International Journal of Plant & Soil Science

32(1): 92-98, 2020; Article no.IJPSS.55241
ISSN: 2320-7035

Physiological Adaptation and Plant Growth Promoting Functional Traits of *Bacillus altitudinis* FD48 under *In vitro* Osmotic Stress

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IJPSS/2020/v32i130238
Editor(s):
(1) Dr. Yong In Kuk, Professor, Sunchon National University, South Korea.
Reviewers:
(1) Moataz Eliw Mostafa, Al-Azhar University, Egypt.
(2) Schirley Costalonga , Universidade Federal do Espírito Santo, Brazil.
Complete Peer review History: http://www.sdiarticle4.com/review-history/55241

Original Research Article

Received 03 January 2020
Accepted 26 February 2020
Published 27 February 2020

ABSTRACT

To develop an osmotolerant microbe, as a bioinoculant to mitigate drought it is vital to understand the impact of osmotic stress on their growth and plant growth promoting functional traits. The present study was aimed to evaluate the physiological adaptations and plant growth-promoting traits of a phyllosphere bacterium *Bacillus altitudinis* FD48 under osmotic stress conditions. The FD48 strain isolated from rice (cultivar ADT43) phyllosphere obtained from Biocatalysts laboratory, Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore. *In vitro* bioassay was conducted to evaluate the osmotolerant potentials of FD48. *B. altitudinis* FD48 grown in LB supplemented with PEG 6000 and grown for 48 hrs. Physiological adaptation to osmotic stress was observed by assessing the osmolytes and free amino acids content produced by FD48 under induced stress. Further the plant growth promoting traits under osmotic stress also asceratined. The growth pattern of FD48 strain decreased with the increase in PEG concentrations. The lower level of osmotic stress enhanced the growth of FD48 but at higher concentration exhibited a decline in growth. Enhanced levels of IAA (25 µg g^{-1} of protein) and EPS (9.76 mg mg^{-1} protein) production were recorded in the FD48 strain at lower levels of osmotic stress. Furthermore, an increase in osmotic stress had a deleterious effect on IAA production and ACC deaminase activity while the exopolysaccharide production was enhanced. Growth of FD48 under osmotic

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stress also increased the accumulation of proline and compatible sugars that will protect the FD48 strain by maintaining the turgor potential of cells and stabilizes the membrane proteins. Hence, the results of our study suggesting that, *B. altitudinis* FD48 strain has the potential to tolerate osmotic stress and might be used as a newer bio-inoculant for triggering moisture deficit stress resilience in plants.

Keywords: PGPB; osmotic stress; Bacillus; plant growth promotion.

1. INTRODUCTION

Drought is considered one of the significant constraints for agricultural productivity worldwide and it is reported to decrease the yield loss of cereals by 9-10% [1]. Hence, there is increasing attention to find a sustainable solution to these drought-related issues and its impact on food security [2]. Recent researches indicated that harnessing the potentials of plant-associated bacteria could help the plants to withstand the osmotic stress [3].The underlying mechanisms include: production of phytohormones, antioxidants, osmolytes and 1-aminocyclopropane-1-carboxylate deaminase (ACCD) that confers microbe induced systemic tolerance (IST). Plant growth-promoting bacteria (PGPB) colonized in the rhizosphere are well known for their role in osmotic stress resilience [4]. However, only fewer studies focused on phyllosphere bacteria for abiotic stress mitigation in plants [5-6].

Bacteria present in sites under water-limited conditions or where dry spells frequently occur have shown to increase the plant growth better than in normal environments. Plant primed with these PGPB helps to cope with osmotic stress was reported in rice, wheat, and tomato [7-8]. Plant associated bacteria acclimatized in the stress-induced environment is crucial for promoting plant growth under osmotic stress. In general osmotic stress also has an impact on plant growth-promoting attributes on beneficial bacteria, either beneficial or deleterious. Previous studies by Sandhya et al. [4] and Manjunatha et al. [9] reported that drought stress adversely affects P solubilization, nitrogen fixation and phytohormone production ability of PGP bacteria. Thus, exploring the potential inoculants for arid and semi-arid regions, it is vital to study the impact of osmotic stress on bacterial growth and PGP traits of the beneficial bacteria. Hence, the present study was aimed to evaluate the effect of osmotic stress on the growth and plant growth-promoting attributes of beneficial bacteria *B. altitudinis* FD48 obtained from the phyllosphere of rice plants under *in vitro* osmotic stress.

2. MATERIALS AND METHODS

2.1 Bacterial Strain and Growth Conditions

Bacterial strain, *Bacillus altitudinis* FD48 [10] used in this study (previously isolated from the phyllosphere of rice cultivar ADT43), was obtained from the Biocatalysts laboratory, Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore. TheFD48 strain was routinely grown in LB broth, incubated at 28°C at 120 rpm.

2.2 Bacterial Growth under *in vitro* Osmotic Stress

Luria Bertani (LB) broth with elevated water potentials (0 to 35%) was prepared by supplementing an appropriate concentration of PEG 6000 (polyethylene glycol) [11]. The broth was inoculated with overnight culture (1%) of *B. altitudinis* FD48 and incubated at 28°C at 120 rpm in an orbital shaker for 48 hrs. The growth was observed at periodical intervals by measuring the absorbance at 600 nm in a spectrophotometer (Spectramax I3X). The growth of FD48 at various water potentials was recorded. Three replicates were maintained for each level of stress and the uninoculated broth served as control. Consequently, the protein content on each level of stress was determined by the Bradford method [12].

2.3 Exopolysaccharides Production (EPS)

Exopolysaccharides (EPS) produced by *B. altitudinis* under induced osmotic stress level (30% PEG) were analyzed and compared with non-stressed conditions. EPS was extracted periodically up to 15th day of culture (FD48) grown in LB with PEG 6000 (-1MPa stress) by centrifugation at 20,000 g for 25 mins, and resultant supernatant was obtained. The pellet was washed twice with 0.85% KCl for the complete extraction of EPS. Protein concentration in the supernatant was determined using Folin's reagent [13]. The supernatant

filtered through nitrocellulose membrane (0.22 µm) was dialyzed against sterile distilled water at 4°C. The Dialyzate was obtained by centrifugation at 20000 x g for 30 mins. In order to eliminate the presence of insoluble material, the supernatant was mixed with 3 volumes of ice-cold ethanol and kept overnight at 4°C for precipitation. EPS precipitate obtained by centrifugation at 10,000 g for 15 min was subjected to further analysis. The mass of both fresh and dry EPS precipitate was measured and expressed in terms of mg.mg^{-1} of protein.

2.4 Total and Free Amino Acids

Bacterial cells inoculated in LB broth supplemented with 30% PEG 6000 (-1MPa drought-stressed) were incubated at 28°C for 48 h and centrifuged at 3000 x g for 5 mins. The collected pellet was added with 70-80% ethanol and boiled in a water bath at 60°C for 45 mins. Then the content was centrifuged at 10,000 x g for 15 mins and the collected supernatant used for further estimation of amino acid as described by Moore and stein [14]. The supernatant also used for the estimation of free proline produced in the bacterial culture [15].

2.5 Total Sugar Content

According to the method of Dubois et al. [15], total sugars were determined. Cell pellets obtained as stated above for total amino acids estimation were dissolved in 4:1 w/v methanol: chloroform and boiled at 60°C in the water bath for 15 mins. Consequently, the mixture was centrifuged at 10000 X g for 10 min, and the supernatant used for total sugars determination. The results are expressed as µ mol g^{-1} DW.

2.6 Functional Traits for Plant Growth Promotion under Osmotic Stress

2.6.1 ACC deaminase production

To assess the presence of ACC deaminase activity, *B. altitudinis* cells grown in 5 ml LB broth at 28 ±2°C for 48 h were harvested by centrifugation and washed thrice with 0.1 M Tris HCl (pH 7.5) for induction of ACC deamination activity [16]. Subsequently, the pellets were dissolved in 2 ml of mODified minimal media (DF minimal media) supplemented with 3mM concentration of ACC with PEG 6000 (-1MPa) for osmotic stress condition and incubated at 28±2°C for 36-72 h in an orbital shaker. ACC deaminase activity was measured by determining the cleavage of ACC by ACC deaminase into α-ketobutyrate and ammonia [17]. The stimulated bacterial cells were collected by centrifugation (3,000 g for 5 min) rinsed twice with 0.1 M Tris-HCl (pH 7.5) and dissolved in 0.1 M Tris-HCl (pH 8.5) followed by cell labilization with 5% v/v toluene, further vortexed for 30s at the highest speed. About 50 µl of labilized cell suspension was mixed with 0.3 M ACC (5 µl) in a microfuge tube, incubated at 30°C for 30 min. Then, 50 µL cell suspension without ACC served as negative control and 50 µl of Tris HCL buffer with 5 µl of 0.3 M ACC were used for blank. Then the samples added with 500 µl of 0.56 N HCl were vortexed and centrifuged (12,000 g for 5 min) to eliminate the cell debris. The supernatant (500 µl) was transferred to a new test tube mixed with 0.056N HCl (400 µl) and 150 µl of dinitrophenylhydrazine (DNF) solution. Then the mixture incubated for 30min at 28°C. One milliliter of 2N NaOH was mixed with the sample, and absorbance measured at 540 nm. The standard curve was plotted using α-ketobutyrate concentration (mM). The protein concentration of the labilized cells was determined by the Bradford method [12]. The activity of ACC deaminase expressed as nmoles of α-ketobutyrate mg^{-1} protein h^{-1}.

2.6.2 IAA production

B. altitudinis FD48 grown in LB broth supplemented with tryptophan and PEG 6000 were assessed for their ability to produce IAA under osmotic stress. The LB broth without PEG served as a control. For each treatment, three replicates were maintained. The culture was allowed to grow up to 3-5 days at 28±2°C. After 5 days of incubation, the culture broth was collected by centrifugation at 6,000X g for 10 mins. The supernatant collected was added with two drops of orthophosphoric acid, followed by 2 ml of Salkowski's reagent (2% of 0.5 M $FeCl_3$ in 35 % perchloric acid) and incubated under dark for 30 min. The absorbance was measured at 530 nm using a spectrophotometer (Spectramax I3X). IAA content was calculated against a standard curve prepared using Indole Acetic Acid and was expressed as µg. mg^{-1} protein.

2.7 P solubilization

P solubilization ability of the bacterial strain *B. altitudinis* FD48 was determined under induced osmotic stress. For induction of osmotic stress, Pikovskya's broth was supplemented with

30% PEG 6000. Pikovskya broth without PEG used as control. Three replications were maintained. Phosphate solubilized in the broth was estimated in the cell-free supernatant by the method of Jackson [17]. Absorbance was recorded at 600 nm using a spectrophotometer (Spectramax I3x). The amount of phosphate solubilized was expressed as µg P solubilized mg^{-1} protein.

2.8 Statistical Analysis

All data were statistically analyzed in Microsoft Excel and add-in with XLSTAT Version 2016.04.325250 (XLSTAT, 2016). Each treatment was performed with at least five replicates, and the standard deviation was calculated and expressed in mean ±SD of five replicates.

3. RESULTS AND DISCUSSION

3.1 Bacterial Growth under *In vitro* Osmotic Stress

In the present investigation, physiological and biochemical modification and adaptations of osmotic stress-tolerant *B. altitudinis* FD48 and its plant growth-promoting traits under *in vitro* osmotic stress was evaluated [18]. Plant growth-promoting bacteria belonging to the different genera are reported to possess abiotic stress tolerance [19]. Osmotic stress is known to severely affect the bacterial growth [8], in order to study the impact of induced osmotic stress on the growth pattern of *B. altitudinis* FD48 was evaluated using the PEG 6000 supplemented in LB broth at elevated concentrations (0-35%). To compare with growth under optimal conditions, control was maintained without PEG 6000. Further, the growth pattern of FD48 strain was monitored by measuring absorbance at OD 600 nm at a periodical interval of up to 72 h of inoculation. An increase in PEG concentration showed a decline in trends in growth (Fig. 1). However, in the presence of 15% PEG, the growth of FD48 was not much affected (1.45), whereas at 30% PEG, the maximum growth obtained was (0.882 OD) observed after 48 h of inoculation. In 35% PEG 6000 the bacterial growth was low and registered (0.47 OD). At 72 hrs of inoculation FD48 strain grown at all the PEG concentrations attains late stationary phase. While osmotic stress affects bacterial growth, no such effect was found on the growth of *B. altitudinis* FD48 up to 30% PEG 6000 (-1Mpa) indicating that FD48 strain tolerant to higher osmotic potential. Similarly, *Bacillus* sp., isolated from maize rhizosphere was able to tolerate -0.73 Mpa and its plant growth-promoting traits were reported [3]. PGPB exposed to adverse environmental conditions are generally acclimatized to such unfavorable conditions without affecting their growth is vital to use as a bio-inoculum to promote plant growth under arid and semi-arid regions [20].

3.2 Physiological Adaptation to Osmotic Stress of *B. altitudinis*

Production of sugars and amino acids by the osmotolerant bacterial strain *B.altitudinis* FD48 was determined under non-stressed (NS) and osmotic stress (S) conditions. EPS, Total soluble sugars, proline, and total free amino acids were significantly increased in both stress level (15% and 35% PEG 600). Whereas the protein content of FD48 was reduced under osmotic stress irrespective of the stress intensity. The protein concentration of the FD48 strain grown under standard conditions showed 25.16 mg protein g^{-1}. Whereas under PEG induced condition, the protein content was considerably reduced (Table 1). At 15% PEG concentration, the protein content was slightly reduced (21.25 mg protein g^{-1}) and a higher concentration of osmotic stress i.e. 35% PEG 6000, protein content was 35% (12.86 mg protein g^{-1}) lowered compared to optimal condition. Under osmotic stress condition bacteria produced a conspicuous amount of exopolysaccharides. In the present study, EPS production was observed maximum at 35% PEG (15.21 mg mg^{-1} protein). EPS production found to be increased with the increase in PEG concentration forms a sheath around the cells creates a microenvironment and protect the cells from adverse environments [21].

Accumulation of compatible solutes and osmolytes has been reported to produce maximum at osmotic stress conditions. Accumulation of osmolytes helps to cope with osmotic stress by maintaining osmotic turgor potential in cells [22]. Accordingly, the present investigation also reported a significant increase in total free amino acid content and free proline in FD48 under osmotic stress (Table 1). Fascinatingly, at a higher concentration of osmotic stress (35% PEG), the maximum free amino acid content of 42.32 µ mol g^{-1} DW was recorded. Furthermore, the total soluble sugars analyzed also significantly increased in both the levels of osmotic stress registering 13.67 and 33.65 µ mol g^{-1} DW for 15 and 35% PEG 6000 respectively. Proline as compatible solute plays a

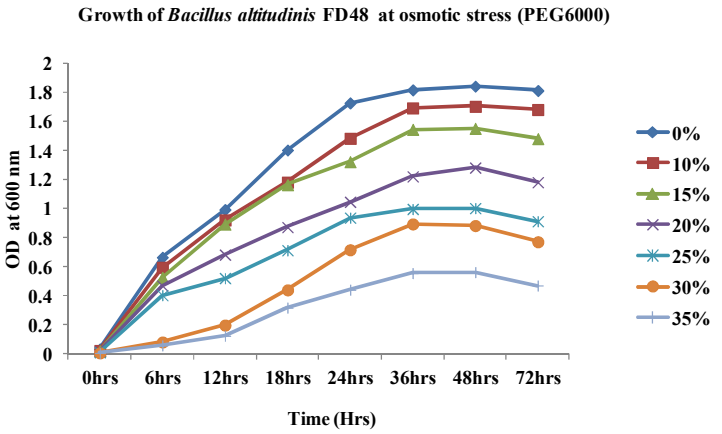

Fig. 1. Impact of osmotic stress (PEG 6000) on growth of *B. altitudinis* FD48

Table 1. Physiological and biochemical adaptations of osmotic stress-resilient *B. altitudinis* FD48 under non-stress and osmotic stress

Treatments	EPS Production mg mg^{-1} protein	Protein mg g^{-1} DW	Total free amino acids µ mol g^{-1}DW	Proline µ mol g^{-1} DW	Total soluble sugars µ mol g^{-1} DW
Control	2.98 ± 0.10	25.16 ± 0.36	18.12 ± 0.14	6.34 ± 0.05	7.65 ± 0.11
15% PEG	9.76 ± 0.03	21.25 ± 0.52	27.65 ± 0.33	11.51 ±0.12	13.67 ± 0.45
35% PEG	15.21 ± 0.35	12.86 ± 0.21	42.32 ± 0.56	21.78 ± 0.54	33.65 ± 0.27

Each treatment was performed with at least five replicates and the standard deviation was calculated and expressed in mean ±SD of five replicates

Table 2. Effect of osmotic stress on plant growth-promoting traits of *B. altitudinis* FD48

Treatments	P solubilization (mg. mg^{-1} protein)	IAA prODuction (µg g^{-1} protein)	ACC deaminase activity n moles α- ketobutyrate $mg^{-1}.h^{-1}$
Control	8.3 ± 0.07	18 ± 0.13	156 ± 0.34
15% PEG	7.9 ± 0.01	25 ± 0.34	185± 1.87
35% PEG	7.6 ± 0.04	19 ± 0.4 3	98± 2.31

Each treatment was performed with at least five replicates and the standard deviation was calculated and expressed in mean ±SD of five replicates

crucial role in scavenging reactive oxygen species (ROS) as well as protects the cells against the adverse conditions such as salinity and drought by maintaining redox homeostasis via stabilization of membrane proteins [23].

3.3 Effect of Osmotic Stress on Plant Growth-promoting Attributes of *B. altitudinis* FD48

PGP traits under osmotic stress can help the plants to cope from drought stress and maintaining the nutritional balance thus enabling the plant-microbial interactions could be used as a standard for the assortment of PGP strains for the crops cultivated under drought-prone regions [3,8]. PGPB induced osmotic stress alleviation attributed to several mechanisms includes the production of hormones, P solubilization, ACC deaminase activity, and EPS production [24]. In the present investigation, the osmotic stress-resilient *B. altitudinis* FD48 was evaluated for their plant growth-promoting activities under induced osmotic stress. The results of this study revealed that osmotic stress significantly affected the plant growth-promoting traits of *B. altitudinis* FD48 strain (Table 2). P- solubilization activity ofFD48 was not affected by osmotic stress. P solubilization was reported to be the most important trait for drought tolerance [25]. However, at 35% PEG concentration, P solubilization was slightly reduced. Whereas IAA production and exopolysaccharide production by FD48 under osmotic stress were increased with

the increase in osmotic stress (18 µg g^{-1} at 15% PEG). Nevertheless, IAA production at 35% PEG was low (19 µg g^{-1}). Generally, IAA triggers stress tolerance by altering the physiological and chemical changes in plants. Our results were in line with Malhotra et al. [25] reported the increased IAA production by *Azospirillum brazilience* under abiotic stress. Moreover, the osmotolerant bacteria used in this study also possessed the ACC deaminase activity which converts ACC into 2-oxoglutarate and Ammonia. These facilitate the plant growth and development by reducing the ethylene levels and protect the plant from osmotic stress [26-27]. However, ACC deaminase activity was found to be increased at 15% PEG 6000 concentration, whereas at 35% PEG, it showed a declining trend. Vejan et al. [28] described that exopolysaccharide and phytohormone production by PGPB also helps to withstand under drought stress.

4. CONCLUSION

In the present investigation, the moisture stress resilient phyllospheric bacteria *Bacillus altitudinis* FD48 strain was assessed for their multi phasic plant growth promoting activities under *in vitro* induced osmotic stress. The results suggest that FD48 strain has the capability to tolerate the detrimental effects of osmotic stress it can be the potential bioinoculant, improves the plant growth in arid and semi-arid region for sustainable agricultural production.

ACKNOWLEDGEMENTS

This study was supported by the Ministry of Human Resource Development, Government of India through MHRD-FAST-CoE (F.No.5–6/2013-TSVII) sanctioned to SU.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/55241