Published in final edited form as:

Sci Total Environ. 2018 December 15; 645: 721–732. doi:10.1016/j.scitotenv.2018.07.187.

Inoculation of *Brevibacterium linens* RS16 in *Oryza sativa* genotypes enhanced salinity resistance: impacts on photosynthetic traits and foliar volatile emissions

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Abstract

The emission of volatiles in response to salt stress in rice cultivars has not been studied much to date. Studies addressing the regulation of stress induced volatile emission by halotolerant plant growth promoting bacteria containing ACC (1-aminocyclopropane-1-carboxylate) deaminase are also limited. The objective of the present study was to investigate the salt alleviation potential of bacteria by regulating photosynthetic characteristics and volatile emissions in rice cultivars, and to compare the effects of the bacteria inoculation and salt responses between two rice genotypes. The interactive effects of soil salinity (0, 50, and 100 mM NaCl) and inoculation with *Brevibacterium linens* RS16 on ACC accumulation, ACC oxidase activity, carbon assimilation and stress volatile emissions after stress application were studied in the moderately salt resistant (FL478) and the salt-sensitive (IR29) rice (*Oryza sativa* L.) cultivars. It was observed that salt stress reduced foliage photosynthetic rate, but induced foliage ACC accumulation, foliage ACC oxidase activity, and the emissions of all the major classes of volatile organic compounds (VOCs) including the lipoxygenase pathway volatiles, light-weight oxygenated volatiles, long-chained saturated aldehydes, benzenoids, geranylgeranyl diphosphate pathway products, and mono- and sesquiterpenes. All these characteristics scaled up quantitatively with increasing salt stress. The effects of salt stress were more pronounced in the salt-sensitive genotype IR29 compared to the moderately salt resistant FL478 genotype. However, the bacterial inoculation significantly enhanced photosynthesis, and decreased ACC accumulation and the ACC oxidase activity, and

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Conflict of Interest Statement

None.

Author Contribution

PC and TS: The conception and design of the study; PC: acquisition of the data; PC, AK, SS, LP ÜN: analysis and interpretation of data, drafting of the article; PC, AK, TS, ÜN: critical revision of the article for important intellectual content.

VOC emissions both in control and salt-treated plants. Taken together, these results suggested that the ACC deaminase-containing *Brevibacterium linens* RS16 reduces the temporal regulation of VOC emissions and increases the plant physiological activity by reducing the availability of ethylene precursor ACC and the ACC oxidase activity under salt stress.

Keywords

ACC deaminase; ACC oxidase; *Brevibacterium linens* RS16; Ethylene; GC-MS; *Oryza sativa*; Photosystem II; Salt stress; Terpenoids; VOCs

1 Introduction

Soil salinity is one of the key abiotic stresses that affect agricultural productivity throughout the world (Munns and Tester, 2008; Hasanuzzaman et al., 2013; Albaladejo et al., 2016; Oyiga et al., 2017;). Nearly 2000 hectares of fertile agricultural land degrades each day due to salinity, resulting in 20% of total land area in the world (Qadir et al., 2014). Salinity induces stress in plants by reducing the osmotic potential of soil water. Salt stress-driven accumulation of cellular Na^+ above a threshold level in chloroplasts causes tissue damage and increases the degree of chlorosis (Flowers et al., 2015). The salinity-induced, decrease in the osmotic potential of mesophyll cells has been shown to curb photosynthetic electron transport activities, ultimately leading to a significant reduction in photosynthesis (Chaves et al., 2009; Sudhir and Murthy, 2004).

Excessive ethylene emission is one of the first signals of stress response in plants (Madhaiyan et al., 2006; Yim et al., 2013), and it is dependent on the level of salt sensitivity or salt tolerance of the plant species (Zapata et al., 2003). However, in addition to ethylene, plants emit a plethora of VOCs (volatile organic compounds) (Copolovici and Niinemets, 2010; Copolovici et al., 2012; Copolovici et al., 2014; Mostofa et al., 2015; Pazouki et al., 2016; Carriero et al., 2016), which either provide for direct defense against the stress by serving as antioxidants (Vickers et al., 2009; Possell and Loreto, 2013) or indirect defense by serving as local and systemic signaling cues for intra- and interspecific communication in the plant community (War et al., 2012). Salt stress has been observed to exhibit a strong effect on volatile emissions (Loreto and Delfine, 2000; Zuo et al., 2012; Possell and Loreto, 2013), thus monitoring the volatile emissions under salinity can provide an illuminative insight into the severity of the stress as well as the induction of various secondary metabolic pathways with the progression of stress. While several studies have investigated the effects of salinity stress on the primary metabolism of plant and on the accumulation of the non-volatile secondary metabolites, there is limited understanding regarding the effects of salinity on the release of VOCs other than ethylene. GLVs (green leaf volatiles) are synthesized from free fatty acids released by phospholipases upon damage to cell membrane due to acute stress (Matsui, 2006; Gigot et al., 2010). Emission of the GLV is a ubiquitous response to various abiotic stresses, including salt stress in *Oryza sativa* (Mostofa et al., 2015), heat and cold shock in *Solanum lycopersicum* (Copolovici et al., 2012), heat shock in *Solanum lycopersicum* (Pazouki et al., 2016), flooding in *Alnus glutinosa*, *Populus tremula*, and *Quercus rubra* (Copolovici and Niinemets, 2010), drought in *Alnus glutinosa*

(Copolovici et al., 2014), and ozone in *Betula pendula* (Carriero et al., 2016). Another class of plant VOCs, monoterpenes are biosynthesized in plastids through 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate pathway (MEP/DOXP pathway), whereas sesquiterpenes are biosynthesized in cytosol via mevalonate-dependent pathway (Rajabi Memari et al., 2013; Rosenkranz and Schnitzler, 2013). Monoterpene synthesis primarily depends on a continuous supply of NADPH and ATP generated via photosynthetic electron transport chain (Niinemets et al., 2002; Rasulov et al., 2016). Other than monoterpenes and sesquiterpenes, stress is also generally associated with the emissions of short-chained oxygenated volatile organic compounds (OVOCs), long-chained saturated aldehydes, benzenoids, and geranylgeranyl diphosphate (GGDP) pathway products (Kask et al., 2016; Kanagendran et al., 2017; Kanagendran et al., 2018).

Rice (*Oryza sativa* L.) is cultivated through warm temperate to tropical regions and is considered as staple food in Asia. Rice is generally, sensitive to salinity, which leads to a reduction in the growth, grain yield and development (Asch and Wopereis, 2001; Zeng et al., 2001). In recent years, various approaches (Deinlein et al., 2014) have been undertaken to assist plants to withstand salinity stress including the development of salt tolerant transgenic plants and also using plant growth-promoting bacteria (PGPB) to increase salinity tolerance in plants. Bacteria that contain 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase) have been demonstrated to decrease the emission of the plant stress hormone ethylene by reducing the level of ethylene precursor ACC, breaking it down to α -ketobutyrate and ammonia (Glick, 2014; Singh et al., 2015; Chatterjee et al., 2017). So far, only a small number of studies have been conducted to investigate the effects of PGPB on volatile emissions in stressed plants (Timmusk et al., 2014; Matsuoka et al., 2016). The mechanism through which a decrease in the ACC level leads to improved stress resistance is still not entirely understood. It is thought that the stress-dependent ethylene in plants occurs in two distinct peaks, one rapid ethylene burst that relies on an endogenous pool of ACC, followed a second longer-term, and typically higher emission burst that requires de novo synthesis of ACC (Pierik et al., 2006; Yim et al., 2013; Glick, 2014). Thus, suppression of the second ethylene emission burst by ACC deaminase-producing bacteria is suggested to be responsible for the improved stress resistance (Yim et al., 2013). So far, there is no information of how salt stress can effect volatile emissions in rice and how bacteria priming can change these emissions under salt stress. It is proposed that monitoring the VOC emission responses throughout the stress period, may support the potential action of the ACC deaminase-containing PGPB.

In the present study, two rice cultivars IR29 (salt-sensitive) and FL478 (moderately salt resistant) were used, to analyze the quantitative responses of carbon assimilation and stress volatile emissions to the combined effects of salinity and inoculation by the halotolerant PGPB *Brevibacterium linens* RS16, which was isolated from coastal soils in South Korea in a study, that aimed at isolating PGPBs to tolerate salt stress (Siddikee et al., 2010). This isolate can grow even at very salty conditions, > 1.7 M NaCl, and has been shown to possess ACC deaminase activity and support plant growth under saline condition (Siddikee et al., 2011; Chatterjee et al., 2018).

In the present study, different concentrations of NaCl were applied in the potting mixture with and without inoculation with *B. linens* RS16 and examined (1) whether the bacterial inoculation improves foliage photosynthetic characteristics throughout the salt-stressed period; (2) how foliage stress volatile emissions respond to individual effects of NaCl and bacterial inoculation, and to the interactive effect of NaCl and bacteria after application of salt stress, until 10th day of the experiment; (3) how salt stress responses of photosynthetic characteristics and volatile emissions and the effect of inoculation with PGPB differ among two rice genotypes with varying degree of salt resistance. It was hypothesized that (1) salt stress affects foliage photosynthetic characteristics and causes the elicitation of stress volatile emissions in rice and all these modifications are more pronounced in the salt-sensitive rice genotype. (2) PGPB inoculation helped in the improvement of foliage photosynthetic characteristics and reduced volatile emissions by regulating the ethylene precursor and the ACC oxidase activity.

2 Materials and methods

2.1 Plant materials and growth conditions

Moderately salt resistant (FL478) and salt sensitive (IR29) rice cultivars (*O. sativa* L.) developed at the International Rice Research Institute (IRRI, Manila, Philippines) and maintained by Rural Development Administration (RDA, South Korea) were used in this study. Rice seeds were surface sterilized for 20 min using 1 % solution of sodium hypochlorite (NaClO) followed by washes in sterile distilled water (250 ml per wash), and dried overnight in a laminar flow. The seeds were evenly placed on moistened paper towel containing Petri dishes (max. ~ fifty seeds per petri dish) for germination (Lee et al., 2006). Initially, the seeds were incubated in the dark for three days to facilitate germination. Subsequently, the seeds were transferred to a Percival AR-95 HIL growth chamber (CLF Plant Climatics GmbH, Wertingen, Germany) and the following environmental conditions were maintained for a week until germination: light intensity of 300-400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 12 h photoperiod, day and night temperatures of 22/18 °C, and relative air humidity of 60%. One week later, the germinated seedlings were transplanted into 350 ml pots (One seedling was transplanted into each pot) filled with commercial potting mixture (Biolan Oy, Kauttua, Finland). The potted soil was fertilized with NPK (5:5:6), and essential micronutrients [B (0.01%), Cu (0.03%), Fe (0.06%), Mn (0.028%), Zn (0.007%)]. The plants were watered to soil field capacity. One week old seedlings were moved to a plant growth room, where they were grown under the light intensity of 400-500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by HPI-T Plus 400 W metal halide lamps (Philips) for 12 h photoperiod, day / night temperature of 28/25 °C, ambient CO₂ concentration of 380-400 ppm, and relative air humidity of 60-70% until the completion of the experiment. 21-day-old seedlings were used for the experiment.

We used six treatments (0 mM, 0 mM + Bacteria, 50 mM, 50 mM + Bacteria, 100 mM, 100 mM + Bacteria), three time points (1, 5, and 10 days), three individual plants as replicates (for each time point, treatment and cultivar). As, we used two cultivars so total 108 individual plants were used in this study.

2.2 Bacterial inoculation

The halotolerant *B. linens* RS16 (Genbank accession no. GU968457) was used in this study. The bacterial suspension was prepared as mentioned in Chatterjee et al. (2017). Bacterial inoculation in the soil was carried out at 7 and 14 days after sowing the rice seeds by adding 10 ml of the bacterial suspension (prepared using 0.03M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) (10^8 CFU ml^{-1}) (Siddikee et al., 2011; Siddikee et al., 2012) near to the root zone of each potted plant and also spraying the suspension over the entire foliage of plants to form a uniform layer on above-ground plant parts (Esitken et al., 2003; Esitken et al., 2006; Esitken et al., 2010). The non-inoculated plants were treated with sterile 0.03M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (carrier solution) at the time of inoculation.

2.3 Salt stress application

Two levels of salt stress, 50 mM and 100 mM were separately applied. In short, 21-day-old rice plants were treated with 10 ml of 50 mM of NaCl solution, which was applied at once. In the case of 100 mM NaCl treatment, 5 ml of 100 mM salt was applied twice in one day interval to prevent an instant osmotic shock. Photosynthesis and volatile emission measurements were started after reaching the desired salt concentration in each case separately (as we applied 50 mM once and 100 mM in interval, so the 1, 5, 10th days are different in each case). Untreated and bacteria treated control plants were well watered throughout the treatment period.

2.4 Leaf gas-exchange and volatile measurement setup

A 1.2l cylindrical doubled-walled glass chamber with glass and stainless steel lower part was used for gas-exchange measurements and volatile sampling. A circulating water bath, was used to control the temperature of water that circulated continuously between the chamber walls. The temperature of the chamber air was maintained at 25 °C and constantly monitored by a thermistor (NTC thermistor, model ACC-001, RTI Electronics, Inc., St. Anaheim, CA, USA), while the leaf temperature was monitored by a thermocouple attached to the lower surface of a representative leaf. Light was provided by four 50 W halogen lamps, and light intensity at the leaf surface was set at 700-800 $\mu\text{mol m}^{-2} \text{s}^{-1}$. A fan enclosed in the chamber was used to maintain high air turbulence to minimize leaf boundary layer resistance. A charcoal-filtered ozone-free ambient air (CO_2 concentration $380 \pm 20 \mu\text{mol mol}^{-1}$) was supplied to the chamber. The measurements were conducted at an air humidity of 60-70% and the air was humidified whenever required using a custom-made humidifier.

All tubing, and connections to and from the glass chamber were made of Teflon[®] and stainless steel. Low-emitting modeling clay was used to seal the site of leaf enclosure. The chamber ports were switched between the reference (ingoing air) and measurement (outgoing air) modes to take gas-exchange measurements. Changes in CO_2 and H_2O concentrations between the reference and the measurement modes of chamber were measured by an infrared dual-channel gas analyzer operated in differential mode (CIRAS II, PP-systems, Amesbury, MA, USA). Further details regarding the gas-exchange system are available in (Copolovici and Niinemets, 2010).

A set of randomly selected fully mature leaves, (ca. 18-25 cm² leaf area) was used for the measurements. Following the hermetic enclosure of the leaves into the glass-chamber, standard environmental conditions were established and the leaves were stabilized for ca. 15-20 min in order to achieve steady-state gas-exchange rates and Gas-exchange and VOC emission measurements were conducted at days 1, 5 and 10 after stress application. Once steady-state gas-exchange rates were achieved, net assimilation and transpiration rate were recorded. The leaves enclosed in the gas-exchange chamber were scanned at 300 dpi to estimate the leaf area using a custom-made software tool. Foliage net assimilation rate (A) and stomatal conductance to water vapour (g_s) were calculated according to von Caemmerer and Farquhar (1981).

2.5 Chlorophyll fluorescence measurements

After gas-exchange measurements and VOC collection, the leaves were removed from the chamber and the dark-adapted chlorophyll fluorescence yield (10 min darkening, F_v/F_m) was measured with an imaging PAM fluorometer (Walz IMAG-MIN/B, Walz GmbH, Effeltrich, Germany). For F_m measurements, blue light (460 nm) was used for saturating light pulses (7000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 1 s).

2.6 VOC sampling, and gas chromatography-mass spectrometry (GC-MS) analysis

VOC sampling was carried out using multi-bed stainless steel adsorbent cartridges filled with a series of Carbotrap adsorbents to quantitatively adsorb all C3-C18 volatiles (Copolovici et al., 2009; Kännaste et al., 2014 for details). The sampling was conducted at a constant air suction rate of 200 ml min⁻¹ for 20 min using a portable suction pump 210-1003 MTX (SKC Inc., Houston, TX, USA). Blank samples were taken from the empty chamber before the collection of leaf-emitted VOC to estimate net leaf VOC emission.

VOC-filled adsorbent cartridges were analyzed with a combined Shimadzu TD20 automated cartridge desorber and Shimadzu 2010 Plus GC-MS system (Shimadzu Corporation, Kyoto, Japan) with Zebron ZB-624 fused silica capillary column (0.32 mm i.d. $\times$ 60 m, 1.8 μm film thickness), (Phenomenex, Torrance, CA, USA) for benzenoids, GGDP, GLV, OVOC, monoterpenes, and sesquiterpenes (Table S1). The compounds were identified based on authentic standards (GC purity, Sigma-Aldrich, St. Louis, MO, USA) and NIST 05 spectral library (see Copolovici et al., 2009; Niinemets et al., 2011; Kännaste et al., 2014) for detailed information about volatile sampling and GC-MS analysis). The emission rates were calculated as described by Niinemets et al. (2011).

2.7 Estimation of foliar ACC accumulation and ACC oxidase activity in terms of ethylene emission in rice cultivars

ACC accumulation and ACC oxidase activity were quantitatively estimated from leaves of *Oryza sativa* L. cv. FL478 and IR29. (Lizada and Yang, 1979; Madhaiyan et al., 2006; Yim et al., 2013).

One gram of fresh leaves was ground using liquid nitrogen. ACC was carefully extracted from the frozen leaf sample in 5 ml of 80% methanol containing butylated hydroxyl toluene as an antioxidant (BHT, 2 mg l⁻¹) and incubated for 45 min. The sample was centrifuged at

2000 *g* for 15 min. The pellet obtained was re-suspended in 4 ml of methanol and centrifuged again under the same conditions. The combined supernatant was dried using rotary vacuum evaporator (Eyela world, Tokyo, Japan).

The collected residues were re-suspended in 2 ml of distilled water and the upper aqueous layer was extracted with dichloromethane mixed with 0.1 ml HgCl₂ (80 mM) in test tube and then the tube was sealed using a rubber septum. 0.2 ml of NaOCl solution (40 ml NaOH, 80 ml NaOCl solution (12.5%), and 30 ml distilled water) was injected carefully into each test tube, shaken and incubated for 8 min. After incubation, one ml of gas was taken and estimated for ethylene emission with a gas chromatograph with Poropak-Q column (dsCHROM 6200, Donam Instruments Inc., Republic of Korea). The ACC accumulation was evaluated carefully by calculating the replicate samples containing internal standards of ACC (Yim et al., 2013).

The ACC oxidase (ACO) activity was assessed from the ground leaf sample, homogenized in 2 ml g⁻¹ of extraction buffer (consisting of 100 mM Tris-HCl (pH 7.2), 10% (w/v) glycerol, and 30 mM sodium ascorbate), then centrifuged at 25704 *g* for 15 min at 4 °C. The ACO enzyme activity was estimated from 1.5 ml of supernatant in 50 mM FeSO₄, 1 mM ACC, and 5% (v/v) CO₂ in 10 ml screw cap tubes fitted with a Teflon® coated septum. The sample was incubated at 30 °C for 15 min and the amount of ethylene released into the headspace was estimated by gas chromatograph (Yim et al., 2013).

2.8 Data analysis

Data were tested for normality of distribution and homogeneity of variance and then the data were log-transformed. Generalized linear model (GLM) based on maximum likelihood model fitting was used to analyze the statistical impact for individual and interactive effects of salt, bacteria, and time on photosynthesis characteristics, volatile emissions, ACC accumulation, and ACO activity. GLM was performed in SPSS v.24 (IBM SPSS, Chicago, IL, USA). Data plots were made using SigmaPlot Version 12.5 (Systat Software Inc, San Jose, CA, USA). All statistical effects were considered significant at $P < 0.05$. Correlation matrix among Na⁺/K⁺ ratio, total volatile emission rates, ACO activity and ACC accumulation in both the rice cultivars was generated using (corrplot) R-package (Wei and Simko, 2013). We used four treatments: 0 mM, 0 mM + Bacteria, 100 mM, 100 mM + Bacteria, and two time points (1 and 10 days) for each rice cultivar. All statistical effects were considered significant at $P < 0.05$ (Figure S1).

3 Results

3.1 Effects of salt stress and bacterial inoculation on foliage photosynthetic characteristics

The maximum dark-adapted photosystem II (PS II) quantum yield estimated by chlorophyll fluorescence (F_v/F_m) decreased in both 50 mM and 100 mM NaCl, compared to controls, and the reduction progressed throughout the salt stress period (Figs 1A and D). The reduction in F_v/F_m was greater for the higher NaCl treatment concentration and in salt-

sensitive cultivar IR29 compared to FL478 (Figs 1A and D). F_v/F_m in plants inoculated with bacteria was not significantly affected by the salt treatment (Figs 1A and D).

Salt stress also reduced the net assimilation rate A , (Figs 1B and E). The bacteria inoculated plants nearly followed the same trend of A as salt stressed FL478 (Fig. 1B) at 10th day since the application of salt stress, but bacteria treatment, enhanced A in untreated and salt treated IR29 (Fig. 1E). The reduction of A and stomatal conductance to water vapour (g_s) (Figs 1C and F) was more pronounced for IR29 throughout the salt-stressed period than for FL478. In FL478, bacterial inoculation improved salt resistance of g_s at the beginning of the salt addition, while in IR29, bacterial inoculation improved g_s of salt-stressed plants in the beginning and at the end of the salt-stressed period (1st day and 10th day since the salt application) (Figs 1C and F; Table S2).

3.2 Effects of salt and bacterial on green leaf volatile (GLV) emission

Through the experiment, no classic unsaturated GLV volatiles and their derivatives such as (*E*)-2-hexenal, (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate were observed, and the emissions of GLV were dominated by hexanal, followed by pentanal in both cultivars in response to all treatments throughout the salt stress period (Figs 2A and 2D; Table S1). Control leaves of FL478 and IR29 emitted total GLV at relatively low levels of ca. $0.017 \pm 0.007 \text{ nmol m}^{-2} \text{ s}^{-1}$ and $0.024 \pm 0.021 \text{ nmol m}^{-2} \text{ s}^{-1}$ respectively at day 1 since the application of salt stress (Figs 2A and D).

The salt treatments resulted in a burst of total GLV emission in a salt stress severity-dependent manner (Figs 2A and D). In non-inoculated plants, once elicited, the GLV emissions continued to remain at high levels, and even increased at the 10th day since the application of the salt stress (Figs 2A, 2D, and Tables S1, S3). The effect of salt, the combined effect of salt and bacteria, and time on total GLV emission were statistically significant ($P < 0.001$), (Table S3). However, the salt-sensitive cultivar IR29 exhibited a higher emission of GLV than FL478 upon salt stress, and the effect of bacterial inoculation on suppression of GLV emission was relatively greater for IR29 for 50 mM salt treatment (average $\pm$ SE through all salt-stress period of 78% in IR29 vs. 30% for FL478), while the suppression was similar for 100 mM salinity treatment (78% throughout the salt-stressed phase in IR29 vs. 74% for FL478); (Figs 2A, 2D).

3.3 Effects of salt and bacteria on mono- and sesquiterpene emissions

Both the rice cultivars were low-level constitutive monoterpene emitters under non-stressed conditions with the average $\pm$ SE emissions of $0.14 \pm 0.07 \text{ nmol m}^{-2} \text{ s}^{-1}$ for FL478 and $0.10 \pm 0.06 \text{ nmol m}^{-2} \text{ s}^{-1}$ for IR29 (Figs 2B and E; Table S1). The individual monoterpenes detected were α -pinene, β -myrcene, β -phellandrene, β -pinene, camphene, limonene, β -carene and 1, 8-cineole in both rice cultivars (Table S1). Monoterpene emission in FL478 was dominated by α -pinene followed by 1, 8-cineole, while in IR29, it was dominated by 1, 8-cineole followed by β -carene and α -pinene.

The 100 mM NaCl treatment, led to a major increase in the monoterpene emission rates in both the cultivars, and was higher in the IR29 ($1.2 \pm 0.7 \text{ nmol m}^{-2} \text{ s}^{-1}$ at day 1 since the application of salt stress) than FL478 ($0.32 \pm 0.17 \text{ nmol m}^{-2} \text{ s}^{-1}$; (Figs 2B and E)). In all

cases, the salt-treated plants inoculated with bacteria emitted lower amounts of total and individual monoterpenes than non-inoculated plants (Figs 2B, 2E, and Table S1), and thus, in both cultivars, effects of salt, bacteria, and salt and bacteria treatments had a statistically significant impact on total monoterpene emission rates (Table S3).

The emissions of sesquiterpenes was very low in non-stressed plants, especially in the bacteria-treated plants (Figs 2C, 2F, and Table S1). In the non-stressed plants, only (*E*)- β -caryophyllene emissions were observed, while under the severe salt stress of 100 mM NaCl emissions of two other sesquiterpenes longifolene and viridiflorene (Table S1) were observed in both the cultivars. Sesquiterpene emissions in the bacteria-inoculated salt-stressed plants were strongly reduced in both the cultivars compared to the non-inoculated salt-stressed plants, throughout the salt-stress period (Table S3). In summary, the salt treatment, the combined treatment of salt and bacteria, and the treatment with bacteria alone, as well as the time after stress application all significantly altered the total sesquiterpene emission rate ($P < 0.001$; Table S3).

3.4 Emission rates of saturated aldehydes, OVOCs, GGDP volatiles, and benzenoids in response to salt and bacterial inoculation

Emissions of long-chain saturated aldehydes were dominated by nonanal, followed by decanal for FL478, while for IR29, they were dominated by decanal, followed by octanal (Table S1). The emissions of saturated aldehydes were enhanced by salt stress in both the cultivars, although the elicitation was much stronger for the IR29 (Figs 3A and C). Bacterial inoculation resulted in reduced saturated aldehyde emissions in the both cultivars, but the bacterial effect was much stronger for salt-sensitive cultivar IR29 (Figs 3A and C). The total saturated aldehyde emissions were significantly affected by salt, combined treatment of salt and bacteria, bacteria alone, and time (Table S3).

Emissions of OVOCs were dominated by acetone (contributing ca. 80 % of total emission rate), followed by acetaldehyde in both cultivars (Table S1). The emissions of OVOC were significantly enhanced upon salt stress, and the emissions were enhanced to a greater degree in the salt-sensitive cultivar IR29 (Figs 3B, 3D; Tables S1 and S3). The salt-stressed plants inoculated with bacteria exhibited lower emission rates in most cases, except the 50 mM treatment at day 1 and 5 since the application of salt stress (Figs 3B, 3D).

The volatile products of the GGDP pathway, (*E*)-geranyl acetone, and 6-methyl-5-hepten-2-one, were emitted from the leaves of both the cultivars, whereas (*E*)-geranyl acetone was the dominant volatile, constituting ca. 60 % of the total GGDP pathway volatiles (Fig. 4A and C; Table S1). The salt treatments enhanced the emission rate of the GGDP pathway volatiles, but the elicitation of emissions was moderate until day 10, since the application of salt stress, especially in more salt-resistant cultivar FL478 (Fig. 4A and C). In both non-inoculated and bacteria treated salt-stressed plants, the emission rate of total and individual GGDP pathway volatiles was lower for the FL478 than that for the IR29 in both salt treatments throughout the salt-stressed phase (Figs 4A, 4C; Tables S1, S3).

The benzenoids detected in the emissions were benzaldehyde, *p*-xylene and *o*-xylene, and the emissions were dominated by benzaldehyde followed by *p*-xylene (Table S1). Salt stress

enhanced the benzenoid emissions in both cultivars, and again more strongly in the salt-sensitive cultivar IR29 (Figs 4B and D; Table S3). Once elicited the total benzenoid emissions remained at a similar level in most of the cases (Figs 4B and D). However, individual compounds, benzaldehyde (Table S1) and *o*-xylene (Table S1) increased with time since treatment in salt-stressed plants for IR29. Salt-stressed plants inoculated with bacteria exhibited lower total benzenoid emission rates in most cases throughout the salt stress period, except the 50 mM treatment for FL478 in the beginning of the salt stress (Fig. 4B).

Overall, analogous emission patterns of total and individual benzenoids was observed as OVOC and GGDP product emission in response to all treatments. The lowest emission of total saturated aldehydes, OVOC, GGDP, and benzenoids was observed when the leaves treated with bacteria (Figs 3 and 4).

3.5 Changes in ACC quantity and ACO activity in response to salt stress and bacterial inoculation

In both the cultivars, treatment with 50 and 100 mM NaCl exhibited enhanced ACC accumulation and ACO activity compared to the control plants throughout the salt-stress period, whereas ethylene emission (as we measured ACC accumulation and ACO activity with respect to ethylene emission) was greater in salt-sensitive cultivar IR29 than in salt-resistant cultivar FL478 (Fig. 5). However, bacterial inoculation decreased the ACC accumulation and ACO activity in time dependent manner both in control and salt treated plants with a progressive reduction observed in salt-treated plants (Fig. 5; Table S3). In addition, the effect of salt, salt-bacteria and time significantly altered the ACC accumulation and ACO activity since the application of treatments ($P < 0.001$) (Table S3).

4 Discussion

4.1 Foliage photosynthetic characteristics of *O. sativa* in response to salinity stress: general patterns

Since enhanced salinity results in a lower physiological soil water availability, it also determines the difference between salt tolerant and salt sensitive cultivars on the basis of their rate of Na^+ and Cl^- ion transport to the leaves (Munns, 2002). Nevertheless, once taken up, high salt concentrations can disrupt cellular ionic homeostasis and negatively impact the rate of key physiological processes (Munns, 2002). The reduction of F_v/F_m in salt-stressed plants is a characteristic stress response and is typically associated with damage at PS II leading to sustained salt stress dose-dependent photoinhibition as observed in previous studies in several crop species such as *Gossypium hirsutum* (Brugnoli and Björkman, 1992), *Sorghum bicolor* (Netondo et al., 2004), and *Helianthus annuus* (Santos, 2004).

Reduction in leaf photochemical activity is often also accompanied by suppression of Calvin cycle and ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) enzyme activity, ultimately resulting in reduced whole leaf photosynthesis rate (Abogadallah and Quick, 2009; Shu et al., 2014) as observed in both the rice cultivars (Figs 1B and E). Salinity stress lowers the osmotic potential, which further reduces the water uptake in salt affected plants,

resulting in reductions in stomatal conductance (Bongi and Loreto, 1989; Romero-Aranda et al., 2001; Flexas et al., 2004), which induces closure of stomata due to decreased water availability and in turn leads to reduced photosynthesis (Mayak et al., 2004). In the present study, stomatal conductance was reduced upon salt stress (Figs 1C and F), partly explaining the reduction in net assimilation rate in both cultivars. Once the reduction in foliage photosynthetic activity was triggered by salinity stress, the reduction continued further in our study (Fig. 1). Such time-dependent after-stress effects have been observed previously in response to severe heat (Hüve et al., 2011), ozone stress (Beauchamp et al., 2005; Kanagendran et al., 2017; Kanagendran et al., 2018), and have been associated with the generation of an oxidative burst in the severely stressed leaves.

Inoculation of rice plants with ACC deaminase-containing *Brevibacterium linens* RS16 alleviated the salt-stress effects on foliage photosynthetic characteristics in our study with especially large improvements observed for net assimilation rate in the salt-sensitive cultivar IR29 (Fig. 1). Maintenance of high level of F_v/F_m in bacterially-inoculated salt-stressed plants suggests that the integrity of the photosynthetic apparatus was maintained may be via controlling the water use efficiency (Mayak et al., 2004) by bacteria. The enhancement of the photosynthetic traits in salt-stressed plants inoculated with *B. linens* RS16 is further associated with inhibited ethylene emission. In addition, *B. linens* RS16 has exhibited its potential to reduce the uptake of Na^+ ions by the roots in red pepper seedlings (Siddiquee et al., 2011). The reduction in the Na^+ uptake is likely associated with the ability of the bacteria to bind Na^+ with surface polysaccharides (Siddiquee et al., 2011), since the sequestration of Na^+ ions into the vacuole is also a key strategy for adjusting the osmotic potential (Shabala, 2013) (Fig. S1; Table S4 and S5).

4.2 Elicitation of foliage green leaf volatiles (GLV) under salinity stress and bacterial inoculation

Typical abiotic stress leads to the generation of reactive oxygen species (ROS), through the modifications in cellular, biochemical and gene expressions, as result of stress response (Doke, 1997; Grant et al., 2000; Wohlgemuth et al., 2002).

ROS accumulation in the salt stressed cells leads to lipid peroxidation, which is considered as an early indicator of salt-induced oxidative damage to the membranes, resulting in the production of GLVs (Mostofa et al., 2015). However, in the present study, the iconic GLVs usually released in response to acute abiotic stress are (*E*)-2-hexenal, (*Z*)-3-hexenol, 1-hexanol, and (*Z*)-3-hexenyl acetate (Heiden et al., 2003; Copolovici and Niinemets, 2010; Copolovici et al., 2012) were not observed even at higher concentration of salt stress (Table S1). As these volatiles are typically associated with the immediate stress response, it might be that we have missed these emissions due to low sampling frequency (Jiang et al., 2017 for high resolution measurements).

The dominant GLV in our study, hexanal, is produced as a result of oxidative cleavage of membrane lipids by lipoxygenase and hydroperoxide lyase enzymes (Heiden et al., 2003), suggesting that leaf oxidative status was enhanced upon salt stress. Hexanal emission was also dominated in GLV emissions upon heat shock in *Solanum lycopersicum* (Pazouki et al., 2016) and ozone stress in *Betula pendula* (Carriero et al., 2016), which are similar to our

study. Higher total GLV emission rate was observed in salt sensitive cultivar IR29 than in moderately salt resistant cultivar FL478 (Figs 2A and D), suggesting that the increase in the oxidative status was comparatively higher in IR29 than FL478.

Usually, GLV emission responses are transient and rapidly decay over time, but in our study, GLV emissions increased throughout the salt-stressed period (Figs 2A and D) and the emission was quantitatively related to the severity of stress (Table S1). In accordance with the hypothesis that salt stress aftereffects are associated with enhanced leaf oxidative status. Substantially reduced time-dependent increase in GLV emissions was observed in salt-stressed plants inoculated with *B. linens* RS16. As suggested above, this can be directly associated with a reduction in ethylene formation (Siddique et al., 2012) during the stress period, and accordingly lack or reduction of endogenous ROS formation in inoculated plants, but the mechanism of this occurrence deserves further study.

4.3 Emission of foliage mono- and sesquiterpenes under salinity stress and bacterial inoculation

Constitutive emissions of mono- and sesquiterpenes from the control leaves may be attributed to the release of terpenoids from the non-specific storage pools in the rice leaves (Hinge et al., 2016), or from the storage in the lipid- phase of the plant cells (Niinemets and Reichstein, 2002) (Table S1). The changes in terpene emissions upon stress might reflect both substrate and gene expression level controls (Copolovici and Niinemets, 2016). However, plant inoculation by *B. linens* RS16 generally increased foliage net assimilation rate in salt-stressed and unstressed plants, but total mono- and sesquiterpene emissions were decreased compared with control. Thus, it is unlikely that the rise in terpene emissions was due to substrate level control, but rather suggests a gene expression level control. However, in the case of oxygenated monoterpene, 1,8-cineole, emission, we cannot rule out the possibility that the 1,8-cineole emission is also controlled by stomatal conductance in our study (Niinemets et al., 2002).

However, this study showed that severity salt stress proportionately increased the terpene emissions in time dependent manner. This temporal emission pattern contradicted terpene emission responses in *Fagus sylvatica* recovering from drought (Šimpraga et al., 2011), in their study, time-dependent reduction of terpenoid emission was observed through the entire recovery phase. Furthermore, in our study, the sustained terpene emission is in accordance with enhanced GLV emission (Fig. 2) and again suggests progression of oxidative stress.

B. linens RS16 inoculation reduced the terpene emission in salt stressed plants, which is further in line with the inhibited ethylene emission. However, there is a rich body of evidence indicating that ethylene emission is directly associated with elicitation of volatiles, including the start of release of terpenoids. For example, Ruther and Kleier (2005) observed that ethylene applied in combination with (Z)-3-hexen-1-ol synergistically enhanced terpene emission in *Zea mays*. Even, the emission of terpenoids was associated with increased ethylene emission in *Medicago truncatula* after beet armyworm (*Spodoptera exigua*) infestation (Arimura et al., 2002). The ethylene emission responses may be regulated at ACC oxidase gene-level, which catalyzes the biosynthesis of ethylene (Arimura et al., 2002; Roeder et al., 2009). In addition, suppression of ethylene biosynthesis pathway showed

reduced terpene emission in transgenic apples (*Malus domestica*) (Defilippi et al., 2005). Furthermore, *Nilaparvata lugens* (brown planthopper (insects feeding on plants)) treatment in *O. sativa* plants had a profound effect on the augmentation of both ethylene signaling and volatile emission (Lu et al., 2006). Further studies addressing the gene expression level controls of the DOXP/MEP and MVA pathway volatiles in response to elevated salt concentration and bacterial inoculation are necessary.

4.4 Induction of other stress-specific compounds in response to salt stress and bacterial inoculation and overall changes in the emission blend

Salinity stress led to enhanced emissions of saturated aldehydes, OVOCs, GGDP pathway volatiles and benzenoids, and these emissions showed dose-dependent relationships in both cultivars.

The emissions of decanal, heptanal, nonanal and octanal were observed under long-chain saturated aldehyde (~C10) category. Though the true biosynthesis pathway is still unknown, but the saturated aldehyde emission might be connected with lipoxygenase or hydroperoxide lyase pathway products (Stenzel et al., 2003) (Figs 3A, 3C). Similar to our findings, the enhanced emission of saturated aldehydes from six different plant species was observed after ozone exposure or pathogen attack Heiden et al. (2003), heat stress (Kask et al., 2016) and methyl jasmonate exposure (Jiang et al., 2017). In all these studies, the enhancement of saturated aldehydes was also associated with enhanced GLV emissions (Feussner and Wasternack 2003).

There were two key volatile compounds observed under the OVOC category acetaldehyde and acetone (Figs 3B, 3D, and Table S1). The biosynthesis of acetaldehyde and acetone, possibly associated with the cellular quenching of ROS through lipid peroxidation (Jardine et al., 2008). In addition, Blokhina et al. (2003) mentioned that acetaldehyde is produced due to oxidative damage in cell membrane, primarily due to oxidative reaction of lipoxygenase enzyme with C18 fatty acids present in cell membranes and acetone emission has been observed under abiotic stresses (Hasanuzzaman et al., 2017). Biosynthesis of the GGDP pathway products might be associated with the oxidative cleavage of the carotenoid pathway products (Kask et al., 2016; Dudareva et al., 2013) or via three prenyl transferases (Schwab et al., 2008) (Figs 4A, C, and Table S1). In this study, GGDP pathway volatiles were significantly enhanced at elevated salt treatments, the entire carotenoid pathway might be responsible for the enhanced emission, which became activated upon salinity stress leading to the production of α - and β -carotene and abscisic acid, a key plant hormone increasing plant tolerance to different abiotic stresses. This is supported by findings that salt stress enhanced carotenoid contents in tomato genotypes (Borghesi et al., 2011).

A substantial quantity of benzenoid emission was dominated by benzaldehyde (Figs 4B, D, and Table S1). Basically, benzenoids are primarily derived from shikimic acid pathway (Pichersky et al., 2006; Yang et al., 2009) and are considered as common stress volatiles under biotic or abiotic stresses (Gentner et al., 2014). Schuurink et al. (2006) demonstrated that benzenoid emission is strongly regulated by ethylene biosynthesis in petunia.

4.5 Impact of *B. linens* RS16 inoculation on enhanced salinity resistance in rice cultivars

The present study was primarily based on a previous study on the ACC deaminase gene transformation, where the ACC deaminase-negative psychrotolerant bacterial strains *Flavobacterium* sp. OR306 and *Pseudomonas frederiksbergensis* OS211 were used to demonstrate that the bacterial ACC deaminase decreases ethylene emission and alleviates stress in tomato plants under cold stress conditions (Subramanian et al., 2015).

In particular, *B. linens* RS16 which has been used in the present study had been screened for nitrogen fixation, indole acetic acid production, thiosulfate production, exopolysaccharide production, protease, cellulase, lipase and ACC deaminase activity (Siddiquee et al., 2010, 2011). The *B. linens* RS16 inoculation has shown to play a significant role in increasing rice plant root length, shoot length and dry weight even under 100 mM salt stress (Chatterjee et al., 2018). However, it was also observed that the foliar Na^+/K^+ ratio has significant positive correlation with total volatile emission, ACC accumulation and ACO activity in both the rice cultivars (Fig. S1; Table S4 and S5). Therefore, it is possible that the inoculation of *B. linens* RS16 in rice plants activated the similar mechanisms reported in the above studies. In addition, the improved photosynthesis characteristics and a decline in the key volatile emissions in bacteria inoculated salt-treated rice plants exhibit that bacterial ACC deaminase suppressed the ACC accumulation (Fig. 5), thus it is associated with reduced volatile emissions, compared to only salt stressed rice plants.

5 Conclusions

In this work, salt stress resulted in a significant decline in foliage photosynthetic parameters in both the rice cultivars with more progressive reduction observed in the salt sensitive cultivar, IR29 than moderately salt resistant, FL478. In addition, salinity enhanced the emission of foliage LOX, mono- and sesquiterpenes and other stress-induced volatiles with pronounced enhancement observed in IR29, which establishes the direct relationship between degree of salt stress and stress volatile emission. However, *B. linens* RS16 inoculation caused significant recovery of the photosynthetic characteristics and reduced the volatile emission in salt stressed rice cultivars. The decrease in the volatile emission upon bacterial inoculation may likely be associated with reduced foliar ACC accumulation and ACO activity catalyzed by ACC deaminase. Taken together, these results indicate that inoculation of bacteria generally promotes salt tolerant capability of rice plants. However, in-depth molecular level studies are needed to get conclusive insight into how these bacteria regulate volatile emission under saline conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2015R1A2A1A05001885) and grants from the European Commission through the European Research Council (advanced grant 322603, SIP-VOL+), and the European Regional Development Fund (Centre of Excellence EcolChange) and the Estonian Ministry of Science and Education (institutional grant IUT-8-3).

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Fig. 1.

Average (+ SE) dark-adapted maximum quantum yield of photosystem II (PS II) estimated by chlorophyll fluorescence (F_v/F_m ; A, D), net assimilation rate (A: B, E) and stomatal conductance to water vapor (g_s ; C, F) in the leaves of *Oryza sativa* L. salt-resistant cultivar FL478 (A, B, C) and salt-sensitive cultivar IR29 (D, E, F) at 1, 5, and 10 days following plant treatment with NaCl. The details of statistical analysis as in Table S2.

Fig. 2.

Average foliage emission rate (+ SE) of total green leaf volatiles (GLV: A, D), total monoterpenes (B, E), and total sesquiterpenes (C, F) from the leaves of control, salt-stressed, bacteria-inoculated and bacteria-inoculated and salt-stressed leaves of *O. sativa* L. cv. FL478 (A-C) and IR29 (D-F) at days 1, 5, and 10 after application of salt stress. The details of statistical analysis as in Table S3.

Fig. 3. Average foliage emission rate (+ SE) of total saturated aldehydes (A, C), light-weight oxygenated volatiles (OVOC) (B, D), in *O. sativa* L. cv. FL478 (A, B) and IR29 (C, D). The details of statistical analysis as in Table S3.

Fig. 4.

Average foliage emission rate (+ SE) of total volatiles of geranyl geranyl diphosphate (GGDP) pathway (mainly the destruction products of carotenoids; A, C), and benzenoids (B, D) in *O. sativa* L. cv. FL478 (A, B) and IR29 (C, D). The details of statistical analysis as in Table S3.

Fig. 5. Average (+ SE) ACC accumulation (A, C) and ACC oxidase activity (B, D) in leaves of *O. sativa* L. cv. FL478 (A, B) and IR29 (C, D) at days 1, 5 and 10 since salt stress applications. The details of statistics are shown in Table S3.