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Contemplating the impact of rhizobacterial two species and multi species consortia for enhancing plant growth and mineral biofortification of *Macrotyloma uniflorum*

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ABSTRACT
The most prevalent form of organic phosphorus is known as phytate, which makes up to 60% of the soil's organic phosphorus, which is poorly utilized by plants. Furthermore, the presence of phytic acid could be a significant barrier to the availability of micronutrients because it chelates many mineral elements, particularly Zn, Fe, Ca, Mg, and Mo, and interferes with their uptake by plants. However, phytic acid can be degraded by the action of the enzyme phytase and bioaugmentation of phytase solubilizing rhizobacteria could be sustainable intervention to increase the bioavailability of micronutrients. In present study, the isolates and their consortia were evaluated for the phytase producing efficiency and other plant growth promoting traits like ammonia production, potassium solubilization etc.. In vitro analysis reveals that two species (NJC4 + NJC 21) and multispecies (NJC1+NJC4+NJC21) consortia have a higher potential for phytase production than single bacteria. These strains were identified as Pseudomonas aeruginosa NJC4 (OP289324), Serratia marcescens NJC21 (OP289323) and Bacillus spp. NJC 1. All the isolates alone and in combination tested for their ability to promote growth of Macrotyloma uniflorum. Results revealed that inoculation with two species and multispecies consortia proved best for stimulating the growth attributes like emergence percentage and seedling vigor index and also enhance the micronutrient content in plant. This study advocate that inoculation of phytate solubilizing rhizobacterial consortia can be potential bio-inoculants for minerals biofortification of Macrotyloma uniflorum plant to alleviate micronutrient deficiency.

Keywords: *Phytase, Biofortification, two species consortia, multispecies consortia, Plant growth promoting rhizobacteria (PGPR).*

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INTRODUCTION

Phosphorus (P) is a key macronutrient in plant nutrition and production of genetic material and cell membranes, as well as the regulator for various enzymes. Soil phosphate deficit is a key issue for farming. Total soil P originates in both organic and inorganic forms. The predominant form of organic phosphorus in soil is phytic acid as phytate (salts of phytic acid), which is not generally accessible to plants as a source of phosphorus since it can either form a complex with cations or adsorbs to diverse soil components. Divalent and trivalent mineral cations in soil, such as Cu^{2+}, Zn^{2+}, and Cd^{2+}, are complexes by it, reducing their bioavailability to plants. The formation of phytate-mineral complexes remains insoluble that limits the mineral ions and eventually crops exhibit the mineral nutrient deficiency [1]. Over fifty percent of the world's population is known to suffer from micronutrient malnutrition, which is regarded as one of the greatest threats to mankind on a worldwide scale. [2]. The mortality of starving populations worldwide should be reduced more quickly as a result of efforts to enrich using environmentally friendly methods. To address this issue Microbial Bio-fortification of crops could be a significant step towards sustainable and environment friendly agricultural techniques, leads to utilization of robust soil microorganisms, plant growth promoting rhizobacteria (PGPR) which are able to utilizing phytate and play an important role in supplying P to plants [3]. They are able hydrolyze this organic form of phosphorus by secreting phytases

[4]. Many different bacterial phytase enzyme known to initiates the sequential releasing of one or more inorganic phosphate groups and releases the cations from phytates [5]. Hence, phytase solubilizing bacteria are being a PGPR members can play an imperative role in the cycling of macro- and micronutrients like iron (Fe), zinc (Zn), copper (Cu), and manganese (Mn) in soil [6]. In this context, bacteria having high phytase activity selected to mineralize phytate in soil, have been selected and inoculated to the rhizosphere of *Macrotyloma uniflorum* (horse gram), which is an annual, herbaceous, protein rich, legume crop belonging from Fabaceae family [7]. This crop accounts for about 5-10% of pulses production in India. In our study, three bacterial isolates were selected on the basis of their phytase producing ability and other plant growth promoting abilities. These strains were evaluated for their synergistic behavior as two species consortia and multispecies consortia. The promising application of co-cultures were further exploited to enhance the major macro and micronutrients in *Macrotyloma uniflorum.* Therefore, the goal of our study was to better understand how PGPR consortia works to increase plant growth and micronutrient concentration in *Macrotyloma uniflorum* in order to achieve biofortification.

MATERIALS AND METHODS

Microbial Strains

In this study, three phosphate solubilizing rhizobacteria, such as *Bacillus spp.*- NJC 1, *Serratia marcescens* - NJC 21, *Pseudomonas aeruginosa* – NJC 4 were isolated and characterized for their phytate solubilizing capacity and were used in the formulation of dual species consortia and multispecies consortia. For further studies, all strains were maintained on the nutrient agar slants at 4 °C [8].

In vitro interaction among the rhizobacterial strains to prepare consortia

All the three isolates were further checked for their antagonist or synergistic activity against each other to prepare their dual and multispecies compatible consortia. For this test, a single isolate will be streaked on nutrient agar plate and rest of the 3 samples will be streaked perpendicularly to the prior streaked isolate in such a way that both the colonies meet each other. This plate is then incubated at 37 ºC for 24 hr. The growth of the isolate or the zone formation around the colonies will indicate us to interpret the antagonist or synergistic behavior of the isolate against each other [9].

Growth profiling of rhizobacteria

To observe the behavior of isolated bacteria in broth growth profiling is performed. 1% of activated inoculum is inoculated in 100 ml of nutrient broth and shaking conditions were provided. For 3 hours at an interval of every 30 min, absorbance was recorded using spectrophotometer at 470 nm. McFarland graph is used as standard and optical density of the unknown samples is plotted against it to obtain the no. of cells/ml. The same protocol is used to generate growth profile of the bi-species and tri-species consortia of the same isolated bacteria. In case of consortia, equal volume of the two and three species which are at their same exponential phase of growth were mixed together in equal amount in nutrient broth. The mean growth of bacteria is calculated using formula constant = 3.322 (logNt- log N_0)/D_t, where N_t = final cell number and N_0 = initial cell number and D_t = Difference in time. The graph of each consortia with respect to the isolate was prepared [9].

Qualitative estimation of phytate solubilization

Phytate solubilizing efficacy of each isolated strains evaluated by spot inoculation of activated culture on phytase screening medium having sodium phytate as sole soul source of organic phosphate. Zone of clearance around the colony observed after 3-5 days of incubation at 28° C. The solubilizing efficacy of insoluble phytate by isolates primarily examined by using following formula.

$$\text{Phytate Solubilizing index} = \frac{\text{Colony diameter} + \text{halo zone diameter}}{\text{Colony diameter}}$$

Quantitative estimation of phytate solubilization

Further, the same experiment was repeated for quantitative estimation of phytase production by individual strains and their consortia. Briefly, 50 mL of phytase screening media without agar was seeded with 1% respective young cultures. The cultures were incubated at 28° C on a rotary shaker for 3 days. Culture supernatants, or crude enzyme fluid, were extracted from the bacterial culture by centrifugation at 10,000 rpm for 15 min. The supernatant was then stored at 4°C for phytase activity testing.

Following the procedure outlined by Quan et al., the amount of liberated inorganic phosphate from phytaye degradation was measured in culture supernatant to determine the phytase activity. The reaction mixture consists, 0.2 M acetate buffer containing 1 mM of sodium phytate at pH 5.5. Inoculate 0.2 ml of culture supernatant in 0.8 ml of acetate buffer and incubate the mixture at room temperature for 30 min. After sufficient incubation add 1 ml of 10% TCA to stop the reaction. To analyze the reaction Harland and Harland

method is followed, where reaction is mixed with ammonium molybdate reagent and after incubation of 30 min at room temperature absorbance at 660 nm in spectrophotometer is determined [10].

Estimation of Ammonia production

By using the procedure suggested by Cappucino and Sharman in 1992, the test for estimation of ammonia production was conducted for single strains as well as developed bi-species and tri-species consortia up to 72 hours. Active isolated bacteria and developed consortia is inoculated in sterile peptone broth and allowed to incubate at 28° C for 24 hours. The occurrence of brown to yellow color after addition of Nessler's reagent in the media were considered as positive for ammonia production by that particular bacterium and/or its consortia [11].

Determination of potassium solubilization efficiency

To determine the potassium solubilizing capacity of bacteria, the isolates were tested on Aleksandrov's medium supplemented with potassium aluminium silicate as insoluble source of potassium. The 24 hour prior activated culture of bacteria were inoculated by making a spot on the media and plates were incubated at 28-30°C for 24 hour. Colonies on the medium supplemented with aluminium silicate which developed hollow zones were indicates the mineral potassium were solubilized by them [12,13].

Seed Bio-priming

Healthy seeds of *Macrotyloma uniflorum* were purchased and disinfected with 0.2% sodium hypochlorite solution and then washed with sterile distilled water till all the residual sodium hypochlorite solution is removed from to seeds. Further, the seeds were covered with 1% carboxy methyl cellulose (CMC) as an adherent and then seeds were bio primed with the isolates and their two species and multispecies consortia [14,8].

Pot assay and seed germination study

Sterilized garden soil was used to fill each experimental pot. Bioprimed seeds (10 seeds/pot) were introduced in pot and watered regularly. The plants were uprooted after 21 days of sowing in order to measure vegetative parameters like root length, shoot length, and root and shoot weight (fresh and dry). As a negative control, few pots are sowed with untreated seeds. Diurnal, the pots are observed for seed germination, root length as well as shoot length and Emergence % and vigor index is estimated [15].

Percent emergence = (Number of seeds emerged ÷ Number of seed sown) × 100

Vigor Index = Emergence (%) × seedling length (cm)

Estimation of micronutrient content in plant roots and shoots

Control and bio primed plants were analyzed for micronutrient content in root and shoot to determine the effect of various treatments on nutrient uptake by the plant. For the precise determination of microelements like Zn, Fe, Mn, and Cu, the plant roots and shoot samples were subjected to acid digestion (a mixture of nitric-hydrochloric acids HNO_3– HCl in a ratio of 1:3). Extracts were analyzed by Atomic absorption spectroscopy [16].

Available phosphate in soil

For the determination of the accessible phosphate in soil, a mixture of 2.5 g of soil, 50 mL of 0.5M $NaHCO_3$ (pH 8.5), and 0.5 mL of 5N H_2SO_4 was prepared and agitated until CO_2 evolution ceased. 4 mL of ascorbic acid were added to a volume of 100 mL of distilled water. Using a spectrophotometer, the intensity of blue color was measured at 760 nm wavelength after 10 minutes of incubation. Without soil, blank readings were taken in the same manner [17].

RESULTS

In vitro interaction of isolates and growth profiling of rhizobacteria

For consortia creation, three distinct PGPR strains were chosen: *Bacillus spp.* - NJC 1, *Serratia marcescens* - NJC 21 *Pseudomonas aeruginosa* – NJC 4. All of the strains were given the opportunity to interact with one another other on plate. All three strains, *Bacillus spp.*- NJC 1, *Serratia marcescens* - NJC 21 *Pseudomonas aeruginosa* – NJC 4 were able to develop concurrently, i.e. they did not hinder each other's growth. As a result, we chose these specific strains for consortia development **(Table 1).**

The growth profiling was already done for the two species consortia. Mean growth rate of isolate and two species consortia was already reported in previous study. Present in vitro interaction under liquid culture condition, mainly focuses to evaluate growth profile behavior for multispecies consortia. The mean growth rate constant of multi species consortia was 0.70±0.01/h. Growth profiling of isolates and developed consortia favors the synergistic behavior among them **(Figure 1)**. In vitro analysis under liquid culture conditions, allows the formulation of two species and tri species consortia. Selected isolates were fast growing and exhibit the commensalism for the mixed species culture.

Phytate solubilization

On a phytase screening medium containing sodium phytate as a sole source of organic phosphorus source, all three strains were evaluated for their capacity to solubilize insoluble organic P. All strains and developed consortiums dissolved sodium phytate, as evidenced by the formation of a halo zone around the spots, signifying the release of free P. Based on the results of plate screening, a strain with a higher solubilizing index value has a higher phytate degradation potential. Present study indicates, the plant growth promoting strain NJC 21 found to be highest solubilizer among the isolates. While, **Figure 2** expresses the maximum solubilizing index was found in consortia tri species consortia.

Phytase activity is a significant metric for estimating degradation potential; the higher the phytase activity, the greater the degradation potential. According to the phytase assay used for quantitative screening, PGPR strain NJC 4 has the maximum phytase activity in the supernatants. **(Figure 3)**.

Phytate solubilization production profile was also valued with phytase screening broth containing *Bacillus spp.* - NJC 1, *Serratia marcescens* - NJC 21 *Pseudomonas aeruginosa* – NJC 4. and their consortiums. The solubilization of phytate began after 24 hours and peaked on the second day of incubation; it was time dependent and increased with incubation duration. When sodium phytate was used as a substrate as an insoluble organic P, the maximal phytase activity was observed in isolate NJC 4. After 2 days of incubation, the highest level of phytase activity was seen in multispecies consortium (2.6 U/mL), followed by two species consortia (2.38 U/mL).

Ammonia production assay

Ammonia production assay was done by using Cappucino and sharman method and estimated for isolates NJC 1, NJC 4 and NJC 21 along with developed bi-species and tri-species consortia at 24hr. Regarding the production of ammonia, all of the strains that were examined were capable of doing so at varying quantities, falling anywhere between 15 to 28.6 µg/mL **(Figure 4)**. The value found to be most significant was found in multi species consortia, while the value found to be the lowest was found in NJC 1.

Determination of potassium solubilization efficiency

For the quantitative measurement of the PGPR's ability to dissolve K, a potassium alumino silicate in Aleksandrov's medium was used. All of the strains were shown to be able to dissolve potassium. All of the bacteria could dissolve K, and the solubilization zone indexed from 0.8 to 1.8 **(Table 2)**. Strain NJC 21 had the strongest ability to dissolve K. The next ones were NJC 4 and NJC 1.

Pot Assay

The seed inoculation experiment on sterile soil with *Macrotyloma uniflorum* established the isolates and consortium's capacity to stimulate plant development. Individually and in formed consortia, *Macrotyloma uniflorum* (horse gram) seeds were bio primed with *Bacillus spp.* - NJC 1, *Serratia marcescens*- NJC 21, and *Pseudomonas aeruginosa*- NJC 4. The current study reveals that bio primed seeds had higher seed emergence in pots than control seeds. Seed emergence ranged from 52-75.77% in isolated bacterial plants compared to controls, and 84-87.66% in consortia. Bacteria NJC 4 had the greatest impact, 75.55%, which is nearly twice that of the control. After 21 days of planting, tri-species consortia had the best results, with 87.77% emergence, nearly double the control. As a result of this research, it has the potential to conclude that co-inoculation can improve seed emergence at a faster pace **(Figure. 5)**.

One of the most important quantitative parameters is called the seedling vigor index. This index takes into account all of the qualities of the seed that have an effect on the possible degree of activity and performance the seed could achieve. The application of NJC 4, which is a PGPR strain, to the seeds exhibits the highest degree of seedling vigor index compared to any other single-species culture application. However, according to the findings of the current investigation, the seedling vigor index was significantly increased when the PGPR strains were combined as a two-species culture, multi species culture and then administered to the seeds. The seedling vigor index of the combination of NJC 1 and NJC 21 was tenfold higher than that of the control and individual treatments combined. This resulted in maximal growth enhancement.

Estimation of micronutrient content in plant roots and shoots

The micronutrient acquirement in *Macrotyloma uniflorum* was examined for microelements like Zn, Fe, Mn, and Cu in plant root and shoot samples. The effect of bacterial treatment on the concentration of plant metal content was derived **(Figure. 6)**. There was clear increase in micronutrient content of plant in presence of isolates but effect was prominent in presence of consortia. Copper was found almost two times higher in the root of consortia treated plants than the control while, we got 16-28% higher Cu in consortia than the highest Cu containing plant treated by isolates. The same occurs in the case of Mn as well as Zn, in which we found respectively 69% and 79% higher content than the control. Consortia treated plants proved to have 33-40% higher Mn than the isolates while this number was at 35-41% in case of Zn. The lowest difference we found was in Iron (Fe). Consortia treated plant have 18% higher Fe content than the control and 8% higher than the isolates which is merely statistical.

Similarly, in shoot micronutrient content we found almost similar trend as root micronutrients. In consortia treated plants, we have got more than two times higher Cu, whopping 106% higher Mn, 55% higher Zn and 16% higher Fe content than the control **(Figure 7)**. As compared to isolates treated plants, consortia treated plants got 10-21% higher Cu, 43-49% higher Mn, 33-41% higher Zn, and 7% higher Fe.

In both, root and shoot micronutrient content, consortia can increase Mn and Zn significantly and differences in Fe content among control, isolates and consortia is only statistical while the difference in Cu content was remain somewhat in the middle range. Thus, these consortia have significantly higher potential than the isolates to increase the micronutrient content of the plant.

Available phosphate in soil

Phosphorus levels in soil were estimated to be 2.78 mg/kg prior to inoculation with bacterial cultures and their consortiums in pot study **(Figure 8)**. After inoculating cultures of bacteria and their consortia, the levels of Phosphorus levels increased with each treatment, with tri species consortia having the highest (5.83 mg/kg), followed by two species (4.67mg/kg) and isolate NJC 4(4.2 mg/kg).

Table 1. Strains and consortia composition

Strains and consortia	Notation
Bacillus spp.	NJC 1
Pseudomonas aeruginosa	NJC 4
Serratia marcescens	NJC 21
Pseudomonas aeruginosa + Serratia marcescens	NJC 4 + NJC 21
Bacillus spp. + Pseudomonas aeruginosa+ Serratia marcescens	NJC 1+ NJC 4 + NJC 21

Table 2. Potassium Solubilization Index

Strains	Potassium solubilizing index
NJC 1	0.8
NJC 4	1.2
NJC 21	1.8

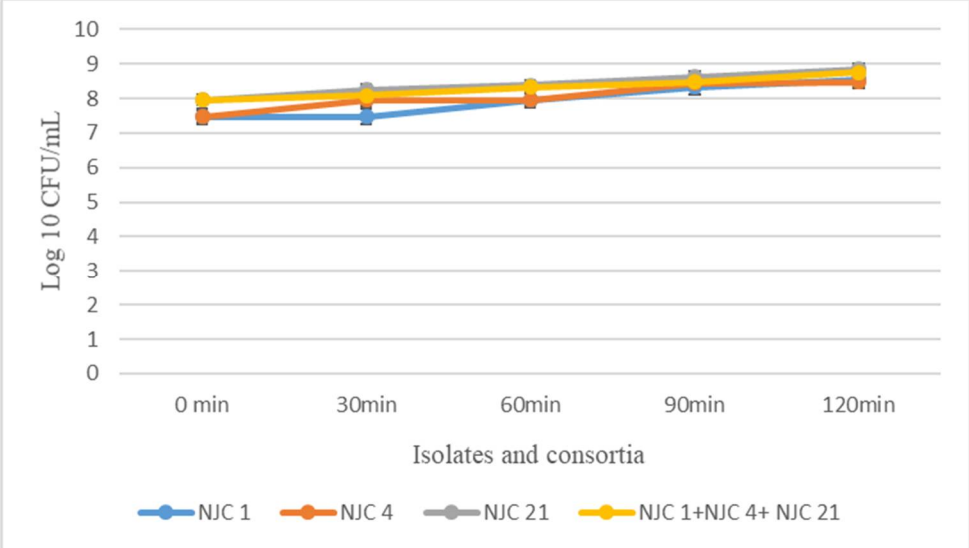

Figure 1. Growth profiling of multi species consortia

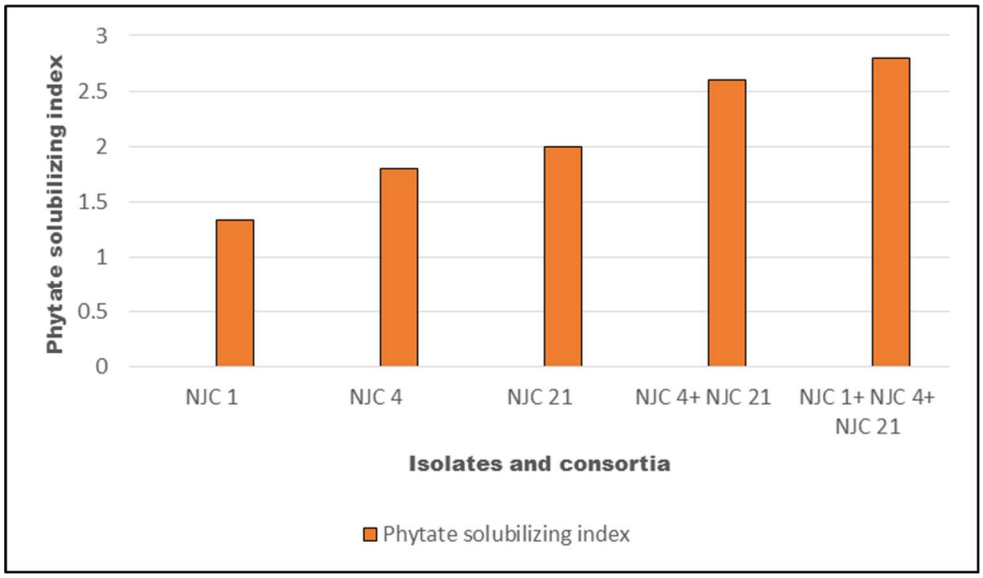

Figure 2. Phytate solubilizing index of isolates and consortia

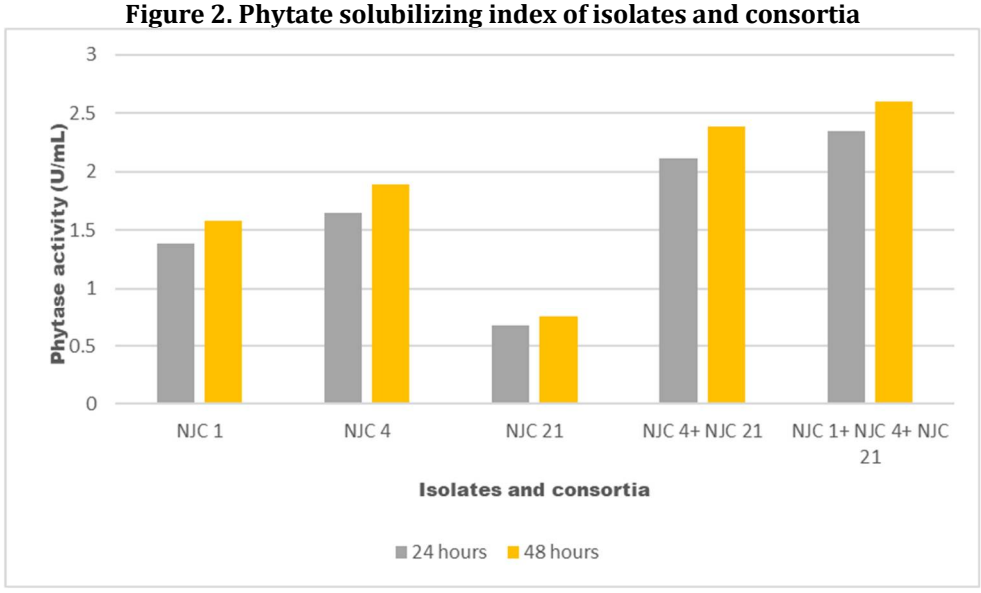

Figure 3. Quantitative analysis of Phytase enzyme by isolates and consortia

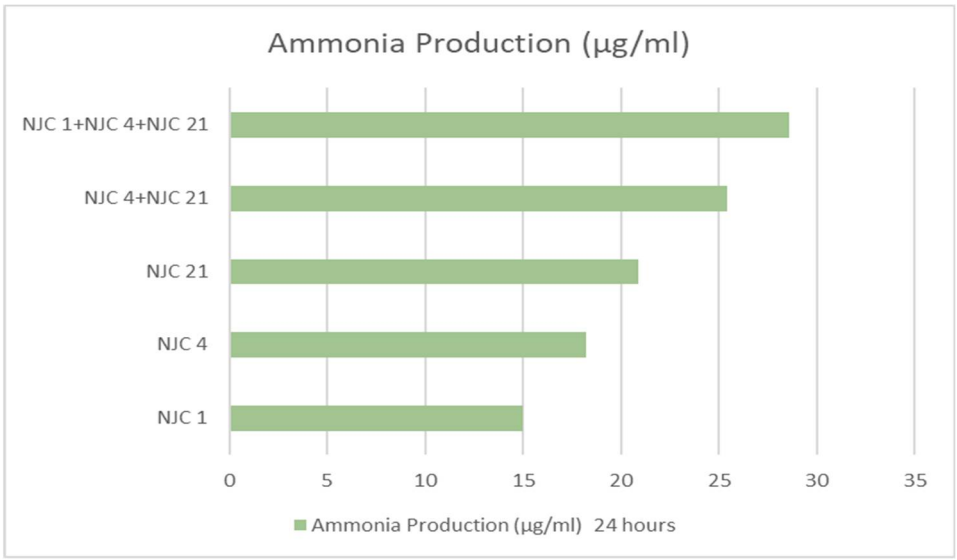

Figure 4. Ammonia production by isolates and consortia

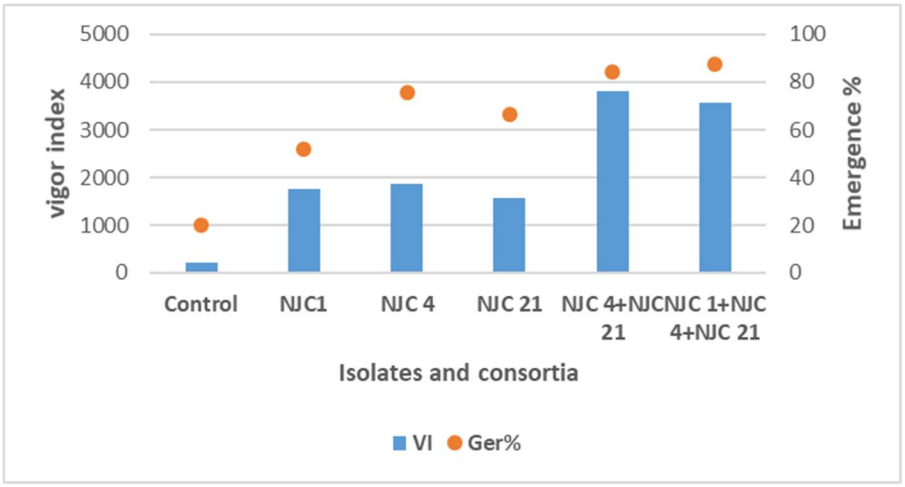

Figure 5. Vigor index and emergence percentage by isolates and consortia in *Macrotyloma uniflorum*

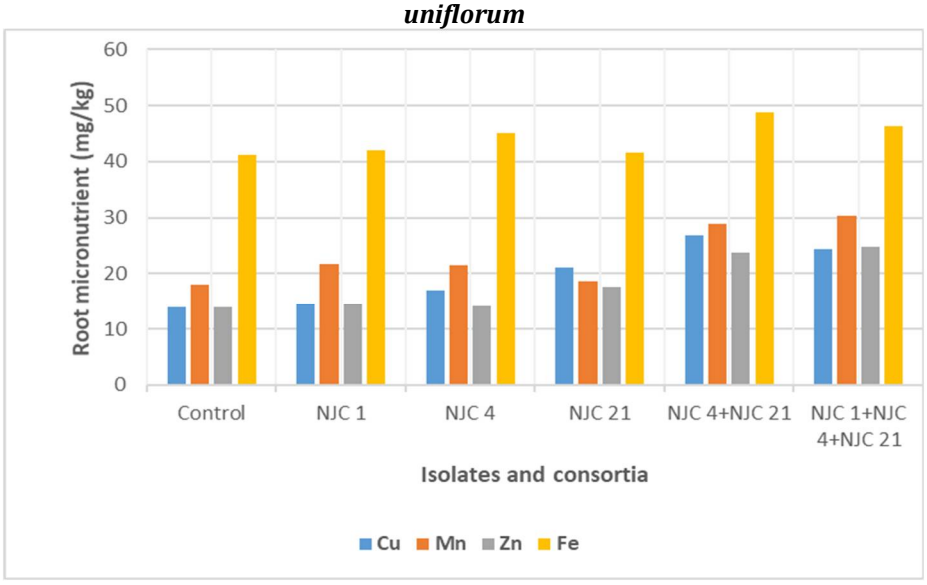

Figure 6. Analysis of Root Micronutrient Content

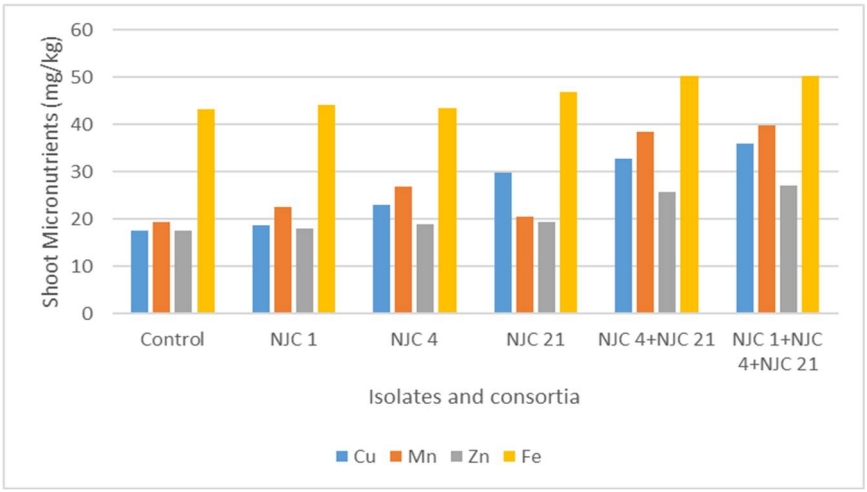

Figure 7. Analysis of Shoot Micronutrient content

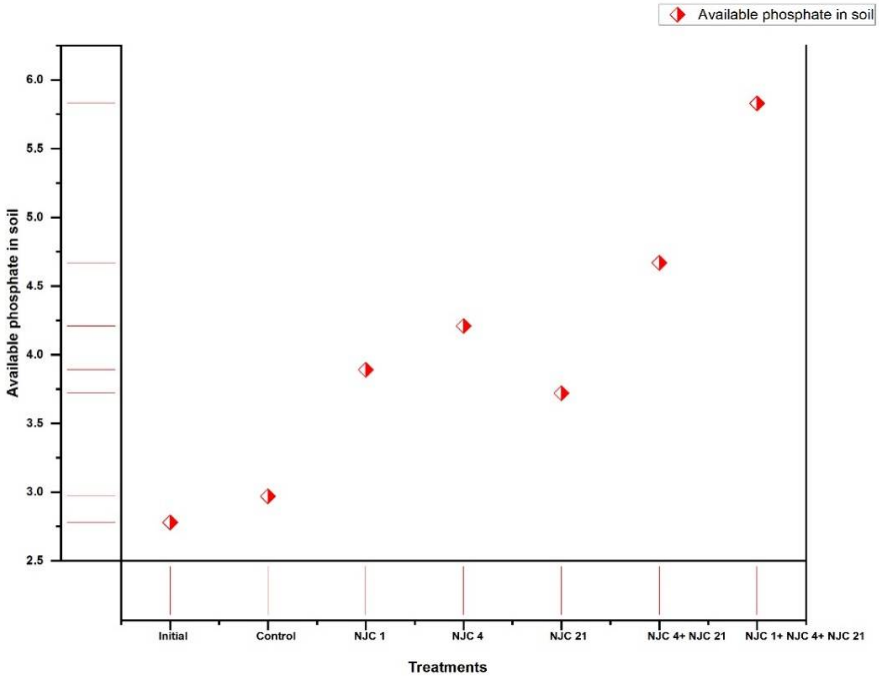

Figure 8. Analysis of Available Phosphate in Soil

DISCUSSION

There is a vast variety of microorganisms that thrive in the soil around plants and are collectively known as plant growth-promoting rhizobacteria [18]. Their importance in enhancing nutrient uptake in difficult soils or deforested regions and maximizing fertilizer efficiency is well known. However, they are less investigated biofortification solutions and should be included in agronomic and breeding approaches to produce efficient crop biofortification strategies. Bio fortification is the technique of supplementing crops or foods with important micronutrients and other health-promoting chemicals to increase their nutritional content.

Our research aimed to improve the bioavailability of micronutrients of a selected legume plant during the pot experiment by employing phytate-solubilizing bacterial strains. Previously isolated strains *Bacillus spp.* - NJC 1, *Serratia marcescens* - NJC 21 and *Pseudomonas aeruginosa* – NJC 4 were reported to have plant growth promoting activities like phosphate solubilisation, HCN production, and IAA production [8]. In present investigation, insoluble phytate (i.e. sodium phytate) was the only source of phosphorus that was employed in the routine screening of phytate solubilizing strains using a plate assay method. According to the findings of a study, *Bacillus spp.* - NJC 1, *Serratia marcescens* - NJC 21 and *Pseudomonas aeruginosa* – NJC 4 and their consortia were able to dissolve insoluble sodium phytate. Quantitative profiling of phytate solubilisation in liquid phytate screening media reveals the highest phytate solubilization achieved in tri species consortia at 48 hours. The strains and developed consortia also able to solubilize potassium and produce ammonia.

It has been observed that the use of bacterial inocula as a form of bio-fertilizer can both boost the growth of plants and increase the yields of plants [19]. In this study, the favourable effect of three different efficient PGPR strains and their consortia on the growth of legume plant *Macrotyloma uniflorum* was studied, and it was found that all of them could greatly improve the growth of selected crop compared to control. It could be attributed to their phytate-solubilizing ability, which helps soils with a low concentration of accessible phosphorus support the uptake of phosphorus nutrients and the growth of plants. After, 21 days of sowing of bio primed *Macrotyloma uniflorum* seeds shows the magnified germination percentage and vigor index compared to control. Highest vigor index was achieved in two species consortia and maximum emergence percentage observed in tri species consortia. Besides the physiological parameters bioprimed seeds also shows higher concentration of microelements in plant roots and shoots compared to control. Among sole inoculations a better increase in manganese concentration was shown by *Bacillus spp.* which was 19%, followed by *Pseudomonas aeruginosa* and *Serratia marcescens* with a rise of 13% & 5% respectively.

Application of tri species consortia showed the maximum root manganese content over the absolute control which was 88%. Micronutrient analysis of root and shoot samples reveals the potential of consortia to aid in delivering the necessary micronutrients with higher concentration compared to control.
Before inoculating the soil with bacterial culture and their consortia, the soil's phosphorus content was estimated and recorded as 2.78 mg/kg. The highest concentration of phosphorus was found in tri species consortia (5.83 mg/kg) treated soil following inoculation with bacterial cultures and their consortiums and evidenced that consortia has more power to increase the availability of macronutrient like phosphorus. The higher concentration of phosphate in soil is also evidence of increased level of phytase activity.
This increased level of phytase activity not only satisfies the plant's demand for phosphate, but it also breaks down the metals that had formed complexes with phytate in the soil and increases the bioavailability of the soil's micronutrients for plant uptake which may be one of the reason of higher micronutrients level in plants [20,21].
Thus, the PGPR isolated here are potential in the plant growth promotion, but when it is applied in the form of consortia, it shows significant impact on the plant growth promotion. Not only plant growth promotion, plant shows significant increment in macro and micronutrient content, such as ammonia production, phosphate solubilisation, HCN production and micronutrient content. All of which can produce nutrient rich crops in other words, biofortified crops which doesn't need any additional fortification.

CONCLUSION

It is evident that strategy of biofortification via bacterial biofertilizer is potential strategy. It is advantageous in many ways such as it can provide better yield, as it can promote the plant growth significantly, Improved seed, emergence percentage and seedling vigor index, and nutrient rich biofortified crop, eliminating the need of additional fortification. Rather than using synthetic fertilizers, biofertilizers are the way of the future and consortia has been proven to be of great potential than the isolates in this regards. There is still a much gap to be filled but the results seems promising that consortia can provide ultimate biofortification solution to us which can be a further step in the sustainable agriculture and ultimately sustainable development.

CONFLICT OF INTEREST

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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