International Journal of Bio-resource and Stress Management 2017, 8(2):309-315
Full Research
Doi: HTTPS://DOI.ORG/10.23910/IJBSM/2017.8.2.1793

Characterization of Abiotic Stress Tolerant Rhizobia as PGPR of Mothbean, Clusterbean and Mungbean Grown in Hyper-arid Zone of Rajasthan

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Article History

Manuscript No. AR1793
Received in 21st January, 2017
Received in revised form 19th March, 2017
Accepted in final form 6th April, 2017

Abstract

The present investigation was focused on isolation and *in vitro* characterization of high abiotic stress tolerance, PGPR traits and dinitrogen fixation efficiency among cultivable rhizobia nodulating mothbean, clusterbean and mungbean. A total of 201 rhizobial isolates were retrieved from 101 different nodule samples collected from various locations of four districts i.e. Churu, Bikaner, Jaisalmer and Barmer situated under hyper-arid zone of Rajasthan, India. Around 70% of rhizobial isolates showed steady growth at 30% of polyethylene glycol 6000 (PEG 6000) and 30% of isolates showed tolerance at 40% PEG when drought tolerance of different rhizobia were assessed. Similarly 70% of the rhizobial isolates showed luxurious growth at temperature of 40 °C and only 30% of isolates showed survival ability at 45 °C. Combined abiotic stress tolerance experiment revealed that 54 rhizobial isolates were tolerant to 30% PEG and 40 °C while only 16 isolates showed high drought tolerance of 40% PEG 6000 and high temperature tolerance at 45 °C. *In vitro* assessment of dinitrogen fixation efficiency showed all 54 stress tolerant isolates possessed dinitrogen fixation ability with different degrees of efficacy. Stress tolerant rhizobial isolates also harbored multiple PGPR traits like phosphate solubilization, IAA production, ammonia excretion, bacteriocin production and ACC deaminase activity. Hence, our result showed a promising percentage (30%) of different rhizobial isolates can be used further *in situ* trials in pot house and field conditions as high abiotic stress tolerant isolate accompanying with multiple PGPR traits for future bioinoculant production.

Keywords: Hyper-arid zone, mothbean, clusterbean, mungbean, rhizobia, PGPR

1. Introduction

Changes in the global climate might increase high drought and temperature-related abiotic stresses with increasing the area of arid and hyper-arid zones and further expected to reduce agricultural crop productivity (Grover et al., 2010; Larson, 2013; Coleman-Derr and Tringe, 2014). Agricultural crops like mothbean, clusterbean and mungbean are the important abiotic stress tolerant *kharif* legumes grown mainly in arid and hyper-arid zones of Rajasthan in India. Abiotic stresses like high temperature and drought affect *Rhizobium*-legume symbiosis and severely reduce legume production (Keneni et al., 2010). Hence the *kharif* legume production in these zones is gradually reducing for prevailing of high abiotic stress conditions. Currently hyper-arid zone of India is situated under four districts of Rajasthan i.e. Churu, Bikaner, Jaisalmer and Barmer. According to recent estimates, Rajasthan possesses the first position in both area and production in mothbean, clusterbean and mungbean production. The highest productivity of these *kharif* legumes is around 1000 kg ha^{-1} but due to severe climatic condition the productivity of legumes of hyper-arid zone is very low approximately 300 kg ha^{-1} (RACP, 2012).

Abiotic stresses like drought, high temperature and salinity affect both plants and microorganisms in a variety of ways (Zahran, 1999; Zahran, 2001). These stress factors have a significant influence on the performance of beneficial microorganisms for crop production. More specifically inoculation of abiotic stress tolerant strains of rhizobia enhances nitrogen fixation and plant biomass yield of legumes under abiotic stress condition (Ogutcu et al., 2010). Agriculture in arid and hyper-arid zones is rainfed and suffers from a poor natural resource base and low productivity (Kumar et al., 2014). Since nitrogen fixation under field conditions is highly sensitive to water stress, the short-term and intermittent dry periodsencountered both in arid and semi-arid environments result insignificant loss of biological nitrogen fixation (BNF) underactual field conditions. To meet such enormous nitrogen requirements through chemical fertilizers would not only be expensive but could also severely degrade soil health. Therefore, the prevalent use of aneco-friendly biological method, like rhizobial inoculants toharness plant production, is a fascinating approach to helpsustainable development in such zones. Besides this, if an efficient rhizobial strain possesses multiple PGPR traits the growth and vigor of the plant enhanced due to improved nutrient uptake, increased survival of macro and micro-symbiont (Ahmad et al., 2011; Bhattacharjee et al., 2012). There are lacuny of high abiotic stress tolerant rhizobial strain in current Indian market for bioinculant production (Mazid and Khan, 2014).

Hence, the present study was undertaken to isolate and characterize high temperature and drought tolerance of different rhizobia from mothbean, clusterbean and mungbean grown in hyper-arid zone of Rajasthan and simultaneous assessment of different PGPR traits i.e. IAA production, ammonia excretion, phosphate solubilization, bacteriocin production and ACC deaminase activity under*in vitro* condition.

2. Materials and Methods

2.1. Sample collection

A total of 101 rhizospheric soil and nodule samples of mothbean, clusterbean and mungbeanfrom hyper-arid zone of Rajasthan were collected from different sites of four districts Churu (28°14′N, 74°52′E), Bikaner (27°47′N, 72°48′E), Jaisalmer (26°50′N, 70°58′E) and Barmer (25°44′N, 71°22′E) during September, 2013 (Table 2). About 100 g of rhizospheric soil sample along with plants were collected. The samples were collected in the sterile plastic bags and kept at 4 °C for further analyses.

2.2. Analysis of soil physico-chemical properties and isolation of rhizobia

The soil samples were analyzed for the physico-chemical properties, i.e. pH, EC, organic carbon (%) using standard method (Jackson, 1958). Isolation of rhizobia from nodules of different legume plants was accomplished as described by Vincent (1970). The isolates were purified on YEMA (Yeast Extract Mannitol Agar) Congo red plates and maintained on YEMA slants at 4 °C (Vincent, 1970). Gram staining of the rhizobial isolates was performed as described by Beveridge (2001). Nomenclature of the isolates was done according to our own convenience.

2.3. In vitro temperature and drought tolerance and combined stress tolerance of rhizobial isolates

Freshly grown cultures of rhizobial isolates were spotted on YEMA plates and incubated at different temperatures of 30, 35, 40 and 45 °C to screen for temperature tolerance. After 3 days of incubation, rhizobial growth was observed and compared to control at 30 °C (Kulkarni and Nautiyal, 2000; Mangla et al., 2014). Drought tolerance of the isolates were accomplished by inoculating YEMA broth with rhizobial isolates using different concentration of Polyethylene glycol 6000 (PEG 6000) i.e. 20%, 30% and 40% (Rehman and Nautiyal, 2002). For assessment of combined stress-tolerance the broth was incubated at 40 and 45 °C depending upon the highest PEG concentration and temperature tolerance of the individual isolate during single stress (Mangla et al., 2014).

2.4. Acetylene reduction assay (ARA) and PGPR traits

Nitrogenase activity was measured by acetylene reduction assay (Boddey, 1987). The acetylene reduction assay (ARA) was expressed as nmol ethylene mg^{-1} protein h^{-1}. Phosphate solubilization capacity of rhizobial isolates were assessed through Pikovskaya medium supplemented with tricalcium phosphate (Kumar et al., 2014), IAA production was determined using Salkowski's reagent (Gordon and Weber, 1951), bacteriocin production, ammonia excretion and ACC deaminase enzyme activity were analyzed using standard method (Kumar et al., 2014).

All the experiments were carried out during the period from October, 2013 to May, 2015.Analyses of variance (ANOVA) technique, Pearson correlation coefficient were applied to analyze the data using SPSS version 22.0. Values of $p<0.05$ were considered as statistically significant.

3. Results and Discussion

3.1. Isolation of rhizobia and physico-chemical properties of soils

A total of 201 rhizobial isolates were obtained from 101 different nodule samples of mothbean, clusterbean and mungbean collected from four different districts namely Churu, Bikaner, Jaisalmer and Barmer of Rajasthan, India (Table 2). The physico-chemical properties (pH, EC, percent organic C) of 101 rhizospheric soil samples are shown in Table 1. The no. and occurrence of rhizobial isolates in legume root nodules showed quite deflated value from the plant roots exposed without abiotic stress as observed in previous study (Keneni et al., 2010). Our study also showed the no. and occurrence of rhizobial isolates adequately augmented in root nodules when organic C (%) in soils simultaneously enhanced. Not only total nos. of root nodulatingrhizobial isolates increased with percent organic carbon but also a strong positive correlation (R^2= 0.8898) found between isolates nos. and organic carbon content of four different districts soils. The reason might be the strong influence of soil free-living rhizobia on the activity of root nodulating rhizobia in legumes whereas soil microbial activity positively correlatedwith soil organic carbon content as reported in

Table 1: Physico-chemical properties of soils of four districts of Rajasthan

Districts	pH	EC (dSm^{-1})	Organic carbon (%)
Churu	8.2±0.04	0.10±0.01	0.25±0.03
Bikaner	8.5±0.04	0.22±0.02	0.15±0.01
Jaisalmer	8.9±0.05	0.18±0.02	0.10±0.01
Barmer	7.7±0.02	0.16±0.01	0.13±0.01
CD (p=0.05)	1.06	0.04	0.03

Data represent the means±SEm in each group

Table 2: Agro-climatic features of study sites and total nos. of rhizobial isolates of mothbean, clusterbean and mungbean in different district wise

Sampling districts	AT	ARA	No. of nodule and soil samples	No. of isolates
Churu	46.1	319.5	20	49
Bikaner	47.1	248.5	30	61
Jaisalmer	47.5	182.8	26	49
Barmer	46.0	320.3	25	42
Total			101	201

AT: Avg. temperature (°C); ARA: Avg. rainfall annum^{-1} (mm) ; Data represent the means±SEm in each group

previous studies (Swanepoel et al., 2011).

3.2. Occurrence of high abiotic stress tolerant rhizobia

In vitro screening for drought tolerance revealed thatall isolates were able to grow up to PEG 20%, 110 isolates were tolerant up to 30% and only 48 isolates were tolerant up to PEG 40% (Figure 3). Hence, it was lucidly observable that around 70% of isolates showed steady growth at PEG 30% and around 30% of isolates showed drought tolerance level of PEG 40% in different crop wise assessment (Figure 4). Similarly, *in vitro* screening for temperature tolerance showed that all isolates were able to grow up to 35 °C, 184 isolates were tolerant to 40 °C and only 82 isolates were tolerant to 45 °C (Figure 1). For different crop wise, around 70% of isolates showed luxurious growth at 40 °C and approximately 30% of isolates were survived up to 45 °C (Figure 2). Hence, the gradual reduction on survival percentage of rhizobial isolates was observed when severity of abiotic stress conditions was enhanced. There were strong negative correlations (R^2= 0.994, 0.924) found between enhanced levels of abiotic stress and total nos. of rhizobial isolates survived under stress. It was also observed that mungbean and clusterbean rhizobia possessed higher percentage of single abiotic stress tolerance

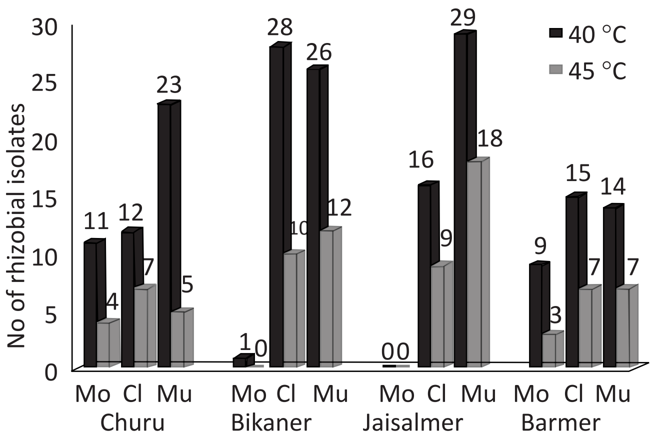


Figure 1: Temperature tolerant (40 °C & 45 °C) rhizobial isolates from four districts of Rajasthan; Mo: mothbean; Cl: clusterbean; Mu: mungbean

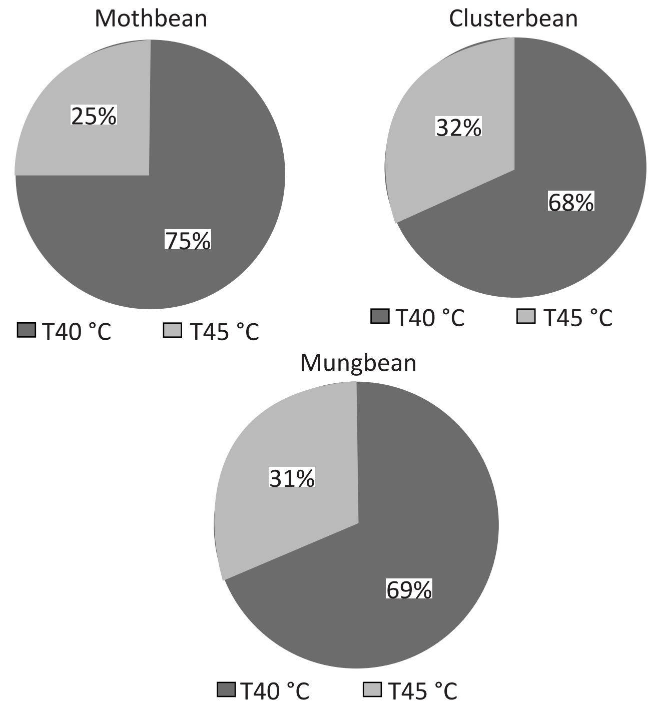


Figure 2: Pie charts representing temperature tolerant rhizobial isolates of mothbean, clusterbean and mungbean; T: Temperature

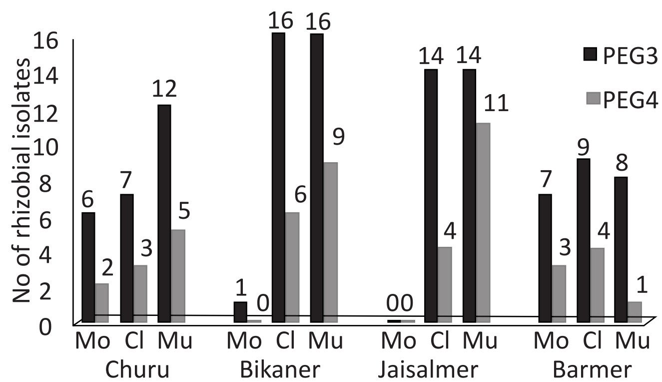


Figure 3: Drought tolerant isolates (PEG 30% and 40%) from four districts of Rajasthan; Mo: mothbean; Cl: clusterbean; Mu: mungbean

as compared to mothbean rhizobia. These findings are in line with previously documented report (Mangla et al., 2014).

Under combined abiotic stress tolerance, 54 isolates were able to grow under moderate drought condition of PEG 30% and temperature at 40 °C. Further 39 isolates were able to grow at PEG 40% and 40 °C, 22 isolates showed growth at PEG 30% and 45 °C and only 16 isolates were able to grow at high drought concentration of PEG 40% and high temperature at 45 °C (data not shown). It was found that mungbean and mothbean acquired higher percentage of coxmbined stress tolerance i.e. practically more significant than clusterbean rhizobia (Figure 5). These findings revealed that high abiotic stress tolerant rhizobia frequently encounter in rhizosphere region of these

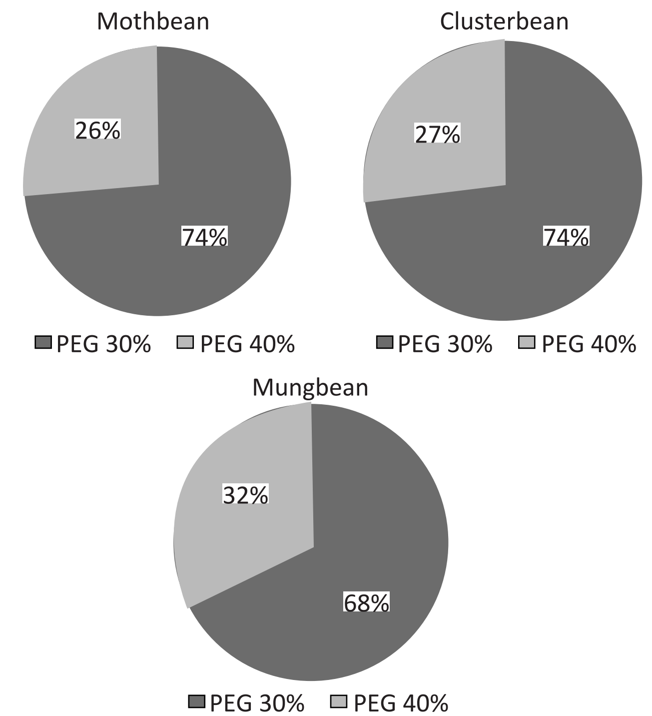


Figure 4: Pie charts representing drought tolerant rhizobial isolates of mothbean, clusterbean and mungbean

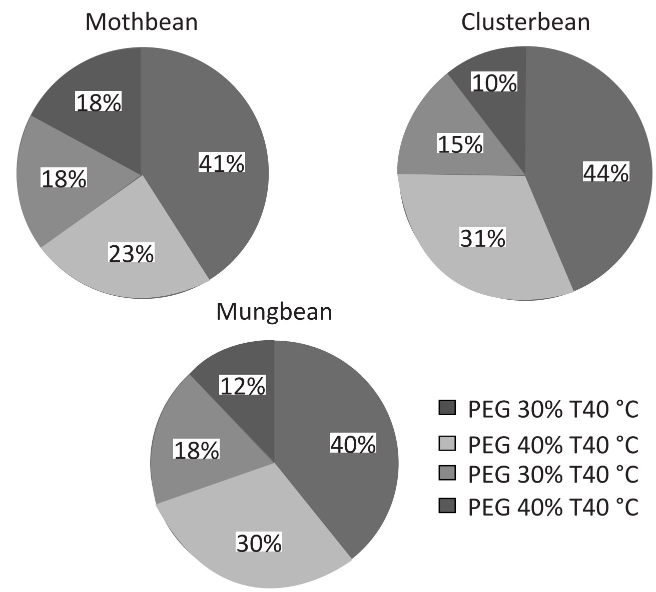
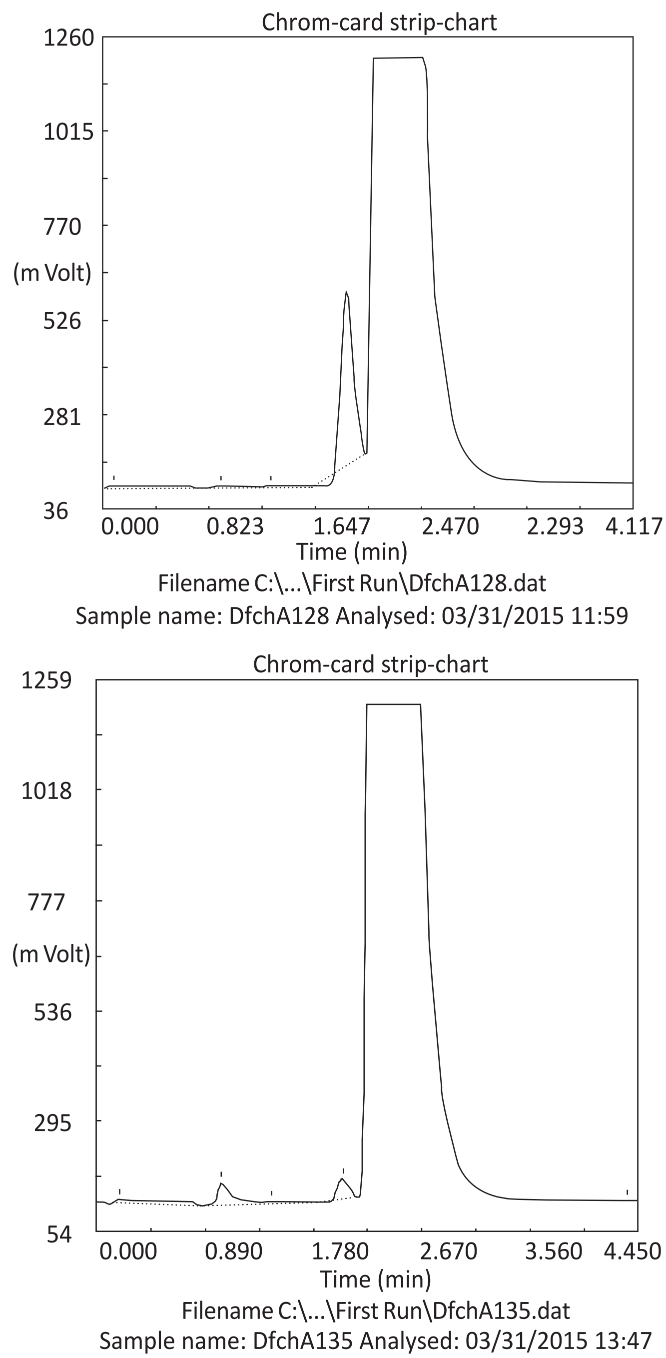


Figure 5: Pie charts representing high temperature and drought tolerant rhizobial isolates of mothbean, clusterbean and mungbean

legumes grown under exposure of higher abiotic stress in soils of Jaisalmer and Bikaner as compared to other two districts where harshness of the climate is considerably abated. This observation is in line with previously documented report that high salinity tolerant rhizobia mainly prevail in soils with high salt content (Zahran, 1999; Keneni et al., 2010). Hence, it established the previous fact on rhizobial occurrence and growth which depends on different physiological factors like temperature, soil moisture regime as reported in previous study (Vriezen et al., 2007).

3.3. In vitro dinitrogen fixation capacity of rhizobia

All 54 stress tolerant isolates showed ARA activity with different degrees of efficacy in nitrogen induction medium with maximum ARA value of 63.12 nmol ethylene mg^{-1} protein h^{-1} from clusterbeanand minimum 0.15 nmol ethylene mg^{-1} protein h^{-1} from mungbean (Figure 6 and Table 3). ARA of the isolates also deciphered that there was a significant variation in *in vitro*nitrogenase activity among 54 stress



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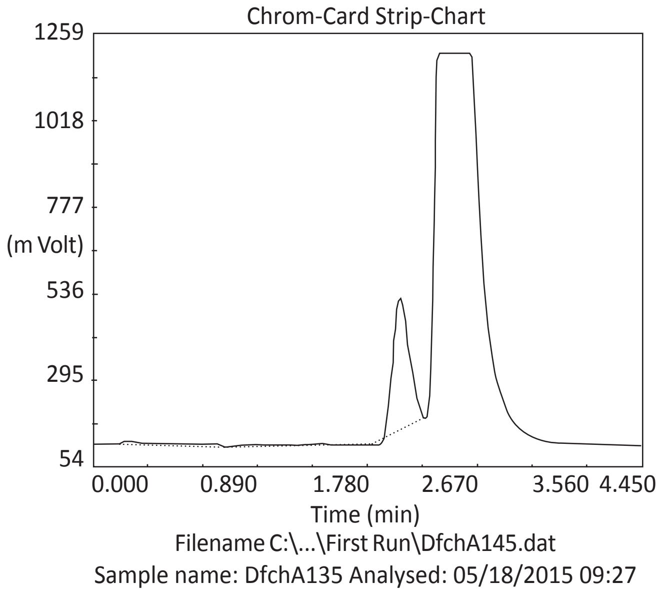

Figure 6: Gas chromatographs (ethylene and acetylene) of ARA of isolates MoBr99b (mothbean), ClBk43a (clusterbean) and MuJs72a (mungbean) after 24 h of incubation

tolerant isolates. It was evident that the rhizobial isolates from Churu have comparatively lower nitrogen fixation ability and the isolates from Bikaner showed higher values of ARA. This also reflects the significant diversity on the capacity to fix atmospheric dinitrogenand higher stress tolerant isolates possessed higher ARA. It might be because of their genetically differences as observed in previous study (Kumar et al., 2014).

3.4. Rhizobia with multiple PGPR traits

All 54 stress tolerant rhizobial isolates possessed multiple PGPR traits but different degrees of diversified traits and efficacy (Table 3) as noted earlier (Marasco et al., 2012). Most of the isolates around 30% of all three legumes showed multiple PGPR traits like ability to solubilize phosphate, ammonia excretion and IAA production (Figure 7). Two recent studies found that more than 25% of bacteria isolated from cultivated crops had plant growth promoting activities (Hassan et al., 2010; Marasco et al., 2012). It also showed there was comparatively reduced level of diversity on the possession of P-solubilization, ammonia excretion and IAA production among rhizobial isolates. Only 5–10% of rhizobial isolates from *kharif* legumes acquired the ability to produce bacteriocin and ACC deaminase enzyme. There was higher level of diversity for bacteriocin production and ACC deaminase activity among all rhizobia but the traits of bacteriocin production and ACC deaminase activity were found in small population of cultivable rhizobia. These results are in line with previous report (Coleman-Derr and Tringe, 2014). Clusterbeanrhizobial isolates showed predominantly as bacteriocin producer and

Table 3: Acetylene Reduction Assay and PGPR traits of high abiotic stress tolerant rhizobia of mothbean, clusterbean and mungbean

Isolate No.	IAA production	Ammonia excretion	P-solublization index	Bacteriocin production	ACC deaminase activity	ARA
MoCh17b	5.54±0.806	-	1.90±0.0023	+	-	0.38±0.0003
MoBr96a	-	4.30±0.0601	5.00±0.0205	-	-	1.80±0.0029
MoBr99b	7.57±0.0903	0.14±0.0011	-	-	+	3.49±0.0306
ClBk31c	2.95±0.0136	1.32±0.0452	1.37±0.0014	-	-	0.24±0.0001
ClBk38c	15.35±0.0963	3.70±0.0352	3.09±0.0125	+	+	1.45±0.0237
ClBk43b	-	0.78±0.0203	5.25±0.0305	-	+++	21.12±1.23
ClJs74b	1.26±0.0102	4.31±0.2251	2.88±0.0964	+	+	31.16±1.96
ClBr87a	14.56±1.02	4.89±0.2356	1.73±0.0056	+	-	10.21±0.6321
MuBk32b	-	4.81±0.3320	1.37±0.0025	-	+	8.12±0.6331
MuBk40c	16.85±1.22	-	1.72±0.0056	-	++	0.23±0.0002
MuBk45b	15.86±1.03	4.32±0.1236	1.30±0.0026	+	-	1.26±0.0045
MuJs52b	-	4.84±0.2246	1.07±0.0056	-	++	3.39±0.2246
MuJs53b	3.40±0.2154	4.89±0.3324	1.11±0.0024	-	-	3.02±0.2213
MuJs69c	7.53±0.6354	-	1.63±0.0027	-	++	0.17±0.0001
MuJs69d	20.35±1.49	4.81±0.3012	2.28±0.1123	-	+	31.64±2.53
MuJs72a	13.90±0.9365	-	2.00±0.0125	-	-	54.83±3.56

Data represent the means±SEm in each group; -: no growth; +: fair; ++: good; +++: profound growth in ACC supplemented medium; Ch: Churu; Bk: Bikaner; Js: Jaisalmer; Br: Barmer; IAA production and ammonia excretion expressed as μg ml^{-1}; P-solubilization as P-SI (P-solubilization index); ARA in nmol ethylene mg^{-1} protein h^{-1}

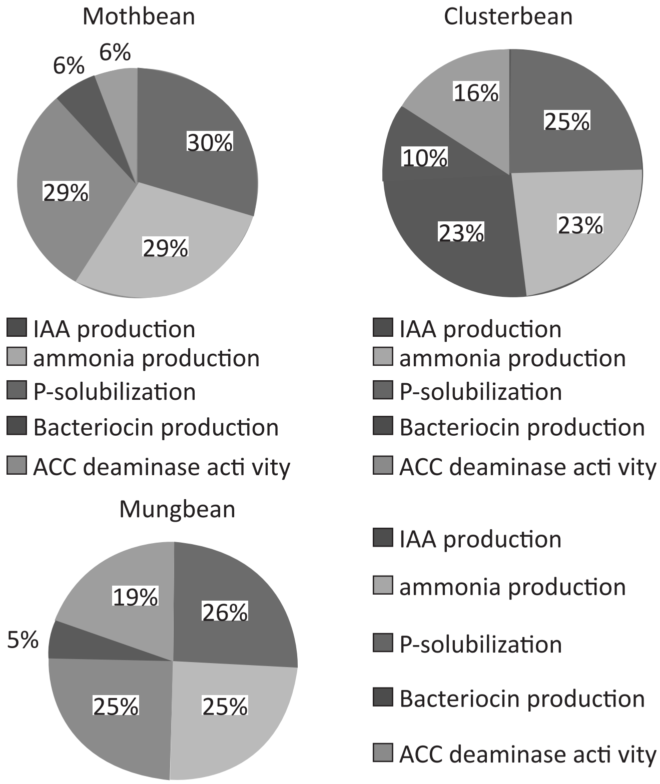

Figure 7: Pie charts depicting multiple PGPR traits of rhizobia of mothbean, clusterbean and mungbean

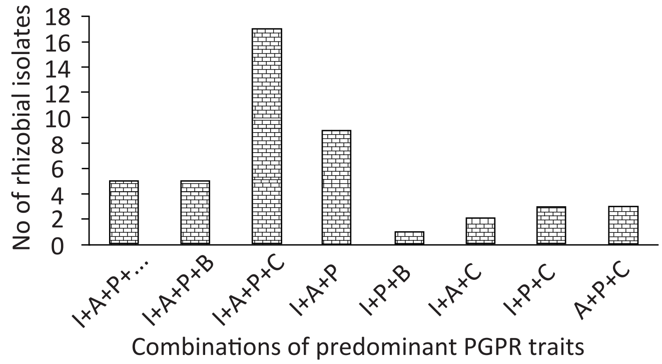

Figure 8: Bar chart representing predominant PGPR trait combinations of rhizobia of mothbean, clusterbean and mungbean; I: IAA production; A: ammonia excretion; P: P-solubiliztion; B: bacteriocin production; C: ACC deaminase activity

mungbean rhizobia were mainly acquired ACC deaminase activity. It was also observed that IAA producing rhizobial isolate simultaneously expressed enhanced level of ACC deaminase activity than others without IAA producer. The reason might be the synergistic interaction between ACC deaminase and both plant and bacterial auxin, indole-3-acetic acid (IAA) as reported in previous study (Glick, 2014).

For possession of combined PGPR traits in rhizobia, it was observed that combination IAA production (I)+Ammonia excretion (A)+P-solubilization (P)+ACC deaminase activity (C) or I+A+P+C was predominant among all three different stress tolerant rhizobial populations (Figure 8). The similar finding was observed in previous study where dominance of IAA production, ammonia excretion and P-solubilization were reported (Kumar et al., 2014). Besides these, one more interest finding was the rhizobial isolates with higher nos. of combined PGPR traits were mainly from the districts of Bikaner and Jaisalmer. It means the higher abiotic stress tolerant isolates acquired more nos. of PGPR traits than others. It might be the rhizobial survival under more severe climatic condition ultimately boosted with more diversity in physiological traits for better adaptation. This result is in line with previous study where higher salt tolerant rhizobia expressed higher level of antibiotic resistance (Keneni et al., 2010). It was observed higher stress tolerant isolates both have higher nitrogenase activity and higher nos. of PGPR traits. Hence, there might be a relationship exists between abiotic stress tolerance, nitrogen fixation capacity and possession of PGPR traits of rhizobia.

4. Conclusion

16 high abiotic stress tolerant rhizobial isolates from mothbean, clusterbean and mungbean were recovered. The isolates also possessed nitrogenase activity and multiple PGPR traits. Hence, these isolates have enormous potential for bioinoculants of arid zone and can be used after pot and field trials.

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