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# Effect of plant growth promoting rhizobia on seed germination and seedling traits in *Acacia senegal*

**S.K. Singh, A. Pancholy, S.K. Jindal, R. Pathak**

**Singh S.K., Pancholy A., Jindal S.K., Pathak R.,** 2011. Effect of plant growth promoting rhizobia on seed germination and seedling traits in *Acacia senegal*. Ann. For. Res. 54(2): 161-169, 2011.

**Abstract.** Among arid zone tree species, *Acacia senegal* and *Prosopis cineraria* are the most important dryland resources of Western Rajasthan desert ecosystem. Due to ecological, biological and molecular similarities, they are often studied together. The climatic conditions in this region restrict the build-up of soil organic matter and soils are generally deficient in nitrogen. Studies were carried out to isolate and molecularly characterize the diverse group of plant growth promoting rhizobacteria from root nodules of native *A. senegal* and *P. cineraria* and their effect on seed germination and seedling traits in two genotypes of *A. senegal*. The direct sequencing of 16S rDNA region resulted in molecular identification of plant growth promoting rhizobacteria as *Bacillus licheniformis, Sinorhizobium saheli* isolated from root nodules of *A. senegal* and *S. kostiense* and *S. saheli* isolated from root nodules of *P. cineraria*. The partial sequences of 16S rDNA were assigned Gen accession numbers HQ738496, HQ738499, HQ738506 and HQ738508. Scarification treatment with sulphuric acid (98%) for 15 minutes was able to break the exogenous seed dormancy and enhanced germination percentage in control treatment to 90% and 92.5% in *A. senegal* in genotypes CAZRI 113AS and CAZRI 35AS, respectively. The treatments with *Bacillus licheniformis* or *S. kostiense,* either inoculated individually or as coinoculants, had positive effect on phenotypic traits of germination. Two *A. senegal* genotypes exhibited significant differences with regard to all the phenotypic traits. On the other hand, treatments with *S. saheli* isolated from either *A. senegal* or *P. cineraria* had negative effects on germination and related phenotypic traits. Values of the coeffivient of determination ($R^2$) over 80% for root length versus shoot length, root/shoot ratio and seedling weight respectively validate that the observed attributes are inter-dependable and linear progression trend can be predicted.
**Keywords** *Acacia senegal, Prosopis cineraria*, scarification, plant growth promoting rhizobacteria, inoculation, germination.

**Author.** Sunil Kumar Singh, Anjly Pancholy, S.K. Jindal, Rakesh Pathak (sksingh1111@hotmail.com) - Central Arid Zone Research Institute, Jodhpur, Rajasthan 342 003, India.
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## Introduction

Among arid zone tree species, *Acacia senegal* (L.) Willd. and *Prosopis cineraria* (L.) Druce are the most important dryland resources of Western Rajasthan desert ecosystem (Tewari et al.1998, Jindal et al. 2000). *A. senegal* is highly drought tolerant multipurpose African tree species and is an important forest resources of gum arabic, fuel wood, human food and fodder for livestock (FAO & UNEP 1983, Dondain & Phillips 1999, Aoki et al. 2007). Recently it is being cultivated in India, Pakistan and Nigeria in arid and semi-arid regions under extreme temperatures (-4°C to 48°C). Whereas, *P. cineraria* is multipurpose leguminous tree, grows very well in dry land agroforestry systems and plays an important role in controlling soil erosion, sand dune stabilization, improving soil fertility, providing fuel energy resources, supplying feed and forage for grazing animals, fire wood, furnishing timber and furniture wood and supplementing food for humans (Tewari et al. 1998, Manzano & Navar 2000, Zare et al. 2011). Due to ecological and biological similarities, *Acacia* and *Prosopis* are very often studied together (Bukhari 1997). The molecular data also suggest that they are closely related (Bukhari et al. 1998).

The climate of western Rajasthan desert ecosystem is often characterized by hot, dry summers, sub-humid monsoon and cold dry winter. The soil is sandy loam with pH > 8.1 and low nutrient levels, with 0.23% organic carbon, 0.03% nitrogen and 0.02% phosphorus (Dhir 1984). The climatic conditions in this region restrict the build-up of soil organic matter and soils are generally deficient in nitrogen (Kackar et al. 1990). 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 (Wolde-Meskel & Sinclair 1998, Bogino et al. 2006, Maia & Scotti 2010). Our knowledge of the symbiotic affinities among tropical tree rhizobia is still limited.

Further, due to hard seeds germination in these most economically and ecologically important species in arid and semi-arid areas is a problem. A number of researchers have investigated methods for breaking seed dormancy to improve seed germination (Dehagan et al. 2003, Linding & Lara-Carbera 2004, Soleiman et al. 2008, Fedrico & Mollard 2009, Zare et al. 2011). The scarification treatment have been reported to be necessary to break seed dormancy in species of *Acacia* and *Prosopis* (Vilela & Ravetta 2001, Sarr et al. 2005, Zare et al. 2011).

The present study was carried out to (i) isolate and molecularly characterize the diverse group of plant growth promoting rhizobacteria (PGPR) from root nodules of native *A. senegal* and *P. cineraria*, (ii) break exogenous seed dormancy following scarification, and (iii) subsequently inoculate with PGPR to find out their effect on seed germination and seedling traits in two genotypes of *A. senegal*.

## Materials and methods

### Isolation of bacteria

Nursery raised plants of *A. senegal* and *P. cineraria* were procured from various nurseries of Jodhpur and Pali districts of Rajasthan and brought to the laboratory during rainy season in the year 2010. The roots were exposed and root nodules were collected, thoroughly washed under tap water and surface sterilized using 0.1% Mercuric Chloride ($HgCl_2$) for 5 minutes. The sterilized root nodules were then rinsed in sterilized water several times to remove the traces of $HgCl_2$. These nodules were treated with 70% ethanol for 2 minutes and allowed to dry for 10 minutes. The nodules were then crushed with the help of sterilized micropestles, serially diluted and 100 µl of the suspension was plated on to Yeast Extract Man-

nitol Agar (YEMA) supplemented with Congo Red (31.83 g/litre; HiMedia Laboratories) using spread plate technique. The plates were incubated at room temperatures (25-30°C) for 5 days. Isolated colonies were picked and transferred to YEMA slants in test tubes and incubated for 5 days at room temperatures. The pure cultures were stored at 4°C until used.

### DNA isolation

Each bacterial culture isolated from root nodules was inoculated separately in conical flasks containing 50 ml of autoclaved Luria broth cultures medium (20 g/Litre; HiMedia Laboratories, Mumbai, India). The inoculated flasks were kept on to rotary shaker for 48 hours. The genomic DNA was isolated following HiPurA™ Bacterial and yeast Genomic DNA purification spin kit (MB 505; HiMedia Laboratories, Mumbai, India) following manufacturer's protocols.

### Molecular identification of bacteria

The genomic DNA of each bacterial 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, 50 ng genomic DNA in $dH_2O$. The reactions were performed in a gradient thermal cycler with following conditions: one min denaturation at 95°C, 30 s annealing at 50°C, 1 min 20 s elongation at 72°C for 36 cycles with a final elongation step of 72°C for 10 min. The PCR products were visualized on 1.5% agarose gel in 1X TAE buffer at 60 V for 100 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 BLAST network services of NCBI, USA database to identify each culture up to species level and accordingly bacterial species designated.

### Bacterial inoculation of *A. senegal* genotypes

The seeds of two *A. senegal* elite genotypes CAZRI 35AS and CAZRI 113AS grown at Central Arid Zone Research Institute, Jodhpur, Rajasthan field 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 15 minutes. 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 coinoculated with phosphate solubilizing bacteria over night. The cultures of *Bacillus licheniformis* (strain AS-4) and *Sinorhizobium saheli* (AS-7) isolated from *A. senegal* 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 hours. The seeds of both the elite genotypes of *A. senegal* were soaked in 50 ml of bacterial cultures as per following treatments.

Bacterial strain and mixtures were noted as follows: T1 = *Bacillus licheniformis* (AS-4); T2 = *Sinorhizobium saheli* (AS-7); T3 = *S. kostiense* (PC-4); T4 = *S. saheli* (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 hours 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 hours after inoculation up to 8 days. The germination energy index and root/shoot ratio were derived from the observation data.

## Results

In order to identify bacteria strains, we use EUB1 (Forward) and EUB2 (Reverse) universal primers to amplify 16S rDNA region from genomic DNA, verified as single prominent band on to agarose gel. After DNA sequencing, the nucleotide sequence comparisons with the available sequences were performed using Basic Local Alignment Search Tool (BLAST) network services of NCBI, USA database which resulted in the maximum score of identities ranging from 1640-1709 with 99 to 100% identities. The direct sequencing of amplified PCR products resulted in molecular identification of growth promoting bacteria as *Bacillus licheniformis* (AS-4, 990 bp); *Sinorhizobium saheli* (AS-7, 928 bp) isolated from root nodules of *A. senegal* and *Sinorhizobium kostiense* (PC-4, 918 bp) and *Sinorhizobium saheli* (PC-6, 925 bp) isolated from root nodules of *P. cineraria*. The partial sequences of 16S rRNA gene of all these four strains were submitted to NCBI, USA, database and Gen accession numbers HQ738496, HQ738499, HQ738506 and HQ738508 assigned to strain AS-4, AS-7, PC-4 and PC-6, respectively.

The inoculation of different bacterial strains and their mixtures significantly influenced seed germination traits. The results of effect of

**Table 1** Effect of different bacterial treatments on seed germination and phenotypic traits in *A. senegal*

| Treatment | Root length (cm) | Shoot length (cm) | Seedling weight (g) | Germination (%) | Germination Energy Index | Root/shoot ratio |
|---|---|---|---|---|---|---|
| V1T1 | 0.94 | 1.28 | 1.63 | 85.00 | 83.93 | 0.73 |
| V1T2 | 0.24 | 0.24 | 1.09 | 15.00 | 14.29 | 1.00 |
| V1T3 | 1.14 | 1.38 | 1.84 | 85.00 | 78.92 | 0.83 |
| V1T4 | 0.72 | 1.20 | 1.45 | 52.50 | 51.78 | 0.60 |
| V1T5 | 0.22 | 0.30 | 1.01 | 15.00 | 13.21 | 0.73 |
| V1T6 | 2.88 | 1.64 | 2.26 | 95.00 | 93.92 | 1.76 |
| V1T7 | 1.18 | 1.02 | 1.46 | 85.00 | 83.57 | 1.16 |
| V1T8 | 1.02 | 1.06 | 1.65 | 90.00 | 84.64 | 0.96 |
| V2T1 | 3.62 | 2.14 | 2.78 | 92.50 | 91.78 | 1.69 |
| V2T2 | 1.04 | 1.28 | 2.21 | 77.50 | 75.35 | 0.81 |
| V2T3 | 3.70 | 2.28 | 2.75 | 97.50 | 97.14 | 1.62 |
| V2T4 | 3.42 | 1.90 | 2.70 | 95.00 | 92.14 | 1.80 |
| V2T5 | 1.82 | 1.80 | 2.64 | 87.50 | 84.28 | 1.01 |
| V2T6 | 4.68 | 2.62 | 3.01 | 97.50 | 97.50 | 1.79 |
| V2T7 | 2.96 | 1.60 | 2.61 | 92.50 | 91.42 | 1.85 |
| V2T8 | 3.38 | 1.90 | 2.25 | 92.50 | 90.00 | 1.78 |
| CD 5% | 0.56 | 0.44 | 0.42 | 0.95 | 11.81 | |

V1 = CAZRI 113AS, V2 = CAZRI 35AS

different bacterial treatments on seed germination and related phenotypic traits in two genotypes of *A. senegal* are shown in table 1. After 8 days of inoculation the results of phenotypic traits showed the maximum root length (2.96 cm), shoot length (2.62 cm), seedling weight (3.01 g) and 97.5% germination with the highest germination energy index of 97.5 in treatment V2T6 wherein seeds of genotypes (CAZRI 35AS) were coinoculated with *Bacillus licheniformis* and *S. kostiense*. Similarly, the maximum root length (2.88 cm), shoot length (1.64 cm), seedling weight (2.26 g) and 95% germination with the highest germination energy index of 93.92 was recorded from the treatment V2T6, wherein seed of genotypes (CAZRI 113AS) were coinoculated with *B. licheniformis* and *S. kostiense*. The data revealed that the treatments with *B. licheniformis* or *S. kostiense* either inoculation individually or as coinoculants had positive effect on phenotypic traits of germination. The *A. senegal* genotypes exhibited significant differences with regard to all the phenotypic traits and genotype (CAZRI 35AS) performed better than genotype (CAZRI 113AS). Whereas, treatments with *S. saheli* isolated from either *A. senegal* or *P. cineraria* had negative effects on germination and related phenotypic traits.

The results of analysis of variance (Table 2) indicated that there were significant differences (at 5% level) not only amongst treatments but also between two genotypes of *A. senegal* with regard to germination traits. In addition, interaction of different treatments on genotypes led to highly significant and meaningful differences among germination properties except for shoot length.

The phenotypic correlation matrix of germination properties are shown in table 3. The data shows that there was the maximum positive correlation between shoot length and seedling weight (0.94) followed by shoot and root length (0.92) and root/shoot ratio (0.91). The minimum correlation of 0.58 was recorded between germination percentage and root/

**Table 2** Analysis of variance between different traits in *A. senegal*

| | df | Germination (%) | Germination Energy Index | Root length (cm) | Shoot length (cm) | Seedling weight (g) | Root/ shoot ratio | Total seedling length (cm) |
|---|---|---|---|---|---|---|---|---|
| Replicates | 4 | 71.29 | 94.93 | 0.21 | 0.1 | 0.24 | 0.22 | 0.38 |
| Genotypes | 1 | 13781.25*** | 14493.34*** | 82.83*** | 17.11*** | 22.89*** | 5.81*** | 192.79*** |
| Treatments | 7 | 3793.53*** | 3805.02*** | 8.94*** | 1.91*** | 0.91*** | 0.96*** | 15.32*** |
| G x T | 7 | 2049.11*** | 1824.61*** | 1.1*** | 0.19 | 0.26* | 0.58*** | 1.26* |
| Error | 60 | 88.997 | 87.19 | 0.2 | 0.12 | 0.11 | 0.125 | 0.4 |
| Total | 79 | 763.351 | 753.313 | 2.1 | 0.50 | 0.49 | 0.316 | 4.22 |

Note: * - significant at 5% ($p < 0.05$), ** - significant at 10% ($p < 0.01$), *** - significant at 1% ($p < 0.001$)

**Table 3** Correlation matrix of germination properties

| | Root length (cm) | Shoot length (cm) | Root/shoot ratio | Seedling weight (g) | Germination (%) |
|---|---|---|---|---|---|
| Root length (cm) | 1.000 | | | | |
| Shoot length (cm) | 0.916 | 1.000 | | | |
| Root/shoot ratio | 0.911 | 0.699 | 1.000 | | |
| Seedling weight (g) | 0.898 | 0.936 | 0.752 | 1.000 | |
| Germination (%) | 0.700 | 0.825 | 0.577 | 0.774 | 1.000 |

shoot ratio. The relationships between the root length, on the one hand and the shoot length, root/shoot ratio, seedling weight and germination (%), on the other hand, are shown in the figure 1. The $R^2$ has values over 80% except for root length versus germination (%).

## Discussion

Most *Acacia* species are characterized by a very hard and impermeable seed coat, which results in temporary dormancy and influences the germination process (Owens et al. 1995, Argaw et al. 1999, Aref 2000, Kassa et al. 2010). 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). Occurrence of this kind of dormancy is because of hard seed coat and the pressure forces resulting from meager water absorption and radicle growth remains insufficient to break dormancy of seeds.

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. Nick et al. (1999) also reported *S. kostiense* from leguminous trees from Sudan and Kenya, as well as Räsänen et al. (2001) reported *S. saheli* and *S. kostiense* from *Acacia* and *Prosopis* nodules in Sudan and Senegal.

A preliminary germination test was performed and very low germination of less than 20% was obtained in both the genotypes of *A. senegal*, indicating that seeds were facing dormancy. Under present study, scarification treatment with sulphuric acid (98%) for 15 minutes was able to break the exogenous seed dormancy and enhanced germination percentage in control treatment up to 90% and 92.5% in *A. senegal* in genotypes CAZRI 113AS and CAZRI 35AS, respectively. The enhancement in the percent germination is attributed to the fact that the sulfuric acid softened the hard seed coat of seed and created a chap on the

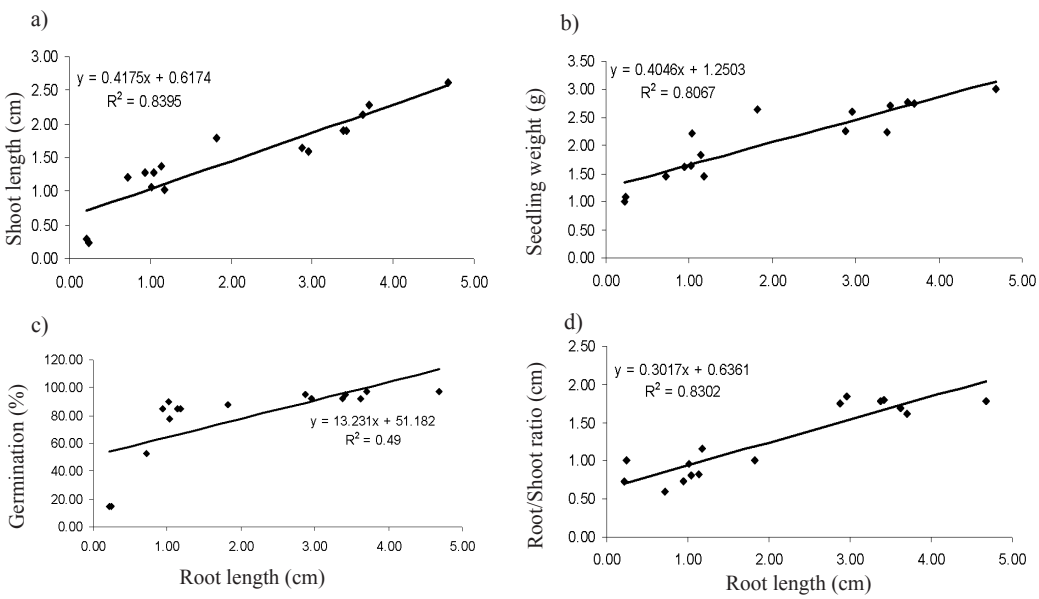


**Figure 1** Relationships between root length and shoot length (a), seedling weight (b), germination (%)(c), root/shoot ratio (d), in germinating seedling of two genotypes of *A. senegal*

coat, as a result, seed treatment with sulphuric acid improved germination.

We observed *B. licheniformis*, *S. saheli* from root nodules of *A. senegal* and *S. kostiense* and *S. saheli* from *P. cineraria* were isolated, owing to the prevalence of these PGPR in their natural habitats. Among the various bacterial inoculation treatments used, the treatments with inoculation of *B. licheniformis* or *S. kostiense* alone and as coinoculants supported the maximum growth of the seeds and were significantly superior to other treatments. It is attributed to the fact that *Bacillus licheniformis* produces gibberellins and that had positive effects on seed germination and seedling traits. Manero et al. (2001) reported that the PGPR *Bacillus pumilus* and *Bacillus licheniformis* produce high amounts of physiologically active gibberellins.

Similar to our study, the growth effect of rhizobia inoculation on some tree species like *Acacia ariculiformes*, *A. mangium*, *Centrolobium tomentosum*, *Dalbergia nigra*, *Inga oestediana* and others has been tested and results were satisfactory (Dela-Cruz et al. 1988, Goncalves et al. 1995, Marques et al. 1997, Santiago et al. 2002, Grossman et al. 2006).

The interaction of $N_2$ fixing bacteria with other bacteria can inhibit or promote their diazotropic activity (Drozdowicz & Santos 1987). In agriculture and forestry inoculation practices mixing of two or more microbial species have been reported to have more positive effect on plant growth than the use of single bacterium (Alagawadi & Gaur 1992, Belimov et al. 1995, Elshanshoury 1995). Under present investigation treatments with coinoculation of phosphate solubilizing bacteria *B. licheniformis* with *S. kostiense* ($N_2$- Fixer) had synergistic effect on seed germination attributes. Whereas, coinoculation of *S. saheli* ($N_2$- fixer) with the same strain of *B. licheniformis*, had negative effect on seed germination attributes. Räsänen et al. (2001) also demonstrated the symbiotic properties of *Sinorhizobia* isolated from *Acacia* and *Prosopis* nodules in Sudan and Senegal. Synergism between *Phyllobacterium* species ($N_2$-fixer) and *Bacillus licheniformis* (P-solubilizer), both from a semi arid mangrove rhizosphere have been demonstrated by Rojas et al. (2001). Our study demonstrates synergistic effect between *B. licheniformis* and *S. kostiense* which had positive effects on phenotypic traits of germination. Whereas, mixed inoculation treatments with *S. saheli* and *B. licheniformis* had negative effects on phenotypic traits of germination. The values of the coefficient of determination ($R^2$) greater than 80% for the relationships between the root lengh, one hand, and length, root/shoot ratio and seedling weight, on the other hand, validate that most of the observed germination attributes are interdependable and linear progression trend can be predicted. This study presents an important significance for laboratory experimentations and for reckoning regression coefficient values before large scale nursery/field inoculations with mixtures of PGPRs.

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