



RESEARCH ARTICLE

Impact of Moisture Stress and *Bacillus altitudinis* FD48 on Physiological Modulation and Seed Germination in Rice (*Oryza sativa* L.)

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ABSTRACT

The impact of moisture stress tolerant *Bacillus altitudinis* FD48 in promoting seed germination and physiological phenomenon under water deficit was evaluated. Rice seeds (Var CO51) biotized with *B.altitudinis* FD48 enhanced the seed germination and seedling vigor index by 51.05% followed by *Methylobacterium* sp., (37.3%) moisture stress seedlings (-0.7 Mpa osmotic potential). Plant biomass, accumulation of proline and compatible sugars also increased in FD48 treated seeds under moisture stress. Further, the alpha amylase activity also accelerated with increase in moisture stress intensity. GC-MS profiling of bacterial primed seed exudates revealed 35 diverse compounds belonging to the class viz., sugars, aminoacids, organic acids, flavones, prenol lipids and fatty acids. The *B. altitudinis* FD48 primed seeds alone released methoxy flavone which may act as a lure for recruiting beneficial rhizospheric bacteria. Hence, this study implies that seed priming with *B.altitudinis* FD48 is a promising approach for enhancing seed germination and seedling establishment in rice for drought prone areas and subsequent recruitment of beneficial bacteria that promotes plant growth and fitness.

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INTRODUCTION

Rice (*Oryza Sativa* L.) is an important cereal crop, serves as a staple food for more than half billion people across the globe contributing 20%-80% of daily calorie requirement. Rice crop is delicate to moisture stress that critically impairs the production of 23 billion hectares of rainfed rice in Asia (Kumar *et al.*, 2015). Unprecedented drought and erratic distribution of rainfall due to climate change are the major bottlenecks in rainfed regions, mainly for aerobic rice cultivation. Among the plant growth and developmental stages of rice plant, seed germination is a critical phase of plant development that plays a pivotal role in seedling establishment. Due to low water availability, the growth phenomenon was affected (Bewley, 1997). Multiple endogenous elements regulate the seed germination, including plant hormones, hydrolytic enzymes and environmental factors viz., temperature, water availability, nutrients and light (Weitbrecht *et al.*, 2011; Cho *et al.*, 2012). The enzyme amylase hydrolyzes endosperm starch into sugars that provide energy for both shoot and root growth (Nauriere *et al.* 1992). However, the activity of amylase is affected by osmotic stress resulting in impaired carbohydrate metabolism (Kaur *et al.*

2000; Zeid & Shedeed 2006). This sheds light on the ability of seeds to combat water deficit conditions. Hence, the successful establishment of crops in drought-prone regions highly depends on the inherent potential of the seed viz., the speed and germination consistency (Arjenaki *et al.* 2011). Germinating seed and its surrounding soil represent an excellent niche for microbial colonization known as the spermosphere. Similar to the rhizospheric region, the spermosphere is formed by the carbon compounds deposited into the soil when the seed initiates the germination process. Such seed exudations influence the microbial activity around the seeds and many of which exert positive impacts on plant growth and fitness. However, the microbial diversity in the spermosphere and their role in plant-microbial interactions remain unexplored (Nelson, 2004).

Plant growth-promoting bacteria (PGPB) has been well established to improve the plant growth and productivity of crop plants under moisture stress (Grover *et al.*, 2014; Punitha *et al.*, 2019). IAA production, enzyme activity and phosphate solubilization are the most common plant growth-promoting mechanisms associated with PGPB to enhance plant growth and productivity (Datta *et al.*, 2011). IAA extrapolates root architectural traits

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such as root length, surface area and root hair intensity, thereby enhances rice seed germination and seedling establishment (Ng *et al.*, 2012). Moreover, phosphorus is an essential macronutrient for rice growth and development. Under moisture stress or in dry soil, P solubilization solely depends on the phosphate solubilizing bacteria through the production of organic acids and phosphatase enzyme (Gyaneshwar *et al.*, 2002). Plant growth-promoting bacterial genera including *Bacillus* (Ng *et al.*, 2012), *Enterobacter* (Gupta *et al.*, 1998) and *Corynebacterium* have been reported to improve the plant growth and fitness under drought. Seed priming with *B. amyloliquefaciens* improved the shoot and root length in *Arabidopsis* under drought and salinity stress (Preeti *et al.*, 2002; Ryu *et al.*, 2004). Therefore, we hypothesize that inoculation of osmotic stress-tolerant PGPB with the potential to solubilize phosphate and IAA production during moisture stress ensures the establishment of rice seedlings.

Our previous studies suggest that *Bacillus altitudinis* FD48 possess plant growth-promoting attributes such as IAA production, P and Zn solubilization, siderophore and ACC deaminase production (Kumar *et al.*, 2017). Besides, it can also able to tolerate the high osmotic potential of -1 Mpa (30% PEG 6000). With this background, the present study aims to demonstrate the efficacy of *B. altitudinis* FD48 on seed germination under induced moisture stress.

MATERIAL AND METHODS

Bacterial strain and culture conditions

Bacterial strain, *Bacillus altitudinis* FD48 used in this study (previously isolated from the phyllosphere of rice cultivar ADT43), was obtained from the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore. The FD48 strain grown in LB broth, incubated at 28 °C at 120 rpm was used for subsequent studies.

Seed biotization

Rice (*Oryza sativa* L.) cultivar C051 seeds obtained from Paddy Breeding Station, Tamil Nadu Agricultural University, Coimbatore, India, was used in this study. De-husked healthy seeds were surface sterilized in 0.1% HgCl₂ for 3 mins and subsequently in 70% ethanol for 1 min followed by five washes with sterile distilled water. Then the surface-sterilized seeds were primed with bacterial suspensions (10⁹ cfu ml⁻¹) of *B. altitudinis* FD48 and *Methylobacterium* sp. TNAU1 (PPFM) (as a positive check) for 1h at 28±2 °C. The treated seeds were placed on sterile filter paper in Petri dishes (9cm) moistened with sterile distilled water or 5,10,15,20, and 25% of polyethylene glycol (PEG 6000) concentration equivalent to osmotic potentials of (-0.30, -0.51,

-0.58 and -0.80 MPa respectively) were incubated for five days in plant growth chamber with a relative humidity of 60% and 12 h light (200 moles m⁻²s⁻¹) at 28 °C. Five replicates with 50 seeds were maintained for each treatment. Seeds immersed in sterile distilled water served as control. Petri dishes were sealed in order to prevent evaporation and seeds were considered to be germinated when the radical length attains for at least 2 mm.

Seed germination analysis

Seed germination percentage is the average number of seeds germinated over the period. In this study, germination percentage was calculated on fifth day using the standard formulae:

Seed Vigour index

Seed vigor index was examined after five days of incubation according to the formula based on the product of germination (%) and seedling length (cm).

Quantitative analysis of enzyme activity during germination

The α-amylase activity in the crude extract of germinated seeds was determined, according to Muscolo *et al.* (2014). Approximately 10 germinated seeds from each treatment with different stress levels with PEG 6000 (0, 5, 10, 15, 20, 25) were homogenized in 1:4 w/v distilled water using chilled pestle and mortar. The extract was centrifuged at 14,000 rpm for 30 min. Collected supernatant filtered through muslin cloth was used for the quantitative assay of α-amylase (EC 3.2.1.1).

Osmolytes content

Free proline content in the sample was detected based on the method described by Bates *et al.* (1973). Approximately 300mg of plant sample homogenized in 5ml of 3% sulfosalicylic acid was centrifuged at 3000g for 20 mins. The collected supernatant was mixed with 2 ml of glacial acetic acid and 2ml of ninhydrin. The mixture was boiled at 100 °C for 25 mins and mixed with 4 ml of toluene. Then the absorbance of the extract was measured in Spectrophotometer (Spectramax I3x) at 535 nm. Anthrone method was followed to determine the total soluble sugar content in the plant sample (Yemn & Willis 1954).

Collection of Seed exudates and GC-MS analysis

To assess the effect of bacterization on seed metabolite pattern, the seed exudates were collected and profiled using GC-MS (Shu *et al.*, 2008). Bacterial primed and control seeds (50 seeds) were placed in 50 ml of sterile MilliQ water and incubated for 12 hrs at 28 °C on a rotary shaker at 120 rpm. The samples were concentrated and passed through a 0.2µm membrane. Consequently, the solution was mixed with an equal quantity of ethyl acetate

followed by overnight. The separated solvent phase was concentrated in a flash evaporator and the crude metabolites extracted in 1 ml methanol was subjected to GC-MS analysis (Perkin Elmer GC-MS Clarus® SQ 8) equipped with DB-5MS (Agilent, USA) capillary standard non-polar column (0.25mm OD x 0.25 µm ID x 30 m length). The instrument was set to an initial temperature of 40 °C and the injection port temperature was ensured at 220 °C, interface temperature set 250 °C, source kept at 220 °C, oven temperature-programmed as 75 °C for 2 min, 150 °C @ 10 °C/min, up to 250 °C at 10 °C per min. The GC conditions were: 1:12 split, helium carrier at 20 psi. The MS conditions were: positive ion mode, electron impact spectra at 70 eV. The mass spectral scan range was set at 50 to 600 Da.

Table 1. Effect of biotization on seed germination, vigor index and total biomass

PEG (%)	Germination Percentage			Vigor Index (%)			Fresh weight (mg)		
	Control	FD48	PPFM	Control	FD48	PPFM	Control	FD48	PPFM
0%	100	100	100	199.66	243.22	239.66	343.33	5.70	370 2.73
5%	94	97	96	185.32	199.16	199.76	329 6.11	362.13	4.36
10%	71	87	88	162.32	191.31	192.14	213 2.73	292.9	4.10
15%	64	71	69	144.89	158.98	155.43	163.82	2.60	208.26 13.72
20%	51	60	58	40.32	51.34	48.65	46.58	4.62	79.27 4.06
25%	22	32	30	35.55	44.32	42.22	6.17	3.18	19.18 1.20
									16.2 0.88

Control – Absolute control without any treatments, FD48- *B.altitudinis* FD48 and PPFM- *Methylobacterium* sp. TNAU1. Values are mean (±standard error) (n=5).

moisture stress. Seed priming with PGPBs significantly promoted the seed germination and seedling establishment under moisture stress (Kalita *et al.*, 2015). Germination started irrespective of all treatments (Table 1 and Fig.1).

Statistical analysis

All data were statistically analyzed in Microsoft Excel and add-in with XLSTAT Version 2016.04.325250 (XLSTAT, 2016). 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 AND DISCUSSION

Germination percentage and vigor index under moisture stress

In the present investigation, biotization of rice seed with *B.altitudinis* FD48 enhanced the germination and seedling growth under induced

However, an increase in PEG concentration (-0.045, -0.14, -0.277, -0.46, -0.7 MPa of osmotic potential) considerably reduced the germination percentage compared to their respective controls. On exposure to sudden moisture stress of -0.27 MPa,

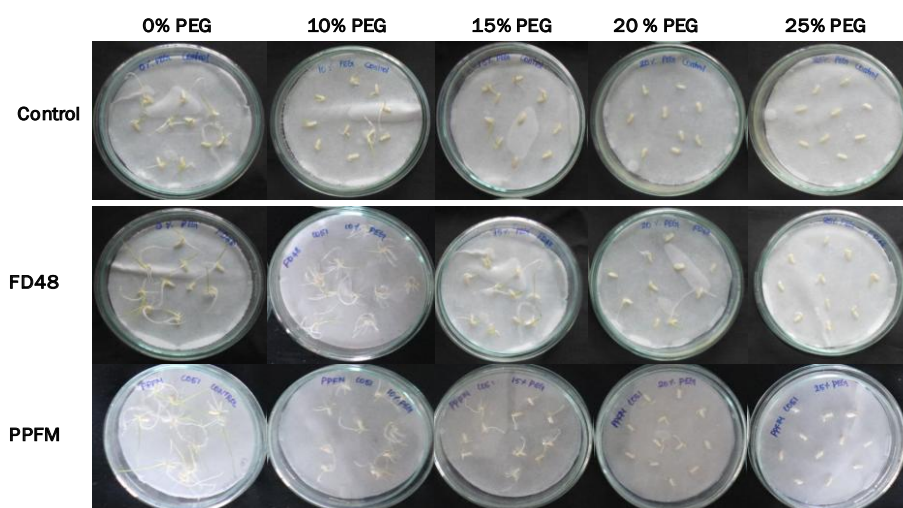


Figure 1. The effect seed biotization on germination of C051 seeds at different PEG 6000 concentration under *in vitro* plate assay.

Control – Absolute control without any treatments, FD48- *B. altitudinis* FD48 and PPFM- *Methylobacterium* sp. TNAU1 (PPFM)

reduction in seed germination was recorded, whereas control seeds registered 100% seed germination after 3 DAS. A drastic reduction was observed in germination percentage on exposure to osmotic potential of - 0.7 MPa (25% PEG 6000). In

general, the seeds primed with strains of *B. altitudinis* FD48 and *Methylobacterium* sp., significantly improved the seed germination over control under induced moisture stress. Among the treatments, osmotic stress imposed seeds treated

with FD48 and *Methylobacterium* sp at -0.7 MPa accounted for 44% and 32% increase in germination percent respectively over uninoculated control. Furthermore, bacterization also increased the seed

vigor index over control under moisture stress. The results suggest that priming seeds with moisture stress tolerant PGP bacterial inoculants accelerated the glucose metabolism of seeds imposed with moisture stress, as explained by Sun *et al.* (2010).

Table 2. Root length (cm) and α -amylase activity (μ moles of reducing sugars formed $\text{min}^{-1} \text{g}^{-1} \text{FW}$) of rice seeds under different concentrations of PEG 6000 on 3days of germination

PEG (%)	Root length (cm)			α -Amylase content (μ moles of reducing sugars formed $\text{min}^{-1} \text{g}^{-1} \text{FW}$)		
	Control	FD48	PPFM	Control	FD48	PPFM
0%	0.95 \pm 0.01 ^c	1.66 \pm 0.03 ^a	1.53 \pm 0.05 ^b	16.44 \pm 0. 51 ^b	18.55 \pm 0.33 ^a	18.33 \pm 0.25 ^a
5%	0.93 \pm 0.01 ^b	1.38 \pm 0.02 ^a	1.33 \pm 0.05 ^a	14.33 \pm 1.15 ^b	18.62 \pm 0.41 ^a	17.69 \pm 0.36 ^a
10%	0.73 \pm 0.01 ^b	1.19 \pm 0.02 ^a	1.17 \pm 0.01 ^a	15.16 \pm 0.20 ^a	16.22 \pm 0.61 ^a	16.15 \pm 0.54 ^a
15%	0.62 \pm 0.01 ^b	0.94 \pm 0.08 ^a	0.87 \pm 0.04 ^a	13.75 \pm 0.56 ^b	15.35 \pm 0.22 ^a	15.12 \pm 0.10 ^a
20%	0.25 \pm 0.01 ^b	0.61 \pm 0.01 ^a	0.56 \pm 0.03 ^a	11. 08 \pm 0.22 ^b	13.18 \pm 0.20 ^a	13.13 \pm 0.16 ^a
25%	0.17 \pm 0.02 ^b	0.25 \pm 0.01 ^a	0.21 \pm 0.00 ^a	8.70 \pm 0.31 ^b	9.4 \pm 0.15 ^a	8.94 \pm 0.20 ^{ab}

Control – Absolute control without any treatments, FD48- *B.altitudinis* FD48 and PPFM- *Methylobacterium* sp. TNAU1. Values are mean (\pm 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 ($p \leq 0.05$).

Moreover, the enzymes involved in the seed germination process shorten the lag and metabolic phase in the presence of growth-promoting phyllosphere bacterium *B. altitudinis* FD48

(Delshadi, 2015). Bandeepea *et al.* (2018) that osmotolerant rhizobacterial strains enhanced the seed germination and vigor index under induced moisture stress disclose similar results.

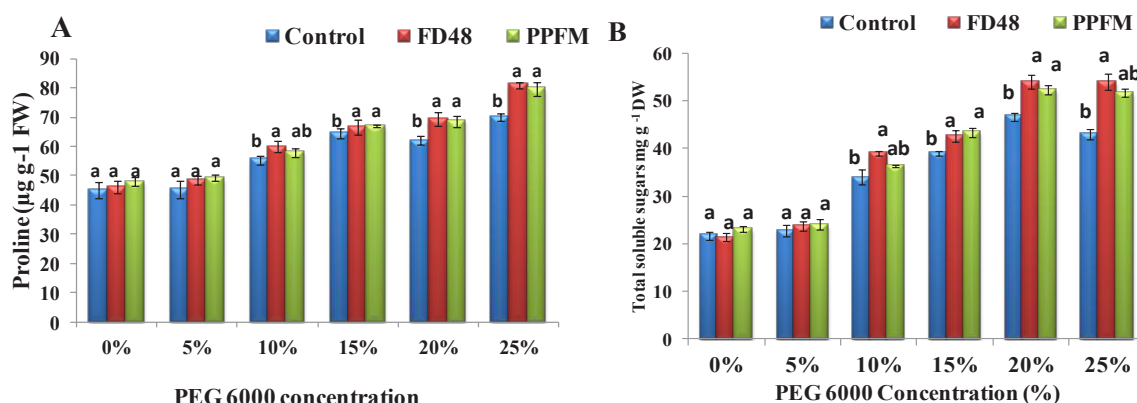


Figure 2. Total proline content (A) and soluble sugars (B) in rice seeds biotized with PGPB at different concentrations of PEG 6000

Control – Absolute control without any treatments, FD48- *B.altitudinis* FD48 and PPFM- *Methylobacterium* sp. TNAU1. Values are mean \pm 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 ($p \leq 0.05$).

Plant biomass

IAA is an essential phytohormone that regulates root architecture and sustains the root shoot growth (Kaufman *et al.*, 1995). The results of data were on variance analysis (Table. 2) showed that seed biotization and different levels of drought stress has a significant effect on the root length ($p < 0.05$). Mean comparison values showed that treatments with FD48 and *Methylobacterium* sp significantly influenced the root length (53% and 48% respectively) over control on 5DAS at the level of -0.7 MPa. A significant increase in root length might be due to endogenous IAA produced by the PGPB strains that enhanced the root growth under osmotic stress (Khalid *et al.*, 2004).

Likewise, the mean values of total biomass showed a steady decline with an increase in the intensity of stress level (Table.1.). At the maximum level of stress (-0.7 MPa), FD48 and *Methylobacterium* sp treated seeds registered 34% and 31% increase of total biomass over control. Also, under non-stressed conditions, FD48 significantly influenced the total biomass followed by *Methylobacterium* sp, when compared to uninoculated control. The present investigation showed that water deficit stress in uninoculated control plants reduced the shoot length. In general, drought stress increases the ethylene concentration of the plant. The crucial factor that prevents auxin transfer and arrests plant height is ethylene acetic

acid (Vacheron *et al.*, 2013). The reduced biomass, because of moisture stress could be attributed to impaired photosynthesis rate due to the dehydration and reduced source-sink relationship (Jamshidi *et al.*, 2012). Hence it can be concluded that priming seeds with PGPB, FD48 and *Methylobacterium* sp in this case triggered auxin production and diminished the ethylene level under moisture stress. Similar results have also been reported by Sarcheshmehpour *et al.* (2013) and Rana *et al.* (2015) on the positive impact of plant-growth-

promoting rhizobacteria on the growth of pistachio, rice and wheat under drought stress respectively.

Osmolytes on seedling establishment

Accumulation of osmolytes and compatible solutes has been reported to increase under water deficit conditions. Osmolytes help the plants to cope with moisture stress by maintaining the osmotic turgor in cells (Grover *et al.*, 2014; Lata *et al.*, 2015). Accordingly, the present study also reported a gradual increase in proline and total soluble sugars

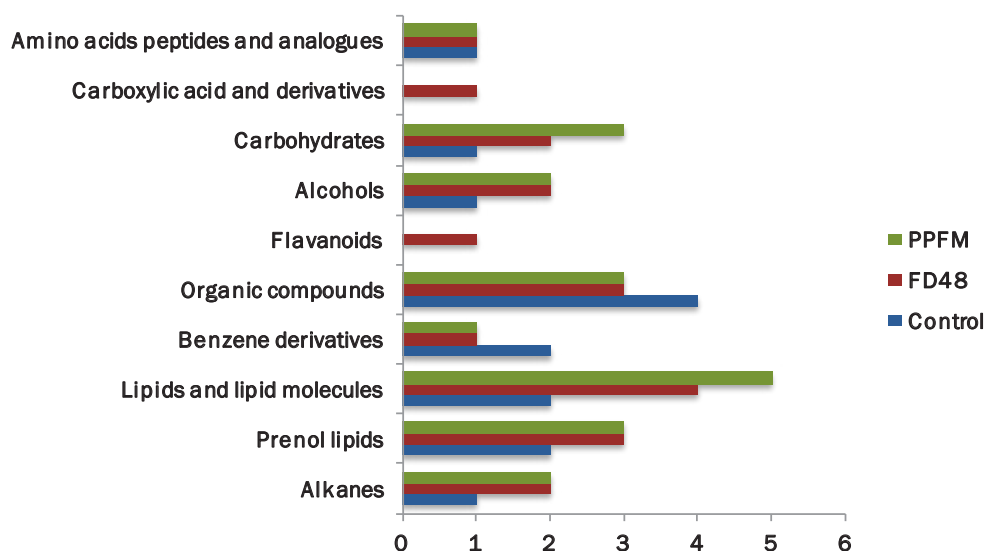


Figure 3. Metabolites detected in seed exudates of biotized rice seeds (cultivar C051) based on their differences in relative abundance.

Control – Absolute control without any treatments, FD48- *B.altitudinis* FD48 and PPFM- *Methylobacterium* sp. TNAU1.

in rice seedlings under different levels of moisture stress. However, the proline and soluble sugar content of PGP bacterial primed seedlings showed a significant increase over uninoculated control (Fig. 2a and 2b). The data on variance analysis showed that there is no significant difference among the PGPB strains on total soluble sugar content in non-stress (NS) treatments.

Interestingly, at high-stress intensity (-0.7 MPa), the maximum soluble sugar content of 54 mg g⁻¹ DW and 51 mg g⁻¹ DW was related to the treatments with FD48 and *Methylobacterium* sp respectively. Meanwhile, FD48 and *Methylobacterium* sp treated seeds accumulated maximum proline content under moisture stress conditions (-0.7MPa) when compared to control on 5 DAS. 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 (Tiwari *et al.*, 2016). Hence, increased seed viability, GP and total biomass in bacterized stressed plants might be

due to osmotic adjustment as a result of increased osmolyte synthesis. Similar observations have been reported in rice seedlings attenuated with *Bacillus pumilis*, subjected to salt stress (Khan *et al.*, 2016) and in maize seedlings under drought stress (Garcia *et al.*, 2017).

Alpha-amylase activity

Alpha-amylase is an essential enzyme involved in the hydrolytic breakdown of starch into simple sugars in order to provide energy for the shoot and root development. Water deficit stress affects α -amylase activity and drastically reduces seed germination. The mean values of α -amylase activity on 3DAS exhibited a gradual decrease with an increase in PEG concentration. However, the data variance analysis suggested no significant difference among the treatments related to NS (Table.2). In comparison to uninoculated moisture stressed seeds, the inoculated seeds enhanced the amylase activity by 8% and 3% (FD48 and *Methylobacterium* sp) respectively. During seed germination, starch hydrolysis is mediated by the enzyme α -amylase, which produces soluble oligosaccharides essential

for other metabolic functions (Kaur *et al.*, 2005). Accordingly, the enhanced α -amylase activity due to bacterial priming suggests better bioconversion of starch into other oligosugars.

Seed exudates profile

Seed exudates are a key to prompt the rhizobacterial colonization, which can impact on the plant growth and health (Nelson, 2004). During germination, seeds exude contain metabolites that influence their immediate biotic and abiotic

environments. The current investigation revealed 35 metabolites belonging to the class viz., (Fig.3) sugars (glycerol, glycine), aliphatic organic acids (fumaric acid), fatty acids (myristic acid, palmitic acid hexadecanoic acid, octadecanoic acid, tridecanoic acid), prenol lipids (squalene) and alcohols (ethanol, propionaldehyde, 1-hexadecanol). The Heat map analysis showed the distribution of seed exuded metabolites among the treatments (Fig.4). Row Z-score (>1) showed an increase in the relative abundance of the metabolites.

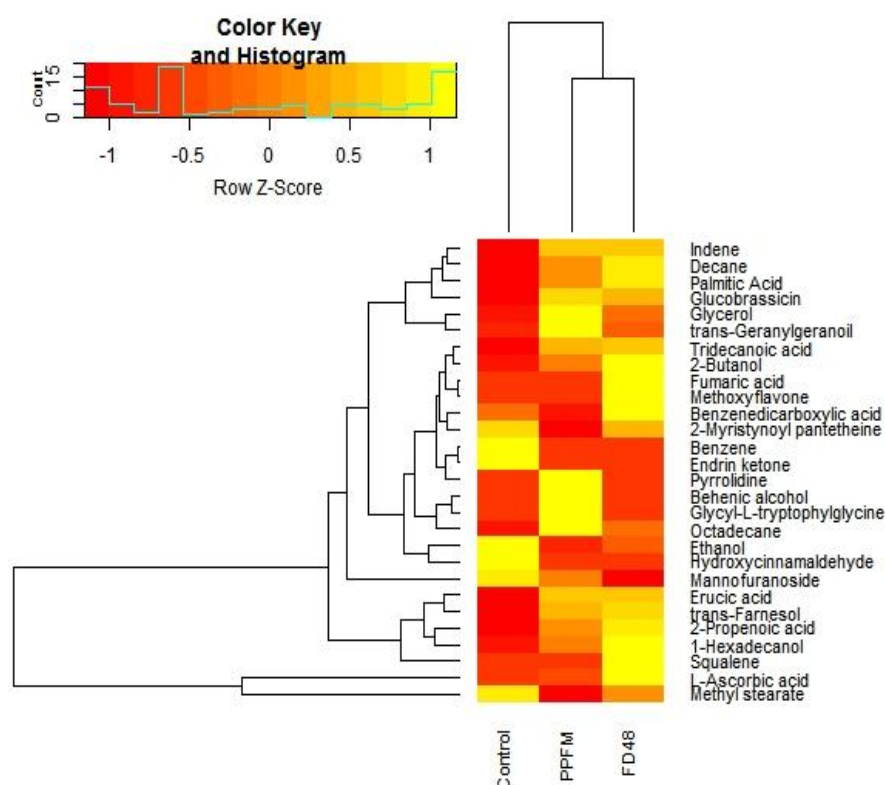


Figure 4. Distribution pattern of metabolites in seed exudates of rice seeds (C051) due to seed priming with *B. altitudinis* FD48 and *Methylobacterium* sp. TNAU1 (PPFM)

Control – Absolute control without any treatments, FD48- *B. altitudinis* FD48 and PPFM- *Methylobacterium* sp. TNAU1.

More diverse compounds were found in treatments of FD48 primed stressed seeds when compared to *Methylobacterium* sp and uninoculated control. The key metabolites with relevance to moisture stress tolerance are glycine, fumaric acid, fatty acids, flavones, squalene and ethanol. More precisely, the enhanced tolerance of bacteria primed moisture stressed seeds might be due to the maintenance of cell turgidity by these osmoregulants mediated by FD48 and *Methylobacterium* sp. Fumaric acid solubilizes phosphorus and makes it more available to the pre-germinating root primordia (Ng *et al.*, 2012). Seed exudates can also be involved as the lure of rhizosphere micro-organisms and can modulate significant bacterial properties that impart the ability to adhere and grow competitively in the vicinity of seeds (Schiltz *et al.*, 2015). Accordingly,

methyl flavones were pinned in the seeds primed with FD48 when compared to *Methylobacterium* sp. These flavone derivatives would help in seedling establishment and recruits beneficial microbial interactions that facilitate plant health and fitness. In addition, Martins *et al.*, (2018) showed that seed exudates of bean seed promoted the growth of *B. amyloliquefaciens* ALB629 and biofilm formation. Herein the present investigation also envisages better colonization of the bioinoculants (*B. altitudinis* FD48 and *Methylobacterium* sp (PPFM)) over the surface of moisture stressed seeds.

CONCLUSION

The present study revealed that rice seed bacterized with moisture stress-resilient phyllosphere bacterium, *Bacillus altitudinis* FD48 promoted seed

germination and vigor index under induced moisture stress. The mechanistic insights further showed the accumulation of osmolytes, compatible sugars and enhanced amylase activity, ensuring root and shoot growth. Further, seed exudates unraveled vital metabolites such as glycine, fumaric acid, squalene, methoxy flavone and other fatty acids that would help in seedling establishment and recruiting other beneficial microbial interactions. Therefore, our finding fosters a bio-inoculant for seed priming that could be adopted in drought-prone regions to increase the seed establishment and plant fitness.

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