International Journal of Microbiology Research
ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 11, Issue 7, 2019, pp.-1651-1654.
Available online at https://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000234

Research Article

PERFORMANCE OF REDGRAM RHIZOBIAL ISOLATES UNDER WATER STRESS CONDITION

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Received: June 30, 2019; Revised: July 24, 2019; Accepted: July 26, 2019; Published: July 30, 2019

Abstract- A favorable ambiance is necessary for Plant-Microbe interaction. For an effective symbiosis, pulses are grown under arid and semi-arid conditions require drought-tolerant rhizobial strains. The efficiency of *Rhizobium* strains was analyzed under water stress conditions by addition of appropriate concentrations of polyethylene glycol 6000 (-0.1 to -1.0MPa water potential). In the present study 3 rhizobial cultures *viz*., CC1, RR3, and RR6 were taken, and the cultures showed good growth and survival under induced water stress with -0.5MPa and they were evaluated for the plant growth promoting traits. PGP traits *viz*., IAA, EPS production, biofilm formation, phosphate solubilization, siderophore production, survivability and seedling vigour under induced water stress was estimated under rhizobial inoculation supplemented with and without PEG and the results are presented in this paper.

Keywords- *Rhizobium, Drought, Polyethylene glycol, Plant growth promoting traits*

Citation: Abina S., *et al*., (2019) Performance of Redgram Rhizobial Isolates Under Water Stress Condition. International Journal of Microbiology Research, ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 11, Issue 7, pp.-1651-1654.

Introduction

The world's food security is affected by drought stress and it is the most significant abiotic stress which increased the intensity over last decades. Recent studies have also shown that declining water levels in the near future could lead to a 25% drop in harvest [1]. Drought stress affects germination, growth, development, flowering pod formation resulting in large economic losses in agriculture [2]. One of the main issues in rain-fed agro-ecosystem is the predominance of abiotic stress such as elevated temperature, salinity and drought where the survival and viability of applied bioinoculants is a significant problem. Water stress creates serious plant growth issues and will affect more than 50% of arable lands by 2050 [3]. Several scientists have evaluated the drought stress tolerance of bacteria simulated by polyethylene glycol (PEG) solution [4,5] and reported the increased production of legumes under water-limited environment [6]. Red gram (*Cajanus cajan* L.) is an important drought tolerant, widely adapted legume crop in Indian rainfed agriculture. PGPR holds promise for plant growth promotion and alleviation of plant drought stress [7, 8] in many agricultural crops and shown to alleviate drought stress in plants by reducing plant ethylene levels that are usually increased during unfavorable conditions [9]. Under adverse conditions, bacterial endophytes can promote plant establishment, enhance germination, seedling emergence, and plant growth [10]. Excellent root colonizing bacterium has the capacity to produce enzymes and metabolites which helps the plant to withstand abiotic stress conditions [11]. Hence the study has been made for evaluating the rhizobial cultures against water stress, so as to develop inoculants with better performance under stress condition.

Materials and Methods

Rhizobial cultures (CC1, RR3 and RR6) which are available in the Department of Agricultural Microbiology, AC and RI, Madurai were used for this study [Fig-1A].

Survival of *Rhizobium* at different PEG Levels

Survival and growth of rhizobial culture under moisture stress was studied using polyethylene glycol (PEG) 6000 at a different concentration ranging from -0.1 to -1.0MPa (megepascal) water potential in yeast extract mannitol (YEM) broth. 1ml of 24 h. old rhizobial culture was inoculated into 10 ml broth supplemented with [Fig-1B] and without PEG [18]. After incubation at 28±2°C for 48 h, the growth was measured spectrophotometrically at OD 420 nm.

Production of PGP traits under water stress

The rhizobial cultures were evaluated for the plant growth promoting traits under PEG induced water stress. Since the above cultures showed growth and survival up to -0.5MPa water potential this stress level was selected for evaluation.

Estimation of IAA production by rhizobial isolates under water stress

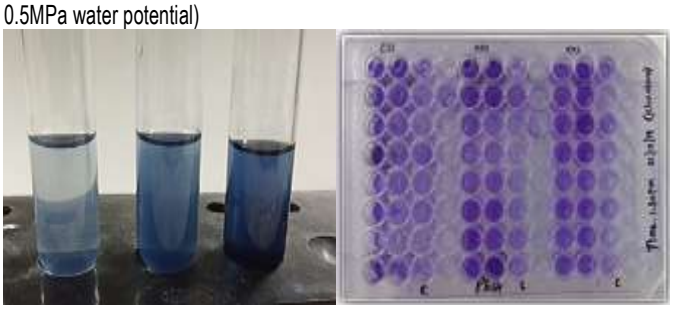
YEM broth was supplemented with tryptophan [8] under the presence (-0.5MPa) and absence of PEG for maintaining water stress. Cultures were inoculated and incubated under dark at 28±2°C for 5days. Incubated cultures were centrifuged for 10min. and then 500 µl of supernatant was taken to which 50 µl 0.1 mM orthophosphoric acid and 2 ml of Salkowaski reagent was added and kept for 30 min. under dark for colour development. Colour intensity was measured spectrophotometrically at OD 530 nm and IAA production was calculated using standard curve and expressed as µg ml^{-1}.

Quantification of biofilm formation by rhizobial isolates under water stress

Biofilm formation assay was done on 96 well microtiter plates [12]. 10µl of cell suspension was inoculated in each well containing 100 µl of YEM broth and supplemented with and without PEG -0.5 MPa to maintain water stress and control conditions. Plates were incubated at 30±2°C for 48 h.

Initial cell turbidity was observed using a microtiter plate reader at an OD of 590 nm. Later, the broth was removed from the wells and washed 2 times with sterile water to remove freely attached cells. Each well in air-dried plates were filled with 100µl of 1% crystal violet solution and kept as such as for 45min. The stained plates were rinsed with sterile water for 2-3 times. Finally, biofilm formation was observed by the purple ring formation on the side wall of each well. Biofilm formation was assessed by adding 200µl of 95% ethanol to destain the wells and colour intensity of crystal violet present in a destained solution was measured at 590 nm using a microtiter plate reader.

Fig-1 Growth of *Rhizobium* without (A) and with PEG induced (B) water stress (-0.5MPa water potential)



(A) Phosphate solubilization (B) Biofilm formation

Fig-2 Production of PGP traits under water stress PEG 6000 (-0.5MPa water potential)

Fig-3 Effect of seed germination and seedling vigor under PEG induced water stress at *in vitro* conditions on 15 DAS (-0.5MPa) *in vitro* condition (1) uninoculated control with no stress (2) uninoculated control with stress (3) *Rhizobium* CC1 with no stress (4) *Rhizobium* CC1 with stress (5) *Rhizobium* RR3 with no stress (6) *Rhizobium* RR3 with stress (7) *Rhizobium* RR6 with no stress (8) *Rhizobium* RR6 with stress.

Quantitative assay of phosphate solubilization by rhizobial isolates under water stress

Phosphate solubilization was assessed according to [13]. The rhizobial cultures were inoculated into 5ml of Pikovskaya's broth supplemented with 0.5% tricalcium phosphate with and without PEG -0.5MPa (20%) to maintain water stress and control conditions and incubated at 30±2°C. After 7 days of incubation, the broth was centrifuged for 5 min. and 1 ml of the supernatant was taken followed by addition of 1 ml distilled water. After that, 2 ml of color reagent was added and the volume was made up to 6 ml by addition of distilled water and incubated for 15 min. to develop blue color and the color intensity was measured in spectrophotometer at OD 882nm. KH_2PO_4 was used as standard and the amount of phosphate solubilization was expressed as total P release mg ml^{-1}.

Quantification of exopolysaccharide production under water stress

Quantification of exopolysaccharide production was done according to [14]. EPS was extracted from 7 days old rhizobial culture grown in YEM broth (supplemented with and without -0.5MPa PEG). 2ml of culture was centrifuged for 10 min. then 1ml of supernatant was taken to which 2ml of 90% ethanol was added and incubated for 24 h. Again, it was centrifuged at 8000 g for 15 min. and then obtained precipitate was dissolved in 2ml of distilled water. Later 200 µl of 5% phenol and 1ml of 93% sulphuric acid were added and kept aside for 10min. to form a yellow color which was an indication as positive for EPS production. The absorbance was recorded at 490 nm using the spectrophotometer. The EPS production was expressed as the release of reducing sugars µg ml-.

Siderophore production

A qualitative analysis of the siderophore production was done on solid CAS universal blue agar plates. The CAS assay was used to test the capability of bacterial isolates to produce iron requisite compounds of siderophore type in solid medium. Chrome Azurol S blue agar media was prepared and poured into Petri plates. On CAS blue agar ten microlitres of 48h. old rhizobial cultures which were grown on broth supplemented with and without PEG were spot inoculated, and uninoculated CAS agar plate was maintained as a control and the plates were incubated in dark for 3 days at 28±1°C. The CAS reaction was determined by measuring the position of the color change from blue to yellow-orange in the CAS blue agar [15].

Effect of *Rhizobium* on seed germination and seedling vigour of redgram under *in-vitro* conditions

The seeds were surface disinfected with 0.05% sodium hypochlorite for 5 min and rinsed with distilled water several times and imbibed in 24 h. old rhizobial suspension for 4 h. Then treated seeds were placed on 1% agar tubes [16] supplemented with and without PEG and incubated for 15 days to assess germination percentage and vigour index. The following formula was used to calculate the vigour index.

Vigour index = germination percentage x plant height

Results and Discussions

The results obtained out of the above experiments were presented. The rhizobial isolates grew well in YEM broth without PEG 6000 at OD 520 nm and showed reduction with increase in PEG levels. The growth of the cultures was good up to -0.4MPa and observed moderate at -0.5MPa. More than-0.5MPa, CC1 showed little growth and other cultures did not show any growth. However, all the three rhizobia *viz*., CC1, RR3, and RR6 were surviving under water potential -0.5MPa with varying levels of growth. Reduction in growth was observed with increased PEG levels at 30% PEG 6000 [1] [22] [Table-1]. Based on these, PGP traits of these cultures were tested at a water potential -0.5MPa.

Table-1 *Survival of rhizobial strains at different PEG 6000 levels*

S	With PEG6000 (MPa)	CC1	RR3	RR6
1	0	+++	+++	+++
2	-0.1	+++	+++	+++
3	-0.2	+++	++	++
4	-0.3	+++	++	+
5	-0.4	++	++	+
6	-0.5	+	+	+
7	-0.6	+	-	-
8	-0.7	-	-	-
9	-0.8	-	-	-
10	-0.9	-	-	-
11	-1	-	-	-

>1.00 +++, 0.5-1.00 ++, <0.5 +, Negative -

PGP traits under water stress

All the three rhizobial cultures were tested for plant growth promotion under stress and unstressed condition PGP traits *viz*., IAA, EPS production, biofilm formation,

Table-2 *Evaluation of Plant growth promotion traits of Red gram rhizobial cultures under water stress condition using Polyethylene glycol (-0.5MPa).*

Isolates	IAA (µg/ml)		PO_4 solubilization (mg/ml)		EPS production (µg/ml)		Biofilm formation	
	Without stress*	Stress**	Without stress*	Stress**	Without stress*	stress**	Without stress*	Stress**
CC1	6.9±0.2^a	6.7±0.2^b	43.8±0.2^c	18.3±0.4^c	5.8±0.1^c	5.3±0.1^c	+	++
RR3	5.1±0.1^b	7.7±0.1^a	60.0±0.5^b	42.5±0.8^a	12.8±0.0^a	14.4±0.2^a	++	+++
RR6	3.0±0.0^c	5.6±0.1^c	64.5±1.0^a	37.5±0.4^b	9.7±0.3^b	11.5±0.1^b	++	++
SEd	0.27	0.18	0.93	0.76	0.18	0.12		
CD(0.05)	0.67	0.45	2.29	1.86	0.46	0.31		

*PEG- Poly ethylene glycol; PEG levels * = 0MPa; ** = -0.5MPa; +++ =Strong; ++ = Moderate; + = Weak;*
Values are mean (± Standard error) (n=3) and column values followed by different letters are significantly different from each other at 5% LSD.

Table-3 *Effect of rhizobia on seed germination, growth and vigour of Red gram seedlings under in vitro conditions under PEG induced water stress (-0.5MPa)*

Isolates	Plant height (cm/plant)		Germination Percentage (%)		Vigour Index	
	Without stress*	Stress**	Without stress*	Stress**	Without stress*	stress**
CC1	35.00±0.05^b	18.00 ±0.32^b	100.00 ±2.13^a	70.00 ±0.91^b	3500.00±32.79^b	1260.00 ±13.9^b
RR3	37.90±0.55^a	22.00 ± 0.44^a	100.00 ± 2.56^a	80.00 ±0.62^a	3790.00±31.56^a	1980.00 ± 15.4^a
RR6	33.50±0.56^c	15.00 ± 0.30^c	90.00 ± 1.26^b	70.00 ±1.16^b	3350.00±17.43^c	1050.00 ± 6.0^c
Control	30.50±0.29^d	6.34 ± 0.24^d	90.00 ± 2.06^b	66.70 ± 3.34^c	3050.00±14.29^d	371.32 ± 18.5^d
SEd	0.59	0.46	2.91	2.61	35.90	20.13
CD(0.05)	1.36	1.08	6.71	6.03	82.80	46.43

*PEG- Poly ethylene glycol; PEG levels * = 0MPa; ** = -0.5MPa, Values are mean (± Standard error) (n=3)*
and column values followed by different letters are significantly different from each other at 5% LSD.

All the three rhizobial cultures were producing IAA under stress as well as unstressed condition. IAA production was reported comparatively higher in bacteria and actinomycetes during stressed conditions [17]. RR3 and RR6 cultures produced more IAA under stressed condition (7.7µg ml^{-1} and 5.6µg ml^{-1}) whereas CC1 recorded higher production of IAA under unstressed condition (6.9µg ml^{-1}). Significant variation in IAA production was observed among the cultures, both at stressed and unstressed condition. Earlier IAA production was studied in *Paenibacillus* under water stress, which was found reduced at increasing PEG levels [18] and similar decrease was reported in Brady *Rhizobium* also [19]. Higher production of IAA by the rhizobial cultures RR3 and RR6 under -0.5Mpa water potential might be due to the drought tolerance of the cultures. Phosphorous is vital for plant growth. Solubilization of phosphate was commonly detected in all the three rhizobial cultures under stressed as well as unstressed conditions [Fig-2A]. The results also showed the reduction in PO_4 solubilization potential of the cultures under stress. However, among which RR3 registered the higher quantity of PO_4 solubilization (42.5 mg ml^{-1}) under stress and RR6 was observed to release high quantity of P (64.5 mg p release per ml) under unstressed condition. Earlier it was reported that the degree of solubilization differed with microorganism with increasing crop yield [20]. All the three cultures registered significant variation in IAA production and solubilization of insoluble phosphate. EPS production was noticed in all three cultures under stressed and unstressed condition. Similar to IAA more EPS production was noticed under stressed condition in RR3 and RR6 cultures. RR3 recorded significantly higher EPS (14.4 µg ml^{-1}) followed by RR6 and the least was noticed in CC1. The result indicates that rhizobial cultures showed significant difference in EPS production which helps the organism to alleviate drought stress. High quantity of EPS production by rhizobia under stress can contribute for increased growth and tolerance in inoculated plants. Exopolysaccharide (EPS) produced by microorganism which protects themselves as well as plants from adverse conditions. Addition of EPS producing microbes to a drought stressed plants helps in drought mitigation [21]. Biofilm formation was assessed for all the three rhizobial culture at -0.5MPa water potential [Fig-2B]. Generally, the tested cultures tend to form strong biofilm under stress compared to unstressed condition. *Rhizobium* RR3 found to form strong biofilm formation under stressed conditions followed by RR6 and CC1 which produced moderate biofilm. Under unstressed condition RR3 and RR6 showed moderate biofilm and CC1 showed poor biofilm. Biofilm provides cell aggregation, which increase population at a specified area. When there is a stress, the cells aggregate and tend to form bioflim, might be due to quorum sensing to potent themselves from stress.

Seed germination and seedling vigour

All the three rhizobial cultures were tested for seed germination and increasing seedling vigour in Redgram under stress and unstressed condition [Fig-3]. Seed germination was 100% in CC1 and RR3 inoculation, which was significantly higher than RR6 inoculation (90.00%) under unstressed condition. But under -0.5MPa water potential, the reduction in seed germination was observed and among them, RR3 had higher germination (80%). Plant growth and vigour index was significantly influenced by rhizobial inoculation. Significant variation among the cultures was noticed both under stress as well as unstressed condition. RR3 culture performed significantly superior in enhancing plant growth both under stress (22.00 cm/plant) as well as unstressed (37.90 cm/plant) condition compared to other cultures tested. It recorded an increase of 24.30% under unstressed and 247% under stressed condition over uninoculated control. Similar results obtained in vigour index also [Table-3], where rhizobial inoculation enhanced the vigour of the seedlings upto 24% under unstressed condition over uninoculated control. A tremendous increase in growth and vigour was noticed at water stress (-0.5Mpa water potential) due to inoculations, which was about 3 folds over uninoculated control. Increase in seedling vigour at early stages of crop growth can contribute for increased redgram production under stress. All the three rhizobial inoculations enhanced plant growth and vigour of redgram to huge extent in stressed condition compared to unstressed condition might be due to the increase in potential of the rhizobial cultures when exposed to stress. Similar results were reported earlier in growth of Chickpea under drought stress with the inoculation of Pseudomonas and *B.amyloliqufaciens* [22, 23].

Conclusion

The increased ability for IAA production, EPS production as well as enhancing seedling vigour by the rhizobial isolate RR3 at induced stress (-0.5MPa) showed the inherent ability and genetic potential of the isolate to express the drought tolerance character as well as the PGP traits. Though the standard culture CC1 showed equivalent growth at stress, it might be lacking for the expression of PGP traits under stress. Hence the potential isolate of RR3 could be exploited for inoculant development suitable for drought prone areas towards sustainable agriculture.

Application of research: *Rhizobium* under drought stress is the need of the hour, due to prevailing drought as well as the reduction in efficiency of rhizobial strains. Hence this study has been proposed to elucidate the efficiency of *Rhizobium* under induced water stress condition. The effective culture of *Rhizobium* (RR3) has been identified for inoculant development towards increasing redgram productivity under moisture stress.

Abbreviations: PGP - Plant growth promotion, YEM – Yeast extract mannitol PEG- Poly ethylene glycol, MPa - Megapascal, EPS - Exopolysaccharides, IAA – Indole acetic acid OD - Optical Density CAS – Chrome Azurol S

Acknowledgement / Funding: Authors are thankful to Agricultural College and Research Institute, Madurai, 625 104, Tamil Nadu Agricultural University, Coimbatore, 641 003, Tamil Nadu, India

***Research Guide or Chairperson of research: Dr. K. Kumutha**
University: Tamil Nadu Agricultural University, Coimbatore, 641 003, Tamil Nadu
Research project name or number: M.Sc. Thesis

Author Contributions: All authors equally contributed

Author statement: All authors read, reviewed, agreed and approved the final manuscript. Note-All authors agreed that- Written informed consent was obtained from all participants prior to publish / enrolment

Study area / Sample Collection: Department of Agricultural Microbiology, Agricultural College and Research Institute, Madurai, 625 104

Cultivar / Variety / Breed name: Nil

Conflict of Interest: None declared

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.
Ethical Committee Approval Number: Nil

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