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Research Article

ALKALI TOLERANT *PENICILLUM ASTURIANUM* AS PLANT GROWTH PROMOTER FOR NUTRIENT DEFICIENT CALCAREOUS SOIL

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ABSTRACT

Objectives: Calcareous soils of India with high soil pH and nutrient deficiency has always been a problem for crop cultivation. Crops grown in these soil record severe nutrient deficiency and yield loss. Biological remediation by the microorganism is a novel approach for problematic calcareous soils and can play a major role in enhancing the crop production.

Methods: A study was conducted for isolation of native microbial flora from areas where soils having pH more than 8.0.

Results: An alkali tolerant *Penicillium asturianum* (IPL/KB/F3) was isolated from soils recorded with high pH (alkali soils). The isolates showed a remarkable amount of alkali tolerance and phosphate solubilizing activity along with other insoluble elements. *In vitro* study showed 54±1.13 to 92±1.16 µg/ml of phosphate solubilization within 3-5 days. The isolate was able to tolerate high alkaline pH up to 11.0 and was able to solubilize micronutrients. The isolated *Penicillium* spp. were allowed to grow at a higher concentration of calcium carbonate and were studied for its ability for nutrient solubilization of different insoluble micronutrients.

Conclusion: The high tolerance to alkaline pH and capability of nutrient solubilization by *P. asturianum* (strain no. IPL/KB/F3) thus identified as novel isolate and can be used as a biological input to improve crop cultivation in alkaline soil.

Keywords: Calcareous soil, Isolation, Alkali tolerant, *Penicillium asturianum*, Acid production, Nutrient solubilization.

INTRODUCTION

Calcareous soils are sick soil suffering from alkalinity within the pH range of 7.5-8.5. These soils generally have 100% base saturation, and the exchange complex is dominated by calcium which locks the essential nutrients in soil thereby making unavailable for plants. Calcareous soils are common in arid and semi-arid climates and occur as inclusions in more humid regions relatively little leaching [16] affecting over 1.5 billion acres of soil worldwide and comprising more than 6.5 Mha land in India. Uttar Pradesh and Bihar consists of more than 1.3 Mha of calcareous soil. These types of soil are distributed in inland and coastal regions associated with arid/semi-arid and humid climates. These soils are commonly found in mainly Punjab, Haryana, Rajasthan, West Bengal, and North East state covering almost 5 Mha of land.

Most of the essential plant nutrients remain in insoluble form in soil. Although soil constitutes 0.5% phosphorous, only a very less amount is available to plant absorption, other remains as insoluble salts and cannot be absorbed by plants [1]. Besides this a large portion of chemical fertilizers with high phosphorous content applied to soil is immobilized rapidly and becomes unavailable to plants [2]. A large number of microorganisms including bacteria, fungi, and actinomycetes are known to produce acidic metabolites which by the change of soil pH or by direct chelation of metal cations, release fixed or insoluble phosphorus into available form [5]. Microorganisms as bacteria and fungi solubilize insoluble phosphate by different mechanisms, producing available phosphate that can be taken up by plants [4]. The available phosphate in these soils is low, and applying P-solubilizing microbial inoculants is a logical approach. *Penicillium* and *Aspergillus* are two important genera of fungus frequently used for phosphate solubilization [10-12]. Previous studies have revealed that inoculation of phosphate solubilizing microorganisms *viz.*, *Penicillium*, *Enterobacter* in soil significantly promote the growth and yield of several important crops like wheat [18], onion [19], alfalfa [20] and soybean [21].

In this study, soil samples were collected from different regions which are alkaline in nature and with low nutrient contents and the native flora was isolated from these soils. The isolated microorganisms were studied for alkali tolerance at pH 8.0-11.00. *In-vitro* analysis was done to check the nutrient solubilizing ability of the isolates at high pH especially for calcareous soil where the calcium carbonate content is high.

METHODS

Soil sample collection

Soil samples were collected from different agricultural field and non-agricultural fields having alkaline pH. About 25 soil samples were collected from different regions of Haryana, Punjab, and Uttar Pradesh. The agricultural fields were under varying cropping system depending on the locations and states. Approximately, 50 g of soil sample was taken from the upper 30 cm of the soil aseptically. The electrical conductivity and the pH of the soil samples were recorded.

Isolation of alkali-tolerant nutrient solubilizers

The soil samples were desiccated, compacted and passed through a 2-mm sieve before being mixed into a single merged sample. These soil samples were then analyzed and the characteristics of the soils were tabulated.

The soil samples were serially diluted using sterile water blanks and plated on modified Pikovskaya's agar medium [14]. The plates were incubated at 28-30°C for 3-5 days. After incubation, the acid producing microorganisms were selected based on the zone of clearing around the colonies. The zone producing isolates were purified by repeated culturing and maintained on potato dextrose agar and nutrient agar slants at 4°C. Further isolates which were recognized as alkali tolerant phosphate solubilizer, were inoculated at pH 9.0. The most alkali tolerant isolate capable of producing maximum solubilization of

phosphate both in plate and in broth were further considered for other nutrient solubilization.

***In-vitro* estimation of phosphate solubilization by the isolate at alkaline pH**

Quantitative estimation of phosphate solubilization in broth culture was performed according to the procedure of Jackson [9]. The inoculated broth was studied for phosphate solubilization after 3, 5 and 7 days. A value of 2 ml of cultures were centrifuged at 10,000 rpm for 10 minutes. 1 ml of this supernatant was mixed with 10 ml of chloromolybdic acid, and the volume was adjusted to 40 ml with distilled water. To this, 1 ml of chlorostannous acid was added and the volume was made 50 ml with distilled water. The absorbance of the developing blue color was measured at 600 nm wave length with ultraviolet (UV) visible spectrophotometer (Thermo scientific). The amount of soluble phosphate was calculated from standard curve of KH_2PO_4.

***In-vitro* study of the isolate for solubilization of insoluble micro and macro nutrients.**

The most potent alkali tolerant isolate thus obtained were further studied for ability to solubilize different insoluble micro and macro nutrients at alkaline pH. Insoluble compound was enriched in modified Pikovskaya agar and broth [15] and pH were adjusted at 8.0, 9.0, 10.0, and 11.0 and were inoculated with the isolate. Growth was observed after 3, 5, and 7 days of incubation. The isolates were grown in enriched medium with 0.5% supplement of insoluble compounds, *viz.,* tri-calcium phosphate (TCP), zinc oxide [13], mica (phyllosilicate), ferric phosphate, calcium borate, manganese carbonate, and calcium carbonate.

RESULTS

Soil sample collection

Soil samples were collected from 25 location including agricultural and non-agricultural soils. The sampling was conducted in soils having pH more than 8.0 from different regions of Haryana, Punjab, and Uttar Pradesh (Table 1).

Isolation of alkali tolerant nutrient solubilizers

About 15 isolates were obtained from the initial screening of the isolates. These contain nine bacterial and six fungal isolates which were able to solubilize insoluble TCP in Pikovskaya's agar plate. These isolates when further inoculated at Pikovskaya's medium at pH 9.0, only 2 bacterial and one fungal isolate were able to show a prominent growth and phosphate solubilization at pH 9.0. Among these, the fungal isolate were able to show most phosphate solubilizing activity at pH 9.0 compare to the bacterial isolates.

The fungal isolates were identified and characterized as *Penicillium asturianum* using phenotypic and molecular characterization and were able to grow with a pH range of 4-9.

The alkali tolerant fungal isolate *P. asturianum IPL/KB/F3* thus considered being the most potent alkali tolerant phosphate solubilizing microbes at alkaline pH. The isolate can be used in agriculture especially in soils with high alkaline pH and nutrient deficiency (Table 2).

Phosphate solubilization by *P. asturianum* IPL/KB/F3

The isolate *P. asturianum* IPL/KB/F3 was able to tolerate pH up to 11.0 and was effectively solubilizes insoluble phosphate (TCP) at pH 7.0,

Table 1: List of soil samples collected from different alkaline regions

S. No.	Location	Crops	Soil composition %		Soil text		Depth (in cm)	Soil pH
			Clay	Sand	Texture	Parent		
01	Palwal, Haryana	Paddy	44	55	Loamy	Alluvium	10-30 cm	8.22
02	Palwal, Haryana	Raddish	42	58	Loamy	Alluvium	10-30 cm	8.43
03	Khedipul, Old Faridabad soil	Onion	39	61	Loamy	Alluvium	10-30 cm	9.16
04	Khedipul, Old Faridabad soil	Spinach	38	62	Loamy	Alluvium	10-30 cm	9.06
05	Khedipul, Old Faridabad soil	Marigold	39	61	Loamy	Alluvium	10-30 cm	8.90
06	Aligarh	Castor	36	64	Loamy	Alluvium	10-30 cm	9.21
07	Flooded agriculture land, Yamuna Basin	No Crops	41	59	Clay	Alluvium	10-30 cm	8.59
08	Flooded agriculture land, Yamuna Basin	No Crops	44	56	Clay	Alluvium	10-30 cm	8.62
09	Flooded agriculture land, Yamuna Basin	No Crops	42	58	Clay	Alluvium	10-30 cm	8.88
10	Flooded agriculture land, Yamuna Basin	No Crops	44	56	Clay	Alluvium	10-30 cm	8.95
11	Khedikala, Haryana	Wheat	38	62	Loamy	Alluvium	10-30 cm	9.20
12	Yamuna River Catchment Area	No Crops	55	45	Loamy	Alluvium	10-30 cm	8.50
13	Yamuna River Catchment Area	No Crops			Loamy	Alluvium	10-30 cm	8.22
14	Kathanai near Allahabad Uttar Pradesh	Vegetables	40	60	Loamy	Alluvium	10-30 cm	10.5
15	Kathanai near Allahabad Uttar Pradesh		38	62	Loamy	Alluvium	10-30 cm	9.00
16	Yamuna Coastal Alluvial soil Site 01	No crop	45	55	Clay	Alluvium	10-30 cm	8.09
17	Yamuna Coastal Alluvial soil Site 02	Vegetables	44	56	Clay	Alluvium	10-30 cm	8.72
18	Bhatinda Punjab	Paddy	38	62	Loamy	Alluvium	10-30 cm	8.43
19	Bhatinda Punjab	Cotton	32	68	Loamy	Alluvium	10-30 cm	9.16
20	Sirsa Haryana	Cotton	41	59	Loamy	Alluvium	10-30 cm	9.06
21	Sirsa Haryana	Paddy	40	60	Loamy	Alluvium	10-30 cm	8.90
22	Dubwali, Haryana	Cotton	42	58	Loamy	Alluvium	10-30 cm	9.21
23	Dubwali, Haryana	Citrus	46	54	Loamy	Alluvium	10-30 cm	8.71
24	Hassi, Punjab	Citrus	44	56	Loamy	Alluvium	10-30 cm	8.41
25	Hassi Punjab	Paddy	41	59	Loamy	Alluvium	10-30 cm	8.46

Table 2: Comparison of isolated microbes for dry mass, pH and zone of solubilization at alkaline pH medium

S. No.	Isolate No.	Dry biomass g/ml			pH			Zone of solubilization (cm)		
		3 d*	5 d*	7 d*	3 d*	5 d*	7 d*	3 d*	5 d*	7 d*
01	(UK NSB 01) IPL/KB/B6	0.009	0.012	0.016	5.16	4.95	5.25	1.9	2.4	2.6
02	(UK NSB 02) IPL/KB/B9	0.010	0.013	0.018	5.38	4.97	5.32	1.8	2.3	2.5
03	*P. asturianum* IPL/KB/F3	0.015	0.018	0.024	3.12	2.78	2. 57	4.3	5.2	6.6

*Number of days, The results are mean of five replicates. *P. asturianum: Penicillium asturianum*

8.0, 9.0, 10.0, 11.0. The results were recorded as the mean of five replicates. Quantitative estimation of phosphate solubilization in broth culture was performed according to the procedure of Jackson, 1973 [9]. Periodic estimation of the broth suggested that the *P. asturianum* isolate IPL/KB/F3 were able to release P from insoluble phosphate sources. Decreased in medium pH were associated with an increase in available phosphate. *P. asturianum* were able to release phosphate at pH 7.0, 8.0, 9.0, 10.0 and 11.0. The amount of available *P* was maximum at pH 8.0-10.0. The isolates *P. asturianum* obtained were the most efficient microbial strain enumerated so far compared to reported strains in terms of phosphate solubilization (Table 3 and Fig. 1).

***In-vitro* study of the isolated alkali tolerant *Penicillium* spp. for solubilization of insoluble micro and macro nutrients**

Alkali tolerant most potent phosphate solubilizing isolate *P. asturianum* was taken for further study for understanding it ability to solubilize inorganic compounds *viz*. TCP, calcium carbonate, ferric phosphate, mica (phyllosilicate), zinc oxide, manganese carbonate, calcium borate.

The solubilization study was conducted at different pH 7.0, 8.0, 9.0, 10.0, 11.0. The results were observed in 3, 5 and 7 days.

P. asturianum IPL/KB/F3 isolated from alkaline soil were able to solubilize insoluble compound at pH 7.0, 8.0, 9.0, 10.0 and 11.0. The optimum solubilization was recorded at pH between 8.0 and 10.0. Maximum biomass was obtained at medium pH of 8.0-10.0. *P. asturianum* was able to solubilize all the insoluble compounds at alkaline pH except mica (phyllosilicate).

Table 3: Amount of phosphate solubilization by *P. asturianum* in 3, 5 and 7 days

S. No.	Number of days of incubation	Available phosphate in the medium µg/ml				
		7.0	8.0	9.0	10.0	11.0
01	3 days	54±1.13	53±1.21	53±1.17	51±1.15	46±1.18
02	5 days	92±1.16	91±1.02	92±1.03	88±1.07	79±1.04
03	7 days	94±1.24	94±1.09	95±1.12	89±1.14	85±1.11

*Values are mean±SD of five replicates. SD: Standard deviation, *P. asturianum: Penicillium asturianum*

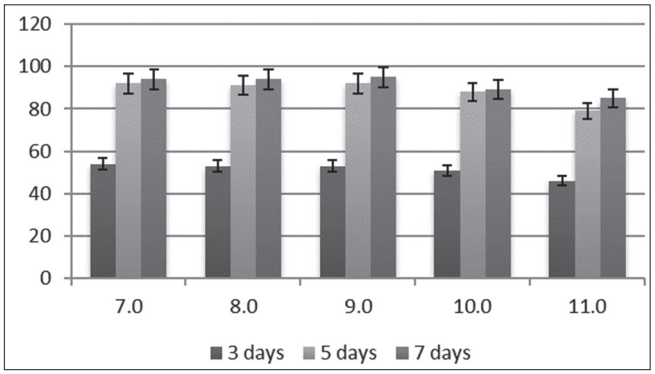

Fig. 1: Amount of phosphate solubilization by *Penicillium asturianum* in 3, 5, and 7 days

Table 4: Growth, pH, zone of solubilization of *P. asturianum* in different insoluble compounds at increasing alkaline pH

S. No.	Insoluble compounds	Initial media pH	Dry biomass g/ml			pH			Zone of solubilization (cm)		
			3 days*	5 days*	7 days*	3 days*	5 days*	7 days*	3 days*	5 days*	7 days*
01	TCP	7.0	0.018	0.021	0.025	2.96	2.51	2.55	0.40	0.49	0.70
		8.0	0.019	0.022	0.026	4.01	3.28	2.98	0.39	0.46	0.70
		9.0	0.019	0.024	0.028	4.61	3.55	3.07	0.39	0.48	0.70
		10.0	0.017	0.023	0.027	4.52	3.78	3.11	0.37	0.44	0.70
		11.0	0.016	0.019	0.022	4.55	3.88	3.21	0.36	0.43	0.70
02	Calcium carbonate	7.0	0.018	0.025	0.029	2.62	2.44	2.33	0.37	0.56	0.70
		8.0	0.017	0.021	0.024	4.24	3.32	2.98	0.13	0.35	0.70
		9.0	0.018	0.022	0.026	4.22	3.23	2.93	0.16	0.33	0.70
		10.0	0.015	0.020	0.024	4.44	3.41	3.01	0.14	0.32	0.70
		11.0	0.012	0.018	0.023	4.42	3.52	3.21	0.13	0.33	0.68
03	Zinc oxide	7.0	0.019	0.022	0.027	3.00	3.21	2.45	0.31	0.48	0.70
		8.0	0.016	0.018	0.026	3.32	2.96	2.53	0.17	0.42	0.70
		9.0	0.016	0.019	0.025	3.55	2.98	2.65	0.16	0.32	0.70
		10.0	0.017	0.020	0.025	3.72	3.32	3.02	0.15	0.24	0.66
		11.0	0.016	0.019	0.022	3.96	3.52	3.19	0.14	0.19	0.63
04	Mica (phyllosilicate)	7.0	0.013	0.016	0.021	4.88	3.84	3.56	0.00	0.00	0.01
		8.0	0.014	0.015	0.020	4.98	4.02	3.78	0.00	0.00	0.01
		9.0	0.013	0.015	0.019	4.82	3.97	3.76	0.00	0.00	0.01
		10.0	0.012	0.014	0.019	4.90	4.14	4.02	0.00	0.00	0.00
		11.0	0.010	0.016	0.018	5.05	4.55	4.14	0.00	0.00	0.00
05	Ferric phosphate	7.0	0.016	0.019	0.025	2.93	2.64	2.57	0.28	0.44	0.70
		8.0	0.016	0.018	0.025	3.23	2.92	2.78	0.22	0.43	0.70
		9.0	0.016	0.018	0.024	3.12	2.99	2.81	0.20	0.37	0.69
		10.0	0.015	0.017	0.022	3.50	3.24	3.12	0.18	0.33	0.69
		11.0	0.014	0.017	0.020	3.55	3.12	3.07	0.18	0.27	0.67
06	Manganese carbonate	7.0	0.015	0.022	0.024	3.24	2.98	2.67	0.32	0.53	0.70
		8.0	0.015	0.020	0.023	3.33	2.99	2.78	0.31	0.42	0.68
		9.0	0.016	0.015	0.019	4.12	3.65	3.25	0.26	0.36	0.66
		10.0	0.015	0.018	0.020	4.23	3.99	3.55	0.24	0.36	0.66
		11.0	0.016	0.018	0.020	4.44	4.01	3.65	0.20	0.34	0.65
07	Calcium borate	7.0	0.014	0.019	0.022	3.56	3.02	2.99	0.31	0.52	0.67
		8.0	0.015	0.019	0.022	3.65	3.15	3.02	0.29	0.49	0.65
		9.0	0.014	0.018	0.021	3.66	3.16	3.00	0.27	0.45	0.62
		10.0	0.013	0.017	0.020	4.02	3.27	3.08	0.24	0.40	0.59
		11.0	0.012	0.016	0.019	4.12	3.48	3.11	0.21	0.38	0.51

*Values are mean±SD of five replicates. SD: Standard deviation, *P. asturianum: Penicillium asturianum*

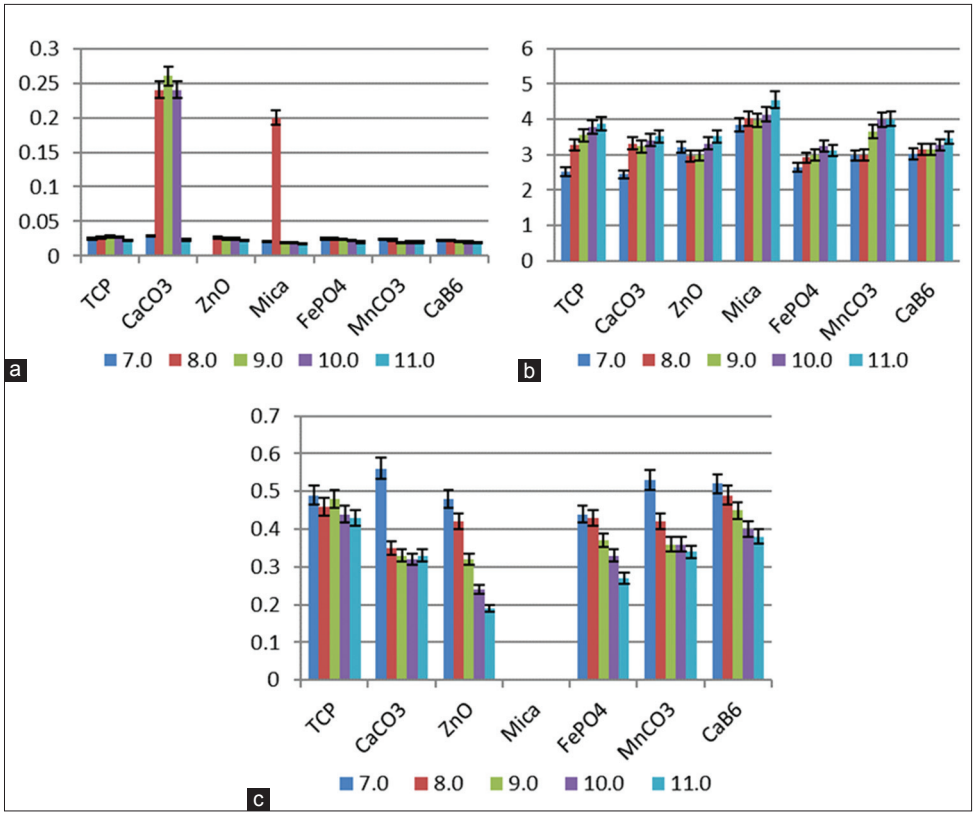

Fig. 2: Growth, pH, zone of solubilization of *Penicillium asturianum* in different insoluble compounds at increasing alkaline pH after 5 days of incubation, (a) Biomass of *P. asturianum* grown in different insoluble compounds at different pH, (b) pH of *P. asturianum* when grown in different insoluble compounds at different pH, (c) zone of solubilization of *P. asturianum* grown in different insoluble compound at alkaline pH

Growth pH was recorded after 3, 5 and 7 days, there was considerable fall in the pH. This may be due to the production of organic acids by the isolates. The isolates also showed prominent solubilization when grown on agar plates with different insoluble organic compounds. The lowering in pH of the medium suggests the release of organic acids by the P-solubilizing microorganisms [7,8]. The zone was measured and was compared at different alkaline pH. The isolates were able to grow and solubilize significantly at all the studied alkaline pH 8.0, 9.0, 10.0, 11.0. The results were studied in replicates and are summarized as the mean of all the replicates. The data was statistically analyzed for a variance by one-way ANOVA at 0.05% level of significance (Table 4 and Fig. 2).

Alkali tolerant *P. asturianum* were able to solubilize all the essential micronutrients at a considerable amount. Qualitatively data was analyzed and recorded. The alkali tolerant *Penicillium* isolates were significantly solubilizes the insoluble compounds except mica (phyllosilicate), produces transparent broth from turbid opaque liquid before inoculation. The isolate was also recorded for the appearance of transparent zone around the colony in all the plates enriched with different compounds. The isolate produces considerable biomass after 3, 5, and 7 days of incubation.

CONCLUSION

Nutrient deficiencies have acquired considerable importance during the recent years. In the light of the above statement, isolation of plant growth promoting microorganisms from novel sources has importance for the development of efficient biofertilizers agents [17]. Chemical management of alkali soil using various existing standard practices are expensive, time consuming and are short term measures. Bioremediation is novel approaches where the alkali tolerant isolates are screened, identified, which can tolerate very high pH and at the same time produce considerable amount of organic acids to solubilize the nutrients which are otherwise remain insoluble form.

If these microbes are made to inhabit the rhizosphere of the plants grown in alkali soils, it can make nutrients available to the plants at the same time reduces the application of chemical fertilizers. *P. asturianum* strain IPL/KB/F3 is an indigenous isolate that is explored and studied for the first time as an alkali tolerant nutrient solubilizing microbe. The unique characteristics make this organism a potential bioremediation agent for nutrient deficient calcareous alkaline soil. Application of *P. asturianum* as a sole or in combination of other biofertilizer expected enhance proper nutrient uptake by the crop plants and, in turn, will open a new vista in organic agriculture.

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