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Mitigating low temperature and draught stress using psychrotolerant rhizobacteria in lentil

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Abstract
Ten psychrotolerant rhizobacterial isolates were evaluated for their PGP traits at 10˚, 20° and 30 °C. Maximum P-solubilization was recorded by LR-5 (66.5, 60.6 and 52.4µg/ml at 10°, 20° and 30° respectively). The highest IAA producer was LR-5 (4.6, 10.8 and 14.8µg/ml at 10 °C, 20 °C and 30 °C respectively) followed by LR-3 (3.6, 7.5 and 12.4 µg/ml at 10 °C, 20 °C and 30 °C respectively). Three isolates LR-1, LR-3 and LR-5 confirmed the presence of ACCD activity.Under axenic conditions the highest root length was recorded in treatment comprising of *Rhizobium* (R)+P LR-57.05, 6.80 and 5.70cm) followed by R+LR-3 (5.60, 4.45 and 4.60cm) at 0.2, 0.4 and 0.6 MPa respectively. Similar pattern was seen in case of shoot length where, highest growth was shown by R+LR-5 (11.0, 8.4 and 6.8cm) followed by R+LR-3 (7.6, 5.8 and 4.9) at 0.2, 0.4 and 0.6 MPa respectively. In case of root and shoot fresh weight also R+LR-5 proved to be the most promising treatment.

Keywords: ACCD activity, IAA, p-solubilization, psychrotolerant

Introduction

The sessile nature of plants renders them susceptible to many environmental stresses and they have to adapt accordingly. Abiotic stresses that effect plant growth involve salinity, heavy metals, draught, flooding, temperature fluctuations, exposure to xenobiotics and ultra-violet radiation. Hence, roots develop defence against these stresses in the form of modification of root system architecture (RSA), therefore, it can be considered as one of the most important defense mechanisms of roots against stresses (Postma and Lynch, 2011) [16]. PGPRs having ACCD activity can bring about modifications in RSA by decreasing the level of stress ethylene by increasing the root surface area (Etesami *et al* 2015) under stress conditions (Glick, 2014) [3]. Reduction of ethylene gives a boost to the root system by increasing the plant access to water and nutrients from deeper layers of soil and, as a result, the plant survives for a longer duration under stress conditions (Glick, 2014; Lim and Kim, 2013) [3, 11]. ACC-deaminase activity has been reported in *Bacillus, Rhizobium, Agrobacterium genomovars, Burkholderia, Pseudomonas, Azospirillum* and *Enterobacter*. These inoculants lower the ethylene level by the virtue of their ACC deaminase activity that breaks ACC into α-ketobutyrate and NH_4^+ (Ali *et al* 2014) [1].

One of the major constraint of plant growth is low temperature which can be overcome by using PGPRs that are metabollicaly active even at low temperature conditions (Nabti *et al* 2010) [15]. Psychrotolerant PGPRs are widely dispersed in the agro-ecosystem and exhibit a variety of plant growth promoting traits. Lentil being a winter crop warrants the search for low temperature adapted microbial species (Mishra *et al* 2008) [14]. Psychrotolerant PGPRs are agronomically important as they are metabolically functional at low temperature conditions and ease mineral uptake by plants.Therefore, we made an attempt to isolate and characterize psychrotolerant rhizobacteria having ACC deaminase activity as a means to provide the best benefit to drought and temperature stressed plants.

Isolation of rhizobacteria from soil samples

Ten grams of soil samples from lentil rhizosphere were heat-treated (80 °C) and then transferred to 90 ml sterile distilled water. For thorough mixing, shaking was carried on a rotary shaker for 15 min. Serial dilution was done and 0.1 ml of the suspension was spread over on Nutrient agar (NA) and Kings B (King *et al* 1954) [9] plates in triplicates. The media was autoclaved at 15 psi and 121 °C for 20 minutes. The petri plates were incubated for 24-48h at 10 °C. Morphologically different colonies were isolated and subcultured. The isolates were further grown at 10°, 20° and 30 °C in respective media and growth in terms of optical density was recorded at 600 nm.

Characterization of phosphate solubilization by Rhizobacteria

Screening of Phosphate Solubilizing rhizobacteria by plate assay

Rhizobacterial cultures were spot inoculated on NBRIP (National Botanical Research Institutes Phosphate growth medium) and Pikovskaya's agar plates. The clearance zone was measured after 48-72 hrs incubation at 10°, 20° and 30 °C.

$$\text{Phosphate Solubilization Index} = \frac{\text{Total diameter (colony+ halo zone)}}{\text{Diameter of colony}}$$

Quantitative estimation of phosphate solubilization
Estimated by method given by Jackson (1973) [6].

Ammonium molybdate-Ammonium vanadate Reagent
22.5 g of ammonium molybdate was added to 400 ml of distilled water. Dissolved 1.25 g of ammonium vanadate in 250 ml of boiling water and then cooled to room temperature. The above two solutions were mixed followed by addition of 250 ml of concentrated nitric acid and then volume was made to 1 litre with distilled water. The total phosphorus solubilized was estimated using vandatomolybdate reagent. The culture broth was digested with 20 ml of triacid mixture, followed by volume make up 50 ml with D.W. To the digested broth 5 ml of the above reagent was added. The yellow colour that developed was read at 470 nm.

IAA Production
IAA production by rhizobacteria was measured by method given by Gordon and Weber (1951) [4]. 0.1 ml of overnight bacterial cultures were inoculated in test tubes containing Luria broth and incubated at 10°, 20° and 30 °C for 5days. Samples were withdrawn on 3rd and 5th day. After centrifugation at 10,000 rpm for 15 minutes, the supernatant was acidified with 2 drops of orthophosphoric acid. Then 2 ml of the Salkowski reagent was added to the supernatant and 25 minutes of incubation at room temperature was given that led to development of pink colour. The absorbance of the pink colour was recorded at 530 nm.

Reagents
Salkowski reagent: 1 ml of 0.4 M $FeCl_3$ in 50 ml of 35% perchloric acid and IAA stock solution: 100µg/ml

Plate assay for ACCD activity
Rhizobacteria grown in LB medium were centrifuged 8000 rpm/5min to collect cell mass. The pellet collected was washed thrice with D.W. and then spot inoculated on sterile DF salt minimal media supplemented with 3 mM ACC. Plates containing only DF minimal medium served as a negative control whereas plates with $(NH_4)_2SO_4$ supplementation served as positive control. The plates were then incubated at 10º, 20º and 30º C for 3-4 days. The isolates that exhibited ACCD activity were selected for quantitative test (Govindasamy *et al* 2009) [5].

Germination of lentil seeds under axenic conditions
Seed bacterization
Seeds of lentil variety LL-931were surface sterilized using 0.1% $HgCl_2$ (v/v) for 30s followed by three washings with 70% alcohol and then washed thoroughly with sterilized distilled water (Khalid *et al* 2004) [8]. Bacterial seed inoculation was done by dipping seeds in inoculum for 30 minutes.

Germination assay
Germination assay was carried in sterile petri plates having germination paper. Ten bacterial inoculated seeds were placed in each petri plate. To simulate drought stress -0.2,-0.4 and -0.6 MPa (Mega Pascals) water treatment was given. Another set of petri plates were treated with water to make a comparison. The petri plates were then incubated at 10±2 °C for 15 days. Three replications of each treatment were used. After 15 days shoot and root length, fresh weight and percent germination was measured.

Results
Five soil samples were obtained from different locations of Punjab during the winter season Isolation of 10 psychrotolerant rhizobacteria from the above soils samples was done at 10 °C. Isolated rhizobacteria were further checked for their growth promoting traits at low temperature.

P-solubilization potential of PGPR isolates
Out of 10 rhizobacterial isolates five rhizobacteria were found to solubilize P which was evident from the halo zone on Pikovskaya's agar plates. In the present study for comparative analysis of P-solubilization, it was found that the highest P-solubilization index was recorded at 30 °C by isolate LR-5 (6.2). As the temperature shifted to 20 °C the P-solubilization index decreased to 5.8 for LR-5 and with a further drop in temperature to 10 °C, the P-solubilization index decreased to 5.2 (Fig 1).

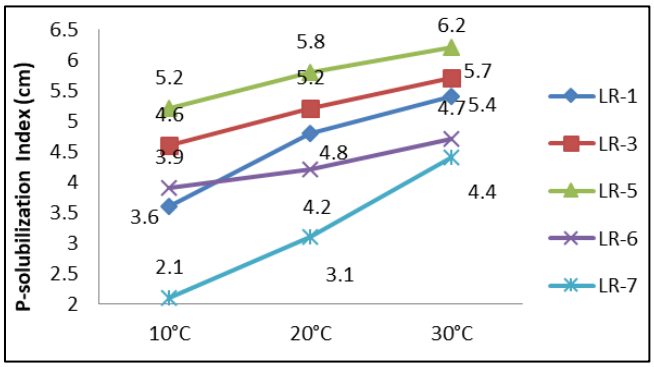

Fig 1: P-solubization index of PGPR isolates

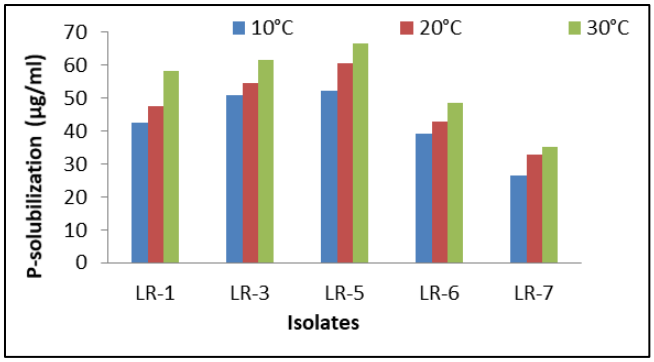

Fig 2: Quantitative P-solubization by PGP isolates

The most promising isolates for this trait at 30 °C were LR-5 (66.5µg/ml) followed by LR-3 (61.6µg/ml), However, at 20 °C a slight decrease in the solubilisation efficiency was observed in the potent isolate LR-5 (60.6µg/ml). The maximum P-solubilization recorded at 10 °C also was exhibited by LR-5 (52.4 µg/ml) followed by LR-3(50.8µg/ml). A comparison of potent P-solubilizers at different temperatures is shown in Fig 2. Although, a large reservoir of phosphorus exists in the soil, however, due to its insoluble nature it cannot support the plant growth. Therefore, PGPRs act as microfactories where the insoluble phosphorus changes to soluble form that is readily utilized by the plant. The incubation period and time duration of incubation has a critical role in mineral solubilisation. The P-solubilisation became evident after 48h and continued upto 12th days after incubation after which it became steady. The most potent P-solubilizer was

LR-5 followed by LR-3.The quantitative studies at 30 °C revealed that P-solubilization showed a gradually increasing trend from 3rd day upto 9th day of incubation after which a steady decrease was observed. This decrease can be attributed to nutrient exhaustion, toxic metabolite production in media or cell death. The potent P-solubilizers were LR-5 and LR-3.

Similarly, Singh and Khanna (2017) [19] worked on the functional attributes of rhizobacteria and reported a wheat rhizobacteria that was a potent P-solubilizser at 10° and 20 °C. Kaur *et al* (2015) [7] reported that psychrotolerant PGPRs exhibited P-solubilization range of 62.5-77.2 µg/ml at 15 °C.

IAA production by rhizobacteria

In the present study it was found that at 10 °C IAA production ranged from 1.6-4.6 µg/ml. At 20 °C IAA equivalents ranged between 3.9-10.8 µg/ml. As the temperature of inoculation was increased to a mesophilic range there was a futher increase in IAA production. At 30 °C, the IAA production range was found to 7.1-14.8 µg/ml after 5 days of inoculation (Fig 3). The highest IAA producer was LR-5 (4.6, 10.8 and 14.8µg/ml at 10℃, 20℃ and 30℃ respectively) followed by LR-3 (3.6, 7.5 and 12.4 µg/ml at 10℃, 20℃ and 30℃ respectively) after 5 days of inoculation in the presence of tryptophan. Our results are in line with Mishra *et al* (2011) [13] who worked on psychrotolerant *Pseudomonas* for cold allevation in wheat and reported that IAA production was in the range of 2.6-6.1 µg/ml at 4 °C and increased to 5.5-15.8 µg/ml at 28 °C. Kaur *et al* (2015) [7] reported that at 15 °C the IAA production by psychrotolerant isolate J-17 was 20.4µg/ml in the presence of tryptophan.

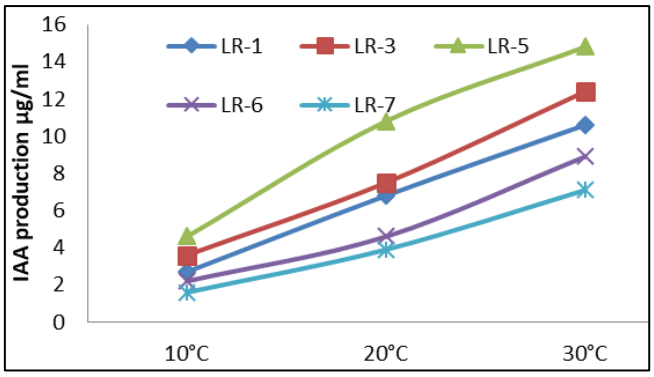

Fig 3: Comparitive analysis of IAA production by promising rhizobacteria at 10°, 20° and 30 °C

Determination of ACC-deaminase production by qualitative assay

Three isolates (LR-1, LR-3 and LR-5) showed growth on ACC and ammonium ammended plates however, no growth was recorded in DF plates due to absence of N source required for growth.

The efficacy of rhizobacteria for utilization of ACC as a N source recorded in terms of optical density (Fig 4) showed that at 30 °C the highest OD was recorded by LR-5 (0.484) followed by LR- 3 (0.456). However, the efficiency of rhizobacteria for ACCD activity differed and was highly temperature dependent. At 20° OD_{600} range was 0.287 to 0.441 that further decreased to 0.244 to 0.338 at 10 °C. It is quite obvious from the above ACC- study that N requirement of bacteria was fulfilled through the deamination of ACC. The most potent ACC-deaminase producer was LR-5.Similarly, Magnucka and Pietr (2015) [12] reported three fluorescent *Pseudomonas* isolates from wheat (PO283 and PO366) and rape (RZ310) rhizosphere that could effectively grow in DF media supplemented with ACC. Kumari and Khanna (2015) reported 29 rhizobacterial isolates that could effectively utilize ACC as N source with OD_{600} varying from 0.205-0.774.

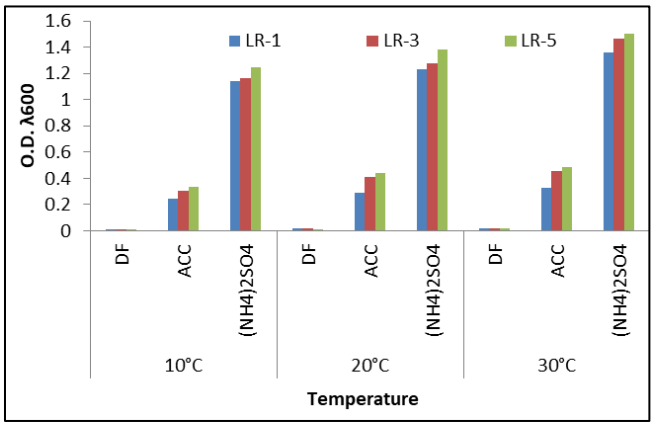

Fig 4: Efficacy of rhizobacteria in growth media with different N-sources under different temperature conditions

Effect of psychrotolerant rhizobacteria on growth of lentil under axenic conditions

Three promising isolates were tested for their effect on germination of lentil under axenic conditions.

The treatments were Control, *Rhizobium* (Recommended by PAU), *Rhizobium*+LR-1, *Rhizobium*+LR-3 and *Rhizobium*+LR-5.The highest root length was recorded by R+LR-5 (7.05, 6.80 and 5.70cm) followed by R+LR-3 (5.60, 4.45 and 4.60cm) at 0.2, 0.4 and 0.6 MPa respectively (Fig 5a).Similar pattern followed in case of shoot length where highest growth was shown by R+LR-5 (11.1, 8.4 and 6.8 cm) that was far higher compared to control (3.8, 3.1 and 2.1 cm) at 0.2, 0.4 and 0.6 MPa, respectively (Fig 5 b). In case of root fresh weight also R+LR-5 proved to be the most promising treatment (Table 1). This was closely followed by R-LR-3. Shoot fresh weight was also found to decrease with increasing drought stress as in case of control where severe drought conditions lead to weight downfall from 280.5mg (water) to 69.7mg (0.6 MPa). In germination assay coinoculation with LR-5 recorded highest growth parameters at all the stress levels followed by LR-3. Our results are in corroboration with Sharma *et al* (2013) who reported that at 0.4 MPa stress condition, the seed treatment of chickpea with *Rhizobium* and *Pseudomonas* outperformed uninoculated treatment. Saini and Khanna (2012) [17] reported that seed treatment of lentil with PGPR isolate resulted in a 66.6% increase in root length with root fresh weight of 178.7 mg/seedling as compared to control (105.5 mg/seedling).

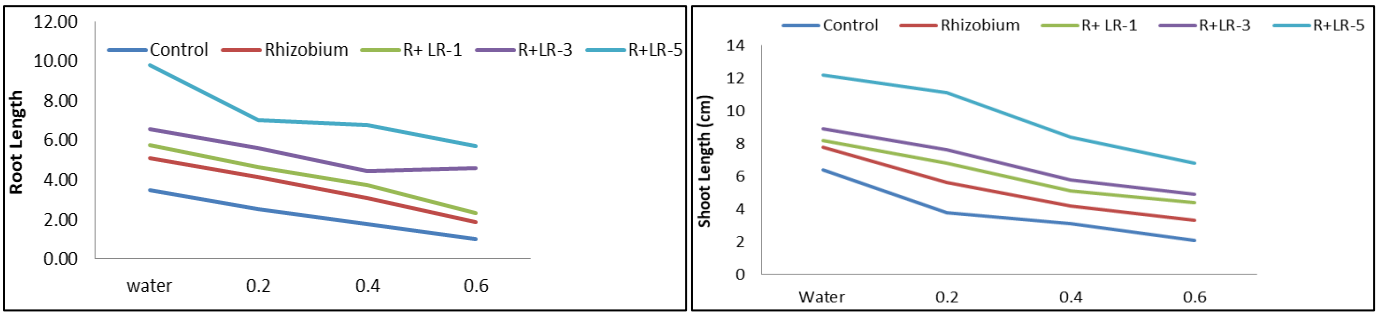

Fig 5: Growth parameters under axenic conditions (a) Root length (b) Shoot Length

Table 1: Root and shoot fresh weight of lentil grown under axenic conditions

Treatments	Root fresh weight (mg)				Shoot fresh weight (mg)			
	Water	0.2 Mpa	0.4 Mpa	0.6 Mpa	Water	0.2 Mpa	0.4 Mpa	0.6 Mpa
Control	100.2	71.6	41.5	19.8	280.5	207.3	119.5	69.7
Rhizobium	223.5	180.4	145.0	114.5	415.6	290.8	210.5	176.2
R+ LR-1	281.6	212.7	165.5	128.5	429.2	398.1	298.4	286.2
R+LR-3	300.7	225.0	187.1	138.0	428.2	381.2	337.1	290.0
R+LR-5	317.0	244.5	204.5	150.6	440.9	410.5	350.6	310.5
CD at 5%	42.5	28.2	34.8	15.8	23.3	23.5	16.1	23.9

Conclusion

The use of plant growth promoting rhizobacteria as bioinoculants is a novel approach to overcome draught stress. The PGPR co-inoculation with *Rhizobium* reported a significant increase in growth parameters of lentil. Our study authenticates the role of microorganisms in improving plant growth parameters.

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