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A Study on the Application and Efficiency of Novel Biofertilizer On Paddy: A Small-Scale Study

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Globally farmers use around 115 million tonnes of chemical fertilizer out of which only 35% is used by plants. The remaining 65% is redundant and is therefore one of the major soil pollutants. Due to their disadvantages, chemical fertilizers are being progressively substituted by bio-fertilizers. The extraordinary advantages and sustainability of bio-fertilizers make them propitious candidates for application in agriculture. Keeping this background, a novel bio-fertilizer was designed and applied to paddy (test crop) under *in-vivo* conditions and its various modes of application were assiduously scrutinized to standardize the quintessential means of treatment implementation for the crop. The selected novel plant growth-promoting bacterial strains (with standardized dosages) were parameterized and investigated in the various modes of application. Different economically suitable modes like the application of bio-fertilizer in suspension or by mixing it in compost are known to provide more victual and nourishment to the plants. Therefore, in this study, a comparative analysis was drawn to standardize the best mode of treatment application. Apart from the known and popular means of application of bio-fertilizer, a new technique of utilization of a proportionate mixture of soil, bio-fertilizer, and bio-synthetic capsules was also tested to ascertain the viability of such setups with synthetic compounds. Treatment was given after 30 days and meticulous observations were taken at a regular interval (7 days). Statistical tools were used for analysis and interpretation of the results of each treatment.

Keywords: Bio-fertilizer, compost, Plant growth promoting bacteria, suspension, rice plants

INTRODUCTION

Rice (*Oryza sativa*), a cereal grain and a monocot, is one of the primary food crops in the world (Mallick *et al*. 2013). It is a complex carbohydrate and acts as a primary source of energy and a staple diet for almost half of the world's population. About more than 500 million metric tons of milled rice were produced an average in the last few harvesting seasons throughout the world. Whilst rice farms are present globally, it's concentrated mainly in Asian developing countries Apart from providing the world with a good nutrient source, the remaining parts of the plant can be re-used as cooking fuel, used for feeding livestock, and reprocessed to manufacture paper (Kaur *et al*. 2017), furniture and upholstery (Sumarno *et al*. 2020). According to crop cultivation, consumption, and export statistics, Asian countries have the most prodigious share of the world's rice production. According to recent official data, with a production quantity of over 212 million metric tons in 2021, China was the world's foremost rice producer, followed by India and Bangladesh (Fig.1). This makes developing countries like India and Bangladesh important contributors to world's food requirements (Shahbandeh, 2023).

The use of chemical fertilizers for the production of rice has been a tradition followed for ages but the detrimental and pernicious effects of chemical fertilizers on the soil, the plant, humans, and the ecosystem have given rise to perturbation and apprehension among agriculturists and environmentalists (Thorat and More, 2022). It has long been recognized that excessive and

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imprudent application of chemical fertilizer use can lead to serious consequences such as soil degradation, emasculation of crops, greenhouse gas emissions, water pollution, health problems, food crisis, and other related catastrophes (Guo *et al*. 2021). The reckless and extravagant use of fertilizers led to a decrease in the yield over the years and this reduction was primarily due to the use of fertilizer to an extent more than the actual demand of agricultural production (Nayak *et al*. 2004). These chemical fertilizers leach into the hydrosphere, and natural lixiviation is very sporadic. The chemical fertilizers also contaminate the atmosphere and lead to deleterious circumstances (Nayak *et al*. 2004). Global warming also affects the production of rice as for its growth a balance between temperature and humidity needs to be maintained. The elevation in the night temperatures due to increasing global warming is one of the paramount causes of the decrease in yields of rice worldwide (Sugiura *et al*. 2012). To curb the exacerbation of the problems caused by the application of chemical fertilizers, scientists have found several alternate options that can substitute the chemical fertilizers without significant compromise in the yield of the crop or the emergence of complications and inconveniences. Apart from using vermicompost as one of the effective methods, other organic methods such as the addition of various potent biofertilizers have also been implemented.

In this investigation, a comparative study on the efficacy of two bacterial strains which are previously reported as novel plant growth-promoting bacteria (PGPB), under different modes of application was performed. The conditions were mimicked and the juxtaposition of the growth of rice plants in different setups after usage of a novel biofertilizer was highlighted. Thus this study aimed at properly comparing the efficacy of biofertilizer under different modes of application.

MATERIALS AND METHODS

Basic Characterization of Bacterial Strains

Two bacterial strains chosen for the preparation of biofertilizer are *Bacillus vallismortis* strain TR01K, and *Bacillus subtilis* strain BRAM_G1. The two strains were reported previously by Maitra *et al*. (2022); Roy *et al*. (2022 , 2023).

The strain *Bacillus vallismortis* T1_TR01K, a tea residual rhizobacteria was isolated from actual tea-growing regions of Dooars, West Bengal. The isolate was characterized elaborately for high nitrogen fixing abilities (both inorganic soil nitrogen via ammonification and nitrification, as atmospheric nitrogen fixation abilities), potassium solubilization abilities, plant growth hormone-like Indole Acetic Acid (IAA), Gibberellic acid (GA_3) production, production of a stress-responsive enzyme like ACC-deaminase, iron-chelating compounds (siderophore), lignocellulolytic enzymes (cellulase, laccase, amylase, lignin peroxidase, pectinase), plant defense enzymes (catalase, peroxidase, beta-1,3-glucanase, proteases) along with high biofilm-forming abilities. (Maitra *et al*. 2022). The other isolate *Bacillus subtilis* BRAM_G1, is a polyextremophilic plant growth-promoting bacterial strain, isolated from high-altitude regions of Gangotri. This gram-positive bacterial strain thrives under a range of single and multi-dimensional stresses. (reported in detail in Roy *et al*. 2022). Furthermore, the plant growth promoting properties like nitrogen fixing abilities, plant growth hormone-like Indole Acetic Acid (IAA), Gibberellic acid (GA_3) production, ACC-deaminase production, siderophore production, production of various lignocellulolytic enzymes (cellulase, laccase, amylase, lignin peroxidase, pectinase), plant defense enzymes (catalase, peroxidase, beta-1,3-glucanase, proteases) of the strain BRAM_G1 were studied in details by Roy *et al*. (2023).

This small-scale pilot study was designed to test and analyze the efficiency of these two extremely potential, plant growth-promoting bacteria under in vivo conditions. The design of the treatment setup and mode of application were tested for optimized plant production.

Design of treatment setups

The modes of application of the biofertilizer were different for all the setups which helped in the realization of the best mode of application.

The different setups used were

(a) Setup 1: An untreated control where only soil was used

(b) Setup 2: A positive control that had soil along with vermicompost which is used in most parts of the world and is readily available.

(c) Setup 3: Solid biofertilizer which is biofertilizer mixed with compost. Such a composition not only encourages the plant to absorb and utilize the nutrients both from the compost and the biofertilizer but also helps the bacteria of the biofertilizer to assimilate and metabolize the nutrients present in the compost thereby enhancing their beneficial attributes and making them more competent.

(d) Setup 4: Bio-fertilizer in suspension which is easy, economical, and used for mass-scale production of biofertilizers all around the world

(e) Setup 5: Bio-fertilizer proportionally mixed with biosynthetic capsules to judge the effect of synthetic compounds on plants. The synthetic compounds had been tested earlier to be non-toxic to the plant. Synthetic capsules are known to help the plant to undergo development due to the consistent release of nutrients.

Preparation of soil

1.5 kg of soil was taken for each replicate of the 5 setups and was mixed with tap water. This soil was grounded and all the impurities in the soil like stones were removed. More water was added with proper stirring and then it was allowed to stand still for 24 hours (Min *et al*. 2001). Each setup was prepared on plastic pots of dimensions 24.2x28.5cm.

For setup 2, and setup 3 the regular garden soil: vermicompost ratio was maintained to be 2:1 (As per IRRI, Rice Knowledge Bank).
Setup 2 was prepared by directly mixing soil: vermicompost as per the recommended ratio.

Setup 3 was prepared by mixing the garden soil with 500 g of vermicompost amended with freshly prepared biofertilizer as per the IRRI recommended aforementioned ratio.

For Setup 4, 1.5kg of regular garden soil was filled in the pot. On the 28th day age of the paddy saplings, the first treatment was applied radially near the vicinity of the rhizosphere, approximately at a distance of 4 cm and at a depth of 0-2cm.

For setup 5, a slurry made up of 8g of bio-synthetic capsules with 80ml of bacterial cultures was prepared and applied on the 28th day age of the paddy saplings approximately at a distance of 4 cm and at a depth of 0-2cm.

Preparation of paddy saplings

The paddy strain PB1692 was chosen for this study. 10 cups (since there are 10 replicates) each having soil of about 250 gms were taken and 10 seeds were added to each cup. When the average length of saplings reached about 18 cm, after 21 days, they were ready to be transferred to the pots (replicates).

Preparation of biofertilizer

The bacterial strains that were used for the production of the biofertilizer were: *Bacillus vallismortis* strain TR01K, and *Bacillus subtilis* strain BRAM_G1.

A total of 5 treatments with two replicates each were prepared. Among them, 3 setups were provided with biofertilizer, and in each of these setups, the amount of bacterial culture given was 8 ml. The biofertilizer was prepared by taking 4ml of each bacteria was taken per replicate.

Bacterial cultures were prepared by inoculating bacterial strains in Luria-Bertani broth (Hi-media) and were incubated for 48 hrs. After 48 hrs these cultures were centrifuged at 10,000 rpm for 15 minutes. The supernatant was discarded and the pellets were dissolved in water. All cultures were half diluted by adding water keeping the final concentration at 10^6-10^7 cells/ml of suspension.

For a solid treatment setup where biofertilizer was added in solid form, 8ml of 48-hour-old bacterial cultures were mixed directly with 750g of compost (for each replicate) and then added to the soil by layering the compost on the soil. For the biofertilizer in liquid form, the bacterial suspension dissolved in water was added directly to the soil prepared. For the treatment amended with biosynthetic capsules, the biofertilizer was added with biosynthetic capsules in a slurry-based mixture. 8g of synthetic capsule was added to 80ml of bacterial cultures prepared to form a slurry which was then added to the soil.

The first treatment was applied at the 28th day (4th week) age of the plants. The treatment was repeated after 3 weeks. The synthetic capsules were procured from the local market of Sealdah (22.5678° N, 88.3710° E), Kolkata.

Estimation of basic bacterial load of soil and vermicompost

To detect the normal flora of the soil, 1 gm of the soil was first dissolved in 10 ml of sterile distilled water. This stock solution was then serially diluted to 10^{-2} and 10^{-4} concentrations. (Ben-David *et al*. 2014). These two concentrations and the stock solution were then plated on *Bacillus* UTI agar and *Bacillus* chrome agar. The plates were then allowed to incubate at room temperature and were observed after 48 hours. The colonies were counted and the characterization of the plates was done. (Alippi, 2019).

To characterize the normal flora of the vermicompost, 1 gm of the vermicompost used was first dissolved in 10 ml of sterile distilled water. This stock solution was then serially diluted to 10^{-2} and 10^{-4} concentrations. These two concentrations and the stock solution were then plated on plates made with *Bacillus* UTI agar and *Bacillus* chrome agar. The plates were then allowed to incubate at room temperature and were observed after 48 hrs. The colonies were counted and the characterization of the plates was done (Alippi, 2019).

Estimation of plant physical data

The observations were taken based on several parameters such as length of leaves, and number of leaves at an interval of 7 days. Post-onset of reproductive parameters, the number of panicles was estimated. Amount of water taken up by the plant etc. observations were taken at a regular interval.

Statistical analysis of data obtained

Analysis of variance (ANOVA) was conducted to estimate the significance of variation between the control and the treatment setups. Boxplot representation of data was prepared.

Estimation of the chlorophyll content in the leaves of the different setups

To estimate the chlorophyll contents of the leaves. About 10mg of leaves were taken from each of the 5 setups. These leaves were then cleaned and then were grinned into small pieces. Acetone was used to prepare about 10 ml of extract from each of these leaves. The extracts were then used to measure the OD by using a spectrophotometer. This reading was an estimate of the amount of chlorophyll present in each of the setups (Johan *et al*. 2014)

Impact of biofertilizer on soil physicochemical properties

To estimate the changes in soil physical properties, the soil samples of all 5 setups post-treatment were estimated for their pH and Electrical Conductivity. The parameters were measured based on Keen-Raczkowski Box measurement. The sample pH level was measured on a systronic glass electrode-bearing pH meter (Jackson, 1967). The electric conductivity of the soil samples was measured on a systronic E.C meter diluting the sample with distilled water at a ratio of 1:2. The soil total organic carbon content and macronutrient (NPK) contents were measured following Jackson (1967) and Tandon (1993) respectively.

RESULTS AND DISCUSSION

Basic Characterization of bacterial strains and application of treatment

The two bacterial strains chosen were elaborately characterized for their various plant-beneficial properties in previous studies by the authors. (Maitra *et al*. 2022; Roy *et al*. 2022, 2023). As per the studies by Maitra *et al*. (2022), the strain *Bacillus vallismortis* TR01K is a rhizobacterial strain having high nitrogen-fixing, growth hormone-producing, and lignocellulolytes-producing abilities. The strain *Bacillus subtilis* BRAM_G1 is a noted polyextremophilic plant growth regulator. (Roy *et al*. 2023). The aforementioned studies showed both strains to have high biofilm-forming abilities and positive interaction with each other, proving them to be efficacious for use as a consortium. For this study, 5 different treatment setups with 2 replicates each were prepared, and 21-day-old paddy saplings were re-transplanted in the pot. The treatment was applied on 4th week of plant growth and subsequent treatment was applied after 3 weeks of the first treatment.

Estimation of basic bacterial load

To presumptively identify the baseline microbial content in the soil both before the application of any alternative bacterial treatment, samples were cultured on chromogenic agar media. The total count of bacterial colonies and the percentage of coliforms and firmicutes present were recorded. All soil samples exhibited a notable presence of both coliforms and firmicutes in the range of 1.62-1.92x10^6 CFU/ml for coliforms and 1.60-1.32x10^6 CFU/ml for *Bacillus* sp.

Among coliforms, *Proteus* spp demonstrated the highest average incidence, followed by *Klebsiella* sp. and *Enterococcus* sp. The elevated occurrence of Enterobacteriaceae members suggests potential fecal contamination in the soil or contamination from the compost used as an amendment. In the compost sample, a percent of *E.coli* growth is noted. Studies have suggested that soil, and in some instances, plant root systems, serve as viable environments for *E. coli* growth in food production systems. Factors such as daily temperature fluctuations, pH levels, and organic carbon content can contribute to the prolonged survival of *E. coli* in bulk soil (Overbeek*et al*. 2021). Firmicutes species are renowned for their diverse and beneficial impacts on composting and sustainable soil health management, as highlighted by Paredes *et al*. (2023). Within the Firmicutes phylum, various species such as *Bacillus subtilis, Bacillus cereus, Bacillus pumilus,* and *Bacillus thuringiensis* among others, were identified, as reported by Alippi (2019).

Plant physiological data and statistical analysis

Physical data of major vegetative parameters like plant height (cm) and the number of leaves were recorded post-application of first treatment at the 4th week of the plant growth span and were subsequently recorded weekly till the onset of panicles at the 12th week. Two treatments were applied in the 4th and 8th week respectively. The changes in the plant height (Fig 1) indicate setups 3 and 4 to have the highest growth in plant height. Setup 5 and the positive control setup both showed comparable plant height more than setup 1. As for the average number of leaves per setup (Fig 2), again highest number was recorded for setups 3 and 4. In this case, the positive control setup performed better than the biosynthetic capsules-based setup. All 4 setups worked better than the untreated control setup.

Post onset of panicle formation, the number of panicles per plant was calculated till maturity (weeks 13th, 14th, and 15th). The highest number of panicles was recorded for setup 4 followed by setup 5. A trend analysis (Fig 3), based on the weekly incidence of several panicles indicates setup 4 has the highest number of panicles in the 13th, 14th, and 15th week. Based on the trend analysis similar results were observed in the case of setup 5 i.e. synthetic capsule amended with bacterial suspension. Both the setups with compost showed a slow onset of panicles with low numbers. All the treatment setups showed a higher number of panicles than the untreated control setup. (Fig 4). Analysis of variance or ANOVA tests were conducted to test the significance level of the differences in setup values. The number of panicles was taken to be the dependent variable while the treatment setups were taken as the independent variable. Based on ANOVA results, the null hypothesis was rejected and an alternate hypothesis was accepted indicating the variance of means within the setups is significant(Table 1).

Estimation of plant pigment was conducted on leaf samples of the setups post-week 15th. Chlorophyll a, chlorophyll b, and total chlorophyll contents of the leaves were measured (Fig 5). Among all the treatment setups highest chlorophyll

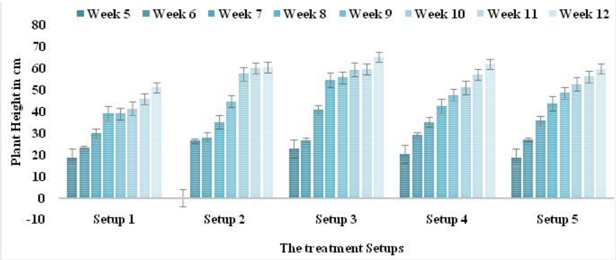

Fig 1: Changes in plant height in cm from application of first treatment (week 5) to week 12 (onset of panicles) of the 5 experimental setups.

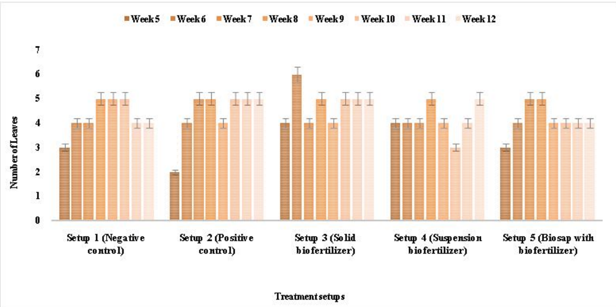

Fig 2: Increase in number of leaves from application of first treatment (week 5) to week 12 (onset of panicles) of the 5 experimental setups.

Fig 3: Number of panicles per plant in different setups. The trend line indicates a statistically significant variation in the incidence of number of panicles.

Fig 4: Treatment setups of plant at 14th week age of plants, where Setup 1 is the negative control or untreated control setup. Setup 2 indicates positive control with only compost setup. Setup 3 is the solid bio-fertilizer which is bio-fertilizer mixed with compost. Setup 4 is Bio-fertilizer in suspension. Setup 5 is Bio-fertilizer proportionally mixed with Bio-sap.

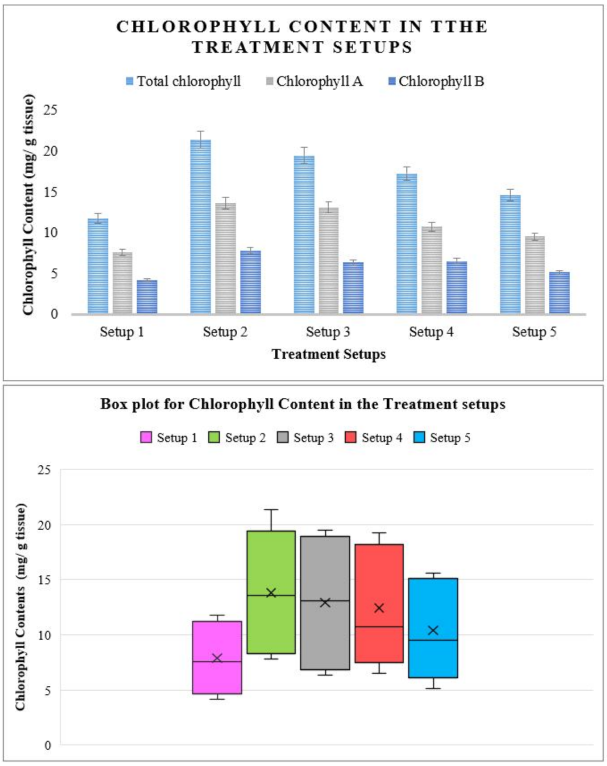

Fig 5: Total chlorophyll, Chlorophyll a and Chlorophyll b content of the leaves post application of treatment. The highest total chlorophyll content was observed in case of setup 2 or the treatment setup amended with compost only as clearly visible from the Box-plot analysis showing signification variation from the rest of the setups.

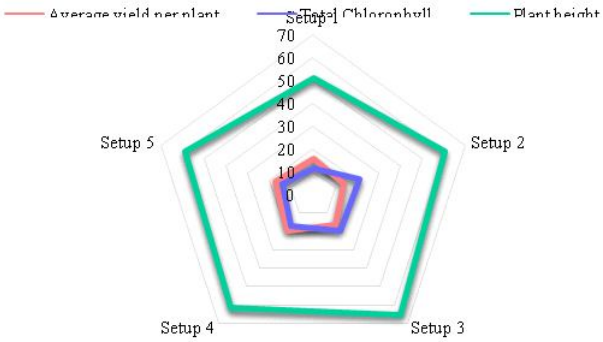

Fig 6 : Radar-plot analysis to estimate the best treatment setup among the 5 based on three major factors plant height (vegetative parameters), average yield per plant (reproductive parameters) and chlorophyll content (physiological parameters), indicating treatment 4 (suspension treatment) to be the best setup.

content was measured for setup 2 and setup 3 i.e the two compost-based setups, followed by setup 4 and setup 5, which could be clearly understood by the boxplot analysis of variance (Fig. 5).

Finally, an attempt was made to estimate the best-performing treatment (Fig 6). A radar-plot statistical analysis was conducted taking three major parameters into account Plant height (major vegetative parameter), average number of panicles/plant (major yield parameter), and total chlorophyll content (major pigment). The plot analysis indicated both the solid treatment setup and bacterial suspension setup to have the highest plant heights, while total chlorophyll content was found to be highest in the case of the compost setup (setup 2). The yield was found to be highest in the case of bacterial suspension setup followed by slurry-based bio-synthetic capsules. Therefore, based on production criteria, the treatment showing the highest yield and plant growth i.e. setup 4 was selected to be the best-performing treatment.

Impact of biofertilizer on soil physicochemical properties

A brief attempt to understand the impact of treatments on soil physicochemical properties was made. (Fig. 7). The pH of the soil in the case of all the treatments was found to be comparable while the electrical conductivity of the samples was found to be lowest in the case of a synthetic-based setup. The total organic carbon content was found to be highest in the case of the setups amended with composts, as composts have a high incidence of C: N ratio. As for the soil macronutrients, nitrogen content was found to be highest for setups 3 and 4 followed by setups 5 and 2. This indicates the high C: N content of the compost used as well as the high nitrogen-fixing ability of the novel bacteria used. (previously reported). As per the soil phosphate content, all the setups showed values>160 kg/ha indicating high phosphate content. As for soil potassium content highest value was recorded for the two compost-based setups followed by setup 4 and setup 5 indicating slower potassium utilization by the plants in the case of compost setups

The two strains of bacteria were tested assiduously for plant growth-promoting and bio-control properties. Both the chosen bacterial strains have high biofilm-forming abilities at approximately 40x times more than regular *Bacillus* sp. laboratory variants. Microbial biofilms represent a collection of single or multi-species bacteria that possess the ability to adhere to diverse surfaces, effectively functioning as a unified system that communicates chemically through quorum sensing. This cooperative interaction is particularly evident in the rhizospheric regions surrounding plant roots, forming a protective dome that shields the root-rhizospheric niche from various biotic and abiotic stresses. Bacterial extracellular polymeric substances (EPS) play multiple roles in this context, including adhesion, cohesion, and aggregation of soil particles. They also retain water molecules and serve as a potential barrier in the rhizospheric regions. Additionally, bacterial EPS facilitates the exchange of ionic and genetic information within the matrix component and contributes to the enhanced production of plant-available nutrients. Thus the bacterial extracellular polymeric substances (EPS) play various roles, such as promoting adhesion, cohesion, and aggregation of soil particles, and are also known to contribute to enhancing the production of plant-available nutrients (Flemming and Wingender, 2010). Therefore, the two strains having high biofilm and subsequent plethora of plant growth-promoting properties production makes them conspicuous choices for the preparation of novel biofertilizers. Besides the characterization of the bacteria, the microflora of the soil and the vermicompost were also identified. The soil was suffused with the bio-fertilizers and the consequent blossoming and manifestations of the concerned parts of the rice saplings were painstakingly studied. For evaluating the abilities of different bio-fertilizers in stimulating the growth, maturation, physiological, and morphological development of rice plants (both in vegetative and reproductive phases), ten saplings (one sapling in each of the two replicates of the 5 setups) were considered and examined under various criteria. Before the experiment, it was ensured that the paddy saplings were 21 days old and had an approximate height of 18 centimeters. The flourishing vegetative growth in the compost amended with bacterial biofertilizer indicates an increased influx of nutrients in the plant root-niche region due to the additional nutritional conditioning of compost in soil along with the two potent bacterial strains solubilizing the excess nutrients. The suspension-based treatment also indicated flourishing vegetative growth indicating the nutrient sequestration abilities of the novel strains

Table 1: Analysis of Variance (ANOVA) of the number of panicles per plant for the 5 treatment setups

	Degrees of freedom (DF)	Sum of square (SS)	Mean sum of square	F-Value	Pr (>F)
S^2	4	67.586	16.896	3.962	0.035
Residuals	10	42.647	4.265		

as has been previously reported by the authors. (Maitra *et al*. 2022; Roy *et al*.2023). In the case of several panicles for the treatment setups highest results were observed in the case of suspension-based treatment followed by bio-synthetic capsules-based slurry treatment. This indicated the high potential and colonizing ability of the novel biofertilizer. The poor performance of the compost-based treatments indicates a lack of essential nutrients required for the onset of flowering along with lacking balance in the auxin cytokinin ratio required for the onset of flowering. Again the leaf photosynthesis rate plays a major role in plant growth, biomass production, and overall yield. Chlorophyll is the major photosynthetic pigment, an increase of which during the heading stage can be critically related to the final yield production. (Takai, 2010) As per the values obtained, the two setups with compost showed the highest chlorophyll content. This indicated higher nutrient availability due to the presence of vermicompost in the soil. The moderate to high range of chlorophyll content in suspension-based biofertilizers can be correlated with the impact of PGPB in breaking down complex polysaccharides and proteins into simpler carbon and amino acid forms which can be easily taken up by the plants. The low yet higher

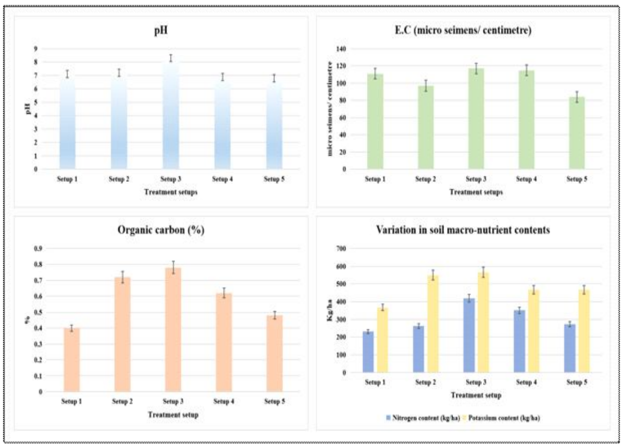

Fig. 7: Soil physico-chemical properties (pH, E.C, % Organic carbon content, macronutrients like nitrogen and potassium) of the treatment setups post application of treatment

chlorophyll content in synthetic slurry-based treatment indicated the masked effect of biofertilizer due to synthetic complexes and slower solubilization of complex molecules in the root-rhizospheric niche region. Finally, a statistically significant radar-plot analysis considering three major parameters of vegetative growth, yield, and pigment was used to determine the best working treatment. Based on the highest number of panicles, plant height, and moderate to high chlorophyll content, the suspension-based biofertilizer was chosen as the best-performing treatment.

CONCLUSION

According to the objective of the experiment, there were indisputable observations from which it could be interpreted that the bio-fertilizers conspicuously invigorated the growth and development of the rice plants. However, among the all forms in which the bio-fertilizers were applied, the best plant growth-promoting characteristics were observed in bio-fertilizer in suspension-based form. The data and evidence obtained from the investigations palpably and understandably depict that, above the congeniality of other forms of bio-fertilizers, however the bio-fertilizer composition in the setup 4 (suspension-based bio-fertilizer) is the most suitable and promising for the prospering of the rice plants.

DECLARATIONS

Conflict of Interest. Authors declare no conflict of interest.

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