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EVALUATION OF NITROGEN FIXERS FOR DROUGHT TOLERANCE AND GROWTH PROMOTION IN TOMATO

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ABSTRACT

Drought stress is one of the most fatal environmental phenomena that affect plant growth and productivity worldwide. In the present study, an attempt was made to screen and characterize nitrogen fixing bacteria for drought tolerance by inoculation in trypticase soy broth with different concentrations of PEG 6000. The strains were further evaluated for induction of drought tolerance and growth promotion in tomato. It was found that inoculation improved the plant physiological parameters of tomato seedlings. Furthermore, plants inoculated with nitrogen fixers *viz*., *Microbacterium*, *Cellulosimicrobium* and *Paenibacillus* showed better growth in terms of plant height, number of leaves, stem girth, root length, fresh and dry weight of plants and yield compared to uninoculated control plants under normal and reduced irrigation. The study demonstrates nitrogen fixers with plant growth promoting traits and drought tolerance can be a useful approach for the development of bioinoculants for drought stress management in crops.

Keywords: Nitrogen fixers, Plant growth promotion, Stress tolerance, Tomato

Plants unlike other living forms are static and faced with numerous biotic and abiotic stresses in the environment. Among abiotic stresses, drought is one of the major constraints that affect the crop production and productivity world-wide. Worldwide extensive research is being carried out for the development of drought tolerant varieties through breeding and biotechnological approaches (Lu *et al*., 2013). But most of these technologies are cost-intensive and genetically modified plants are not well accepted in some regions of the world (Wahid *et al*., 2007).

The role of microbes in plant growth, nutrient supply and biocontrol activity is very well established (Souza *et al.,* 2015). The beneficial microorganisms/ PGPR colonize he rhizosphere of plants and promote the growth of the plants through various direct and indirect mechanisms (Grover *et al*., 2011). Recent studies indicate that rhizosphere microorganisms can help plants to cope with stress through its metabolic activities and plant interactions (Schmidt *et al*., 2014).

The mechanism of plant drought tolerance induced by rhizobacteria include: (1) production of phytohormones like abscisic acid (ABA), gibberellic acid, cytokinins, and IAA (2) ACC deaminase to reduce the level of ethylene in the roots (3) induced systemic tolerance by bacterial compounds (4) bacterial exopolysaccharides or biofilms (Timmusk *et al*. 2014, Vurukonda *et al*., 2016). Inoculation with rhizobacteria influences the physiology of the host plant under stress conditions and thereby enhances the tolerance of plants to drought stress. Monitoring of physiological parameters in plant could give better understanding on plants drought tolerance.

Solanum lycopersicum L. (tomato) is an important vegetable crop and is quite sensitive to drought stress during its growth period (Foolad, 2007). There are reports on alleviation of stress tolerance by *Pseudomonas* sp (Vurukonda *et al*., 2016). However, reports on stress tolerance by nitrogen fixing organisms like *Cellulosimicrobium*, *Peanibacillus* and *Microbacterium* are limited. Hence in the present study an attempt was made to screen the plant growth promoting nitrogen fixing microorganism for stress tolerance and evaluation of stress tolerance by using tomato as test crop under controlled conditions.

MATERIALS AND METHODS

Fifteen nitrogen-fixing isolates previously isolated from the rhizosphere of black pepper from Wayanad, maintained at the Department of Agricultural Microbiology, Kerala Agricultural University, Thrissur were used in the present study. The cultures were purified on Jensen's agar medium and further characterized for their drought tolerance.

Screening of bacterial isolates for drought tolerance *in vitro*

In order to screen the isolates for drought tolerance, trypticase soya broth (TSB) with different water

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potentials (-0.65 M Pa, -1.57 MPa and -2.17 MPa) was prepared by appropriate concentrations (10, 20 and 30%) of polyethylene glycol (PEG 6000) (Jain *et al*., 2013) and was then inoculated with 1% overnight raised bacterial cultures in TSB. Three replicates of each isolate with each concentrations of PEG were prepared. After incubation at 28°C for 24-48 h, the growth and population were estimated by measuring the optical density at 600 nm using a UV-VIS spectrophotometer (Biomate) and spreading the culture on Jensen's agar medium, respectively.

PGP traits of the selected isolates

The *in-vitro* plant growth promoting traits *viz*. nitrogen fixation (Bremner, 1960) ammonia (Cappuccino and Sherman, 1992), siderophore (Schwyn and Neilands, 1987), IAA (Nguyen *et al.*, 1992) and hydrocyanic acid (HCN) production (Bakker and Schipper, 1987) of the selected isolates were estimated using standard protocols. The isolates were also tested for their antagonistic activity against major plant pathogens *viz*., *Sclerotium rolfsii, Fusarium*, *Pythium* and *Rhizoctonia solani* by dual culture method (Skidmore and Dickinson, 1976).

Evaluation of the isolates for drought tolerance in tomato

The isolates *Cellulosimicrobium* sp. MG800341.1, *Paenibacillus* sp MG800342.1 and *Microbacterium* sp KY963636.1, with drought tolerance and multiple plant growth promoting traits were selected for evaluation of drought tolerance.

A pot culture experiment was carried out using tomato (variety "Anagha") as a test plant under greenhouse conditions. The experiment was laid in completely randomized design with five treatments and four replications per treatment. Seeds of tomato were sown in pot tray and after 3 weeks of growth they were transplanted in to a polybag contain sterile potting mixture of 15 kg. After one week, the plants were inoculated with 2 ml of cultures corresponds to 3×10^8 cells.

Water deficit was imposed by withholding water for several days. Before the start of experiment, water requirement of plants was standardized by gravimetric method (Earl, 2003). The pots were watered to field capacity and after one day, the pot weight was recorded and served as a reference point for the further experiment.

To estimate the daily water requirement of control plants, the pots were weighed before the photoperiod daily. For stress treatments, soil water status was gradually brought down to specific FC (70 %) by weighing the pots daily at a fixed time of the day. Soil moisture content at 100% field capacity (FC) was determined using the formula; Soil moisture content at 100% FC = [(SW – DW)/DW] × 100; Where SW-saturated soil weight taken after flooding the soil followed by overnight gravitational drainage; DW-oven dry soil weight. Two weeks after transplanting, water deficit stress was imposed to tomato seedlings simulating field condition. Progressive stress was applied for a set of plants over a period of 8 days to reach 70% field capacity (FC) by gravimetric method.

Physiological measurements

Relative water content (RWC) was measured at 10 and 30 days after drought imposition. The third fully expanded leaf from the top of the main stem was sampled between 9:00 h to10:00 h in the morning. After recording the fresh weight, turgid weight was measured by placing the leaf sample in water for 8 h, blotting dry and weighing. Leaf dry weight was determined by drying the leaves in an oven at 60°C for 48 h until a constant weight was obtained (Galmes *et al*., 2007). RWC was computed by the following formula:

RWC = fresh weight – dry weight/ turgid weight – dry weight x100

Leaf membrane stability index (MSI) was measured as described by (Sairam et al., 1997): 0.1 g of leaf discs was thoroughly washed in running tap water and deionized water in two sets. One set was subjected to 40°C for 30 min, and the electrical conductivity of the solution was measured by a conductivity meter (C1). The second set was placed in a boiling water bath (100°C) for 10 min, and its electrical conductivity was measured as above (C2). The membrane stability index (MSI) was calculated as:

MSI = 1 – C1 /C2 × 100

C1= electrical conductivity after exposure to 40°C; C2= electrical conductivity after exposure to 100°C

To measure the content of chlorophyll, 0.5 g of fully expanded leaves was completely ground in 10 ml of 80% acetone and centrifuged for 15 min at 3000 rpm. After making up the solution to 25 ml with acetone, the absorbance at 665 and 645 nm was recorded using a UV–vis spectrophotometer (Biomate). The concentrations of chlorophyll a and b and total chlorophyll were determined (Lichtenthaler and Buschmann, 2001).

Chlorophyll stability index (CSI) in leaf tissues was estimated using a spectrophotometer following the method of Koleyoreas (1958). It was calculated as the difference in light transmission percentage between treated and untreated leaf samples at 652 nm. Treated sample: 1g of leaf sample kept in a test tube containing water at 55°C for 1 h, Untreated/ control :1g of leaf

Table 1. Survival of bacterial isolates at different concentrations of PEG 6000

Bacterial isolate	Microbial population (cfu/ml)		
	PEG Concentration (%)		
	10	20	30
Cellulosimicrobium sp	26 x10^6	19 x10^6	15 x 10^6
Paenibacillus sp	43 x10^6	22.5 x10^6	21 x10^6
Microbacterium sp	58 x10^6	36 x10^6	19 x10^6

sample kept in a test tube containing water at room temperature

CSI = OD at 652 nm of treated sample / OD at 652 nm of control X 100

Measurements of the third expanded leaf counted from the uppermost leaf were performed 7 and 30 days after inducing drought (before 10.am.). Measurements of net photosynthesis were made with a Li-6400 portable photosynthesis system, fitted with a custom-made leaf chamber (Clough et al 1997). Repeated measurements were made on tagged leaves at various levels from the top to the bottom of the canopy throughout the day, over a period of several days.

Plant height, numbers of leaves per plant, stem girth were determined at 30 days interval after inoculation up to 90 days. Root length, fresh weight, dry weight of plants and yield per plant were also recorded according to standard protocols. The data were then analyzed using ICAR GOA (WASP 2.0) programme.

RESULTS AND DISCUSSION

Although the beneficial roles of microbes in plant growth promotion, nutrient management, and disease control are well known, their roles in the management of abiotic stress such as drought have more recently gained importance (Yang et al., 2009; Dimpka *et al*., 2009; Grover *et al*., 2010).

Screening of nitrogen fixing bacteria for drought tolerance

The addition of PEG 6000 in the medium bounds the water molecule and stimulates drought stress in microbes. In the present study, nitrogen fixing microbes isolated from black pepper rhizosphere soils of Wayanad region of Kerala were screened for drought stress tolerance using varying concentrations of PEG 6000. Among the fifteen different isolates tested, only three isolates *viz*., *Cellulosimicrobium* sp. MG800341.1, *Paenibacillus* sp MG800342.1 and *Microbacterium* sp KY963636.1were able to grow at maximum water potential of -2.17 Mpa with the highest tolerance observed in *Paenibacillus* (21x 10^6 cfu/ml). The microbial count was in agreement with the cell density (Fig 1). Generally, optical density values were linearly related to water potential contained in the medium. Reduction of water potential values would decrease number of bacterial populations. Isolates that had capability to grow on medium with a certain potential water value and OD value ≥ 0.4 classified as drought tolerant isolates (Alikhani and Mohamadi, 2010).The water deficit stress tolerant nitrogen fixing bacteria could improve the growth, physiology, and yield contributing parameters of crops under varying levels of field capacity.

Screening of bacterial isolates for invitro plant growth promotion trait

Growth promotion by PGPR may be attributed to mechanisms such as production of PGP hormones and other PGP activities (Glick, 1995). In the present investigation, the strains which were able to tolerate maximum drought stress were screened for plant growth promoting traits. The nitrogen fixing ability was found to be maximum for *Cellulosimicrobium* (40.60 mg of N/g of sucrose utilized). All the three isolates were found to be positive for IAA and ammonia production. Furthermore, HCN production was observed in the isolates

Table 2. Screening of bacterial isolates for *in vitro* plant growth promotion traits

Bacterial isolate	PGP CHARACTERS				
	Amount of nitrogen fixed (mg of N/g of carbon source	IAA (µg/ml)	Ammonia	HCN	Siderophore
Cellulosimicrobium sp	40.60	1.8	+	+	-
Paenibacillus sp	36.86	2.0	+	-	-
Microbacterium sp	18.40	2.5	+	+	-

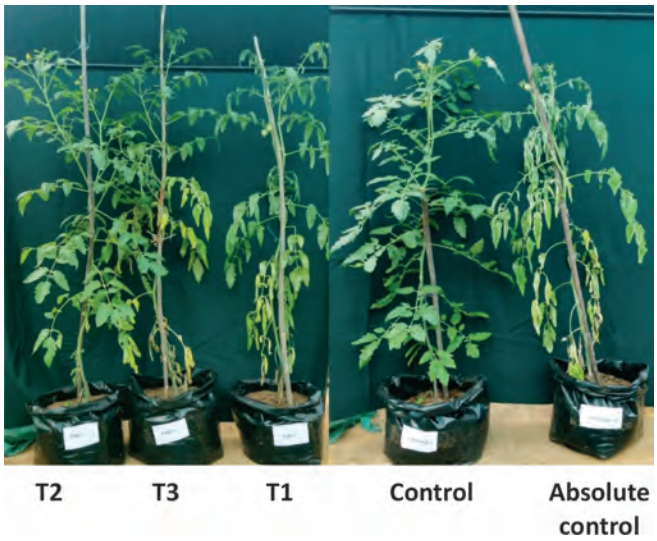

Plate 1. Effect of drought stress in tomato after 60 days

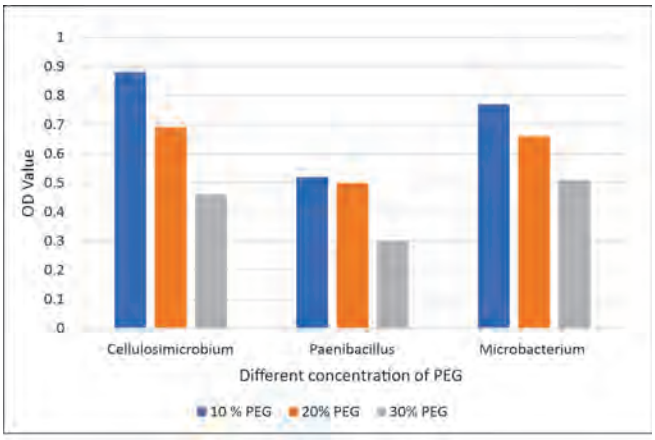

Fig. 1. Tolerance of bacterial isolates to different concentrations of PEG

Cellulosimicrobium and *Microbacterium* and none of the isolates were good producers of siderophore (Table 2). Among the three strains, antagonistic activity against plant pathogens was found only in *Cellulosimicrobium*. The isolate showed 14.4% inhibition against *Sclerotium rolfsii* and 31.1% inhibition against *Rhizoctonia solani.* It has been suggested that microbes producing high amount of IAA resulted in increased root growth and help the plant to uptake water and nutrients to cope with drought stress (Vurukonda *et al*., 2016). In the present study, all the three isolates were good producers of IAA with maximum observed in *Microbacterium* (2.5 µg/ml). Similarly microbes with HCN production could control the growth of phytopathogens (DeCoste *et al*., 2010).

Changes in physiological parameters of tomato seedlings under drought stress

The response of plants to water deficit has been evaluated based on morpho-physiological traits. The leaf gas exchange, relative water content (RWC), photochemical efficiency of PSII, chlorophyll content, and regulation of the electron transport have been used as indicators of plant stress (Burling *et al*., 2013). Inoculation of tomato seedlings with *Cellulosimicrobium* sp, *Paenibacillus* sp and *Microbacterium* sp had influenced the plant physiological parameters to a greater extent. The parameters like RWC, membrane stability index, chlorophyll stability index, total chlorophyll content and photosynthetic rate were measured after eight days of withholding irrigation, 30 DAP and 60 DAP. (Table 3 and Table 4).

RWC is a key indicator of plant water status and one of the most reliable indicators for defining water retention in plants (Shi *et al*., 2015). In this study, we observed that induction of drought stress in plants reduced the relative water content in both uninoculated and inoculated plants, however inoculation significantly increased the RWC of tomato leaves compared to the uninoculated control plants. It was found that inoculation with *Paenibacillus* resulted in maximum increase in RWC of tomato (77.03%) which was on par with control plants irrigated daily (74.33%) and was followed by

Table 3. Effect of drought stress on physiological parameters in tomato

Treatment	RWC (%)		Membrane stability index (%)		Chlorophyll stability index (%)		Total chlorophyll content (µg/ml)		Photosynthetic rate (micromol $Co_2/m^2/sec$)	
	30 DAP	60 DAP	30 DAP	60 DAP	30 DAP	30 DAP	30 DAP	60 DAP	30 DAP	60 DAP
T1 (*Cellulosimicrobium*)	60.79^{b}	53.05^{b}	55.37^{a}	44.90^{ab}	38.01^{c}	33.00^{c}	893.15^{b}	726.50^{b}	9.618^{a}	15.33^{c}
T2 *(Paenibacillus*)	77.03^{a}	67.25^{a}	64.55^{a}	59.30^{a}	86.29^{a}	77.05^{a}	408.87^{c}	347.50^{c}	8.842^{a}	20.70^{a}
T3 (*Microbacterium* F)	58.46^{b}	52.50^{b}	60.07^{a}	61.20^{a}	74.25^{b}	67.50^{ab}	1045.87^{a}	888.83^{a}	5.984^{b}	17.20^{b}
Control	74.33^{a}	71.25^{a}	57.00^{a}	58.25^{a}	43.23^{c}	64.75^{b}	1115.12^{a}	979.75^{a}	9.384^{a}	11.63^{d}
Absolute control	42.40^{c}	36.70^{c}	24.78^{b}	37.45^{b}	25.50^{d}	21.25^{c}	251.02^{d}	208.75^{c}	2.534^{c}	6.98^{e}
CD (0.05)	4.72	6.39	11.15	16.66	9.17	12.26	147.36	109.25	1.97	0.79

DAP – Days after planting

Table 4. Effect of drought tolerant isolates on growth parameters in tomato after 90 days

Treatment	Plant height (cm)	No.of leaves per plant	Stem girth (cm)
T1 (*Cellulosimicrobium*)	91.60^{b}	20.60^{ab}	2.96^{ab}
T2 *(Paenibacillus*)	91.10^{b}	20.68^{ab}	2.91^{b}
T3 (*Microbacterium* F)	103.32^{a}	21.84^{a}	3.07^{a}
Control	81.80^{c}	18.60^{b}	2.84^{bc}
Absolute control	76.40^{c}	14.20^{c}	2.76^{c}
CD (0.05)	8.84	2.37	0.135

Table 5. Effect of drought tolerant isolates on growth parameters and yield in tomato after 90 days

Treatment	No.of fruits/ plant	Yield/plant (g)	Fresh weight of plant (g)	Dry weight of plant (g)	Root length(cm)
T1 (*Cellulosimicrobium*)	9.58^{ab}	38.40^{b}	109.10^{b}	62.80bc	33.10^{a}
T2 *(Paenibacillus*)	11.90^{a}	49.40^{a}	116.10^{ab}	73.00ab	33.50^{a}
T3 (*Microbacterium* F)	9.80^{ab}	39.80^{b}	124.10^{a}	76.10a	38.80^{a}
Control	8.56^{b}	42.40^{ab}	109.10^{b}	56.30cd	24.30^{b}
Absolute control	5.00^{c}	25.00^{c}	88.50^{c}	48.50d	14.80^{c}
CD (0.05)	2.32	9.25	14.57	12.13	7.16

Cellulosimicrobium (60.79%) and *Microbacterium* (58.46%) inoculated plants respectively after 30 days of planting. Similar increase in RWC was observed 60 DAP also. The increase in RWC may be due to the reduction in the inhibitory effect of drought on roots and the development of a more effective root system in the inoculated plants (Dodd *et al.*, 2010) (Table 3).

The degree of integrity and stability in cell membranes under water deficit conditions is correlated with the tolerance of plant to drought stress (Premachandra *et al.,* 1992). The results showed that the uninoculated plants under water stress conditions had lowest membrane stability index while the inoculated plants and irrigated control plants were on par with each other. The results clearly indicated that cell membranes were sensitive to the reduced water content in the plants. However, inoculation of drought tolerant microbes could reduce the membrane injury to a greater extent. A positive correlation between drought stress sensitivity and membrane damage (EL) were observed by Vardharajula *et al.* (2011) and Sandhya *et al.* (2010), and they reported that bacterial inoculation reduced the membrane damage in plants stressed by drought, which was in agreement with our results (Table 3).

High chlorophyll content has been linked with drought tolerance in many plants such as pea, maize, wheat, sorghum (Arunyanark *et al*., 2008; Minakshi *et al*., 2013). In the present study, it was found that chlorophyll stability index of inoculated plants was higher compared to uninoculated plants. A high CSI value of 86.29 and 77.05 per cent was present in *Paenibacillus* inoculated plants 30 DAP and 60 DAP, respectively (Table 3). It indicates that the stress did not have much effect on chlorophyll content of plants and could withstand stress through better availability of chlorophyll.

The photosynthetic rate of control plants (uninoculated) markedly dropped because of drought stress, and was considerably higher in the inoculated plants. It was observed that after 30 days, photosynthetic rate of plants inoculated with *Cellulosimicrobium* and *Paenibacillus* were on par with control plants which were given irrigation daily (Table 3) However, after 60 days of planting, photosynthetic rate was significantly higher in *Paenibacillus* inoculated plants and lower in uninoculated control plants (Table 3).

Evaluation of bacterial isolates on growth promotion in tomato

In uninoculated seedlings, drying drastically affected the growth as indicated by stunted growth, less plant vigor and wilting the leaves. However, the inoculated seedlings showed better growth in terms of plant growth, as compared to respective uninoculated control treatments (plate 1). Bacterial inoculation significantly enhanced the seedling growth in terms of plant height, stem girth and number of leaves, root length, plant fresh weight, dry weight and yield parameters (Table 4 and Table 5) as compared to uninoculated control under drying stress as well as under control condition (plants watered daily).

Among the different treatments, inoculation with *Microbacterium* showed an increment of 35.23 % ,53.80 % and 11.23 % in plant height, number of leaves and stem girth respectively, compared to control under drought stress. While *Cellulosimicrobium* and *Paenibacillus* inoculation resulted in 19.89 and 19.24% increase in plant height, 45.07 ad 45.63 % increase number of leaves and 7.24 and 5.43 % increase in stem girth over uninoculated plants with drought stress. Likewise, inoculation increased the root length, fresh and dry weight of plants and yield compared to non-inoculated control under normal and reduced irrigation (Table 4 and Table 5) (Plate 1).

In conclusion, this work showed that nitrogen fixers isolated from Wayanad region displayed a higher tolerance to drought stress. Further, inoculation of the microbial isolates in tomato plants helped seedlings to tolerate drought stress to a higher level as compared to uninoculated plants and it resulted in a better response under water deficit condition. Further studies will be needed to determine the behavior of these isolates in field trials in view of developing new inoculants suitable for areas with water deficit.

Authors' contribution

Conceptualization of research work and designing of experiments (DG), Execution of field/lab experiments and data collection (PPS), Analysis of data and interpretation (PPS, ST); Preparation of manuscript (PPS, SG, ST)

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