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Assessing Stress Tolerant Rhizobial Isolates of Clusterbean (*Cymopsis tetragonoloba* (L.) Taub.) Retrieved from Semi- Arid Regions of Haryana, India

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A B S T R A C T

An attempt has been made to evaluate the effect of abiotic constraints (drought and temperature) on the growth of rhizobia isolated from clusterbean grown in semiarid regions of Haryana with a view to screen out stress tolerant rhizobial isolates. A total of 158 rhizobial isolates have been used for screening their stress tolerating ability with contrast to environmental abiotic soil conditions commonly prevailing in semi-arid regions of Haryana. All the isolates were phenotypically and biochemically characterized. Growth of pure rhizobial isolates on Yeast Extract Mannitol Agar (YEMA) medium having variable range of temperature (30, 35, 40 and 45) and different concentrations of PEG (0, 10, 20, 30, 40 and 50 %) were recorded at 420 nm using UV-VIS spectrophotometer after incubation at 30◦C for five days. On the basis of comparison of growth under varied stress conditions, fifty four rhizobial isolates from combined (temperature as well as drought) stress study were screened out. The stress tolerant traits of these rhizobia are of potential value from the point of view of biofertilization of legume seedlings during a forestation of degraded areas in semi-arid tropics of Haryana.

Introduction

India and Pakistan are the main producers of clusterbean which accounting for 80% production of the world's total, while Haryana and Rajasthan occupies the largest area (82.1%) in India (Pathak *et al.*, 2010). In India, 3.34 million hectares of the farmable land was under guar cultivation during the year 2006-2007 (Ministry of Agri. and Co-op GOI, 2010). It is cultivated in arid zones of Rajasthan, some arid and semi-arid regions of Haryana and Madhya Pradesh. The productivity of guar ranges from 474 kg/ha in Rajasthan to 1200 kg/ha in Haryana. In the recent years, clusterbean cultivation has become an attractive option with the farmers due to availability of high yielding varieties with high gum (30 to 35% of whole seed) content (galactomannans) in its endosperm which has great value as an enhancer of viscosity in food industry, like whipped cream substitutes a stabilizer for cheese, stiffner in soft ice-cream and instant pudding and as a metal binder. It is widely used from cosmetic and paper to mining and explosive industry (Whistler and Hymowitz, 1979; Mudgil *et al.*, 2014). Like other legumes, clusterbean is nodulated by *Rhizobium* or *Bradyrhizobium* strains belonging to cowpea miscellany group. Though, legume-*Rhizobium* symbiosis has been extensively studies in many crops but despite of its multipurpose use, no systematic work has been done to exploit the nitrogen-fixing ability of this crop and for increasing its production. Poor nodulation of clusterbean (guar) in Haryana may be due to environmental conditions such as high temperature and drought conditions. The poor survival of the inoculated culture and low density of rhizobial population in the field soil may also result in poor noduation of this crop. Since an adequate number of effective nodules can provide all the nitrogen requirement of this crop but it is desirable to have *Rhizobium* strains that would effectively nodulate cluster bean and fix nitrogen under agroclimatic conditions of Haryana.

Drought stress is one of the major limitations to crop productivity. It is the most common stress affecting plant growth in arid and semi-arid regions. Thus, it is necessary to improve the level of efficiency in plant capture and use of water and nutrients. Inoculation of plants with native beneficial microorganisms may increase drought tolerance of plants growing in arid or semi-arid areas (Marulanda *et al.*, 2007). Abiotic stress tolerance in soil microorganisms has been studied to provide a biological understanding of the adaptation and survival of living microorganisms in extreme environments. Shortage of water compromises plant and rhizobial growth and is a major cause of nodulation failure and low N_2 fixation. Water stress affects rhizobial morphology, survival, growth and population structure in soil (Guerin *et al*., 1991). Symbiotic N_2 fixation of legumes is also highly sensitive to soil water deficiency. A number of temperate, tropical and shrub legumes exhibit a reduction in nitrogen fixation when subjected to soil moisture deficit. This is due to the fact that water stress affects the formation and longevity of nodules, synthesis of leghaemoglobin and nodule function (Guerin *et al*., 1991). In general, the wide range of moisture levels characteristic of ecosystems where legumes have been shown to fix nitrogen suggests that rhizobial strains with different sensitivity to soil moisture can be selected. Studies have shown that sensitivity to moisture stress varies for a variety of rhizobial strains. Thus, it can be assumed that rhizobial strains can be selected with moisture stress tolerance within the range of their legume host. The modification of rhizobial cells by water stress will eventually lead to a reduction in infection and nodulation of legumes (Zahran, 1999). Optimization of soil moisture for growth of the host plant, which is more sensitive to moisture stress than bacteria, results in maximal development of fixed-nitrogen inputs into the soil system by the legume-*Rhizobium* symbiosis (Tate, 1995), the clusterbean crop growing environment in the Semi-Arid Tropics (SAT) is highly variable due to erratic spacing and timing of season rainfall. Therefore, this legume grown under semi-arid lands require temperature and drought tolerant rhizobia for effective symbiosis (Singh *et al*., 1999). The present study aimed to isolate temperature and drought tolerant Rhizobia that nodulate clusterbean crop under high temperature and drought conditions.

Materials and Methods

Collection of root nodules and isolation of rhizobia

Sixty seven soil samples were collected from Bhiwani, Hisar and Mahendergarh districts of Haryana. The seeds of clusterbean variety HG-563 were sown in 67 pots containing 2 kg of each soil sample and each pot was containing 3 or 4 clusterbean plants. All the pots supported the growth of clusterbean plants and the nodule formation was observed in all pots. After 45 days of growth, when nodule formation took place on the roots of clusterbean plants, 2 or 3 healthy pink nodules were collected from each plant and surface sterilized by using 0.1% $HgCl_2$ and ethanol as described in material and methods section.

The nodules were crushed and streaked on Yeast extract Mannitol medium (YEMA) medium plates containing Congo red dye. The colonies from each nodule were purified by streaking 2-3 times on same media. In total 158 rhizobial isolates were obtained, out of which 92 isolates obtained from Bhiwani, 30 and 36 isolates from Hisar and Mahendergarh, respectively. Pure cultures were obtained with one or more further sub-culturing steps. These rhizobial isolates were further purified and maintained on YEMA slants and were stored at 4 ^{0}C on slants for further studies.

Authentication of rhizobia by plant infectivity test

All the 158 rhizobial isolates were authenticated by plant infection test using clusterbean seeds (HG-563) under sterilized conditions in coffee cups (Giri and Dudeja, 2013). Seeds were surface-sterilized with a 0.2% $HgCl_2$ followed by 70% ethanol and finally rinsed in five changes of sterile water. Sterilized seeds were inoculated with log phase growing rhizobial cultures (10^4 – 10^5 cfu/seed) and sown in sterilized coffee cups containing sand in triplicate. Seedlings were watered with sterilized tap water. Nodule formation was scored after 45 days.

Stress tolerance studies

A total 158 clusterbean rhizobial isolates were screened for abiotic stress tolerance particularly drought tolerance with respect to 0, 10, 20, 30, 40 and 50% polyethylene glycol (PEG) and temperature tolerance on YEMA medium plates at 30, 35, 40 and 45 ^{o}C in BOD incubator.

Determination of temperature tolerance rhizobia

The effect of temperature on rhizobia-growth was studied using different temperature. Ten microliters of YEM overnight culture was spotted on YEMA medium plates, after incubation at 30, 35, 40 and 450C. After 5 days of incubation, rhizobial growth was recorded by visual observation compared to control treatments incubated at 30ºC.

Determination of drought tolerance rhizobia

The effect of drought on rhizobia-growth was studied using polyethylene glycol (PEG) 6000. One hundred microliters of YEM overnight culture was transferred to 10ml of the same YM broth supplemented with 10, 20, 30, 40 and 50% PEG, after incubation at 30^{0}C with shaking at 120 rpm for five days the bacterial growth was measured spectrophotometrically The growth was measured spectrophotometrically at OD 420 nm (Abdel-salam *et al*., 2011).

Determination of combined stress tolerance rhizobia

The YEM broth supplemented with 20, 30 and 40% polyethylene glycol (PEG) concentration were prepared. Log phase grown culture was inoculated in YEM broth supplemented with different concentration polyethylene glycol (PEG).

The broth were incubated at 40 or 45^{o}C depending upon the highest PEG concentration and temperature tolerance of the individual isolate during single stress.

The growth was measured spectrophotometrically at OD 420 nm (Abdel-salam *et al*., 2011).

Results and Discussion

Isolation and authentication of rhizobia

A total of 158 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 negative with small rods. Moreover, the isolates obtained from same soil samples showed identical cell shape and size. For peptone water test, all the isolates were inoculated in test tubes containing 5ml peptone water broth and incubated at 30^{o}C for 3-4 days to observe the growth of the isolates. Most of the rhizobial isolates did not show the growth in peptone water broth with control, however, only a few were able to grow in the above broth indicating the doubt about its authenticity. Thus, on the basis of Gram staining and peptone water test, 158 rhizobial isolates were selected for abiotic stress tolerance.

Table.1 Number of cluster bean rhiobial isolates obtained from different districts along with their isolates number

S. No.	District	Rhizobial isolates number	No. of isolates
1	Bhiwani	GB-1a, GB-1b, GB-1c, GB-2a, GB-2b, GB-3a, GB-4a, GB-5a, GB-5-b, GB-5c, GB-7a, GB-7b, GB-8a, GB-8b, GB-9a, GB-10a, GB-10b, GB-10c, GB-10d, GB-11a, GB-11b, GB-11c, GB-12a, GB-12b, GB-13a, GB-14a, GB-14b, GB-14c, GB-15a, GB-16a, GB-16b, GB-16c, GB-16d, GB-17a, GB-17b, GB-17c, GB-17d, GB-18a, GB-18b, GB-19a, GB-19b, GB-20a, GB-21a, GB-22b, GB-22c, GB-23a, GB-23b, GB-23c, GB-24a, GB-24b, GB-25a, GB-25b, GB-25c, GB-25d, GB-26a, GB-26b, GB-26c, GB-26d, GB-26e, GB-27a, GB-27b, GB-27c, GB-27d, GB-28a, GB-28b, GB-28c, GB-29a, GB-29b, GB-29c, GB-30a, GB-30b, GB-31a, GB-31b, GB-32a, GB-32b, GB-32c, GB-32d, GB-33a, GB-33b, GB-33c, GB-34a, GB-35a, GB-35b, GB-35c, GB-36a, GB-36b, GB-38a, GB-38b, GB-38c, GB-39a, GB-39b, GB-39c	92
2	Hisar	GH-1a, GH-1b, GH-1c, GH-1d, GH-2a, GH-2b, GH-2c, GH-3a, GH-4a, GH-4b, GH-4c, GH-4d, GH-5a, GH-5b, GH-5c, GH-5d, GH-6a, GH-6b, GH-6c, GH-6d, GH-7a, GH-7b, GH-7c, GH-7d, GH-8a, GH-8b, GH-9a, GH-9b, GH-9c, GH-9d	30
3	Mahendergarh	GM-1a, GM-2a, GM-2b, GM-3a, GM-3b, GM-3c, GM-4a, GM-5a, GM-5b, GM-6a, GM-7a, GM-7b, GM-7c, GM-8a, GM-9a, GM-9b, GM-9c, GM-10a, GM-11a, GM-11b, GM-11c, GM-12a, GM-13a, GM-13b, GM-13c, GM-14a, GM-14b, GM-14c, GM-14d, GM-15a, GM-15b, GM-15c, GM-16a, GM-16b, GM-16c, GM-16c, GM-16d	36
		Total	158

Table.2 Effect of temperature as well as drought on the growth of rhizobial isolates

S. No.	Rhizobial isolates	40°C		45°C
		PEG concentration (%)		PEG concentration (%)
		40	30	20
1	GB-1a	ND	0.028	ND
2	GB-1b	ND	0.129	ND
3	GB-3a	ND	0.089	ND
4	GB-5c	ND	ND	-
5	GB-7a	ND	0.054	ND
6	GB-10b	ND	0.008	ND
7	GB-10c	ND	0.066	ND
8	GB-14a	ND	0.012	ND
9	GB-14c	ND	0.194	0.103
10	GB-16a	ND	0.161	-
11	GB-17a	ND	0.085	ND
12	GB-18b	0.099	0.181	ND
13	GB-19b	ND	0.111	ND
14	GB-24b	ND	ND	0.064
15	GB-25d	ND	0.016	ND
16	GB-26b	ND	0.078	ND
17	GB-26d	0.132	ND	0.155
18	GB-27b	ND	0.029	ND
19	GB-29a	ND	ND	0.131
20	GB-30a	ND	0.118	ND
21	GB-31b	ND	0.065	ND
22	GB-32a	ND	0.046	-
23	GB-32c	0.071	0.116	0.080
24	GB-32d	ND	0.024	0.052
25	GB-36b	ND	0.143	ND
26	GB-38b	ND	0.099	0.007
27	GH-1a	ND	ND	-
28	GH-2b	ND	0.107	ND
29	GH-2c	ND	0.031	ND
30	GH-4b	ND	0.051	0.042
31	GH-4d	ND	ND	-
32	GH-5a	ND	0.145	ND
33	GH-5b	0.122	ND	ND
34	GH-5d	ND	0.036	ND
35	GH-6d	ND	0.111	ND
36	GH-7d	0.067	0.080	ND

37	GH-8a	0.086	0.100	ND
38	GH-9a	ND	0.044	ND
39	GH-9b	ND	0.106	ND
40	GM-1a	ND	0.002	ND
41	GM-3c	0.142	ND	ND
42	GM-5a	0.064	0.092	0.052
43	GM-6a	ND	0.038	ND
44	GM-7c	ND	0.003	-
45	GM-8a	ND	ND	ND
46	GM-10a	ND	0.101	0.041
47	GM-11b	ND	0.099	0.058
48	GM-11c	ND	0.024	ND
49	GM-13a	ND	0.131	ND
50	GM-14b	ND	0.088	ND
51	GM-14c	ND	ND	-
52	GM-15b	ND	ND	-
53	GM-15c	ND	ND	0.070
54	GM-16b	ND	ND	ND

Fig.1 Isolation of rhizobia nodulating cluster bean using trap plants from different districts of south-western Haryana

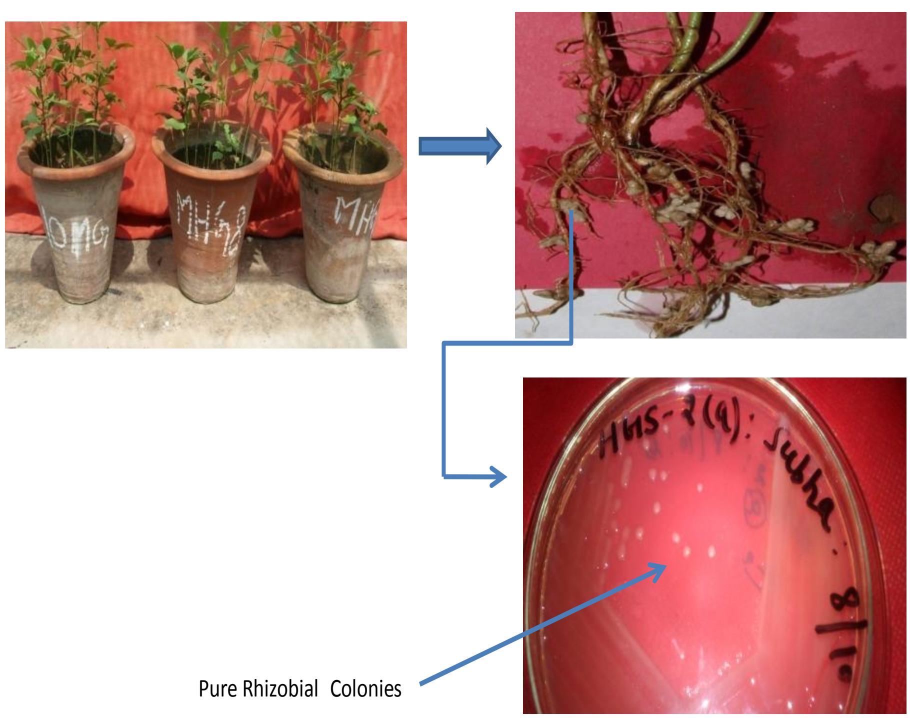


Fig.2 Morphological characterization of cluster bean rhizobial isolates using Gram staining and peptone water test

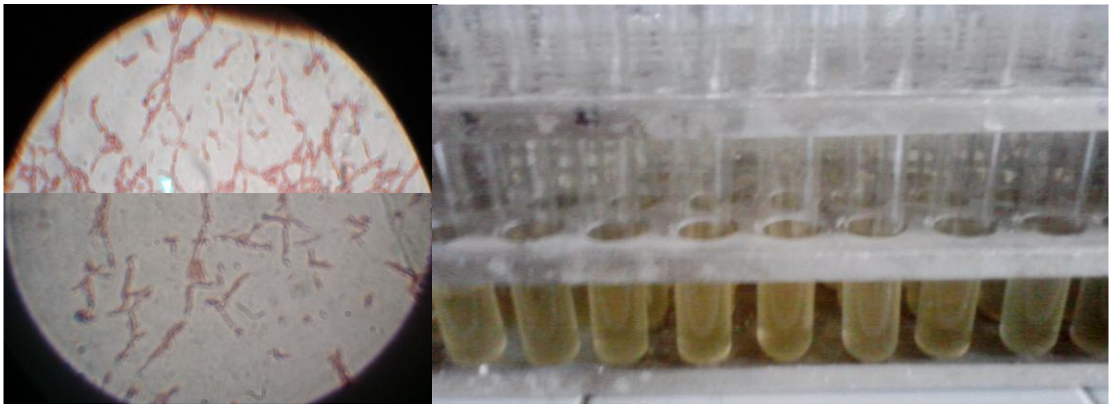


Fig.3 Combined (drought and temperature) stress tolerance by cluster bean rhizobial isolates

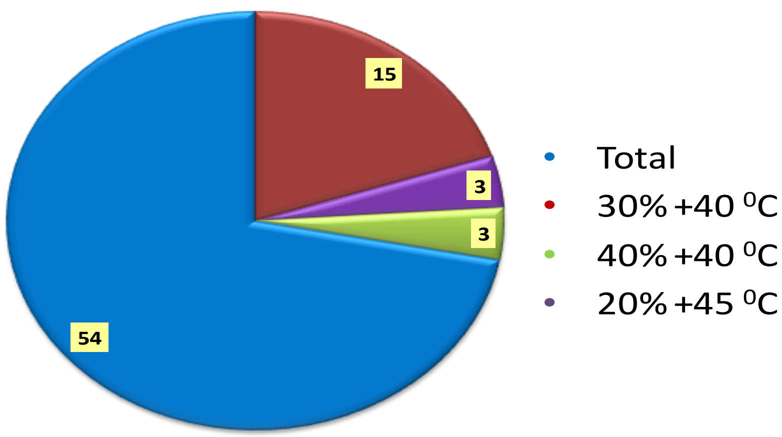


Fig.4 Temperature tolerance by cluster bean rhizobial isolates

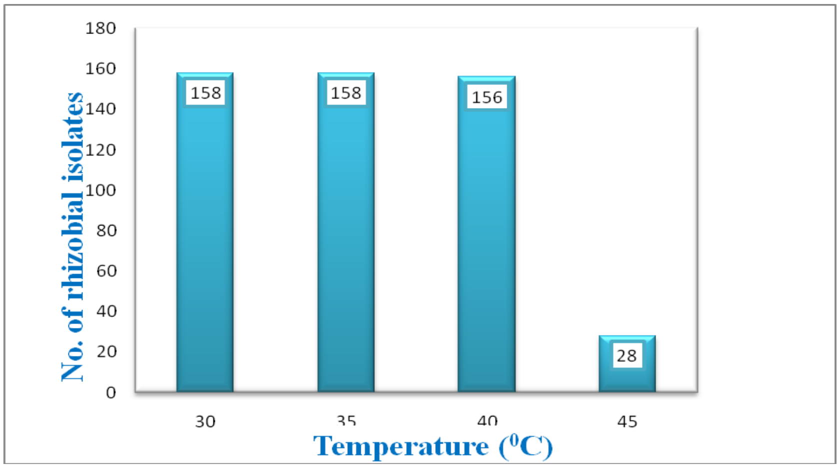
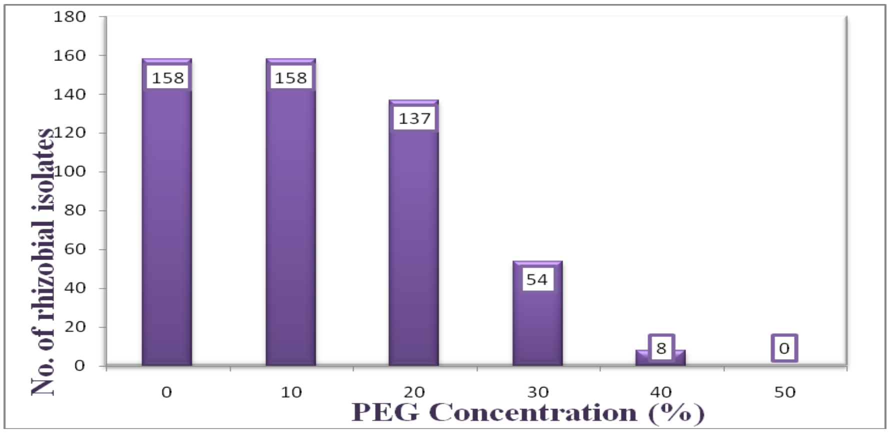


Fig.5 Drought tolerance by cluster bean rhizobial isolates



Temperature tolerance

In general, majority of the isolates exhibited luxuriant growth at the temperature ranging from 25^{o}C-35^{o}C. In present study, it was observed that all the rhizobial isolates were able to grow at temperatures i.e. 30, 35^{o}C. However, 156 rhizobial isolates showed growth at 40^{o}C, except few isolates where growth was slightly suppressed, while only 28 were able to grow at 45^{o}C, out of which some showed good, moderate and poor growth. Further increase in temperature led to noticeable decline in growth. These findings agreed with the results of previous studies on *Rhizobium leguminosarum* strains isolated from Nile Valley of Egypt which showed tolerance to temperatures ranging between 35-40°C (Moawad and Beck, 1991) and *Cicer arietinum* rhizobial isolates, which grew at 45°C (Maatallah *et al*., 2002). Ours results are also agreement with that of Graham (1992) who reported that rhizobia can grow better from 10 to 37^{o}C and maximum temperature for growth in free-living rhizobia ranged between 35-45°C (Zhang *et al*., 1991; Zahran *et al*., 1994). However, Kulkarni *et al*., 2000 observed survival of rhizobial strains from *Sesbania aculaeta* at 50 and 65°C on YEMA plates at pH 7.0 for up to 2 and 4 hours.

Drought tolerance

In the present study rhizobial strains growth were measured after their exposure to 10% to 50% PEG 6000, for five days (Fig. 5). Tolerance to drought stress is a very complex phenotype that involves not only the bacterial ability to tolerate the stress but also the swiftness to respond and adapt to the environmental change. In the current study, decreased growth of rhizobial isolates with increasing PEG concentration was registered. As much as 60% of legume production in the developing world occurs under conditions of significant drought stress (Graham and Vance, 2003; Zhang *et al*., 2007). The effect of drought on rhizobia-growth was studied using polyethylene glycol (PEG) 6000. So all the rhizobial isolates were tested for drought tolerance in YEM broth supplemented with 0, 10, 20, 30, 40 and 50% PEG. Most of the rhizobial isolates were able to grow up to 20% PEG, however, there was drastic decrease in their growth rate with increasing concentration of PEG. Out of 158 rhizobial isolates, only 54 and 8 isolates were able to grow at 30 and 40 % PEG, respectively and none of them was able to grow at 50% PEG concentration. (Fig.5). Uma *et al*., (2013) studied 30 isolates using YEM broth supplemented with PEG. All the 30 isolates grew well in YEM broth without PEG. As the concentration of PEG increased, the growth was found to decrease. The isolates SBJ-2, SBJ-10, SBJ-14 and SBJ-23 were found to grow at 30% PEG 6000. These results are conformity with the results of Abdel-Salam *et al*., 2011. The growth and persistence of *Rhizobia* and *Bradyrhizobia* in soils are negatively impacted by drought conditions (Cytryn *et al*., 2007).

Combines stress (temperature as well as drought) tolerance

All the selected abiotic stress tolerant clusterbean rhizobial isolates were also tested for combined stress tolerance i.e. drought as well as temperature. In the presence of combined stress, out of 54 clusterbean rhizobial isolates, 15 were able to grow in presence of 30% PEG at 40^{o}C, whereas 3 and 3 clusterbean rhizobial isolates were able to grow in presence of 40% PEG at 40^{o}C and 20% PEG at 45^{o}C, respectively (Fig. 3). This study showed that there was considerable variability in the level of stress tolerance of rhizobial isolates obtained from clusterbean plants native to semi-arid regions of Haryana. Based upon the comparative assessment, we have screened three isolates from combined stress tolerant study i.e. high temperature as well as drought (GB-14c, GB-26d and GB-32c which could further be utilized for their symbiotic effectiveness determination under field conditions in semi-arid regions of Haryana.

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