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Effect of co-inoculations of native PGPR with nitrogen fixing bacteria on seedling traits in *Prosopis cineraria*

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Abstract

Prosopis cineraria significantly contribute to sand dune stabilization, soil fertility rejuvenation and is an integral component of agro-forestry systems in arid regions of India. Effect of different rhizobacterial seed treatments on seed germination and seedling traits in two genotypes of *P. cineraria* (HPY-1) and (FG-1) were tested. Observations on seed germination (%) and seedling traits viz., root length (cm), shoot length (cm), seedling weight (g) and seedling length of different treatments were recorded. Whereas, germination index (GI), seedling vigour index (SVI) and root/shoot length ratio were derived from the observed data. The scarification treatment with sulphuric acid for 10 minutes substantially enhanced germination from <20% to 80-82% in control treatments.Treatments with co-inoculations of *Bacillus licheniformis* and *Sinorhizobium kostiense* or *S. saheli* supported the maximum seed germination and seedling growth and vigour. The maximum germination per cent (92.5%), seedling length (10.94 cm), seedling vigour index (10.12) and germination index (7.97) were recorded with treatment (V2T6) wherein seeds of high pod yielding genotype were co-inoculated with *Bacillus licheniformis* and *S. kostiense*. The higher positive correlations of seedling length v/s shoot length followed by SVI v/s seedling length, SVI v/s root length and seedling length v/s root length is a fair indicative of inter dependency of these characteristics. Higher R^2 values of root length v/s shoot length followed by that of SVI v/s GI indicates that a regression line fits the data well and future outcomes of observed seedling traits are likely to be predicted by the model.

Key words

Germination index, *Prosopis cineraria*, Scarification, Seed treatment, Seedling vigour,

Introduction

Prosopis cineraria (L.) Druce grows naturally in the dry arid regions of India, Pakistan, Afghanistan, Iran, and Arabia. It is extremely drought tolerant and can withstand as little as 75 mm annual rainfall (F'FN, 1991). Slightly frost hardy and tolerant of high temperatures up to 50°C, it grows at altitudes from sea level to 600 m. The tree is found in alluvial and coarse, sandy, alkaline soils where pH may reach 9.8. Being a multipurpose leguminous tree it is a symbol of socio-economic development in Western Rajasthan as it provides fodder, fuel, vegetable, fruits, shelter, gum etc. It is significantly contributing towards sand dune stabilization, rejuvenation of soil fertility and is an integral component of agro-forestry systems (Kaushik and Kumar, 2003). Low and erratic rainfall combined with dry hot summers and cold dry winters restrict the build-up of soil organic matter and sandy loam soils are generally deficient in nitrogen (Kackar *et al*., 1990).

About 60% of the Rajasthan state falls within the desert region and covers the largest area (61%) of hot arid regions of the Indian Desert (DST, 1994). The natural rhizobia of wild legumes growing in arid zones exhibit higher tolerance to prevailing adverse conditions like salt stress, elevated temperature and drought (Sharma *et al*., 2013; Wei *et al*., 2008). Selection of effective, efficient and compatible stress tolerant rhizobial strains could help in ecological rehabilitation of degraded soils and increase soil fertility thereby improving the growth of associated plants of this region. However, only few studies on this aspect have been carried out in dry regions of Rajasthan (Ali *et al*., 2009; Gehlot *et al*., 2012; Mahobia and Mahna, 2002).

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Symbiotic nitrogen fixation is carried out by selective species of bacteria specific to particular legume species (Chandrasekar *et al*., 2005; Qureshi *et al*., 2009). Several authors have shown that it is possible to improve the growth of leguminous trees by inoculation with effective rhizobia (de Carvalho *et al*., 2012; Maia and Scotti, 2010). Aggarwal *et al*. (1993) observed higher available nitrogen (250 kg ha^{-1}) content in soil underneath *P. cineraria* than in the adjacent open soil.

Seed germination in *Prosopis* suffers with the seed coat-imposed dormancy (inhibition of germination imposed by the embryonic envelopes) which must be broken. Removal of or injury to the seed coat endocarp enables a non-dormant embryo to germinate upon imbibition of water and exchange of gases. A number of methods have been reported to improve seed germination (Dehagan *et al*., 2003; Linding and Lara-Carbera, 2004; Soleiman *et al*., 2008). The scarification treatment have been reported to be necessary to break seed dormancy in species of *Acacia* and *Prosopis* (Sarr *et al*., 2005; Vilela and Ravetta, 2001; Zare *et al*., 2011). Germination index values not only provide a good indication of dormancy but also facilitate differentiation of rate of germination among the varieties and treatments (Frančáková *et al.,* 2012). Seed vigor comprises those seed properties that determine the potential for rapid, uniform emergence, and development of normal seedlings under a wide range of field conditions (AOSA, 2002).

To exploit the potentials of native rhizobacteria in early stages of seedling establishment, studies were carried out to isolate PGPRs (Plant Growth Promoting Rhizobacterias) from rhizospheric soils of *Acacia senegal* and *P. cineraria*, improve seed germination by scarification and to find out their effect on per cent seed germination and seedling traits (Root length, shoot length, seedling weight, germination index, root/shoot length ratio, seedling length and seed vigour index) in two distinct genotypes of *P. cineraria*.

Materials and Methods

Bacteria and DNA isolation : One month old nursery raised plants of *P. cineraria* and *A. senegal* were procured from various nurseries of Jodhpur and Pali districts of Rajasthan in the year 2010. The roots were exposed and root nodules collected, washed under tap water and surface sterilized using 0.1% mercuric chloride ($HgCl_2$) for 5 min. To remove the traces of $HgCl_2$, the root nodules were rinsed in sterilized water several times and treated with 70% ethanol for 2 min and allowed to dry for 10 min. The treated nodules were then crushed with the help of sterilized micro-pestles, serially diluted to 10^{-6} dilution and 100 µl of the suspension was plated on to Yeast Extract Mannitol Agar (YEMA) supplemented with Congo Red (31.83 g l^{-1}; HiMedia Laboratories) using spread plate technique. The plates were incubated in BOD incubator at 25°C for 3 days. Isolated colonies were picked and transferred to YEMA slants in test tubes and incubated for 48 hrs at 25°C. The pure cultures were stored at 4°C until used.

Each bacterial culture isolated from root nodules was inoculated separately in conical flasks containing 50 ml of autoclaved Luria broth cultures medium (20 g L^{-1}; HiMedia Laboratories). The inoculated flasks were kept on to rotary shaker for 48 hr. The genomic DNA was isolated following HiPurATM Bacterial and yeast Genomic DNA purification spin kit (MB 505; HiMedia Laboratories) following manufacturer's protocols and protocol suggested by Birren and Lai (1993) and Sambrook *et al.* (1989).

Molecular identification of bacteria : The genomic DNA of each rhizobacterial strains was used for amplification and sequencing of 16S rRNA gene. The PCR primers EUB 1 (5' AGA GTT TGA TCC TGG CTC A 3') (19 bp) and EUB 2 (5' GCT CGT TGC GGG ACT TAT CC 3') (20 bp) were used to amplify the 16S ribosomal DNA. Amplification by PCR was performed in a total volume of 50 µl containing: 1U Taq DNA polymerase (Bangalore Genei), 2.5 mM $MgCl_2$, 160 µM dNTP mix (Bangalore Genei), 50 pmol of each EUB 1 and EUB 2 primers, 40 ng genomic DNA in dH_2O. The reactions were performed in a thermal cycler with following conditions: Initial denaturation for 2 min at 95°C, followed by 36 cycles (one min denaturation at 95°C, 30 sec annealing at 50°C, 1 min 20 sec elongation at 72°C) with a final elongation step of 72°C for 10 min. The PCR products were visualized on 1.6% agarose gel in 1X TAE buffer at 60 V for 10 min. Agarose gels were stained with ethidium bromide and photographed under UV light.

The PCR products were directly sequenced using EUB 1 (forward) and EUB 2 (reverse) primers by Big dye terminator method in ABI prism DNA sequencer. The sequenced data of EUB 2 reverse primer was inverted and complimented using Gene doc software and aligned with sequence data of EUB 1 primer to obtain continuous sequence of amplified products. Nucleotide sequence comparisons were performed using Basic Local Alignment Search Tool (BLAST) network services of National Centre for Biotechnological Information (NCBI), USA database to identify each culture up to species level and accordingly bacterial species designated.

Bacterial inoculation of *P. cineraria* genotypes : The seeds of two *P. cineraria* elite genotypes PCFG-1 (fast growing) and PCHPY-1 (high pod yielding) growing at Central Arid Zone Research Institute, Jodhpur, Rajasthan fields were collected in the month of January, 2010. The seeds were thoroughly washed with sterilized distilled water and subjected to scarification with sulphuric acid (98%) for 10 min. Again the seeds were washed several times with sterilized distilled water to remove the traces of H_2SO_4. The scarified seeds were then treated with different plant growth promoting bacteria separately and co-inoculated with phosphate solubilizing bacteria over night. The cultures of *Bacillus licheniformis* (strain AS-4) and *Sinorhizobium saheli* (AS-7)

isolated from *P. cineraria* and *S. kostiense* (PC-4) and *S. saheli* (PC-6) isolated from *P. cineraria* were raised on Luria broth culture medium on to a rotary sacker for 48 hrs. The seeds of both the elite genotypes of *P. cineraria* were soaked in 50 ml of bacterial cultures as per following treatments.

Bacterial strains and their combinations were noted as follows: T1= *Bacillus licheniformis* (strain AS-4); T2 = *Sinorhizobium saheli* (strain AS-7); T3 = *S. kostiense* (strain PC-4); T4= *S. saheli* (strain PC-6); T5= T1 + T2; T6= T1 + T3; T7= T1 + T4; T8 = un-inoculated control. Eight seeds per Petri plate were placed on top of the double layered filter paper impregnated with sterile distilled water. In all eight treatments had 40 seeds per treatment with five replications in randomized block design for each genotype. The Petri plates were exposed for 8 hrs to white light daily in an incubation room maintained at 25±2°C.

Germination observations : Observations were recorded for germination (%), root length (cm), shoot length (cm), seedling weight (g) and total seedling length, 24 hrs after inoculation up to 8 days. The germination index, root/shoot length ratio and seedling vigour index were derived from the observation data. The germination index (GI) was calculated from the results of the germination energy determination according to EBC (1998) method. Seedling vigour index was calculated according to ISTA (1996).

Results and Discussion

The molecular identification studies revealed that PGPR namely, *B. licheniformis* and *S. saheli* were found associated with the root nodules of *A. senegal* and *S. kostiense* and *S. saheli* with root nodules of *P. cineraria* in this region (Pancholy *et al*., 2012). Nick *et al*. (1999) also reported *S. kostiense* from leguminous trees from Sudan and Kenya. Boivin *et al*. (1997) reported *S. Saheli* from Senegal and Räsänen *et al*. (2001) reported *S. saheli* and *S. kostiense* from *Acacia* and *Prosopis* nodules in Sudan and Senegal.

A preliminary germination test resulted in very low germination of less than 20% in both the genotypes of *P. cineraria*. The scarification treatment with sulphuric acid (98%) for 10 min enhanced germination percentage in control treatments up to 80% in genotype (PC FG-1) and 82% in genotype (PC HPY-1). Owing to the lack of any other effective mode of regeneration in *Prosopis cineraria* except through seed, it is essential that seeds used in nursery raising or afforestation possess high germinability. Seed traits like seed weight and size have been found in other tree species like *Alangium lamarckii Thwaites* (Ahirwar, 2012), *Leucaena leucocephala* (Lam.) De Wit (Alam *et al*., 2005), and *Jatropha curcas* L. (Singh and Saxena, 2009) *Azadirachta indica* A. Juss (Kumar, 2007) to improve germination.

Most of the *Prosopis* species are characterized by hard and impermeable seed coat, which result in temporary dormancy and retard the germination process (Aref, 2000; Kassa *et al*., 2010; Vilela and Ravetta, 2001). The exogenous seed dormancy occurs when water and air are not permitted to enter the seed and seed fails to imbibe water and consequently resulting in decrease in seed germination (Bewley, 1997). Under present study, scarification treatment with sulphuric acid was able to break the exogenous seed dormancy and enhanced germination percentage. The enhancement in the germination is attributed to the fact that the sulphuric acid softened the hard seed coat of seeds and created a chap on the coat, as a result, germination improved by rendering seed coat porous that eventually

Table 1: Effect of different bacterial treatments on seedling and germination traits in *P. cineraria*

Treatment	Root length (cm)	Shoot length (cm)	Root/shoot ratio	Seedling weight (g)	Germination (%)	Seedling length (cm)	SVI	GI
V1T1	3.36	3.46	0.97	1.22	87.5	6.82	5.97	4.73
V1T2	2.76	2.62	1.05	1.17	90.0	5.38	4.84	5.00
V1T3	3.38	4.30	0.79	1.30	77.5	7.68	5.95	4.77
V1T4	3.62	4.40	0.82	1.41	85.0	8.02	6.82	5.47
V1T5	2.72	2.72	1.00	1.14	77.5	5.44	4.22	3.87
V1T6	3.92	4.48	0.88	1.29	90.0	8.4	7.56	7.00
V1T7	3.78	5.06	0.75	1.48	82.0	8.84	7.23	5.90
V1T8	2.68	2.90	0.92	1.16	80.0	5.58	4.46	3.83
V2T1	3.76	4.98	0.76	1.46	85.0	8.74	7.43	4.73
V2T2	3.18	3.86	0.82	1.40	87.5	7.04	6.16	4.93
V2T3	3.60	4.66	0.77	1.39	82.0	8.26	6.77	4.70
V2T4	3.98	5.04	0.79	1.43	85.0	9.02	7.67	5.27
V2T5	3.46	4.48	0.77	1.27	92.5	7.94	7.34	5.17
V2T6	5.26	5.68	0.93	1.66	92.5	10.94	10.12	7.97
V2T7	5.00	5.96	0.84	1.84	87.5	10.96	9.59	6.43
V2T8	3.34	3.94	0.85	1.22	82.0	7.28	5.97	4.60
CD 5%	0.83	1.09	-	0.89	0.99	-	-	-

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facilitated absorption of water. Further, remarkable increase in germination percentage in control treatments validate that *P. cineraria* was suffering from exogenous seed dormancy which can be removed by scarification.

The maximum root length (5.26 cm), shoot length (5.68 cm), seedling weight (1.66 g), germination per cent (92.5%), seedling length (10.94 cm), seedling vigour index (10.12) and germination index (7.97) were recorded with treatment (V2T6) wherein seeds of genotype (PC HPY-1) were co-inoculated with *Bacillus licheniformis* (AS-4) and *S. kostiense* (PC-4). Similarly, the maximum root length (3.92 cm), per cent germination (90%), seedling vigour index (7.56) and germination index (7) were recorded with treatment (V1T6) wherein seeds of genotype (PC FG-1) were co-inoculated with *B. licheniformis* (AS-4) and *S. kostiense* (PC-4). Treatment T7 wherein seeds of both the genotypes were co-inoculated with *B. licheniformis* (AS-4) and *S. saheli* (PC-6) positively influenced the root length, shoot length, seedling weight, seedling length, seedling vigour index and germination index. The data (Table 1) revealed that the rhizobacterial treatments T6 and T7 had positive effect on the phenotypic traits of the germination in both the genotypes of *P. cineraria*. The genotype (PC HPY-1) of *P. cineraria* performed better than genotypes (PC FG-1).

Significantly superior effect of T6 and T7 treatments irrespective to *P. cineraria* genotypes under present investigation were due to co-innoculations of *B. licheniformis* with either S. *kostiense* or S. *saheli* wherein *B. licheniformis*, a known PGPR supplied physiologically active gibberellins and *Sinorhizobium* species have established role of atmospheric nitrogen fixation (Manero *et al*., 2001). Gibberellic acid seed treatment have been reported to enhance growth in lupine (Dehagan *et al*., 2003) Further, Drozdowicz and Santos (1987) reported that the interaction of nitrogen fixing bacteria with other bacteria can inhibit or promote their diazotropic activity. The synergistic effect of application of mixed bio-inoculants as PGPRs or bio-control agents have proved more remunerative to improve plant health (Ladwal *et al*., 2012; *Saber et al., 2012*). The higher SVI and GI values of T6 and T7 treatments validated the concept and synergistic effects of co-inoculations of PGPRs with nitrogen fixing bacteria under present investigation.

The data on phenotypic correlation matrices revealed that there was the maximum positive correlation between seedling length and shoot length (0.985) followed by SVI and seedling length (0.978), SVI and root length (0.977) and seedling length and root length (0.971). The minimum positive correlation of 0.126 was recorded between germination percentage and root/shoot length ratio (Table 2). The higher positive correlations of seedling length v/s shoot length followed by SVI v/s seedling length, SVI v/s root length and seedling length v/s root length is a fair indicative of inter dependency of these characteristics and predictability with time (Guler *et al*., 2001; Singh and Singh, 2012).

The analysis of variance presented in Table 3 indicates that most of the characters for different treatments were highly

Table 2: Correlation matrix of germination properties

	Root length (cm)	Shoot length (cm)	Root/Shoot ratio	Seedling weight (g)	Germination (%)	Seedling length (cm)	SVI	GI
Root length (cm)	1.000							
Shoot length (cm)	0.914	1.000						
Root/shoot ratio	-0.302	-0.656	1.000					
Seedling weight (g)	0.900	0.893	-0.408	1.000				
Germination (%)	0.459	0.305	0.126	0.315	1.000			
Seedling Length	0.971	0.985	-0.519	0.915	0.378	1.000		
SVI	0.977	0.943	-0.413	0.888	0.558	0.978	1.000	
GI	0.855	0.701	-0.083	0.679	0.646	0.782	0.849	1.000

Table 3 : Mean sum of square for different seedling traits in *P. cineraria*

Source of variation	Df	Root length	Shoot length	Seedling weight	Germination (%)	GI	R/S Ratio	Seedling length (cm)	SVI
Replication	4	1.24	1.83	0.03	319.34	3.68	0.06	4.67	0.73
Total treatment	15	2.57**	4.92**	0.18**	111.98	5.97**	0.04	13.99**	12.86**
Variety	1	8.98**	23.44**	0.69**	195.31	3.26*	0.10	61.43**	60.07**
Treatment	7	3.80**	6.68**	0.24**	132.81	11.52**	0.02	20.03**	17.49**
V x T	7	0.43	0.51	0.04*	79.24	0.80	0.05	1.18	1.47
ERROR (C)	60	0.45	0.77	0.02	100.59	0.84	0.03	1.92	1.74
TOTAL	79								

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significant in both the genotypes of *P. cineraria* studied except germination (%) and root/shoot length ratio. Interestingly both the varieties did not show any significant difference with regards to different rhizobacterial treatments and/or for seed germination and seedling traits except for seedling weight.

The maximum coefficient of determination (R^2) value of 0.9272 was recorded from linear equation of root length v/s shoot length, which was followed by the value of 0.7393 from data of SVI v/s GI. Whereas, the least R^2 value of 0.3192 was recorded from linear equation of germination (%) v/s SVI. The coefficient of determination (R^2) is most often seen as a number between 0 and 1, used to describe how well a regression line fits a set of data (Hafez and Kobata, 2012). An R^2 near 1 indicates that a regression line fits the data well, while an R^2 closer to 0 indicates a regression line does not fit the data very well. R^2 represented as squared correlation between the outcomes and the values of the single regressor were used for prediction (Fig. 1-3). Accordingly, higher values of R^2 (0.9272) of root length v/s shoot length followed by that of 0.7393 obtained from the linear equation of SVI v/s GI indicates that a regression line fits the data well and future outcomes of observed seedling traits are likely to be predicted by the model i.e. increase in root length is directly proportional to shoot length and SVI is directly proportional to GI per unit time.

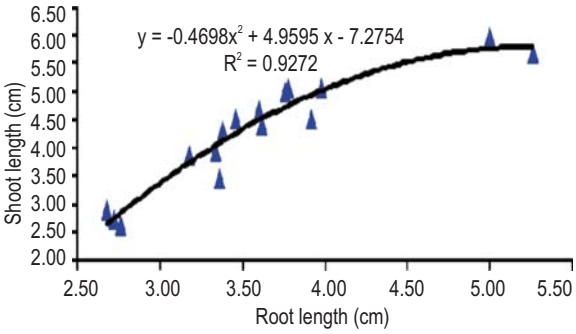

Fig. 1 : Effect of different treatments with rhizobacteria on inter-relationship of root length and shoot length in *P. cineraria*

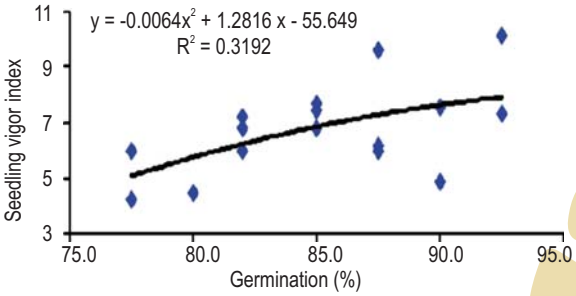

Fig. 2 : Effect of different treatments with rhizobacteria on inter-relationship of germination percentage and seedling vigour index in *P. cineraria*

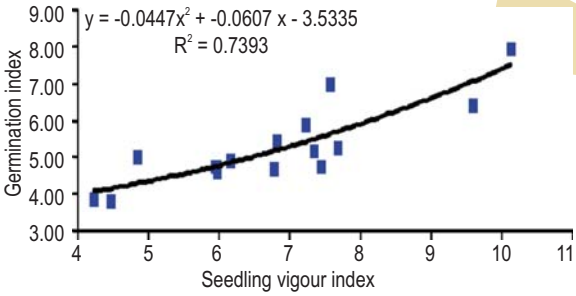

Fig. 3 : Effect of different treatments with rhizobacteria on inter-relationship of seedling vigour index and germination index in *P. cineraria*

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