International Journal of Ecology and Environmental Sciences
www.ecologyjournal.in
Online ISSN: 2664-7133; Print ISSN: 2664-7125; Impact Factor: RJIF 5.28
Received: 06-06-2020; Accepted: 20-06-2020; Published: 07-07-2020
Volume 2; Issue 3; 2020; Page No. 37-42

Drought tolerant *Rhizobium* sp. VRE1 induced osmotic stress tolerance, seed germination and seedling vigor in blackgram (*Vigna mungo* L.)

Brundha Annadurai[1], Z John Kennedy[2], Sivakumar Uthandi[3*]
[1] Biocatalysts Laboratory, Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India
[2] Centre for post-harvest technology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Abstract
In the present study, we evaluated the mechanism of osmotic stress-resilient nodule endophytic *Rhizobium* sp. VRE1 grown in simulated osmotic stress conditions. The results showed that *Rhizobium* sp. VRE1 tolerated up to -0.73 MPa and produced a detectable amount of IAA (2.0µg mL^{-1}). A significant increase in the concentration of total free amino acids, proline, and total soluble sugar content due to inoculation of *Rhizobium* sp. VRE1 was observed moisture stressed conditions. However, the protein content had reportedly higher under non - stress condition (19.04 mg g^{-1} dry weight) but decreased under stress condition due to inoculation of Rhizobium sp. VRE1 (7.20 mg g^{-1} DW). Blackgram seeds biotized with *Rhizobium* sp. VRE1 showed germination efficiency of 60% and vigor index of 1190 under induced moisture stress (-0.49MPa). *Rhizobium* sp. VRE1 produced higher levels of EPS (62.12 mg) under drought stress compared to non- stressed (11.37 mg). Hence, the study suggests the scope of enhancing the yield potential and fitness of blackgram by bio-augmenting *Rhizobium* sp. VRE1 as a biofertilizer for sustainable pulse production in rice fallow and rain-fed areas.

Keywords: Drought, exopoly saccharides, vigor index, black Gram

Introduction
Abiotic stresses, more precisely drought, causes significant crop yield losses worldwide and affects crop production (Vinocur and Atman 2005, Wani *et al*. 2016) [30, 31]. The physiological drought caused due to the unprecedented occurrence of drought spells harms crop growth and suffers significant yield loss of 20-30% (Sai *et al*. 2019) [23]. Severe moisture- stress affected metabolic functions of crop plants, such as reduced photosynthetic pigment synthesis, accumulation of osmoprotectants including proline, decreased cell membrane stability, and changes in physiological parameters, including plant height, leaf area, and cell membrane stability (Baroowa *et al*. 2011) [2]. Legume crops such as blackgram (*Vigna mungo* L.) and green gram (*Vigna radiata* L.) are the two most common pulse crops grown for plant protein sources in India. Legume-rhizobia symbiotic association plays a vital role in the maintenance of soil fertility by fixing atmospheric nitrogen. However, nitrogen fixations by legumes are hampered due to recurrent drought episodes and subsequently deprive the soil health. There is evidence that the genotypic nature governed the ability to fix N_2 under drought conditions (Pimentel *et al*. 1990). In order to improve osmotic stress tolerance of a drought-sensitive common bean cultivar (COCOT), plants were inoculated by the reference strain *Rhizobium tropici* CIAT 899. However, rhizobial strains isolated from native soils, where drought occurs frequently, hold a better option in combating moisture stress and increasing pulse productivity.
Certain *Rhizobia* are likely to develop antioxidants (catalase), osmolytes, stress proteins, and exopolysaccharides for survival in especially drought-stricken environments (Goyal *et al*. 1986, Vanderlinde *et al*. 2010). Nevertheless, *Rhizobium* has been found to thrive up to -3.5 MPa under drought stress (Abolhasani *et al*. 2010). Such rhizosphere bacteria's ability to withstand extreme water stress conditions can be unveiled to ameliorate drought impacts (Goyal *et al*. 1986). Specific stress ameliorating and plant growth-enhancing mechanisms of rhizobia have been observed including the development of chaperons and sugars (Berjak 2006), synthesis of stress enzyme 1- aminocyclopropane 1-carboxylic acid (Zahir *et al*. 2009) [10], exopolysaccharides (Alami *et al*. 2000), organic compounds of low molecular weight such as trehalose (Zahran 1999), and enhanced root respiration (Volpin and Phillips 1998) by influencing host physiology. For instance, *Rhizobium leguminosarum*, *Mesorhizobium cicero*, and *Rhizobium phaseoli* associated with wheat under moisture stress enhanced the biomass and drought tolerance index under polyethylene glycol (PEG 6000) simulated drought environments (Hussain *et al*. 2014) [10]. Few rhizobia such as *Rhizobium tropici*, *Paenibacillus polymyxa* and *Paenibacillus taichungensis* produced indole acetic acid (IAA) and improved the root length of the seedlings in wheat and common beans (Hussain *et al*. 2014, Figueiredo *et al*. 2008 and Raja *et al*. 2019) [11, 6, 21].
A rhizobial strain, *Rhizobium* sp. VRE1 previously isolated from the nodules of blackgram (Thanuja *et al*. 2017, Thanuja *et al*. 2020) [27] tolerated up to -1MPa (-10bars). The present investigation aims to explore the plant growth-promoting ability of *Rhizobium* sp. VRE1 and its possible mechanism to tolerate moisture stress in simulated drought environments using PEG 6000. The influence of *Rhizobium* sp. VRE1 on the seedling vigor of blackgram was also studied.

Materials and Methods

Inoculum preparation

Rhizobium sp. VRE1 (Acc. No. MF133350), used in the present investigation, was obtained from Biocatalysts lab, Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore. The strain was maintained in yeast extract mannitol agar (YEMA) and grown in 100 ml Tryptone yeast extract (TY) broth. The inoculated flasks were incubated at 28 °C in a rotary shaker for 2-3 days until the logarithmic phase (~ 1 x 10^9 viable cells mL^{-1}) (Woomer *et al*. 2011) [32] and the desired population was ensured prior to seed biotization (Thanuja *et al*. 2020) [28].

Quantification of IAA by *Rhizobium* sp. VRE1 under simulated osmotic stress

TY broth containing L - Tryptophan (0.2%) was prepared at 25% (- 0.73 bars) level of PEG 6000 and inoculated with *Rhizobium* sp. VRE1. The pH was maintained at 7.0 and incubated at 28 ˚C under shaking condition (125 rpm) for 7 d. Two mL of cell-free extract collected by centrifuging at 10,000 rpm for 20 min was mixed with two drops of orthophosphoric acid (0.1mM) and 4 mL of Salkowski reagent (1 mL of 0.5 M $FeCl_3$ in 50 mL of 35% perchloric acid). The mixture was incubated at room temperature for 30 min, and the OD value was measured at 535 nm (Gordon and Paleg, 1957) [8]. Uninoculated Tryptone yeast extract medium served as control.

Adaptive mechanisms of *Rhizobium* sp. VRE1 under *in-vitro* osmotic stress

Total free amino acids

An aliquot of 0.1 mL of culture supernatant prepared above was added with 1 mL of ninhydrin reagent and mixed well. The volume was made up to 2 mL with water and boiled in a water bath for 20 min. Five mL of diluent (Equal volumes of water and n-propanol) was added while still on the water bath. After 15 min of boiling, the tubes were cooled under running tap water and the absorbance of the purple color against a reagent blank (0.1 mL of 80% ethanol instead of extract) at 570 nm (Green filter) was measured. The standard curve was prepared from leucine by pipetting out 0.1 - 1.0 mL (10 - 100 µg range) of working standard (Moore and Stein, 1948) [17].

Total proline content

Free proline accumulation was determined in the culture supernatant according to the method of Bates *et al*. (1973) [3]. Exactly 2 mL of supernatant was added with 2 mL of glacial acetic acid and ninhydrin and mixed well. It was then placed in a boiling water bath for 1 h, and then the reaction was terminated by placing on an ice bath. Four mL of toluene was added and mixed vigorously for 20-30 sec. The chromophore (toluene) layer was aspirated and warmed to room temperature. The absorbance of red color was measured at 520 nm against a reagent blank. The amount of proline in the sample was calculated using a standard curve prepared from pure proline (range 0.1-36 µm) and expressed on a dry weight basis.

Total sugar content

Rhizobium sp. VRE1 cells were centrifuged at 6000 rpm for 5 min and boiled at 60 ºC in a water bath with a mixture of Methanol: Chloroform (4:1 v/v) for 15 min and centrifuged at 10,000 rpm for 15 min. The supernatant was used for the quantitative estimation of sugars by the method described by Dubois *et al*. (1956) [5]. To 1 mL of sample solution (0.2 mL supernatant + 9.8 mL water), 1 mL of phenol, and 5 mL of 96% sulphuric acid was added and shaken well. After 10 min, the contents were shaken in the tubes and placed in a water bath at 25-30 °C for 20 min. The color was measured at 490 nm using a UV spectrophotometer.

Total protein content

Pellets of non-stressed and moisture stressed cultures were suspended and washed in 0.02 M $MgCl_2$, followed by sterile distilled water. Each time the cells were obtained by centrifugation at 6000 rpm, 4 °C for 15 min. The pellets were re-suspended in 500 mL lysis buffer (Bangalore Genei, India) and incubated for 30 min at room temperature. Finally, extracts were centrifuged at 10,000 rpm for 15 min at 4 ºC, and the supernatant was used for the estimation of protein content by the Bradford method (1976).

Exopolysaccharide production

Exopolysaccharide (EPS) produced by *Rhizobium* sp. VRE1 under induced moisture stress (25% PEG) was analyzed and compared with non-stressed conditions. EPS was extracted periodically in cultures grown in TY with PEG 6000 (-1 MPa stress) (15DAI) by centrifugation at 20,000 g for 25 mins, and resultant supernatant was obtained. The pellet was washed twice with 0.85% KCl for complete extraction of EPS. Using Bradford method (1976), protein content in the supernatant was determined. Then the collected supernatant was filtered through nitrocellulose membrane (0.45 µm) and dialyzed against sterile distilled water at 4 ˚C. Dialyzate was obtained by centrifugation at 2000 g for 30 mins. In order to eliminate the presence of insoluble material, the supernatant was mixed with 3 volumes of ice-cold ethanol (absolute alcohol) and kept overnight at 4 ˚C for precipitation. EPS precipitated was obtained by centrifugation at 10,000 g for 15 mins. Then the precipitate was dissolved in 1 mL of sterile distilled water. The total carbohydrate present in the EPS was determined based on the method described by Dubois *et al*. (1956) [5]. EPS produced by *Rhizobium* sp. VRE1 was detected using FT-IR (FT-IR 6800 JASCO, Japan), and the functional groups analyzed from the FT-IR spectral bands in the spectral range between (400-4000 cm^{-1}).

Seed biotization

Blackgram seeds (var. VBN6) obtained from (National Pulses Research Centre, Vamban - 622 303, Pudukottai district, Tamil Nadu) were surface sterilized in 0.1% $HgCl_2$ for 2 min and rinsed five times with sterile water. The seeds were then treated with overnight grown cultures of *Rhizobium* sp. VRE1 (10^9 CFU ml^{-1}). Similarly, blackgram seeds treated with sterile water served as control. *Rhizobium* sp. VRE1 treated seeds were placed on a filter paper in Petri dishes, moistened with (0, 10, 15, 20% of PEG 6000) corresponding to the osmotic potentials of 0, -0.05, -0.14, -0.29, -0.49 MPa. The Petri plates were sealed with parafilm to prevent evaporation and incubated at room temperature (28±2 ºC). Germination percentage was assessed after 72 hrs calculated using the standard formulae (Praveen Kumar *et al*. 2015) [20].

"Germination % =Number of seeds germinated/ Number of seeds placed ×100" he seed vigor index was examined after five days of incubation according to the formula based on the product of

germination (%) and seedling length (cm) (Muscolo *et al*. 2014) [18].

Statistical analysis

All data were statistically analyzed in Microsoft Excel and add-in with XLSTAT Version 2016.04.325250 (XLSTAT, 2010). Significant differences among the treatments were statistically analyzed using analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) at $p < 0.05$ significance level.

Results

IAA production by *Rhizobium* sp. VRE1 under simulated moisture stress

In the present investigation, the osmotic stress-tolerant *Rhizobium* sp. VRE1 was evaluated for IAA production under a simulated drought stress using PEG 6000 (ψ of -0.29, -0.49 and -0.73 MPa). The results revealed that osmotic stress significantly affected the IAA production of *Rhizobium* sp. VRE1 and it ranged from 8.14 µg.ml^{-1} to 2 µg. mL^{-1} (Fig.1). However, at 25% PEG (-0.73MPa) concentration, IAA production of 2.0 µg. mL^{-1} was observed. It was also observed that IAA production declined with an increasing osmotic stress intensity.

Biochemical insights of osmotic stress resilience in *Rhizobium* sp. VRE1

Sugars and free amino acid production by the osmotolerant bacterial strain *Rhizobium* sp. VRE1 were determined under osmotic stressed conditions. The total soluble sugars and total free amino acids were significantly increased in all stress levels (ψ of -0.29, -0.49, and -0.73 MPa PEG 6000). At higher stress intensity of 25% (-0.73 MPa) total soluble sugars and free amino acids of 21.23 µmol g^{-1} DW and 25.20 µmol g^{-1} DW of were observed, respectively. On the other hand, the protein content of VRE1 was reduced under osmotic stress irrespective of the stress intensity. The protein concentration of the *Rhizobium* sp. VRE1 grown under PEG induced stress conditions, registered 7.20 mg protein g^{-1} at 25% (- 0.73 MPa). However, at 20% (-0.49 MPa) PEG 6000, the protein content was slightly reduced (6.62 mg protein g^{-1}), and at lower osmotic stress *i.e*. 15% (-0.29 MPa) PEG 6000, protein content was (5.27 mg protein g $^{-1}$). The protein content was greatly increased under non-stress condition (NS) condition when compared to moisture-stress induced condition.

Likewise, under osmotic stress conditions, *Rhizobium* sp. VRE1 produced a conspicuous amount of osmolytes. In the present study, proline was observed maximum at 25% PEG (14.20 µmol g^{-1} DW). Proline content found to be increased with the increase in PEG concentration, while at non-stress conditions, it was lesser compared to the moisture-stress environment (Table 1).

Exopolysaccharide production by osmotic stress-resilient *Rhizobium* sp. VRE1

Rhizobium sp. VRE1 produced EPS of 62.12 mg at moisture stress-induced condition (25% PEG). On the contrary, the EPS production was comparatively very low (11.37 mg) under non-stress conditions (Table 1). FTIR absorption spectra of exopolysaccharides produced by *Rhizobium* sp. VRE1 is presented in Fig 2. The major absorption peaks observed in the ranges between 4000 and 3500 cm^{-1}, 3000 and 2500 cm^{-1}, 2000 and 1500 cm^{-1}, 1000, and 500 cm^{-1}. The wavenumbers were recorded between 5, 10, and 15- day intervals of both stress and non-stress conditions. The wavenumbers 3900.84 cm^{-1}, 2652.06 cm^{-1}, 2530.53 cm$^{-1,}$ and 1200.94 cm^{-1} represents the alkanes functional group due to CH_3/CH_2/CH stretching, C-H stretch of aldehyde group and C-O stretch of alcohols group, respectively. The same functional groups were also observed during 15 DAI in *Rhizobium* sp. VRE 1 grown in simulated osmotic stress. Other functional groups corresponding to the wavelengths such as 1514.41, 1276.93, 1100.23, 1023.12, and 421.20 cm^{-1} under non-stressed environments represents ketone group due to C=O stretch, amine group due to NH_2 scissoring, C-N stretching, C-O stretching, and O-H bending respectively.

Germination percentage and vigor index of blackgram seeds under *in-vitro* simulated drought stress

Considerable reduction in seed germination was observed as the concentration of PEG increased i.e., (0, 5, 10, 15, and 20%). Howbeit the effect of PEG was significantly reduced by treating the blackgram seeds with *Rhizobium* sp. VRE1. At -0.05 MPa (5% PEG infusion), the seeds treated with VRE1 showed 90% germination efficiency and vigor index of 3150. Nonetheless at higher stress intensity of 0.49 MPa (20% of PEG infusion) VRE1 sustained 60% of germination efficiency and showed a vigor index of 1190 when compared to uninoculated control (Table 2).

Discussion

***Rhizobium* sp. VRE1 protects blackgram under induced moisture stress by producing IAA and osmolytes**

Abiotic stress responsive increase in IAA production has been observed in *Azospirillum brasilense* (Malhotra and Srivastava 2008) [16]. In contrast, (Sandhya *et al*. 2010) [24] reported deferred expression of different PGP traits such as P-solubilization, production of phytohormones - IAA, gibberellic acid and cytokinins under osmotic stress conditions as compared to non-stressed conditions. Osmotolerant bacterial strains exhibited better PGP activities under osmotic stress conditions as compared to the bacterial strains, which were susceptible to osmotic stress (Vejan *et al*. 2016). Likewise, in our present study, PEG 6000 treated (-0.05, -0.14, -0.29, -0.49, and 0.73 MPa of osmotic potential) *Rhizobium* sp. VRE1 showed a considerable reduction in IAA level at the stressed condition.

The present study also reported a gradual increase in proline and total soluble sugars under different levels of osmotic stress. However, the proline and total soluble sugar content showed a significant increase over un -inoculated control. The results of the study suggested that the accumulation of proline and compatible solutes under moisture stress guard the plants against osmotic stress, by maintaining the redox homeostasis through stabilizing membrane proteins, and ROS scavenging (Garcia *et al*. 2017).

Rhizobacteria of the genera *Bacillus*, *Pseudomonas*, and *Acinetobacter*, amongst others, are effective in conferring abiotic stress tolerance to plants and withstand harsh environmental conditions due to their EPS producing abilities. In the study of Sandhya *et al*. (2009) [25], *Pseudomonas putida* GAP-P45, when inoculated on sunflower plants under drought stress, produced high levels of EPS (63.30). Likewise, in the present investigation, FT-IR spectra of EPS revealed significant wavenumbers that coincides with the functional group of polysaccharides. Hence, the *Rhizobium* sp VRE1 produced EPS in response to induced osmotic stress, as previously reported by Sah *et al*. (2016) [22].

Seed biotization of *Rhizobium* sp. VRE1 improves germination, seedling vigor of blackgram

In the present investigation, biotization of blackgram seeds with *Rhizobium* sp. VRE1 enhanced the germination percentage under induced moisture stress conditions. Seed priming with plant growth-promoting microbial inoculants significantly promoted the seed germination and seedling establishment under moisture stress (Kalita *et al*. 2015) [12]. In the present study, initiation of the germination was uniform among the treatments (Table 2). However, at higher PEG concentration, the germination was reduced considerably compared to their respective controls. On exposure to sudden moisture stress of -0.27 MPa, reduction in seed germination was recorded, whereas control seeds registered 100% seed germination after 2 days after sowing. A drastic reduction in germination percentage was observed on exposure to osmotic potential of - 0.46 MPa (20% PEG 6000). In general, the seeds primed with inoculum of *Rhizobium* sp. VRE1 significantly improved the germination percentage over uninoculated control under induced moisture stress. Similar to the findings of Sun *et al*. (2010) [26], where priming seeds with moisture stress-tolerant plant growth-promoting microbial inoculants accelerated the glucose metabolism of seeds imposed with moisture stress (Kanchiswamy *et al*. 2015) [13].

Table 1: Biochemical adaptations of *Rhizobium* sp. VRE1 under induced moisture stress

Parameters	Non-stress (NS)	Drought stress (DS)			
	Un - inoculated control	Control (0%)	15% PEG	20% PEG	25% PEG
Proline (µ mol g^{-1} DW)	3.81±0.89^{e}	7.30±1.03^{d}	9.21±0.98^{c}	12.19±0.32^{b}	14.20±0.21^{a}
Total soluble sugars (µ mol g^{-1} DW)	10.20± 1.11^{e}	12.54±0.97^{cd}	14.29±2.32^{c}	16.62±1.76^{b}	21.23±2.13^{a}
Protein (mg g^{-1} DW)	19.04± 2.32^{a}	5.23±0.32^{d}	5.27±0.65^{d}	6.62± 0.12^{c}	7.20±0.33^{b}
Total free amino acids (µ mol g^{-1} DW)	8.45±0.67^{d}	12.21±1.21^{c}	19.35±2.09^{b}	22.25±2.13^{a}	25.20±1.87^{a}
Exopolysaccharides (EPS)	11.37±0.03^{d}	15.62±0.30^{d}	40.21±0.75^{c}	50.26±1.60^{b}	62.12±2.06^{a}

Values are mean (±standard error) (n=5) and values followed by the same letter in each column are not significantly different from each other on the observation day as determined by DMRT ($p \leq 0.05$).

Table 2: Germination percentage and vigor index of blackgram seeds under moisture - stress condition

Germination attributes	Un inoculated Control	*Rhizobium* sp. VRE1				
		Control 0% PEG	5% PEG	10% PEG	15% PEG	20% PEG
Germination %	98	100	90	88	68	60
Vigor Index	4130	4245	3150	1458	1291	1190

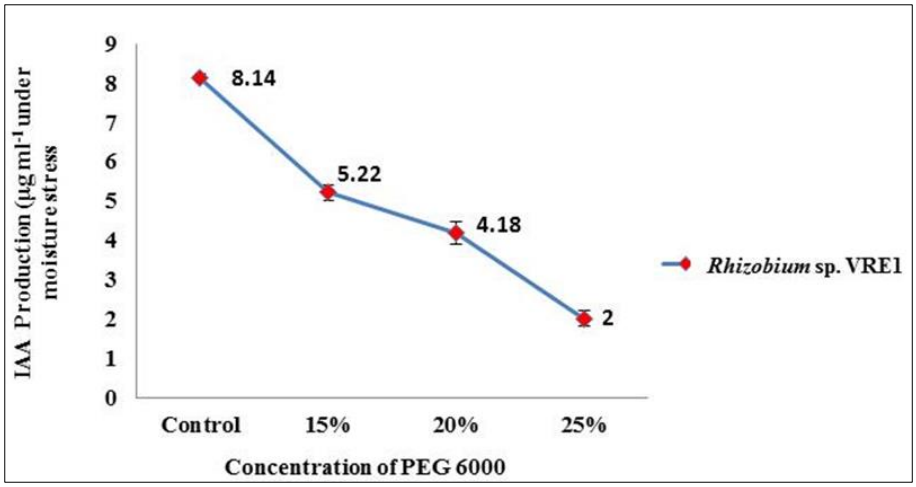

Fig 1: IAA production of *Rhizobium* sp. VRE1 under moisture - stressed condition at different PEG 6000 concentrations

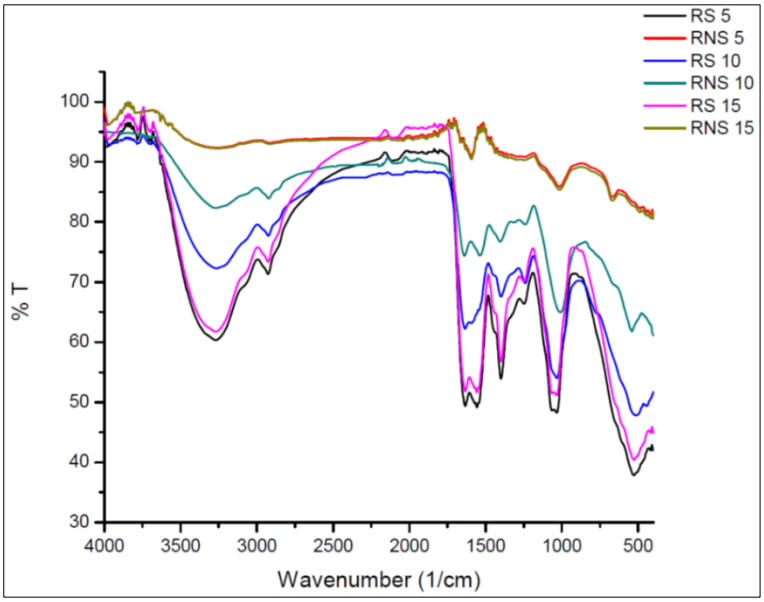

Fig 2: FT-IR spectra of the EPS from *Rhizobium* sp. VRE1 on induced moisture stressed conditions at a different time interval. RS 5 - *Rhizobium* sp. VRE1 stress at 5 days intervals; RNS 5 - *Rhizobium* sp. VRE1 under non - stress at 5 days intervals; RS 10 - *Rhizobium* sp. VRE1 stress at 10 days intervals; RNS 10 - *Rhizobium* sp. VRE1 under non - stress at 10 days intervals; RS 15 - *Rhizobium* sp. VRE1 stress at 15 days intervals; RNS 15 - *Rhizobium* sp. VRE1 under non - stress at 15 days intervals

Conclusion

The present study concludes, that the inherent ability of Rhizobium sp. VRE1 to tolerate increased osmotic potential is another paradigm in legume-rhizobia symbiosis. Further, the plant growth-promoting traits under a stressed environment may offer ample scope for an inexpensive, eco-friendly, and promising bio-inoculant to mitigate drought, especially in the rain shadow and rain-fed areas.

Acknowledgments

The Ministry of Human Resource Development, Government of India, financially supported this research through MHRD-FAST-CoE (F.No.5-6/2013-TSVII) sanctioned to SU. We acknowledge Professor and Head, Pulse Research Institute, Vamban, for offering quality seed materials.

Conflict of Interest: The authors declare no conflict of interest.

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