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Evaluation of promising soybean rhizobia for conferring drought tolerance in soybean under pot

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Abstract
The present investigation was conducted under pot condition with the objective to see the effect of inoculation of soybean, so as to find the most effective strain for growing soybean under moisture stress condition. The experiment was laid out in Factorial Complete Randomized Design taking Soybean crop, Var- Indira Soya9 with 4 (inoculation type) ×2 (Stress type) treatments replicated three times The treatments were inoculation of soybean with the *Bradyrhizobium* strains from the Department Culture Collection of Microorganisms, i.e., T1(Control), T2 ((*local SB-116)*), T3 (*Bradyrhizobium daqingense*), T4 (*Bradyrhizobium liaoningense*) with normal and moisture stress condition. The study involved characterization of isolates i.e., they were gram negative, positive with respect to catalase test, while in amaylase test (SB 116) was positive, fast growing and *Bradyrhizobium* were slow in amylase production. Triple sugar iron test showed fermentation of glucose, lactose and sucrose occurred by local strain and fermentation of Glucose only seen in *Bradyrhizobium* cultures While gas production was observed in *Bradyrhizobium liaoningense* and there was No H_2S evolution was observed in SB 116 and *Bradyrhizobium daqingense* isolates. Investigation showed that inoculation of soybean with the *Bradyrhizobium daqingense* strains positively affected soybean growth and yield in normal and moisture stress condition. The treatment of *Bradyrhizobium daquingense* showed significantly maximum plant height(31.42cm), number of leaves(21.68/seedling), shoot dry biomass accumulation (2.07 g/ seedling), at flowering stage in Soybean under normal and moisture stress condition respectively. Significantly maximum chlorophyll content at flowering stage was recorded under T3 (*Bradyrhizobium daqingense*) while minimum chlorophyll content was recorded under T1 (Control). It was concluded that the treatment i.e., inoculation of *Bradyrhizobium daquingense* was most effective and may be adopted to improve germination, vegetative growth and yield of Soybean under normal and stress condition followed by local strain of SB-116. The results of experiments revealed that *Bradyrhizobium daquingense* was effective in conferring drought tolerance in pot grown soybean".

Keywords: *Rhizobium, bradyrhizobium,* soybean, nitrogen fixation

Introduction
Drought is currently one of the most stressful environmental factors affecting crop yield. Drought stress in legume nodules has been known to have a negative impact on nitrogenase activity (Sprent 1972). Drought stress is a major factor which affects symbiosis resulting decreased nodule formation, nodule size and N_2 fixation (Napoles, MC. *et al.* 2009) [6]. Greenhouse and field experiments have shown that drought stress has resulted in a significant reduction in seed yield (24 ~ 50%). So formation of effective nodules in soybean may be possible by enhancing BNF under stress condition when inoculated with compatible stress tolerant Rhizobium. Maximum yield loss is observed when drought stress occurs, mainly throughout the seed development period (R5-R7), resulting in a 45 per cent and 88 per cent respectively reduction. the most extreme effect of moisture stress on Soybean crop was observed during the seed filling time (Desclaux D; *et al.* 2000) [7]. Inoculation of promising microbes to Soybean seed increases yield and yield component of soybean crop (Rajput, L.S *et al.* 2001). Coating soybean seeds with rhizobial inoculants has also been studied to improve N_2 fixation under stress condition (Dobereiner, J. *et al.*1995) [8].

Materials and Methods

Colony Morphological Characteristics of *Rhizobium* and *Bradyrhizobium* isolates

The colony characters viz. shape of the colony, size, margin, elevation, surface, color and motility were observed on YEMA medium and recorded. 1.0 ml of appropriate dilution of *Rhizobium* was transferred into the Petri plates. The plates were incubated at 28 °C temperature for 24-48 hours. The phenotype and growth pattern of *Rhizobium* isolates were observed.

Biochemical characterization of *Rhizobium* and *Bradyrhizobium* isolates

Gram staining

The cultures of *Rhizobium* and *Bradyrhizobium* isolates were put for gram staining for more specific identification of the colonies. The gram staining test was done in a laminar airflow hood. The slides were first of all washed with ethanol then colonies were marked on the slides with the help of inoculating needle and were heat fixed. Then smears were stained in the following steps: 1) Firstly, crystal violet was applied on each slide and kept for 1 min., 2) Washed with distilled water, 3) Then applied iodine on the slides as mordant for 1 min and 95% alcohol was used for washing for 30 sec. then washed with distilled water, 4) Safranin was applied on the slides then washed with distilled water and 5) air-dried the slides.

Starch hydrolysis test

For starch utilization, Starch Agar Medium was inoculated with *Rhizobium* then iodine was added to determine the capability of microbes to use starch. A drop of iodine (0.1N) was spread on 24 hours old culture that showed a clear zone of inhibition around bacterial colonies.

Catalase test

Firstly, a smear of strain was made on a clean and dry glass slide, then a few drops of H_2O_2 were added to the slide. Production of gas bubbles and effervescence showed a positive test.

Triple sugar iron agar test

In this test organism that ferments glucose, sucrose, lactose and produces acids, changes the color of the medium from red to yellow. After inoculation and incubation, the color was observed on the butt and the slant.

Pot experiment

A random collection of surface soil was done from a 15cm (6-inch) depth from the fields of real agricultural land near the College of Agriculture, Raipur and was thoroughly blended with compost samples. This soil was sieved and processes from a 2mm sieve. A 5Kg well-mixed sample in a ratio 3:1:1 for Soil: Sand: Compost For facilitating proper drainage of water, at the bottom of every pot, four holes were made. Few of the samples of the soil collected are then preserved in a plastic bag to analyse the microbial and physicochemical properties of the sample, and hence were stored and tagged properly. For this experiment, the var. Indira soya9 soybean seeds were used in healthy condition. A 95% ethanol was used for rinsing these seeds all of uniform size, and were then submerged in 0.1% solution of mercuric chloride. After which these seeds are thoroughly washed using sterilized double distilled water for a minimum of 5-times. Local strain *Rhizobium* as well as *Bradyrhizobium* biofertilizers are then applied for treating the seed placed in the pots. Using the triangular technique three-holes were marked on each pot 2-3cm deep. These holes are made with a help pf a gloss rod, properly sterilized, with a proper equal hole to hole distant.

Result

Common morphological characteristics of native *Rhizobium* (RhiSB-116) and *Bradyrhizobium* isolates

Rhizobium and *Bradyrhizobium* isolates produced translucent, raised, circular and mucilaginous colonies which varied in size between 2.00 to 4.00 mm and off white in their appearance. *Bradyrhizobium daqingense* and *Rhizobium* (Local, Rhi-SB 116) isolates showed off white colour colonies but colonies of *Bradyrhizobium liaoningense* looked creamier and darker as compared to other two isolates. *Rhizobium*, *Bradyrhizobium daqingense* and *Bradyrhizobium liaoningense* colony morphological characteristics can also be observed in Plate-4.1. After the gram-staining test, the bacteria assumed a red colour which indicates that they were Gram-negative. Gram staining is based on their growth and colony morphology. Gram staining of the cultured isolate was done to provide information as presumptive tests of the isolates. The authentication of the isolates was performed using sub culturing method.

Biochemical characterization of *Rhizobium* isolates

As per the results of the characterization study of native *Rhizobium* isolates (Table 1), and *Bradyrhizobium* isolates showed a gram-negative reaction and while a positive result for result for amaylase test and catalase test, was observed.

Triple Sugar Iron Agar Test (TSI)

As per triple sugar iron test showed fermentation of glucose, lactose and sucrose occurred by *Rhizobium* and fermentation of Glucose only seen in *Bradyrhizobium* cultures. While gas production was observed in *Rhizobium* and *Bradyrhizobium liaoningense* and There was No H_2S evolution was observed in *Rhizobium* and *Bradyrhizobium isolates*

Pot experiment in soybean plant

Plant height study

On 15 DAS, the treatment T2 (*Rhizobium* (SB-116)) recorded significantly higher (18.10cm) height of plant whereas the minimum height of plant (15.36 cm) was recorded in the treatment T1 (Control). The treatments T3 (17.00cm) and T4 (17.16 cm) were at par with each other. The treatment T2 (*Rhizobium* (SB-116)) recorded significantly maximum (32.20cm) height of plant on 30 DAS, whereas the minimum height of plant (28.00cm) was recorded in the treatment T1 (Control). On 45 DAS, the treatment T3 (*Bradyrhizobium daqingense*) recorded significantly higher (40.16 cm) height of plant whereas the minimum height of plant (33.37 cm) was recorded in the treatment T1 (control). The treatments T2 (38.00 cm) and T4 (36.62 cm) were at par with each other in stress free condition, while in stress condition T3 (*Bradyrhizobium daqingense*) recorded significantly higher (40.33) height plant whereas the minimum height of plant was recorded in the treatment T1 (control). The treatments T2 (38.34cm) and T4(37.37cm) were at par with each other. On 60 DAS, the treatment T3(*Bradyrhizobium daqingense*) recorded significantly higher (39.33 cm) height of plant whereas the minimum height of plant (31.0 cm) was recorded in the treatment T1 (control). The treatments T2 (37.33cm) and T4(36.66 cm) were at par with each other in stress free condition, while in stress condition T3(*Bradyrhizobium*

daqingense) recorded significantly higher(37.66cm) height plant whereas the minimum height of plant was recorded in the treatment T1 (control). The treatments T2 (36.66 cm) and T4(35.33 cm) were at par with each other. Similar result was observed by Alain Ndoli1 *et al.* (2013) [1] in Soybean under pot condition with microbial inoculation.

Chlorophyll content

The data regarding total chlorophyll content are given in Fig.1. The inoculants significantly affected the total chlorophyll content. At flowering stage, the maximum total chlorophyll content (15.50mg/g tissue) was recorded under T3 (*Bradyrhizobium daqingense*) while minimum (11.83mg/g tissue) chlorophyll content was recorded under T1 (Control). At no moisture stress condition in S0 level, the maximum total chlorophyll content (16.33mg/g tissue) was recorded under T3 (*Bradyrhizobium daqingense)* while minimum (12.33mg/g tissue) chlorophyll content was recorded under T1 (Control) and when moisture stress was given, at S1 level, total chlorophyll content (8.33mg/g tissue) was recorded as maximum under T3 (*Bradyrhizobium daqingense*) while minimum (5.15mg/g tissue) chlorophyll content was recorded under T1 (Control). In un inoculation there is decrease in chlorophyll content due to the changing green color of the leaf into yellow (Schelmmer *et al*., 2005). It was observed that the effect of inoculants on total chlorophyll content was found to be significant in Table 4.7. There was increase in total chlorophyll content due to application of inoculants, because plants were photosynthetically more activeby inoculation, Similar results were obtained by (MinobuKasai *et al.*, 2012) [12].

Nitrogen uptake in shoot of Soybean

N uptake by shoot of At moisture stress condition S0 T3 (*Bradyrhizobium daqingense*) recorded the maximum nitrogen uptake in shoot of soybean (21.12 mg/plant) which was at par with treatment T2(16.74 mg/plant) followed by T4 (12.8mg/plant). However, the minimum (9.39) nitrogen uptake in shoot of soybean was recorded in the treatment T1 (Control). And At moisture stress condition in S1 level The maximum nitrogen uptake in shoot of soybean (17.86mg/plant) was recorded under T3 (*Bradyrhizobium daqingense*) which was at par with treatment T2(13.24mg/seedling) followed by T4 (10.68mg/seedling). while minimum (7.49mg/plant) nitrogen uptake in shoot of soybean was recorded under T1 (Control). It was observed that the effect of inoculants on nitrogen concentration was found to be significant in nitrogen uptake in shoot of soybean observations were in close agreement with Morshed *et al.* (2008) [3]

Shoot biomass study

Similar to plant growth attributes in the present study, plant fresh and dry weight differed significantly among the treatments. At flowering stage, The treatment T2 (*Rhizobium SB-116*) recorded the maximum fresh weight of shoot (3.16 g) which was at par with treatment T3(3.05g) followed by T4 (2.20g). However, the minimum (1.55g) fresh weight of shoot was recorded in the treatment T1 (Control). At moisture stress condition in S0 level The maximum fresh weight of shoot (2.48g) was recorded under T3 (*Bradyrhizobium daqingense*) while minimum (1.16g) fresh weight of shoot was recorded under T1 (Control) and in S1 level The maximum fresh weight of shoot (2.40) was recorded under T3 (*Bradyrhizobium daqingense*) while minimum (1.03) fresh weight of shoot was recorded under T1 (Control)It was observed that the effect of inoculants on no. of shoot (g)was found to be significant in Table Plant dry weight differed significantly among the treatments. In flowering stage Maximum plants dry weight was recorded in plants treated with Rhizobium SB-116 T2 (1.26 g / plant), which was statistically on par with the treatment of T3(*Bradyrhizobium daqingense*) (1.20g/plant) and the minimum seedling dry weight was seen in uninoculated control treatment T1 (0.62 g /plants).At moisture stress condition in S0 level The maximum dry weight of shoot (0.96g/plant) was recorded under T3 (*Bradyrhizobium daqingense*) while minimum (0.53g/plant) fresh weight of shoot was recorded under T1 (Control) and At moisture stress condition in S1 level The maximum dry weight of shoot (0.94g/plant) was recorded under T3 (*Bradyrhizobium daqingense*) while minimum (0.51g/plant) dry weight of shoot was recorded under T1 (Control)It was observed that the effect of inoculants on no. of shoot (g)was found to be significant in fig2

Rhizobial population dynamics

At no moisture stress condition (S0 Level), population was found maximum in the treatment of T3 (*Bradyrhizobium daqingense*) (10.16 x 10^4 cfu g^{-1} soil). The next best treatment which had more population was found in the treatment of T2(Rhizobium SB-116) (8.87 X 10^4 cfu g^{-1} soil). Minimum population was recorded in the uninoculated control treatment T1 (5.43 x 10^4 cfu g^{-1} soil). In S0 Level Highest population was seen in T3 ((10.16 x 10^4 cfu g^{-1} soil) followed by T2 (8.87 X 10^4 cfu g^{-1} soil) and T4 (7.12 x 10^4 cfu g^{-1} soil). At moisture stress condition S1 Level, population was found maximum in the treatment of T3 *(Bradyrhizobium daqingense*) (7.6 x 10^4 cfu g^{-1} soil). The next best treatment which had more population was found in the treatment of T2(Rhizobium SB-116) (6.42 X 10^4 cfu g^{-1} soil). Minimum Rhizobium population was recorded in the uninoculated control treatment T1 (3.26 x 10^4 cfu g^{-1} soil). similar observation was done by singh *et al*. (2000) in table 2

Conclusion and Future Prospects

Keeping in view the finding related to stress tolerance in Rhizobium and Bradyrhizobium chlorophyll content during stress condition and Biological nitrogen fixation parameters like an accumulation of biomass and nitrogen, performance of *Bradyrhizobium daqingense* isolate of soybean was found to superior among all rhizobial isolates taken for study. These isolates accumulated 3.16 mg/plant extra amount of atmospheric nitrogen over uninoculated control plants. However, the result indicative and required further experimentation to arrive at a more consistent result. Since, the result of present investigation belongs to pot experiment, this type of experiment in field condition is recommended to ensure the long term growth performance of soybean in the response of inoculation.in stress tolerance.

Table 1: Effect of inoculationwith *Rhizobium* and *Bradyrhizobium* on plant height of Soybean plant

Plant height(cm)									
Treatments	**Treatments detail**	**15 DAS**	**30 DAS**	**45 DAS**			**60 DAS**		
				S0	**S1**	**mean**	**S0**	**S1**	**Mean**
T1	Control	15.36	28.00	33.37	31.00	32.17	31.00	28.33	29.67
T2	*Rhizobium* (SB-116)	18.10	32.20	38.00	38.34	38.17	37.33	36.66	37.00
T3	*B. daqingense*	17.00	31.43	40.16	40.33	40.25	39.33	37.66	38.50
T4	*B. liaoningense*	17.16	30.36	36.62	37.37	37.33	36.66	35.33	36.00
Mean		16.90	30.47	37.21	36.75	36.98	36.08	34.50	35.29
C.D		0.56	0.80	C.D(S):0.572 C.D(T):0.810 C.D(S×T):1.145			C.D(S):0.637 C.D(T):0.901 C.D(S×T):1.274		

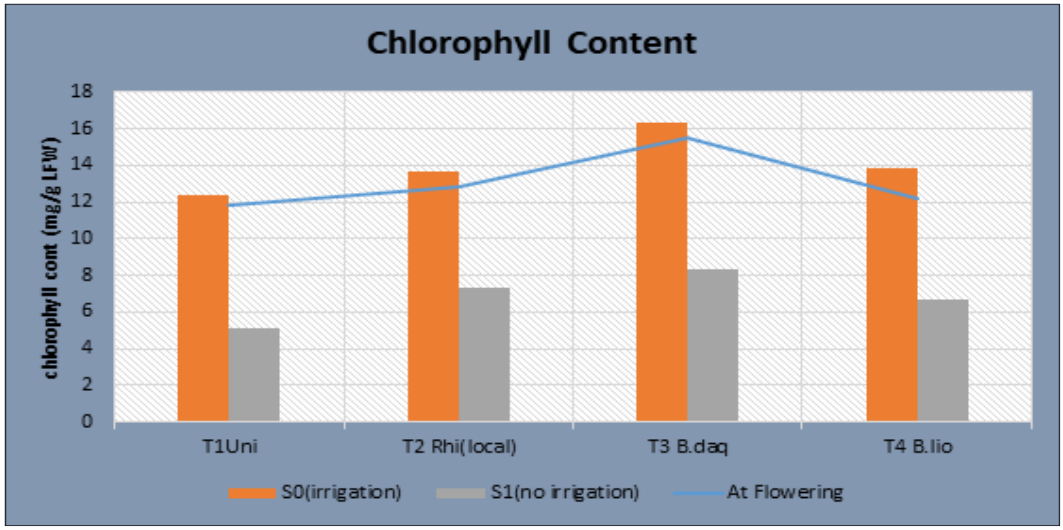

Fig 1: Effect of Soybeanrhizobiaon chlorophyll content in leaves of Soybean plant

Table 2: Effect of inoculants on Microbial population count in YEMA mediain rhizosphere soil after harvest of Soybean

Treatments	**Treatments detail**	**Microbial population count in YEMA media (No. x 10^4) CFU/g of rhizosphere soil**		
		S0	**S1**	**Mean**
T1	Control	5.43	3.26	4.35
T2	*Rhizobium(SB-116)*	8.87	6.42	7.65
T3	*Bradyrhizobium yuanmingense*	10.16	7.66	8.91
T4	*Bradyrhizobium liaoningense*	7.12	5.81	6.47
	Mean	7.89	5.79	6.92
	CD(p=0.05)	C.D(S):0.188 C.D(T):0.267 C.D(S×T):0.377		

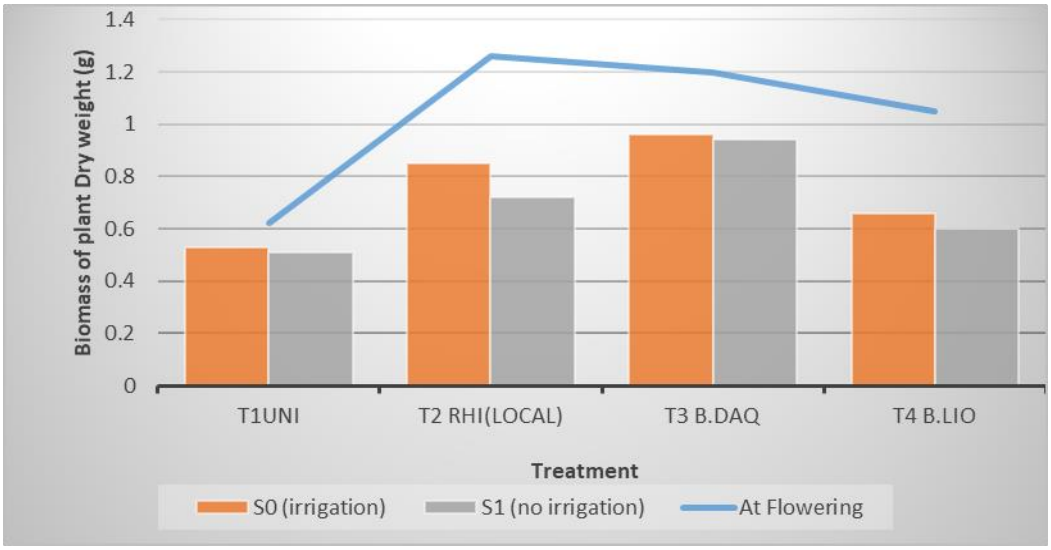

Fig 2: Effect of *rhizobial inoculation* on biomass accumulation (dry) of Soybean plant

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