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Isolation and Screening of Novel Microorganisms from the Minor Millets

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A B S T R A C T

Minor millets are drought-resistant crops, normally grown under rainfed conditions. Reducing the application of chemical fertilizers will reduce the cost of cultivation drastically. This study aimed to isolate and screening the novel microorganisms from the rhizosphere soil of the minor millets for supplementing the major plant nutrients. Totally 18 different isolates were isolated from the rhizosphere of minor millets. Out of which, 12 isolates were screened based on their performance. Among them, isolates D6, D5 and D1 were exhibited better performance in ARA activity (840±14.3p mol^{-1} mg^{-1} h), phosphorous solubilization (3.08 ± 0.005 cm^2), and potassium solubilization (6.59 ± 0.027 cm^2) respectively. The isolates D6, D5, and D1 were found to be promising in supplying the major nutrients to the crops and could be used for further research to develop suitable inoculants for millets.

Introduction

Millets are the world's most important ancient domestic crops. Before the popularity of rice and wheat, millets are the stable foods in the semiarid regions of East Asia and even in the entire Euro-Asian continent (Weber *et al.*, 2008). Millets are referred to as 'Nutri-cereals' (Ministry of Agriculture, India. 2018), because of the nutrition quality and its stover is an important animal feed. Millets can withstand severe biotic and abiotic stress with fewer inputs (Padulosi *et al.*, 2015). In general, millets do not require any external inputs, but to increase the productivity of the minor millets, we should supply the major nutrients in the form of fertilizers. Instead of regular usage of chemical fertilizers, we can supplement nutrients through the application of biofertilizers. Biofertilizers are low cost, effective, and renewable source of plant nutrients (Borast*et al*., 2009). Biofertilizers can supplement 25% of the crop fertilizer requirement (Chauhan *et al.*, 2015 and Rekha *et al.*, 2018). Thilagar *et al.*, 2016 reported as application selective microorganisms reduce

chemical fertilizer usage by up to 50%.To supplement the chemical fertilizers and to supply the major nutrients to these crops, it was planned to isolate and screen the novel microorganisms from minor millets grown in various places of Tamil Nadu.

Materials and Methods

Soil sample collection

Totally 12 rhizosphere soil samples were collected from different locations of Tamil Nadu, India by carefully uprooting the root system of the minor millets *viz*., Barnyard millet, pearl millet, and finger millet. Soil samples were stored at 4℃ for future use.

Isolation from the rhizosphere soil

For isolation of novel microbes, the sieved-soil plate method (Jensen *et al.,* 1960) was used. Soil particles were evenly distributed over the previously prepared Nutrient agar media and incubated at 30℃ for 48 hr. Those isolated cultures were purified and stored in agar slants for future use. Among 18 different isolates were screened based on their morphological and biochemical characteristics and they were named according to their place of isolation (Boothakudi, Madurai:B; Paramathivelur:P; Dharmapuri:D).

Characterization and screening

For characterization, biochemical tests were performed. ARA activity was used for screening the N fixing microorganisms. Pikovaskaya's media and Aleksandrov´s media were used for screening the phosphate and potassium solubilizing microorganisms.

Nitrogen-fixing efficiency

Before testing the ARA activity of the isolates, they were grown in NFB media Dobereiner., (1995) and observed for the colour changes (green into blue colour) which indicate the growth of the isolates in the nitrogen-free media.

Acetylene reduction assay (ARA)was used to determine the nitrogenase activity of the isolates Hardy *et al.,* (1968). Each bacterial isolate was grown in a 20-ml test tube containing 10 ml of nitrogen-free semi-solid medium for 72 h at 30°C. Each test tube was sealed with a rubber stopper, and 1 ml of acetylene gas was replaced to the air in the headspace (10 ml). The test tubes were incubated at 30°C for 24 h. One ml of each gas sample from the headspace was assayed for ethylene production by gas chromatograph (Varian GC (CP-3800)) equipped with a hydrogen flame ionization detector (FID) and a Porapack N column. Nitrogenase activity was calculated as nmol of ethylene per tube per hr.

Screening for P solubilizing microorganisms

For screening of phosphorus solubilizing microorganisms, Pikovaskaya's media was used. For that 24hr old cultures were inoculated in the Pikovaskaya's agar plates supplemented with 0.5% tricalcium phosphate by using spot inoculation technique and incubated at 28±1℃ for 4 days (Gaind, 2013). The area of phosphate solubilization zone formation (cm^2) was recorded after 4 days of incubation (Pikovskaya., 1948).

Screening for K solubilizing microorganisms

Totally three different Aleksandrov's media was prepared for assessing the K solubilization. Plates of Aleksandrov's media and modified Aleksandrov's media (Saha *et al.,* 2016) were prepared by using KH_2PO_4 and K_2HPO_4. Then 48 hr old cultures (from

NA broth) are inoculated in the plates and incubated at 28±2℃ for 3days. The area of the solubilization zone formed around the colonies after the incubation period was measured (Aleksandrov *et al.*, 1967).

The area of the solubilization zone was expressed in (cm^2), calculated by using the area of a circle formula.

$$A = \pi r^2$$

Where,

A - Area, □ - 3.14, and r - Radius.

Statistical analysis

The experiment was conducted in a completely randomized block design. The results presented are the mean of three replicates. Sample variability was estimated by the standard deviation of the mean. Analysis of variance on the data at CD 5%.

Results and Discussion

Totally 18 different isolates were isolated from the rhizosphere soil of the minor millets and characterized based on the morphology and biochemical tests. All the isolates were rod-shaped and half of the isolates were gram-positive, the remaining isolates were gram-negative (table:1).Isolates B1, B3, B7, P2, D2, D3-I, D4-II, and D5 were positive for citrate utilization test, they produced blue colour in Simmon's citrate agar. Among the 18 isolates, 12 isolates were positively performed in the above screening methods.

Table.1 Morphological and biochemical characterization of rhizosphere isolates of minor millets

Isolates	Gram reaction	Colony Colour and morphology	Catalase test	MR test	VP test	Citrate utilization test	Starch hydrolysis	Cellulase production
B1	-	Raised white	+	-	+	+	-	-
B3	+	Raised Dull yellow	+	+	+	+	-	-
B4	+	Transparent slimy	+	-	-	-	-	-
B7	+	Dull brown	+	-	+	(+)	-	-
B8	-	Translucent and uneven edges	-	+	+	-	-	-
B9	-	Brown colonies with white edges	-	-	-	-	-	-
P1	+	White slimy	+	-	+	-	+	+
P2	-	Raised white slimy	+	-	+	+	-	-
P3	+	White slimy	+	-	-	-	-	-
D1	-	Dull yellow smooth colonies	+	-	+	-	-	-
D2	+	Flat dull white	+	-	+	+	-	-
D3-I	-	White slimy	+	-	+	+	-	-
D4-II	+	Dull yellow, translucent	+	-	-	(+)	-	(+)
D5	+	Flat white smooth	+	-	+	(+)	+	+
D6	+	White slimy with uneven edges	-	-	+	-	-	-
D8	-	Slimy white with yellow spots	+	+	+	-	-	-
D9	-	Dull brown colour	+	-	+	-	-	-
D12	-	Slimy white	+	-	+	-	-	-
All the isolated cultures are rod-shaped; +, Positive; (+), weakly positive; -, negative								

Table.2 Qualitative assay of P and K solubilization for the isolates of minor millets

Isolates	Phosphorus solubilization zone in cm^2	Potassium solubilization zone in cm^2		
		Potassium aluminosilicate	KH_2PO_4	K_2HPO_4
B1	-	5.2 ± 0.075^{b}	2.07 ± 0.034^{c}	1.81 ± 0.034^{c}
B3	-	-	1.51 ± 0.014^{d}	-
P2	2.5 ± 0.008^{b}	5.27 ± 0.031^{b}	3.2 ± 0.019^{b}	2.05 ± 0.030^{b}
P3	1.32 ± 0.003^{c}	3.2 ± 0.014^{d}	1.45 ± 0.026^{d}	-
D1	-	6.59 ± 0.027^{a}	5.53 ± 0.115^{a}	4.59 ± 0.062^{a}
D3-I	-	1.22 ± 0.012^{e}	1.32 ± 0.011^{e}	-
D5	3.08 ± 0.005^{a}	-	-	-
D6	-	3.98 ± 0.074^{c}	-	1.06 ± 0.020^{d}
D8	0.44 ± 0.005^{d}	-	-	-
Grand mean	0.816	2.828	1.676	1.057
SEd	0.006	0.066	0.073	0.046
CD (0.05)	0.014	0.140	0.152	0.097

Values in each column are means of three replicates (±standard error) (n=3) and column values followed by different letters are significantly different from each other at 5% LSD

Fig.1 Estimation of Acetylene reduction activity (ARA) for the isolates of minor millets

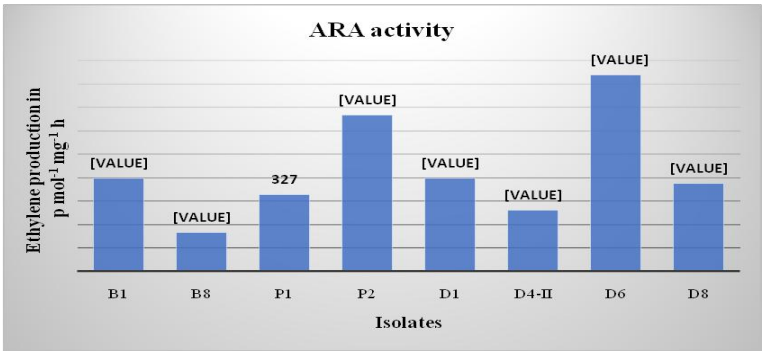
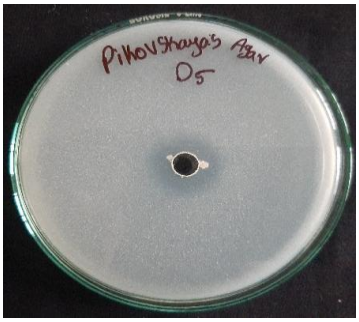
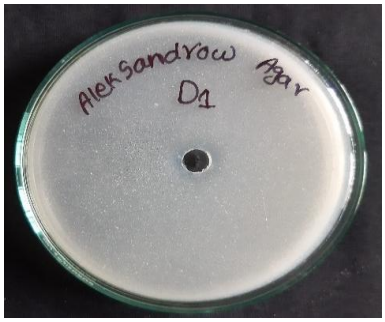


Plate.1 Growth of Nitrogen-fixing isolates in Nitrogen free broth and solubilization zone formation by the P and K solubilizing isolates

NFB broth



Phosphorus solubilization



Potassium solubilization

ARA activity is used to indirectly identify the presence of nitrogenase enzyme which is responsible for the fixation of atmospheric nitrogen. In this study, among the 18 isolates, 8 cultures were showed positive for nitrogen fixation.

Based on the obtained results, the bacterial isolate D6 exhibited higher nitrogen-fixing efficiency (840±14.3 nmol of ethylene produced h^{-1} mg^{-1} protein) (Fig:1) followed by P2 (667.2±9.6 p mol^{-1} mg^{-1} h) and B1(397.8±6.7 p mol^{-1} mg^{-1} h).

Isolate B8 exhibited the least nitrogen-fixing efficiency (165.6±0.3 p mol^{-1} mg^{-1} h) compared to the other isolates. Similarly, Tiwari *et al.*,(2003)isolated and characterized diazotrophs by using ARA activity, from the rhizosphere soil of the pearl millet (*Pennisetum glaucum*). Isolate D6 changed the NFB broth colour, green into blue (Plate:1).

The ability of the Phosphorus solubilization was estimated by Pikovaskaya's media supplemented with 0.5% tricalcium phosphate. From the results, the isolateD5 formed the higher clear zone area, 3.08±0.005 cm^2 (Table:2) around the colonies followed by P2 and P3, which formed2.5±0.008 cm^2 and 1.32±0.003 cm^2 solubilization zones respectively.

Among the isolates, D8 forms the least solubilization zone (0.44±0.005 cm^2) (Plate:1).Similar studies were done in foxtail millet (*Setaria italica* L.) by Kour*et al.*, (2020) and reported as the application of P solubilizing microorganisms useful for stress environmental conditions.

In the case of screening for Potassium releasing ability,isolateD1performed well(Plate:1). Isolate D1 solubilized all three different potassium sources, *viz.,* Potassium alumina silicate (6.59±0.027cm^2), KH_2PO_4 (5.53±0.115 cm^2), and K_2HPO_4 (4.59±0.062 cm^2) (Table.2). Previous study revealed that rhizosphere isolates (*Bacillus sp*) from the common bean, solubilized the potassium sources in the Aleksandrov´s medium (Kumar *et al.*, 2012).

Among the isolates, B1, P2, and D1 solubilized the three sources of K which were supplemented with Aleksandrov´s medium. Isolates D5 and P1 both can hydrolysis the starch and the cellulose (Table:1), which proved the production of amylase and cellulase enzymes. Similar results were also reported by Onah and Tseea, (2003).

From this study, it was concluded that isolate D6 exhibited higher nitrogen-fixing efficiency, isolate D5 showed better solubilization in phosphorous, and D1 solubilized all the three sources of potassium (Potassium aluminosilicate, KH_2PO_4, and K_2HPO_4).

The isolates D6, D5, and D1 were found to be promising in supplying the major nutrients to the crops and could be used for further research to develop suitable inoculants for millets.

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