Annals of Arid Zone 64(3): 313-328, 2025

Amelioration of Salinity Stress and Enhancing Growth in Wheat Seedling using Halotolerant *Arthrobacter* sp. NIMD28

Kamlesh K. Meena*, Devendra Singh, Saritha M., Shravan Kumar, Priyanka Verma, Pooja Gahlot and Dheeraj Singh

ICAR-Central Arid Zone Research Institute, Jodhpur 342 003, India

Received: June 5, 2025 Accepted: June 17, 2025

Abstract: Growth promotion and salinity stress-attenuation ability of the metabolites secreted by plant growth promoting, halotolerant *Arthrobacter* sp. NIMD28 (LC189176) was evaluated on germination, establishment, and development of wheat. The isolate produced diverse types of biomolecules in varying concentrations. Metabolites produced by the isolate also showed presence of phenolic compounds with significant non-enzymatic antioxidant activity. The gnotobiotic seed germination experiment revealed interesting results, where despite of the halotolerant nature, the isolate seems lagged in the plant growth promotion (PGP) over the *in vitro* induced metabolites of its own in terms of the phenotypic, biochemical and enzymatic characters. The Pearson's correlation coefficient and hierarchical clustering analyses also endorsed the performance of metabolites under saline conditions. The results strongly endorse needful initiatives towards keen optimization of the dose-response of the metabolites at larger scale, to develop next generation, sustainable bio-inoculants for abiotically stressed habitats.

Key words: *Arthrobacter* sp., bacterial metabolites, halotolerant rhizobacteria, salinity stress, induced systemic tolerance.

Correspondence
Kamlesh Kumar Meena
kkmeenamicro@gmail.com

Citation
Meena, K.K., Singh, D., Saritha M., Kumar, S., Verma, P., Gahlot, P. and Singh, D. 2025. Amelioration of salinity stress and enhancing growth in wheat seedling using halotolerant Arthrobacter sp. NIMD28. Annals of Arid Zone 64(3): 313-328

https://doi.org/10.56093/aaz.v64i3.167571

https://epubs.icar.org.in/index.php/AAZ/article/view/167571

Soil microbes; including fungi, bacteria, and archaea inhabit extreme abiotic environments. The ability of such organisms to promote plant growth under the influence of edaphic stresses in the natural soil environments has been well demonstrated (Ramadoss *et al.*, 2013; Sorty *et al.*, 2016). The crop productivity frequently suffers owing to, biotic, abiotic and global environmental changes (Hasanuzzaman *et al.*, 2013). Considering the overall influence of abiotic stressors on global agriculture, it is critical to have a sustainable, green technology to empower the crop under stress conditions. Resource management and crop improvement can help in recovering of losses, but such strategies being long time and costly. However, the time demands use of simple and cost-effective alternatives such as microorganisms and their biological products that can withstand on a short-term basis, and offer safe, cost effective and environmental friendly route to cope-up with the situation. The use of plant-beneficial microbes for development of stress tolerance in plants has been therefore thought upon. Plant associative, beneficial microbes synthesize the plant growth

promoting biomolecules, help plant to combat the stress situation, and encourage the other beneficiary interactions with neighbor plants and microbes (Shrivastava and Kumar, 2015). Though some salt tolerant bacteria in agricultural soil are able to produce PGP biomolecules even under saline conditions and also develop symbiotic and beneficiary interactions with plants (Chookietwattana and Maneewan, 2012; Bharti *et. al.,* 2013; Patil, 2015), major fraction of rhizosphere microbes loses the ability due to influence of the stress. The microbes also respond to plant root exudations that include diverse molecules, including organic acids, amino acids, sugars, mucilage, etc. which serve as chemotactic factors for the microbes and encourage the colonization (Yuan *et al.,* 2015), however the composition of root exudations markedly change under saline conditions, thus affecting the signaling mechanism. Under such circumstances, the augmentation bacterial primary and secondary metabolites in the rhizosphere can be helpful for plants, however, little is known about the *in-situ* PGP ability of the *in-vitro* derived microbial secretions under stress environment. Encompassing the need, we evaluate the response of the metabolites secreted by a PGP, halotolerant *Arthrobacter* sp. in salt affected wheat. Ability of *Arthrobacter* sp. to mitigate salinity stress in wheat has been reported in few studies (Upadhyay *et al.,* 2012) under moderate saline environment; however, reports concerning the behavior of same organism under highly saline environment are rare. Moreover, the knowledge regarding PGP performance of metabolites produced by *Arthrobacteria* under saline environments is scarce; the present study thus focuss on PGP performance of the bacterium and its metabolites under highly saline conditions.

Materials and methods

Collection of samples

Luxuriantly growing, healthy *Sesbania bispinosa* plants were collected from saline, barren land patches at Sangvi and Khandaj villages, located in the vicinity of Baramati, India. The plants were uprooted along with the block of rhizospheric soil, including the whole root system, and collected in sterilized polyethylene bags. The samples were transferred to the laboratory under ambient conditions and processed immediately.

Characterization of samples and isolation of halotolerant bacteria

A fraction of rhizosphere soil samples was sieved, oven dried at 110°C till constant weight and used for determination of electrical conductivity ($EC_{1:2}$) and pH (Corwin and Lesch, 2005). The isolation of bacteria from rhizoplane and rhizosphere was done as described elsewhere (Supanekar and Sorty, 2013); except for the higher salt content of the medium that ranged from 5-20% w/v, and the pH of the medium, that varied as *in situ*. The isolates were maintained on nutrient agar slants supplemented with the respective salt concentrations at 4°C, and also preserved in 20% glycerol at -80°C for long-term storage.

Biochemical characterization of isolates

The salt tolerance ability of the isolates was determined using NaCl gradient on nutrient agar from 0-30% with steps from 1% (Sorty *et al.,* 2016). The PGP traits viz., nitrogen fixation (Reinhold, *et. al.,* 1986, 87), Exopolysachharide (EPS) production in Tryptic soy agar, phosphate solubilization (Nautiyal, 1999) and siderophore production (Schwyn and Neilands, 1987) were evaluated at the respective salt concentrations obtained during the salt tolerance experiment. Quantitative IAA production ability was determined by Salkowski's method (Ehmann, 1977). The detailed biochemical characterization of the potential PGP isolates was achieved by implementing BIOLOG Gen III plates with the inoculating fluid (IF) 'B'.

Molecular identification of the candidate isolates

The candidate isolate (D28) was grown on nutrient agar with 5% NaCl at 28±2°C for 24 h. Single colonies were washed twice with 0.5 ml of TE buffer (10 mM Tris-HCl; 1 mM Na-EDTA; pH 8.2). The genomic DNA of isolates was extracted using Ultra Clean DNA isolation kit (MoBio) following the manufacturer's instructions. The 16S rRNA gene was PCR amplified and sequencing was done as described earlier (Sorty *et al.*, 2016). The isolates were identified based on the 16s rRNA gene sequence-similarity with the type sequences from the GenBank database. The alignment and comparison of the sequences were performed using CLUSTAL W; followed by constructing the phylogenetic tree using the

neighbor-joining method in MEGA 6.0 (Tamura *et al.,* 2013).

Extraction and characterization of metabolites

The isolate was cultivated in 300 ml of nutrient broth with 5% NaCl at 30±2°C for 120 h, at 180 rpm. The cells were harvested at 7800 rpm for 10 min in an Eppendorf (*5430R*) centrifuge; the metabolite extraction from the culture filtrate was achieved both at native pH (7.5) and at pH 2.5 (adjusted using 6M HCl). The extraction was done using preconditioned XAD16 resin. Briefly, twenty grams of the preconditioned resin was mixed with 300 ml batch of culture filtrate and the slurry was agitated for 30 min at 180 rpm. Finally, the slurry was poured in a glass column and the liquid was drained at a flow rate of 2 mL min^{-1}. The resin was washed with 3 bed volumes of milli Q water and the trapped metabolites were eluted using 3 bed volumes of absolute acetone. The organic phase was then evaporated at 30°C under vacuum (*Concentrator Plus*, Eppendorf) and stored at -20°C till further use.

UHPLC profiling of metabolites

The metabolites profiling was achieved using reversed phase UHPLC equipped with a photo diode array (PDA) detector (*Nexera* X2, Shimadzu, Japan); and a C18 column (250 mm X 4.6 X 3), with a flow rate of 0.9 ml min^{-1} using the mobile phase comprising 0.2% ortho-phosphoric acid in water: methanol (30:70) at 30°C. The pelleted metabolites were resuspended in 20 ml of mobile phase, passed via 0.2 µm syringe filter and 20 µL aliquots were injected with the help of an auto-sampler (SIL-30 AC). The chromatograms were monitored at 254 nm and the diversity of metabolites was determined using retention time (R_t).

Antioxidant potential and phenolics content in metabolites

The pelleted metabolites were resuspended in 20 ml methanol: water (2:1); 1 ml aliquot of the same was mixed with 4 ml of DPPH (0.6 µM L^{-1} in methanol), the absorbance was read at 517 nm at 0 min and 30 min; the mixture was kept in the dark in the meantime (Yen and Duh, 1994). The phenolics content were determined as described by Bray and Thorpe, 1954.

Gnotobiotic pot trials under simulated saline conditions

Gnotobiotic experiment was carried out in pots filled with sterilized garden soil (autoclaved for 1h at 121°C). The electrical conductivity (EC) of respective pots (Neutral (0), 5, 10 and 15 dS m^{-1} respectively) was adjusted by draining the pots till saturation, with the crude NaCl solution of corresponding EC. The isolate for seed inoculation was raised in 200 ml batch of nutrient broth with 5% NaCl at 30±2°C for 36 h with constant agitation at 180 rpm. The cells were pelleted at 7800g for 10 min, washed three times with phosphate buffered saline (PBS) and used immediately.

The wheat seeds, var. *Netravati* (NIAW 1415) were surface disinfected using 2% sodium hypochlorite followed by several times washing with sterile Milli Q water to remove the traces of disinfectant. The sticker solution for seed coating contained 1.2% of gum aerobic; 0.2% of carboxy methyl cellulose (CMC); 0.5% of jaggary; and 20% v/v of the isolate (~10^9 cfu). The seeds were coated by mixing with sticker solution containing the isolate, and dried under the shed. Uninoculated seeds served as control. The seeds were then sown in respective pots. For the metabolites treatment, extracted metabolites (as earlier) were diluted 20 X with sterile MQ water and applied via irrigation (0.005% v/v) at weekly intervals. All the pots were irrigated with sterile tap water and incubated at 12 h dark and light cycles under ambient conditions (~25°C). The samples were collected weekly.

Physicochemical parameters

The physicochemical parameters including germination percent, root and shoot length, biomass, vigor index, chlorophyll content, total phenol, sugar content, total protein, superoxide dismutase (SOD) (Beauchamp and Fridovich, 1971), peroxidase (GPX) (Reddy *et al.,* 1995), and catalase (CAT) (Luck, 1974) were recorded.

Estimation of phenolic compounds

The phenolics content of the samples were extracted as described by Annabelle Damerum *et al.,* (2015). Briefly, 0.1g leaf samples were crushed in liquid nitrogen and resuspended in 2 ml methanol: water: formic acid (25:24:3) and vigorously agitated in the dark at 4°C for 30 min. The samples were then centrifuged for 20 min, at 7800 rpm. The residues were re-extracted and the supernatants were pooled.

The phenolics content were estimated as described by Bray and Thorpe (1954).

Estimation of chlorophylls

50 mg of leaf samples were kept immersed in 10 ml of 80% acetone in dark, for 24 h. The absorbance of organic phase was read at 640 and 665 nm on a spectrophotometer (UV 3000+; Lab India). The chlorophyll content was calculated as described by Harborne (1973).

Protein content and antioxidant enzymes activity

The total protein content was estimated using Lowry's assay (Lowry *et al*., 1951). Leaf samples (0.5 g) were crushed in 2ml of chilled phosphate buffer (100 mM; 1 mM EDTA; pH 7.8). The supernatant (containing enzymes) was obtained by centrifugation at 12000 X g at 4°C and stored at -20°C for total protein, SOD, Catalase, and peroxidase determination. The SOD, CAT and GPX activities were estimated as described by Chaitanya and Naithani (1994); Aebi (1984) and Putter (1974), respectively.

Statistical Analyses

All the numerical data were statistically analyzed through analysis of variance (ANOVA), with subsequent Ducan's multiple range test (DMRT), Pearson's correlation coefficient and cluster analysis using SPSS 16.0. The Pearson's correlation coefficients and hierarchical clustering were also done using SPSS 16.0. The differences at 95% confidence were considered significant during analysis.

Results and Discussion

The characterization of rhizosphere soil revealed the presence of alkaline pH in addition to the highly saline conditions (Table 1), where the $EC_{(1:2)}$ was found 13.62 dS m^{-1} and pH 8.34; indicating the presence of double-stressor situation. This also encouraged the prediction of probable isolation environment for trapping the candidate PGP microbes from the samples. The isolate appeared resilient upon exposure to diverse grades of individual as well as combined stressors *in-vitro*. The PGP traits tested under different salt concentrations revealed characteristic production of siderophores, fixation of atmospheric nitrogen, solubilization of phosphate, and production of IAA by the isolate. The isolate failed to produce siderophores and solubilize phosphate; but produced exopolysaccharide, which is considered one of the important traits needful to tolerate high saline conditions (Vyride and Stuckey, 2009), the same is also useful for adherence and colonization (Fujishige *et al*., 2006). The IAA production ability of the isolate differed considerably both in the presence and absence of specific substrate (tryptophan), and

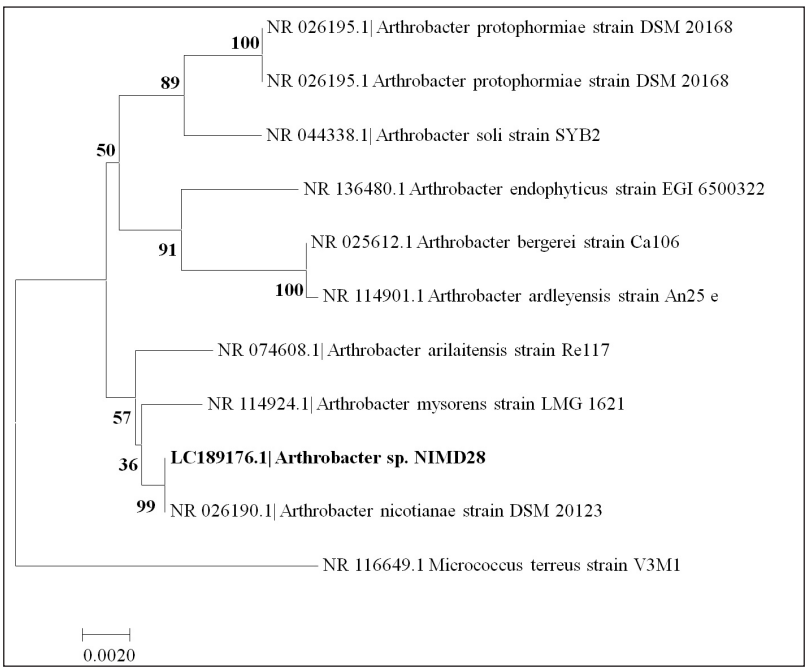

Fig. 1. Phylogenic positions of the isolates based on 16S rRNA gene sequences.

Table 1: Characterization of samples and metabolic plasticity of the isolates

Character/Trait		Result
Habitat		Rhizosphere of *Sesbania bispinosa*
Electrical Conductivity ($EC_{1:2}$)		13.62
pH		8.34
Molecular identity of the isolate		*Arthrobacter*sp. *NIMD28* (LC189176)
Siderophore production	1% NaCl	-
	5% NaCl	-
	10% NaCl	-
	10% PEG	-
	20% PEG	-
Nitrogen Fixation	1% NaCl	+
	5% NaCl	-
	10% NaCl	-
IAA Production (µg/ml)	With tryptophan	3.134
	Without tryptophan (at 5% NaCl)	0.469
	10% PEG	0.086
	20% PEG	-
	30% PEG	-
Phosphate Solubilization	1% NaCl	-
	2% NaCl	-
	5% NaCl	-
	10% NaCl	-
EPS production		+
Sugar Fermentation (Dextrose)	0.5% NaCl, pH 7	-
	0.5% NaCl, pH 8	-
	0.5% NaCl, pH 9	++ (48h)
	5% NaCl, pH 7	+ (48h)
	5% NaCl, pH 8	-
	5% NaCl, pH 9	++ (24h)
	10% NaCl, pH 7	-
	10% NaCl, pH 8	+ (24h)
	10% NaCl, pH 9	+ (24h)
	15% NaCl, pH 7	-
	15% NaCl, pH 8	++ (48h)

(+) Positive test; (++) Strongly positive; (+) Partially positive; (-) Negative test

under decreasing water potential (Table, 1). The influence of stressor on sugar metabolism of the isolate was also determined using increasing pH and salt concentration, where both the isolates showed characteristic stress-time relationship during the fermentation. The time required for detectable fermentation increased, especially with the increase in salt concentration. This highlighted the influence of stressors on the metabolic potential of the isolate. The hindered metabolism at higher magnitude of stress clearly indicated the probable lowering of PGP activity under stress-prone environments (Table 1).

The detailed metabolic characters of the isolate were studied using the BIOLOG GEN III assay, where the isolate successfully utilized variety of carbon substrates, showing diverse metabolic configuration (Table 2). The molecular identification of the isolates based on 16S rRNA gene sequences revealed the phylogenic relationship of the isolates with the genus *Arthrobacter*, based on which the isolate was identified as *Arthrobacter* sp. NIMD28 (LC189176) (Fig. 1). The metabolite production experiment was designed based on the overall metabolic characters of the isolate. The UHPLC analysis of the harvested metabolites revealed the chromatograms with diverse peak-profiles, with multiple biomolecules having different retention times (R_t) and absorption maxima, indicating presence of diverse compounds in the harvested metabolite-pool (Fig. 2).

Table 2. Diversity of metabolic profile of the isolate revealed by BIOLOG GEN III assay.

Sugars			Amino acid			Carboxylic acids, easters and fatty acids			Hexose acid			Chemical sensitivity		
	M	G		M	G		M	G		M	G		M	G
D-Raffinose	-	-	Gelatin	++	+	P-Hydroxy-Phenylacetic Acid	++	+	Pectin	+	-	pH 5	-	-
α-D-Glucose	+++	+	Glycyl-L-Proline	+	-	Tween 40	-	-	D-Galacturonic Acid	+	-	pH 6	+++	++
Dextrin	+++	+	L-Alanine	++	-	Methyl Pyruvate	++	+	L-Galactonic Acid Lactone	+	-	1% NaCl	+++	++
α-D-Lactose	-	-	L-Arginine	+	-	α-Amino-Butryric Acid	-	-	D-Gluconic Acid	+++	+	4% NaCl	+++	+
D-Mannose	-	-	L-Aspartic Acid	+	-	D-Lactic Acid Methyl Ester	-	-	D-Glucuronic Acid	+	-	8% NaCl	+++	+
D-Maltose	+++	-	L-Glutamic Acid	++	-	α-Hydroxy-Butyric Acid	+	-	Glucuronamide	+	-	1% Sodium Lactate	+++	+
D-Melibiose	-	-	L-Histidine	++	-	L-Lactic Acid	++	+	Mucic Acid	+++	+	Troleandomycin	-	-
D-Fructose	++	+	L-Pyroglutamic Acid	++	-	β-Hydroxy-D,L-Butyric Acid	++	+	Quinic Acid	+++	+	Lincomycin	-	-
D-Trehalose	+	+	L-Serine	++	+	Citric Acid	++	+	D-Saccharic Acid	+++	++	Vancomycin	-	-
α-Methyl-DGlucoside	++	-	D-aspartic acid	-	-	α-Keto-Butyric Acid	+	-				Nalidixic Acid	+++	+
D-Galactose	++	+	D-serine	-	-	α-Keto-Glutaric Acid	++	+				Aztreonam	+++	++
D-Cellobiose	+++	+				Acetoacetic Acid	++	-				Fusidic Acid	-	-
D-Salicin	++	-				D-Malic Acid	-	-				Rifamycin SV	+++	++
3-Methyl Glucose	-	-				Propionic Acid	+++	+				Guanidine HCl	+++	++
Gentiobiose	+++	++				L-Malic Acid	+++	+				Tetrazolium Violet	+	-
N-Acetyl-DGlucosamine	-	-				Acetic Acid	++	-				Lithium Chloride	+++	++
D-Fucose	-	-				Bromo-Succinic Acid	++	-				Sodium Butyrate	+++	++
Sucrose	+++	+				Formic Acid	+	-				D-Serine	+++	+
N-Acetyl-b-DMannosamine	-	-										Minocycline	-	-
L-Fucose	-	-										Niaproof 4	-	-
D-Turanose	++	++										Tetrazolium Blue	-	-
N-Acetyl-DGalactosamine	-	-										Potassium Tellurite	+++	+++
L-Rhamnose	++	+										Sodium Bromate	+++	+++
Stachyose	-	-												
N-Acetyl Neuramic Acid	++	+												
Inosine	+++	+												
D-Sorbitol	-	-												
D-Mannitol	-	-												
D-arabitol	++	+												
Myo-inositol	-	-												
Glycerol	+++	+												
D-glucose6-phosphate	-	-												
D-fructose 6-phosphate	++	-												

M = Metabolism; G = Growth

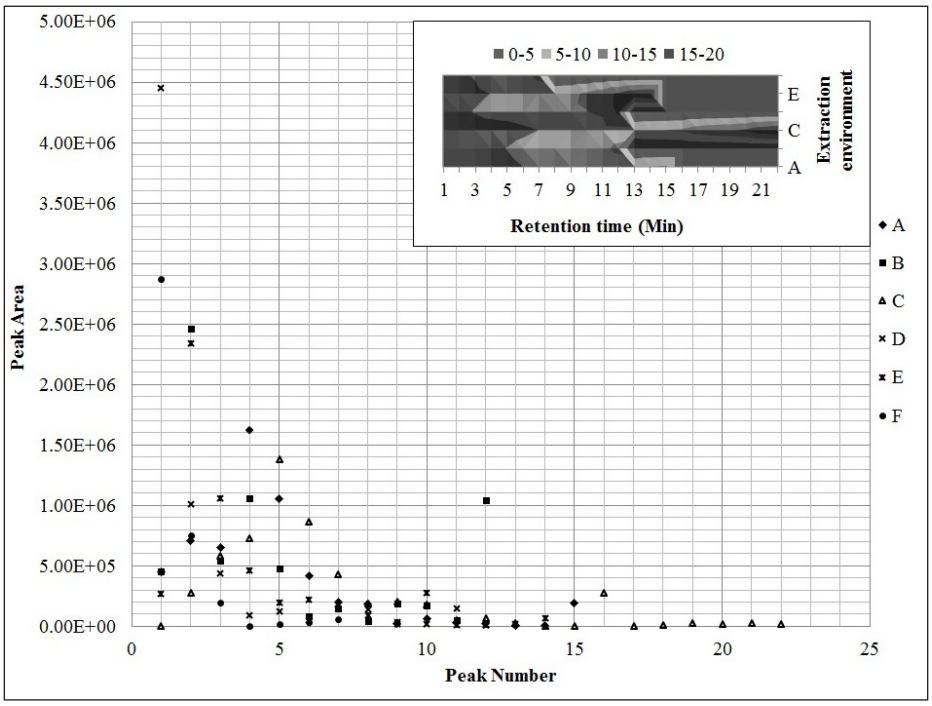

Fig. 2. UHPLC analysis of diverse biomolecular profiles in harvested metabolites.

The non-enzymatic antioxidant potential of the harvested metabolite-pool was measured to detect the presence of reducing power where, almost every metabolite fraction tested, exhibited exceptional reduction potential, indicating presence of high quantity of antioxidant metabolites. This could prove beneficial to both the host as well as the associating organism, as under the influence of salinity stress the elevated levels of oxidative stress have been frequent (Bose *et al.*, 2014; Habib *et al.*, 2016). Moreover, this mechanism could also potentially work as an add-on to the enzymatic antioxidant system under stress situation. Similarly, the content of phenolic compounds from the metabolites also found significantly higher. The role of phenolics in plants under stress situation is well known. Elevated levels of phenolics in

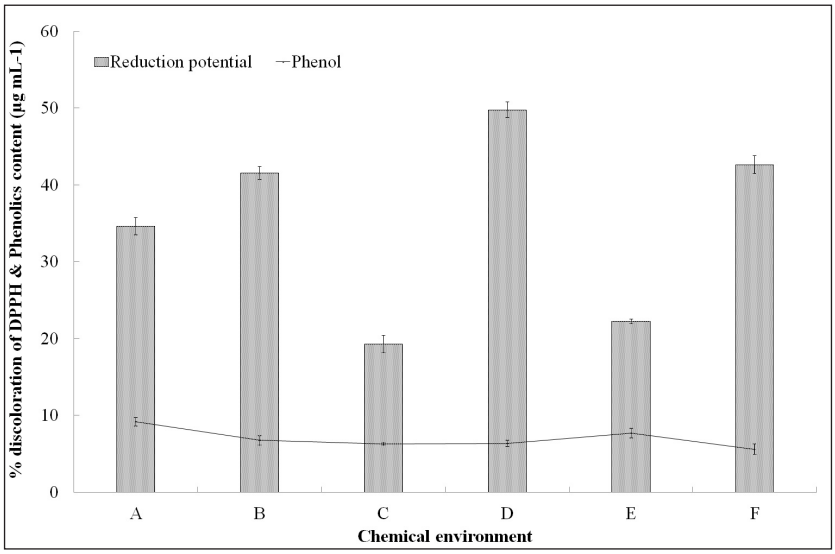

Fig. 3. Anti-oxidation potential and phenolics content in the metabolite-mix of the isolates produced and extracted under different chemical environments (X axis indicates the % NaCl during the growth of the isolate and pH during extraction of metabolites. A: 0.5%, pH 7.5; B: 0.5%, pH 2.5; C: 5%, pH 7.5; D: 5%, pH 2.5; E: 10%, pH 7.5; F: 10%, pH 2.5).

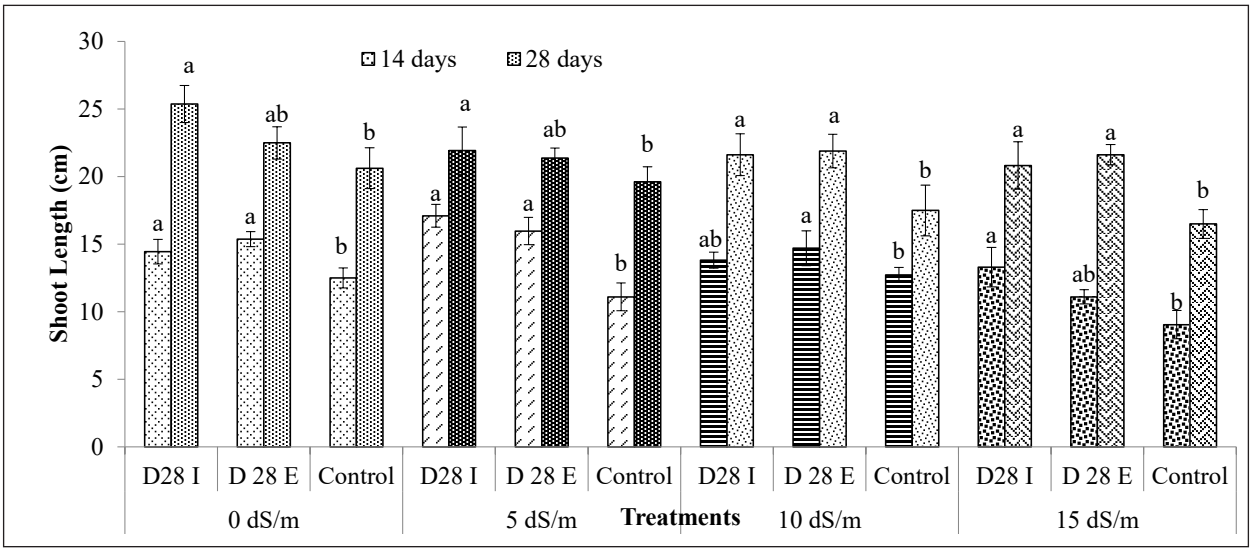

Fig. 4. Effect of isolates (I) and metabolites (E) on shoot length of wheat under saline stress in gnotobiotic assay.

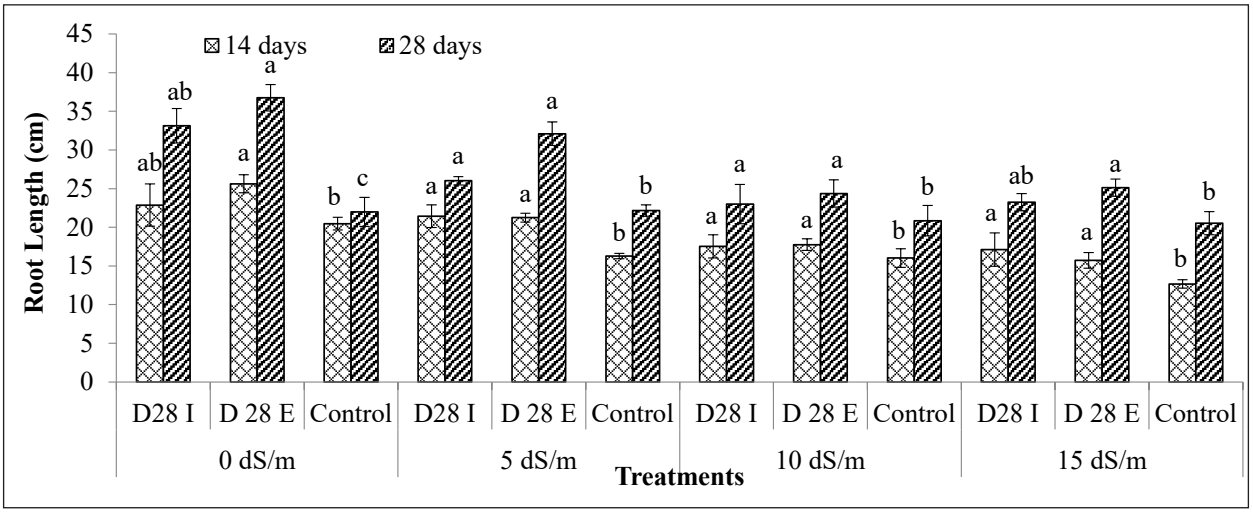

Fig. 5. Effect of isolates (I) and metabolites (E) on root length of wheat under saline stress in gnotobiotic assay.

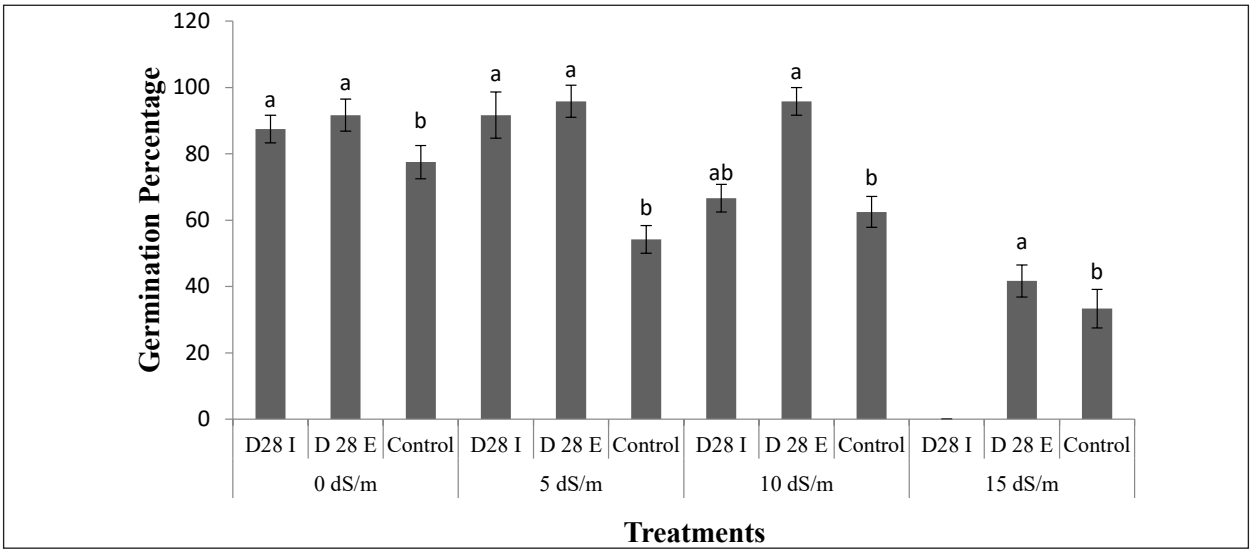

Fig. 6. Effect of isolates (I) and metabolites (E) on germination percentage of wheat under saline stress in gnotobiotic assay.

Table 3. Biochemical parameters of the wheat seedlings under the influence of the isolate and metabolites

Treatments		Shoot sugar		Root sugar		Total Chlorophyll	Total Phenol	Total proteins
		14 Days	28 Days	14 Days	28 Days			
0 dS m^{-1}	Isolate	5.5996±1.97852^{a*}	1.4724±.07311^{b}	4.0218±2.52768^{ab}	3.9230±.03241^{a}	2.0325±.25708^{a}	0.1881±0.00616^{a}	4.3722±0.86504^{c}
	Metabolites	4.8806±1.18502^{b}	**2.0844±.43895^{a}**	**4.8933±2.28084^{a}**	3.6471±.19484^{a}	1.2125±.09570^{ab}	0.1806±0.01022^{a}	**5.312±0.70342^{c}**
	Control	3.3574±.60867^{c}	1.4222±.05657^{c}	3.9329±1.75347^{b}	2.2648±.10169^{b}	0.9375±.12633^{b}	0.1647±0.00587^{b}	3.7726±0.71226^{a}
5 dS m^{-1}	Isolate	4.1133±2.24541^{b}	1.8230±.06267^{a}	5.8435±3.16905^{a}	2.0646±.03633^{b}	1.6650±.31257^{ab}	0.1824±0.00569^{b}	4.2542±0.87246^{bc}
	Metabolites	**6.0950±2.97125^{a}**	1.8084±.04270^{a}	**5.9400±2.62871^{a}**	**2.2681±.07312^{a}**	**1.8175±.30674^{a}**	**0.2091±0.00609^{a}**	**4.9027±0.67651^{ab}**
	Control	1.9100±.46383^{c}	1.0676±.04274^{b}	4.1546±2.84093^{b}	1.2016±.06063^{c}	1.0800±.13711^{c}	0.1280±0.00323^{c}	3.749±0.90993^{ab}
10 dS m^{-1}	Isolate	5.8689±2.73998^{a}	1.7040±.06754^{b}	3.9643±2.49980^{a}	2.9520±.08432^{ab}	1.225±.17746^{a}	0.1969±0.00697^{b}	3.1155±0.93136^{bc}
	Metabolites	5.8587±3.16802^{a}	**3.9135±.09068^{a}**	**4.2505±2.49043^{a}**	**4.1555±.09007^{a}**	**1.4500±.02708^{b}**	**0.2167±0.00481^{a}**	**3.772±0.42612^{bc}**
	Control	3.3283±1.58392^{b}	1.1525±.42469^{c}	3.1678±2.24854^{b}	1.3695±.07052^{b}	1.0000±.03000^{c}	0.1857±0.00811^{c}	2.3565±0.75349^{b}
15 dS m^{-1}	Isolate	4.1845±1.78662^{b}	4.0922±.12648^{ab}	3.8364±2.44836^{ab}	4.1024±.11525^{a}	1.6725±.22336^{a}	0.2071±0.00744^{b}	2.5485±0.57619^{bc}
	Metabolites	**4.4131±2.82613^{a}**	**4.6570±.14453^{a}**	**4.4461±1.61883^{a}**	**4.3202±.06830^{a}**	**1.7300±.26090^{a}**	**0.2278±0.00530^{a}**	**4.2461±0.72490^{a}**
	Control	2.2801±.61018^{c}	3.6634±.16273^{b}	3.373±2.16197^{b}	3.2684±.15840^{b}	1.4625±.17212^{a}	0.12061±0.00430^{c}	2.181±1.34026^{b}

*Values from the same column represented by different superscribed letters are with significant difference (p = 0.05) in the Duncan's multiple range test

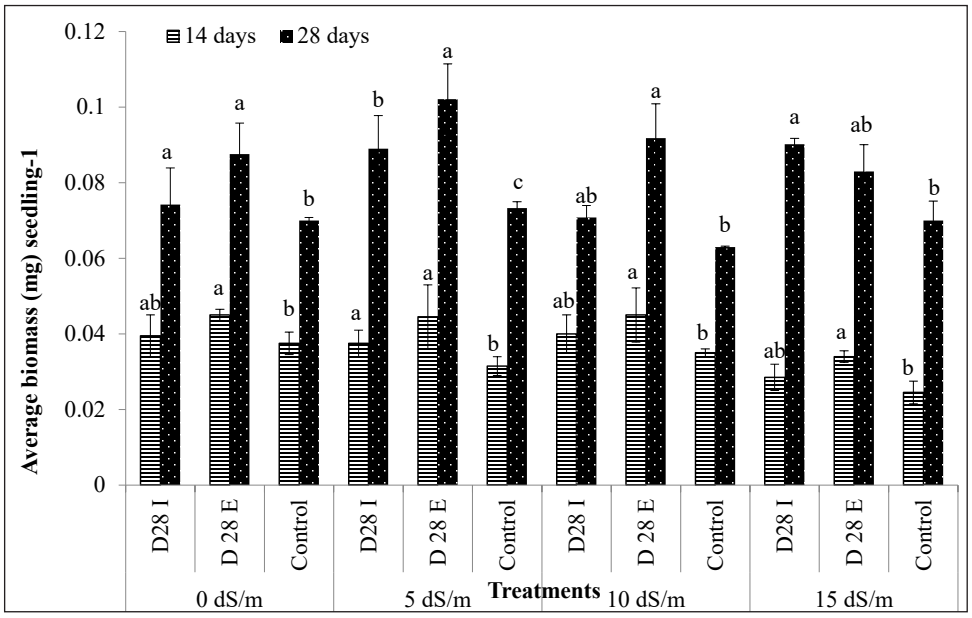

Fig. 7. Effect of isolates (I) and metabolites (E) on average biomass of wheat under saline stress in gnotobiotic assay.

Table 4. Antioxidant enzyme activity in the wheat seedlings following the treatment with isolate and metabolites

Treatments		GPX	CAT	SOD
0 dSm^{-1}	Isolate	1.0963±0.13984^{b*}	1.6294±0.14389^{a}	0.4581±0.14932^{b}
	Metabolites	**1.1918±0.36505^{a}**	1.4264±0.14772^{b}	0.4325±0.08060^{b}
	Control	0.3771±0.14913^{c}	1.2466±0.08352^{c}	0.2127±0.02739^{c}
5 dSm^{-1}	Isolate	0.7860±0.12562^{a}	1.5449±0.04454^{ab}	0.3489±0.05386^{a}
	Metabolites	0.6072±0.15908^{b}	**1.9946±0.05098^{a}**	0.3063±0.05120^{ab}
	Control	0.4607±0.15402^{c}	1.4667±0.06716^{b}	0.1983±0.03544^{b}
10 dSm^{-1}	Isolate	1.7004±0.25751^{a}	1.3701±0.13381^{b}	0.5943±0.13164^{a}
	Metabolites	1.3664±0.49235^{b}	**1.6619±0.20388^{a}**	0.4770±0.12890^{b}
	Control	0.5182±0.05517^{c}	0.8603±0.18366^{c}	0.2071±0.02835^{c}
15 dSm^{-1}	Isolate	0.7048±0.11934^{b}	1.7735±0.09939^{a}	0.4328±0.06414^{a}
	Metabolites	**0.8480±0.17249^{a}**	1.5136±0.19108^{bc}	0.3003±0.04304^{bc}
	Control	0.6659±0.12513^{b}	1.3108±0.05300^{c}	0.2412±0.10768^{c}

*Values from the same column represented by different superscribed letters are with significant difference (p = 0.05) in the Duncan's multiple range test

Table 5. Correlation analysis between different parameters of isolates and metabolites

	Biomass	SL	RL	DSW	DRW	Phenol	Protein	GPX	SOD	Catalase	Chl	SS	RS	VI	GP
Isolate															
Biomass	1														
SL	0.397	1													
RL	0.298	0.894**	1												
DSW	0.817*	0.268	0.115	1											
DRW	0.865**	0.635	0.546	0.558	1										
Phenol	0.361	0.533	0.375	0.559	0.184	1									
Protein	0.27	0.788*	0.707	-0.028	0.68	0.063	1								
GPX	0.038	0.486	0.376	0.269	0.132	0.48	0.105	1							
SOD	0.339	0.639	0.484	0.52	0.332	0.685	0.147	0.920**	1						
Catalase	0.817*	0.618	0.542	0.632	0.821*	0.209	0.402	0.25	0.511	1					
Chl	0.593	0.606	0.790*	0.399	0.613	0.33	0.3	0.348	0.523	0.729*	1				
SS	0.384	0.415	0.454	0.288	0.244	0.399	-0.099	0.438	0.638	0.627	0.733*	1			
RS	0.479	-0.261	-0.199	0.408	0.117	0.051	-0.57	-0.011	0.176	0.433	0.398	0.68	1		
VI	-0.137	0.643	0.641	-0.382	0.283	0.155	0.821*	0.184	0.105	-0.066	0.151	-0.163	-.719*	1	
GP	-0.242	0.468	0.473	-0.424	0.192	0.035	0.744*	0.183	0.017	-0.233	-0.009	-0.372	-.804*	0.959**	1
Metabolites															
Biomass	1														
SL	0.746*	1													
RL	0.740*	0.7	1												
DSW	0.923**	0.638	0.808*	1											
DRW	0.924**	0.705	0.548	.789*	1										
Phenol	0.608	0.638	0.385	0.559	0.413	1									
Protein	0.770*	0.915**	0.870**	0.747*	0.686	0.459	1								
GPX	0.551	0.55	0.482	0.455	0.456	0.494	0.378	1							
SOD	0.707*	0.683	0.619	0.6	0.624	0.561	0.539	.964**	1						
Catalase	0.917**	0.611	0.531	0.789*	0.947**	0.374	0.662	0.328	0.478	1					
Chl	0.728*	0.288	0.345	0.629	0.576	0.534	0.323	0.333	0.372	0.751*	1				
SS	0.454	0.468	0.31	0.216	0.324	0.507	0.295	0.791*	0.738*	0.337	0.554	1			
RS	0.281	0.16	-0.046	0.047	0.186	0.412	-0.029	0.582	0.469	0.279	0.656	0.891**	1		
VI	0.597	0.698	0.738*	0.59	0.575	0.37	0.713*	0.374	0.581	0.378	-0.05	0.081	-0.327	1	
GP	0.644	0.668	0.627	0.637	0.658	0.449	0.623	0.415	0.617	0.436	0.009	0.055	-0.287	0.961**	1

SL=Shot length; RL=Root length; DSW=Dry shoot weight; DRW= Dry root weight; Chl=Total chlorophylls; SS=Shoot sugars; RS= Root sugars; GP=Germination percentage

* Correlation is significant at the 0.05 level (2-tailed); ** Correlation is significant at the 0.01 level (2-tailed).

plants have been demonstrated under the influence of abiotic stressors (Lim *et al.*, 2012). The phenolic compounds also contribute for non-enzymatic anti-oxidation activity. Thus, the metabolite-antioxidant potential of the isolate highlighted the probability of effective attenuation of salinity stress by the application of metabolites (Fig. 3).

The gnotobiotic seed germination experiment revealed a marked influence of stress conditions on seed germination and development of wheat. Both the inoculation with isolate and its metabolites exerted characteristic growth promoting effect under saline conditions. The overall growth pattern of control seedlings seems aligned with previous reports documenting the declined shoot elongation under elevated salinity (Puniran-Hartley *et al*., 2014). However, the isolate enhanced shoot length under no stress to moderate stress (5 dS m^{-1}) conditions, whereas the metabolites appeared dominant over both the isolate and control, at higher degree of stress (10 and 15 dS m^{-1}) (Fig. 4). The root length also was seen enhanced over the control by both the isolate as well as metabolites; however, the metabolites further dominated over the isolate irrespective of the intensity of the stress intensity (Fig. 5). Saline conditions raise the osmotic potential of the surrounding solution, thus generating conditions similar to water deficit (Munnus, 2002); this further affects the processes of cell division and elongation, which ultimately reduce the growth and development of plants. The control from higher salinity exhibited similar impact, however as evident, the treatments with isolate and the metabolites successfully alleviated these effects with different intensities. The germination and vigor index was also seen enhanced with both the treatments over the control; the treatment metabolites found exceptionally dominant at higher stress conditions (10 and 15 dS m^{-1}). Under moderate and no saline conditions there exhibited no significant difference in the enhancement of germination by both the isolate and metabolites; however, there was significant enhancement in germination under highly saline conditions (10 and 15 dS m^{-1}), under the influence of metabolites. Delayed germination (after 7 DAS) under the influence of treatment with isolate at 15 dS m^{-1} further endorsed the significance of the treatment with metabolites. These results also validated our assumption of salinity-induced relegation in the PGP activity of microbes. The vigor index also increased in alignment with germination percent and seedling length. The metabolites predominantly enhanced vigor index over both the treatment with isolate and the control (Fig. 6). The treatments also enhanced the biomass accumulated over the control; the metabolites were found dominating to increase the biomass under lower as well as high stress conditions,

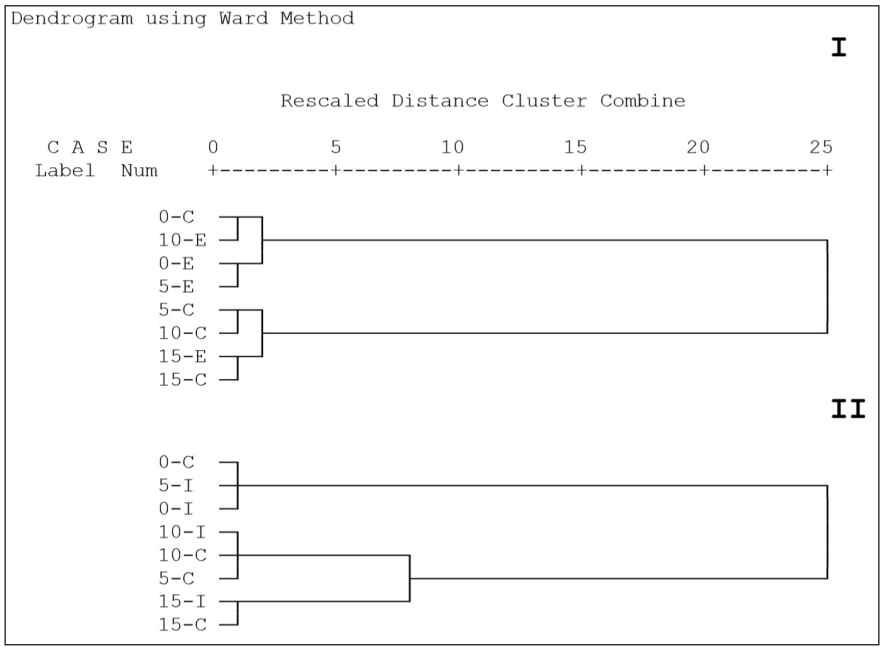

Fig. 8. Grouping of the treatments under high saline environments (10 and 15 dS m-1 respectively)

however only at 15 dS m^{-1}, the isolate found dominant over the metabolites (Fig. 7).

Protein and phenolic compounds from the seedlings were recorded significant enhancement over the control when seeds were treated with either of the treatments. However, at 5, 10 and 15 dS m^{-1}, the metabolites found dominant in enhancing both of these traits over the treatment with the isolate. There was slight (insignificant) increase only in the content of phenolic compounds by treatment with the isolate under no stress conditions (Table, 2). The sugar content in shoot and roots of the seedlings was analyzed to investigate the mounting of defense system in terms of accumulated sugars to combat the increasing salinity stress. Both the treatments significantly induced the accumulation of sugars in shoot as well as roots of the seedlings. With a few exceptions (14 DAS at 0 and 10 dS m^{-1}, 28 DAS at 5 dS m^{-1} in shoot, and 28 DAS at 0 dS m^{-1} in roots, where treatment with the isolate induced accumulation of more sugars), the treatment with metabolites predominantly induced accumulation of sugars in both shoot and roots (Table 3).

Total chlorophylls were also found positively influenced by both the treatments over the control. Treatment with the isolate actively raised the content of total chlorophylls over both the treatment with metabolites and the control, under no stress conditions. However, though the response of the treatment with isolate dominated over the control under higher degree of stress, it progressively faded over the treatment with metabolites (Table 3). These results highlighted the ability of the treatments to protect and uphold the carbon fixation system in the seedlings under saline conditions.

The assessment of antioxidant enzymes activities permitted the overview of the enzyme-based management of oxidative stress under the influence of the treatments. Both the treatments performed better over the control, while mounting the antioxidant enzyme-based response to combat oxidative stress. The GPX levels were raised more efficiently by the treatment with metabolites at 0 and 15 dS m^{-1}; however, at 5 and 10 dS m^{-1}, the isolate dominated (Table 4). Similarly, the CAT activity was up-regulated by the treatment with the isolate at 0 and 15 dS m^{-1}; whereas unlike that of GPX, the CAT activity was enhanced by the treatment with metabolites at 5 and 10 dS m^{-1} (Table 4). Unlike both the former enzymes, the levels of SOD were found raised by the treatment with the isolate over that with the metabolites, irrespective of the degree of stress imposed (though the difference was insignificant, the SOD levels were higher at 0 dS m^{-1} in case of the treatment with the isolate). Increased salinity generates oxidative stress in plants, which is major cause of membrane damage, and ultimately become lethal to the cell (Qureshi *et al*., 2005; De Pinto *et al*., 2012). The increased activity of SOD has been also reported earlier in the plants suffering from abiotic stresses (Eyidogan and Oz, 2005).

Correlation analysis of the measured parameters revealed intertwining relationships among the measured parameters. The treatment with isolate resulted in a significantly positive correlation between the biomass and dry shoot weight ($r = 0.817$), dry root weight ($r = 0.865$), catalase ($r = 0.817$); shot length and root length ($r = 0.894$), protein content ($r = 0.788$); root length and total chlorophylls ($r = 0.790$); dry root weight and catalase ($r = 0.821$), protein content and vigor index, and germination percentage ($r = 0.821$ and 0.744 respectively), GPX and SOD ($r = 0.920$); catalase and total cholorophyll ($r = 0.729$); and vigor index and germination percent ($r = 0.959$). Only instances of significantly negative correlation were evident between root sugars and vigor index, and germination percentage ($r = -0.719$ and -0.804 respectively) (Table 5). The positive relationship of biomass, dry root weight and chlorophyll with catalase highlighted the significance of CAT-mediated management of oxidative stress; also the positive correlation between GPX and SOD indicated the co-dominant expression of both the enzymes.

The treatment with metabolites yielded higher digit of significantly positive correlations, between biomass and shoot length, root length, dry shoot weight, dry root weight, protein content, SOD, catalase and total chlorophyll ($r = 0.746, 0.740, 0.923, 0.924, 0.770, 0.707, 0.917,$ and 0.728 respectively); shoot length and protein content ($r = 0.915$); root length and dry root weight, protein content, and vigor index ($r = 0.808, 0.870,$ and 0.738 respectively); dry shoot weight and dry root weight, protein content, and CAT, ($r = 0.789, 0.747,$ and 0.789

respectively); dry root weight and CAT (r = *0.947*); protein content and vigor index (r = *0.713*); GPX and SOD, and total chlorophylls (r = *0.964* and *0.791* respectively); SOD and shoot sugars (r = *0.738*); CAT and total chlorophylls (r = *0.751*); shoot sugars and root sugars (r = *0.891*); and between the vigor index and germination percent (r = *0.961*) (Table 5).

The hierarchical clustering of the measured parameters under the influence of the treatments with the isolate and its metabolites also differed significantly. The treatment with metabolites under 10 and 5 dS m^{-1} found clustered with that of control at 0 dS m^{-1}, thus supporting the above observations (Fig. 8). However, the same in case of the inoculation with isolate found restricted only to 5 dS m^{-1} (Fig. 8), thus the efficiency of the metabolites to attenuate the higher salinity stress in wheat was further endorsed.

The involvement of microorganisms in mitigating the abiotic stresses in plants is well established. Variety of plant associated as well as free living organisms contribute their role in plant growth promotion under both the normal and, stress-prone situations. Many wild plants are well known for their comprehensive lifestyle, even under the influence of extreme environmental situations like drought, and salinity. It is quite natural to expect the microorganisms associated with such plants thriving under increasingly unfavorable circumstances adapted to the continuous presence of stressor(s) (Siddikee *et al.,* 2010; Sorty *et al.,* 2016). In an endorsement of the same fact, we successfully isolated and screened a candidate PGP, halotolerant bacterium (the isolate D28; *Arthrobacter* sp.) from the phyllosphere of *Sesbania bispinosa*, thriving in highly saline habitat. Soils, in general are diverse with respect to pH and physicochemical factors that not only influence the survival and metabolism of soil microflora but also the germination, growth and physiology of the plants thriving therein (Bashan *et al.,* 2013). Moreover, these factors can also influence the process of rhizosphere colonization and development of symbiotic associations between plant and the soil microbes. However, our assumption being selection of adaptive bacterial population, selective isolation conditions were maintained to enhance the chances of obtaining more resilient isolates. Furthermore, most of the times such isolates effectively manage to maintain the routine metabolic traits operative under stress conditions, thus enabling them express PGP traits even under high-stress conditions. Our current as well as earlier results have endorsed this fact (Sorty *et al.,* 2016). Use of metabolites produced by PGP isolate D28 proved superior in promoting the growth and salinity tolerance in wheat, over the inoculation with bacterial cells. The physico-chemical parameters shown by wheat seedlings under the influence of the treatments with metabolites also found improved. Negative influence of salinity on germination and vigor index is well known (Khan and Webber, 2008), however, the treatment-induced enhancements can be considered an indicative of successful attenuation of the stress-impact on both the germination percentage and vigor index. Significant enhancement of shoot as well as root lengths were observed with the metabolites treatment, even under saline conditions. Inoculation with live cells also performed better over the control; however, it remained significantly lower when compared with that of metabolites treatment.

Up-regulated expression of phenolics compounds is advantageous to the plants pertaining to the multi-faceted role of this class of molecules in plant biochemistry, particularly during combat with biotic and abiotic stress circumstances (Al-Wakeel *et al.,* 2013). Higher levels of phenolic compounds in the seedlings not only aligned with this fact, but also endorsed fitness of the treatments with respect to the intensity by which they mount the response. Elevated levels of sugars were also recorded in the seedlings, which may offer benefit in terms of maintaining osmotic balance under saline conditions, helping the plant retain the appropriate levels of moisture (Kerepesi and Galiba, 2000). Additionally, the sugars constitute a major fraction of plant root exudations, the salinity-induced down-regulation of sugar content may also affect root exudations, which may probably hinder plant-microbe signaling in rhizosphere region, and consequently the process of rhizosphere colonization.

Activity of antioxidant enzymes also found enhanced under the influence of treatments with live cells as well as metabolites. Salinity promotes the accumulation of superoxide

radicles into the plants, which ultimately damage the cellular components and induce apoptosis. However, the situation can be restricted by two possibilities, either preventing or efficiently managing the increasing oxidative stress inside the plant cell. Both the treatments successfully increased the levels of antioxidant enzymes over the control; however, at high salinity level, the treatment with metabolites resulted in relatively lower levels of antioxidant enzymes over that of the inoculation with live cells. This may indicate restriction of the production of superoxide radicles itself, or probable presence of an alternative mechanism, since the overall growth pattern of germination and establishment seedlings appeared sound in case of the treatment with metabolites.

Pearson's correlation analysis and the hierarchical clustering also highlighted the significant improvements in physico-chemical status of wheat seedlings under saline conditions. The evidence of positive relationship found extended with the metabolites treatment. Strong positive relation of phenotypic parameters like shoot, root length, dry weights, with antioxidant enzymes further endorsed the significant attenuation of oxidative stress. The correlation between dry root weight and CAT also underlined the contribution of CAT activity in root development under salt stress.

Conclusions

The results strongly emphasized plant growth promotional ability of *Arthrobacter* sp. NIMD28 and its metabolites secreted *in* vitro, under elevated saline conditions; however, the metabolites of the isolate found superior, even under the situations where live isolates baffled. Priming of wheat with the metabolites produced by *Arthrobacter* sp. NIMD28, thus seem beneficial under saline conditions; moreover, the prominence of metabolites could not be neglected considering the quantity implemented for the act. These results therefore strongly endorse the need to execute keen, *in situ* trials relating to optimization of dose response during the application of pooled metabolites of PGP strains for effective alleviation of salinity stress.

Acknowledgements

Authors gratefully acknowledge the Science and Engineering Recruitment Board-Department of Science and Technology (SERB-DST) Young Scientist scheme and Indian Council of Agricultural Research (ICAR), Govt. of India under Application of Microorganisms in Agriculture and Allied Sectors (AMAAS) for financial assistance.

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Printed in September 2025