Physiology and Molecular Biology of Plants (June 2023) 29(6):815–828
https://doi.org/10.1007/s12298-023-01328-2

RESEARCH ARTICLE

Role of ACC-deaminase synthesizing *Trichoderma harzianum* and plant growth-promoting bacteria in reducing salt-stress in *Ocimum sanctum*

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Received: 11 April 2023 / Revised: 12 June 2023 / Accepted: 14 June 2023 / Published online: 23 June 2023

Abstract

Salinity is a significant concern in crop production, causing severe losses in agricultural yields. *Ocimum sanctum*, also known as Holy Basil, is an important ancient medicinal plant used in the Indian traditional system of medicine. The present study explores the use of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase-producing strains of plant-growth-promoting bacteria (PGPB) namely Str-8 (*Halomonas desiderata)*, Sd-6 (*Brevibacterium halotolerans)*, Fd-2 (*Achromobacter xylosoxidans*), Art-7 (*Burkholderia cepacia)*, and Ldr-2 (*Bacillus subtilis)*, and *T. harzianum* (Th), possessing multi-functional properties like growth promotion, stress alleviation, and for enhancing *O. sanctum* yield under salt stress. The results showed that co-inoculation of Th and PGPBs enhanced plant height and fresh herb weight by 3.78–17.65% and 7.86–58.76%, respectively; highest being in Th + Fd-2 and Th + Art-7 compared to positive control plants. The doubly inoculated plants showed increased pigments, phenol, flavonoids, protein, sugar, relative water content, and nutrient uptake (Nitrogen and Phosphorous) as compared to monocultures and untreated positive control plants. In addition, co-inoculation in plants resulted in lower Na^+, MDA, H_2O_2, CAT, APX activities, and also lower ACC accumulation (49.75 to 72.38% compared to non-treated salt- stressed plant) in *O. sanctum*, which probably played a significant role in minimizing the deleterious effects of salinity. Finally, multifactorial analysis showed that co-inoculation of Th and PGPBs improved *O. sanctum* growth, its physiological activities, and alleviated salt stress compared to single inoculated and positive control plants. These microbial consortia were evaluated for the first time on *O. sanctum* under salt stress. Therefore, the microbial consortia application could be employed to boost crop productivity in poor, marginalized and stressed agricultural fields.

Keywords Plant growth-promoting bacteria · *Trichoderma harzianum* · *Ocimum sanctum* · Salinity · ACC accumulation

Introduction

Ocimum sanctum (Family: Lamiaceae), also called the queen of all medicinal herbs, is grown throughout the tropical Asian region, including India, for the last 3000 years (Cohen 2014). Various chemical constituents have been identified in *O. sanctum* essential oil including eugenol, cinnamyl acetate, *β*-elemene, rosmarinic acid, flavonoids, ursolic acid, linolenic acid (Dharsono et al. 2022; Wakchaure et al. 2017). Different plant parts of *O. sanctum*, like stem, root, seeds, flower, and leaves contain some phytochemicals which have therapeutic potential and are being used in anticancer, antiasthmatic, antidiabetic, hepatoprotective, hypolipidaemic and antistress drugs (Singh and Chaudhuri 2018; Wakchaure et al. 2017). The medicinal potential of the crops is heavily reliant on the secondary metabolites found in them. However, under extreme abiotic stress conditions (cold, drought, waterlogging, salt), the *Ocimum* plant revealed a decrease in secondary metabolite and lowered root growth (Rastogi et al. 2019).

Among the variety of abiotic stresses, salinity is the primary threat to agriculture, causing the most significant loss in crop production worldwide (Shrivastava and Kumar 2015). The major components affected by salt stress are plant yield and secondary metabolite content (Sarri et al.

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2021; Lucini et al. 2016). Several researchers have previously reported that alkalinity and salinity have a negative impact on the growth and yield of various crops (Shrivastava and Kumar 2015; Kamran et al. 2020; Ondrasek et al. 2022). According to the latest statistics, saline lands affect about 10% of the world's land surfaces (Hassani et al. 2021). The use of chemical fertilizers and irrigation with salty water in agricultural field are the main cause of soil salinity (Hailu and Mehari 2021). The resultant main effects of salinity are ionic imbalance and hyperosmotic stress. The effect of ionic imbalance causes molecular damage resulting in impairment in plant growth. Under severe salinity conditions, the amount of potassium, calcium, and magnesium in the tissues of many plant species gets reduced. Consequently, this leads to cell and plant death (Gupta and Huang 2014; Ma et al. 2020). Barbieri et al. (2012) have reported that basil showed a reduction in stomatal density, delay in abscisic acid, and proline accumulation under salt stress conditions. Treating the plants with PGPB (plant growth- promoting bacteria) is known to induce growth and defense systems in plants against various stresses modulating their secondary metabolites (Ali et al. 2014; Barnawal et al. 2017; Ahmad et al. 2015; Cho et al. 2015).

Trichoderma spp. has been formerly used as biofertilizer and biocontrol agent to enhance crop production and tolerance to environmental stresses (Mastouri et al. 2010; Shoresh et al. 2010). Likewise, various PGPBs are found useful in enhancing crop growth, and tolerance to environmental stresses in various plants via various mechanisms including synthesis of growth-enhancing compounds like phytohormones, siderophores, certain enzymes, and activities such as nitrogen fixation and solubilization of phosphates (Barnawal et al. 2012; 2013; 2014; 2017; Abd-Allah et al. 2018). Several species of halotolerant bacteria such as *Brevibacterium* sp. (Rahman et al. 2022*), Burkholderia* sp (Pinedo et al. 2015; Luo et al. 2022), *Halomonas* sp (Bharti et al. 2014, 2015) and *Achromobacter* sp (Sultana et al. 2020; Shahid et al. 2020) have been reported to ameliorate salt stress in crops (Arora et al. 2020).

Some physiological changes that could be induced by certain enzymes in plants can carry out these functions. Among these enzymes, ACC (1-aminocyclopropane-1-carboxylate) deaminase is essential for ethylene regulation (Valluru et al. 2016). ACC can be metabolized by some PGPBs possessing ACC-deaminase activity into ammonia and α-ketobutyrate; or else, ACC can be transformed into ethylene (Barnawal et al. 2012). The ethylene produced in plants, when combined with auxins, can promote plant growth as well its development, especially the development of roots (Gschwendtner et al. 2016). The strains of bacteria having ACC-deaminase activity can mitigate harmful effects on plants induced by stress-induced ethylene. Previous studies have demonstrated that inoculating bacteria with ACC deaminase can lessen the effects of salt-stress in a variety of plants (Barnawal et al. 2013; Himadri and Tapan 2021; Subhan et al. 2020). Although there are many reports on the application of *T. harzianum* and PGPB alone to increase salinity tolerance in various plants; no or limited attempts on co-inoculation of ***T. harzianum*** and PGPB to enhance salt stress tolerance in ***O. sanctum*** have been made. Therefore, the present study focuses on the effect of combined inoculations of ACC-deaminase producing *T. harzianum* and PGPBs in *O. sanctum* under salt stress.

Materials and methods

Culture selections and compatibility study

Five PGPB strains Str-8 (*Halomonas desiderata*; GenBank accession no. JQ436849), Ldr-2 (*Bacillus subtilis*; GenBank accession no. JX996197), Fd-2 (*Achromobacter xylosoxidans*; GenBank accession no. JQ 975,414), Art-7 (*Burkholderia cepacia*; GenBank accession no. KP 198,544), Sd-6 (*Brevibacterium halotolerans*; GenBank accession no. NR042638) were obtained from Crop Protection Division, CSIR- Central Institute of Medicinal and Aromatic Plants, Lucknow, India. Plant growth-promoting activities of these selected strains have been previously evaluated on various aromatic and medicinal crops (Barnawal et al. 2012, 2013; Maji et al. 2013; Bharti et al. 2015). These selected strains were further characterized for various plant growth-promoting activities such as ACC-deaminase production, nitrogen fixation, solubilization of phosphates, IAA, and siderophore production. All the strains were checked for their compatibility with *T. harzianum* (US Patent No. 6,475,772).

In vitro testing was performed to determine the compatibility of all PGPBs with *T. harzianum* by following the method of Singh et al. (2019). Freshly prepared bacterial cultures (24 h old) were streaked at the periphery of a Petri plate, poured with {NA + PDA (1:1)} growth media, and incubated for 24 h at 26 ± 2 °C. After one day, 5 mm disc of *T. harzianum* (5 days old culture) was placed in the center of the plate and further incubated at 26 ± 2 °C for 5 days. The absence of an inhibition zone indicated the compatibility between the bacterial and fungal strains. Each test was independently replicated four times.

Biofilm study

For in vitro biofilm study, compatible microbes were used for the crystal violet assay (Triveni et al. 2012; Singh et al. 2019). Three ml of *T. harzianum* suspension (3.0×10^8 spores ml^{-1}; 5 days old) and 5 ml of bacterial culture (cfu count 3×10^8 ml^{-1} ; 2 days old) were mixed in 250 ml broth and incubated for 16 days at 30 °C. Fungal-based

bacterial biofilm was observed under an LCD microscope US3 model (BR Biochem Life Sciences Pvt. Ltd.) as per the procedure of Alexander (2009).

Preparation of bacterial and *T. harzianum* inoculums.

PGPB strains and *T. harzianum* were grown separately in nutrient broth (24 h) and potato dextrose broth (7 days), respectively. Bacterial cells were harvested by centrifugation (8,000 x g at 4° C for 10 min), washed thoroughly, and re-suspended in sterilized NaCl solution (0.85%). The bacterial suspension's CFU (colony forming unit) count was maintained at 3×10^8 ml^{-1} (Bharti et al. 2013). *T. harzianum* culture was filtered through cheesecloth into a sterile glass bottle and suspended in sterile saline solution. The spore count has adjusted to approximately 3.0×10^8 spores ml^{-1}.

Glasshouse experiment

The experiments in pots were conducted with *O. sanctum* cv CIM "Ayu" to assess the effect of selected PGPBs and *T. harzianum*, either alone or in various combinations, on the growth and secondary metabolite status of plants under salt stress conditions. The glasshouse experiment was designed and performed according to the method followed by Bharti et al. (2016). Before planting, roots were dipped for 30 min in microbial cultures, and the remaining culture (5 ml pot^{-1}) was poured into the same pot (Maji et al. 2013). The potting mixture without inoculation served as a control (Bharti et al. 2013; Singh et al. 2019). Plantlets were inoculated with the same microbial cell suspension (3×10^8 CFU/ml) after 15 days.

Treatments applied were as given below:

1. Negative control (untreated non-salt stressed);
2. Positive control (untreated salt-stressed),
3. *T. harzianum* (Th) + salt-stressed.
4. *Halomonas desiderata* (Str-8) + salt-stressed.
5. *Bacillus subtilis* (Ldr- 2) + salt-stressed.
6. *Achromobacter xylosoxidans* (Fd-2) + salt-stressed.
7. *Burkholderia cepacia* (Art-7) + salt-stressed.
8. *Brevibacterium halotolerans* (Sd-6) + salt-stressed.
9. Th + Str-8 + salt-stressed.
10. Th + Ldr-2 + salt-stressed.
11. Th + Fd-2 + salt-stressed.
12. Th + Sd-6 + salt-stressed.
13. Th + Art-7 + salt-stressed.

All the pots were watered three times a week with SDW for the first 15 days after inoculation of cultures and then periodically supplemented with NaCl (salt) solution at concentrations steadily increasing from 50 mM to 500 mM NaCl solution till harvesting. Plants were harvested after 90 days of planting (Bharti et al. 2016). Negative control plants were not supplemented with saline solution (EC = 0.587 mS at 20.5 °C).

Plant growth measurement, electrolyte leakage, and relative water content

Growth parameters like the height of the plant and herb weight (fresh), were recorded before harvesting of the plants. The plants were harvested after 90 days of treatment. The samples for biochemical parameters were collected just before harvesting and kept in deep freezer at − 80 °C for performing biochemical tests. Electrolyte leakage and relative water content were also determined by following the methods of Hnilickova et al. (2019) and Medici et al. (2003), respectively.

Photosynthetic pigments

Chlorophyll and carotenoid contents were determined by previously described methods of Lichtenthaler and Buschmann (2001) and Singh et al. (2019). A UV-VIS Spectrophotometer (Spectra Max plus 384) was used to measure absorbance at 665.2, 662.4, and 470 nm.

Proline, malondialdehyde (MDA), hydrogen peroxide, and sugar content

Proline content in the leaf was determined, as illustrated by Bates et al. (1973). The concentration of MDA, a lipid peroxidation product, was analyzed by the TBA method in leaf samples (Hodges 1999). To determine hydrogen peroxide content, fresh leaf tissues were extracted in TCA (0.5 ml; 0.1%), and then centrifuged. H_2O_2 content was calculated as illustrated by Velikova et al. (2000). The total soluble sugar in leaf samples was determined as described previously by Irigoyen et al. (1992).

Antioxidant enzyme activities

Leaf tissues were extracted in100 mM phosphate buffer (4 ml; pH: 7.2) using mortar and pestle, as Aroca et al. (2003) reported. After centrifugation (18000x *g* for 10 min at 4 °C), the supernatant was used for evaluating enzyme activity.

Ascorbate Peroxidase (APX): The enzyme's activity was assessed in a 1ml mixture of potassium phosphate buffer (100 mM; pH: 7.0), hydrogen peroxide (0.5 mM), Ascorbate (5 mM), and enzyme extract. To start the reaction, H_2O_2 was added, and the reduction in the absorbance was recorded at 290 nm for 1 min (Nakano and Asada 1981).

Catalase (CAT) For assessing the catalase activity method of Aebi (1984) was followed. The activity of CAT

was determined by recording the decline in absorbance at 240 nm in a reaction mixture containing 10 mM phosphate buffer, 150 mM hydrogen peroxide, and enzyme extract and calculating the rate of decomposition H_2O_2.

Total protein content It was analyzed using the Lowry et al. (1951) method, and Bovine Serum Albumin was used as a standard.

Total flavonoid and phenol content

Fresh leaves were extracted in 0.5 ml ethanol (80%) thrice and centrifuged. Supernatant was then pooled, and the total phenolic content was calculated using the Folin–Ciocalteu method, as previously reported by Zhang et al. (2006), using gallic acid as a standard. The total flavonoid content was measured using the $AlCl_3$ colorimetric method described by Chang et al. (2002) and quercetin as the standard.

ACC level determination in the plant tissues

1-aminocyclopropane-1-carboxylic acid (ACC) concentrations in root samples were estimated by following Barnawal et al. (2012). Frozen leaf samples were extracted in 80% methanol (5 ml) containing butylated hydroxytoluene (2 mg l^{-1}) thrice and samples were pooled. After centrifugation (12000x g for 15 min at 4 °C), the supernatants were combined and evaporated using a rotary evaporator. ACC level was estimated by gas chromatography as reported by Barnawal et al. (2012). The supernatant was mixed with 0.1 ml $HgCl_2$ (80 mM) in vials and air tightened with rubber septa. Afterward, sodium hypochlorite solution (0.2 ml) was injected in the vials through septa, shaken well, and incubated for 8 min, and analyzed by gas chromatography for ethylene determination (Barnawal et al. 2012, 2013, 2014).

Foliar nutrient uptake

In air-dried samples, sodium, nitrogen, and phosphorous content were estimated by wet digestion with $HNO_3 + H_2O_2$. The Kjeldahl method was used for nitrogen estimation (Singh et al. 2009). Flow Inject Analyzer (Foss FI- ASTAR 5000) was used for phosphorus and flame photometer for sodium estimation (Singh et al. 2009, 2019).

Root colonization through Microscopy (TTC staining)

The endophytic colonization of PGPB strain was examined using TTC (2,3,5-triphenyl tetrazolium chloride) staining of the root. Roots were surface sterilized in 3% NaOCl for 5 min, followed by three times washing in sterilized water. After that roots were placed for 3 days in TTC malate buffer. The thin sections of root were detected for pinkish red spots of bacterial colonies at 100X magnification under a light microscope (LCD microscope; US3 model; BR Biochem Life Sciences Pvt. Ltd.) (Ray et al. 2016).

Statistical analysis

Statistical analysis was done by ANOVA method with IBM SPSS Statistics 20 software. Means and standard errors were determined for 4 replicate values, and means were compared using DMRT's significance level under 0.05. The principal component analysis was applied using XLSTAT 2021.1.1 Add in soft to reveal the correlation among treatments and parameters.

Results

Compatibility study

Microscopic and crystal violet assay of in vitro biofilm formed by individual and combined bioinoculants showed that all the five strains Str-8, Ldr-2, Fd-2, Sd-6, and Art-7 were compatible with *T. harzianum* (Fig. S1 A-E and F, Supplementary file). Dark blue color of crystal violet in combined inoculation of 96-well plates showed compatibility of Th with PGPBs in comparison to single strains (Fig S1 F).

Effect on plant growth, electrolyte leakage, and relative water content

No effect of single and dual inoculations was noticed on plant height compared to positive control (untreated salt-stressed) plants. Of the monocultures, Sd-6 inoculated plants showed increased fresh herb weight (41.81%) compared to positive control. On the other hand, in doubly inoculated plants, Th + Art- 7, Th + Ldr-2, and Th + Fd-2 (58.77, 48.90, 46.74%, respectively) inoculations significantly increased fresh herb weight compared to positive control. These consortia also showed significant increase in fresh herb weight over their respective monocultres (Table 1). Highest enhancement in plant growth was noticed in Th + Art-7, (58.77% compared to control), while in doubly inoculated plants Th + Ldr-2 showed highest increase in fresh herb weight compared to respective monoculture i.e., 38.05%. The plants inoculated with monocultures exhibited a significant decrease in electrolyte leakage, which was reduced further with dual inoculation of Th + Art-7 compared to monocultures and positive control. Str-8, Fd-2, and Sd-6 inoculated plants significantly enhanced relative water content compared to positive control in monocultures. Th + Ldr-2 followed by Th + Sd-6 and Th + Fd-2 increased the relative water content of inoculated plants further compared to monocultures and positive control (Table 1).

Table 1 Effect of bio inoculants on height, fresh herb weight, electrolyte leakage and relative water content in *O. sanctum* under salt stress

Treatments	Height (cm)	Fresh herb weight (g)	Electrolyte Leakage (dS/m)	Relative Water content (%)
Positive control	59.5 ± 2.90^{ab}	36.67 ± 1.44^{d}	80.94 ± 0.54^{a}	69.12 ± 1.13^{g}
Th	64.25 ± 5.72^{ab}	42.61 ± 5.54^{cd}	69.28 ± 1.16^{d}	74.72 ± 0.42^{f}
Str-8	55.75 ± 2.25^{b}	42.11 ± 3.50^{cd}	63.79 ± 1.09^{e}	78.34 ± 0.81^{e}
Ldr-2	57.25 ± 1.89^{ab}	39.55 ± 3.38^{d}	65.66 ± 0.63^{e}	74.60 ± 1.13^{f}
Fd-2	67.5 ± 5.58^{ab}	42.59 ± 6.54^{cd}	41.38 ± 1.59^{g}	82.45 ± 0.42^{cd}
Sd-6	70 ± 0.71^{a}	52.00 ± 3.88^{abc}	65.60 ± 1.22^{e}	80.03 ± 0.78^{de}
Art-7	61.75 ± 2.59^{ab}	45.68 ± 1.36^{bcd}	60.25 ± 0.84^{f}	72.75 ± 0.57^{f}
Th + Str-8	62.75 ± 5.91^{ab}	42.17 ± 0.99^{cd}	72.34 ± 0.69^{c}	80.43 ± 1.28^{de}
Th + Ldr-2	67.25 ± 1.31^{ab}	54.60 ± 2.37^{ab}	71.56 ± 0.50^{cd}	88.44 ± 0.57^{a}
Th + Fd-2	70 ± 2.86^{a}	53.81 ± 2.51^{ab}	69.98 ± 1.02^{cd}	84.11 ± 0.92^{bc}
Th + Sd-6	67.75 ± 2.29^{ab}	52.04 ± 3.12^{abc}	78.14 ± 0.70^{b}	86.43 ± 1.02^{ab}
Th + Art-7	68 ± 5.12^{ab}	58.22 ± 2.95^{a}	39.14 ± 0.42^{g}	81.22 ± 1.00^{d}
Negative control	60 ± 4.71^{ab}	52.56 ± 2.32^{abc}	28.04 ± 0.75^{h}	87.00 ± 0.43^{a}

Different letters denote significant difference among treatments according to Duncans Multiple Range Test ($P < 0.05$)

Th *Trichoderma harzianum*, Sd-6 *Brevibacterium halotolerans*, Art-7 *Burkholderia cepacia*, Ldr-2 *Bacillus subtilis*, Fd-2 *Achromobacter xylosoxidans*, Values represents means ± SE (n = 4); positive control: untreated salt-stressed plants; negative control: untreated non-salt-stressed plants; Statistical analysis was done by ANOVA method with IBM SPSS Statistics 20 software

Effect on total chlorophyll and carotenoid content

All the monoculture treatments, except Sd-6, increased total chlorophyll content compared to positive control, while among dual inoculations, Th + Fd-2 and Th + Art-7 treated plants recorded a significant increase in total chlorophyll content compared to positive control (Fig. 1). Inoculation of four monocultures out of five resulted in enhanced carotenoid content in plants. Th + Fd-2 showed highest increase in carotenoid content; however, the increase was not significant compared to monocultures (Fig. 1).

Effect on antioxidant enzymes

Irrespective of the single and dual inoculated plants, salt-stressed plants (positive control) recorded significantly

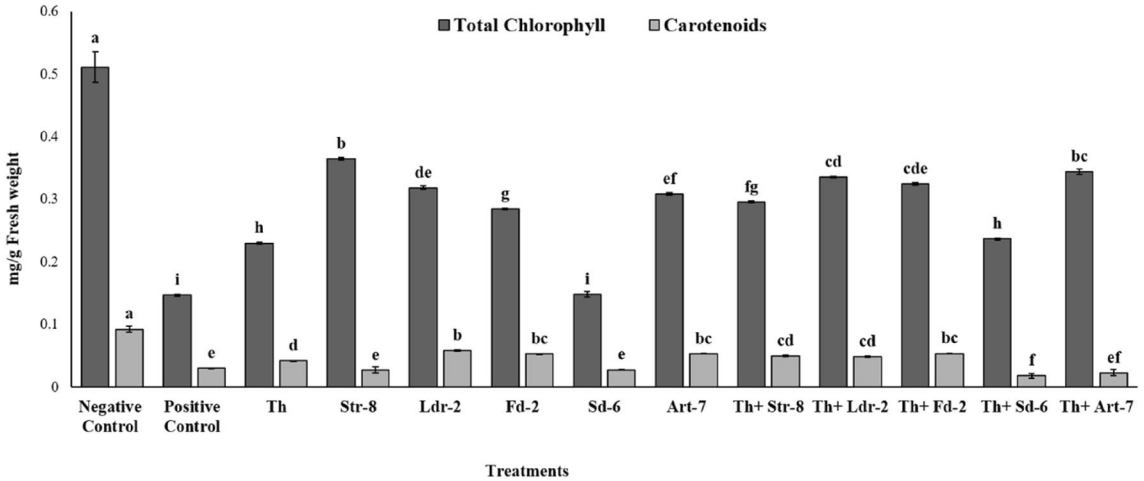

Fig. 1 Effect of bioinoculants on total chlorophyll and carotenoids content under salt stress in *O. sanctum*; Th: *Trichoderma harzianum*; Sd-6: *Brevibacterium halotolerans*; Art- 7: *Burkholderia cepacia;* Ldr-2: *Bacillus subtilis;* Fd-2: *Achromobacter xylosoxidans;* positive control: untreated salt-stressed plants; negative control: untreated non-salt-stressed plants; Error bars represents means ± SE (n = 4); Statistical analysis was done by ANOVA method with IBM SPSS Statistics 20 software; Different letters denote significant difference among treatments according to Duncan's Multiple Range Test ($P < 0.05$)

higher CAT and APX activities. However, all the single and dual inoculated plants recorded considerably greater CAT and APX activities compared to uninoculated plants (negative control). In the case of dual inoculations, Th + Art-7 inoculated plants recorded a increase in CAT but not in APX activity compared to monocultures (Fig. 2).

Effect on protein, Sugar, H_2O_2, MDA, and proline content

All monocultures recorded an increase in protein content compared to positive control except Ldr-2 inoculated plants. Application of Th + Sd-6 and Th + Ldr-2, in case of dual inoculations, recorded a significant increase in protein content (i.e., 142.57% and 137.63%, respectively) compared to positive control plants; however, no dual treatment was found significantly superior over monocultures. Likewise, the sugar content was enhanced by all single and dual inoculated plants as compared to positive control plants. Again, no dual inoculated plants could significantly enhance sugar content in comparison to the monocultures (Table 2). Proline content was not significantly increased by any monoculture, while in dual inoculations, it was significantly higher in Th + Ldr-2 inoculated plants (i.e., 10.53% compared to the positive control plants). All the monocultures recorded a reduction in MDA content, and in dual inoculated plants, it was significantly reduced by Th + Ldr-2 inoculated plants (i.e., 48.05% compared to the positive control plants) followed by Th + Fd-2 and Th + Art-7 as compared to monocultures and positive control. Similarly, all the monocultures recorded a reduction in H_2O_2 content compared with positive control. In the case of dual inoculations, namely Th + Ldr-2, Th + Sd-6 and Th + Art-7, plants showed reduction in H_2O_2 content compared to monocultures and positive control. The highest reduction was recorded in Th + Sd-6, Th + Fd-2, and Th + Art-7 inoculated plants; a reduction of 71.38%, 64.51%, and 73.54%, respectively, as compared to positive control plants (Table 2).

Effect of various bioinoculants on nutrient uptake

In salt-stressed plants, sodium concentration was reduced by all single and dual inoculated plants compared to the positive control. All the monocultures recorded a reduction in sodium content, while in the case of dual inoculations, all inoculated plants showed significantly reduced sodium content compared to monocultures and positive control plants. A slight increase in nitrogen content was recorded by all monocultures, except Sd-6, compared to positive control. In contrast, dual inoculation of Th + Sd-6 recorded a highest increase in nitrogen content over monocultures and positive control plants. Th and Str-8 significantly increased phosphorous content among all monocultures compared to the positive control. Also, in plants with dual inoculation of Th + Str-8 and Th + Art-7, significantly higher phosphorous content was recorded compared to monocultures and positive control (Fig. 3).

Effect on ACC content

All treatments significantly decreased 1-aminocyclopropane-1-carboxylic acid (ACC) content, with the highest reduction in *T. harzianum* inoculated plants compared to the untreated salt-stressed plants. However, all the dual

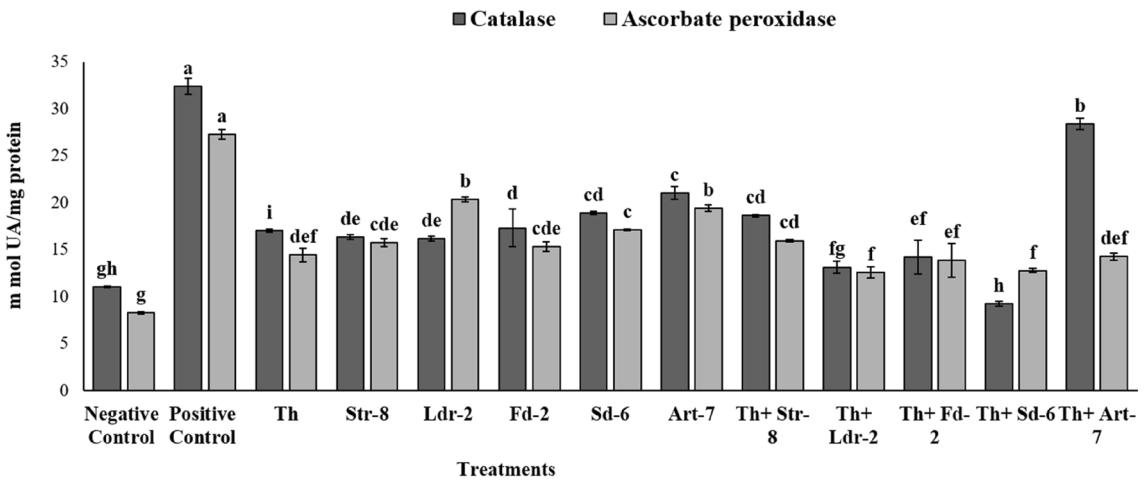

Fig. 2 Effect of bioinoculants on Catalase and Ascorbate peroxidase content under salt stress in ***O. sanctum***; Th: *Trichoderma harzianum*; Sd-6: *Brevibacterium halotolerans*; Art- 7: *Burkholderia cepacia;* Ldr-2: *Bacillus subtilis;* Fd-2: *Achromobacter xylosoxidans;* positive control: untreated salt-stressed plants; negative control: untreated non-salt-stressed plants; Error bars represents means ± SE (n = 4); Statistical analysis was done by ANOVA method with IBM SPSS Statistics 20 software; Different letters denote significant difference among treatments according to Duncan's Multiple Range Test ($P < 0.05$)

Table 2 Effect of bioinoculants on biochemical parameters in *O. sanctum* under salt stress

Treatments	Protein (mg g^{-1} FW)	Sugar (µg g^{-1} FW)	Hydrogen peroxide (mg g^{-1} FW)	MDA (nmol g^{-1}FW)	Proline (mg g^{-1} FW)
Positive control	225.16 ± 4.47^{g}	191.35 ± 14.15^{d}	9.75 ± 0.04^{a}	4.87 ± 0.04^{a}	12.54 ± 0.08^{b}
Th	530.48 ± 17.39^{b}	498.22 ± 15.46^{ab}	4.50 ± 0.17^{d}	3.31 ± 0.01^{de}	8.02 ± 0.21^{d}
Str-8	427.53 ± 6.93^{de}	512.85 ± 4.87^{ab}	7.88 ± 0.15^{b}	3.41 ± 0.06^{cd}	4.79 ± 0.02^{f}
Ldr-2	250.24 ± 1.32^{g}	347.12 ± 6.76^{c}	3.60 ± 0.06^{fg}	3.27 ± 0.04^{de}	3.74 ± 0.04^{gh}
Fd-2	410.47 ± 11.31^{de}	336.00 ± 43.95^{c}	6.14 ± 0.35^{c}	3.44 ± 0.08^{cd}	6.17 ± 0.44^{e}
Sd-6	381.95 ± 3.84^{def}	515.32 ± 0.60^{ab}	4.38 ± 0.02^{d}	4.26 ± 0.02^{b}	5.73 ± 0.06^{e}
Art-7	343.37 ± 5.06^{f}	523.18 ± 2.00^{ab}	8.17 ± 0.10^{b}	3.59 ± 0.06^{c}	2.82 ± 0.05^{i}
Th + Str-8	436.02 ± 2.30^{cd}	520.87 ± 1.97^{ab}	4.06 ± 0.02^{de}	3.30 ± 0.02^{de}	7.75 ± 0.04^{d}
Th + Ldr-2	535.05 ± 26.67^{b}	470.16 ± 13.17^{b}	3.24 ± 0.09^{g}	2.53 ± 0.02^{h}	13.86 ± 0.17^{a}
Th + Fd-2	493.17 ± 61.08^{bc}	348.34 ± 10.03^{c}	3.46 ± 0.30^{fg}	3.18 ± 0.05^{ef}	12.43 ± 0.05^{b}
Th + Sd-6	546.16 ± 3.69^{b}	527.12 ± 1.22^{ab}	2.79 ± 0.03^{h}	3.42 ± 0.06^{cd}	8.90 ± 0.32^{c}
Th + Art-7	370.16 ± 8.58^{ef}	538.90 ± 2.21^{a}	2.58 ± 0.02^{h}	2.81 ± 0.18^{g}	4.31 ± 0.20^{fg}
Negative control	838.54 ± 8.89^{a}	491.60 ± 48.21^{ab}	3.72 ± 0.04^{ef}	3.05 ± 0.04^{f}	3.29 ± 0.07^{hi}

Different letters denote significant difference among treatments according to Duncans Multiple Range Test ($P < 0.05$)

Th *Trichoderma harzianum*, Sd-6 *Brevibacterium halotolerans*, Art-7 *Burkholderia cepacia*, Ldr-2 *Bacillus subtilis*, Fd-2 *Achromobacter xylosoxidans*, positive control: untreated salt-stressed plants; negative control: untreated non-salt-stressed plants; Values represents means ± SE (n = 4); Statistical analysis was done by ANOVA method with IBM SPSS Statistics 20 software

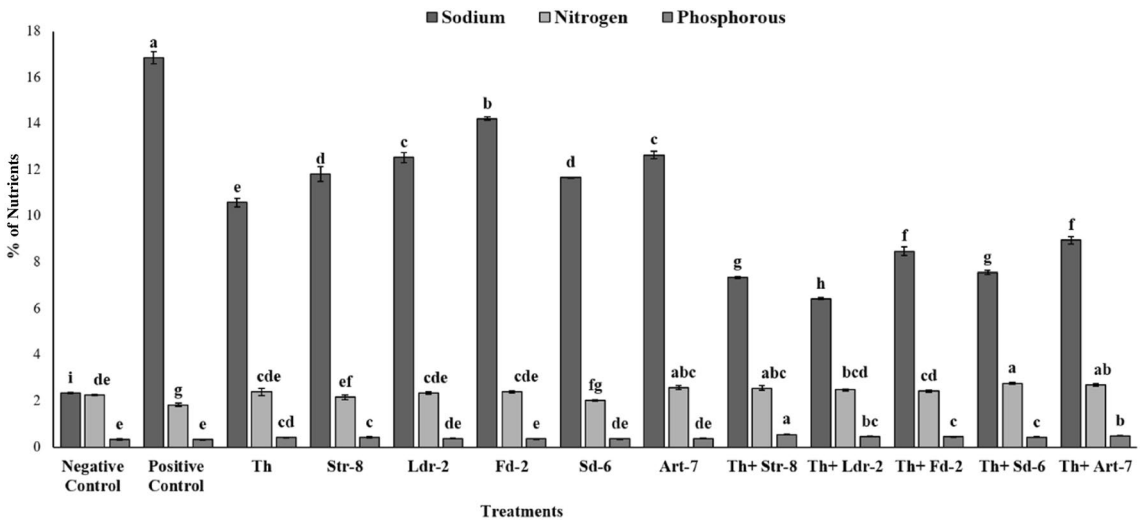

Fig. 3 Effect of bioinoculants on nutrient uptake under salt stress in *O. sanctum*; Th: *Trichoderma harzianum*; Sd-6: *Brevibacterium halotolerans*; Art-7: *Burkholderia cepacia;* Ldr-2: *Bacillus subtilis;* Fd-2: *Achromobacter xylosoxidans;* positive control: untreated salt-stressed plants; negative control: untreated non-salt-stressed plants; Error bars represent means ± SE (n = 4); Statistical analysis was done by ANOVA method with IBM SPSS Statistics 20 software; Different letters denote significant difference among treatments according to Duncan's Multiple Range Test ($P < 0.05$)

inoculated plants showed reduction in ACC content. A significant reduction was found in Th + Fd- 2 (61.88%), Th + Sd-6 (56.99%), and Th + Ldr-2 (38.95%) compared to their respective bacterial monocultures (Fig. 4).

Effect on total phenol and flavonoid content

All monocultures recorded an increase in total phenol and flavonoid contents compared to the positive control. In dual

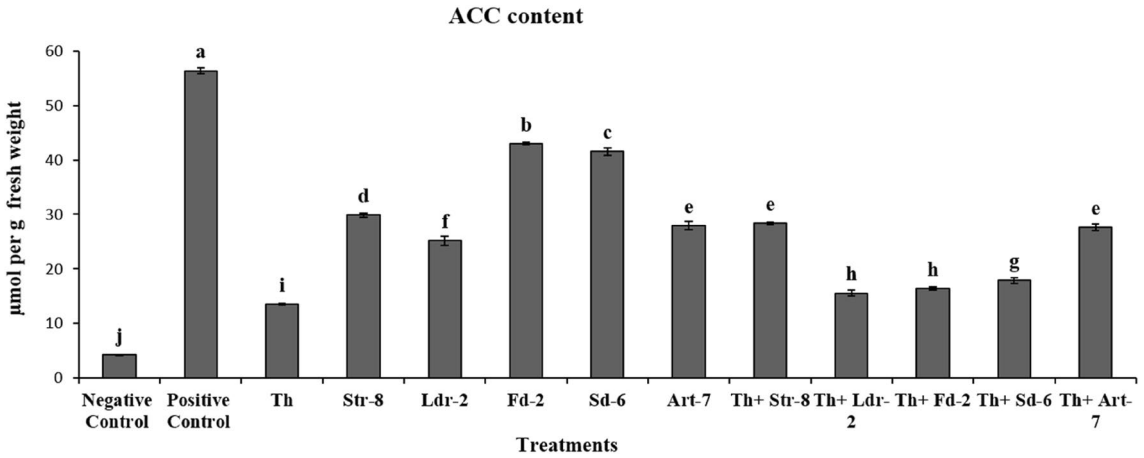

Fig. 4 Effect of bioinoculants on ACC content under salt stress in *O. sanctum*root; Th: *Trichoderma harzianum*; Sd-6: *Brevibacterium halotolerans*; Art-7: *Burkholderia cepacia;* Ldr-2: *Bacillus subtilis;* Fd-2: *Achromobacter xylosoxidans;* positive control: untreated salt-stressed plants; negative control: untreated non-salt-stressed plants; Error bars represents means ± SE (n = 4); Statistical analysis was done by ANOVA method with IBM SPSS Statistics 20 software; Different letters denote significant difference among treatments according to Duncan's Multiple Range Test ($P < 0.05$)

inoculations, total phenol content was significantly increased by all the doubly inoculated plants as compared to monocultures and positive control. Flavonoid content was also significantly enhanced by all the dual inoculations over monocultures and positive control plants (Fig. 5).

Microbial colonization analysis

TTC malate-stained roots were observed under Light microscope after cutting their section and observed that the pink color of strains detected the PGPBs existed inside the cortical section of roots (Fig. S2, supplementary file).

Multifactorial analysis

The PCA (principal component analysis) and their correlation analysis specified the relationship between measured parameters with the application of different treatments (Fig. 6). The first factor showed 46.80%, whereas the second factor represented 13.71% of the variance of data. In view of this PCA, three clusters were created in the biplot,

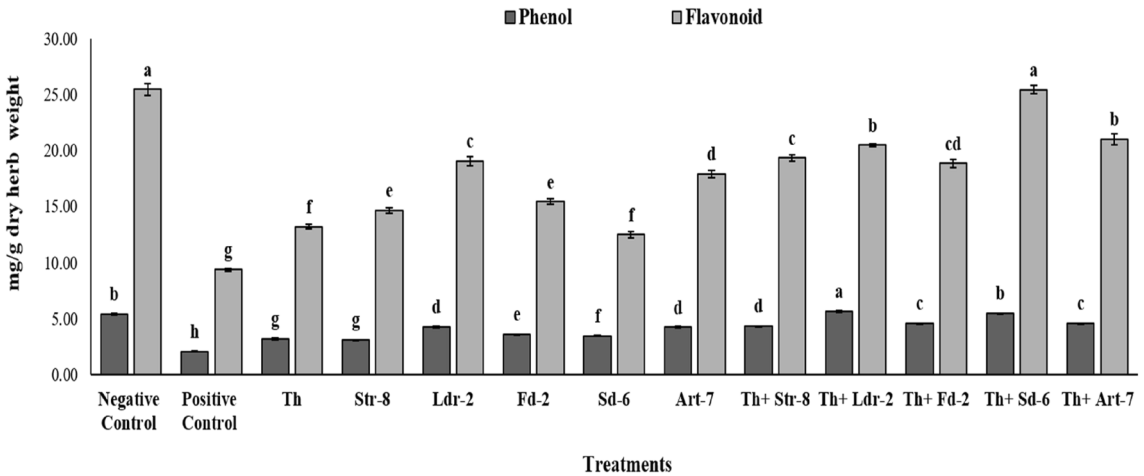

Fig. 5 Effect of bioinoculants on Phenol and Flavonoids content under salt stress in *O. sanctum*; Th: *Trichoderma harzianum*; Sd-6: *Brevibacterium halotolerans*; Art-7: *Burkholderia cepacia;* Ldr-2: *Bacillus subtilis;* Fd-2: *Achromobacter xylosoxidans;* positive control: untreated salt-stressed plants; negative control: untreated non-salt-stressed plants; Error bars represents means ± SE (n = 4); Statistical analysis was done by ANOVA method with IBM SPSS Statistics 20 software; Different letters denote significant difference among treatments according to Duncan's Multiple Range Test ($P < 0.05$)

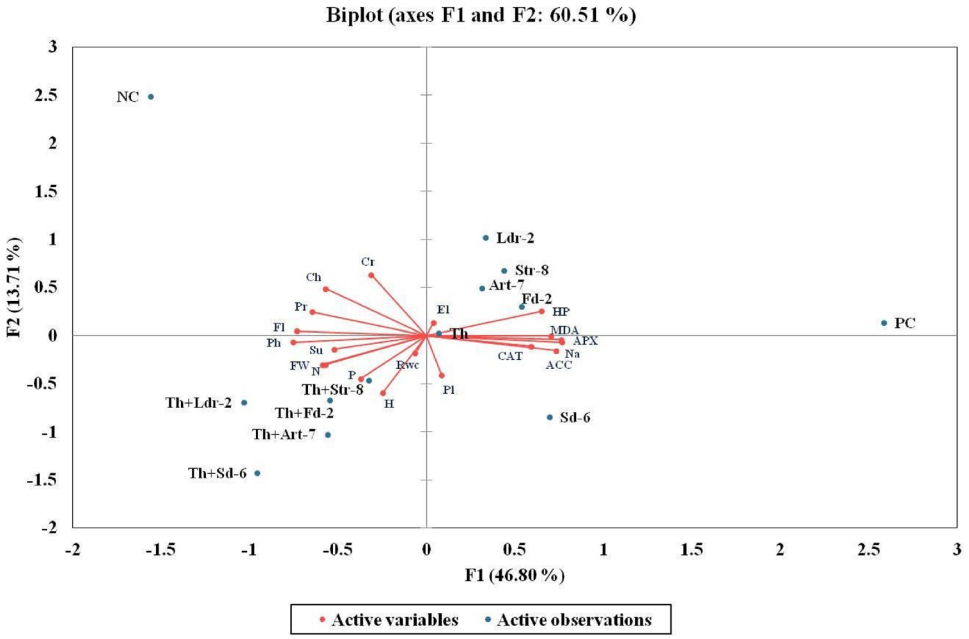

Fig. 6 Biplot showing parameters and treatments using Principal component analysis. Correlation between treatments and various parameters; Parameter codes H: Height; P: Phosphorous; FW: Fresh weight; Rwc: Relative water content; Su: Sugar; Ph: Phenol; N: nitrogen; Fl: Flavonoids; Pr: protein; Ch: Chlorophyll; Cr: Carotenoids; HP: Hydrogen peroxide; MDA: Malondialdehyde; APX: Ascorbate peroxidase; CAT: Catalase; ACC:1- amino-cyclopropane-1-carboxylic acid; EL: Electrolyte leakage; Pl: Proline; PC: Positive control; NC: negative control; Str-8: *Halomonas desiderata*; Sd-6: *Brevibacterium halotolerans*; Fd-2: *Achromobacter xylosoxidans;* Ldr2: *Bacillus subtilis;* Art-7: *Burkholderia cepacia;* Th: *Trichoderma harzianum*

which included chlorophyll, carotenoids, protein, flavonoids as one cluster. Phenols, sugars, fresh weight, nitrogen, phosphorous, relative water content, and height formed the second cluster, while electrolyte leakage, hydrogen peroxide, catalase, ascorbate peroxidase, ACC content, sodium, proline fell in the third cluster. The parameters in each cluster were closely related to each other. Similarly, treatments also had 2 clusters: cluster 1, which had Th + Ldr-2, Th + Art-7, Th + Str-8, Th + Fd-2, and Th + Sd-6, while the 2nd cluster had Ldr-2, Art-7, Str-8, Fd-2, Th, and Sd-6. However, negative and positive controls were located far-away from the other treatments showing different responses from other clusters. Based on the factorial analysis, cluster 1 and cluster 2 parameters were closely related to all the dual inoculations, while cluster 3 parameters were closely related to the monocultures.

Discussion

Microbial consortia are gaining strong acceptance by agriculturists around the globe as a possible alternative to chemical fertilizers and are thus being incorporated into important nutrient and disease management programs (Kaushal et al. 2019). However, the combination of strains/organisms with multifunctional activities like *T. harzianum* and selected PGPBs in plant growth promotion and abiotic stress alleviation has not been focused much, especially in the case of *O. sanctum* under salt stress. We previously conducted a biochemical study using these bioinoculants on *O. sanctum* plants under cold stress (Singh et al. 2020). Therefore, further efforts were made to enhance the plant growth under salt stress with combined inoculation of organisms that can reduce stress, improve the nutrient uptake and increase the yields under salinity stress in *O. sanctum*. Previous reports on the association of bacteria and fungi residing in roots, leaves, and seeds of *Withania somnifera* have been reported to improve plant growth and photosynthetic efficacy (Pandey et al. 2018). In the present study, inoculation of *T. harzianum* and PGPBs, both possessing 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activities, considerably increased the fresh herb weight, total chlorophyll, proline, total sugar, protein, phenol, and flavonoids in *O. sanctum* as compared to positive control plants under salt stress. It is suggested that the improved growth and biochemical parameters indicating stress alleviation are due to the synergistic effects of microbial partners, i.e., *T. harzianum* and PGPBs (Kushwaha et al. 2019; Singh et al. 2020). Moreover, *Trichoderma* having ACC deaminase activity has previously been reported to improve wheat growth, minimize ionic toxicity, and provide tolerance to salt stress (Zhang et al. 2019). Likewise, ACC deaminase-producing bacteria have been shown

in studies to improve plant growth during environmental stresses that increase ethylene production (Glick et al. 2007; Belimov et al. 2009; Barnawal et al. 2014, 2017).

In the present study, ACC deaminase producing *T. harzianum* and PGPBs effectively reduced electrolytic concentrations than non-treated salt-stressed plant. The lesser permeability of the plasma membrane chiefly pointed towards the integrity and constancy of cellular tissues by the microbial consortia interaction over the non-treated salt-stressed plant (Garg and Manchanda 2009). In addition, the RWC of dual inoculated plants was recorded higher than that of non-treated salt-stressed plants, indicating the positive impact of microbial consortia in reducing salt stress by fetching more water from sources available to control plants (Singh et al. 2013). Other parameters i.e., MDA, hydrogen peroxide, antioxidant enzymes (CAT, APX), and ACC accumulation, were reduced by different single and dual inoculated plants; results were more encouraging in dual inoculated *O. sanctum* plants under salt stress. For example, *B. cereus* inoculation in *Vigna radiata* decreased salt-induced oxidative damage in terms of MDA and H_2O_2 (Islam et al. 2016). In our investigation, the lower MDA and H_2O_2 level in dual inoculated plants validated the previous findings that plants were protected from salt-induced oxidative damage by various plant beneficial microorganisms (Bharti et al. 2014; Barnawal et al. 2014). The present work found that Th + Ldr-2 treated plants had the highest increase in proline content and Th + Art-7 treated plants had the highest increase in sugar content compared to positive control plants. During salt stress, osmolytes including proline, sugar, and amino acids play a key role in maintaining the water balance of cells by regulating cellular metabolism (AbdAllah et al. 2016; Agami et al. 2016).

According to one study, PGPR strains *Serratia* sp. and*Rhizobium* sp. reduced soil salinity and *Lactuca sativa* growth through modulating antioxidant enzymes, chlorophyll content, and nutrient uptake (Han and Lee 2005). Our results showed that soil salinity increased the enzyme activity in non-treated salt-stressed plant which is similar to the results of Haider et al. (2019), while PGPBs and Th inoculated plants decreased the CAT and APX enzyme activity. Previous studies have shown that microbial consortium treatment reduces CAT and APX enzymes in several plants under stress conditions (Upadhyay et al. 2012; Han and Lee 2005; Kumar et al. 2018). On the contrary, in some studies, inoculation with PGPR in plants improved enzyme activities under saline conditions (Singh et al. 2017). These studies, although valuable, are rather inexplicable. It has been observed that in plant defense mechanism, ROS does not depend merely on a few enzyme activities but on the total antioxidant defense system (Liang et al. 2003). It may be concluded that each PGPR strain utilizes its own distinct mechanism to affect different plant genotypes for alleviation of abiotic stress (Akbari et al. 2020). Another study showed a decrease in antioxidant enzyme activities in PGPR inoculated paddy, signifying a slightly lower ROS scavenging and dissimulating plant's capability, suggesting the role of PGPR in salt tolerance (Jha and Subramanian 2014).

For reducing the stress, another major mechanism used by plant growth-promoting microbes consists of minimizing ethylene levels through hydrolyzing the ACC through an enzyme ACC deaminase. It is known that certain PGPB containing ACC deaminase enzymes can convert ACC (immediate precursor of ethylene) to ammonia and α-ketobutyrate (Gamalero and Glick 2015; Raghuwanshi and Prasad 2018; Singh et al. 2015). We used selected PGPBs and a *T. harzianum* strain possessing ACC deaminase activity in the present study to improve plant growth by increasing the photosynthetic rate while decreasing xylem equilibrium pressure (Wang et al. 2016).

Nitrogen and phosphorous are essential macronutrients for plant growth as well as development, and their levels increase the plant stress tolerance (Averina et al. 2014). Another study found that a consortium consisting of *B. japonicum* and *P. putida* improved salt tolerance in soybean, facilitating better nutrient uptake in the plants (Egamberdieva et al. 2017). Our results were also on a similar line as a significant increase in nitrogen and phosphorus content was observed over positive control in doubly inoculated plants. In addition, numerous studies have reported that salt stress can increase the toxic levels of cellular Na^+ concentration in plants while restricting K^+ uptake, disturbing the intracellular ionic equilibrium. Thus, a reduction in Na^+ accumulation in plants provide tolerance to salt stress and induces plant growth (Deng et al. 2016; Chen et al. 2007). The current investigation found that Na^+ ion concentration was reduced in leaves of all single and dual inoculated plants; most significantly in Th + Ldr-2 treated plants compared to positive control. The inoculation of these strains in the *Ocimum* plant could effectively reduce salinity-induced damage due to high nutrient uptake by dual inoculated plants compared to single inoculations, which is similar to the finding of Zhou et al. (2011). This probably happened because of the substantial reduction in the content of the ACC (direct substrate for ethylene biosynthesis), thereby reducing salt-induced damage mediated by ethylene. ACC content in the individual application of Th was reduced and further decreased by all dual inoculations except Th + Art-7 compared to bacterial monocultures, which may represent the basal cellular ethylene level needs to be present for cellular homeostasis. This also suggests that other growth-promoting activities of selected microbes (like phosphate solubilization, IAA production, siderophore production, and fixation of nitrogen) play a major role in the effect found in this study. Hence, it is apparent that PGPB that produce ACC deaminase have the

potential to reduce abiotic stress-induced ethylene levels and its damaging effect on plants (Glick 2014). Another study supporting our results showed that consortium of *Aneurini bacillus aneurinilyticus* and *Paenibacillus* spp. inoculation in French beans significantly reduced ethylene levels by 60% and improved the biomass and total chlorophyll content under salt stress (Gupta and Pandey 2019).

In the current study, it has been revealed that a compatible combination of *T. harzianum* and PGPBs sustained better growth and abiotic stress tolerance compared to single strains or non-inoculated salt-stressed plants. According to the studies, it has been observed that microbial consortia play a more prominent role in salt tolerance than individual treatments. Signal transduction modulation is the mechanism that is involved in plant microbe interaction when salt stress is applied. This mechanism allows for the upregulation or downregulation of genes that are responsible for salt stress tolerance. This is demonstrated by the upregulation of the expression of transcription factor from the wrky and myb family, which is then accompanied by an increase in the expression of a number of stress-related genes, such as ABA, bHLH, bZIP, CaM, AP2EREBP (Rastogi et al. 2019). Modulation in myb and wrky transcription factor created by these microbial consortia in plants confers salt tolerance and improves growth. These transcription factors drive adaptive responses by increasing the gene expression mechanisms which encode transporters and ion channels to remove harmful ion formation in the cytosol, osmoprotectants, and antioxidant defense mechanisms to lower ROS and MDA levels (Bharti et al. 2016). In general, our findings suggest that the treatment of salt tolerance by microbial consortiums is a complex process that may include the modification of ABA signalling, the ion transporters, SOS pathway, and antioxidant machinery. To confirm the role of these transcription factors and other possible pathways in PGPBs mediated salt tolerance in plants, more evidence and data is needed.

Conclusion

The current findings of the dual culture compatibility assay, crystal violet assay, and pot study showed that all the PGPBs were compatible with Th and showed signs of their cumulative growth promotion activity in dual culture. It has been observed that microbial consortia having a syntrophic relationship put forth favourable and improved results in plant growth and secondary metabolite content. Therefore, exploring *T. harzianum* and PGPBs possessing plant growth- promoting and stress alleviation and biocontrol potential may thus play an important role in developing bioformulations useful in integrated nutrient management and stress management programs. Therefore, this study strengthens the viewpoint that the application of microbial consortia-based bio-formulations can be successfully used for increasing agricultural production, especially in salt-affected areas.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12298-023-01328-2.

Acknowledgements The author (SS) is thankful to the Department of science and technology (Ref no. DST/DISHA/SoRf-PM-016/2013) for financial support and Director, CSIR-Central Institute of Medicinal and Aromatic Plants for providing necessary facilities for the present work.

Authors contribution Designing the research SS and AK, performing the experiments, data curation, writing manuscript SS, ACC analysis CSC, Review and editing AS, Supervision AK.

Declarations

Conflict of interest The authors declare that they have no conflict of interest regarding the publication of this paper.

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