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Impact of microbial consortium along with biochar and pressmud on mashbean yield and seed quality in field conditions

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Abstract

Application of organic matter sources along with microbial formulations stimulates microbial activities and their potential to support plant growth, and enhances plant survival under field conditions. We evaluated the impact of different rhizobial and rhizobacterial strains in sustaining plant growth under field conditions by using Vigna mungo as test crop. Unlike most previous research that examine single microbial inoculants or overlook the role of local organic amendments, current study evaluated the consortium of indigenous rhizobium and PGPR with biochar and pressmud to synergistically improve mash bean growth, yield and quality. 16S rRNA sequencing revealed Rhizobium sp., 'Stenotrophomonas rhizophila' yielding most efficient outcomes followed by Bacillus subtilis, and Bradyrhizobium japonicum. Co-inoculation of bacteria (S. rhizophila and Rhizobium sp.) along with 0.5% orange peel biochar and 5 tons/ha pressmud proved to be the most efficient in this regard. Treatment (T5) exhibited the greatest increase in plant height (51%), plant fresh weight (127%), plant dry weight (174%), 1000-grains weight (37.4%) than control. Next best increase Bacillus subtilis, Bradyrhizobium japonicum, 0.25% biochar, 10 tons/ha pressmud yielded the next best increase in grain yield (37.4%). Maximum crude protein (54%) was observed with inoculation of strain 'PKA-18, RKA-47, 0.5% biochar, 5 Tons ha^{-1} pressmud as compared to un-inoculated control. Among all the tested isolates, co-inoculation of T5 followed by T4 was the most efficient treatment in improving mash bean growth, yield, and seed quality.

Keywords: Mash bean, co-inoculation, rhizobium, PGPR, seed quality, biochar, pressmud, field trial

Introduction

Agricultural productivity is a critical factor to ensure world food security, particularly for a rising population estimated to reach 9 billion people in 2050 (Bahar et al., 2020). According to global agriculture productivity (GAP) and world health organization (WHO), 80 g/capita/day of food is required for a healthy person but currently insufficient to reach (Anonymous, 2016; Zeigler and Steensland, 2022). The use of biological nitrogen fixing leguminous crops offers a viable solution to this problem by converting unavailable atmospheric N to plant useable form, thereby improving soil fertility (Bochalya *et al*., 2021). Pulses, belonging to the Fabaceae family, are self pollinated, yellow flowering, auxiliary inflorescence and have a unique place in cropping classification as a main staple food for 90 million people. It is a crucial part of diet that contains 3 times more protein, vitamin, carbohydrate and minerals, and is also used as catch, cover, green manure, fodder and as intercrop.

A useful tool of modern sustainable agriculture is the use of bio-inoculants along with organic matter sources such as composts or biochar from household wastes. Biofertilizers contain bacteria which ensure adequate supply of nutrients (through different mechanisms) to the host plants to improve their growth and development, and regulate their physiology. Despite the known biological nitrogen fixation benefits of rhizobia and pulses in improving soil fertility, there remains a significant gap in our understanding of how different bacterial strains in combination with biochar and organic materials such as press mud can be optimized to improve crop yield and soil quality under various environmental conditions. Rhizobia exist in root nodules and as free-living, while within the vicinity of the plant root i.e., rhizosphere, rhizobacteria reside. Plant growth promoting rhizobacterial strains can increase plant development through various mechanisms such as phosphorus solubilization, siderophore, phytohormone, exopolysaccharides and ACC-deaminase activity (Cardinale *et al*., 2015), but the potential benefits of

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using a consortium of bacteria in combination with organic amendments like biochar remain underexplored, particularly in heterogeneous field conditions. Legume rhizobial symbiosis might be a good source of nitrogen (N) in place of inorganic N sources and could reduce N requirements and improve vegetative growth, maturity, fixation soil capacity and crop yield (Allito *et al*., 2020; De-Carvalho *et al*., 2020; Kebede *et al*., 2021). The most vital benefit of rhizobium N fixation is as a renewable source and there is scant probability of leaching and volatilization (Sanchez-Navarro *et al*., 2020). Both amendments are cost effective, locally available by products. Pakistan produces 2.29 million tons of citrus which produces significant amount of peel, and pressmud as by-product of sugarcane in tons (Imam *et al*., 2021). Often these sources are discarded and unutilized, making them low cost inputs and easily accessible to farmers.

Vigna mungo is an important pulse in Pakistan, because it provides 25-28% protein, essential amino acids, mineral contents in significant amount, while improving soil fertility through N-fixation. Characters of short lifecycle marginal lands adaptability make it more suitable for smallholder farmers, since most farmers in Pakistan are smallholders. However, its productivity is constrained due to Pakistani soil condition, nutrient deficiencies, and poor nodule formation. As Pakistan is a net pulses importer, increasing local production can lower this import, which can improve food self-sufficiency as well as farmers livelihood. To address these challenges bacterial strains were selected from local mash been growing area of Punjab. These strains of rhizobia and PGPR were screened from huge number of isolates on the basis of their demonstrated characters of nutrient solubilization ultimately increasing availability, stress tolerance, enhancing nodulation. Thereby offering a low-cost, eco-friendly approach to strengthen Pakistan's pulse sector and agricultural economy. Benefits of bacterial application for legumes are well documented, most prior studies focused on single, not evaluated with combination of these locally available organic matter sources. Synergistic potential of these strains with organic matter sources in Pakistan's alkaline soils remain largely unexplored in heterogenous conditions.

This study aims to fill and investigate the knowledge gap in how mash bean growth and yield under field conditions can be improved through coinoculation of rhizobium, and rhizobacteria along with amendment of commonly available organic matter sources. The experimental hypothesis is that this combination will enhance plant growth under varying environmental conditions. The current research involved isolation and characterization of indigenous rhizobium and PGPR from mash bean growing area in Pakistan, assessing their phylogenetic diversity through 16S rRNA gene analysis, and evaluating their effects on plant growth promotion. Obtained bacterial strains after growth optimization will maintain their activity in heterogenous soil conditions, thus contributing to sustainable plant growth and yield. The application of bacterial consortia can play a critical role in crop growth by enhancing soil and plant health, where single bacteria may not thrive (Yaduwanshi *et al*., 2021; Karuppiah *et al*., 2022). Coinoculation improves resource utilization, plant growth, and biocontrol capabilities, addressing issues of bacterial stability in field conditions (Alves *et al*., 2022). This novel approach may help maintain long-term strain functionality. These findings may provide new insights into the role of bacterial consortia in improving crop productivity and soil health, offering a more sustainable alternative to chemical fertilizers.

Material and Methods

Plant growth promoting rhizobacteria and rhizobia were isolated from mash bean sown in three different areas of Faisalabad Punjab, Pakistan, and were screened based on their potential for plant growth promotion abilities (earlier study). Rhizobia and PGPR isolates showing optimum growth in yeast extract mannitol (YEM) and Luria bertani (LB) media, respectively were further assessed for plant growth promotion. For the estimation of Gram staining (Claus, 1992), exopolysaccharide (Ashraf and McNeilly, 1992), phosphate solubilization (Pikovskaya, 1948), siderophore (Schwyn and Neilands, 1987), qualitative ACC-deaminase activity (Dahiya *et al*., 2019), catalase (McFaddin, 1980) and oxidase methods were followed. In the same way, congo red, glucose peptone agar, ketolactose, and growth on alkaline medium were measured by using the methods employed by Somasegaran and Hoben (1994), Deora and Singal (2010), Bernaertz and Daley (1963) and Kumar and Pindi (2021) respectively.

Based on initial characterization, four strains (PKA-18, PKA-25, RKA-46, and RKA-47) were selected to check their impact on growth, seed quality and yield of mash bean plant under field condition. For microbial identification, stab culture of isolates was prepared and send to Macrogen Korea for 16S rRNA identification. Microbial identification indicates these strains are (*Stenotrophomonas rhizophila,* PKA-18; *Bacillus subtilis,* PKA-25; *Bradyrhizobium japonicum,* RKA-46; *Rhizobium sp.* RKA-47).

Field trial

A field trial was performed to determine the effect of bacterial consortium along with biochar, pressmud levels on mash bean growth, yield, and seed quality, by using factorial

RCBD design with levels of amendments, rhizobial and PGPR strains at the University of Agriculture Faisalabad, Pakistan (31.26◦ N, 73.06◦ E). Five treatments mentioned in (table 1) were tested in three replicates. Plots were randomized by following randomized completely block design (RCBD). Plant Growth, seed quality, and yield attributes were recorded at crop harvest. The treatment plan of the field trial mentioned in (Table 1). Orange peel was collected and air dried for biochar preparation. Pyrolysis was performed at 350ºC in a closed vessel. Sieved and used.

Inoculum preparation and seed inoculation

For fresh inoculum, YEM agar (for 1L is 10g mannitol, 0.5g yeast extract, 0.5g K2HPO4, 0.2g MgSO4.7H2O, 0.1g NaCl, 15g agar (for agar plates), and congo red), LB agar media (1L is 10g tryptone, 5g yeast extract, 10g NaCl, 15g agar) for rhizobium and PGPR strains respectively was prepared. Broth were inoculated for each strain, LB was incubated for 48 hours while YEM for 64 hours at 120 rev/min and 28 ± 2 °C on an orbital shaker. Culture with (10^6–10^8 CFU mL^{-1}) was used for inoculation. In laminar flow hood seeds are surface sterilized and inoculated with cultures. For inoculation 10% sugar solution and peat-based slurry was used.

Plant analysis

Digestion

In a digestion flask 0.5g of plant samples, conc. sulfuric acid (H_2SO_4) and hydrogen peroxide (H_2O_2) were added, then heated at 350◦C till the colorless endpoint was obtained. After dilution, the digested material was used for nutritional analysis (Wolf, 1982).

Table 1: Treatment plan for field trial

Indicated By	Treatment Details
Control	Control
KC1	Combination 1 (PKA-25, RKA-46)
KC2	Combination 2 (PKA-18, RKA-47)
KC1-B1P2	Combination 1+B1+P2 (PKA-25, RKA-46 +0.25%+10 Tons ha^{-1})
KC2-B2P1	Combination 2+B2+P1 (PKA-18, RKA-47 + 0.5%+ 5 Tons ha^{-1})

Strains: Combination 1=(*Bacillus subtilis + Bradyrhizobium japonicum*), Combination 2=(*Stenotrophomonas rhizophila + Rhizobium sp.*). **Biochar:** B0=(Control), B1=(0.25%), B2=(0.50%). **Pressmud:** P0=(Control), P1=(5 tons ha^{-1}), P2=(10 tons ha^{-1})

Table 2: Characterization of rhizobacterial isolates

Isolates	Phosphate Solubilization	Siderophores production	Catalase	Gram staining	Oxidase	Exopolysaccharides	Qualitative ACC-deaminase
Control	-	-	-	-	-	-	-
PKA-18	+	+	-	+	++	+	-
PKA-25	+	+	+	-	+	+	+

Note: –, no reaction; +, moderate production; ++, good production: +++ high production.

Table 3: Characterization of rhizobial isolates

Isolates	Congo red test	Glucose peptone agar test	Ketolactose test	Gram staining	Phosphate solubilization	Siderophore production	Catalase	Oxidase	Exopolysaccharide	ACC-Qualitative
Control	+	-	+	-	+	+	++	++	+	+
RKA-46	+	+	+	-	+	+	+	++	++	+
RKA-47	+	-	+	-	+	+	++	++	+	+

Note: –, no reaction; +, moderate production; ++, good production: +++ high production.

Table 4: Interactive effect of Consortium and organic matter sources on NPK uptake of mash bean

	NPK uptake		
Treatments	N %	P %	K %
Control	0.99 ± 0.068b	0.14 ± 0.027c	0.84 ± 0.068d
KC1	1.65 ± 0.136a	0.29 ± 0.027a	1.60± 0.068bc
KC2	1.10 ± 0.068b	0.25 ± 0.027ab	1.55 ± 0.068c
KC1-B1P2	1.68 ± 0.136a	0.19 ± 0.027bc	1.75 ± 0.068ab
KC2-B2P1	1.70 ± 0.136a	0.23 ± 0.027ab	1.10± 0.068a

Means sharing similar letter (s) are statistically at par with each other at $p \leq 0.05$, n=3. **Strains:** (KC1) Combination 1=(*Bacillus subtilis+Bradyrhizobium japonicum*), (KC2) Combination 2=(*Stenotrophomonas rhizophila+Rhizobium sp.*).
Biochar: B0=(control), B1=(0.25%), B2=(0.50%). **Press mud:** P0=(control), P1=(5tons ha^{-1}), P2=(10 tons ha^{-1}).

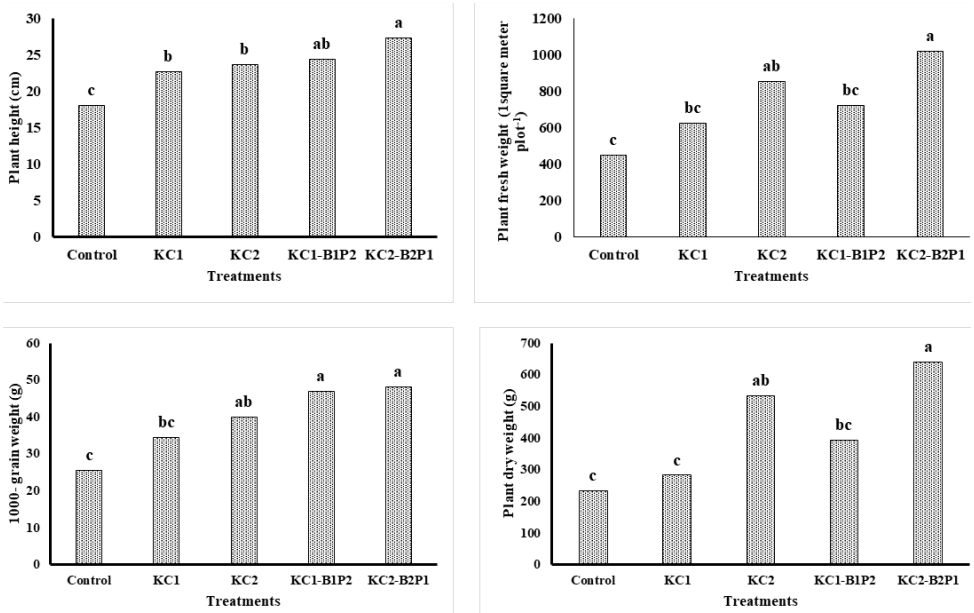


Figure 1. Effect of microbial inoculum and biochar and press mud amendment on plant height, plant fresh weight, plant dry weight and 1000-grain weight (g) of mash bean under field trial

Bars exhibiting same letters are statistically non-significant at ($p \leq 0.05$)

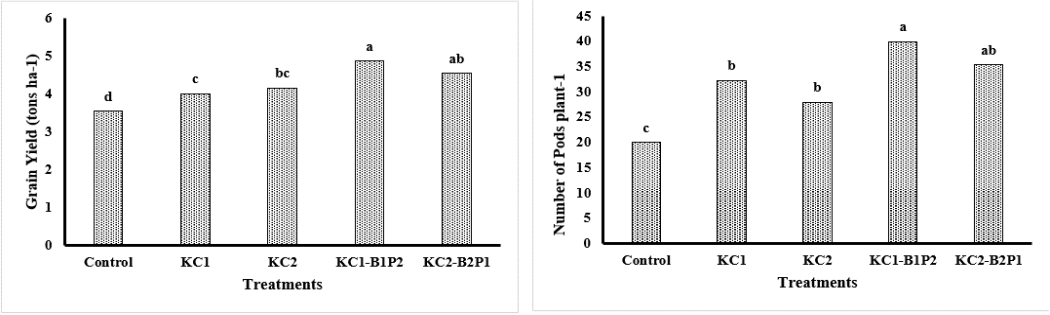


Figure 2: Interactive effect of microbial inoculant and organic matter sources on yield parameters of mash bean

Mean separation was determined using the Tukey HSD test at a significance level of $p \leq 0.05$ and means sharing similar letters are statistically at par with each other at n=3.

Strains: KC1=(*Bacillus subtilis* + *Bradyrhizobium japonicum*), KC2=(*Stenotrophomonas rhizophila* + *Rhizobium sp.*). **Biochar:** B0=(Control), B1=(0.25%), B2=(0.50%). **Pressmud:** P0=(Control), P1=(5 tons ha^{-1}), P2=(10 tons ha^{-1})

NPK determination

N was determined through a Kjeldahl ammonium distillation method (Jackson, 1962). In a distillation flask, 10 ml NaOH was added to a 5ml sample. A conical flask of 100mL with 4% boric acid was used to collect the distillate (NH_4^+). Distillation was stopped when a final volume of 50ml in in the receiver flask was obtained. After few minutes cooling, the contents were then titrated with H_2SO_4 (0.1 N) and an indicator (bromocresol green mixed in ethanol) to the appearance of the light pink end point color. The N contents in the titrated samples were determined by following standard calculations. For P determination, 5ml sample was added with 10 ml Barton reagent (Ashraf *et al*., 1992) and after making volume up to mark with distilled water and after 30 min analyzed on a spectrophotometer (SA-162800 NRTL/C UV-Vis, Thermo Electron Corporation) at 410nm. P concentration was determined by drawing a curve against standards. For determination of K in leaves which is an essential element Flame Photometer (Model BWB-XP, BWB Technologies, UK) was used (U.S. Salinity Laboratory Staff, 1954).

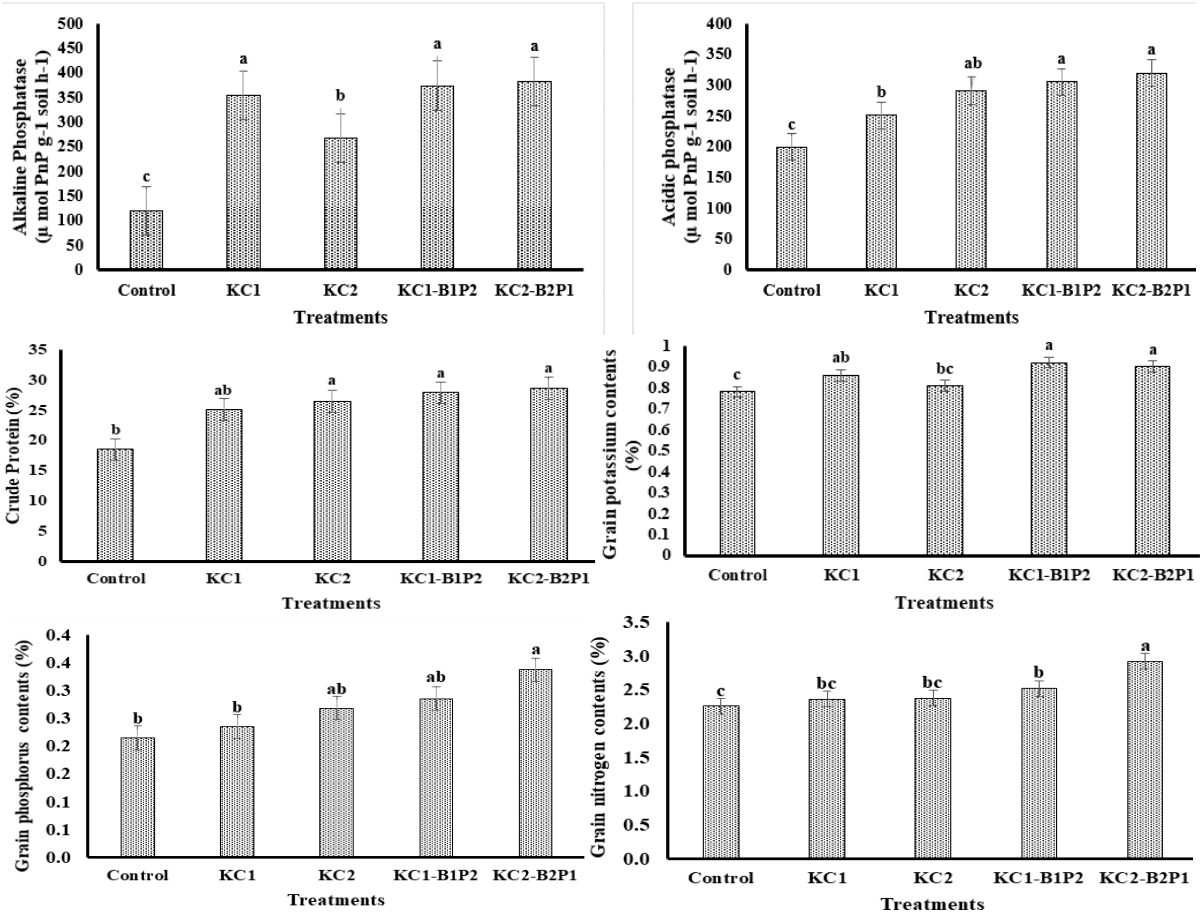


Figure 3: Interactive effect on Seed contents of mash bean i.e (A) Grain P , (B) Grain K %, (C) Grain N %, (D) Crude Protein %

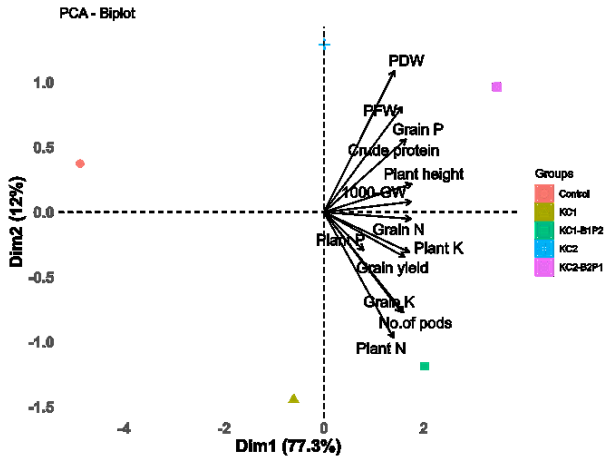


Figure 4: Principal Component Analysis (PCA) of consortium and along with different biochar and press mud levels under field conditions

Seed quality

Mineral contents

The mineral contents of seeds were measured after incineration (450°C for 12 hours) in a muffle furnace (Zia-Ul-Haq *et al*., 2012). After incineration, diacid (HNO_3:$HClO_4$) digestion was done. K was determined with a flame photometer, Kjeldahl method was used for Nitrogen, while phosphorus was determined by spectrophotometry.

Crude protein

Kjeldhal method was used to determine seed crude protein contents (Gharibzahedi *et al*., 2012) in which 0.5 g ground sample was mixed with conc. acid and digested on hot plate. After digestion, the mixture was cooled down and volume was made up to mark. In distillation, 5ml sample and 10ml sodium hydroxide were mixed. Conical flask with 2% boric acid was used. Distillation start in Kjeldhal apparatus. After distillation stopped and processed. Observed N values were multiplied by 6.25 to give protein content (Salo and Koivistoinen, 1996).

Statistical analysis

Data regarding *Vigna mungo* growth were analysed by Statistix 8.1 (Analytical Software) by following a RCBD. Significant differences among treatments were analysed by using analysis of variance (ANOVA) at 5% confidence level. Tukey's Honestly Significant Difference (HSD) post hoc test was used to differentiate among treatments. Tables and graphs were generated by using Microsoft Word and Excel 2021® (Office 365, Microsoft Cooperation, USA). Principal component analysis (PCA) analysis was performed by using built in R package.

Results

Results of the compatibility and pot trial signified that all the investigated strains (*Stenotrophomonas rhizophila,* PKA-18; *Bacillus subtilis,* PKA-25; *Bradyrhizobium japonicum,* RKA-46; *Rhizobium sp.* RKA-47*)* were compatible to each other and able to grow. In current field trial inoculation of strain was done either in combination, individually or along with various levels of orange peel biochar and pressmud, to assess their potential for improving enzymatic activity, the uptake of essential plant nutrients, seed contents conferring heterogenous field conditions. Properties of the experimental soil is clayey loam soil, with 8 pH, 0.59% organic matter and 0.09% total nitrogen contents. Application as a consortium was more effectual than individual application. Isolates were isolated and tested for different growth promoting mechanisms such as siderophore, P-solubilization etc. (Tables 2 and 3). Above mentioned 4 strains are screened on the basis of their capacity for phosphate solubilization, exopolysaccharide, siderophore production (Table 2 and 3). The results of parameters are provided below.

Microbial identification

Based on 16S rRNA sequencing by Macrogen Korea, 'PKA-18' was identified as *Stenotrophomonas rhizophila*, and 'PKA-25' as Bacillus subtilis. 'RKA-46', and RKA-47 were identified as *Bradyrhizobium japonicum,* and *Rhizobium sp*., respectively.

Growth and yield attributes

A strong interaction was revealed between co-inoculation of different strains along with different biochar, press mud levels and different plant attributes. Among different inoculants, 'KC2-B2P1' (*Stenotrophomonas rhizophila+Rhizobium sp* +0.50% biochar+ 5 tons ha^{-1} pressmud) led to a maximum increment in plant height (51%), plant fresh weight (1.3 fold), plant dry weight (1.74 fold), 1000-grain weight (37%) followed by KC1-B1P2, KC1, KC2 (Figure 1 and 2). In addition, KC1-B1P2 (*Bacillus subtilis+Bradyrhizobium japonicum* + 0.25% biochar+ 10 tons ha^{-1} pressmud) yielded more significant outcomes in terms of grain yield by (37.4%), when compared with control treatment (Figure 2).

Plant and Seed nutrients

Among different inoculants, application of '*Stenotrophomonas rhizophila+Rhizobium sp.*'+ 0.5% biochar + 5 tons ha^{-1} press mud increased N quantity in leave and grain by 71 and 29%, respectively, compared to un-inoculated control (Table 4). Among all treatments maximum P contents was observed in leaves by 'KC1' *Bacillus subtilis+Bradyrhizobium japonicum* (0.29%) and in seed 'KC2-B2P1' (56%) inoculation (Figure 3). Similarly, maximum increment in K^{+1} content was determined in leaves by KC2-B2P1 (1.8%), and by KC1-B1P2 (17%). Principal component analysis (PCA) analysis was performed (Figure 4) based on all the measured parameters (growth, physiological and seed quality) using software 'R-Studio'. Best performing treatment was T5 (combination 1+biochar 2+press mud 1) based on the result of PCA analysis. The PCA scoring indicated that maximum efficient treatment is T5.

Discussion

Mash bean is a vital crop for sustainable agriculture due to its dietary benefits, high protein content, and N-fixing

ability. However, challenges such as poor nodule formation, soil degradation from prolonged overuse of inorganic fertilizers and severe nutrient deficiencies in alkaline soils hinder *Vigna mungo* production (Vida *et al*., 2020). Under such circumstances, growth and development of plants is adversely affected. *Rhizobium* and PGPR improve plant growth through various mechanisms by exerting positive impacts on plant via different growth promotion mechanisms such as EPS production, Auxin production, ACC-deaminase activities, phosphate solubilization and siderophore production (Shabaan *et al*., 2022). Bacterial co-inoculation along with organic matter source improves plant growth and yield attributes through various mechanisms (Sanda *et al*., 2022). Enhancement in aboveground biomass might be due to the availability of organic carbon, nutrients and higher water retaining capacity of the biochar and press mud. Our results are in accordance with the results of studies conducted in literature by Shete *et al*. (2021) and Das *et al*. (2021). Microbial secretion of certain organic substances such as cytokinin, gibberellins, and auxin improves plant growth as these substances are known as growth regulators. Hypocotyl cell division induction and low dead cell accumulation in root cortex can significantly improves root area and biomass (Alam *et al*., 2015).

Co-inoculation of rhizobium and PGPR improve protein content in mash bean by forming new bands. Crop yield is mediated through nutrient solubilization which is reflected in plant nutrient concentrations. Higher NPK contents were observed due to BNF leading to more solubilization ultimately absorption and N translocation for plant uptake, in inoculated treatments compared to absolute control (Das *et al*., 2021). P solubilization is known as the main mechanism in PGPR which enhances BNF (Bargaz *et al*., 2018; Bhowmik and Das, 2018). Higher nodulation observed in inoculated plants was due to the role of P in nodule energetic transformations (Gomez *et al*., 2013). Reason for improvement in protein content with the combined effect of co-inoculation with organic matter sources, might be due to nitrogen fixing ability and number of nodules, results of this study were aligned with results of studies conducted by (Donohoe, 2017; Khalid *et al*., 2023). Improved N-fixation synergistically enhances other nutrients status by stimulating root growth, development, and rhizosphere activity. Extensive root development results in improvement of phosphorus and potassium. Increase in N enhances plant phosphorus uptake by regulating root environment (Schleuss *et al*., 2020). Nitrogen promotes secretion of root exudation such as organic acid, phosphatases. Energy by nitrogen assimilation supports the active uptake of potassium. Thus, optimum availability of nitrogen fulfills primary requirement of plant, but also positively influences other nutrients availability. More nitrogen and other nutrients uptake by grains are due to nodule fixing ability of N and co-inoculated bacterial capability to promote plant development (Dayoub *et al*., 2017; Barbosa *et al*., 2021; Araujo *et al*., 2021; Kaschuk *et al*., 2022).

Conclusion

Application of different organic matter sources to soil stimulates microbial activities, which in turn, improve plant growth by following different growth promoting mechanisms. However, among different PGPR strains PKA-18, PKA-25, while RKA-46, and RKA-47 rhizobia strains showed tremendous potential in terms of plant growth promoting characters. Here, we performed a field experiment to investigate the synergistic potential of different plant growth promoting rhizobacterial and rhizobial consortia and organic amendments (biochar and pressmud) in improving mash bean growth and yield. We observed that co-application of '*Stenotrophomonas rhizophila*' and *Rhizobium spp*. in association with orange peel biochar @ 0.5% and press mud @ 5 tons ha^{-1} yielded the most significant outcomes in terms of improving the growth and yield attributes of mashbean in addition to the crude protein contents as compared to control treatment. Moreover, based on PCA analysis T5 was found to be most significant treatment followed by T4 (*Bacillus subtilis* and *Bradyrhizobium japonicum* along with biochar @ 0.25%, and pressmud @ 10 tons h^{-1}). Co-inoculation of bacterial strains and organic amendments proved to be more significant as compared to individual application of either of these. In developing countries where the non-judicious and frequent application of agrochemicals increase the cost of operation, application of environmentally friendly and cost-effective innovations could be a way forward in helping the farmer community in raising their standard of living. In this way, outcomes of current study could urge the farming community to opt for more sustainable and economically viable solutions for enhancing their productivity. There is a need to adopt these outcomes in more diverse areas utilizing potential of other organic amendments and bacterial consortia.

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