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Species Specific *Rhizobium* Inoculation on Seedling Growth of *Albizia lebbeck* and *Acacia catechu* Under Water Stress Conditions

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ABSTRACT
Background: Diverse group of rhizobia nodulate tree legumes. These nodulating bacteria have great potential with these tree legumes which are usually exposed to arid or water stress environmental conditions. Plant Growth Promoting Rhizobacteria (PGPR) can have positive effects on vigor and productivity, especially under stress conditions. **Methods:** The effect of water stress on four selected strains of *Rhizobium* species isolated from *Albizia lebbeck*, *Delbergia sissoo*, *Acacia catechu* and rhizospheric soils of legume crops were studied on *A. lebbeck* and *A. catechu* seedlings growth. The water stress (0, -3, -5, -8 and -10 bars) were obtained using Polyethylene glycol 6000 (PEG-6000) solutions. **Results:** A decrease in water potential produced a marked reduction in germination percentage, seedling germination and seed vigor index. Out of the four isolates tested, *Rhizobium* isolated from *A. lebbeck* and *A. catechu* performed better in respect of germination percentage, Seedling Vigor Index (SVI), root and shoot length, number of leaves, secondary root formation and total biomass yield, compared to other strains. **Conclusion:** Growth responses varied between the different strains, these differences in germination ability of *A. lebbeck* and *A. catechu* might be attributed to intraspecific interaction resulting from the effects of natural selection and selected strains could assist in the rehabilitation of agroforestry by overcoming the effect of water stress.

Key words: *Rhizobium*, *A. lebbeck*, *A. catechu*, water stress, germination

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INTRODUCTION
Leguminous nitrogen fixing tree species can play a major role in improving productivity of degraded forest soils. Nitrogen fixing tree are important in afforestation of degraded lands and are widely grown in India. These stabilize sandy and eroded soil and exploit deep underground water by virtue of its extensive root system[1].

Of the different factors limiting the forest productivity, drought is the major important one, affecting the world food security and sustainability in agricultural production. The microbiotic, abiotic and biotic factors can all manipulate *Rhizobium* populations[2] but it is the abiotic cause which is the most acknowledged and of these, drought is often quoted as one of the most imperative factors potentially limiting the *Rhizobium*-legume symbiosis[3]. Rhizobia are affected by water stress in two ways, during drying and in any following rewetting phase. During drought, the thickness of water films around soil particles and the neck diameter of water filled pores decrease. As rhizobia are motile, this affects their movement and size reduction[4].

The legume-*Rhizobium* symbiosis in general is known to be more sensitive to environmental stress (especially drought) than the uninfected legume[5], suggesting that *Rhizobium* is the more sensitive of the two partners. In forest tree practices, seed inoculation with *Rhizobium* is normally not adopted in the areas where *A. lebbeck* or *A. catechu* and other leguminous tree species are growing luxuriantly due to good inoculation while in the wasteland area the microbial population is supposed to be very low or totally absent, the practice of artificial inoculation of *Rhizobium* from the strain of same species will give better results by boosting the rhizobial population of such area and to achieve healthy plant growth[6]. A large number of studies have been carried out on the effects of water stress on the germination of forest tree species However, in none of the studies was the

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selection of *A. lebbeck* or *A. catechu* with *Rhizobium* species under different water potential conditions. Keeping the above in view, the present investigation was undertaken to study the effects of *Rhizobium* inoculation on *A. lebbeck* and *A. catechu* seedling germination under decreased water potential conditions imposed by PEG-6000.

MATERIALS AND METHODS

Isolation of test bacteria: Rhizobia were cultured from nodules from a minimum of five seedlings each of *Albizia lebbeck*, *Acacia catechu*, *Delbergia sisso* by surface-sterilizing each nodule with 5% hypochlorite, crushing and smearing onto Yeast Extract Mannitol (YEM) agar medium and incubating the plates at 25°C for 3 days. The cultures were transported to Aberdeen on mannitol agar slopes and cultured again on mannitol agar plates. A single colony microscopically identified as rhizobial after staining was excised from each plate to establish pure cultures (tested by streak plating and replicate plating on mannitol agar). Stock cultures were made up from these isolates to provide the inocula for further studies. The four bacterial strains (fourth one isolated from the rhizosphere of leguminous crop soil) used in this study were selected on the basis of nitrogen fixation and phytohormone Indole Acetic Acid (IAA) production (Table 1).

Seed sterilization and incoculation: The healthy and uniform seeds of *A. lebbeck* and *A. catechu* were sterilized by exposing to 70% alcohol in a beaker for one minute and then dipping in 0.2% $HgCl_2$ solution for 50 sec, finally washed 5 times with sterile distilled water. The surface sterilized seeds were treated with different *Rhizobium* isolates (T1-*Albizia* isolate, T2-*Dalbergia* isolate, T3-*Acacia* isolate and T4-rhizosphere isolate), all the isolates were grown in YEM broth having count of 10^9 cfu mL^{-1}.

Experimental setup: Polyethylene glycol 6000 (PEG), HiMedia, Mumbai, India has been used in this study because of its ability as nonpermeating solute to lower the external water potential without penetrating the cell wall. The PEG was dissolved in sterile distilled water to prepare osmoticum solution of -3.0, -5.0, -8.0 and -10.0 bars water potential as per the method of [7]. Three replicates of 20 seeds of each cultivar were germinated in two rolled Whatman filter papers with 10 mL of respective test solutions. The papers were replaced every 2 days to prevent accumulation of salts[8]. In order to prevent evaporation, each rolled paper was put into a sealed plastic bag. Seeds were allowed to germinate at 20±1°C in the dark for 16 days. To determine the toxic effects of the PEG solutions on germination, non-germinated seeds in each treatment were transferred to distilled water and counted for an additional 3 days. Germination percentage was recorded every 24 h for 9 days. For interpretation of results, average mean data has been used in the present study. Seedling Vigor Index (SVI) was calculated by multiplying germination (%) and seedling length (mm). Each treatment was analyzed with at least three replicates and Standard Deviation (SD) was calculated using Microsoft excel program.

Table 1: Nitrogenease activity and IAA production by *Rhizobium* isolates used in present study

Rhizobial isolates	Nitrogenase activity (nmole C_2H_2 h^{-1} mg^{-1} protein)	IAA production (µM)
T1	65.4	73.3
T2	67.6	67.4
T3	59.5	79.2
T4	47.9	65.7

Table 2: Effect of rhizobial isolates and moisture stress levels on germination (%) of *A. lebbeck* and *A. catechu*

Rhizobial isolates	Stress levels (bars)	*A. lebbeck* (day)		*A. catechu* (day)	
		3	9	3	9
T1	-3	50.3±5.6	90.3±7.1	76.7±4.2	94.0±9.1
	-5	42.6±5.1	87.0±6.5	71.2±6.4	90.0±3.7
	-8	24.3±3.4	62.3±4.3	66.6±7.3	85.4±5.8
	-10	18.7±3.0	55.5±4.7	62.4±3.5	82.2±7.3
T2	-3	44.6±4.2	88.3±8.3	80.2±8.3	96.6±8.2
	-5	36.3±3.8	76.0±6.4	76.5±4.8	93.0±4.7
	-8	20.3±3.4	62.3±4.2	70.0±6.2	90.5±6.9
	-10	10.5±2.1	50.6±3.8	67.7±4.3	85.5±5.2
T3	-3	38.6±3.9	72.4±7.0	82.6±5.7	100.0±9.5
	-5	30.3±3.1	60.5±6.8	79.3±4.2	97.7±7.1
	-8	24.6±2.7	52.8±5.3	73.6±6.2	90.2±7.6
	-10	1.0±0.2	38.4±4.9	70.2±4.0	90.5±6.7
T4	-3	30.0±3.8	63.3±6.8	73.0±6.9	90.2±6.7
	-5	20.2±3.0	52.8±6.2	70.2±4.7	84.6±5.6
	-8	16.6±2.4	45.0±3.8	64.8±5.6	82.2±6.4
	-10	5.3±1.1	32.3±4.1	62.0±4.2	78.6±5.7
Control	-0	40.3±4.3	84.6±7.5	72.5±6.1	92.5±8.6

Statistical method: The standard deviation and mean in the tables have been calculated using Microsoft excel.

RESULTS

In this study, different *Rhizobium* isolates were tested with seeds of *A. lebbeck* and *A. catechu* under various water potential levels. The criterion for seed germination was taken as the emergence of 2 mm radicle at the time of observation. The germination percentage was found to decrease with increase in moisture stress levels in *Albizia* and *Acacia* species (Table 2). Transfer of non-germinated seeds from PEG solution to the distilled water resulted in 100% germination regardless of osmotic potential (data not shown), therefore, It showed that PEG was non toxic to seeds. The germination of seedlings of both the species were more with rhizobial isolates from same plant species i.e., *A. lebbeck* by T1 and *A. catechu* by T3 rhizobial isolate (Table 2). *Rhizobium* isolated from test species i.e., *A. lebbeck* T1 was found to be the more

resistant in terms of overcoming the different stress levels and tolerated maximum moisture stress of -10 bars compared to control and followed by other treatments T2, T3 and T4 (Table 2). Similarly the *A. catechu* seeds showed maximum germination (%) at all moisture tension levels in presence of Rhizobial isolate T3 followed by T2, T1 and T3 (Table 2). The SVI and biomass yield was also influenced by species specific rhizobial isolates. Although, the inhibition effect on germination percentage of seed increased with increase in water potential, the inhibitory effect was overcome by inoculation of rhizobial isolates (Table 3). The root and shoot formation was also found to decrease with increasing water stress levels, although *Rhizobium* inoculation could bring about a increase in root and shoot formation in water stressed *A. lebbeck* and *A. catechu* seedling over control seedlings where no stress was exerted. *Rhizobium* from the test species of *A. catechu* (T3) exhibited resistance with different stress levels and similar results were obtained with *A. lebbeck* (Table 4). The number of leaves and secondary root formation in *A. lebbeck* were also found to decrease with increase in moisture stress levels. Inoculation of rhizobial strains were able to form leaf emergence maximum to -5 bars and no growth was observed at -8 and -10 bars. The secondary root formation was observed up to -8 bars in T1 and T2 treatments (Table 5). In *A. catechu* no leaf formation was observed till 10th day of experiment. Isolates T1 and T3 and control showed leaf formation on 13th day, while in T2 no leaf formation observed till 16th day of study. Secondary roots formation was observed at -3 and -5 bars in T1 and T2, -3, -5 and -8 bars in T3 while only at -3 bars in T4 isolates (Table 5, 6).

Table 3: Effect of rhizobial isolates and moisture stress levels on Seedling Vigor Index (SVI) and biological yield of *A. lebbeck* and *A. catechu*

		A. lebbeck		*A. catechu*	
Rhizobial isolates	Stress levels (bars)	SVI	Biomass yield 14th day	SVI	Biomass yield 14th day
T1	-3	112.9 ± 18.2	0.86 ± 0.03	104.36 ± 14.4	0.39 ± 0.04
	-5	96.0 ± 14.5	0.70 ± 0.04	84.56 ± 12.6	0.28 ± 0.06
	-8	67.4 ± 9.30	0.59 ± 0.08	68.94 ± 14.5	0.20 ± 0.04
	-10	28.1 ± 3.70	0.44 ± 0.02	57.03 ± 9.50	0.12 ± 0.01
T2	-3	103.8 ± 9.80	0.72 ± 0.04	99.88 ± 10.3	0.46 ± 0.06
	-5	77.5 ± 10.5	0.58 ± 0.09	82.08 ± 8.40	0.36 ± 0.04
	-8	46.8 ± 7.60	0.50 ± 0.03	65.77 ± 12.3	0.26 ± 0.05
	-10	34.1 ± 5.90	0.41 ± 0.03	58.06 ± 9.50	0.15 ± 0.02
T3	-3	78.9 ± 8.10	0.64 ± 0.02	118.49 ± 19.4	0.57 ± 0.06
	-5	63.1 ± 11.5	0.51 ± 0.06	93.74 ± 11.2	0.42 ± 0.04
	-8	34.8 ± 5.80	0.44 ± 0.05	77.80 ± 6.80	0.33 ± 0.07
	-10	19.5 ± 4.70	0.35 ± 0.07	65.90 ± 6.20	0.22 ± 0.03
T4	-3	67.9 ± 10.1	0.52 ± 0.06	71.29 ± 7.90	0.24 ± 0.02
	-5	41.6 ± 6.40	0.40 ± 0.03	61.96 ± 6.80	0.16 ± 0.01
	-8	27.0 ± 5.30	0.22 ± 0.02	51.10 ± 4.60	0.10 ± 0.03
	-10	19.0 ± 4.10	0.17 ± 0.05	43.28 ± 6.20	0.06 ± 0.01
Control	-0	95.94 ± 8.7	0.60 ± 0.07	82.06 ± 11.4	0.38 ± 0.04

Table 4: Effect of rhizobial isolates and moisture stress levels on root and shoot length of *A. lebbeck* and *A. catechu*

		A. lebbeck		*A. catechu*	
Rhizobial isolates	Stress levels (bars)	12th day root	12th day shoot	12th day root	12th day shoot
T1	-3	2.66 ± 0.72	5.90 ± 1.02	1.92 ± 0.39	3.05 ± 0.82
	-5	2.24 ± 0.45	5.58 ± 0.98	1.76 ± 0.65	2.97 ± 0.52
	-8	1.75 ± 0.38	4.90 ± 0.54	1.61 ± 0.25	2.40 ± 0.61
	-10	1.51 ± 0.25	4.26 ± 0.51	1.39 ± 0.20	2.13 ± 0.37
T2	-3	2.02 ± 0.36	5.22 ± 0.89	1.79 ± 0.42	2.84 ± 0.68
	-5	1.89 ± .032	4.90 ± 0.53	1.67 ± 0.40	2.66 ± 0.57
	-8	1.67 ± 0.28	4.43 ± 0.41	1.51 ± 0.72	2.30 ± 0.69
	-10	1.49 ± 0.24	3.62 ± 0.29	1.22 ± 0.25	2.03 ± 0.71
T3	-3	1.91 ± 0.31	4.77 ± 0.99	2.02 ± 0.36	3.29 ± 0.51
	-5	1.77 ± 0.24	4.02 ± 0.58	1.81 ± 0.89	3.01 ± 0.82
	-8	1.40 ± 0.21	3.97 ± 0.39	1.64 ± 0.25	2.80 ± 0.49
	-10	1.29 ± 0.18	2.81 ± 0.34	1.40 ± 0.30	2.53 ± 0.67
T4	-3	1.78 ± 0.39	3.74 ± 0.39	1.52 ± 0.19	2.61 ± 0.91
	-5	1.60 ± 0.28	3.02 ± 0.32	1.34 ± 0.75	2.37 ± 0.65
	-8	1.31 ± 0.21	2.66 ± 0.21	1.21 ± 0.31	1.96 ± 0.39
	-10	1.27 ± 0.13	2.48 ± 0.18	1.11 ± 0.26	1.72 ± 0.76
Control	-0	2.00 ± 0.72	5.50 ± 1.04	1.89 ± 0.87	2.90 ± 0.62

Table 5: Effect of rhizobial isolates and moisture stress levels on leaf and secondary root formation of *A. lebbeck*

		A. lebbeck							
		Leaf formation (No.)				Secondary roots formation (No.)			
Rhizobial isolates	Stress levels (bars)	7th day	10th day	13th day	16th day	7th day	10th day	13th day	16th day
T1	-3	1.0 ± 0.10	1.30 ± 0.23	2.30 ± 0.89	2.30 ± 0.87	12.1 ± 2.30	19.6 ± 5.20	26.7 ± 7.20	29.3 ± 7.10
	-5	-	1.00 ± 0.11	1.00 ± 0.96	1.30 ± 0.72	9.60 ± 2.10	16.1 ± 3.80	21.3 ± 6.30	23.1 ± 6.80
	-8	-	-	-	-	5.20 ± 1.10	11.4 ± 6.50	15.4 ± 4.80	16.5 ± 4.70
	-10	-	-	-	-	-	-	-	-
T2	-3	-	1.00 ± 0.13	1.30 ± 0.90	1.3 ± 0.14	8.30 ± 2.50	13.2 ± 3.50	20.5 ± 5.90	21.5 ± 6.60
	-5	-	-	-	-	4.70 ± 1.00	09.7 ± 3.80	14.3 ± 7.10	18.1 ± 5.30
	-8	-	-	-	-	-	-	2.40 ± 0.84	02.6 ± 0.54
	-10	-	-	-	-	-	-	-	-
T3	-3	-	0.66 ± 0.15	1.00 ± 0.12	1.0 ± 0.11	6.30 ± 1.00	11.3 ± 2.70	15.1 ± 3.50	15.1 ± 3.60
	-5	-	-	-	-	2.50 ± 0.95	08.4 ± 3.00	12.3 ± 2.70	13.2 ± 2.80
	-8	-	-	-	-	-	-	-	-
	-10	-	-	-	-	-	-	-	-
T4	-3	-	0.30 ± 0.09	0.66 ± 0.10	0.66 ± 0.16	5.70 ± 1.40	10.6 ± 3.50	13.4 ± 3.90	13.4 ± 4.10
	-5	-	-	-	-	-	02.7 ± 0.52	5.60 ± 1.70	05.6 ± 1.10
	-8	-	-	-	-	-	-	-	-
	-10	-	-	-	-	-	-	-	-
Control	-0	-	0.66 ± 0.07	1.00 ± 0.11	1.30 ± 0.19	10.1 ± 3.50	16.6 ± 4.10	24.1 ± 4.20	26.1 ± 7.80

-: No growth

Table 6: Effect of rhizobial isolates and moisture stress levels on leaf and secondary root formation of *A. catechu*

		A. catechu							
		Leaf formation (No.)				Secondary roots formation (No.)			
Rhizobial isolates	Stress levels (bars)	7th day	10th day	13th day	16th day	7th day	10th day	13th day	16th day
T1	-3	-	-	0.33 ± 0.10	0.33 ± 0.09	4.1 ± 0.87	6.30 ± 1.60	8.20 ± 3.40	9.20 ± 2.50
	-5	-	-	-	-	-	1.50 ± 0.43	3.40 ± 1.00	3.50 ± 0.72
	-8	-	-	-	-	-	-	-	-
	-10	-	-	-	-	-	-	-	-
T2	-3	-	-	-	-	7.3 ± 2.10	9.40 ± 2.80	10.3 ± 2.80	10.4 ± 2.60
	-5	-	-	-	-	3.4 ± 1.00	5.30 ± 1.60	7.50 ± 2.60	8.10 ± 1.30
	-8	-	-	-	-	-	-	-	-
	-10	-	-	-	-	-	-	-	-
T3	-3	-	-	1.60 ± 0.69	2.00 ± 0.08	8.3 ± 2.80	11.7 ± 3.10	12.5 ± 3.10	16.3 ± 3.90
	-5	-	-	0.33 ± 0.57	0.33 ± 0.05	5.6 ± 1.80	9.20 ± 2.20	11.0 ± 2.70	13.4 ± 2.70
	-8	-	-	-	-	3.2 ± 0.57	6.10 ± 1.40	7.20 ± 1.40	7.00 ± 1.50
	-10	-	-	-	-	-	-	-	-
T4	-3	-					2.40 ± 0.26	4.70 ± 0.95	6.20 ± 0.83
	-5	-	-	-	-	-	-		
	-8	-	-	-	-	-	-	-	-
	-10	-	-	-	-	-	-	-	-
Control	-0	-	-	0.30 ± 0.04	0.66 ± 0.05	5.1 ± 1.20	8.10 ± 2.70	12.3 ± 3.40	15.6 ± 4.10

-: No growth

DISCUSSION

Germination studies of ecologically important nitrogen fixing trees needs priority attention to include them in plantation program. Seed dormancy is an important constraint faced in the majority of hard coat species. Thus, enhancing seed germination by treating seeds is an important aspect[9]. The time required for effective treatment differs between species and related with seed coat thickness. In the present study, different rhizobial isolates were tested under various stress levels for their effectiveness in root colonization and promoting plant growth parameters. The retardation or suppression of various seedlings growth was observed proportionally to the increasing moisture stress levels (Table 2-6). This can be due to the retardation of mobilization of reserves of carbohydrates and proteins which are easily assessable when there is no moisture stress. *Rhizobium* isolated from test species i.e., *A. lebbeck* (T1) and *A. catechu* (T3) were found to be resistant against various water potential compared to other (T2 and T4) rhizobial species.

The various physiological response of plant to water scarcity varies with the duration of stress. Large number of processes is altered even by a very mild stress[10]. It was observed that artificial inoculation of *Rhizobium* from the strain of same species gave better results in most of the

parameters to overcome water stress levels. The seeds which were treated with *Rhizobium* were able to tolerate the stress or duration of stress to a greater extent than control and other treatments (Table 3, 4). Any treatment that is used to overcome physical seed dormancy is designed mainly to soften, puncture, wear away or split the seed coat in order to render it permeable without damaging the embryo and endosperm within it[11]. It is quite possible that *Rhizobium* treatment has softened the seed coat and made it permeable. Reduced germination under water stress conditions may be attributed to the effect that seeds seemingly develop an osmotically enforced "dormancy" under water stress conditions which may be an adaptive strategy of seeds to prevent germination under stressful environment thus ensuring proper establishment of the seedlings[12,13].

Osmotic stress caused a significant reduction in seedling germination and other plant growth parameters, indicating that seedlings were under stress. Similar observations under stress conditions were made by[14] in *Sorghum* spp., wheat[15], *Chenopodium* spp .[16] The germinability of seeds decreased with increasing the level of water stress, not only the germination was inhibited but the extension growth of the seedlings was also obstructed. These results show that radicle and plumule growth of the seedlings was greatly adversely affected by water stress. Slow and poor germination under water stress is obviously due to decreased water potential of the germination medium which restricts the water availability to the seeds[17,18]. As water moisture is one of the primary requirement in seed germination the water stress developed by PEG reduced germination greatly in this study.

The application of selected rhizobial strains resulted in adverting the effect of moisture stress, these positive effects of bacteria on seed germination might be attributed to increased water use efficiency, stimulation of root growth by production of phytohormones and/or softening of seed coat by enzymatic activities and lowering of plant ethylene concentrations. Bacterial IAA production under water stressed conditions may explain their effectiveness in promoting plant growth and shoot water content increasing plant drought tolerance[19,20]. The differences in these results may also be due to the adaptation of tree legumes when inoculated with their specific rhizobia. The non-species specific rhizobia were not able to make interaction with selected tree legume seeds, thus resulted in less tolerance of increasing water stress levels. Although the rhizobial strains T2 and T4 have nitrogen fixing and IAA producing capabilities, yet they were not able to tolerate the increasing water potential levels compared to species specific strains T1 and T3. The selected *Rhizobium* strains obtained in this study are excellent models to study the precise mechanism(s) of such interaction of adoptation and to elucidate the role of genetics of drought tolerance.

CONCLUSION

The drought tolerant pattern found among the indigenous rhizobial strains are reflecting the environmental stresses pressure predominant in their locations and are very good examples of the importance of using efficient-indigenous rhizobial strain for plant inoculation in each specific area. *Rhizobium* with the genetic potential for increased tolerance to drought and/or salinity could enhance production of food and forage in legume in semiarid and arid regions of the world.

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