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Isolation and characterization of Nitrogen fixing, Phosphate and Potash solubilizing bacteria and their inoculation effect on Maize (*Zea Mays* L.)

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Abstract
The present study focused on isolating and characterizing nitrogen-fixing bacteria, phosphorus-solubilizing bacteria (PSB), and potash-mobilizing bacteria (KSB) from the rhizospheric soil of different fruit crops, including Ber, Pomegranate, Orange, Aonla, and Jamun. The study involved the identification of five nitrogen-fixing bacterial isolates (PRN-1, PRN-2, ORN-1, ORN-2, ARN-1), three phosphorus-solubilizing isolates (PRP-1, ORP-1, ARP-1), and three potash-mobilizing isolates (PRK-1, PRK-2, ORK-1). These isolates were then tested for their effectiveness in promoting plant growth. The most efficient isolate was selected for further evaluation of its impact on maize growth and yield under field conditions.

Keywords: Maize, *Azotobacter*, PSB, KSB

Introduction
Maize (*Zea mays* L.), a member of the *Poaceae* family and Panicoideae sub-family (Kumar and Jhariya, 2013) [12], is also known by various names such as zea, silk maize, makka, and barajovar. It is considered one of the most adaptable and promising crops, capable of thriving in a wide range of agro-climatic conditions. Maize, commonly known as corn, plays a vital role in global agriculture and ranks as the third most widely cultivated cereal crop after wheat and rice (Sandhu *et al*., 2007) [18]. The United States of America (USA) stands as the leading producer, contributing approximately 35% of the total global maize output. In India, the primary maize-producing states include Uttar Pradesh, Rajasthan, Madhya Pradesh, Punjab, Haryana, and Andhra Pradesh, which together account for more than 95% of the country's total maize production (Milind and Isha, 2013) [15]. Maharashtra is becoming one of the key maize-producing states in India, covering about 10% of the nation's total maize cultivation area and contributing significantly to overall production.

It serves as a valuable source of carbohydrates and B-complex vitamins, along with vitamins C, A, and K. Additionally; it contains high levels of beta-carotene and a moderate amount of selenium, which supports thyroid gland function (Saritha *et al.*, 2020) [19]. Maize is also packed with other nutrients, including vitamins E, B1 (thiamine), B2 (niacin), B3 (riboflavin), B5 (pantothenic acid), B6 (pyridoxine), folic acid, selenium, and compounds like N-p-coumaryl tryptamine and N-ferrulyl tryptamine. Potassium, a key nutrient found in maize, holds particular importance as it is often lacking in the average human diet (Kumar *et al*., 2013) [12].

As heavy feeder crop, maize needs high amount of nutrients from soil and give response to added manures and fertilizers. After green revolution, uncontrolled use of chemical fertilizers affects soil health and it directly affects sustainable development of agriculture. The soil inhibiting microbes such as nitrogen fixing, phosphorous solublizing and potash mobilizing bacterial are best solution for the said issues. The study assessed, the nitrogen-fixing, phosphate-solubilizing, and potash-solubilizing capabilities of the bacterial isolates, as well as investigate the effects of their inoculation on the growth and yield of maize.

Materials and Methods

Experimental site

A field experiment was carried out during the Rabi season of 2024-25 at the Experimental Farm of the Department of Plant Pathology and Microbiology, Post Graduate Institute, Mahatma Phule Krishi Vidyapeeth (MPKV), Rahuri, Maharashtra, India. The experimental site is located in the water-scarce region of Western Maharashtra, which experiences a semi-arid climate with an average annual rainfall of 520 mm, mostly occurring in two peaks (July and September). The soil at the site was medium black in texture with a neutral pH of 7.72. The organic carbon content of the soil was 0.55%.

Plant material and fertilizers

Seeds of the maize variety, Phule Rajarshi required for the field experiment was obtained from the Maize breeder, All Coordinated Research Project (AICRP), Kasaba Bewada, Kolhapur. The recommended dose of fertilizers used @ 120:60:40 N: P_2O_5: K_2O kg ha^{-1} and 10 t of FYM per hectare. Nitrogen, phosphorus and potassium applied through urea, single super phosphate and muriate of potash, respectively for the experimental crop.

Collection of Rhizospheric Soil Samples and Isolation of Nitrogen fixing, PSB and KSB

From field of Horticulture department, Mahatma Phule Krishi Vidyapeeth, Rahuri, soil samples from the rhizosphere of fruit crops *i.e.* Ber, Pomegranate, Orange, Aonla, Jamun were taken. For isolating nitrogen fixing, P-solubilizing and K-solubilizing bacteria by serial dilution and the pour plate method. One gram of soil was serially diluted up to 10^{-6} using sterile distilled water, and 0.1 ml aliquots were plated on selective media: Jensen's medium for *Azotobacter*, Pikovskaya's medium for phosphate solubilizing bacteria, and Aleksandrow's medium for potassium solubilizing bacteria. Plates were incubated at 28-30 °C for 7 days, and distinct colonies were purified by streak plate method.

Morphological and physiological characterization

Isolates were characterized based on colony morphology, pigmentation, Gram reaction and cell shape. The selected isolates were screened for temperature tolerance by observing the growth on respective medium by keeping at different level of temperature such as 30°C, 40°C, 50°C. For salt tolerance (NaCl) by observing their growth on respective plates supplemented with NaCl. Ability of isolates to tolerate salt *in vitro* conditions were tested at 5 different concentration of salt *i.e.* 1, 2, 3, 4, 5 per cent NaCl weight by volume (w/v).

Biochemical characterization

Standard assays were performed for catalase, urease, starch hydrolysis, methyl red, Voges-Proskauer, and gelatin hydrolysis following Cappuccino and Sherman (1987) [2].

Functional potential

The nitrogen fixing bacterial isolates screened on nitrogen free malate agar media containing bromothymol blue (BTB) as an indicator. Phosphate solubilization was assessed by halo zone formation on Pikovskaya's medium. Potassium mobilization was determined qualitatively on Aleksandrow's medium.

Experimental design and treatments

The field trial was laid out in a randomized block design (RBD) with seven treatments replicated three times. Each gross plot measured 4.0 x 3.0 m with a net plot of 2.8 x 2.8 m, maintaining 60 x 20 cm plant spacing.

Table 1: The treatments were as follows

Treatment No.	Treatment details
T_1	Untreated control
T_2	100 % RDF alone
T_3	100 % RDF + *Azotobacter* + PSB + KSB
T_4	75 % RDF + *Azotobacter* + PSB + KSB
T_5	50 % RDF + *Azotobacter* + PSB + KSB
T_6	25 % RDF + *Azotobacter* + PSB + KSB
T_7	Alone application of *Azotobacter* + PSB + KSB

Crop management

Seeds were sown per plot in September 2024. Standard agronomic practices were followed. A light irrigation was given immediately after sowing, followed by irrigation at 10-15 days intervals. Hand weeding and intercultural operations were carried out as required. Observations recorded from three randomly selected plants per plot:germination percentage,plant height, number of leaves per plant at 50 % flowering, number of days to 50 % flowering, cob length, cob girth, root length, test weight, grain yield and dry fodder yield.

Microbial population count

Soil samples (0-15 cm depth) were collected at sowing and harvesting to assess microbial populations using serial dilution and plate count technique (Alexander, 1977) [1].

Statistical analysis

Experimental data were analyzed using analysis of variance (ANOVA) for randomized block design as per Panse and Sukhatme (1967) [16]. Treatment means were compared at 1% and 5% levels of significance using critical difference (CD).

Results and Discussions

Isolation and characterization of *Azotobacter*, PSB and KSB

Isolates were obtained from the rhizosphere of fruit crops and designated as *Azotobacter* (Isolates PRN-1, PRN-2, ORN-1, ORN-2, ARN-1), *Bacillus* (Isolates PRP-1, ORP-1, ARP-1) and *Pseudomonas* (Isolates PRK-1, PRK-2, ORK-1). Functional analysis confirmed that *Azotobacter* isolates nitrogen-fixing ability in selective agar medium by precise blue zone formation described by several studies (Gothwal *et al.*, 2008 and Ghorai and Ghosh, 2023) [6-7]. *Bacillus* isolates by clear halo zone around each colony on Pikovaskaya's medium supplemented with insoluble tricalcium phosphate as the only P source and *Pseudomonas* isolates produced solubilization zone on Aleksandrov's medium. These traits validate their potential as biofertilizers for nutrient mobilization. Morphologically, *Azotobacter* colonies were brown pigmented and rod-shaped, *Bacillus* isolates were light green with wavy margins, while *Pseudomonas* formed yellow to cream yellow colonies with entire margins. Gram staining confirmed *Azotobacter* and *Pseudomonas* as Gram-negative, and *Bacillus* as Gram-positive. Physiological assays revealed that all isolates grew well at 30 °C, moderately at 40 °C, However, both PSB and

KSB isolates displayed poor growth at 50°C, indicating limited tolerance to higher temperatures confirming their mesophilic nature. It was discovered that PSB-isolatePRP-1showed growth at 1 % NaCl concentration, moderate growth at 2 % and low growth at 4 % and 5 % NaCl concentration. *Azo*-isolate PRN-2 and KSB-isolate ORK-1 showed full growth up to 5 % concentration of NaCl and thrived on all salt concentrations. Each isolate's designation as a halophile was based on its NaCl content. Biochemical characterization showed that *Pseudomonas* and *Azotobacter* were positive for catalase, gelatin hydrolysis, amylase Production, hydrogen Sulphide Production and citrate Test while *Bacillus* isolates showed strong positive Voges-Proskauer reaction, reflecting their metabolic diversity.

Table 2: Isolate obtained from rhizospheric soil samples along with their designation

Location	Source of rhizospheric soil sample	Isolate obtained			Designation of isolate		
		Azotobacter	PSB	KSB	*Azotobacter*	PSB	KSB
Horticultural department field of Mahatma Phule Krishi Vidyapeeth, Rahuri.	Ber	-	-	-	-	-	-
	Pomegranate	+	+	+	PRN-1, PRN-2	PRP-1	PRK-1, PRK-2
	Orange	+	+	+	ORN-1, ORN-2	ORP-1	ORK-1
	Aonla	+	+	-	ARN-1	ARP-1	-
	Jamun	-	-	-	-	-	-

Table 3: Efficacy of bacterial isolate for nitrogen fixing

Bacterial isolate	Colony diameter (mm)	Colourization zone (mm)	Nitrogen fixing zone diameter (mm)
PRN-1	6	12	6
PRN-2	5	23	18
ORN-1	3	19	16
ORN-2	3	18	15
ARN-1	5	17	12

Table 4: Phosphate solubilizing zone of bacterial isolates

Bacterial isolate	Clear zone diameter (mm)	Colony growth diameter (mm)	Width of the clear zone (mm)	Phosphate solubilising efficiency (%)	Phosphate solubilization index (SI)
PRP-1	13	3	10	433	5.33
ORP-1	10	3	7	333	4.33
ARP-1	8	4	4	200	3

Table 5: Value of Potassium solubilization zone of bacterial isolates

Bacterial isolates	Clear zone diameter (D) (mm)	Colony growth diameter (d) (mm)	D/d (ratio)
PRK-1	15	7	2.14
PRK-2	15	8	1.88
ORK-1	19	4	4.75

Table 6: Characterization of isolates based on morphology

Morphological Characterization	PRN-2	PRP-1	ORK-1
Size of colony (mm)	5	3	4
Colour Pigmentation	Brown	Light Green blue	Yellow to cream yellow
Margin	Entire	Wavy	Entire
Elevation	Raised convex	Flat	Convex
Opacity	Opaque	Opaque	Translucent
Consistency	Mucoid	Mucoid slimy	Mucoid
Motility	Motile	Motile	Motile
Gram reaction	-ve	+ve	-ve
Shape	Rod	Rod	Short rod

Table 7: Biochemical characterization of isolates

Biochemical Test	PRN-2	PRP-1	ORK-1
Catalase Test	+	+	+
Urease Test	-	-	+
Starch Hydrolysis Test	+	+	-
MR Test	+	-	-
VP Test	+	+	-
Gelatin Hydrolysis Test	+	+	+
Amylase Production	+	+	+
Casien Hydrolysis	+	+	-
Hydrogen Sulphide Production	+	-	+
Oxidation formation of Glucose	+	-	-
Citrate Test	+	+	+

Effect of inoculants of *Azotobacter*, PSB and KSB on germination percentage of Maize

There was not any significant difference for germination percentage due to inoculants of *Azotobacter*, PSB and KSB along with graded levels of RDF tried for maize.

Plant height

Among the various inoculation treatments,T_3 *i.e.* inoculation with *Azotobacter*, PSB and KSB along with 100% RDF was determined to be the most effective as it recorded maximum plant height about 175.11cm compared to the other treatments. however, it was statistically at par with treatment T_4 *i.e.* 75% RDF + *Azotobacter*+ PSB + KSB and treatment T2 *i.e.* 100% RDF alone (100:60:40 NPK kg ha^{-1}). The minimum plant height 151.20 cm was recorded in treatment T1 *i.e.* uninoculated control plot. All treatments demonstrated significant plant growth-promoting activity compared to the untreated control. Action of biofertilizers, such as *Azotobacter*, enhances the availability of nitrogen and various growth substances, including auxins, gibberellins, vitamins, and organic acids secreted by bioinoculants. This was in conformity with the findings of Tiwari and Kulmi (2003), Meena *et al.* (2012), Gayatri *et al*. (2021), Kumar *et al*. (2021) [21, 14, 5].

Number of leaves per plant at 50 % flowering

The T_3 treatment (100 % RDF + *Azotobacter* + PSB + KSB) recorded the highest number of leaves per plant at 50 % flowering, averaging 9.59 and was significantly superior to the other treatments. This was followed by T_4 (75 % RDF + *Azotobacter* + PSB + KSB) and T_2 - (100 % RDF alone), which recorded 9.00 and 8.52 leaves per plant, respectively and and where statistically at par with T_3. The untreated control, T_1, recorded the lowest number of leaves at 5.68 per plant. Kujur *et al.* (2020) [9], found that growth parameters like plant height, number of leaves and chlorophyll content effect of dual inoculation of bioinoculants was significant for these parameters.

Number of days required for 50 % flowering

Respect with the number of days required for 50 % of flowering. Among different inoculation treatments, the treatment T_1 *i.e.* untreated control recorded the lowest days, *i.e.*56.33 days over rest of the treatments, The most delayed 50 % of flowering was observed in treatment T_3 *i.e.* 100 % RDF + *Azotobacter* + PSB + KSB. The application of the biofertilizers recorded the non-significant differences for number of days required for 50 % flowering. Dudhade *et al*. (2021) [3], Number of days to 50 % flowering significantly differed with the use of bio-fertilizers in Rabi sorghum. Seed treatment with MPKV *Azotobactor*, PSB and KMB Consortium + 100 % RDF recorded significantly more days to 50 % flowering.

Root length

At harvesting, the treatment T_3 -(100 % RDF + *Azotobacter* + PSB + KSB) recorded maximum root length 20.08 cm and found at par with T_4 - (75 % RDF + *Azotobacter* + PSB + KSB) and T_2 - (100 % RDF) recorded root length 18.03 cm and 15.92 cm respectively. The absolute control treatment recorded at least root length 8.03 cm.

Table 8: Observations recorded

Tr. No.	Treatment details	Germination (%)	Plant height (cm)	Leaves at 50 % flowering	Days for 50 % flowering	Root length (cm)
T_1	Untreated control	88.30	151.20	5.68	56.33	8.03
T_2	100% RDF alone	89.50	169.90	8.52	59.66	15.92
T_3	100% RDF + *Azotobacter* + PSB + KSB	90.40	175.11	9.59	61.33	20.08
T_4	75% RDF + *Azotobacter* + PSB + KSB	90.00	171.80	9.00	60.00	18.03
T_5	50% RDF + *Azotobacter* + PSB + KSB	89.23	165.62	7.82	58.67	13.88
T_6	25% RDF + *Azotobacter* + PSB + KSB	89.01	161.20	7.16	57.66	11.83
T_7	Alone application of *Azotobacter* + PSB + KSB	88.87	159.13	5.79	56.66	9.54
	S.Em. $\pm$	1.51	2.40	0.48	1.52	1.64
	CD at 5%	NS	7.41	1.50	NS	5.07

Yield attributes

Cob length

The data revealed significant variations in cob length across different treatment combinations involving *Azotobacter*, PSB, KSB and recommended fertilizer doses. The treatment T_3 - (100 % RDF + *Azotobacter* + PSB + KSB) produced a significantly greater cob length of 21.90 cm, which was statistically at par to T_4 - (75 % RDF + *Azotobacter* + PSB + KSB) and T_2 - (100 % RDF), with cob lengths of 20.73 cm and 19.50 cm respectively. In contrast, the lowest cob length of 12.42 cm was observed in T_1, the untreated control (without inoculants).

Cob girth

The results regarding the cob girth showed significant differences due to various treatment combinations of *Azotobacter*, PSB, KSB and recommended dose of fertilizers. The treatment T_3 - (100 % RDF + *Azotobacter* + PSB + KSB) showed the higher cob girth of 18.26 cm at the harvesting stage over the treatment T_4 - (75 % RDF + *Azotobacter* + PSB + KSB) had cob girth of 17.28 cm while, the treatment T_1 - Absolute control (without inoculation), recorded shortest cob girth of 9.00 cm. Meena *et al*. (2012) [14], Different treatments had a significant impact on maize plant cob length and cob girth. *Azotobacter* and farmyard manure (FYM) contribute notably to nutrient mineralization in the soil, thereby aiding in the nutrient supply to the plants.

Test weight

Test weight was significantly influenced because of various treatment combinations of *Azotobacter*, PSB and KSB inoculants, along with varying levels of RDF. The treatment T_3 - (100 % RDF + *Azotobacter* + PSB + KSB) recorded much higher test weight 31.60 g as compared to the treatment T_4 - (75 % RDF + *Azotobacter* + PSB + KSB) recorded test weight 30.33 g and T_2 - (100 % RDF) recorded test weight 29.04 g. Among the all treatments, the treatment T_1 - Absolute control (without inoculation), recorded minimum test weight 21.43 g. Similar results were obtained by Game *et al.*, (2020) [4] in wheat. Kumar *et al*. (2021) [5], Significantly higher yield parameters were recorded under application of *Azotobacter* and KSB Inoculation as it increased the biomass may have favorably contributed for the test weight.

Grain yield

The significance difference in grain yield (q ha^{-1}) was noticed in the treatment T_3 - (100 % RDF + *Azotobacter* + PSB + KSB) *i.e*, 81.15 q ha^{-1} with the other treatments. In general treatment T_3 was noted maximum yield whereas treatment T_1 - Absolute control (without inoculation), recorded lowest yield among all the treatments. The results obtained from treatment T_4 (75.39 q ha^{-1}) and T_2 (72.30 q ha^{-1}) were found statistically at par to each other followed by treatments T_5 (69.15 q ha^{-1}). The results of interaction were found to be significant. Whereas, minimum yield was found at treatment T_1 - Absolute control *i.e*, 38.87 q ha^{-1}.

Dry fodder yield

Among all the treatments, treatment T_3 *i.e.* inoculation with *Azotobacter*, PSB and KSB along with 100 % RDF show highest dry fodder yield (8.24 t ha^{-1}) over rest of the treatments, however it was statistically at par with treatment T_4*i.e.* 75 % RDF + *Azotobacter* + PSB + KSB (7.74 t ha^{-1}) and treatment T_2 *i.e.* 100 % RDF alone (120:60:40 NPK kg ha^{-1}) (7.26 t ha^{-1}). The untreated control recorded lowest dry fodder yield (3.53 t ha^{-1}) among all the treatments. Meena *et al*. (2012) [14] different treatments significantly influenced grain and stover yields of maize. The increase was due to conjoint use of chemical and organic fertilizers. Dudhade *et al*. (2021) [3], The application of biofertilizers significantly increased the yield of sorghum. The grain, fodder and biological yields were significantly affected due to use of different bio-fertilizers formulations. The result showed positive impact of combined application of both biofertilizer and chemical fertilizer on herbage yield which showing the conformity of result revealed by Han *et al.* (2006),Singh *et al.* (2007), Gayatri *et al*. (2021) andKumar *et al*. (2021) [8, 18, 5].

Microbial population

Soil analysis at harvest revealed higher available nitrogen, phosphorus and potassium in treated plots compared to control. Inoculation not only improved nutrient uptake by plants but also enhanced microbial population in the rhizosphere, suggesting positive microbial dynamics.The results are in agreement with the findings of earlier researchers. Kumaeasan *et al*. (2019) reported that the highest population (98.65 *cfu*×10^6) was recorded in maize. Similarly, Patil *et al.* (2020) [17] noted that *Azotobacter* populations were higher during the vegetative stage than at maturity.

Table 9: Observations recorded

Tr. No.	Treatment details	Cob length (cm)	Cob girth (cm)	Test weight (g)	Grain Yield (q ha^{-1})	Dry fodder Yield (t ha^{-1})
T_1	Untreated control	12.42	9.00	21.43	38.87	3.53
T_2	100% RDF alone	19.50	16.28	29.04	72.30	7.26
T_3	100% RDF + *Azotobacter* + PSB + KSB	21.90	18.26	31.60	81.15	8.24
T_4	75% RDF + *Azotobacter* + PSB + KSB	20.73	17.28	30.33	75.39	7.74
T_5	50% RDF + *Azotobacter* + PSB + KSB	18.30	15.28	27.72	69.15	6.92
T_6	25% RDF + *Azotobacter* + PSB + KSB	17.21	14.31	26.45	66.30	6.643
T_7	Alone application of *Azotobacter* + PSB + KSB	14.10	10.73	22.92	50.28	5.097
	S.Em. ±	0.92	0.75	0.98	3.26	0.37
	CD at 5%	2.86	2.33	3.02	10.06	1.16

Conclusion

Among all the treatments, the combined application of *Azotobacter* spp. (nitrogen fixer), *Bacillus* spp. (phosphate-solubilizing bacteria) and *Pseudomonas* spp. (potash-solubilizing bacteria) along with 100 % of the recommended dose of fertilizers (RDF) showed notable improvements in plant height, number of leaves per plant at 50 % flowering, root length, cob length, cob girth, test weight, grain yield and dry fodder yield in maize. Additionally, it resulted in the highest levels of available nitrogen, phosphorus and potassium in the soil at the time of harvest. However, no significant differences were observed among treatments concerning germination percentage and the number of days to reach 50 % flowering. The treatments involving 75 % RDF + *Azotobacter* + PSB + KSB and 100 % RDF alone were found to be statistically comparable to the 100 % RDF + biofertilizer combination. Based on statistical analysis from the present study, it can be concluded that treatment with 75 % RDF combined with *Azotobacter*, PSB and KSB is effective. This approach offers a 25 % saving in chemical fertilizers (nitrogen, phosphorus and potassium) while still achieving maximum grain and dry fodder yield in maize.

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