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Unveiling the Consortial Role of Heterotrophic Nitrogen Fixing, Phosphate Solubilizing and Potash Mobilizing Bacteria: Augmenting Soil Health, and Growth and Yield of Wheat Crop (*T. aestivum*) through Integrated Nutrient Management Strategy

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A B S T R A C T

The dependence on chemical fertilizers for meeting plant nutrient needs has precipitated declines in soil health and microbial diversity degradation. Enhanced soil health and diverse rhizosphere microbial populations are said to contribute for sustainable agricultural ecosystem. Among the nutrients, nitrogen (N), phosphorous (P), and potassium (K) are crucial and are considered as primary elements. Although there is presence of N, P, and K in the environment, most of it is unavailable for plant absorption. Hence, microbes play a pivotal role in rendering these elements accessible to plants by their natural processes, reducing reliance on chemical fertilizers. Amid diverse experiments subjecting various microorganisms for crop growth and yield, selection of highly compatible and productive microbial consortia holds significant importance. The present investigation was aimed to isolate nitrogen fixing bacteria, phosphate-solubilizing bacteria, and potash mobilizing bacteria from wheat rhizosphere soil, assessing for their *in vitro* capabilities to fix, solubilize or mobilize, and function as a compatible consortium. They were subjected to biochemical and molecular characterizations for identification. Further, the consortium was used to assess its efficacy to influence wheat crop growth, nutrient uptake, crop yield, and soil health. The distinguished isolates- AZ-II, P-III, and K-II with highest nitrogen fixing capability (148.65 µg of nitrogen/mg of carbon used), phosphate solubilizing ability (33.45%), and potash mobilizing ability (41.33 µg ml^{-1}) respectively were identified as *Azotobacter chroococcum, Bacillus subtilis*, and *Pseudomonas fluorescens*. The consortium was formulated and 2 x 10^7cfu g^{-1} of lignite powder was added as carrier material for seed treatment. The results showed that among eight treatments, the treatment with consortium of *Azotobacter sp.*, PSB, and KMB + 75% recommended dose of fertilizer showed significant influence on crop growth, yield, soil health and microbial population. It showed highest germination, plant height, root length, dry weight of shoot and roots at earhead formation and harvest stage of the crop, number of panicles, 1000 seed weight, seed yield, NPK uptake and population of *Azotobacter*, PSB and KMB at flowering stage of wheat.

Introduction

Wheat (*Triticum aestivum L.*) is the second most important crop after rice in country, which contributes nearly one third of the total food grain production. Wheat is a staple food crop and is consumed by nearly 65 per cent across the globe in the form of chapatti and different bakery products. Wheat is grown all over the world as a food security crop as it is having a great potential of producing good yield per unit area and grows well in almost all agro-climatic conditions. India is the largest wheat producing country in the world after China and accounts for more than 13 per cent of the world's wheat production.

Nearly ninety per cent area under wheat cultivation is spread under irrigated conditions in the states of Punjab, Haryana, UP, MP, Rajasthan and Gujarat state. During 2018-19 Rabi season, India harvested record wheat production of 101.20 million tons from an area of 29.55 million hectare with the record average productivity of 34.24 q/ha (ICAR-IIWBR, 2019).

Applying fertilizers plays an important role in maximizing crop yield, hence, farmers in generally apply a high dose of chemical fertilizers during wheat production to harvest high grain yields. However, the heavy use of chemical fertilizers in agricultural farming has hazardous environmental impacts including degradation of soil fertility, lowering of microbial activity, and organic matter absorption and decreased water holding capacity, nutrient mobilization and uptake by the root zone (Xiao *et al.,* 2019).

Due to the harmful effects on the environment and human health, there is a new trend toward minimizing the use of chemical fertilizers and adopting bio-organic farming technology, which is also known as sustainable agriculture. Bio-organic farming uses organic matter and beneficial microorganisms to provide crops associated with high quantity and quality while maintaining the soil environment (Mallik and Williams, 2008).

Organic manure and biofertilizer amendments are feasible ways to reduce chemical fertilizer application in wheat production without decreasing grain yields. Biofertilizers are the live formulations of microorganisms that have the ability to acquire, mobilize, and transport plant nutrients in the soil and offer a cheap, low capital intensive, non-bulk and eco-friendly source to boost productivity (Kloepper *et al.,* 1989).

Among biofertilizers, *Azotobactersp.* plays a key role in the nitrogen cycle in nature that binds atmospheric nitrogen inaccessible to plants and releases it in the form of ammonium ions available to plants in the soil fixing an average of 20kg N/ha per year. It is able to grow at a pH range of 4.8–8.5 and fixes N at an optimum pH of 7.0–7.5 (Dilworth *et al.,* 1988). *Azotobactersp.* Increases the yield of all the agriculture crop plants by approximately 10-12 % (Jaga and Singh, 2010).

Azotobacter sp., along with phosphate solubilizing bacteria and potash mobilizing bacteria increases the grain yield of wheat and increases the availability of micronutrients such as Fe, Mn, and Zn in the soil. Hence, the combined application of biofertilizers can be considered beneficial for the growth and yield of wheat (Noreen and Noreen, 2014).

Biofertilizers are cost-effective relative to chemical fertilizers. They have lower manufacturing costs, especially regarding nitrogen and phosphorus use. In view of these facts, an experiment was carried out to study the effect of biofertilizers viz., *Azotobacter*, phosphate solubilizing bacteria, potash mobilizing bacteria alone and in combination with different doses of inorganic fertilizers on the growth and yield of wheat.

Materials and Methods

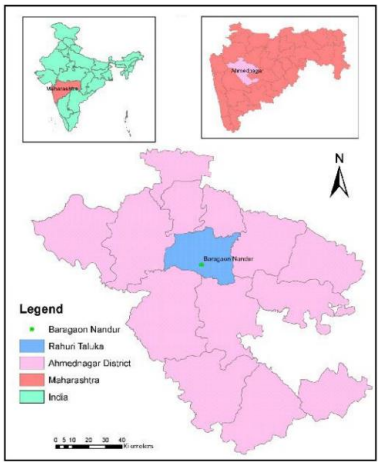
A field experiment was conducted to study the effect of a consortium of *Azotobacter,* PSB and KMB on the growth, nutrient uptake and yield of wheat during *rabi,* 2021. Detailed information on the laboratory procedures, material used and

experimental techniques adopted for the study is presented in this chapter.

Experiment Location

The current investigation was carried out at the Post Graduate Institute Farm, Mahatma Phule Krishi Vidyapeeth, Rahuri, Maharashtra, India, located at 19^0 48' N and 19^0 57' N latitude and between 74^0 32' E and 76^0 19' longitude and at altitude of 657 meters above mean sea level (Plate 1). This area falls in the semi-arid tropics with an annual rainfall ranging from 317 and 619 mm. About 80 % of the average annual rainfall is received during the months of October and November and practically negligible rains received during summer. The soils are light to medium clay loamy black soils called as *regur* soils. They are classified into Vertisols with montmorillonite minerals. The soil has a slightly basic pH of 7.2.

Plate.1 Experimental location of the study area on the map marked in blue (Pavan *et al.*, 2017)



Seeds

The seeds of the wheat variety *Trimbak* needed for the experiment were obtained from the Seed Unit, MPKV, Rahuri.

Rhizosphere soil samples of wheat

Rhizosphere soil samples at the flowering stage of wheat were collected from the field and used for isolation of *Azotobacter,* PSB and KMB isolates.

Manures and fertilizers

Well decomposed farmyard manure was procured from the Department of Animal Husbandry, MPKV, Rahuri. Urea, SSP and MOP were bought and applied to the research plot as per the recommendation.

Isolation of *Azotobacter* from rhizospheric soil of wheat

The *Azotobacter* sp. were isolated on Asbhy's agar medium by the serial dilution pour plate technique given by Subba Rao (1986) and three bacterial isolates were selected as nitrogen fixers.

Biochemical characterization of pure isolate

Pure culture of the isolate will be made and then subjected to Gram reaction. The gram-negative isolates were further subjected to biochemical tests including catalase, gelatin hydrolysis, growth on carbon sources and growth at various temperatures for confirmation.

Gram staining

The isolate was characterized by Gram staining as per the procedure given by Singh *et al.*, (2016). A loopful culture was taken on a cleaned dry slide; smear was prepared, air dried and heat fixed. Two drops of crystal violet were added and incubated for 30 seconds. The slide was washed with distilled water. Then, 1-2 drops of Gram's iodine were added and kept for 60 seconds. The slide was washed with 95 % ethyl alcohol. Later, safranin was added and the samples were kept for 30 seconds and then washed with distilled water. The slide was dried with blotting paper and observed under a microscope. The pink colonies indicate gram-negative bacteria and the purple colonies show the gram-positive bacteria.

Catalase test

A small sample of culture organisms was transferred to a labelled slide. A drop of 3 % hydrogen peroxide was added to the slide. The immediate presence of bubble formation on the slide indicated a positive catalase test.

Gelatine hydrolysis

Gelatine deep tube cultures were placed in a refrigerator at 4°C for 30 minutes. The cultures were examined to determine whether the medium was solid or liquid. Two day and seven day incubation periods were followed to determine whether the organism was capable of hydrolyzing gelatine.

Utilization of different carbon sources

Three different carbon sources viz. Glucose, sucrose, and mannitol were used to evaluate the ability of the isolate to grow on different carbon sources(Table.1) (Rojas *et al.*, 2011).

Table.1

Sr. No.	Microorganism	Culture medium	Carbon source utilization
1.	*Azotobacter*	Ashby's Agar medium	Glucose, sucrose, and mannitol

The culture medium was amended with different carbon sources and autoclaved. Sterilized Petri plates were poured with 15-20 ml medium with different carbon sources and allowed to solidify. After solidification, 10 µl of a 24 hr-old culture of the test organism was spotted on plates with each carbon source. The plates were incubated for 48 hrs at 28 ±2°C and the ability of the isolate to grow on different carbon sources was noted.

Temperature tolerance

The culture organism was inoculated in slants and was allowed to grow at different temperatures viz. 10°C, 20°C 30°C, 40°C, 50°C (Islam *et al.*, 2008).

Nitrogen fixing ability

Nitrogen estimation by micro-Kjeldahl method

A 48-hour old culture of a freshly isolated *Azotobacter* strain will be inoculated into 5 ml of Ashby's agar medium and incubated for 48hrs. One milliliter of this broth will be inoculated into 50 ml of Ashby's agar medium and incubated for 15 days. Ten milliliters of this culture will be used for N estimation by following the standard procedure of the MicroKjeldahl technique (Reis *et al.*, 1994). The formula for N_2 estimation is:

$$N_2 \text{ (mg/g)} = \frac{\text{ml of } H_2SO_4 \text{ in the sample} \times \text{Normality of } H_2SO_4 \times 14.01}{\text{Weight of the sample (carbon used in grams)}}$$

Isolation of phosphate solubilizing bacteria (PSB) from rhizosphere soil of wheat

The isolation of phosphate-solubilizing bacteria on Pikovskaya's medium will be carried out by serial dilution of soil and the agar plating method (Aneja, 2003). Ten grams of rhizosphere soil sample was suspended in 90 ml of sterilized water blank. Serial dilutions will be made from 10^{-1} to 10^{-6}. 1 ml aliquot of dilutions from 10^{-3} to 10^{-5} will be transferred to sterilized petriplates separately. This was followed by pouring sterilized Pikovskaya's medium before solidification (45°C temperature) and mixing the contents in plates by rotating the plates gently taking care that the medium should not touch the lid. After solidification, plates will be kept at 28±2°C in BOD incubator for 4-5 days. All plates will be observed for the appearance of bacterial colonies showing a clear zone of solubilization of tricalcium phosphate (TCP).

Characterization of PSB isolates

Colonies forming clear zone of P-solubilization on Pikovskaya's medium were selected, purified by

subculturing and maintained on the slants of Pikovskaya's agar for further use.

Phosphate solubilizing ability of the bacterial isolates

The ability of the bacterial isolates to solubilize insoluble inorganic phosphate was tested by spotting 10 µl of overnight cultures on Pikovskaya's agar plates and incubating at 28-30^{o}C for 2-3 days. The isolates showing a clear zone of solubilization of tricalcium phosphate (TCP) around the colony were be noted as phosphate solubilizers. The diameter of the zone of TCP solubilization is measured and expressed in millimeters.

Quantitative estimation of Pi released from tricalcium phosphate for bacterial isolates

The bacterial isolates positive for P solubilization on Pikovskaya's agar medium were subjected to quantification of Pi released from TCP in broth medium. The Erlenmeyer flasks containing 50 ml Pikovskaya's broth (Pikovskaya, 1948) will be inoculated with 500 µl overnight culture of each isolate in two replicates and incubated for 10 days at 28 ± 2°C. The amount of Pi released in the broth was estimated at 10 days of incubation in comparison with the uninoculated control. The reduction in pH of the broth from the initially adjusted pH of 7.0 will also be noted after 10 days of incubation to monitor the amount of acidity produced and study its correlation with the Pi released. The TCP broth cultures were spun at 10,000 rpm for 10 minutes to separate the cells and insoluble phosphate and the available P content of the supernatant was estimated by using the phosphomolybdic blue color method (Jackson, 1973).

Reagents used

Chloromolybdic acid

To prepare this reagent, 7.5 g of ammonium molybdate was dissolved in 150 ml distilled water followed by the addition of 162 ml conc. HCl. The total volume was made to one liter with distilled water and the reagent was stored in an amber color bottle at 4^{o}C.

Chlorostannous acid

This reagent was prepared by dissolving 25 g of $SnCl_2.2H_2O$ in100 ml conc. HCl and making the total volume to one liter with distilled water. It was also stored in an amber color bottle at 4°C.

Procedure

One ml of the TCP broth culture supernatant was dispensed in a 50 ml volumetric flask. To this, 10 ml of chloromolybdic acid was added, and mixed thoroughly, and the volume was made to approximately 3/4th with distilled water.

Chlorostannous acid (0.25 ml) was added and the final volume was made to 50 ml with distilled water and mixed thoroughly. The flasks were kept for15 minutes for colour development and the blue colour developed was read in a spectrophotometer at 610 nm using a reagent blank.

Preparation of the standard curve

Potassium-dihydrogen phosphate (0.2195g KH_2PO_4) dried at 40^{o}C for 3-4 hrs was dissolved in 400 ml distilled water. After the addition of 25 ml of 7 N H_2SO_4, the volume was adjusted to one liter with distilled water and mixed thoroughly. 20 ml of this solution was diluted further to 500 ml with distilled water to obtain a 2 ppm stock P solution. The standards of P (0 to 2 ppm P) were prepared by using this stock 2 ppm P solution. These standards were also subjected to colour development as above and read in a spectrophotometer at 610 nm. The standard curve of P was plotted and the amount of P solubilized (percent Pi released) by the isolates from TCP was calculated by using the following formula:

$$\% \text{ Pi released} = \frac{R \times \text{Volume of chloromolybdic acid} \times \text{Final volume made}}{\text{Volume of chlorostannous acid} \times \text{Aliquot taken}} \times \frac{1}{100}$$

where,

R = O.D. x slope factor

O.D. = Optical density (absorbance at 610 nm)

$$\text{Slope factor} = \frac{\text{Sum of standards of P conc.}}{\text{Sum of OD}}$$

Volume of chloromolybdic acid used = 10 ml

Volume of chlorostannous acid used = 0.25 ml

Final volume made = 50 ml

Aliquots = 1 ml of TCP broth culture supernatant.

Isolation of potash mobilizing bacteria from the rhizosphere of wheat

One gram of rhizosphere soil was mixed thoroughly in 100 ml of sterile water and processed following the serial dilution agar plate technique (Aneja, 2003). Suitable dilutions (10^{-5} and 10^{-6}) of both rhizosphere and rhizoplane solutions are plated on Aleksandrov medium (Hu *et al.,* 2006). Aleksandrov medium is a selective medium for the isolation of potassium solubilizers, containing insoluble potassium bearing minerals (mica). The plates were incubated at room temperature (30±1°C) for 3 days and the colonies exhibiting clear zones were selected, and purified by the four-way streak plate method.

Characterization of potash mobilizing bacteria

Morphological characterization

All the selected isolates were examined for colony morphology, cell shape, Gram reaction and ability to form spores as per the standard procedures given by Cappuccino and Sherman (1987).

Biochemical characterization

The biochemical characterizations of the isolates were carried out as per the procedures outlined by Cappuccino and Sherman (1987). Gelatin hydrolysis test, catalase test, H_2S production test, growth on carbon source and growth at various temperatures were performed and recorded.

Molecular characterization

The molecular characterization of the distinguished isolates were carried out at Cellbioases Pvt. Ltd., Pune, Maharashtra. The procedure followed was partial sequencing of the most conserved 16S rRNA of all three isolates, using aforward primer sequence comprising 27F (5'- AGAGTTTGATCCTGGCTC AG-3') and a reverse primer with 1492R (5'-CGGTTACCTTGTTACGACTT-3').

For *Pseudomonas fluorescens,* forward primer sequence comprising Psmn 289 (5'-GGTCTGAGAGGATGATCAGT-3') and reverse primer with Psmn 1258 (5'-TTAGCTCCACCTCGCGGC-3') were chosen. The sequences obtained were identified using BLAST software of National Centre for Biotechnology Information (NCBI).

Quantitative estimation of 'K' solubilization

The isolates showing zone of solubilization on Aleksandrov agar medium will be further examined for their ability to release K from broth media. The amount of K released from muscovite mica in the broth by the isolates should be studied at 7, 15 and 20 days after incubation (DAI) in laboratory conditions (Parmar *et al.,* 2016).

Procedure for quantitative estimation of potassium release

A loopful of 48-hour old grown culture was inoculated into 25 ml Alexandrov medium broth in a 50 ml capacity flask containing either of different sugars: glucose, sucrose, mannitol, and mannose. All inoculated flasks were incubated at 28±2°C for 10 days.

The growth suspension was centrifuged at 7,000 g for 10 minutes in a Hettick Micro Rapid centrifuge (Tuttlingen) to separate the supernatant from cell

growth and insoluble potassium. One ml of the supernatant was taken in a 50 ml volumetric flask and the volume was made to 50 ml with distilled water and mixed thoroughly. The solution was fed to atomic absorption spectrophotometer to determine the K content. A standard curve was prepared using various concentrations of 10 ppm KCl solution *i.e*., 0.5, 1.0 and 1.5 ppm. The amount of potassium solubilized by the bacterial isolates was calculated from the standard curve.

Selection of culture medium

The culture media of various compositions (M I, M II, M III, M IV) were formulated and screened for the growth of nitrogen fixing, phosphate-solubilizing and potash-mobilizing bacteria in broth by using various carbon sources such as glucose, sucrose and nitrogen sources such as ammonium sulphate and yeast extract in different concentrations along with different micronutrients (Table 3.2.13). The pH of all culture media was maintained in the range of 6.9 to 7.1.

Invitro studies

Broth of each culture medium *viz*., MS I, MS II, MS III and MS IV was inoculated with efficient strains of *Azotobacter*, PSB and Potash mobilizing bacteria separately as well as in consortia and kept for incubation at 28±2^{o}C for 5 days. The cfu count of *Azotobacter*, PSB and potash mobilizing bacteria was recorded after an incubation period of 5 days by using the direct plate count technique. Before the development of the consortium, all strains were examined *in vitro* for their compatibility on selective medium by the cross-streak method. Observations of the growth and cfu count of *Azotobacter*, PSB and Potash mobilizing bacteria in each culture medium were recorded.

Inoculum preparation

Inocula of *Azotobacter chroococcum*, *Bacillus subtilis* and *Pseudomonas fluorescens* were prepared in MS III selective medium. The media were inoculated in 500 ml conical flasks containing 150 ml medium and incubated at 28 ± 2^{o}C under shaking at 100-150 rpm for three days to give an optical density of 0.5 recorded at 535 nm. Lignite powder used as carrier was sterilized at 121^{o}C and 1.04 kg/cm^{2} pressure for one hour and inoculated with broth cultures of *Azotobacter chroococcum*, *Bacillus subtilis* and *Pseudomonas fluorescens* (100 ml per 500 g of lignite powder). Lignite powder-based inoculum was incubated at 28 ± 2^{o}C for three days by adding 10% solution to increase the population of respective microbe. Inocula of *Azotobacter chroococcum*, *Bacillus subtilis* and *Pseudomonas fluorescens* having cfu of 2 x 10^{7} per gram of lignite powder were applied to wheat as seed coating.

Table.2 Composition of culture media for consortium of *Azotobacter*, PSB and KMB

Sr. No.	Chemicals	Composition of culture media (g)			
		M I	M II	M III	M IV
1	Glucose	35	20	10	9
2	Iron chloride	0.005	0.1	0.1	0.1
3	Tri calcium phosphate	5	5	4.5	4
4	Ammonium sulphate	0.5	0.5	0.4	0.3
5	Yeast extract	0.5	1	1	1
6	Magnesium sulphate	0.1	0.6	0.5	0.3
7	Potassium chloride	0.2	0.2	0.2	0.2
8	Manganese sulphate	1	0.1	0.1	0.1
9	Ferrous sulphate	0.2	0.1	0.1	0.1
10	Calcium carbonate	2.1	0.1	0.1	0.1
11	Potassium alumino silicates	2	2	2	2
12	Dipotassium hydrogen orthophosphate	1	0.5	0.4	0.3
13	Sodium Chloride	0.5	0.1	0.1	0.1
14	pH	6.9	7.1	6.9	7
15	Distilled water	1000 ml	100 0 ml	100 0 ml	100 0 ml

Methods used for soil analysis

Soil samples were collected from the experimental plot before and after harvesting the wheat for analysis of the nutrient status of the soil. The collected soil samples were air dried, and crushed in wooden mortar and pestle. Soil was sieved through 2 mm sieve. Then the soil samples were analyzed for their chemical properties by using standard analytical methods.

Determination of available nitrogen

Transferred 20 g sieved soil into one liter round bottom flask, two to three glass beads and 1 ml of liquid paraffin was added to prevent frothing. Furthermore, 100 ml of $KMnO_4$ and 100 ml of NaOH was added to it.

Distill and collect the distillate in a beaker containing 20 ml of boric acid working solution. Then, 150 ml of distillate is collected.

Titrated distillate with 0.02 N H_2SO_4 till color changed from green to red.

Determination of available phosphorus

1. 25 g soil was taken in 250 ml of conical flask.
2. Added one spoon carbon black or activated charcoal.
3. Added 50 ml $NaHCO_3$ (pH 8.5).
4. Simultaneously, blank was run without soil.
5. Flask was kept on shaker for 30 minutes.
6. Filtered through filter paper.
7. Pipette out 5 ml $NaHCO_3$ extract in 25 ml of volumetric flask.
8. Added two drops of Para nitrophenol.
9. Added 5N H_2SO_4 drop by drop till yellow color disappears at pH 5.0.
10. Added 4 ml solution B and made volume to 25 ml with distilled water.
11. After 30 minutes, the blue color intensity was measured using spectrophotometer at 882 nm.

Determination of Potassium

1. A 5 g soil sample was taken in a 250 ml of conical flask.
2. Next, 25 ml of neutral nitrogen ammonium acetate solution was added.
3. The flasks were kept on a shaker for 5 minutes.
4. The filtrate was collected.
5. Atomized using a flame photometer.

Table.3 Analysis of soil samples

Sr. No	Parameter	Method	Reference
1.	Available Nitrogen	Alkaline permanganate method	Subbiah and Asija (1956)
2.	Available Phosphorus	0.5 $NaHCO_3$	Olsen *et al*. (1954)
3.	Available Potassium	Ammonium acetate extractable method	Knudsen *et al*. (1982)

Plant sample analysis

The plant samples for total N, P, and K analysis were collected separately from each treatment plot at the harvesting stage. The plant parts were then kept in paper bags and dried in hot air oven at 70^{o}C for 48 hours.

The dried plant parts were finely ground in a mixer. This fine powder was again dried in an oven at 60^{o}C for a couple of hours and stored properly until the samples were used for chemical analysis of nutrients.

Total nitrogen

The nitrogen content of plants was estimated by following modified Kjeldahl's process as described by Jackson (1967) and expressed in terms of percent on dry weight basis.

Total phosphorus

The phosphorus content of plants was determined by the colorimetric method as suggested by Jackson (1973) employing the vanadomolybdate phosphoric

yellow color colorimetric method. Yellow color intensity was read in a spectrophotometer at 470 nm.

Total potassium

Potassium content of plant was recorded with the help of flame photometer as described by Jackson (1973). The results were expressed as percent on dry weight basis.

Field Experiment

A field experiment was conducted during *Rabi* 2021 at the Post Graduate Institute Farm, Mahatma Phule Krishi Vidyapeeth, Rahuri. The wheat variety *Trimbak* was used as a test crop.

Experimental details

- Experimental design : Randomized Block Design (RBD)
- Replications : 3
- Season : *Rabi* 2021
- Spacing : 22.5 x 10 cm
- Plot size : Gross: 3.60 x 3.0 sq.m.
- Net: 3.30 x 2.80 sq.m.
- Variety : *Trimbak*
- Recommended fertilizer (RDF): 120:60:40 N: P_2O_5:K_2O, kg ha^{-1}

Treatment details

The wheat seeds were inoculated before sowing as follows.

T_1 : Consortium of *Azotobacter*, PSB and KMB
T_2 : Consortium of *Azotobacter*, PSB and KMB + 100 % RDF
T_3 : Consortium of *Azotobacter*, PSB and KMB + 75 % RDF
T_4 : *Azotobacter* + 75 % recommended N + 100 % recommended P_2O_5 and K_2O
T_5 : PSB + 75 % recommended P_2O_5 + 100 % recommended N and K_2O
T_6 : KMB + 75 % recommended K_2O +100 % recommended N and P_2O_5
T_7 : 100 % RDF
T_8 : Absolute control

Note

FYM @ 5 t ha^{-1} (as per recommendation) will be applied to all the treatments except absolute control.

Sowing of seeds

The treated and untreated seeds were dibbled to a depth of 2-3 cm and spacing of 22.5 cm x 10 cm was maintained.

Crop management

Seedling thinning operation was carried out within 15 days from the date of sowing and maintained only one healthy seedling per hill. To check weeds, intercultural operations were carried out twice, at 15 and 30 days after sowing by using weeding hook and cycle hoe. The plots were subsequently irrigated at an interval of 2-4 days depending on prevailing weather conditions.

Harvesting

Wheat was harvested manually when it turned yellow and started drooping and color of the head turned yellowish gold.

Experimental observations

Five randomly selected plants per plot were tagged for recording observations.

Growth attributes

Germination percentage

The germination percentage was recorded 15 days after sowing.

Plant height at ear head formation and harvesting stage

Plant height was measured from ground level to tip of the plant. It was recorded at head formation (60

DAS) and at harvesting stage (90 DAS) of the crop.

Root length at ear head formation and harvesting stage

Root length was recorded at ear head formation (45 DAS) and harvesting stage (90 DAS) of crop.

Dry weight of wheat roots and shoots at ear head formation and harvesting stage

Five randomly selected plants from each plot were uprooted at the ear head formation stage (60 DAS) and harvest stage (90 DAS) of the crop. Roots were washed thoroughly with tap water and sun dried initially followed by oven drying at $80^{o}C \pm 5^{o}C$ for 72 hours until a constant weight was attained. The weight of oven dried plant sample was recorded.

Yield attributes

Number of panicles per plant

The number of panicles per plant was recorded from five randomly selected plants at harvesting (90 DAS) stage.

1000 seed weight (g)

A total of 1000 seeds from each plot were taken after harvesting and their weight was recorded in g.

Wheat seed yield (q ha^{-1})

Wheat seed yield was recorded from each plot after threshing and computed per ha.

Physiological parameters

Initial and after harvest available NPK kg ha^{-1} in soil

The initial and after harvest available nitrogen, phosphorus and potassium in soil were estimated as per the procedures stated in 3.2.16

NPK uptake by wheat at harvest

The nitrogen, phosphorus and potassium uptake by wheat plants at harvest was estimated as per the procedures stated in 3.2.17.

Microbial count of *Azotobacter*, PSB and KMB at harvest stage of wheat

Fresh soil of wheat at the earhead formation stage was analyzed for the *Azotobacte*r population on Ashby's agar medium. Moreover, rhizospheric soil samples at the ear head formation stage of wheat were analyzed for microbial population of phosphate-solubilizing bacteria (PSB) and potash-mobilizing bacteria (KMB) using serial dilution of soil and agar plating method (Aneja, 2003).

The PSB and KMB population were enumerated on Pikovskaya's medium and Alexandrov's agar medium, respectively, at 10^{6} dilutions. The plates were incubated at $28\pm2^{o}C$ temperature for 72 hours and colonies were counted. The population was expressed as cfu g^{-1} soil.

Statistical analysis

The data recorded on various parameters were subjected to statistical analysis by following the standard method of analysis of variance. The level of significance used in 'F' and 't' tests was P = 0.05. Critical difference (CD) values were calculated where the 'F' test was found to be significant (Panse and Sukhatme, 1985).

Results and Discussion

Isolation of Nitrogen Fixing, Phosphate Solubilizing and Potash Mobilizing Bacteria

Isolation of *Azotobacter* from rhizosphere soil of wheat

All the isolates of *Azotobacter* from rhizosphere soil of wheat (var. *Trimbak*) cultured on Ashby's agar

medium, using serial dilution method (Subba Rao, 1986) and incubated at 30°Cfor 2-3 days, gave creamy/ white transparent, shining, mucoid, smooth and circular colonies. (Plate 4.1)

Isolation of phosphate-solubilizing bacteria from rhizosphere soil of wheat

The isolates of phosphate-solubilizing bacteria cultured on Pikovskaya's medium carried out by serial dilution of soil and agar plating method (Aneja, 2003) resulted in the appearance of bacterial colonies showing a clear zone of solubilization of tricalcium phosphate (TCP) on the medium and named as P I, P II and P III. The phosphate solubilization varied from 3.3 mm to 6 mm among the different PSB isolates. The maximum zone was produced by isolate P-III. The bacterial isolate thus obtained was maintained on the slants of Pikovskaya's agar for further use. (Plate 4.3 and 4.4).

Isolation of potash-mobilizing bacteria from rhizosphere soil of wheat

The isolates of potash-mobilizing bacteria cultured on Alexandrov agar medium, by using serial dilution and agar plating techniques (Aneja, 2003) were purified, identified and maintained. Colonies exhibiting clear zone of potassium solubilization were selected. Three bacterial isolates were named K I, K II and K III (Table 4.3). The diameter of the zone of solubilization ranged from 2 mm to 5 mm at 72 hours after incubation (HAI). Among the isolates, K II recorded a maximum solubilization of 5 mm followed by K III with a solubilization of 3 mm and a minimum solubilization zone (2 mm) was observed in isolate K I (Table 4.3). The bacterial isolates thus obtained were maintained on slants of Alexandrov's agar medium for further use. (Plate 4.5 and 4.6).

Colony and morphological characterization of *Azotobacter*, PSB and KMB

All the *Azotobacter* isolates (AZ I, AZ II and AZ III) formed circular, smooth and mucoid colonies with convex elevation, exhibited light brown pigmentation for cultures in the late logarithmic phase of growth and reported unevenness in colony texture in subculture and unevenness in color as dull to clear white.

The fresh isolates were gram negative in reaction with approximately spherical cells, in short chains or clumps, arising by shortening of rod-shaped cells and the old isolates gave gram positive reactions. The old cultures of gram negative *Azotobacter* became spherical, producing a cyst with a thick layer of mucous. This thick layer resisted decolorization by alcohol or acetone leading to a false gram-positive reaction. The gram-positive cells were macroscopically indistinguishable from the gram-negative cells. They resembled the gram-positive *Azotobacter* described by Löhnis and Smith (1916, 1923), having the general appearance of the larger types of *Bacillus*. (Plate 4.2)

The phosphate-solubilizing isolates (P I, P II and P III) formed cream, circular colonies with entire margins and had convex elevation on Pikovskaya's agar medium. The isolates gave gram-positive reactions with rod-shaped bacterial cells.

The potash mobilizing isolates (K I, K II and K III) showed round shaped colonies with convex elevations. All isolates exhibited white pigmentation and gave gram-negative reaction with rod shaped cell appearance.

Functional Diversity of Nitrogen Fixing, Phosphate Solubilizing and Potash Mobilizing Bacterial Isolates

Nitrogen fixing ability of the *Azotobacter* isolates

The bacterial isolates were subjected to estimation of nitrogen fixation by micro-Kjeldahl method. The N_2 fixing ability of *Azotobacter* isolates is different for each isolate. The isolate AZ-II fixed the highest amount of nitrogen (17.4 mg of nitrogen/g of sucrose used) compared to AZ-I isolate (12.6 mg of

nitrogen/g of sucrose used) followed by AZ-III (8.26 mg of nitrogen/g of sucrose used). The wide variations in the nitrogen fixing capacity of different *Azotobacter* isolates depend on the nitrogenase enzyme and have been reported by Sanoria and Sundara Rao (1975).

Phosphate solubilizing ability of PSB isolates

The three isolates of PSB were tested for their ability to solubilize inorganic phosphate both qualitatively and quantitatively and their results are presented in the Table 4.2.

Quantitative estimation of Pi released from TCP

The amount of Pi released from tricalcium phosphate by PSB isolates was estimated at 10 days after inoculation (DAI). The amount of Pi released from TCP by isolates at 10 DAI ranged from 27.11 to 33.45%. Isolate P III recorded the highest P-solubilization (33.45 %) compared with remaining isolates.

Decrease in pH of medium during phosphate solubilization

The pH was initially adjusted to a value of 7.0, whose reduction was recorded after 10 DAI. A significant reduction in the pH of the medium was observed in P III (pH 2.92) followed by isolate P II (pH 3.32) and P I which reduced the pH of medium to 3.46 (Table 4.2).

Quantitative estimation of 'K' solubilization

The KMB isolates K I, K II and K III were examined for their ability to solubilize muscovite mica on Alexondrov agar media supplemented with mica. The amount of K released in broth from muscovite mica by the isolates was studied at 7, 15 and 20 days after incubation (DAI) in laboratory conditions and was found in the range of 30.96 mg/l to the of 41.33 mg/ml. The results indicated that the amount of 'K' increased as the days of incubation increases and the highest was observed at 20 DAI. The highest solubilization after 20 DAI was observed for K II (41.33 mg/ml), followed by isolate K-I (30.96 mg/ml) and the least was observed in K-III (27.27 mg/ml) (Table 4.3).

With the results thus obtained on the basis of nitrogen fixing, phosphate solubilizing and potash mobilizing ability, highly efficient nitrogen fixing *Azotobacter* isolate (AZ-II), Phosphate solubilizing isolate (P-III) and Potash mobilizing isolate (K-II) were further tested for various biochemical tests.

Biochemical characterization and identification of nitrogen fixing, phosphate solubilizing and potash mobilizing bacterial isolate

The efficient *Azotobacter* isolate among the three (AZ-II) was tested for various biochemical characteristics *viz.*, Gram staining, catalase test, growth on carbon sources and growth at various temperatures. The cells of *Azotobacter* isolate AZ-II chosen for formulation of consortium were oval or spherical shaped and gram negative in reaction (Plate 4.2). This isolate gave positive for catalase test, while negative for gelatin hydrolysis. Glucose, sucrose, and mannitol were used as the sole carbon sources for growth by the bacterial isolate. Bacteria growth was positive at various concentrations (5, 10, 20, 30 %) of sugar. The growth of bacterial isolate was positive at 20^{o}C, 30^{o}C and 40^{o}C temperature with maximum (+++) growth observed at 30^{0}C, but was negative (-) at 10^{0}Cand 50^{0}C. The results obtained are in close agreement with the results of Islam *et al.*, (2008). The biochemical characteristics of *Azotobacter* isolate AZ-II are presented in Table 4.5.According to Bergey's Manual of Systematic Bacteriology (Holt and Krieg, 1984), the isolate was identified as *Azotobacter chroococcum* which is also used for the formulation of microbial consortium.

The efficient PSB isolate among the three P-III was tested for various biochemical characteristics *viz*., Gram staining, catalase test, gelatin hydrolysis, growth on various carbon sources, growth at various temperatures. The cells of isolate P-III which were used for the formulation of consortium were rod

shaped and gram positive in reaction. The isolate P-III was reported to be positive for the catalase test and gelatin hydrolysis. The biochemical results of the highly efficient PSB isolate, P-III are presented in the table 4.5. The most efficient PSB isolate among the three showing the highest solubilization of phosphate in TCP medium (P-III 33.45 %) was identified by morphological identification, cultural studies and biochemical characterization as *Bacillus subtilis* according to method given by Buchanon and Gibbons (1974) and used for formulation of microbial consortium.

The efficient isolate of potash mobilizing bacteria K-II was tested for various biochemical characteristics *viz*., Gram staining, catalase test, gelatin hydrolysis, growth on different carbon sources and growth at different temperatures (table 4.5). The efficient isolate K-II gave gram-negative reaction in Gram's test (Plate 4.8.). The same isolate was reported to be positive for catalase activity and gelatin hydrolysis. The sole sources for carbon are maltose, sucrose and mannitol for growth by bacterial isolate. The growth of bacteria at different concentrations of sugar (5, 10, 20 and 30 %) was positive. The growth of bacteria at various temperatures showed less (at room temperature) or no growth. The biochemical characteristics of isolate K-II are presented in table 4.5. The isolate K-II having the highest efficiency to solubilize potassium mineral (mica) in liquid medium (K-II 41.33 µg/mL) was identified as *Pseudomonas fluorescens* by morphological observation, cultural studies and biochemical characterization, which is later used for formulation of consortium.

Molecular characterization and identification of nitrogen fixing, phosphate solubilizing and potash mobilizing bacterial isolate

The efficient *Azotobacter spp.* isolate (AZ-II), PSB isolate (P-III) and KMB isolate (K-II) were subjected to DNA isolation using modified CTAB method. The integrity and concentration of purified DNA were determined by agarose gel electrophoresis. The total genomic DNA extracted was dissolved in molecular grade water and stored at 4ºC. Universal primers 27F and 1492R were utilized for sequencing the DNA. For *Pseudomonas,* the specific primers used for forward and reverse primers are Psmn 289 and Psmn 1258 respectively. The DNA of the isolates was sequenced using Sanger's dideoxy sequencing and subjected to PCR for amplification. The resulting sequences obtained in FASTA format files were tested for identification in BLAST software on the NCBI website. The test resulted showed that the nitrogen fixing isolate (AZ-II) was *Azotobacter chroococcum*, PSB isolate (P-III) was *Bacillus subtilis*, and KMB isolate (K-II) was *Pseudomonas fluorescens.* The strains were named as VAS98, Parunandi and mani respectively. All the sequences along with an uncultured *Azotobacter* were deposited in the NCBI repository and accession numbers were obtained. The accession numbers allotted were, *Azotobacter chroococcum* VAS98(OP764015), *Bacillus subtilis* parunandi (OP767523), *Pseudomonas fluorescens* mani (OP769224) and the uncultured *Azotobacter sp.* (OP763991).

Microbial count of *Azotobacter*, PSB and KMB in a consortium on different culture media

Among all the culture media, MS III culture medium recorded the maximum count of *Azotobacter*, PSB and KMB ($10x10^7$, $5 \times 10^7$and 7×10^7cfu g^{-1}, respectively).

Formulation of *Azotobacter*, PSB and KMB consortium

One of the primary sources of nutrition for metabolic activity and growth of the microorganisms is carbon, whereas nitrogen sources help in enhancing the growth of bacterial isolates in the medium. The sources of carbon such as glucose, and sources of nitrogen such as ammonium sulphate and yeast extract, proved to be best for the growth of *Azotobacter,* PSB and KMB. The data from the findings of Kore *et al.*, (2020) revealed that the maximum growth was observed in M-III culture medium (Table 3.2) containing glucose (10g/L),

ammonium sulphate (0.4g/L) and yeast extract (1g/L). All three beneficial organisms were found on culture medium. The results also showed that since the medium M-III contained all the essential nutrients in sufficient amounts required by bacteria for growth *i.e.,* it contained $MgSO_4$, $CaCO_3$ and K_2HPO_4 etc. It has been reported that $MgSO_4$ prolongs the viability of microorganisms that are suspended in potassium buffer (Gunter, 1954). Rojas *et al.,* (2011) reported the optimum growth of *Azotobacter* when supplemented with medium containing sucrose 13.06 g/L and yeast extract 3.70 g/L.

The microbial consortium was formulated with *Azotobacter chroococcum* (isolate AZ-II), *Bacillus subtilis* (isolate P-III) and *Pseudomonas fluorescens* (isolate K-II) as nitrogen fixing, phosphate solubilizing and potash mobilizing bacteria, respectively on MS III culture medium, and an inoculum containing 2 x 10^7cfug^{-1}with lignite powder was used to study the inoculation effect of consortium on the growth and yield of wheat *cv. Trimbak*. (Plate 4.7)

Field experiment

The results of field experiments with respect to various attributes of wheat as influenced by inoculation of a consortium of *Azotobacter*, PSB and KMB under graded levels of recommended doses of fertilizers are discussed further under major heads. The data related to various growth and yield attributes were subjected to statistical analysis using RBD. In support of tabular representation of data, graphical representation has also been presented for better comprehension of the characters.

Growth attributes

Germination percent

The results with respect to the germination of wheat as influenced by *Azotobacter*, PSB and KMB consortium under graded levels of recommended doses of fertilizers are presented in Fig. 1. A significant difference among treatments was observed for germination percentage. Among the different inoculation treatments, T_3 *i.e.,* seed inoculation with consortium of *Azotobacter*, PSB and KMB + 75 % recommended dose of fertilizers (RDF) was found to be the most effective as it recorded the highest germination (97.50 %) over the remaining treatments. It was followed by T_4 *i.e.*, inoculation with *Azotobacter* + 75 % recommended N + 100 % recommended P_2O_5 and K_2O which recorded the second after highest at 96.28 per cent germination. However, it was statistically on par with T_6 *i.e.,* inoculation with KMB + 75 % recommended K_2O+ 100 % recommended N and P_2O_5 (95.70 %), T_2 *i.e.,* consortium of *Azotobacter*, PSB and KMB + 100 % recommended dose of fertilizer (95.33 %) and T_1 *i.e.,* consortium of *Azotobacter*, PSB and KMB (95.04 %). It was followed by T_5 *i.e.,* PSB + 75 % recommended P_2O_5 + 100 % recommended N and K_2O (94.28 %), T_7*i.e.*, 100 % recommended dose of fertilizer (92.157 %).

The minimum germination percentage (85.18 %) was noticed in the uninoculated control plot T_8. The application of recommended dose of fertilizers along with biofertilizers (*Azotobacter*, PSB and KMB) alone and in combination contributed for enhancing germination in wheat. This parameter was noted at 15 DAS. The observed highest germination might be due to biofertilizer treatment which stimulated germination and growth by excreting phytohormones and enhancing the nutrient mobilization from the seed.

Plant height at earhead formation and harvesting stage

The results of plant height recorded at earhead formation and harvesting stage of wheat as influenced by *Azotobacter*, PSB and KMB consortium under graded levels of recommended dose of fertilizers are presented in Fig 2 and Plate 4. Among the different inoculation treatments, T_3 *i.e.*, inoculation with consortium of *Azotobacter*, PSB and KMB + 75 % recommended dose of fertilizers (RDF) was found to be the most effective as it

recorded maximum height at both earhead formation and harvest stages (65.80 cm and 83.60 cm, respectively) over the rest of the treatments. It was followed by T_7 *i.e.*, 100 % recommended dose of fertilizer (63.50 cm and 79.0 cm) and T_2 *i.e.*, consortium of *Azotobacter*, PSB and KMB + 100 % recommended dose of fertilizer (62.50 cm and 79.70 cm) at earhead formation and harvesting stage, respectively.

The minimum plant height was recorded in treatment T_8 *i.e.*, uninoculated control plot (56.0 cm and 69.30 cm) at ear head formation and harvest stage, respectively. The increase in height of the plants may be attributed to adequate availability of nitrogen, phosphorous, potassium owing to application of inorganic fertilizers along with biofertilizers. The action of biofertilizers such as *Azotobacter* can result in more availability of nitrogen and certain growth substances such as auxins, vitamins, gibberellins and organic acids secreted by bioinoculants.

Nitrogen, is also a constituent of proteins which is essential for formation of protoplasm, enhancing cell division, cell enlargement and thereby increasing the plant height. Potassium also plays an important role in maintaining cell turgor, enhances photosynthesis and essential for protein synthesis.

Root length at earhead formation and harvest stages

The results in respect of length of roots (cm) in wheat both at earhead formation and harvest stage as influenced by inoculation of *Azotobacter*, PSB and KMB consortium under graded levels of recommended dose of fertilizers are presented in Fig 2.

Among the different inoculation treatments, T_3 *i.e.*, inoculation of consortium of *Azotobacter*, PSB and KMB + 75 % RDF was found to have significantly highest root length (8.60 cm and 16.73 cm) at earhead formation and harvest stage, respectively. The minimum root length was observed in T_8 *i.e.*, Absolute control (3.9 cm and 7.4 cm) at earhead formation and harvest, respectively.

Dry weight of wheat shoot at earhead formation and harvesting stage

The results of dry weight of wheat shoot recorded at earhead formation and harvesting stage of wheat as influenced by *Azotobacter*, PSB and KMB consortium under graded levels of recommended dose of fertilizers are presented in Table 4.9 and Fig 4.4. Among the different inoculation treatments, T_3 *i.e.*, seed inoculation with consortium of *Azotobacter*, PSB and KMB + 75 % recommended dose of fertilizers (RDF) was found to be the most effective as it recorded significantly highest shoot dry weight at both earhead formation and harvest stage (6.35 and 10.64 g plant^{-1}, respectively). However, it was statistically on par with T_2 *i.e.*, Consortium of Azotobacter, PSB and KMB + 100 % RDF (6.16 and 10.43 g plant^{-1}, respectively) and T_4 *i.e.*, Azotobacter + 75 % recommended N + 100 % recommended P_2O_5 & K_2O (6.11 and 10.26g plant^{-1}, respectively) at earhead and harvesting stages. The minimum dry weight of shoot at both earhead and harvesting stages (4.8 and 6.45) was observed in T_8 *i.e.*, absolute control.

It was observed that the treatment T_4 *i.e.*, Azotobacter + 75 % recommended N + 100 % recommended P_2O_5 and K_2O (10.2 g plant^{-1}) resulted in more dry weight compared to treatment T_5 *i.e.*, PSB + 75 % recommended P_2O_5 + 100 % recommended N and K_2O (9.51 g plant^{-1}), which is probably due to the presence of *Azotobacter* with deficiency of phosphate.

The application of FYM during the land preparation has relation with the dry matter levels at different stages. The results thus obtained are not only due to the biofertilizers present, but also due to the FYM, added at 5 t/ha *i.e.*, 7.12 kg/plot.

Dry weight of wheat root at earhead formation and harvesting stage

The results of dry weight of wheat root recorded at earhead formation and harvesting stage of wheat as

influenced by *Azotobacter*, PSB and KMB consortium under graded levels of recommended dose of fertilizers are presented in Table 4.10 and Fig 4.5.

Among the different inoculation treatments, T_3 *i.e.*, seed inoculation with consortium of *Azotobacter*, PSB and KMB + 75 % recommended dose of fertilizers (RDF) was found to be the most effective as it recorded significantly highest root dry weight (866.48^{-1} and 1761.45 mg $plant^{-1}$, respectively) at both earhead formation and harvest stage over the rest of the treatments.

The minimum dry weight of root was observed in T_8 *i.e.*, absolute control treatment (535.10and 1021.30 $plant^{-1}$, respectively) at earhead and harvesting stage of the crop. It was observed that the treatment T_4 *i.e.*, Azotobacter + 75 % recommended N + 100 % recommended P_2O_5 and K_2O (1729.47g $plant^{-1}$) resulted in more dry weight compared to treatment T_5 *i.e.*, PSB + 75 % recommended P_2O_5 + 100 % recommended N and K_2O (1650.66mg $plant^{-1}$), which is probably due to the presence of *Azotobacter* with deficiency of phosphate.

The application of FYM during the land preparation has relation with the dry matter levels at different stages. The results thus obtained are not only due to the biofertilizers present, but also due to the FYM, added at 5 t/ha *i.e.*, 7.12 kg/plot.

The increase in dry weight of roots due to application of bacterial consortium may be attributed to improved availability of nutrient content due to microbial activity.

Number of panicles per plant

The results of number of panicles per plant of wheat as influenced by inoculation of *Azotobacter*, PSB and KMB consortium under graded levels of recommended dose of fertilizers are presented in Table 4.11 and Fig. 4.6.

Among the different treatments, T_3 *i.e.*, seed inoculation of consortium of *Azotobacter*, PSB and KMB + 75 % RDF was found to be most effective as it recorded maximum number of panicles per plant (9.57). However, it was statistically on par with T_2 *i.e.*, consortium of *Azotobacter*, PSB and KMB + 100 % RDF (8.11). The least number of panicles per plant was observed in absolute control treatment T_8, where no fertilizers or bacterial consortium application was done.

The increment in number of panicles per plant in treatment with inoculation and fertilizer application may be attributed to increase in availability of absorbable forms of nitrogen, phosphorous and potassium along with direct application of recommended dose of fertilizers.

Table.4 Nitrogen fixing ability of *Azotobacter* isolates of wheat by Microkjeldahl method

Sr. No.	*Azotobacter* isolate	Nitrogen fixing ability (µg of nitrogen/mg of carbon)
1.	AZ-I	**12.6**
2.	AZ-II	**17.4**
3.	**AZ-III**	**8.26**

Table.5 Zone of 'P' solubilisation on Pikovskaya's agar and *per cent* Pi released fromTCP broth by the PSB isolates

Sr. No.	PSB isolate	Zone of solubilisation on TCP (mm)	%Pi released from TCP after 10 days	Decrease in pH of medium (from initial pH 7.0) after 10 days
1	P-I	3.3	27.11	**3.46**
2	P-II	4.5	30.83	**3.32**
3	**P-III**	**6**	**33.45**	**2.92**

Table.6 Solubilization of muscovite mica by the KSB isolates

Sr. No.	KMB isolate	Zone of solubilization (mm)	7 DAI ($\mu g\ ml^{-1}$)	15 DAI ($\mu g\ ml^{-1}$)	20 DAI ($\mu g\ ml^{-1}$)
1	K-I	2	14.43	26.65	**30.96**
2	K-II	5	24.08	35.16	**41.33**
3	**K-III**	**3**	**16.24**	**22.21**	**27.47**

Table.7 Microbial count of *Azotobacter*, PSB and KMB in a consortium on different culture media

Sr. No.	Culture media	*Azotobacter* (*cfu* g^{-1})	PSB (*cfu* g^{-1})	KMB (*cfu* g^{-1})
1.	MS I	1×10^3	1×10^3	1×10^3
2.	MS II	1×10^5	1×10^3	-
3.	MS III	10×10^7	5×10^7	7×10^7
4.	MS IV	1×10^3	-	1×10^7
5.	MS V	-	1×10^3	1×10^7

Table.8 Biochemical characterization of nitrogen fixing, phosphate solubilizing and potash mobilizing isolate

Sr. No.	Biochemical tests	*Azotobacter isolate* AZ-II	PSB isolate P-III	KMB isolate K-II
1.	Cell shape	Oval or Spherical	Rod shaped	**Rod shaped**
2.	Gram's reaction	-	+	-
3.	Catalase test	+	+	+
4.	Gelatin Hydrolysis	-	+	+
5.	Growth on Carbon sources			
a.	Glucose	+	+	
b.	Sucrose	+	-	+
c.	Mannitol	+	-	+
d.	**Maltose**	-	-	+

Table.8 contd….

Sr. No.	Biochemical tests	*Azotobacter isolate* AZ-II	PSB isolate P-III	KMB isolate K-II
6	Growth at Various Temperatures			
a.	10^0C	-	-	-
b.	20^0C	++	+	-
c.	30^0C	+++	++	+
d.	40^0C	++	+++	-
e.	**50^0C**	-	-	-

Table.9 Effect of inoculation of *Azotobacter,* PSB and KMB consortium on dry matter of wheat shoot at ear head formation and harvest stages (g plant^{-1})

Sr. No.	Treatment details	Dry weight of shoot (g plant^{-1})	
		Earhead formation	Harvest
T_1	Consortium of Azotobacter, PSB and KMB	5.80	**9.32**
T_2	Consortium of Azotobacter, PSB and KMB + 100 % RDF	6.16	**10.43**
T_3	Consortium of Azotobacter, PSB and KMB + 75 % RDF	6.35	**10.64**
T_4	Azotobacter + 75 % recommended N + 100 % recommended P_2O_5 and K_2O	6.11	**10.26**
T_5	PSB + 75 % recommended P_2O_5 + 100 % recommended N and K_2O	5.76	**9.48**
T_6	KMB + 75 % recommended K_2O +100 % recommended N and P_2O_5	5.55	**9.53**
T_7	100 % RDF	5.11	**8.36**
T_8	Absolute control	4.46	**6.55**
	SE±	0.17	**0.16**
	CD at 5 %	**0.52**	**0.50**

Table.10 Effect of inoculation of *Azotobacter*, PSB and KMB consortium on dry matter of wheat root at earhead formation and harvest stages (mg plant^{-1})

Sr. No.	Treatment details	Dry weight of root (mg plant^{-1})	
		Earhead formation	Harvest
T_1	Consortium of Azotobacter, PSB and KMB	683.15	**1629.56**
T_2	Consortium of Azotobacter, PSB and KMB + 100 % RDF	774.44	**1729.47**
T_3	Consortium of Azotobacter, PSB and KMB + 75 % RDF	866.48	**1761.45**
T_4	Azotobacter + 75 % recommended N + 100 % recommended P_2O_5 and K_2O	735.24	**1633.31**
T_5	PSB + 75 % recommended P_2O_5 + 100 % recommended N and K_2O	747.00	**1650.66**
T_6	KMB + 75 % recommended K_2O +100 % recommended N and P_2O_5	632.37	**1571.65**
T_7	100 % RDF	744.37	**1721.47**
T_8	Absolute control	535.10	**1021.30**
	SE±	2.10	**8.05**
	CD at 5 %	**6.44**	**24.67**

Table.11 Effect of inoculation of *Azotobacter*, PSB and KMB consortium under graded level of recommended dose of fertilizers on number of panicles per plant

Sr. No.	Treatment details	Number of Panicles/plant
T_1	Consortium of Azotobacter, PSB and KMB	**7.58**
T_2	Consortium of Azotobacter, PSB and KMB + 100 % RDF	**8.11**
T_3	Consortium of Azotobacter, PSB and KMB + 75 % RDF	**9.57**
T_4	Azotobacter + 75 % recommended N + 100 % recommended P_2O_5 and K_2O	**6.32**
T_5	PSB + 75 % recommended P_2O_5 + 100 % recommended N and K_2O	**6.01**
T_6	KMB + 75 % recommended K_2O +100 % recommended N and P_2O_5	**5.40**
T_7	100 % RDF	**7.45**
T_8	Absolute control	**4.42**
	SE±	**0.21**
	CD at 5 %	**0.67**

Table.12 Effect of inoculation of *Azotobacter*, PSB and KMB consortium under graded level of recommended dose of fertilizers on 1000 seed weight (g)

Sr. No.	Treatment details	1000 seed weight (g)
T_1	Consortium of Azotobacter, PSB and KMB	**41.70**
T_2	Consortium of Azotobacter, PSB and KMB + 100 % RDF	**42.67**
T_3	Consortium of Azotobacter, PSB and KMB + 75 % RDF	**44.40**
T_4	Azotobacter + 75 % recommended N + 100 % recommended P_2O_5 and K_2O	**38.45**
T_5	PSB + 75 % recommended P_2O_5 + 100 % recommended N and K_2O	**39.33**
T_6	KMB + 75 % recommended K_2O +100 % recommended N and P_2O_5	**36.64**
T_7	100 % RDF	**32.72**
T_8	Absolute control	**27.62**
	SE±	**0.13**
	CD at 5 %	**0.40**

Fig.1 Germination percentages of different treatments, with treatment containing consortium and 75% recommended fertilizer dose showing highest germination %

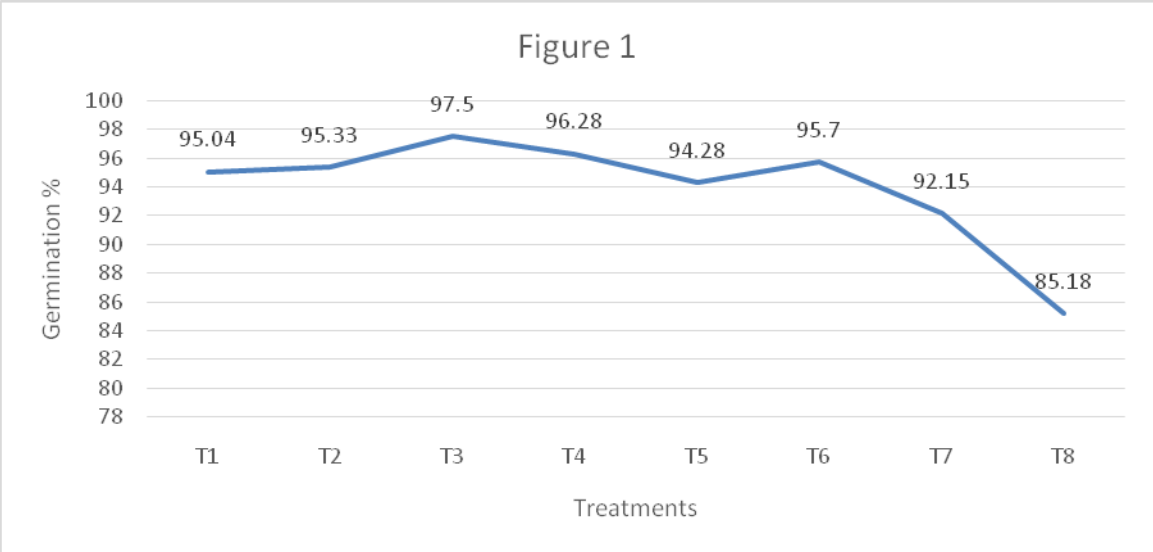


Fig.2 Effect of inoculation of consortium along with graded amounts of chemical fertilizers on plant height and root length at ear head formation and at harvest stages, measured in centimeters.

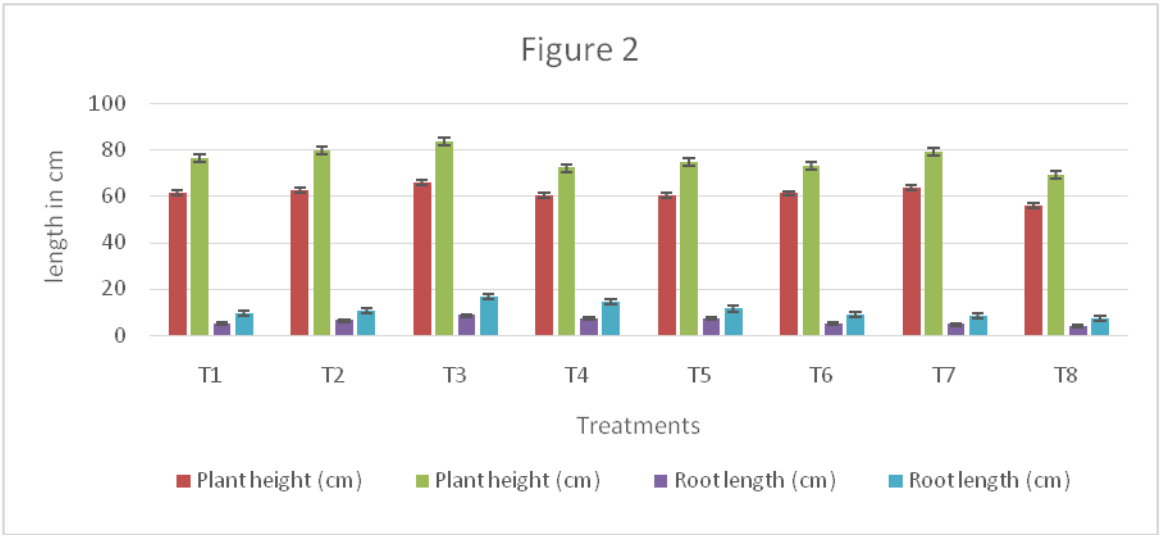


Fig.3 Effect of inoculation of *Azotobacter*, PSB and KMB consortium under graded levels of recommended dose of fertilizers on wheat seed yield (q ha^{-1})

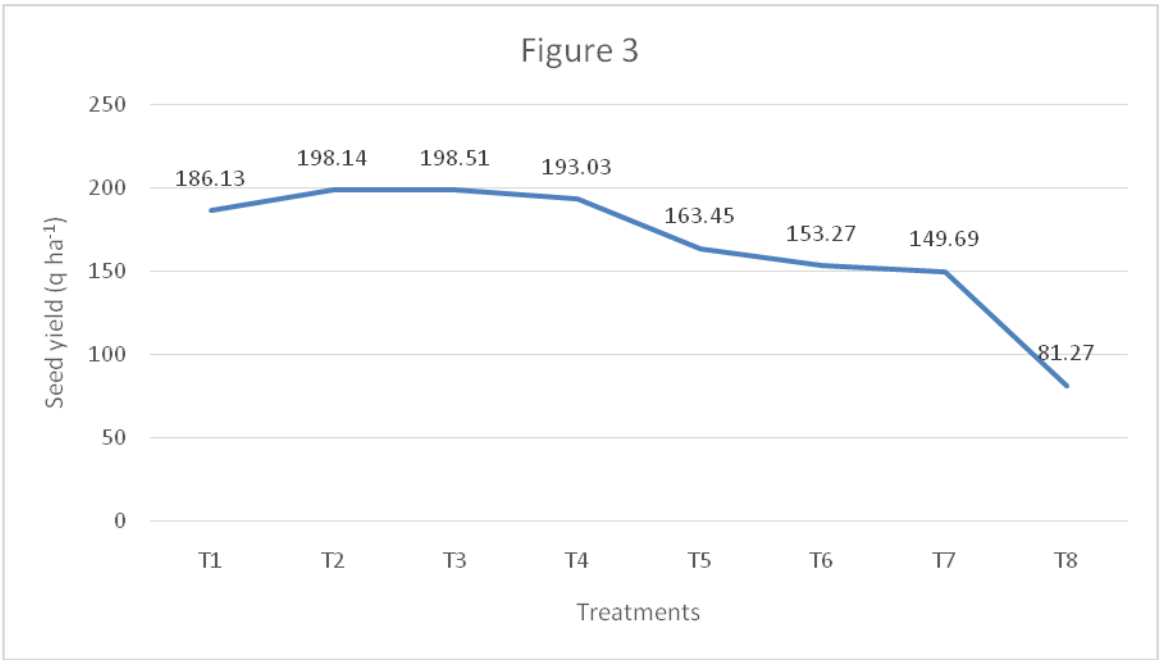


Fig.4 Effect of inoculation of *Azotobacter*, PSB and KMB consortium under graded levels of recommended dose of fertilizers on NPK uptake by wheat and available nutrients in soil at harvest

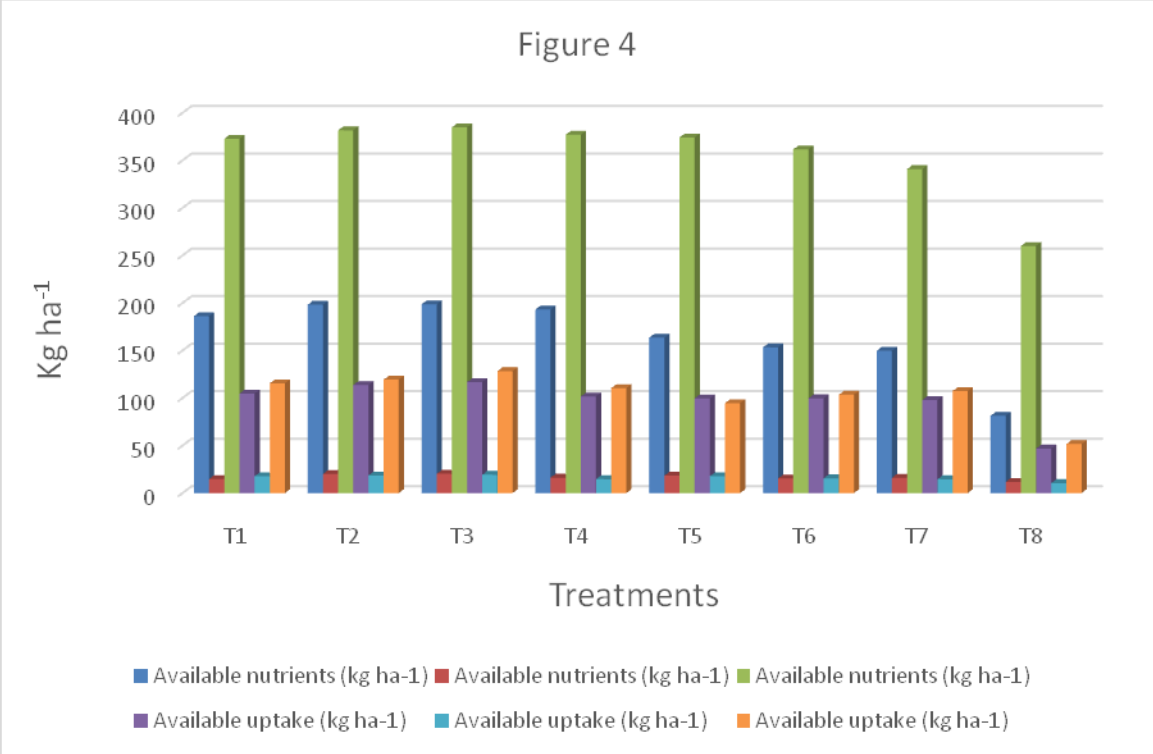


Fig.5 Effect of inoculation of *Azotobacter*, PSB and KMB consortium under graded levels of recommended dose of fertilizers on microbial population at harvest

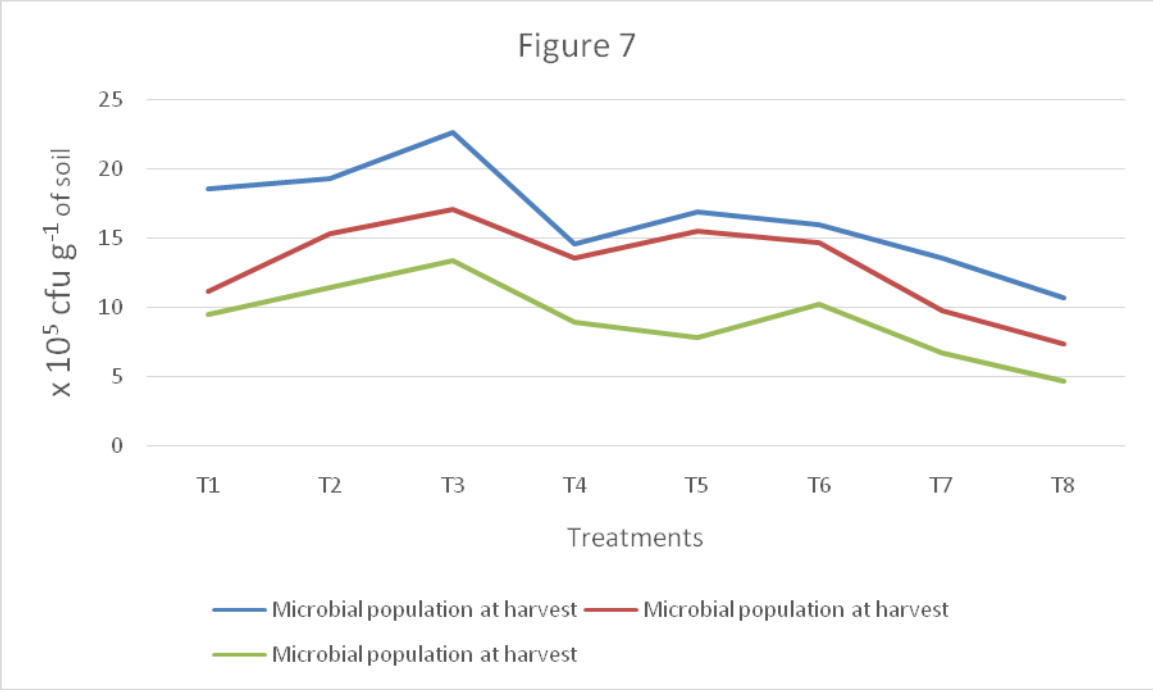


Plate.2 Pure colony of *Azotobacter chroococcum* isolated from rhizosphere soil of wheat on Ashby's Agar media

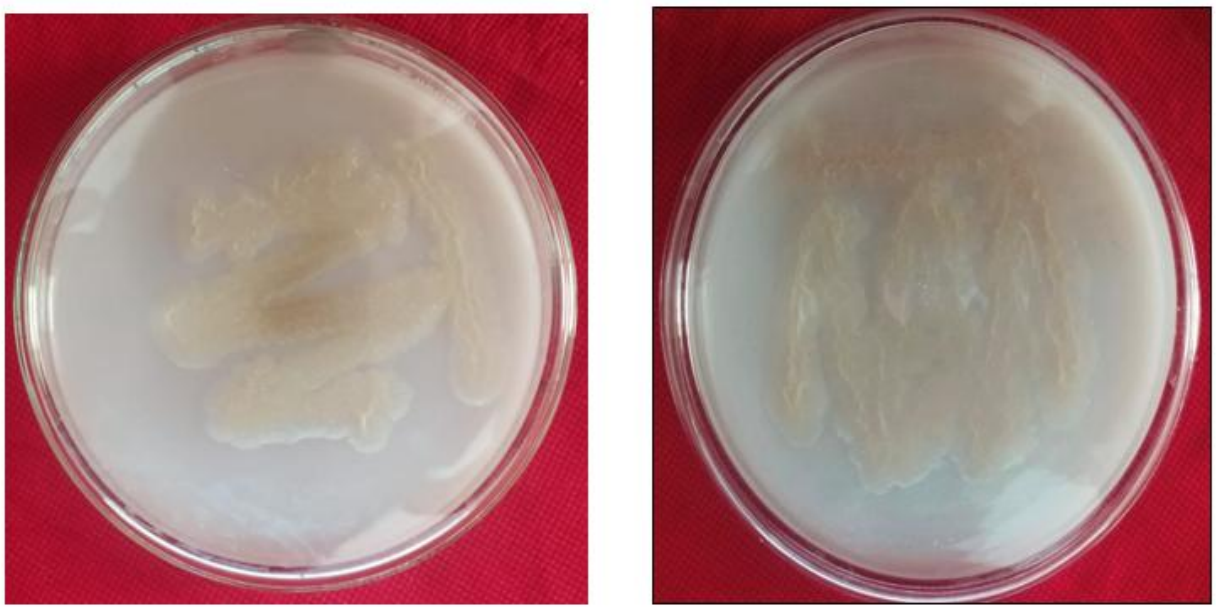


Plate.3 Cell morphology of *Azotobacter* isolated from rhizosphere soil of wheat Oval or Round shaped, Gram negative bacteria

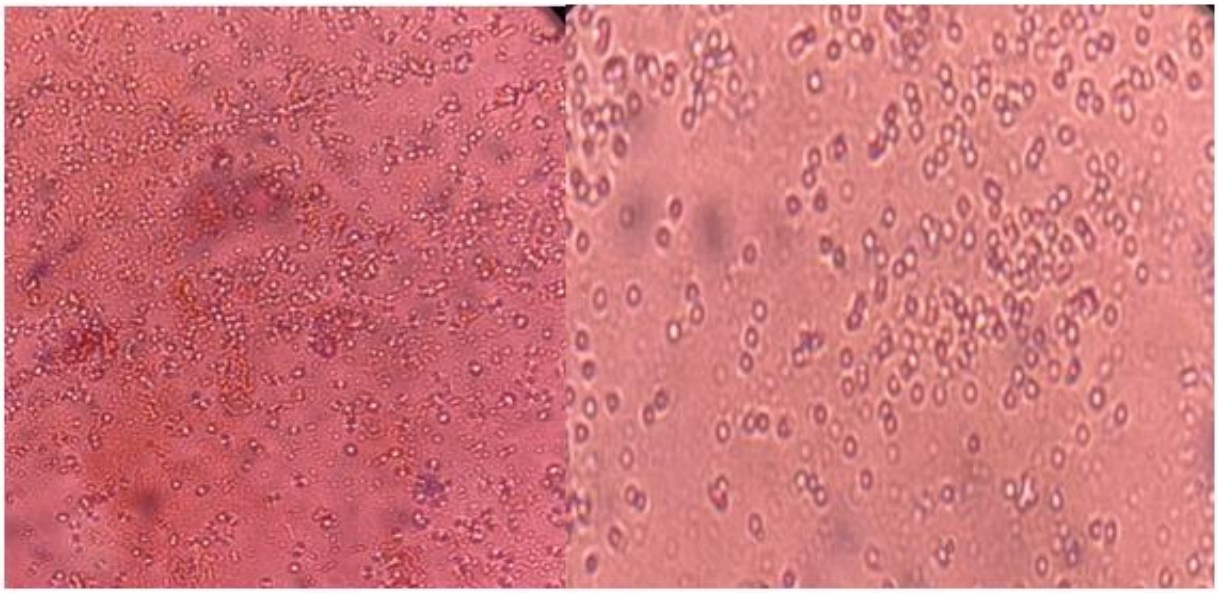


Plate.4 PSB isolate showing clear zone of solublization of TCP on Pikovskaya's medium

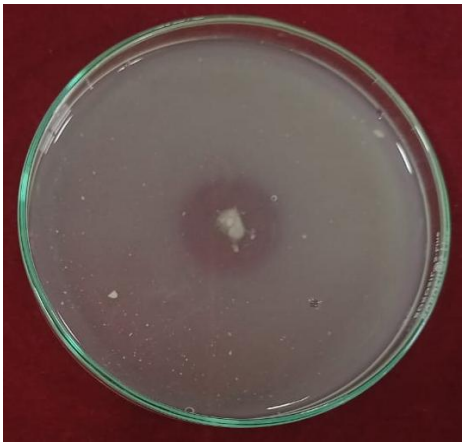


Plate.5 Pure colony of PSB isolated from rhizosphere soil of wheat

Plate.6 KMB isolate showing clear zone of solublization of insoluble potassium bearing mineral (mica) on Aleksandrow medium

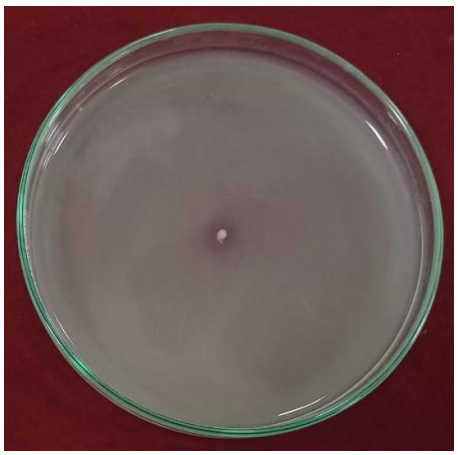


Plate.7 Pure colony of KMB isolated from rhizosphere soil of wheat

Plate.8 Compatibility of *Azotobacter*, PSB and KMB on MS III medium

Plate.9 Inoculation Effect of Consortiumon Shoot length at earhead formation

T_1 : Consortium of *Azotobacter*, KMB and PSB,
T_2 : Consortium + 100% RDF,
T_3 : Consortium + 75% RDF,
T_4 : *Azotobacter* + 75% recommended N + 100% recommended P_2O_5& K_2O,
T_5 : PSB + 75% recommended P_2O_5 + 100% recommended N and K_2O,
T_6 : KMB + 75% recommended K_2O +100% recommended N and P_2O_5 ,
T_7 : 100% RDF,
T_8 : Absolute control

Plate.10 Inoculation Effect of Consortiumon Root Length at earhead formation of wheat

T_1 : Consortium of *Azotobacter*, KMB and PSB,
T_2 : Consortium + 100% RDF,
T_3 : Consortium + 75% RDF,
T_4 : *Azotobacter* + 75% recommended N + 100% recommended P_2O_5& K_2O,
T_5 : PSB + 75% recommended P_2O_5 + 100% recommended N and K_2O,
T_6 : KMB + 75% recommended K_2O +100% recommended N and P_2O_5,
T_7 : 100% RDF,
T_8 : Absolute control

1000 seed weight (g)

The results of 1000 seed weight *i.e.*, test weight of wheat as influenced by inoculation of *Azotobacter*, PSB and KMB consortium under graded levels of recommended dose of fertilizers are presented in Table 4.12 and Fig. 4.7. Among the different inoculation treatments, T_3 *i.e.*, seed inoculation with consortium of *Azotobacter*, PSB and KMB + 75 % RDF was found to be most effective as it recorded significantly highest 1000 seed weight (44.40 g) over rest of the treatments. It was followed by T_2 *i.e.* seed inoculation with consortium of *Azotobacter*, PSB and KMB + 100 % RDF (42.67 g). The minimum 1000 seed weight (27.62 g) was observed in treatment T_8 *i.e.* absolute control.

The 1000 seed weight value depends on weight of grains themselves as well as the biological characteristics of the genotype and to a greater or lesser extent on the environmental conditions.

Wheat seed yield (q ha^{-1})

The results in respect to seed yield of wheat as influenced by inoculation of *Azotobacter*, PSB and KMB consortium under graded levels of recommended dose of fertilizers are presented in Table 4.13 and Fig.4.8. Among the different inoculation treatments, T_3 *i.e.* seed inoculation with consortium of *Azotobacter*, PSB and KMB + 75 % RDF was found to be most effective as it recorded significantly highest seed yield (24.21 q ha^{-1}) over rest of treatments, however it was statistically at par with T_2 *i.e.* consortium of *Azotobacter*, PSB and KMB + 100 % RDF (23.62 qha^{-1}). The minimum seed yield (17.83 q ha^{-1}) was observed in treatment T_8 *i.e.*, absolute control.

The increase in the seed yield may be due to adequate availability of nitrogen, phosphorous and potassium in combination with biofertilizers. This may be due to ability of biofertilizers to produce growth promoting substances like IAA, vitamins and riboflavin etc., which might have helped to increase seed yield.

Available NPK kg ha^{-1} after harvest in soil

The results of available NPK in wheat soils recorded

at harvest as influenced by *Azotobacter*, PSB and KMB consortium under graded levels of recommended dose of fertilizers are presented in Fig 4. The initial available N, P, and K in the soil was 128.41 kg ha^{-1}, 11.63 kg ha^{-1} and 298.05 kg ha^{-1} respectively. Among the different inoculation treatments, T_3 *i.e.* seed inoculation with consortium of *Azotobacter*, PSB and KMB + 75 % RDF showed significantly higher amount of available NPK after harvest (198.51, 20.27 and 384.80 kg ha^{-1} N, P and K respectively) over rest of the treatments. However, it was statistically on par with T_4 *i.e. Azotobacter* + 75 % recommended N + 100 % recommended P_2O_5 and K_2O with 198.14, 19.94, 383.70 kg ha^{-1} of available N, P and K at harvest. The lowest amount of available NPK was found in T_8 *i.e.* absolute control plot (81.27, 11.70, 259.93 kg ha^{-1}).

The increase in nitrogen content in soil at harvest may be attributed to the application of chemical nitrogenous fertilizers in combination with *Azotobacter* which fixed atmospheric nitrogen in soil using nitrogenase enzyme. The fixation improved availability of nitrogen and subsequently more uptake of nutrients.

The increase in phosphorous and potassium content in the soil at harvest may be attributed to balanced application of phosphatic and potassic chemical fertilizers in combination with biofertilizers PSB and KMB, which produce organic acids like lactic acid, glycolic acid, citric acid and acetic acid that may be responsible for quick solubilisation of chemically fixed potassium and phosphorous into available form and release it near plant vicinity. The differential response of one element in combination with varying levels of second applied *i.e.*, both the elements together produce an enhanced effect. This effect is not because of one of them alone. The enhanced uptake of phosphorous and potassium may be attributed to application of biofertilizers which increased solubilisation of phosphorous and potassium into available forms, which resulted in higher availability of potassium and phosphorous and subsequently more uptake of nutrients.

NPK uptake by wheat at harvest

The results of amount of NPK taken by wheat as influenced by *Azotobacter*, PSB and KMB consortium under graded levels of recommended dose of fertilizers are presented in Fig 4. Among the different inoculation treatments, T_3 *i.e.*, seed inoculation with consortium of *Azotobacter*, PSB and KMB + 75 % RDF was found to have highest NPK uptake (116.71, 19.25 and 128.31 kg ha^{-1}, NPK, respectively) followed by T_2 *i.e.*, Consortium of *Azotobacter*, PSB and KMB + 100 % RDF (109.617, 18.47 and 119.31 kg ha^{-1}, respectively). The lowest uptake was observed in T_8 *i.e.* absolute control, where there was less available and less uptake.

Increase in nutrients may be the result of microbial activity which improved uptake of N, P and K. Increase in nitrogen fixation, phosphate solubilisation and potash mobilization increased nutrient availability which encouraged plants to uptake enough.

Microbial count of *Azotobacter*, PSB and Potash Mobilizing Bacteria (KMB) at harvest stage of the crop

The results of microbial population at harvest stage of wheat as influenced by *Azotobacter*, PSB and KMB consortium under graded levels of recommended dose of fertilizers are presented in Table 4.16 and Fig. 4.11. Among the different inoculation treatments, T_3 *i.e.*, seed inoculation with consortium of *Azotobacter*, PSB and KMB + 75 % RDF was found to have significantly highest microbial population at flowering stage (22.66, 17.06 and 13.38 x 10^5 cfu g^{-1} of soil of *Azotobacter*, PSB and KMB, respectively over rest of treatments and was followed by T_2 *i.e.* seed inoculum with consortium of *Azotobacter*, PSB and KMB + 100 % RDF at flowering stage (19.33, 15.34and 11.39 x 10^5cfu g^{-1} of soil of Azotobacter, PSB and KMB, respectively). The lowest microbial population of *Azotobacter*, PSB and KMB (10.62, 7.35 and 4.66 x 10^5cfu g^{-1} of soil) at harvest was noticed in treatment T_8 *i.e.* absolute control plot.

The microbial count of *Azotobacter*, PSB and KMB was recorded on Ashby's agar medium, Pikovskaya's agar medium and Alexandrov agar medium, respectively. It was observed that there is a considerable improvement in bacterial population where microbial consortia were observed. The results denote that the microbial activity continued to elevate where there is inoculation of *Azotobacter*, PSB and KMB, without addition of chemical fertilizers.

Azotobacter sp. is a free living N2 fixing bacterium, it can successfully grow in the rhizosphere zone of wheat, maize, rice, cotton, tomato, lady's finger and many others and fixed 10 - 20 kg N ha^{-1} cropping season-1.

The present investigation was conducted with the aim of isolating *Azotobacter sp.*, phosphate-solubilizing bacteria (PSB) and potash-mobilizing bacteria (KMB) from the rhizosphere soil of wheat, and testing their nitrogen fixing, phosphate solubilizing and potash mobilizing abilities and also their mutual compatibilities, to make formulations of consortium of *Azotobacter*, PSB and KMB. Furthermore, the consortium was applied to wheat crop in different combinations with chemical fertilizers to observe for growth, nutrient uptake and yield of wheat during *rabi* 2021. Additionally, soil health was also measured after harvesting the crop.

The nitrogen fixing bacteria, phosphate solubilizing bacteria and potash mobilizing bacteria from rhizosphere soil of wheat were isolated and further tested for different tests. Based on biochemical, physiological and molecular characterization, nitrogen fixing isolates were identified as *Azotobacter chroococcum* (OP764015), phosphate solubilizing bacterial isolates identified as *Bacillus subtilis* (OP767523) and potash mobilizing bacterial isolates identified as *Pseudomonas fluorescens* (OP769224).

The nitrogen fixing isolate AZ-II fixed the highest amount of nitrogen (148.65 µg of nitrogen/mg of carbon used) than the other isolates tested. Moreover, the phosphate solubilizing bacterial isolate P-III recorded the highest P-solubilization (33.45 %) than the other isolates tested. Furthermore, potash mobilizing bacterial isolate K-II recorded the maximum solubilization of muscovite mica (41.33 $\mu g ml^{-1}$) than the other isolates tested.

The pure cultures of *Azotobacter* (*Azotobacter chroococcum*), PSB (*Bacillus subtilis*) and KMB (*Pseudomonas fluorescens*) were found compatible with each other on MS III culture media and showed maximum count of *Azotobacter*, PSB and KMB (10 x 10^7, 5 x $10^7$and 7 x 10^7cfu g^{-1}, respectively) on MS III culture medium. The consortium of *Azotobacter* (*Azotobacter chroococcum*), PSB (*Bacillus subtilis*) and KMB (*Pseudomonas fluorescens*) was prepared on MS III culture medium and 100 ml of broth culture of consortium was mixed in 500 g lignite powder. The consortium of *Azotobacter chroococcum*, *Bacillus subtilis* and *Pseudomonas fluorescens* having2 x 10^7cfug^{-1} of lignite powder was applied to wheat as seed coating.

The results of the present investigation revealed that among the different inoculation treatments, seed inoculation with consortium of *Azotobacter*, PSB and KMB + 75 % RDF was found to be the most effective as it recorded significantly highest germination (97.50 %), plant height (65.80 and 83.60 cm), root length (8.60 and 16.73 cm), dry weight of shoot (6.35 and 10.64 g $plant^{-1}$) and dry weight of root (866.48 and 1761.45mg $plant^{-1}$) at earhead formation and harvest stage of the crop, number of panicles (9.57 $plant^{-1}$), 1000 seed weight (44.40 g), seed yield (24.21 q ha^{-1}), NPK uptake (116.71, 19.25 and 128.31 kg ha^{-1}, respectively) and population of *Azotobacter*, PSB and KMB (22.66, 17.06 and 13.38 x 10^5cfu g^{-1} soil, respectively) at flowering stage of wheat, however it was statistically at par with the treatment of seed inoculation with consortium of *Azotobacter*, PSB and KMB + 100 % RDF for growth parameters, microbial population, nutrient uptake and seed yield of wheat.

The same treatment has shown an improvement in available soil N, P and K, indicating an

improvement in soil health aiding next crop. Thus, these treatments were found to be significantly superior over rest of the treatments in ameliorating growth parameters, microbial population, nutrient uptake and seed yield of wheat.

In Conclusion,

1. The nitrogen fixing bacteria, phosphate solubilizing bacteria and potash mobilizing bacteria from rhizosphere soil of wheat were isolated and further tested for different tests. Based on biochemical, molecular and physiological characterization, nitrogen fixing isolates were identified as *Azotobacter chroococcum* (OP764015), phosphate solubilizing bacterial isolates identified as *Bacillus subtilis* (OP767523) and potash mobilizing bacterial isolates identified as *Pseudomonas fluorescens* (OP769224).
2. The nitrogen fixing isolate AZ-II fixed the highest amount of nitrogen (148.65 µg of nitrogen/mg of carbon used) than the other isolates tested. Moreover, the phosphate solubilizing bacterial isolate P-III recorded the highest P-solubilization (33.45 %) than the other isolates tested. Furthermore, potash mobilizing bacterial isolate K-II recorded the maximum solubilization of muscovite mica (41.33 μgml^{-1}) than the other isolates tested.
3. Among the different inoculation treatments, seed inoculation with consortium of *Azotobacter*, PSB and KMB + 75 % RDF was found to be the most effective as it recorded significantly highest germination, plant height, root length, dry matter production, nutrient uptake, available NPK after harvest in soil and seed yield of wheat.
4. Seed inoculation with consortium of *Azotobacter*, PSB and KMB + 75 % RDF was found to be the most effective as it recorded significantly highest population of *Azotobacter*, PSB and KMB (22.66, 17.06 and 13.38 x 10^5 $cfug^{-1}$ soil, respectively) at flowering stage of wheat over rest of the treatments.
5. From the present investigation it can be concluded that seed inoculation with consortium of *Azotobacter*, PSB and KMB + 75 % RDF was found to be the most beneficial for getting higher seed yield of wheat with 25 % saving of nitrogen, phosphorus and potassium dose of chemical fertilizers to wheat.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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