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The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; SP-12(11): 1033-1036

www.thepharmajournal.com
Received: 08-08-2023
Accepted: 11-09-2023

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Isolation of drought tolerant phosphorus solubilizing bacteria from rhizosphere of sorghum

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Abstract
Among the abiotic factors affecting the plant growth, drought is the significant one. It is known to affect the plant water relations there by causing specific and non specific damages. Increased ethylene production caused by drought is the primary factor in early senescence of leaves and flowers in plants, which has a negative impact on productivity and yield (9–10%). Some microorganisms have the ability to produce an enzyme called 1-Aminocyclopropane-1-Carboxylate (ACC) de-aminase that has been known to cleave 1-Aminocyclopropane-1-Carboxylic acid (precursor of Ethylene) to form α-ketogultarate. This coversion will reduce Ethylene levels in plant and its adverse effects. Some of the P- Solubilizing microorganisms are also known to produce ACC de-aminase and this study focuses on isolation of such drought tolerant ACC de-aminase producing P-solubilizers to mitigate drought stress and increase P-availability in plants. This may be the cost effective and most efficient solution for the damages caused the water stress in plants. Among the 110 strains isolated from 34 soil samples from different part of Kalaburagi district, 14 P-solubilizing isolates showed positive results for drought tolerance and ACC de-aminase production. 10 isolates have shown positive results for production of IAA. Among these isolates C4-B produced highest amount of ACC de-aminase and solubilized maximum quantity of phsphorus followed by the isolate C1-A.

Keywords: ACC de-aminase, drought, P-solubilizers

Introduction
Phosphorous (P) is one of the primary elements that plants require in larger quantities. It has an important in photosynthesis, respiration, cell membrane synthesis, glycolysis, and enzyme activity in plants. According to Wu C. *et al*. (2005) [1], phosphorus promotes seed formation, strengthening of stem, crop maturity, root development, and nitrogen fixation in plants. Soils lack easily available orthophosphate despite containing higher levels of total phosphorus. Due to which the global need of Phosphorus fertilizer is dependant on Rock phosphate which is a finite source like oil, coal and petroleum. The existing worldwide P reserves are expected to be drained by 70% during the next 100 years, and given the rise in global demand, a considerable fall in worldwide production is anticipated by 2070. Phosphorus is one of the major limiting nutrients for plants, due to its least mobility and availability. Phosphorus (P) is rapidly fixed in the soil, unlike nitrogen (N), and even hours after application, it's accessibility to plants diminishes. Depending on the type of soil, added P interacts with soil organic molecules including calcium, iron, aluminum, and aluminum to create complexes that make it available to plants. Up to 80% of applied phosphorus is fixed in soil, and the release of this enormous fixed phosphorus pool is very concerning from a point of view of making optimal use of these finite resources. Re-dissolution of Phosphorus in soil and bioavailability largely depends on the soil type, cultural practices and soil microorganisms. Use of phosphate solubilizing microorganisms is a potential area as these microorganisms contribute in soil P cycle and mineralise P from rock phosphate and Tri-calcium phospahte by releasing low molecular weight organic acids. Through nutrient release, mineralization, microbial storage, and decomposition, they can improve soil fertility. Therefore, these microbes can be considered as cost effective options to increase the availability of P in plants (Owen D. *et al.*, 2015) [2]. According to Roopa B. *et al.*, 2012 [3], P-solubilzers are involved in important activities such as organic matter decomposition, formation of soil structure, cycling of elements such as carbon, phosphorus, nitrogen, potassium, sulfur and removal of toxins.
Drought is one considered as of the major abiotic stresses limiting the agricultural productivity worldwide (Mina *et al*. 2019; Tomer *et al*. 2015) [4, 5].

Drought limits growth of plant and Reduce yield. The synthesis of main plant hormone Ethylene, which is known as the stress hormone is stimulated by stress signals such as drought, high temperature and floods etc. Only at lower quantities can the plant growth inhibitor ethylene benefit the plant, but at greater concentrations it can prevent seed germination, root elongation, and legume nodulation (Ahamd *et al*. 2017) [6]. Plant growth Promoting microorganisms have been known to possess an enzyme, ACC-deaminase which cleaves the precursor ACC to form α-ketoglutarate before it can get transformed into ethylene, thereby reducing the ethylene levels in plants under stress (Glick 2004) [7]. The importance of ACC Deaminase enzyme in high temperature and rainfed areas is more and isolating the phosphorus solubilizing microorganisms having the ability to produce ACC Deaminase not only help them survive in stress conditions but also help in enhancing plant growth and yield in drought area. The current study is therefore focused on isolating drought tolerant phosphorus solubilizing bacteria for mitigating harmful effects of stress on plants in dry areas.

Materials and Methods

Collection of soil samples

Soil samples were collected from sorghum rhizosphere of various dry regions of Kalaburagi. pH and electrical conductivity (EC) of the collected soil samples were assessed by using, pH meter and EC meter respectively. Other soil characters such as colour and type were noted for detailed study.

Isolation of drought (osmotic) tolerant rhizobacteria

For isolation of drought tolerant bacteria from the rhizosphere, collected roots were shaken to remove the bulk soil and dipped in sterile distilled water and shaken to collect soil adhering to the roots. The soil suspension was serially diluted and plated on pikovaskaya's agar media added with 110gm/L of PEG (6000) to induce lower water activity(-0.15MPa).

Screening of isolates for drought tolerance

Trypticase soya broth with different water potentials was used by adding the different concentrations of poly ethylene glycol (PEG 6000) - 0.15 MPa, -0.30 Mpa, - 0.49 Mpa and - 0.73 MPa for screenin. It was then inoculated with 1% of bacterial cultures cultivated overnight in TSB. After a 24 hour incubation period at 28 °C with 120 rpm continuous shaking, growth was calculated by measuring the optical density at 600 nm with a spectrophotometer. Growth at different levels of stress was noted (Ali S. Z. *et al.*, 2014) [9].

Screening of isolates for P-solubilization

An assay was carried out for all the drought tolerant isolates by spotting 10 µl of bacterial suspension of the culture on sterile Pikovskaya's medium for screening P solubilizing isolates. The plates were incubated for one week at 28 ± 2 °C. The diameter of the colony and zone was noted and zone of solubilization, solubilization index (SI) was calculated by using the formula (Amrutha G *et al.*, 2018) [10].

$$\text{Zone of solubilization} = \frac{\text{Diameter of the zone - diameter of the colony}}{\text{Diameter of the colony}}$$

$$\text{SI} = \frac{\text{Colony diameter + halo zone diameter}}{\text{Colony diameter}}$$

Quantification of P-solubilzation by isolates in stress conditions

Phosphate solubilized by isolates was measured using the phosphomolybdate blue color method. Pikoviskaya's broth (pH 7) and solution of tricalcium phosphate (0.3 g/100 ml) was autoclaved mixed and loop full of phosphate-solubilizing strains were inoculated in each of the flask under aseptic conditions. Different concentrations (20%, 40%, 60%) of PEG (6000) was added to induce stress condition. The culture in the flask was placed on the rotary shaker at 120 rpm for 12 days. Thereafter, the inoculum suspension was centrifuged at 10,000 rpm for 15 min. The resulting supernatant was used to measure the available phosphorus using a spectrophotometer at 882 nm and calibrated against the phosphorus standard curve (Amrutha G *et al.*, 2018) [10].

Screening for exopolysaccharide production

Exopolysaccharide production by the selected isolates was tested with the method given by Paulo *et al.* (2012) [8]. 5-mm diameter paper discs disposed in a medium (2% yeast extract; 1.5% K_2HPO_4; 0.02% $MgSO_4$; 0.0015% $MnSO_4$; 0.0015% $FeSO_4$; 0.003% $CaCl_2$; 0.0015% NaCl; 1.5% agar) were inoculated by selected strains. Media was modified by the addition of 10% of sucrose and pH was adjusted to 7.5. Based on the size of the halo produced and its slime appearance the production of EPS was quantified. Confirmation of EPS production of was done by mixing a portion of the mucoid substance in 2 mL of absolute ethanol, the formation of a precipitate indicated the presence of EPS.

Screening for ACC-deaminase activity

The ACC-deaminase activity of drought-tolerant P-solubilizing isolates was determined based on the ability of the respective isolate to use ACC as a sole nitrogen source. All drought-tolerant P-solubilizing isolates were grown in 5 ml of TSB medium at 28 °C for 24 h with shaking (120 rpm). The cells were harvested by centrifugation at 3,000 rpm for 5 min, washed twice with sterile 0.1 M Tris–HCl (pH 7.5), resuspended in 1 ml of 0.1 M Tris–HCl (pH 7.5), and spot inoculated on petri plates containing modified DF (Dworkinand Foster) minimal salts medium supplemented with 3 mM ACC as the sole nitrogen source. Plates containing only DF minimal salts medium without ACC was used as the negative control and those with $(NH_4)_2SO_4$ (0.2% w/v) was used as the positive control. The plates were incubated at 28 °C for 72 h. Growth of isolates on ACC-supplemented plates were compared to the negative and positive controls and were selected based on growth by utilizing ACC as the nitrogen source (Ali S. Z. *et al.*, 2014) [9].

Results and Discussion

Isolation of drought tolerant rhizobacteria

34 Rhizosphere soil samples along with the roots of Sorghum from dry lands were collected from 5 different taluks of Kalaburagi district (Kalaburagi, Jewargi, Chittapur, Sedam and Shahbad). A total of 110 rhizobacteria were isolated from the samples on nutrient agar media and were further studies for their colony characteristics. Among 110 bacterial isolates 14 showed positive results for P-solubilization at -0.15 Mpa water activity.

Screening for Drought tolerance

Further the 14 P-solubilizing isolates were tested for their efficacy in survivng drought by inducing different levels of water activity. The growth of all the isolates decreased with increase in concentration of PEG in the media and among 14 isolates C_4-B, C_1-A and C_4-D were able to grow comparitively more at lowest water activiy (-0.73 MPa) than other isolates. Among the 3 isolates C_4-B showed highest growth (OD_{600}=0.69 at -0.73 MPa) (Table 1).

Table 1: Drought tolerancy test of 14 isolates on Pikovskaya's broth

Sl. No.	Sample Code	Optical Density (600 nm)			
		-0.15	-0.30	-0.49	-0.73
1	K2-A	1.48	0.74	0.48	0.10
2	K2-D	1.37	0.81	0.51	0.08
3	F4-E	1.13	0.45	0.14	0.02
4	SH2-A	1.42	0.48	0.39	0.04
5	SH2-B	1.39	0.52	0.42	0.10
6	SH4-A	1.26	0.37	0.26	0.04
7	SH5-C	1.41	0.60	0.35	0.07
8	C1-A	1.50	0.82	0.55	0.56
9	C1-D	1.33	0.75	0.31	0.10
10	C3-B	1.45	0.68	0.29	0.08
11	C3-C	1.34	0.57	0.150	0.04
12	C4-B	1.59	0.92	0.740	0.69
13	C4-D	1.52	0.91	0.570	0.40
14	C5-B	1.22	0.76	0.210	0.05
	CD@1%	0.231	0.129	0.194	0.103
	SEM	0.055	0.043	0.046	0.024

Qualitative and Quantitative estimation of P-Solubilization

Isolates were grown on Pikovskaya's agar and Pikovskaya's broth media for qualitative and quantitative estimation of P-solubilization respectively. Zone of P-solubilization was measured for qualtitive measurement where, C4-B showed highest zone of solbilization followed by C1-A (Table 2, Fig 1). In quantitative estimation the same isolates showed highest and lowest solubilization at 600 nm.

Table 2: Zone of solubilization formed by isolates on Pikovskaya's agar media

Sl. No.	Sample Code	Solubilization Diameter (in mm)	Growth Diameter (in mm)	Solubilization index
1	K2-A	15	13	2.15
2	K2-D	11	09	2.22
3	F4-E	12	10	2.20
4	SH2-A	11	08	2.37
5	SH2-B	14	10	2.40
6	SH4-A	18	13	2.38
7	SH5-C	14	11	2.27
8	C1-A	10	06	2.67
9	C1-D	11	07	2.57
10	C3-B	09	07	2.28
11	C3-C	12	09	2.33
12	C4-B	14	08	2.75
13	C4-D	14	13	2.08
14	C5-B	16	12	2.33
			CD@1%	0.312
			SEM	0.074

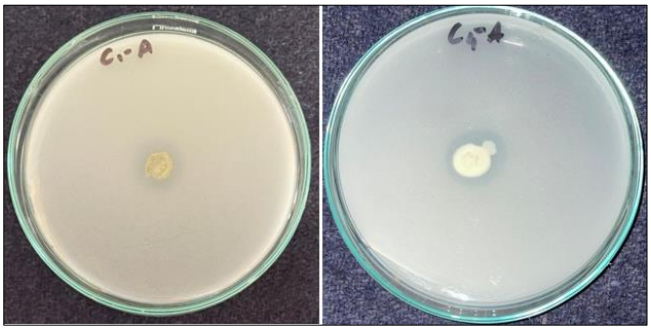

Fig 1: Zone of solubilization on Pikovskaya's agar by the isolates

Test for production of Indole Acetic Acid

Drought tolerant P-solubilizing isolates were tested for the production of growth hormone Indole Acetic Acid using TSB (Trypticase Soy Broth) with 5mM 1-tryptophan. Readings were taken at 530 nm in spectrophotometer. 10 out of 14 isolates showed positive for IAA production (Table 3).

Table 3: IAA production by P solubilizing isolates

Sl. No.	Sample Code	Qualitative assay	IAA production (in µg/ml)
1	K2-A	+	21
2	K2-D	-	0
3	F4-E	-	0
4	SH2-A	+	22
5	SH2-B	-	0
6	SH4-A	+	25
7	SH5-C	+	13
8	C1-A	+	29
9	C1-D	+	17
10	C3-B	+	20
11	C3-C	-	0
12	C4-B	+	31
13	C4-D	+	27
14	C5-B	+	23

ACC Deaminase activity

ACC-Deaminase has been found to cleave the precursor ACC into α-ketoglutarate before it can get converted into ethylene, which ultimately lowers the ethylene levels in plants under stress. ACC-Deaminase is very important character which helps in overcoming drought stress in plants. The isolate C4-B (1.52 µmol) showed highest ACC-Deaminase activity followed by C1-A (1.39 µmol), (Table 4).

Table 4: ACC Deaminase activity by the isolates on TSB medium

Sl. No.	Sample Code	µmol/g biomass/ h
1	K2-A	0.47
2	K2-D	0.64
3	F4-E	0.25
4	SH2-A	0.71
5	SH2-B	1.08
6	SH4-A	0.30
7	SH5-C	0.78
8	C1-A	1.39
9	C1-D	1.03
10	C3-B	0.21
11	C3-C	0.95
12	C4-B	1.52
13	C4-D	1.22
14	C5-B	0.18
	CD@1%	0.204
	SEM	0.048

Conclusion

Phosphorus solubilizing microorganisms are good producers of ACC-deaminase enzyme which is known to help the plants in water stress conditions. Harnessing these microorganisms can alleviate the harmful effects of drought on plants and there by improving their growth and yield potentials. Further pot culture and field experiment studies on these microorganisms must be done to know their survivability and efficiency in *in vivo* condition.

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