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PRELIMINARY INVESTIGATIONS ON SELECTION OF SYNERGISTIC HALOTOLERANT PLANT GROWTH PROMOTING RHIZOBACTERIA FOR INDUCING SALINITY TOLERANCE IN WHEAT

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Abstract

Halotolerant bacteria having 1-aminocyclopropane-1-carboxylate (ACC)-deaminase activity were isolated from wheat rhizosphere and further screened for their plant growth promoting potentials in *planta* by using wheat as test crop under salinity stress. ACC-deaminase activity ranged from 85 to 399 nmol α-ketobutyrate mg^{-1} hr^{-1} of these rhizobacteria. In absence of L-tryptophan, out of 25 rhizobacteria, 16 were able to produce IAA equivalents while nine did not produce auxins as IAA equivalents. Production of IAA equivalents ranged from 1.45 to 12.32 µg mL^{-1} in absence of L-tryptophan. While, in the presence of L-tryptophan, all the 25 rhizobacteria produced auxins as IAA equivalents and it ranged from 3.10 to 34.76 µg mL^{-1}. Out of 25 rhizobacterial isolates, seven isolates were recognized as plant growth promoting rhizobacteria (PGPR) which were statistically significant for improving growth of wheat under saline conditions. These seven halotolerant PGPR were tested for their compatibility of growth and synergism with each other. Out of 7, only three isolates were found synergistic and they showed abilities to coexist. Sequencing of *rrs* (*16S rRNA*) gene of these PGPR strains and phylogenetic analysis confirmed that these 3 PGPR strains are *Bacillus cereus* strain Y5, *Bacillus sp.* Y14 and *Bacillus subtilis* strain Y16. It is concluded that rhizobacteria varying in ACC-deaminase activity differentially respond to influence plant growth under salinity stress.

Key words: PGPB; Synergism; Bacillus; ACC-deaminase.

Introduction

World population is likely to reach 9.1 billion till 2050 with increasing demand of 70% more food production (Anon., 2009a). Need of the cereals is expected to reach 3 billion tonnes by 2050; nearly 2.1 billion tonnes up from present cereals' demand (Anon., 2009a). In this situation, current agricultural production needs to be raised which can only be possible by expanding agricultural land area or by increasing yield per acre (Anon., 2009b). On the other hand, our agricultural lands are under threat due to loss of soil organic carbon, nutrient depletion, soil erosion, soil sealing and soil salinization (Anon., 2015). Soil salinization is an evil of the global climate change which is undermining our global food security. According to an estimate, approximately 900 million hectares of arable land of the globe is salt affected (Flowers, 2004). Furthermore, soil salinization is increasing day by day and 30% of arable land is likely to become saline by 2025 (Munns, 2002). Soil salinization may cause loss of about 12 billion US$ to annual income of the globe (Ghassemi *et al.*, 1995). Soil salinity is one of the major abiotic stresses which are putting down crop production especially in arid and semi-arid regions of the world (Yadav *et al.*, 2011). It hampers the soil productivity and crop yields by disturbing the chemical, physical as well as biological properties of soil (Benlloch-Gonzalez *et al.*, 2005). It affects the plant growth mainly due to osmotic stress, specific ion toxicity as well as nutrient imbalances (Tester & Davenport, 2003). Elevated level of salts in soils disturbs the plant biochemical as well as physiological processes which ultimately results in impaired growth and yield of crop plants (Lynda *et al.*, 2016). It has been observed that salinity stress causes perturbation in gaseous exchange processes of plants like photosynthesis, sub-stomatal CO_2 concentration, net CO_2 assimilation, stomatal conductance and transpiration rate (Shaheen *et al.*, 2013). Moreover, salinity stress has also been known to cause disturbance in normal hormonal balance and lipid and protein metabolism of plants (Shabala & Munns, 2012).

Therefore, eco-friendly and affordable ways are required to bring the salt affected soils under cultivation to fulfill the food demands of increasing population. Reclamation of salt affected soils through chemical amendments and/or improved irrigation practices are not permanent solution and often expensive (Selvakumar *et al.*, 2014). Moreover, development of salt tolerant plant through plant breeding and transgenic approaches is time consuming and complex process due to multi-gene control of salt tolerance in plants and extensive genetic diversity amongst different genotypes (Cabota *et al.*, 2014; Selvakumar *et al.*, 2014). Biotechnological and plant breeding techniques remained unsuccessful for improving salt tolerance in rice, wheat and corn (Dionisio-Sese & Tobita, 2000) even after gene improvement (Ottow *et al.*, 2005).

Plant growth promoting bacteria (PGPB) are well known to improve plant growth under normal as well as stressed environment. Plenty of scientific literature has revealed the importance of microbial inoculants for improving plant growth under stress conditions (Nadeem *et al.*, 2010; Glick, 2014; Nadeem *et al.*, 2014; Ahmad *et al.*, 2016; Nadeem *et al.*, 2016). Plant growth promoting bacteria improve plant growth under stressful environment through their various mechanisms like regulation of ethylene biosynthesis in plants by 1-aminocyclopropane-1-

carboxylate (ACC) deaminase enzyme (Glick, 2014), enhancing the nutrients availability i.e., nitrogen, phosphorus, iron, potassium and zinc (Han & Lee, 2005; Wu *et al.,* 2005; Iqbal *et al.,* 2016), production of exopolysaccharides (Upadhyay *et al.,* 2012), and biosynthesis of phytohormones i.e., auxins, cytokinins, and gibberrellins (Nadeem *et al.,* 2014). Plant growth promoting bacteria also elicit induced systemic tolerance (IST) against abiotic stresses (Yang *et al.,* 2009) and induced systemic resistance (ISR) against biotic stresses (Kloepper *et al.,* 2004; Rahman *et al.,* 2017).The objective of this investigation was to search out new synergistic halotolerant bacteria with their abilities to promote plants growth under salinity stress. This is a preliminary investigation on selection of synergistic halotolerant bacteria having plant growth promoting traits.

Materials and Methods

Isolation of bacteria from rhizosphere of wheat: Wheat samples were collected for isolation of rhizobacteria from different agro-ecological zones of Punjab, Pakistan, grown under salt affected fields. For this, wheat plants were up-rooted and placed in polyethylene bags before bringing to the laboratory. Plant roots were gently shaken to remove bulk soil (non-rhizospheric soil). The soil from rhizosphere of wheat was collected by gentle shaking the plant roots under aseptic conditions in sterilized distilled water. The dilution plate technique was used to isolate rhizobacteria from the suspension of soil closely adhering to plant roots(Wollum II, 1982) by using DF minimal salt medium (Dworkin & Foster, 1958) containing ACC as a sole source of nitrogen. Serial dilutions were prepared from the suspension of rhizospheric soil with sterilized NaCl (0.85%) solution. One milliliter of suspension from each dilution was poured in the sterilized Petri plate and thoroughly mixed with sterilized DF minimal salt medium containing ACC as a sole source of nitrogen. Petri plates were incubatedat 28±2°C for bacterial growth. After incubation for 72 h, the rhizobacterial colonies with prolific growth on nutrient agar medium were further purified by repeated streaking method using the same medium. The pure isolated rhizobacterial colonies were selected for evaluating their osmoadaptation potential and these rhizobacterial isolates were preserved by using 40% glycerol in eppendorf tubes at -40°C.

ACC-deaminase enzyme activity, indole 3-acetic acid assay and production of exopolysaccharides: ACC-deaminase activity of the bacterial strains was determined by following the modified method of Honma & Shimomura (1978) and Penrose & Glick (2003).Ability of all rhizobacterial isolates to produce IAA equivalents was determined spectrophotometrically with and without use of substrate (L-TRP) by following the method adopted by Sarwar *et al*. (1992). These rhizobacteria were cultured on RCV media containing mannitol for visual assessment of exopolysaccharide production (Ashraf *et al.,* 2004).

Osmoadaptation assay: These rhizobacterial isolates were tested for their osmoadaptation potential at various salinity levels (0, 5 and 10% of NaCl) in nutrient broth medium. Osmotic potential of broth medium was measured by osmometer (OSMOMAT-030-D, Gonotec, Germany) at respective salinity level. Bacterial suspension of about 10^3 CFU mL^{-1} for each rhizobacterial isolates were prepared and 1 mL of that suspension were inoculated in test tubes containing nutrient broth of respective salinity level. After 96 hours of incubation in mechanical shaking incubator at 28±1ºC temperature and 100 revolutions per minute (rpm), optical density (OD) was measured at λ550 nm by densitometer (Den-1, Densitometer, McFarland, UK). Moreover, population counts (CFU mL^{-1}) were also determined at each salinity level by following the procedure adopted by Vincin (1970).

Screening of rhizobacteria for plant growth promoting activity under gnotobiotic conditions: These rhizobacterial isolates were also scrutinized for their potential to improve wheat growth under salinity stress in axenic conditions. A jar experiment was conducted for screening of these rhizobacterial isolates for plant growth promotion as described by Asghar *et al.* (2002). For this, inocula of each rhizobacterial strains were prepared by using nutrient broth (NB) medium. The broth medium was sterilized by autoclaving at 121°C temperature and 15 psi pressure for 20 minutes. Each flask containing broth were inoculated with respective rhizobacterial strains and incubated at 28±2°C for 72 h in an orbital shaking incubator at100 rpm. After incubation, optical density (OD) was measured at λ 550 nm by densitometer. The OD for each bacterial strain was adjusted by dilution with sterilized distilled water to achieve their required population growth (10^7-10^8CFU mL^{-1}). Two sterilized filter paper sheets were soaked in inoculum and these filter papers were saturated with inoculum while in case of control filter paper sheets were dipped in autoclaved inoculum suspension. Wheat seed were surface sterilized by dipping in 70% ethanol for 1 minute and 3.5% sodium hypochlorite for 3-5 minutes and followed by 3-4 washings with autoclaved distilled water (Long *et al.,* 2008). Surface sterilized seeds of wheat were placed in sterilized Petri plates enfolded in two sheets of filter paper moistened with sterilized distilled water. These Petri plates were incubated at 25±2°C for germination in an incubator under darkness for two days. Three fully sprouted seeds of wheat were sandwiched between soaked filter paper sheets, which were then rolled and placed in autoclaved glass jars. To ensure aseptic conditions and to minimize chances of contamination all the inoculation and sowing of seeds in glass jars were performed in a laminar flow hood. Two salinity levels i.e., 0 and 100 mM of NaCl were maintained by using required amount of NaCl salt in sterilized Hoagland solution (half strength). Ten milliliter of sterilized Hoagland solution per glass jar was applied thrice in a week for nutrient supply as well as to maintain salinity stress in respective glass jars. The glass jars were arranged in the growth chamber by following the completely randomized design with three replications. The light intensity in growth chamber was adjustedto350 µmol m^{-2} s^{-1} with 8 h dark and 16 h light period and temperature was maintained at 25±2°C. Data regarding growth attributes of wheat seedling like fresh and dry biomass as well as shoot and root lengths were recorded after 21 days.

Compatibility test of salinity tolerant plant growth promoting rhizobacteria (PGPR): On the basis of osmoadaptation assay and jar experiment, efficient salinity tolerant and plant growth promoting rhizobacterial strains were tested for their compatibility by cross streak assay on nutrient agar medium to be used in multi-strain inoculation (Raja *et al.*, 2006). One bacterial strain was streaked on the solidified nutrient agar plate and incubated at 28±2°C for 24 h. The counter bacterial strain was streaked vertically to the growth of already streaked bacterial strain. The Petri plates were observed for bacterial growth at 28±2°C for 48 h. The growth suppression of counter bacterial strain by already streaked bacterial strain were considered as non-compatible and mixing of bacterial growth of both strains were considered as compatible. All dual combinations of selected PGPR strains were evaluated for their compatibility to be used in multi-strain inoculation.

Synergism/antagonism assay of halotolerant PGPR: The synergism/antagonism between selected plant growth promoting rhizobacterial isolates was assessed. For synergism/antagonism assays, the selected bacterial isolates were cultured at 28±2°C for 24 h in nutrient broth. The aliquots bacterial cultures (10 µL) were spot-inoculated on nutrient agar (NA) plates pre-seeded with tested bacterial strain (100 µL). The growth of bacteria was observed on Petri plates after 48 h of incubation at 28±2°C and formation of any clear zones of inhibition were observed (Naveed *et al.*, 2014).

Identification and phylogenetic analysis of selected PGPR by sequencing *rrs* (*16S rRNA)* gene: Most efficient rhizobacterial isolates were identified by *rrs* (*16S rRNA*) gene sequence analysis. CTAB/Chloroform-isoamyl alcohol method was followed for extraction of genomic DNA of the bacteria (Wilson, 1987). The purified DNA was then subjected to PCR amplification with *16S rRNA* gene universal primer sets consisting of 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) to target the *16S rRNA* gene. Phusion® High-Fidelity DNA Polymerase kit (BioLabs, New England) was used for PCR amplification. PCR amplification was performed in a thermocycler (Primus 96 advanced®, PEQLAB Biotechnologie GmbH, Germany) according to following program: an initial denaturation at 98ºC for 30 seconds followed by 29 cycles of denaturation at 98ºC for 10 seconds, annealing at 58 ºC for 30 seconds, and extension at 72 ºC for 30 seconds; and a final extension at 72ºC for 7 minutes. PCR products were purified and sequenced by Macrogen Inc., Korea. The sequences of *16S rRNA* gene were compared with the known nucleotide sequences using BlastN accessed at: http://www.ncbi.nlm.nih.gov/BLAST. ClustalX software was used for the multiple alignments of sequences (Thompson *et al.*, 1997) and then data were processed by using NJ-Plot for neighbor joining method (Perriere & Gouy, 1996) to construct phylogenetic tree. The sequences of *16S rNRA* gene of rhizobacterial isolates Y5, Y14 and Y16 were submitted in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) under the accession number KM652420, KM652421 and KM652422, respectively.

Statistical analysis: Standard errors were estimated by using Microsoft Excel 2007® (Microsoft Cooperation, USA) for the data regarding ACC-deaminase activity, IAA biosynthesis, and osmoadaptation assay. Data regarding screening trial were converted to Z-score by using Microsoft Excel 2007®and R software (R Core Team, 2013) was used to generate Heatmap and Redundancy Analysis (RDA).

Results

In present study, rhizobacteria with ACC-deaminase activity were isolated from wheat rhizosphere and tested for osmoadaptation as well as plant growth promotion. Selected plant growth promoting rhizobacterial (PGPR) isolates were further tested for their synergism/ antagonism and compatibility of their growth to be used in combinations. Results of these experiments are given below:

ACC-deaminase activity, biosynthesis of auxins as indole acetic acid (IAA) equivalents and exopolysaccharides (EPS) production by rhizobacteria: Total 25 rhizobacteria were isolated from wheat rhizosphere by dilution plate technique using DF minimal medium having ACC as sole source of nitrogen. These 25 rhizobacterial isolates were further tested for quantitative ACC-deaminase assay. ACC-deaminase activity ranged from 84.67 to 399.33 nmol α-ketobutyrate mg^{-1} biomass hr^{-1} (Table 1). The maximum ACC-deaminase activity was observed by isolate Y22. The 11 rhizobacterial isolates (Y2, Y3, Y5, Y7, Y9, Y10, Y14, Y15, Y16, Y22 and Y24) showed ACC-deaminase activity more than 200 nmol α-ketobutyrate mg^{-1} biomass hr^{-1} while 5 isolates (Y1, Y4, Y11, Y18 and Y20) showed ACC-deaminase activity less than 100 nmol α-ketobutyrate g^{-1} biomass hr^{-1}.

These 25 rhizobacteria were further tested for their ability to produce auxins as indole acetic acid (IAA) equivalents in the presence and absence of L-tryptophan. In absence of L-tryptophan, from 25 rhizobacteria, 16 were able to produce IAA equivalents while 9 rhizobacteria did not produce auxins as IAA equivalents (Table 1). Production of IAA equivalents ranged from 1.45 to 12.32 µg mL^{-1} in absence of L-tryptophan and maximum IAA equivalents were produced by rhizobacterial isolate Y22. While, in presence of L-tryptophan, all the 25 rhizobacteria produced auxins as IAA equivalents and it ranged from 3.10 to 34.76 µg mL^{-1} and maximum IAA equivalents were produced by Y22. In presence of L-tryptophan, 10 rhizobacteria (Y1, Y2, Y5, Y7, Y9, Y13, Y14, Y16, Y20 and Y22) had ability to produce IAA equivalents more than 20 µg mL^{-1} while only 6 rhizobacteria (Y8, Y11, Y15, Y18, Y21 and Y25) were able to produce less than 10 µg mL^{-1} of IAA equivalents. These 25 rhizobacterial isolates were also tested qualitatively for their ability to produce exopolysaccharides (EPS) (Table 1) and 16 rhizobacteria were able to produce EPS while other 9 rhizobacteria did not produce EPS.

Table 1. Plant growth promoting traits (ACC-deaminase, IAA equivalent and Exopolysaccharide production) of rhizobacteria isolated from rhizosphere of wheat. Data are means ± SE of 3 replicates.

Rhizobacterial isolate	ACC-deaminase activity (nmol α-ketobutyrate mg^{-1} hr^{-1})	IAA equivalents (µg mL^{-1})		EPS
		Without L-TRP	With L-TRP	
Y1	93.33 ± 3.85	1.45 ± 0.10	22.53 ± 1.18	+
Y2	218.00 ± 9.30	2.16 ± 0.11	30.70 ± 0.83	++
Y3	339.00 ± 9.30	ND	15.20 ± 1.19	-
Y4	84.67 ± 6.18	2.34 ± 0.07	14.30 ± 1.25	-
Y5	**392.67 ± 10.43**	**7.96 ± 0.28**	**34.60 ± 1.54**	**++**
Y6	124.00 ± 6.36	ND	10.93 ± 1.03	+
Y7	**232.33 ± 14.51**	**1.46 ± 0.06**	**24.80 ± 1.91**	**-**
Y8	100.00 ± 6.67	ND	3.10 ± 0.21	+
Y9	209.33 ± 8.02	5.21 ± 0.12	35.00 ± 1.73	-
Y10	**356.00 ± 11.56**	**9.07 ± 0.56**	**17.07 ± 0.52**	**++**
Y11	87.67 ± 6.39	ND	4.05 ± 0.44	+
Y12	131.33 ± 9.15	7.93 ± 0.23	15.64 ± 0.63	++
Y13	124.67 ± 5.21	7.06 ± 1.06	25.17 ± 0.90	-
Y14	**338.33 ± 9.22**	**11.02 ± 1.16**	**27.93 ± 1.03**	**+**
Y15	290.00 ± 7.24	ND	6.67 ± 0.36	++
Y16	**269.33 ± 9.22**	**9.72 ± 0.27**	**22.53 ± 1.18**	**++**
Y17	187.33 ± 6.70	7.07 ± 0.52	18.33 ± 0.88	-
Y18	**87.67 ± 6.39**	**ND**	**7.56 ± 0.87**	**+**
Y19	160.33 ± 9.22	1.67 ± 0.12	18.03 ± 0.73	-
Y20	**84.67 ± 8.46**	**4.41 ± 0.31**	**25.67 ± 1.20**	**++**
Y21	104.33 ± 3.76	ND	6.32 ± 0.71	-
Y22	**399.33 ± 6.39**	**12.32 ± 0.65**	**34.76 ± 0.91**	**+++**
Y23	157.00 ± 10.71	3.21±0.25	15.87 ± 0.94	+
Y24	244.67 ± 6.37	ND	11.82 ± 0.74	++
Y25	177.33 ± 6.94	ND	6.63 ± 0.19	-

ND indicates not detectable

Osmoadaptation of rhizobacteria: All the rhizobacterial isolates were tested for their osmoadaptation potential at 5 and 10% of NaCl level. Rhizobacterial isolates showed variable response for adopting osmotic stress (Table 2). At 5% of NaCl, minimum reduction in CFU of rhizobacteria was observed by isolate Y22 which was upto 5% less compared with CFU at 0% of NaCl (where no salt stress was applied). The decreases in CFU by isolates Y22, Y11, Y12, Y21 and Y5 were upto 5, 6, 6, 7 and 8%, respectively, at 5% of NaCl as compared with CFU of these rhizobacterial isolates at 0% of NaCl. It was observed that all the rhizobacteria showed less than 40% decreases in CFU at 5% of NaCl compared with 0% of NaCl except only one isolate Y17 that showed 60% reduction in CFU. However, growth of these rhizobacterial isolates was further decreased when concentration of NaCl was increased upto 10%. The reduction in CFU by all these rhizobacterial isolates ranged from 41 to 93% at 10% of NaCl compared with their CFU at 0% of NaCl. At 10% of NaCl, maximum decrease in CFU was recorded by rhizobacterial isolate Y15 and minimum reduction in CFU was observed by rhizobacterial isolate Y16 compared with their CFU at 0% of NaCl. Out of 25 rhizobacterial isolates, only 3 isolates (Y6, Y10 and Y16) showed less than 50% reduction in CFU at 10% of NaCl compared with their CFU at 0% of NaCl while reduction in CFU by all other rhizobacterial isolates was more than 50%. The data regarding osmoadaptation ability of these rhizobacterial isolates showed great variations among different isolates at various levels of NaCl concentrations.

Screening of rhizobacteria for plant growth promoting activity under axenic conditions: All the 25 rhizobacterial isolates tested for osmoadaptation were further scrutinized for their potential to improve wheat growth under normal and salinity stress in axenic conditions. The data depicted that inoculation with rhizobacterial isolates had variable effect on growth of wheat seedling under normal as well as in saline conditions (Fig. 1). Under normal conditions (0 mM NaCl/ where no salt added), out of 25 isolates, 11 isolates (Y4, Y5, Y7, Y9, Y11, Y14, Y15, Y16, Y18, Y20 and Y25) significantly improved the shoot length of wheat compared with un-inoculated control. However, 3 rhizobacterial isolates (Y3, Y6 and Y24) negatively affected the shoot length and statistically significant decreases in shoot length were observed compared with un-inoculated control. The remaining 11 isolates (Y1, Y2, Y8, Y10, Y12, Y13, Y17, Y19, Y21, Y22 and Y23) were statistically non-significant with un-inoculated control. Under saline conditions (100 mM NaCl), shoot length was significantly improved by 14 rhizobacterial isolates, 6 isolates caused negative effect and significantly decreased the shoot length of wheat as compared to un-inoculated control. However, 5 isolated remained statistically at par with un-inoculated control by their effect on shoot length. The isolates which remained statically non-significant in their effect on shoot length under saline conditions were Y2, Y9, Y15, Y21 and Y23.

Table 2. Osmoadaptation abilities of rhizobacteria isolated from wheat rhizosphere.

Rhizobacterial isolates	Optical density			Population count (CFU mL^{-1} X 10^3)		
	0% NaCl (-0.06 MPa)	5% NaCl (-0.65 MPa)	10% NaCl (-1.23 MPa)	0% NaCl (-0.06 MPa)	5% NaCl (-0.65 MPa)	10% NaCl (-1.23 MPa)
Y1	5.03 ± 0.10	3.16 ± 0.03	2.67 ± 0.15	27.27 ± 0.60	22.86 ± 0.17	12.91 ± 0.77
Y2	4.16 ± 0.09	3.13 ± 0.15	1.03 ± 0.07	18.49 ± 0.94	12.71 ± 1.00	8.30 ± 0.64
Y3	6.86 ± 0.12	6.20 ± 0.35	2.23 ± 0.19	30.40 ± 0.99	25.56 ± 0.94	3.81 ± 0.59
Y4	3.90± 0.22	2.46 ± 0.15	2.00 ± 0.12	23.62 ± 0.32	16.83 ± 0.54	7.74 ± 0.32
Y5	6.96 ± 0.15	6.00 ± 0.23	1.20 ± 0.12	37.82 ± 0.47	34.83 ± 0.76	10.11 ± 0.89
Y6	3.10 ± 0.12	2.86 ± 0.24	2.73 ± 0.15	18.78 ± 0.46	11.28 ± 0.64	9.97 ± 0.73
Y7	5.53 ± 0.15	4.83 ± 0.31	1.07 ± 0.10	22.57 ± 0.45	17.89 ± 0.66	3.55 ± 0.51
Y8	3.10 ± 0.07	2.26 ± 0.12	1.70 ± 0.09	14.11 ± 0.61	10.54 ± 0.52	4.78 ± 0.58
Y9	6.13 ± 0.20	5.66 ± 0.33	5.53 ± 0.15	48.62 ± 0.73	44.64 ± 0.54	20.74 ± 1.45
Y10	6.23 ± 0.30	5.73 ± 0.15	4.73 ± 0.15	28.95 ± 1.16	24.59 ± 0.66	15.08 ± 0.58
Y11	7.16 ± 0.12	5.33 ± 0.21	0.63 ± 0.03	38.28 ± 0.44	36.11 ± 1.48	2.89 ± 0.38
Y12	6.46 ± 0.09	5.26 ± 0.29	0.10 ± 0.03	41.35 ± 0.61	38.74 ± 0.74	3.84 ± 0.30
Y13	3.96 ± 0.07	3.40 ± 0.17	0.73 ± 0.07	22.75 ± 1.16	18.36 ± 0.40	2.38 ± 0.41
Y14	7.96 ± 0.09	5.83 ± 0.25	3.23 ± 0.15	45.55 ± 0.63	32.24 ± 1.15	20.60 ± 1.35
Y15	6.70 ± 0.20	5.43 ± 0.10	0.50 ± 0.07	38.18 ± 1.16	33.32 ± 1.17	2.74 ± 0.52
Y16	6.46 ± 0.15	5.46 ± 0.15	1.47 ± 0.12	31.52 ± 0.91	27.83 ± 0.64	18.73 ± 0.59
Y17	7.16 ± 0.12	4.76 ± 0.12	0.70 ± 0.18	38.94 ± 1.57	15.64 ± 1.21	3.15 ± 0.41
Y18	5.86 ± 0.17	5.00 ± 0.19	0.47 ± 0.06	29.47 ± 0.67	26.53 ± 0.64	2.86 ± 0.15
Y19	3.26 ± 0.13	3.16 ± 0.15	0.53 ± 0.03	20.30 ± 1.78	15.82 ± 0.89	4.49 ± 0.27
Y20	2.93 ± 0.06	2.00 ± 0.15	0.67 ± 0.12	17.22 ± 1.66	13.04 ± 1.16	4.84 ± 0.33
Y21	6.20 ± 0.69	6.10 ± 0.09	0.97 ± 0.21	40.90 ± 2.20	37.92 ± 0.65	3.37 ± 0.46
Y22	7.30 ± 0.09	6.20 ± 0.13	2.60 ± 0.19	45.50 ± 1.30	43.41 ± 1.22	19.85 ± 0.90
Y23	3.30 ± 0.18	2.76 ± 0.15	0.27 ± 0.12	23.41 ± 0.54	14.62 ± 0.24	5.55 ± 0.37
Y24	3.90 ± 0.18	3.36 ± 0.33	1.23 ± 0.09	14.52 ± 0.72	9.83 ± 0.44	5.51 ± 0.47
Y25	3.93 ± 0.21	3.03 ± 0.17	1.57 ± 0.12	25.69 ± 0.94	16.52 ± 0.29	6.10 ± 0.19

Data are means ± SE of 3 replicates

Inoculation with different rhizobacterial isolates showed variable effect on root length of wheat under normal as well as saline conditions. Under normal conditions (0 mM NaCl), the effect of 17 rhizobacterial isolates was significantly positive and 4 rhizobacterial isolates caused significant negative effect on root length while 4 rhizobacterial isolates remained statically non-significant in their effect on root length in comparison to un-inoculated control. The rhizobacteria Y1, Y3, Y6 and Y17 were statistically non-significant in their effect on root length in comparison with un-inoculated control. Under saline conditions (100 mM NaCl), there was also great variability among different rhizobacterial isolates for influencing root length. Out of 25 isolates, 16 isolates positively influenced the root length under saline conditions as compared to un-inoculated control. Increase in root length ranged from 48 to 116% over un-inoculated control by these 16 isolates. However, isolates Y3, Y22, Y24 caused statistically significant decreases in the root length of wheat compared with un-inoculated control. Moreover, rhizobacterial isolates Y1, Y6, Y8, Y17, Y19 and Y21 were statically at par compared with un-inoculated control for influencing the root length.

Under normal conditions, total fresh biomass was significantly increased by 16 rhizobacterial isolates, 7 isolates significantly decreased the total fresh biomass, while 2 isolates remained statistically non-significant for influencing the total fresh biomass as compared to un-inoculated control. Under saline conditions (100 mM NaCl), total fresh biomass was significantly improved by 16 rhizobacterial isolates, 5 isolates caused negative effect and significantly decreased the total fresh biomass of wheat seedlings as compared to un-inoculated control. However, 4 isolated remained statistically at par with un-inoculated control.

The data depicted that inoculation with rhizobacterial isolates had variable effect on total dry biomass of wheat seedlings under normal as well as saline conditions (Fig. 1). Under normal conditions (0 mM NaCl), out of 25 isolates, 18 isolates (Y2, Y4, Y5, Y7, Y9, Y10, Y11, Y12, Y13, Y14, Y15, Y16, Y17, Y18, Y20, Y21, Y23 and Y25) significantly improved the total dry biomass of wheat seedlings as compared to un-inoculated control. However, 6 isolates (Y1, Y3, Y6, Y8, Y19 and Y24) negatively affected the total dry biomass and statistically significant decreases in total dry biomass were observed compared with un-inoculated control. The remaining one rhizobacterium (Y22) was statistically non-significant with un-inoculated control. Under saline conditions (100 mM NaCl), there was also great variability among different rhizobacterial isolates for influencing total dry biomass of wheat seedlings. Out of 25 isolates, 18 isolates positively influenced the total dry biomass and significantly improved the total dry biomass of wheat seedlings under saline conditions compared with un-inoculated control. However, rhizobacterial isolates Y1, Y3, Y6, Y8 and Y24 significantly decreased the total dry biomass compared with un-inoculated control. Moreover, rhizobacteria Y19 and Y22 were statically at par compared with un-inoculated control for influencing the total dry biomass of wheat seedlings under saline conditions.

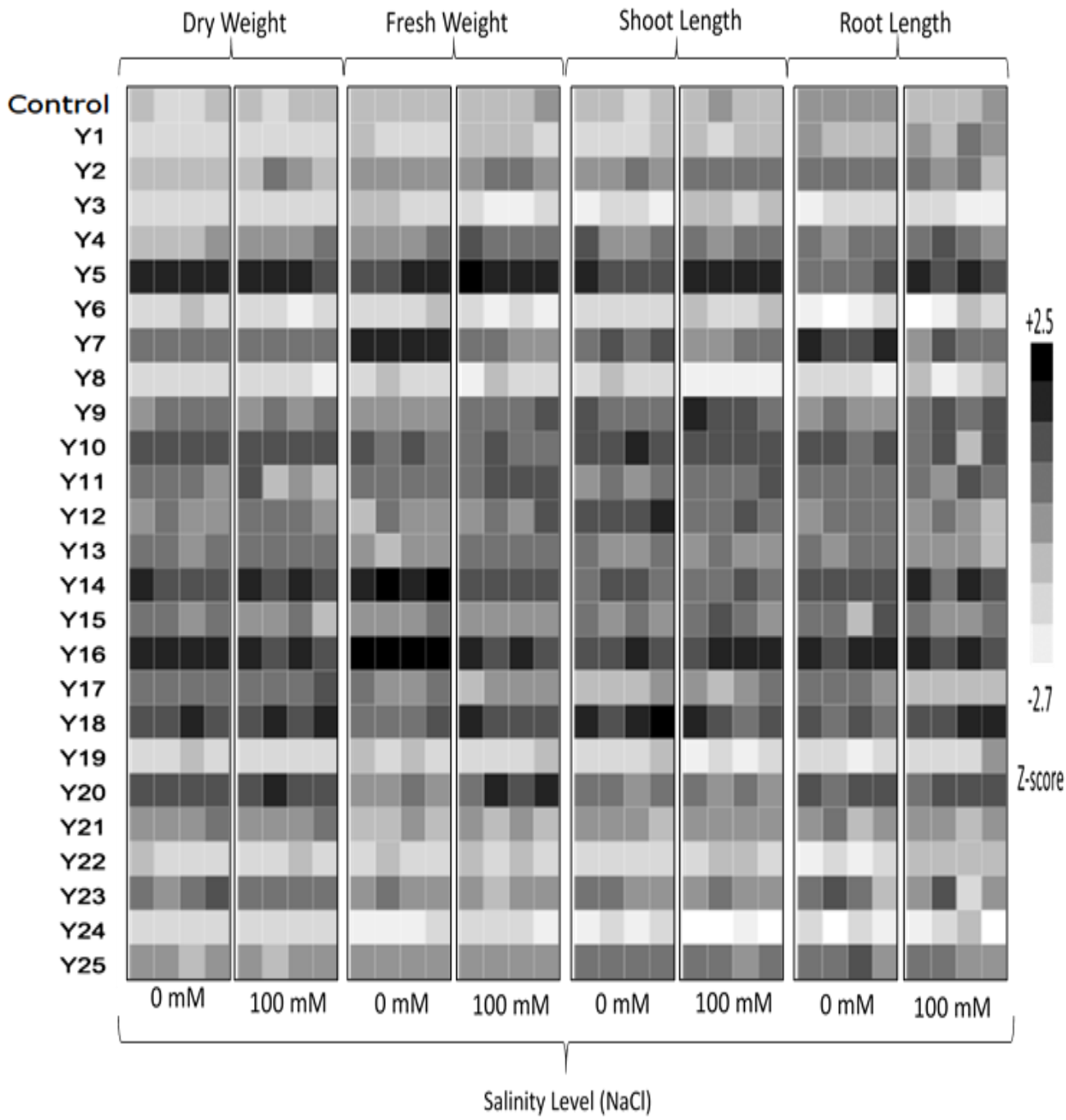

Fig. 1. Heatmap showing effect of rhizobacterial isolates on shoot length, root length, fresh weight and dry weight of wheat seedling in normal (0 mM of NaCl) as well as saline (100 mM of NaCl) conditions. Data are Z-score values indicating 4 replications of each treatment.

Selection of halotolerant PGPR strains: On the basis of screening trial, the efficient rhizobacteria were selected for further assays of compatibility of growth and synergism/ antagonism. The PGPR isolates Y5, Y7, Y10, Y14, Y16, Y18 and Y20 were selected for compatibility test and synergism/antagonism assay. These selected PGPR isolates were most effective for improving growth of wheat under normal as well as saline conditions. Redundancy analysis (RDA) further confirmed these rhizobacterial isolates in a distinct group from other two groups (Fig. 2). Three distinct groups of rhizobacterial isolates were plant growth promoters, plant growth inhibitors and neutral. The rhizobacterial isolates Y1, Y3, Y6, Y8, Y19, Y22 and Y24 were in a group of plant growth inhibitors. However, other rhizobacterial isolates (Y2, Y4, Y9, Y11, Y12, Y13, Y15, Y17, Y21, Y23 and Y25) were neutral in their effect on plant growth and remained statistically at par with control for most of the parameters studied.

Synergism/antagonism between PGPR isolates: Results of compatibility test showed that out of 7 PGPR isolates, only 3 isolates (Y5, Y14 and Y16) were compatible for growth. Synergism/ antagonism assay further confirmed that PGPR isolates Y5, Y14 and Y16 were also synergistic in their effect (Fig. 3)

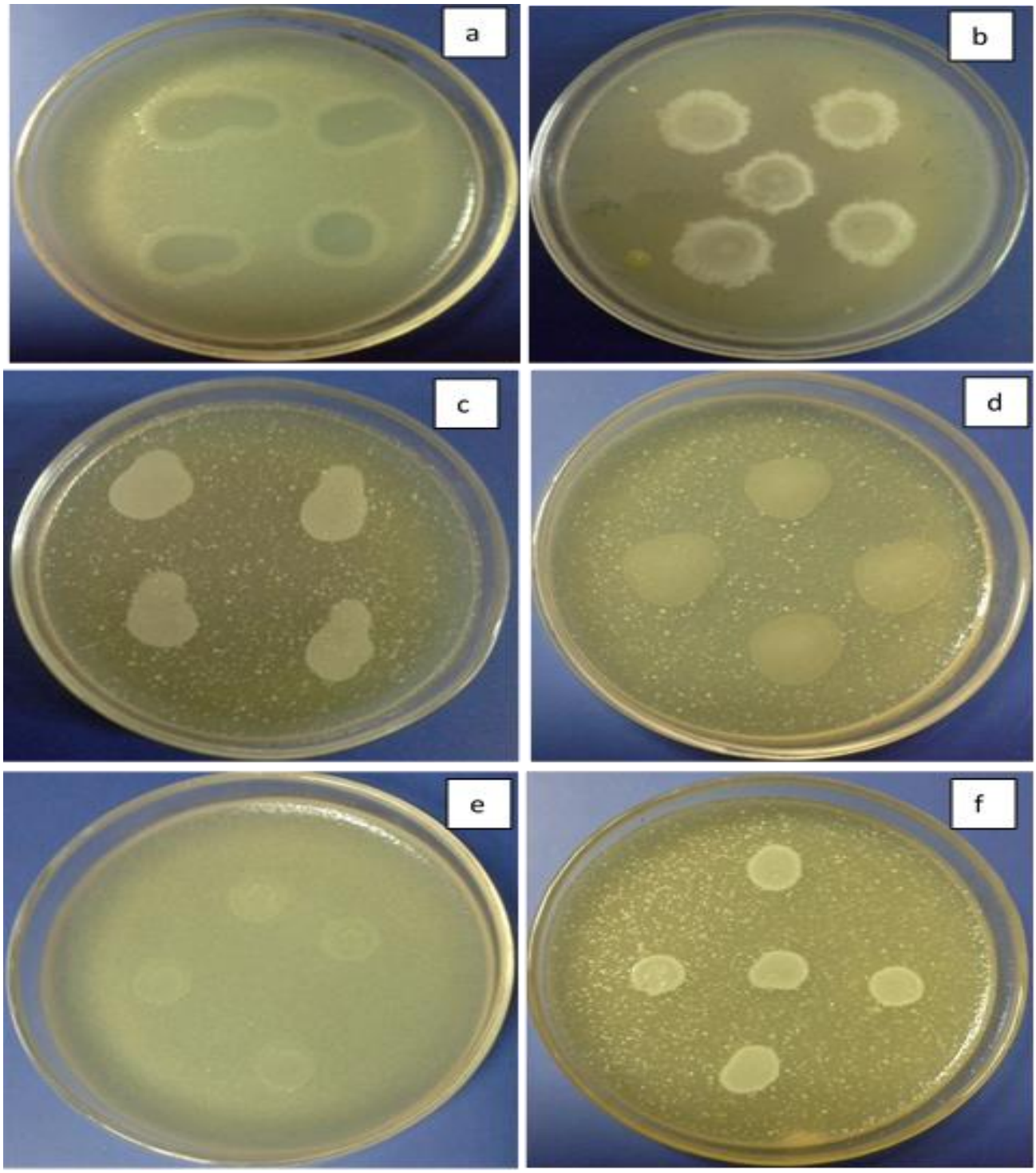

Fig. 2. Synergism/antagonism between selected PGPBR strains. a) Antagonism between Y5 and Y18, b) Antagonism between Y16 and Y18, c) Synergism between Y5 and Y15, d) Synergism between Y5 and Y16, e) Synergism between Y14 and Y16 and f) Synergism between Y14 and Y5.

Phylogenetic relationship of efficient PGPR strains based on sequences of *rrs* (*16S rRNA*) genes: Selected compatible PGPR isolates and synergistic in their effect, were identified through sequencing of *rrs* (*16S rRNA*) gene. BlastN analysis showed that sequences of *rrs* (*16S rRNA*) gene of isolates Y5, Y14 and Y16 had more than 99% similarity with *rrs* (*16S rRNA*) gene sequences of the genus *Bacillus.* In-silico bioinformatics analysis of *rrs* (*16S rRNA*) gene sequences of these PGPR were performed by making phylogenetic tree by neighbor-joining method. These PGPR strains were situated in the group comprising of bacteria belonging to the genus *Bacillus* (Fig. 4). On the basis of phylogenetic analysis, selected PGPR strains were named as *Bacillus cereus* strain Y5, *Bacillus* sp. Y14 and *Bacillus subtilis* strain Y16. In brackets, Genbank accession numbers of *rrs* (*16S rRNA*) gene sequences of the bacterial strains are given and bootstrap values are also given, values greater than 900% are marked as black dot.

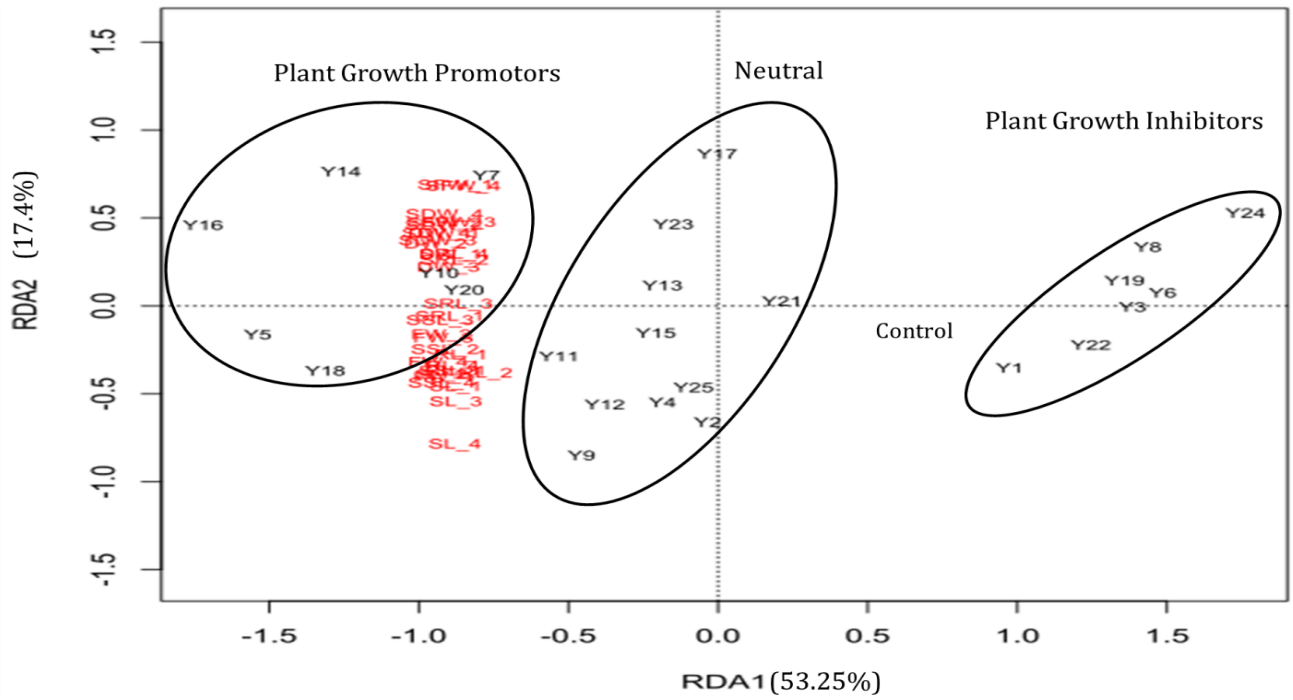

Fig. 3. RDA graph showing three distinct groups of rhizobacteria

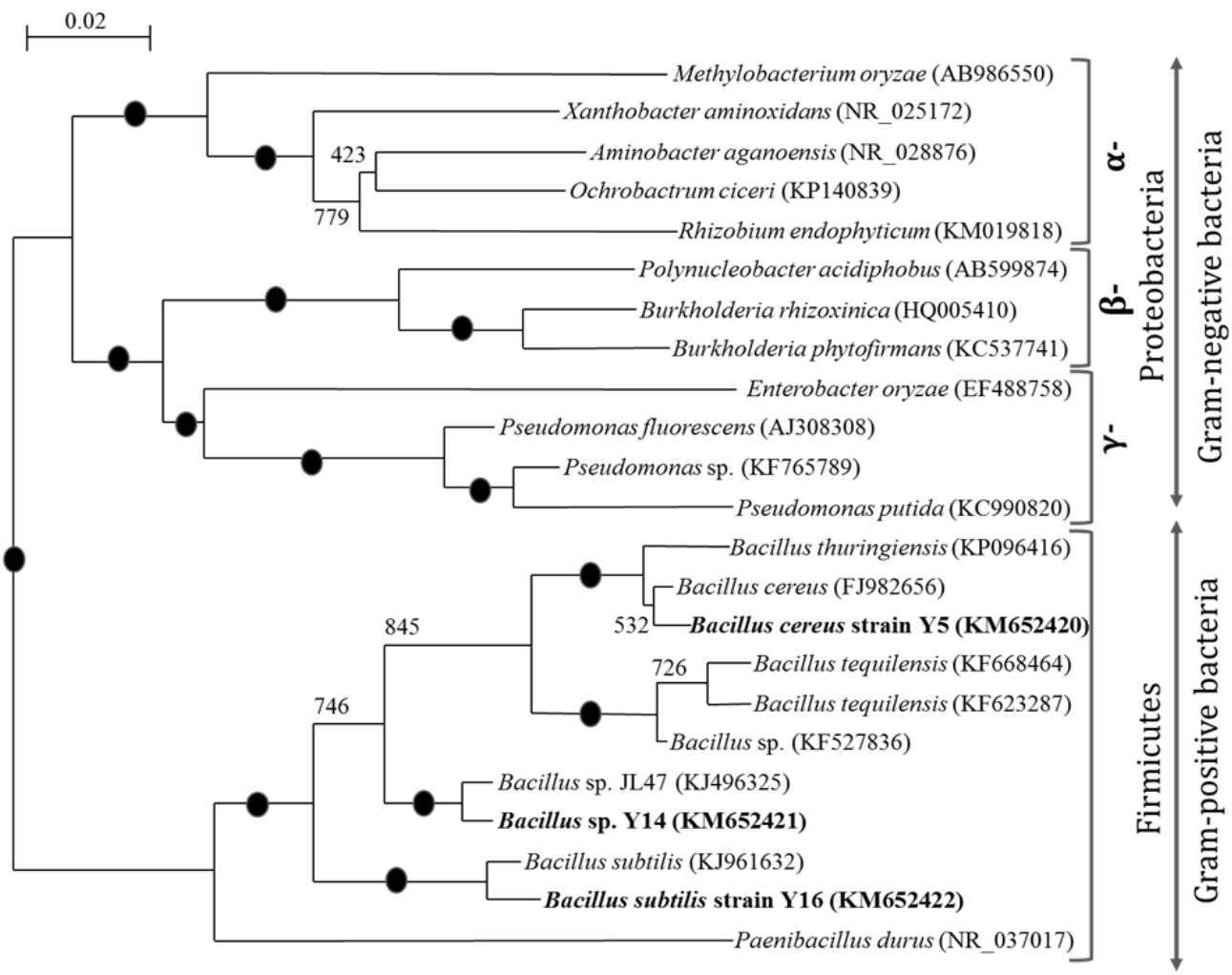

Fig. 4. Neighbor-joining phylogenetic analysis resulting from the multiple alignment of *16S rRNA* gene sequence of *Bacillus* sp. with those of other bacterial strains found in the GenBank database. Bootstrap values more than 1000 are marked as black dot and phylogenetic distance is shown on a scale bar. Accession numbers of *16S rRNA* gene used in phylogenetic analysis are given in parenthesis

Discussion

In present study, bacteria were isolated from rhizosphere of wheat by using 1-aminocyclopropane-1-carboxylate (ACC) as sole source of nitrogen. Only those rhizobacterial isolates were selected which had ability to utilize ACC as sole source of nitrogen. Further, quantitative ACC-deaminase activity of these rhizobacteria was also determined and these rhizobacterial isolates showed variability in their ACC-deaminase activity. Variation in ACC-deaminase activity in rhizobacteria might be attributed to variable induction process of enzyme (ACC-deaminase) due to differential expression of *AcdS* gene of these rhizobacteria (Singh *et al.,* 2015). ACC-deaminase is present differently in species of Gram positive as well as Gram negative bacteria (Nascimento *et al.,* 2014). This enzyme is involved in deamination of ACC by bacteria, which is an immediate precursor for biosynthesis of ethylene in plants. Bacteria convert ACC into NH_3 and α-ketobutyrate and use NH_3 as a nitrogen source for their growth (Glick, 2007). ACC-deaminase activity is encoded by an *AcdS* gene; this is an inductive enzyme and its activity is induced in the presence of its substrate ACC (Singh *et al.,* 2015). Induction of ACC-deaminase in bacteria is controlled by *AcdS* gene which showed differential expression under variable environmental conditions (Li & Glick, 2001). Expression of *acdS* gene is also regulated by LRP protein encoding regulatory genes which are differentially expressed under aerobic as well as anaerobic conditions (Cheng *et al.,* 2008). Some other regulatory genes like *AcdB* and *LysR* could also play role in expression of *AcdS* gene under different conditions (Singh *et al.,* 2015). Similarly, in our study, variation in ACC-deaminase activity was observed in different rhizobacteria.

These rhizobacterial isolates further showed variability in their ability to produce auxins as indole acetic acid (IAA) equivalents in the presence and absence of L-tryptophan. Biosynthesis of phytohormones is considered one of the most important mechanisms adopted by PGPR for facilitating the plant growth (Khalid *et al.,* 2004). Amongst the phyotohormones, auxins play pivotal role in plant growth and development both under normal as well as stressed conditions (Naveed *et al.,* 2014). Plants exudate different organic compounds via their roots in rhizosphere which include different types of sugars, proteins and lipids as well as different types of amino acids (Walker *et al.,* 2003). Plant associated bacteria utilize tryptophan present in root exudates and produce the auxins (IAA) in the root region. Indole acetic acid (IAA) absorbed by plant from rhizosphere along with endogenously plant-produced IAA can influence the plant growth and development (Glick, 2014). Synergy between bacterially produced ACC-deaminase and auxins is necessary for PGPR having ACC-deaminase activity to improve plant fitness under stressed conditions (Glick, 2014). In agreement with our findings, variation in biosynthesis of IAA equivalents by different PGPR both with and without L-tryptophan has been observed by Asghar *et al.* (2002) and Khalid *et al*. (2003).

Rhizobacteria having ACC-deaminase activity were further tested for their potential of salt tolerance. All the rhizobacterial isolates varied in their abilities to tolerate high salt concentrations. The growth of all rhizobacteria was decreased with increasing salt concentrations and maximum reduction in growth was observed at 10% of NaCl (w/v). Variability in growth of rhizobacteria in varying levels of salts was also noticed by Naz *et al.* (2009) and Sgroy *et al.* (2009). These growth variations of bacteria in varying salt concentrations might be due to variation in their diversity in genetic makeup (Trabelsi *et al.,* 2009). Bacteria survive in hostile high salt concentrations by osmoregulation, producing compatible solutes like ectoine, trehalose, glycine betaine as well as production of extracellular protease (Das *et al.,* 2015).

Results of jar experiment for screening of rhizobacteria also showed that these rhizobacteria had variable influence on wheat growth under normal (0 mM of NaCl/no added salts) as well as saline (100 mM of NaCl) conditions. Redundancy analysis (RDA) showed that rhizobacterial isolates were in three distinct groups of plant growth promoters, plant growth inhibitors and neutral. Out of 25 rhizobacterial isolates, 28% rhizobacteria were plant growth promoters and 44% rhizobacteria were neutral in their effect on plant growth. While, 28% rhizobacteria showed negative impact on plant growth. Therefore, bacterial population in rhizosphere could be beneficial, neutral as well as harmful for plant growth. The improvement in plant growth might be due to ability of these rhizobacteria to produce auxins and ACC-deaminase enzyme which regulate biosynthesis of ethylene in plants (Glick, 2014). Similar findings were observed by other researchers (Gosh *et al.,* 2003; Dodd *et al.,* 2004; Sergeeva *et al.,* 2006; Shaharoona *et al.,* 2006) that rhizobacteria with ACC-deaminase activity improved the plant growth especially under stressed conditions. While, negative impact of rhizobacteria on wheat growth might be due to production of phytotoxic metabolites by these bacterial isolates (Nehl *et al.,* 1996; Klopper *et al.,* 2004). Interestingly, one salt tolerant rhizobacterial isolate Y22 having maximum ACC-deaminase activity (399.33 nmol α-ketobutyrate mg^{-1} h^{-1}) and highest biosynthesis of IAA equivalents both in absence (12.32±0.65 µg mL^{-1}) as well as presence of L-tryptophan (34.76±0.91 µg mL^{-1}) showed negative effect on wheat growth. This negative effect of Y22 on wheat growth might be due to over production of auxins (Spaepen *et al.,* 2007) and/or higher levels of ACC-deaminase activity. Similarly, Penrose & Glick (2003) reported that bacteria having ACC-deaminase activity higher than 300 to 400 nmol α-ketobutyrate mg^{-1} h^{-1} do not essentially improve plant growth.

Further, the 7 isolates of plant growth promoting rhizobacteria from screening trials were tested for compatibility and synergism/antagonism between them. Out of 7 PGPR isolates, only 3 isolates were found synergistic in effect. Moreover, selected 3 PGPR strains (Y5, Y14 and Y16) were also identified on the basis of *rrs* (*16S rRNA*) gene sequencing. All the selected PGPR were belonging to genus *Bacillus* and named as *Bacillus cereus* strain Y5, *Bacillus sp.* Y14 *and Bacillus subtilis* strain Y16. Bacteria from genus *Bacillus* are globally used to formulate biofertilizers and biopesticides due to their ability of better survival in rhizosphere and multiple mechanism of plant growth promotion (Idris *et al.,* 2002). It was believed that bacteria from phylum Firmicutes have ACC-deaminase activity but *AcdS* gene was not reported including genus *Bacillus* (Nascimento *et al.,* 2014). Nevertheless, in few reports it has been reported that

bacteria from genus *Bacillus* also have *AcdS* gene for encoding ACC-deaminase enzyme (Madhaiyan *et al.*, 2010; Chen *et al.*, 2013). Sequence of *AcdS* gene of *Bacillus cereus* was submitted in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) under the accession number JN625726 (Chen *et al.*, 2013).

Conclusions

It is concluded from present investigation that bacteria residing near as well as on the roots of wheat have variable potential to utilize ACC as source of nitrogen. These rhizobacteria varying in ACC-deaminase activity also variably influence the plant growth under salinity stress. Further, these rhizobacteria also have variable synergism as well as antagonism between them. Synergistic halotolerant PGPR strain could be further investigated in their consortium to improve plant fitness under salinity stress. Multi-strain consortia of synergistic PGPR need to be assessed for development of biofertilizers.

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