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# Screening, Characterising and Selection of Efficient ACCD (1-Aminocyclopropane-1-Carboxylate Deaminase) Producing Bradyrhizobial Isolates for Nodulation in Soybean under Drought Stress Condition

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ABSTRACT

Drought is one of the most important abiotic stresses which have a huge impact on soybean production, especially in India as most of the soybean growing belts falls under rainfed condition. To overcome drought stress, researchers have so far applied wide range of drought mitigation strategies ranging from development of stress tolerant cultivars to application of modern genetic engineering tools. In the end there is always a limitation to these kinds of methods and here comes the role of drought tolerant PGPRs. Keeping in mind the multifarious roles of PGPRs, we isolated 24 Bradyrhizobial isolates and screened them for their ACCD producing ability and drought tolerance capacity, besides studying their morphological and biochemical characteristics. Among the 24 Bradyrhizobial isolates, 12 Bradyrhizobial isolates were found to produce ACCD. Further, these 12 isolates were tested for their drought tolerance capacity under different PEG concentrations, where four isolates (1R7, 1R8, 1R15 and 2B7) were able to tolerate -0.38 MPa (25% PEG) of water stress. Therefore, these four promising isolates can be used against drought stress for nodulating soybean.

*Keywords* : *Bradyrhizobium*, Drought, ACCD (1-Aminocyclopropane-1-carboxylate deaminase), PEG (Polyethylene glycol)

PLANTS are mostly subjected to biotic and abiotic stresses throughout their cropping period. However unlike biotic stresses, abiotic stresses are very difficult to control manually with drought stress being a major one among them. It is reported that drought is one of the most important natural factor affecting crop productivity globally (Wang *et al*., 2020). In the case of soybean, drought plays a major role in reduction of its production and productivity (Kunert *et al*., 2016). This situation can also be attributed to the fact that most of the Soybean growing areas fall under rainfed condition. Traditionally, the approach has been more concentrated on developing stress tolerant varieties, which has changed in course of time towards the use of PGPRs (Rama and Naik, 2017). PGPRs (Plant Growth Promoting Rhizobacteria) besides producing several other plant growth promoting compounds, also produce ACCD (1 aminocyclopropane-1-carboxylate deaminase) enzyme which proves to be quite essential in regulating the ethylene level of plants under drought stress (Gurumurthy and Shivaprakash, 2017).

Soybean is nodulated by diverse groups of rhizo bacteria (*Bradyrhizobium* sp., *Sinorhizobium fredii* and *Mesorhizobium tianshanense*). However, *Bradyrhizobium* is the most dominant genus with *B. japonicum* and *B. elkanii* being the two most reported species (Hafiz *et al*., 2021). Broadly we can classify soybean nodulating rhizobia into two groups: fast growers (*Sinorhizobium fredii* ) and slow growers (*Bradyrhizobium* sp.). For better understanding their cellular metabolism, it is needed to characterise them and the easiest techniques are through biochemical methods (Bromothymol blue test and Carbohydrate metabolism). *Bradyrhizobium* besides being a symbiont which fixes atmospheric nitrogen in soybean also harbours several plant growth promoting properties (Swarnalakshmi *et al*., 2020). However when it comes to drought stress, ACCD

enzyme produced by this bacterium plays a crucial role in regulating ethylene level and thus is helps in increasing the productivity of soybean (Murset *et al*., 2012). Utilisation of drought tolerant, ACCD producing Bradyrhizobial isolates is one of the most effective strategies for raising the productivity of soybean under rainfed condition. Therefore, the main objective of this study was to isolate, characterise and screen efficient ACCD producing Bradyrhizobial isolates tolerating drought stress.

## Material and Methods

### Collection of Root Nodules from Soybean Plants

Soybean root samples were collected from fields of UAS Bengaluru and UAS Raichur campuses. From the fields, plants were uprooted by digging upto 20cm along with their nodules and soil intact. These plants along with their soil clump were carefully placed inside polythene bags and brought to laboratory. Roots were carefully washed under gentle stream of water and nodules were carefully removed from the roots along with some root tissue without damaging the nodules using a sterile scalpel just before isolation of rhizobia.

### Isolation of Bradyrhizobial Isolates

Bradyrhizobial isolates were isolated from soybean root nodules by using the method of Somasegaran and Hoben (1994). From each sample, five healthy nodules were picked up and washed thoroughly with sterile distilled water. After washing, nodules were surface sterilized using 70 per cent alcohol for 30 sec to remove wax coating if any and were subsequently immersed in 4 per cent (w/vol.) sodium hypochlorite for 3 min followed by immediate washing (5 times) with sterile distilled water to remove traces of sodium hypochlorite. The surface sterilized nodules were then transferred to sterile tubes containing 100 µl of sterile distilled water. Nodules were crushed using a sterile glass rod and a loopful of milky suspension was streaked on Congo red yeast extract mannitol agar media (CRYEMA) after which plates were incubated at 28°C in dark condition until growth appears. Single discrete colonies were picked up and re-streaked on CRYEMA media until pure colonies were obtained.

### Screening of Bradyrhizobial Isolates based on their Biochemical Properties

### Bromothymol Blue (BTB) Agar Test

Yeast extract mannitol agar supplemented with Bromothymol blue (BTB) was used as a medium for differentiating *Bradyrhizobium* sp. from *Rhizobium* sp. BTB agar was made by adding 5 ml of 0.5 per cent BTB in ethanol (0.5g/100ml) to 1 liter of YEMA medium (Somasegaran and Hoben, 1994). The pH of the medium was adjusted to 6.8 till green colour appears. Cultures were streaked on BTB agar plates (green colour) and incubated at 28°C in dark condition until colour change was observed.

### Hofer's Alkaline Broth Test

Hofer's alkaline broth was prepared and its pH was adjusted to 11. Five mL of this broth was taken in 15 ml capacity test tubes and sterilized at 121°C for 15 min. The test isolates were inoculated in broth tubes and incubated at 28°C for 7-10 days. Tubes were observed for the presence or absence of the growth in the broth and observations were recorded (Hofer, 1935).

### Glucose Peptone Agar Test

Test isolates were streaked on freshly prepared Glucose-Peptone agar plates added with Bromocresol purple and incubated at 28°C for 7-10 days. Change in pH and presence or absence of growth was recorded to determine the identity of the isolates (Vincent, 1970).

### Ketolactose Test

Test isolates were streaked on lactose medium in the centre and incubated for 7-10 days at 28°C. Then 5 mL of Benedict's reagent was poured in each Petri plate and kept at room temperature for 1-2 h. The plates were observed for the formation of yellow ring around the colonies indicating the ability of the organism to convert lactose to 3-ketolactose (Bernaerts and Deley, 1963).

**Qualitative Estimation of ACCD Activity of Bradyrhizobial Isolates**

The Bradyrhizobial isolates were inoculated to Dworkin-Foster salts minimal media, with ammonium sulphate as the nitrogen source and was incubated in a shaker at 200 rpm at 28°C. One mL aliquot of the culture was transferred to 50 mL sterile DF salts minimal medium in a 250 ml flask containing 3 mM ACC (instead of $(NH_4)_2 SO_4$) as the source of nitrogen. A 0.5 M solution of ACC was filter-sterilized through a 0.2 mm membrane and the filtrate was collected, aliquoted and frozen at -20°C. Just prior to inoculation, the ACC solution was thawed and 300 µl of aliquot was added to 50 mL sterile DF salts minimal medium; following inoculation, the cultures were placed in a shaker at 200 rpm and incubated at 28°C. Just before inoculation on DF-ACC agar, the plates were spread with ACC (30 µM per plate) and was allowed to dry fully. The final culture in DF-ACC broth were spot inoculated onto solid DF salts minimal agar medium and was incubated at $28^0$C for 7-10 days. Isolates that grow on DF-ACC media were scored as positive for ACCD activity (Dworkin and Foster, 1958).

**Morphological and Biochemical Characterization of ACCD producing Bradyrhizobial Isolates**

All the ACCD producing Bradyrhizobial isolates were subjected to morphological (colony morphology-form, elevation, margin and colour) and biochemical characterization tests.

**Gram's Staining**

Gram reaction was performed for all the isolates and the stained preparations were observed under the microscope through oil immersion.

**Casein Hydrolysis Test**

Casein hydrolysis was performed on Skim milk agar and the zone formation around the colonies was observed (Deshmukh *et al.,* 2013).

**Catalase Test**

Pure cultures of test isolates were placed on a cleansed glass slide to which a drop of 3 per cent hydrogen peroxide was poured and observed for gas bubbles (Blazevic and Ederer, 1975).

**Citrate Utilization Test**

To the Simmon's citrate agar slants, pure cultures of the test isolates were streaked and incubated at $28^o$C for 7 days. The colour change from green to blue was taken as positive for the test (Baron and Finegold, 1990).

**Starch Hydrolysis Test**

Pure cultures of test isolates were streaked on the plates containing starch agar medium and incubated at 28°C for 7 days. Plates were observed for the formation of a clear hallow around the line of growth after flooding with few drops of iodine for 10-15 minutes (Macfaddin, 2000).

**Urease Test**

Pure cultures of test isolates were inoculated into the sterilized urea broth and incubated for 7 days at 28°C. Change in the colour of the broth from orange to pink was taken as positive for the test (Delost, 1997).

***In vitro* Screening for Drought Stress Tolerance of Bradyrhizobial Isolates**

For assessing the drought stress tolerance of the isolates, a known quantity of yeast extract mannitol broth (YEMB) was dispensed at the rate of 30 mL in 100 mL conical flasks and amended with different concentration of polyethylene glycol (PEG) 6000 (0, 5, 10, 15, 20, 25%). Fresh cultures of twelve test isolates grown in the YEM broth on a shaker incubator was used as initial inoculum to the PEG amended medium. After incubation at 28 $^o$C under shaking conditions (120 rpm) for 7 days, growth of these isolates was estimated by measuring the optical density (OD) at 600 nm in a spectrophoto meter (Thermo Scientific™ BioMate 3S UV-Visible spectrophotometer) with uninoculated medium as blank.

The experiment was carried out in Completely Randomized Design in triplicates for each level of PEG 6000 for all the twelve isolates. The Osmotic Pressure (OP) of PEG 6000 solutions was calculated using the given formula (Michel and Kaufmann, 1973).

$$OP = -(1.18 \times 10^{-2}) \times C - (1.18 \times 10^{-4}) \times C + (2.67 \times 10^{-4}) \times C \times T + (8.39 \times 10^{-7}) \times C^2T$$

Where,

C=PEG Concentration (%), T=Temperature ($^0$C)

## Results and Discussion

A total of 120 rhizobial isolates were obtained from soybean root nodules, out of which 90 isolates were from nodules collected from fields of UAS-B campus (coded as 2B and 3B) and 30 isolates were obtained from nodules collected from UAS-Raichur campus (coded as 1R). After screening all these isolates in BTB agar, it was found that 80 per cent of these isolates were found to be fast growing *i.e.* they turned the colour of BTB agar plates from green to yellow (Fig. 1 (a). This was due to the fact that fast growing rhizobial isolates produces an acidic reaction and thus changes the colour of media to yellow (Sharma *et al*., 2010). However, it was quite surprising to obtain such high number of fast growing rhizobial isolates. One of the reasons could be that soybean can be nodulated by wide range of rhizobacteria (*i.e. Bradyrhizobium* sp., *Sinorhizobium fredii* and *Mesorhizobium tianshanense*) out of which *Sinorhizobium fredii* is a fast grower (Hafiz *et al*., 2021). Moreover, recently researchers from India have reported the abundance of fast growing native rhizobial isolates instead of slow growing ones from soybean nodules (Sharma *et al*., 2010; Ansari and Rao, 2014).


Fig. 1 : (a) Yellow colour production by fast growing rhizobial isolates on BTB plate due to acidic reaction

The remaining 24 isolates produced blue colour on BTB agar plates due to production of alkaline reaction (Fig. 1(b)). BTB agar test is one among the simplest and widely used method for distinguishing slow growers from that of fast growing rhizobacteria and have been previously used by many researchers for screening of Bradyrhizobial isolates (Shahzad *et al*. 2012; Deshmukh *et al*., 2013).


Fig 1: (b) Blue colour production by slow growing Bradyrhizobial isolates on BTB plate due to alkaline reaction

Further, all the 24 isolates were subjected to different biochemical tests to confirm their identity. Absence of growth in Hofer's alkaline and Glucose peptone agar confirms that *Agro bacterium*, which often is a major contaminant, is not present along with Bradyrhizobial isolates. *Bradyrhizobium* cannot grow at higher alkaline pH (above 10) while *Agrobacterium*, a common contaminant, grows well. Similarly, none of the 24 Bradyrhizobial isolates showed growth on glucose peptone agar with a neutral or alkaline reaction, while *Agro bacterium* sp. can grow very



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fast on this medium. Further conformity of Bradyrhizobial isolates was assured by performing ketolactose agar test in which none of the isolates produced a yellow ring precipitate of cuprous oxide ($Cu_2O$) around the colonies of bacterial plates when flooded with Benedict's reagent as they were negative for the production of 3-ketolactose.

Screening of all 24 Bradyrhizobial isolates was done for production of ACCD enzyme on DF media with ACC as sole nitrogen source. Out of 24 isolates, 12 isolates grew well on the DF media as compared to rest other isolates; thus confirming the production of ACCD enzyme (Fig 2). ACCD enzyme is produced by several PGPRs, which

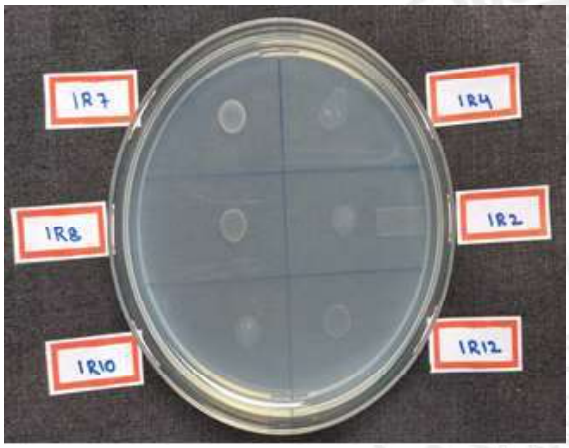


Fig. 2 : Bradyrhizobial isolates inoculated on DF media with ACC : Isolates 1R7, 1R8, 1R10 and 1R12 showed good growth as compared to isolates 1R4 and 1R2

helps the plant in alleviating drought stress. The mechanism present in these PGPRs is their ability to cleave ACC to a-ketobutyrate and ammonia. Since ACC is degraded which being the precursor of ethylene and produced in larger concentrations during drought stress, protects the plant from the harmful effects of ethylene (Saikia *et al*., 2018). The ability of *Bradyrhizobium* to able to synthesise ACCD enzyme attributes to the presence of *acd*S gene (Singh *et al*., 2015). Presence of *acd*S gene in *Bradyrhizobium* was previously reported by many groups of workers, which reaffirms our result (Murset *et al*., 2012; Nascimento *et al.,* 2018).

The morphological features of ACC producing Bradyrhizobial isolates were observed and gram staining was performed to study its gram's reaction and shape (Table 1).

TABLE 1

Morphological characteristics of ACCD producing Bradyrhizobial isolates

| Morphological Characters | Description |
|---|---|
| Cell shape | All were Rod-shaped |
| Colony Size (after 7 days of growth) | < 1mm |
| Colony colour and nature | Creamy white and translucent, glistening and slimy |
| Elevation and Margin | Convex and Circular |
| Gram's reaction | Negative |

All the ACCD positive isolates were then subjected to different biochemical tests for understanding their metabolism (Table 2). It was found that all 12 isolates were positive for catalase and urease tests, while they were found negative for citrate, starch and casein hydrolysis. All the results obtained are in favour with Bergey's Manual of Systematic Bacteriology.

TABLE 2

Biochemical characteristics of ACCD producing Bradyrhizobial isolates

| Isolates | Starch hydrolysis | Casein hydrolysis | Urease activity | Citrate utilization | Catalase activity |
|---|---|---|---|---|---|
| Control | - | - | - | - | - |
| 1R7 | - | - | + | - | + |
| 1R8 | - | - | + | - | + |
| 1R10 | - | - | + | - | + |
| 1R12 | - | - | + | - | + |
| 1R15 | - | - | + | - | + |
| 1R17 | - | - | + | - | + |
| 2R5 | - | - | + | - | + |
| 2R7 | - | - | + | - | + |
| 2R8 | - | - | + | - | + |
| 2R9 | - | - | + | - | + |
| 2R11 | - | - | + | - | + |
| 3B3 | - | - | + | - | + |

Note : (+) Positive, (-) Negative

TABLE 3

Growth of ACCD positive *Bradyrhizobial* isolates as influenced by different concentrations of PEG 6000

| Isolates | 0% | 5% (-0.03 MPa) | 10% (-0.08 MPa) | 15% (-0.16 MPa) | 20% (-0.26 MPa) | 25% (-0.38 MPa) |
|---|---|---|---|---|---|---|
| 1R7 | 0.87 ab | 0.76 abc | 0.56 abc | 0.38 bc | 0.26 ab | 0.18 ab |
| 1R8 | 0.62 cde | 0.71 bc | 0.52 bcd | 0.33 bcd | 0.24 abc | 0.11 b |
| 1R10 | 0.55 de | 0.53 cd | 0.37 de | 0.21 d | 0.11 de | 0 c |
| 1R12 | 0.53 e | 0.46 d | 0.26 e | 0.17 d | 0 e | 0 c |
| 1R15 | 1.02 a | 0.98 a | 0.73 a | 0.54 a | 0.33 a | 0.21 a |
| 1R17 | 0.57 de | 0.46 d | 0.27 e | 0.18 d | 0 e | 0 c |
| 2R5 | 0.85 abc | 0.73 bc | 0.52 bcd | 0.37 bc | 0.17 bcd | 0 c |
| 2R7 | 0.9 ab | 0.85 ab | 0.68 ab | 0.42 ab | 0.26 ab | 0.12 b |
| 2R8 | 0.63 cde | 0.56 cd | 0.37 de | 0.21 d | 0.1 de | 0 c |
| 2R9 | 0.72 bcde | 0.69 bcd | 0.49 cd | 0.29 bcd | 0.13 cd | 0 c |
| 2R11 | 0.62 cde | 0.47 d | 0.28 e | 0.18 d | 0 e | 0 c |
| 3B3 | 0.78 bcd | 0.55 cd | 0.36 de | 0.26 cd | 0.12 de | 0 c |
| SEm± | 0.016 | 0.016 | 0.010 | 0.007 | 0.004 | 0.002 |
| CD <0.05) | 0.047 | 0.042 | 0.032 | 0.020 | 0.012 | 0.006 |

*Note*: Means with same superscript in a column do not differ significantly as per Duncan Multiple Range Test (DMRT). Values represented are mean ± SE (n=5)

For testing the efficiency of ACCD producing Bradyrhizobial isolates under drought condition, they were grown on YEMB medium with PEG (polyethylene glycol) to artificially induce water stress (Table 3). PEG is a high molecular weight compound which depicts water stress condition by preventing water uptake into bacterial cells (Pavli *et al*., 2020). It was observed that most of the isolates were able to tolerate 15 per cent PEG, while the growth of rest of the isolates decreased at 20 per cent. The ability of isolates to tolerate 20 per cent PEG concentration can be attributed to the fact that *Bradyrhizobium* is a slow growing bacterium. Moreover, osmotic stress has a negative impact on rhizobia and alters its morphology, causing cell dehydration (Niste *et al*., 2013). When the stress level was increased upto 25 *Bradyrhizo bium* PEG, only four isolates 1R7, 1R8, 1R15 and 2R7 were able to survive. Among them, 1R15 was the most promising Bradyrhizobial isolate which showed significant growth at -0.38 MPa (25% PEG) of water stress showing an OD value of 0.21. Ability of the above four isolates to survive at 25 *Bradyrhizobium* PEG concentration can be related to their inherent drought tolerant mechanisms like production of exopolysaccharides, osmolytes and small heat shock proteins (Gopalakrishnan *et al*., 2015). Swaine *et al*. (2005) tested the efficiency of *Bradyrhizobium elkanii* isolates under water stressed condition and found that most of these isolates could achieve a maximum growth of 0.2-0.1 OD at -0.4 Mpa, above which there was no significant growth recorded in these isolates. Further, to support our data, similar results were obtained by Abdel-Lateif *et al*. (2016) who found that most of the rhizobial isolates were not able to tolerate 20 per cent PEG. Due to the drought tolerance ability of the above four isolates at 25 per cent PEG, they can used for nodulating soybean to mitigate drought stress.

Several strategies have been explored and applied before to increase the productivity of soybean but the introduction of drought tolerant Bradyrhizobial isolates is one among the most promising method to increase its productivity. By screening over 24 Bradyrhizobial isolates, we found four ACCD

producing isolates (1R7, 1R8, 1R15 and 2R7) having drought tolerance capability at 25 per cent PEG concentration which can be effectively used for nodulating soybean to overcome drought stress.

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