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EVALUATION OF RHIZOBIAL STRAINS FOR ABIOTIC STRESS TOLERANCE IN PIGEON PEA FROM ARID AND SEMI-ARID ZONES OF HARYANA, INDIA

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KEYWORDS

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ABSTRACT

In the present investigation, 196 Pigeon pea [*Cajanus cajan* (L.) Millspaugh] rhizobial isolates were obtained from 84 soil samples collected from South-Western Haryana using trap plant method. All the rhizobial isolates were able to grow at 30, 35 and 40ºC temperatures. However, only 44 rhizobial isolates were able to grow at 45ºC, out of which some showed good, some moderate and few showed poor growth. Most of rhizobial isolates were able to grow upto 20% polyethylene glycol (PEG 6000) concentration. However, with increase in PEG concentration only 71 and 3 rhizobial isolates were able to grow at 30 and 40% PEG concentration, respectively. On the basis of combined stress tolerance when 49 pigeon pea rhizobial isolates were analysed, only 18 showed growth at 30% PEG concentration while they were reduced to 5 when concentration of PEG was increased from 30 to 40% at 40ºC temperature. With 20% PEG concentration at 45ºC, 5 isolates showed growth and only 4 isolates when temperature was reduced to 40ºC at same PEG concentration. However, 4 and 17 rhizobial isolates were able to grow at 30% PEG concentration at 45ºC and 20% PEG concentration at 40ºC, respectively.

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INTRODUCTION

Pulses are the most favoured crops in semi-arid tropics on account of their less input requirement, intrinsic capacity of biological nitrogen fixation, less water requirement deep rooting-system and high temperature tolerance (Singh *et al.*, 2015). Pigeon pea [*Cajanus cajan* (L.) Millspaugh] commonly known as toor dal or arhar dal is a perennial member of the family Fabaceae. It is an important legume mainly grown as hardy and drought tolerant crop. Environmental stress is a major component of natural selection in soil ecosystem. Stresses both abiotic and biotic, are major constraints to agricultural production. Under stressed environment, plant growth is affected by a number of factors such as physiological disorders, susceptibility to diseases, hormonal and nutritional imbalance, ion toxicity, etc. (Nadeem *et al.*, 2014; Kachhap *et al.*, 2015). Among several environmental conditions, the most prominent problematic abiotic factors in the arid and semi-arid regions are drought and high temperature. For successful functioning of microbial bioinoculants and their influence on plant growth and soil health, exhaustive efforts have been made to expose microbial diversity, their distribution and behaviour. Pigeon pea is a diploid (2n = 22) belonging to the *Cajaninae* subtribe of the tribe *Phaseoleae* (Young *et al.,* 2003). Since, it is principal green manuring crop supplying upto 40 kg nitrogen per hectare besides possessing the capability to fix large amount of nitrogen (N) in a process called biological nitrogen fixation. Therefore, it plays a crucial role in enrichment of the soil (Kuykendall *et al.,* 2000; Domoguen *et al.,* 2010). The biological nitrogen fixation by legume rhizobia symbiosis system is one of the wonders in nature and is important for sustenance of agricultural productivity. The legume nodulating bacteria, commonly called as rhizobia, form a group of soil bacteria belonging to α-subclass of the Proteobacteria (Young and Haukka, 1996). Pigeon pea is commonly nodulated by the cowpea miscellany group of rhizobia, which are indigenous in tropical soils (Troedson *et al.,* 1990).

In biological nitrogen fixation, various environmental conditions are restraining factors to the growth and activity of the nitrogen-fixing plants. Generally, the problematic environs for rhizobia are marginal lands with low rainfall, extremes of temperature and poor water-holding capacity (Bottomley, 1991). In arid and semiarid regions the most frequent stress affecting plant growth is water deficit. Therefore, it is imperative to enhance the level of effectiveness in plants to capture and usage of water and nutrients. In these regions, the inoculation of indigenous beneficial microorganisms may improve drought tolerance of plants (Marulanda *et al.*, 2007, 2009). Biological nitrogen fixation by legumes in tropical areas is profoundly influenced by the high soil temperatures (Micheals *et al.*, 1994). The microbial population is influenced by the cropping pattern and management practices like fertilization, crop rotation and addition of organic amendments. However, under rainfed conditions the crop production relies on microbial resources for nutrients (Gera *et al.*, 2012). For maintaining the functionality of the symbiosis the bacteria need to have better survival ability even under unfavourable ecological conditions. In the soil, the rhizobia often confront varied stresses that disturb their growth, preliminary steps in symbiosis and the capacity of nitrogen fixation (Zahran, 1999;

Ali *et al.*, 2009). Populations of *Rhizobium* and *Bradyrhizobium* species differ in their tolerance to main environmental stresses, therefore, screening for tolerant strains is imperative for decreasing soil loss and ameliorating adverse edaphic conditions. Moreover, rhizobia show several plant growth promoting activities as they are the important members of growth promoting rhizobacteria (Glick *et al.,* 1994).

The study was undertaken in the south-western parts of Haryana because this region is having water scarcity, low rainfall and high temperature. Moreover, the legumes occupy a major position in the cropping system of the region. Keeping in view the aforementioned facts, the present study was attempted to evaluate the rhizobial strains for abiotic stress tolerance.

MATERIALS AND METHODS

Isolation of native rhizobia nodulating pigeon pea using trap plant method

Seeds of pigeon pea (*Cajanus cajan* L.) were grown in pots, each containing 2 kg soil samples collected from South-Western Haryana to trap the rhizobia nodulating pigeon pea. After 45 days of growth when proper nodule formation took place the healthy pink nodules were removed separately from each host plant and were surface sterilized by using 0.1% $HgCl_2$ and 70% ethanol. After that the nodules were washed (5-6 times) with sterilized distilled water and crushed. A loopful of nodule sap was streaked on YEMA plates containing congo a red dye. The plates were incubated at 30ºC and growth was observed daily for 3-7 days. The rhizobial isolates were picked up from the plates and were restreaked for purification. Single rhizobial pure isolates were picked up from the plates and maintained on YEMA slants. The slants were stored at 4ºC in a refrigerator for further studies.

Characterization of rhizobia by Gram's staining and Peptone water tests

All the 196 pigeon pea rhizobial isolates obtained from nodules were characterized for Gram staining and peptone water test to check the authenticity of rhizobia. The isolates were inoculated individually in different peptone water containing tubes and incubated at 30ºC for 3-4 days.

Drought tolerance of pigeon pea rhizobial isolates

YEM broth supplemented with 0, 10, 20, 30, 40 and 50% polyethylene glycol (PEG 6000) concentration was prepared. Log phase grown culture was inoculated in YEM broth supplemented with different concentration of polyethylene glycol (PEG 6000). The broths were incubated at 30ºC for 4 days and growth was measured spectrophotometrically by taking absorbance at 600 nm.

Temperature tolerance of pigeon pea rhizobial isolates

YEMA medium plates containing Congo red were prepared. Log phase grown culture was spotted on YEMA medium plates. These plates were incubated at 30, 35, 40 and 45 degree temperature. Growth was observed daily for 4-7 days. A good amount of visible growth on the plates indicates tolerance at that particular temperature.

Combined stress (drought and temperature) tolerance of pigeon pea rhizobial isolates

The YEM broth supplemented with 20, 30 and 40% polyethylene glycol (PEG 6000) concentration were prepared. Log phase grown culture was inoculated in YEM broth supplemented with different concentration of polyethylene glycol (PEG 6000). The broth were incubated at 40 or 45ºC depending upon the highest PEG concentration and temperature tolerance of the individual isolate during single stress. The growth was measured spectrophotometrically by taking absorbance at 600 nm after 4 days of incubation.

RESULTS AND DISCUSSION

Nitrogen fixing bacteria (free-living and symbiotic) improve the soil fertility by supplying fixed nitrogen to the plants. Their population is dependent on the host plants, soil chemical parameters and management practices. Agricultural crops have created highly selective and homogenous environments that determine the bacterial diversity. In the present investigation because of poor nodulation in the fields, a total of 84 soil samples were collected from South-Western Haryana. These soil samples were collected from pigeon pea fields of Hisar, Bhiwani, Mahendergarh and Rewari districts.

Physico-chemical properties of soil samples collected from semi-arid zones of Haryana

It is necessary to check the chemical properties of the soil samples as they affect the growth and nutrient uptake of the plants. These soil samples were analyzed for pH, EC, organic C and available N. Soil pH ranged from 6.7-8.5 while EC was in the range of 0.07-0.67 d Sm^{-1}. The organic C and available N were in the range of 0.15-0.67% and 80-145 kg ha^{-1}, respectively (Table 1). For above purpose, soil and nodule samples were tried to be collected from pigeon pea field from different villages of Hisar, Bhiwani, Mahendergarh, and Rewari districts of Haryana State because of the poor nodulation in fields due to environmental conditions like high temperature, water stress and other factors dependent on the host plant and the invading microsymbiont *Rhizobium*, there was very poor nodulation in the field crops. Therefore, soil samples were collected to trap pigeon pea rhizobia under pot house conditions. A total of 84 soil samples were collected from the

Table 1: Physico-chemical properties of the study area

District	pH	EC (d Sm^{-1})	OC (%)	N (kg ha^{-1})
Hisar	6.7-7.5	0.07-0.65(0.36)	0.33-0.48(0.39)	84-140(115)
Bhiwani	6.8-8.5	0.10-0.67(0.30)	0.15-0.40(0.28)	80-112(90)
Mahendergarh	6.8-8.5	0.08-0.66(0.33)	0.18-0.67(0.38)	82-145(111)
Rewari	6.9-7.8	0.08-0.59(0.31)	0.24-0.63(0.46)	82-133(106)
Overall range	6.7-8.5	0.07-0.67	0.15-0.67	80-145
Overall mean	-	0.32	0.35	103

Table 2: Number of pigeon pea rhizobial isolates obtained from different districts along with their isolate number

Sr. No.	District	Rhizobial Isolates number	No. of isolates
1.	Hisar	PPH-1A, PPH-1B, PPH-2A, PPH-2B, PPH-3A, PPH-3B, PPH-4A, PPH-4B, PPH-4C, PPH-4D, PPH-4E, PPH-5A, PPH-5B, PPH-6A, PPH-6B, PPH-7, PPH-8A, PPH-8B, PPH-8C, PPH-8D, PPH-8E, PPH-9A, PPH-9B, PPH-9C, PPH-10A, PPH-10B, PPH-10C, PPH-10D	28
2.	Bhiwani	PPB-1, PPB-2A, PPB-2B, PPB-3, PPB-4, PPB-5, PPB-6, PPB-7, PPB-7A, PPB-8, PPB-8A, PPB-8B, PPB-9A, PPB-9B, PPB-10, PPB-11, PPB-11A, PPB-12, PPB-13, PPB-14, PPB-15A, PPB-15B, PPB-15C, PPB-16, PPB-17, PPB-18A, PPB-18B, PPB-18C, PPB-19A, PPB-19B, PPB-20, PPB-21A, PPB-21B, PPB-22A, PPB-22B, PPB-22C, PPB-22D, PPB-23A, PPB-23B, PPB-24A, PPB-24B, PPB-25A, PPB-25B, PPB-25C, PPB-26A, PPB-26B, PPB-27A, PPB-27B, PPB-28, PPB-29A, PPB-29B, PPB-29C, PPB-30A, PPB-30B, PPB-30C, PPB-30D, PPB-31, PPB-32A, PPB-32B, PPB-32C, PPB-33A, PPB-33B, PPB-33C, PPB-34A, PPB-34B, PPB-34C, PPB-34D, PPB-35A, PPB-35B, PPB-35C, PPB-36A, PPB-36B, PPB-36C, PPB-36D, PPB-37A, PPB-37B, PPB-37C, PPB-38A , PPB-38B	79
3.	Mahendergarh	PPM-I, PPM-2, PPM-2A, PPM-2B, PPM-3, PPM-4, PPM-5, PPM-6, PPM-7, PPM-8, PPM9, PPM-10, PPM-11, PPM-11A, PPM-12, PPM-13, PPM-13A, PPM-14, PPM-15, PPM-15A, PPM-16, PPM-16A, PPM-17, PPM-18, PPM-19, PPM-20, PPM-21A, PPM-21B, PPM-21C, PPM-22A, PPM-22B, PPM-23A, PPM-23B, PPM-24, PPM-25A, PPM-25B, PPM-25C, PPM-26A, PPM-26B, PPM-26C, PPM-27, PPM-28, PPM-29A, PPM-29B, PPM-30A, PPM-30B, PPM-31A, PPM-31B, PPM-32A, PPM-32B, PPM-33A, PPM-33B, PPM-33C, PPM-34A, PPM-34B, PPM-34C, PPM-34D, PPM-35A, PPM-35B, PPM-36A, PPM-36B, PPM-37A, PPM-37B, PPM-37C, PPM-37D, PPM-38A, PPM-38B, PPM-38C, PPM-39, PPM-40, PPM-41, PPM-42, PPM-43	73
4.	Rewari	PPR-1A, PPR-1B, PPR-1C, PPR-2, PPR-3A, PPR-3B, PPR-4A, PPR-4B, PPR-5A, PPR-5B, PPR-5C, PPR-6A, PPR-6B, PPR-7A, PPR-7B, PPR-7C	16
TOTAL			196

(PP = Pigeon pea, H = Hisar, B = Bhiwani, M = Mahendergarh, R = Rewari)

Figure 1: Isolation of pure pigeon pea rhizobial isolates using trap plant method on YEMA media

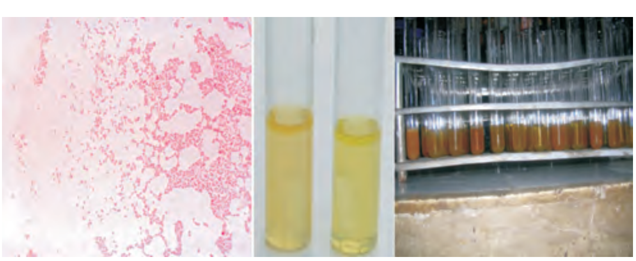

Figure 2: Morphological characterization of pigeon pea rhizobial isolates using Gram's staining and Peptone water test

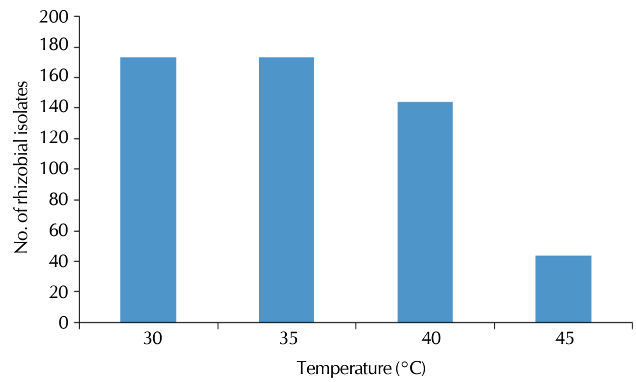

Figure 3: Temperature tolerance by pigeon pea rhizobial isolates

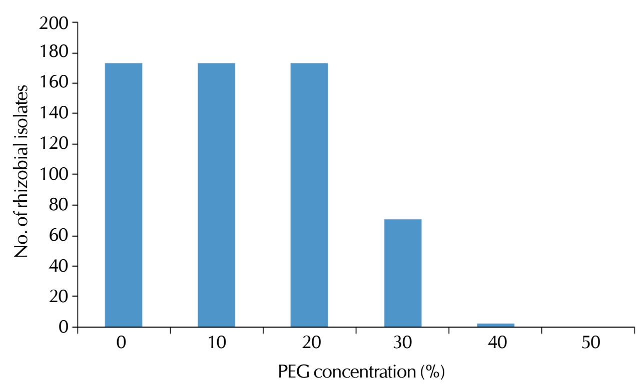

Figure 4: Drought tolerance by pigeon pea rhizobial isolates

rhizosphere of pigeon pea crops from 4 districts of Haryana.

Isolation of rhizobia nodulating pigeon pea using trap plants

The seeds of pigeon pea (Paras variety) were sown in 84 pots containing 2 kg of each soil sample and each pot was containing 3 or 4 pigeon pea plants. Out of these, the nodule formation was observed only in 82 pots. After 45 days of growth, when nodule formation took place on the roots of pigeon pea plants, 2 or 3 healthy pink nodules were collected from each plant and surface sterilized by using 0.1% $HgCl_2$

Table 3: Growth in terms of absorbance at 600 nm of pigeon pea rhizobial isolates supplemented with different concentration of polyethylene glycol (PEG 6000) at different temperature

Sr. No.	Isolates No.	40°C temperature + different concentration (%)			45°C temperature + different concentration (%)		
		40	30	20	40	30	20
1	PPH-1B	ND	0.047	0.107	ND	ND	ND
2	PPH-2A	ND	0.80	0.135	ND	0.017	ND
3	PPH-2B	0.067	0.110	ND	ND	0.068	ND
4	PPH-4A	ND	0.025	0.075	ND	ND	ND
5	PPH-5A	ND	0.032	0.078	ND	ND	ND
6	PPH-8A	0.054	0.118	ND	ND	0.047	ND
7	PPH-8C	0.115	0.156	ND	0.015	0.065	ND
8	PPH-8E	0.087	0.150	ND	ND	0.075	ND
9	PPH-9B	0.102	0.167	ND	ND	0.105	ND
10	PPH-10A	ND	0.093	0.145	ND	ND	0.103
11	PPH-10B	ND	0.096	0.145	ND	ND	ND
12	PPR-2	0.075	0.120	ND	0.056	0.105	ND
13	PPR-4B	0.043	0.109	ND	ND	0.034	ND
14	PPR-7B	ND	0.097	0.163	ND	0.015	0.114
15	PPB-1	ND	0.062	0.114	ND	ND	ND
16	PPB-3	ND	0.087	0.136	ND	0.031	ND
17	PPB-7A	ND	0.015	0.038	ND	ND	ND
18	PPB-8	ND	0.093	0.147	ND	ND	ND
19	PPB-8A	0.122	0.157	ND	0.048	0.113	ND
20	PPB-8B	ND	ND	0.122	ND	ND	0.100
21	PPB-13	ND	0.076	0.156	ND	ND	0.107
22	PPB-14	ND	0.105	ND	ND	0.042	ND
23	PPB-21B	0.100	0.156	ND	ND	0.056	ND
24	PPB-22A	0.115	0.169	ND	ND	0.062	ND
25	PPB-22B	ND	0.007	0.098	ND	ND	0.035
26	PPB-23B	ND	0.106	ND	ND	0.062	ND
27	PPB-25A	ND	0.068	0.097	ND	ND	ND
28	PPB-25C	0.067	0.125	ND	ND	0.084	ND
29	PPB-26A	ND	0.079	0.118	ND	ND	ND
28	PPB-27B	ND	0.087	0.124	ND	ND	ND
31	PPB-30B	ND	ND	0.178	ND	ND	0.137
32	PPB-32C	ND	0.073	0.132	ND	0.059	ND
33	PPB-33A	ND	0.096	0.148	ND	ND	ND
34	PPB-34B	ND	0.120	ND	0.042	0.072	ND
35	PPB-34C	ND	0.105	ND	ND	0.074	ND
36	PPB-34D	ND	0.045	0.075	ND	ND	ND
37	PPB-35A	ND	ND	0.015	ND	ND	ND
38	PPB-37C	0.093	0.136	ND	0.026	0.102	ND
39	PPB-38B	ND	0.087	0.119	ND	ND	ND
40	PPM-14	ND	0.046	0.078	ND	ND	ND
41	PPM-21A	ND	0.023	0.078	ND	ND	ND
42	PPM-21B	ND	ND	0.065	ND	ND	ND
43	PPM-22A	ND	0.068	0.097	ND	ND	ND
44	PPM-23A	ND	ND	0.112	ND	ND	0.035
45	PPM-30A	ND	0.107	ND	ND	0.075	ND
46	PPM-33C	ND	0.084	0.126	ND	ND	ND
47	PPM-35A	ND	ND	0.095	ND	ND	ND
48	PPM-37A	ND	0.120	ND	ND	0.078	ND
49	PPM-37D	0.035	0.076	ND	ND	0.047	ND

(ND = Not detected)

and 70% ethanol as described in Material and Methods section. The nodules were crushed and streaked on YEMA medium plates containing Congo red dye. The colonies from each nodule were purified by streaking 2-3 times on same media. In total 196 rhizobial isolates were obtained out of which 28 isolates were obtained from Hisar, 79 from Bhiwani, 73 from Mahendergarh and 16 isolates were obtained from Rewari districts. These isolates were further purified and maintained on YEMA slants for further studies (Fig. 1; Table 2).

Characterization of rhizobia using Gram's staining and Peptone water test

A total of 196 rhizobial isolates obtained from different nodules were characterized by using Gram staining and peptone water test. It was observed that all the isolates were found to be Gram -ve with small rods shape. For peptone water test, all the isolates were inoculated in test tubes containing 5 ml peptone water broth and incubated at 30°C temperature for 3-4 days to observe the growth of the isolates. Out of 196 pigeon pea rhizobial isolates, only 23 isolates showed growth in the above broth indicating the doubt about their authenticity (Fig. 2). Thus, on the basis of Gram staining and peptone water test, 173 rhizobial isolates were selected for further studies.

Screening of rhizobial isolates for temperature tolerance

All the 173 rhizobial isolates were tested for temperature tolerance at 30, 35, 40, and 45°C temperature**.** Almost all the rhizobial isolates showed considerable growth at 30 and 35 °C while at 40°C some of the isolates showed moderate and poor growth. However, only 44 rhizobial isolates (25%) were able to grow at 45°C out of which some showed good, moderate and poor growth, respectively (Fig. 3). High tolerance to heat shock is common in in chickpea rhizobia and other species also (Kulkarni and Nautiyal, 2000; Rodrigues *et al.*, 2006). So, all 173 rhizobial isolates were also tested for their temperature tolerance. It was observed that all the rhizobial isolates were able to grow at temperatures i.e. 30, 35°C. However, 41% rhizobial isolates showed growth at 40°C, except few isolates where growth was slightly suppressed, while only 2% were able to grow at 45°C, out of which some showed good, moderate and poor growth, respectively (Fig. 3). These results are agreement with that of Graham (1992) who reported that rhizobia can grow better from 10 to 37°C. Lindstrom and Lehtomaki, (1988) reported the range of Temperature maximum 32.5-34.5°C for *Rhizobium leguminosarum* var *viceae* HAMBI 499, HAMBI 1125, and MPI 6001 isolated from faba bean and field pea from USA, UK, and the Netherlands. Karanja and Wood (1998) obtained several strains of *Rhizobium japonicum* tolerant upto 40°C.

Screening of rhizobial isolates for drought tolerance

The drought tolerance of all the 173 pigeon pea rhizobial isolates was tested in YEM broth supplemented with 0, 10, 20, 30, 40 and 50% polyethylene glycol (PEG 6000) concentration. Most of the pigeon pea rhizobial isolates were able to grow up to 20% PEG concentration. However, there was drastic decrease in their growth rate with increasing concentration of PEG. Out of 173 isolates only 71 and 3 isolates were able to grow in YEM broth supplemented with 30 and 40% PEG concentration, respectively (Fig. 4) and while not even a single isolate was able to grow at 50% PEG concentration. The growth and persistence of rhizobia and bradyrhizobia in soils are negatively impacted by drought conditions (Cytryn *et al.*, 2007). Uma *et al.* (2013) studied 30 isolates using YEM broth supplemented with PEG. All the 30 isolates grew well in YEM broth without PEG addition. As the concentration of PEG increased, the growth of rhizobial isolates was found to decrease. Abolhasani *et al.* (2010) grouped *Sinorhizobium* sp. in two clusters: sensitive and tolerant based on their growth rate in YEM broth containing different concentrations of PEG.

Screening of selected rhizobial isolates for combined abiotic stress tolerance

The combined abiotic stress *i.e.* temperature and drought tolerance of selected rhizobial isolates was studied which showed growth at high temperature (40 or 45°C) and high PEG concentration (30 or 40%). The YEM broth supplemented with 30 or 40% PEG concentration was inoculated with pigeon pea rhizobial isolates and incubated at 40 or 45°C depending upon the highest PEG concentration and temperature tolerance of the individual isolate during individual stress. The growth was measured spectrophotometrically by measuring absorbance at 600 nm after 4 days of incubation. As shown in table 3 out of 49 isolates tested only, 5 showed growth at high combined stress, however their growth was delayed as compared when grown with individual stress. All the selected abiotic stress tolerant pigeon pea rhizobial isolates were also tested for combined stress tolerance i.e. drought as well as temperature. In the presence of combined stress, out of 49 pigeon pea rhizobial isolates, 18 were able to grow in presence of 30% PEG at 40°C, whereas 5 and 5 pigeon pea rhizobial isolates were able to grow in presence of 40% PEG at 40°C and 20% PEG at 45°C, while 4 and 17 were able to grow in presence of 30% PEG at 45°C and 20% PEG at 40°C, respectively (Table 3).

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