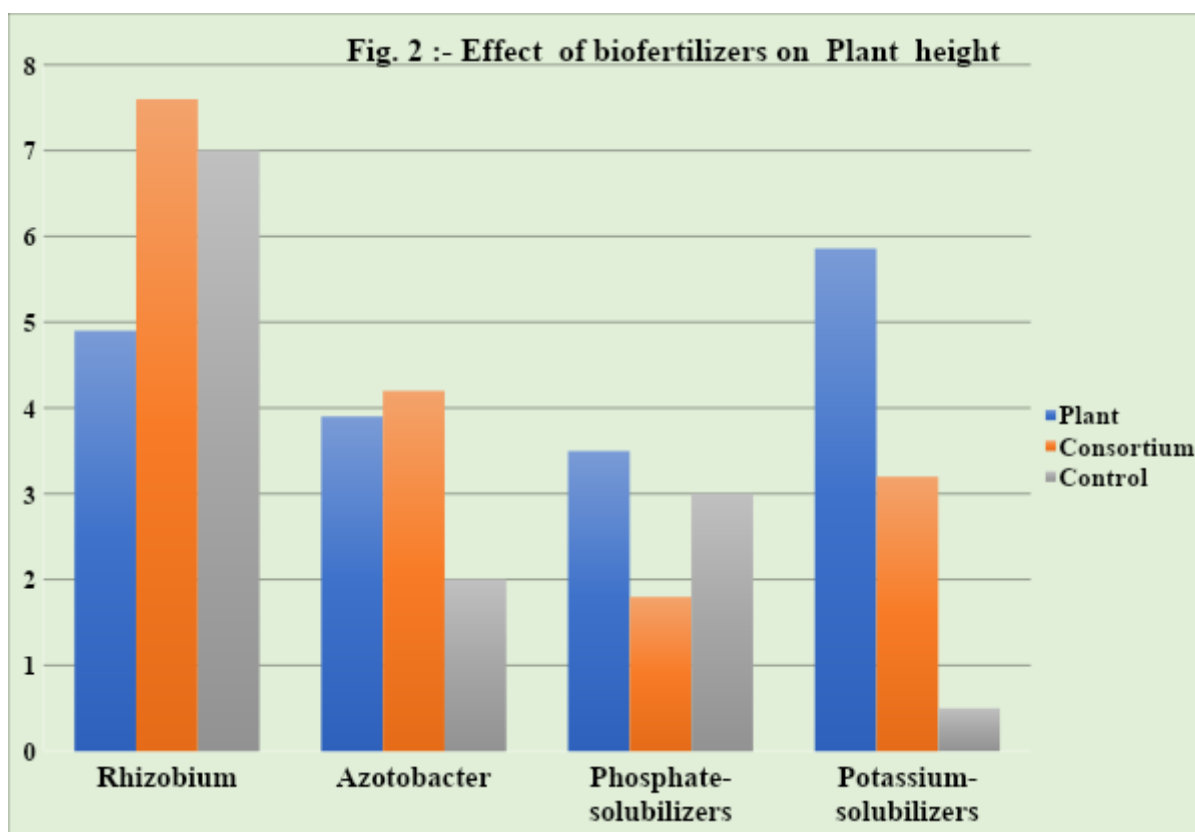
Statistical analysis of the data obtained from crop growth monitoring. Comparison with control groups (e.g., untreated soil, chemical fertilizer treated soil.)

IV. STATISTICAL DATA ANALYSIS

ANOVA single factor was analysed by the study of parameters observed in crop development by the use of bio fertilizers with microbial cultures (Ahmad et al., 2021).

The following are the graphical presentation for the same





V. RESULTS AND DISCUSSIONS

V.I. RHIZOBIUM SPP

Gram-negative, rod-shaped bacteria were observed by mucilaginous colonies isolated on Congo Red Yeast Mannitol Agar plate. Its synergistic-antagonistic activity did not show any zone of inhibition against Rhizobium culture (Takil et al., 2023).

Increased nitrogen availability due to symbiotic nitrogen fixation leads to healthier and more vigorous plant growth. This enhances crop yield, especially in leguminous plants such as peas, beans, and lentils.

The fenugreek plants were treated with the Rhizobium bio fertilizer and their growth parameters were observed for a period of 5 days. The test plants on the 5th day showed a shoot height of 8.6 ± 1.4 cm and the number of leaves observed was 2. The plants treated with the consortium showed a shoot height of 12.7 ± 1.1 and leaves were 2. The control showed a growth of 5.7 ± 1.4 with 2 leaves.

V.II. AZOTOBACTER SPP

Gram negative, oval shaped bacteria were observed by the colonies isolated on Ashby's Mannitol Agar Plate and the characteristic feature of Azotobacter spp. i.e. cyst formation was shown by the culture. Its synergistic-antagonistic activity did not show any zone of inhibition against Azotobacter culture.

Increased nitrogen availability in the soil leads to enhanced plant growth, increases yield, and better quality of crops. Additionally, Azotobacter can also produce growth-promoting substances, contributing to overall plant health.

The growth of the chickpea plants were examined over the course of 5 days and their physical parameters (height and amount of leaves) were carefully tracked. The plants treated with Azotobacter had a height of 4.5 ± 1.5 cm. 4 plants had 4 leaves and the rest had 2 leaves. Plants treated with consortium had a shoot height of 4.8 ± 8 cm. 1 plant showed 6 leaves, 2 had 4 leaves and 3 plants had 2 leaves respectively. The control showed a shoot height of 2 ± 0.5 cm where 2 plants had 5 leaves, 2 had 4 leaves and 2 plants had 2 leaves (Abdiev et al. 2019).

V.III. PHOSPHATE-SOLUBILISING BACTERIA

Gram negative, dispersed slender rods were observed by the colonies isolated on Pikovaskaya's Agar plate. The colonies showed a clear zone indicating their potent in solubilising phosphorous in media. This zone of clearance might be due to production of organic acids or through phosphatase enzyme activity. Its synergistic-antagonistic activity did not show any zone of inhibition against phosphorous solubilising bacteria (Soumare et al., 2020).

Improved phosphorous uptake by plants leads to better root development, flowering, and fruiting. This results in increased crop yield and better overall plant development.

The test plants i.e. application of PSB bio fertilizer showed a shoot height of 1.4 ± 0.42 cm and the number of leaves was 2 (Mukhtar et al., 2017). The plants treated with consortium bio fertilizer showed a shoot height of 2.6 ± 0.55 cm and the number of leaves was 2. In the control, only one seed germinated as no bio fertilizer was used. Hence, control plants are not included in the analysis (Pande et al., 2017).

V.IV. POTASSIUM SOLUBILISING BACTERIA.

Gram-negative, cocci in clusters were observed by the colonies isolated on Aleksandrov's agar plate giving zone of clearance around colonies indicating the potent to solubilise potassium in the media by enhancing the chelation of cations bounded to potassium and acidolysis of the surrounding compound of microorganisms. Its synergistic-antagonistic activity did not show any zone of inhibition against Potassium solubilising bacteria.

Enhanced potassium uptake by plants promotes better water and nutrient uptake, improved tolerance to environmental stresses, and increased resistance to diseases. This results in higher crop yields and better quality production.

Wheat plants were inoculated with the KSB biofertilizer and their growth parameters including plant height and number of leaves were checked. The height of the wheat plants were found to be 10 ± 2 cm.

VI. CONCLUSION

From the above results, it can be concluded that the Nitrogen-fixing bacteria *Azotobacter* and *Rhizobium* have the capacity to fulfil a plant's nutritional requirement individually and the plant doesn't need other microbes to survive. On the other hand, these nitrogen-fixing bacteria did very well in a consortium as well, indicating that they are synergistic with almost everything in their ecosystem (Basalingappa et al., 2018). Nitrogen is a very essential nutrient for every plant but it cannot be taken up by the plant easily. *Azotobacter* and *Rhizobium* solved this problem and that is why a vast difference was observed between plants that grew with nitrogen-fixing bacteria and those that grew without them. The other nutrients like potassium and phosphates are not the core nutrients for the plant and hence the impact of Potassium-solubilising bacteria and Phosphate-solubilising Bacteria on the plants was negligible. The consortium worked well for all those plants (wheat and moong) because of the presence of the nitrogen-fixing bacteria. These observations demonstrate that integrating bio-fertilizers into farming and agricultural development, they harness the power of beneficial microorganisms to improve soil health, nutrient availability, and plant growth, thereby reducing the reliance on synthetic fertilizers and minimizing environmental impact. Through continued research and implementation, microbial biofertilizers can play a pivotal role in advancing sustainable agricultural practices, fostering food security, and mitigating the challenges posed by conventional farming methods.

VII. ACKNOWLEDGEMENT

We are thankful to all the students of S.Y.BSc from Department of Microbiology of PTVA's Sathaye College (Autonomous), Vile Parle, Mumbai for their help in research.

VIII. FUTURE PROSPECTS

Biofertilizers not only help plant growth but also make the plant more nutritious. They enhance the uptake of nutrients by the plant and hence make the plant more nutritionally beneficial. Biofertilizers can be implemented in countries having nutritionally poor soil and conditions of malnutrition so that the population could be nutritionally fit overall. Since Biofertilizers contain living organisms, it is difficult to store them for longer periods of time. If this problem is overcome, biofertilizer production would be economically beneficial. In order for biofertilizers to have a preference over chemical fertilizers, they need to have a faster, easier and economical production process. Biofertilizers are the future and there is a huge scope to work on their enhancement.

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