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Marine Algal-Derived Endophytic Bacteria: Induced Tolerance to Salinity Stress in Mexican Lime Seedlings

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ABSTRACT

Bacterial endophytes grow symbiotically inside plants and improve the growth of their hosts. We evaluated the effects of inoculating macroalgae bacterial endophytes, introduced formerly by our group, *Bacillus aquimaris* strain OD14, *B. megaterium* strain AM25, *B. zhangzhouensis* strain Tv91C, individually and in combination with each other on the reactive oxygen species (ROS) of scavenging and antioxidant functions, as well as growth characteristics of Mexican lime seedlings under salinity stress. Accordingly, Mexican lime (*Citrus aurant -ifolia* Swingle.) seedlings were subjected to four salinity levels, i.e. 0, 2000, 4000 and 6000 µs cm^{-1} in the presence or absence of bacterial endophytes. The results indicated that salinity stress significantly reduced growth, chlorophyll, and carotenoid content of plants lacking endophytes. Combinatory applications with bacterial endophytes significantly improved the above-mentioned parameters under salinity stress. Lipid peroxidation levels were significantly reduced in plants inoculated with bacterial endophytes. Salinity stress significantly increased the activities of ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione reductase (GR), peroxidase (POD), and catalase (CAT) in salinity conditions. Overall, the inoculation with bacterial endophytes improved salinity tolerance and reduced the accumulation of ROS by increasing their scavenging via an enhanced redox state of glutathione and more effective antioxidant enzyme activities.

Abbreviations: Ascorbate peroxidase (APX), Catalase (CAT), Dry weight (DW), Fresh weight (FW), Glutathione reductase (GR), Nitro blue tetraz olium (NBT), Peroxidase (POD), Poly vinyl pyrrolidone (PVP), Reactive oxygen species (ROS), Superoxide dismutase (SOD), Soil water content (SWC), Thiobarbituric acid (TBA), Trichloroacetic acid (TCA).

Introduction

Various environmental factors can have negative effects on growth and yield of plants in arid and semi-arid regions of the world (Arbona et al., 2017). Salinity stress is as a major stress factor that seriously decreases plant growth and yield (Negrao et al., 2017). Currently, 800 million hectares of land are salt-affected and about 45 million hectares have been adversely affected by salinity. Plants can adapt to unfavorable environmental conditions by regulating defense signaling pathways and improving their

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antioxidant systems and metabolism (Khan et al., 2017). Plants by various enzymatic and non-enzymatic antioxidants, defend against ionic fluxes (Mittler, 2002; Turkan and Demiral, 2009). Under stress conditions, ROS such as superoxide anion (O^{2-}) and hydrogen peroxide (H_2O_2) are generated (Mittler, 2002). To eliminate ROS and maintain redox homeostasis, plants employ antioxidant defense system, consisting of antioxidant molecules and antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD) (Mittler, 2002). Over-expression of these antioxidant enzymes, reduction of oxidative damages induced by abiotic stresses (Talaat and Shawky, 2014; Talaat, 2014), either by reacted directly with H_2O_2 or catalyzed by ascorbic peroxidase reaction (APX, EC 1.11.1.11) involved using ascorbate as an electron donor. In modern agricultural strategies, via biotechnological methods, attempts have been made to improve salinity stress tolerance in plants by the production of transgenic plants (Lu et al., 2013). But since abiotic stress tolerance is multigenic, it is difficult to introduce new tolerant varieties. Also, modified plants are not welcome in some countries (Naveed et al., 2014).

Bacterial endophytes are an important associating group of microorganisms existing within plant tissues (Douanla-Meli and Langer, 2012; Tadych et al., 2012; Soltani and Moghaddam, 2015; Soltani, 2017; Compant et al., 2019; Khan et al., 2020; Glick and Gamalero, 2021; Adeleke et al., 2022; Olmo et al., 2022), which are in interaction with their hosts without any visible disease symptoms (Clay et al., 2016). Some bacterial endophytes, can produce some metabolites that are similar or identical to the host plant's metabolites and it helps to the hosts plants to producing lacking biologically active compounds (Seifi et al., 2013). Advantages of using these compounds in agriculture, industry, and medicine has been reported (Soltani and Moghaddam, 2015; Soltani et al., 2016; Soltani, 2017). Endophytes occur in ecological niches in host plants and are capable of improving stress tolerance indirectly and increase growth parameters of plant host (Azad and Kaminskyj, 2016).

Citrus species are among the most important evergreen fruit trees, cultivated in many countries worldwide, including south of Iran (Santini et al., 2012). There are several obstacles in citrus production in southern Iran, limiting the continuity of citrus production. Severe soil salinity is one of the main challenges, threatening citrus industry of southern Iran. Similar to other citrus species, lime trees have suffered drastically from salinity stress. Accordingly, this study aimed at elucidating the role of bacterial endophyte inoculation in Mexican lime seedlings under salinity stress to improve antioxidant enzyme activity, growth factors, and photosynthetic pigment content. Recent studies have indicated that endophytic microbes isolated from extreme environments can possess significantly higher plant growth-promoting traits. Therefore, the present study was carried out to explore bacterial endophyte applications of the macroalgae to protect plants against salinity stress. The importance of this research is that these marine plants comprise one of the most productive ecosystems in the world. The bacterial endophyte strains were isolated from macroalgae and could be helpful to mitigate salinity stress in susceptible plants such as Mexican lime.

Material and Methods

Plant materials and growth conditions

Mexican lime (*Citrus aurantifolia*) seedlings were used as host plants. Seeds of Mexican lime were curated from a citrus orchard in Hormozgan province, Iran. For the sterilization of seeds, NaOCl 5% was used by thoroughly rinsing with deionized water. The seeds were cultivated in pots (diameter 21 cm, depth 22 cm), containing autoclaved (0.1 MPa, 121 °C, 1 h) substrates of peat moss, coco peat and sand (2:1:1, v/v/v). The salinity stress treatments were administered on eight-month-old plants. The plants were grown under greenhouse conditions (16 h photoperiod, day/night temperatures of 40 °C/30 °C), relative humidity of 65–75% and photon flux density of 400 µ mol m^{-2} s^{-1}, and were irrigated every three times a week, in addition to the provision of fertilizers by 20 ml fertilizer solution 2.0% (w/v) (20:20:20, NPK) every two weeks.

Endophytic bacteria

Endophytic bacteria were recovered and identified from seaweed tissues in Hormozgan University Central lab. Three out of 33 isolates that have the best tolerance to salinity test were choice to plant inoculation. The isolates were recorded as *Bacillus aquimaris* strain OD14 (Accession number of MT278260), *B. megaterium* strain JX285 (Accession number of MN626631) and *B. zhangzhouensis* strain Tv91C (Accession number of MN611359) in NCBI gene bank.

Bacterial inoculation and plant stress treatment

The experiment of plant inoculation comprised treatments in triplicates: (1) *B. aquimaris* strain OD14 (B1), (2) *B. megaterium* strain AM25 (B2),

(3) *B. zhangzhouensis* strain Tv91C (B3), (4) B1+B2, (5) B1+B3, (6) B2+B3, (with cell density of $1x10^8$ CFU/ml) were applied to eight-months-old Mexican lime seedlings. Moreover, an equal volume of nutrient broth was used as control. The plants were inoculated with soil drenching and foliar spraying by 1×10^8 CFU suspensions of each bacterial endophyte in combination and individual forms. To spray leaves, each Mexican lime plant was completely sprayed by 10 ml suspension from each microorganism. For soil drenching, 5 ml of the suspensions were spread on the soil, surrounding the crown of the plant. The plants were irrigated with sterile distilled water.

Salinity stress treatments

Three months after establishment of endophytes in plants, the plants were exposed to four salinity intervals, i.e. 0, 2000, 4000 and 6000 µs cm-1. Soil water content (SWC) was determined using the weight fraction as:

$$SWC(\%) = [\frac{FW - DW}{DW}] \times 100$$

Where FW was the fresh weight of the soil from the inner area of each pot and DW was measured by weight loss of soil after oven-drying at 75 °C for 3 days (Meggio et al., 2014).

Plant harvesting

Two months after salinity stress, the effect of bacterial endophytes on salinity tolerance in seedlings was evaluated using morphological, biochemical, and antioxidant characteristics. Upon stress completion, all plants were immediately harvested and suitable leaves were frozen in liquid nitrogen and stored at –80 °C. During harvest, growth parameters, root length, root width, shoot length, trunk width, leaf and branch number were documented.

Plants growth measuring

Root length, root width, leaves fresh/dry weight, root fresh/dry weight, shoot fresh/dry weight, shoot length, trunk width, leaf and branch number were measured. Leaf, shoot, and root dry weight (DW) were measured after oven-drying at 70 °C for 48 h until a fixed weight was achieved.

Photosynthetic pigments quantification

Carotenoids, chlorophyll a, b and total chlorophyll were estimated by extracting 0.2 g leaf in 10 ml 80% acetone and determined spectrophotometrically according to Agrawal and Rathore (2007).

Measurement of malondialdehyde (MDA) levels

The MDA concentration of leaves was determined using the protocol described by Hodges et al. (1999). For this purpose, 200 mg of leaf powder was mixed with 2.5 ml of trichloroacetic acid (TCA) 0.1%, centrifuged at 10,000 × g for 15 min at 4 °C, then 1 ml of the supernatant was blended with 4 ml of 0.5% of thiobarbituric acid (TBA) and 20% trichloroacetic acid (TCA). The above blend was heated in a water bath at 90 °C for about 20 min and after cooling in an ice bath, the mixture centrifuged at 15, 000 × g for 10 min. The absorbance was calculated at 532 nm.

Antioxidant enzyme activities assay

For all antioxidant enzyme, 200 mg of leaf powder was homogenized in an extraction buffer (50 mM potassium phosphate buffer), pH 7.5 and 1% (w/v) poly vinyl pyrrolidone (PVP). The mixture was centrifuged at 10,000 × g for 15 min at 4 °C. The supernatant was stored in -20 °C and used for antioxidant enzyme assay.

Superoxide Dismutase (SOD, EC 1.15.1.1)

Superoxide dismutase (SOD, EC 1.15.1.1) activity was assessed on the basis of its capability to inhibit the photoreduction of nitro blue tetrazolium (NBT) as explained by Becana et al., (1986). The reaction mixture (1 ml) contained 50 µl enzyme extract, 50 mM potassium phosphate buffer (pH 7.5), 14.3 mM methionine, 82.5 mM NBT, 0.1 mM EDTA and 2.2 mM riboflavin. The reaction was placed under 15 W fluorescent lamps. The reaction was terminated after 10 min. The absorbance was measured at 560 nm.

Catalase (CAT, EC 1.11.1.6)

Catalase (CAT, EC 1.11.1.6) activity was measured according to Chance and Maehly (1955). The reaction mixture (1 ml) contained 50 mM potassium phosphate buffer (pH 7.0), 4.4 mM H_2O_2 and 100 µl of enzyme extract. The absorbance was calculated at 240 nm (ε= 39.4 mM^{-1} cm^{-1}).

Ascorbate peroxidase (APX, EC 1.11.1.11)

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was assessed according to procedure of Asada (1984). The reaction mixture (1.0 ml) contained 50 mM potassium phosphate buffer (pH 7.0), 33 µl of enzyme extract and 0.17 mM ascorbate. The reaction was initiated when 5 mM H_2O_2 was added. The absorbance was calculated at 290 nm for 3 min (ε= 2.8 mM^{-1} cm^{-1}).

Peroxidase (POD, EC 1. 11. 1.7) activity

For POD, a mixture of 3 ml contained 30 µl of enzyme extract, 2970 µl of guaiacol (45 mM) and H2O2 (100 Mm) which was prepared in 50 mM potassium phosphate buffer pH 7.0 containing 0.5 mM EDTA was used. POD activity was determined by measuring the oxidation of guaiacol in the presence of H_2O_2 (extinction coefficient of 26.6 mM^{-1} cm^{-1}) at 470 nm over 2 min intervals (Chance and Maehly, 1955).

Glutathione reductase (GR, EC 1.6.4.2)

Glutathione reductase (GR, EC 1.6.4.2) activity was assessed using the protocol described by Smith et al. (1988). The reaction mixture (1.0 ml) contained 50 mM potassium phosphate buffer (pH 7.5), 100 µl of crude enzyme extract, 1 mM GSSG, 0.75 mM DTNB (5, 5′ dithiobis-2nitrobenzoic acid). 0.1 mM NADPH was added to initiate the reaction. The increase in absorbance, due to the formation of TNB (5-thio-2-nitrobenzoic acid), was determined at 412 nm (ε= 14.15 $mM^{-1}cm^{-1}$).

Statistical analyses

Data were analyzed statistically based on a completely randomized design, with a two-way factorial method. Significant differences were calculated using the least significant difference (LSD) test at the level of $P<0.05$ using SAS.9.4 software.

Results

Growth parameters

The interaction effect of salinity level and endophyte colonization on shoot, leaf, and root fresh weight (FW) and dry weight (DW) was significant ($P<0.001$). In some treatments, leaf, shoot and root FW/DR weight decreased by increased salinity level. All endophyte-inoculated plants showed significantly higher leaf, shoot and root FW/DW weight compared to endophyte-free plants, either in salinity stress or in non-stress conditions. Also, shoot dry weight was significantly higher ($P<0.001$) in plants inoculated with a combination B2 and B3. A combination of B1 and B3 endophytes had highest leaves, branch count, trunk diameter, root width and root/leaf fresh weight. The ANOVA analysis of morphological parameters are descripted in Table 1. Most of the measured parameters were significant ($P<0.001$).

Table 1. The ANOVA analysis of interaction of salinity and endophytes on morphological parameters in inoculated Mexican lime seedling compared with the control.

S.O.V	df	Mean of Squares											
		T D	L no	B no	SL	LFW	LDW	RFW	RDW	SFW	SDW	RL	RW
Salinity	3	1.37^{***}	102.51^{*}	2.96^{ns}	243.14^{***}	3.79^{*}	1.41^{***}	29.38^{***}	1.52^{***}	8.02^{***}	2.49^{***}	193.54^{***}	529.19^{***}
Endophytes	6	11.72^{***}	4309.28^{***}	47.91^{***}	1159.44^{***}	120.62^{***}	12.3^{***}	309.77^{***}	13.87^{***}	75.9^{***}	17.34^{***}	410.21^{***}	947.99^{***}
S*E	18	1.64^{***}	610.05^{***}	4.89^{**}	77.80^{***}	12.2^{***}	0.74^{***}	14.96^{***}	0.69^{***}	5.02^{***}	2.10^{***}	50.60^{***}	37.30^{***}
Error	-	0.09	27.29	2.00	10.91	1.52	0.11	2.61	0.13	0.55	0.19	17.00	10.31
***F*-value**		41.97	50.4	7.12	30.83	23.16	30.16	31.35	26.65	37.75	28.12	8.61	28.54
C.V	-	5.60	9.47	18.64	8.91	12.56	15.46	13.18	13.78	10.46	15.9	10.92	8.09

TD, L no, B no, SL, LFW, LDW, RFW, RDW, SFW, SDW, RL and RW means; trunk diameter, leaf number, branch number, shoot length, leaf fresh weight, leaf dry weight, root fresh weight, root dry weight, shoot fresh weight, shoot dry weight, root length and root width respectively.

In Table 2, the comparison of mean values pertaining to morphological parameters were described. As shown, the interaction effect of salinity level and endophyte colonization on shoot and root dry weight was significant ($P<$ 0.001). Shoot and root dry weight were decreased by the drought level (Table 2). All endophyte-inoculated plants showed significantly higher shoot dry weight compared with endophyte-free plants, either in drought stress or in non-stress

conditions. Also, shoot dry weight and shoot length were significantly higher ($P<0.001$) in plants inoculated with a combination of two bacterial endophytes [*B. megaterium strain* AM25 (B2), *B. zhangzhouensis* strain Tv91C (B3)] in highest salinity level (Table 2). The highest amount of root fresh weight was obtained in salinity 4000 ms cm^{-1}, and plants inoculated with a combination of two bacteria endophytes (*B. aquimaris* strain OD14 and *B. megaterium* strain AM25), with 480.53% (4/8-fold) difference, compared to control (Table 2). The plants associated with a combination of *B. megaterium* strain AM25 and *B. zhangzhouensis* strain Tv91C bacteria endophytes had significantly higher leaf number with 526.14% (5.2-fold) compared with non-endophyte associated, in 4000 µs cm^{-1} water salinity level. Plant inoculated with *B. megaterium* strain AM25 bacteria endophytes had significantly higher root length compared with other treatments under 6000 µs cm^{-1} water salinity (Table 2). Overall, bacteria can improve morphological parameters in inoculated seedlings compared with the control (Fig. 1).

Table 2. Changes in the fresh/dry weight of shoot, root and leaves in plant^{-1} (g), root length and width^{-1} (cm), shoot length^{-1} (cm), trunk diameter^{-1} (cm), leaves and branch number in Mexican lime inoculated with endophytic bacteria, *B. aquimaris* strain OD14 (B1), *B. megaterium* strain AM25 (B2) and *B. zhangzhouensis* strain Tv91C (B3), individual inoculation and in combination with each other under different salinity stress (0, 2000, 4000 and 6000 µs cm^{-1}). The control (N) was treated with distilled water.

	Treats	TD (mm)	L no (no)	B no (no)	SL (cm)	LFW (gr)	LDW (gr)	RFW (gr)	RDW (gr)	SFW (gr)	SDW (gr)	RL (cm)	RW (cm)
S0	N	3.58^{k}	21.00^{jk}	3.66^{gh}	20.00^{k}	4.99^{hi}	0.45^{no}	5.03^{klm}	1.36^{lm}	3.11^{p}	1.53^{fghi}	29.5^{hij}	27.00^{ijkl}
	B1	6.54^{b}	64.66^{de}	10.00^{a}	35.00^{fghi}	9.08^{ef}	1.74^{kl}	8.77^{i}	2.03^{jkl}	5.61^{mno}	1.76^{fgh}	53.33^{a}	37.33^{fgh}
	B2	4.9^{ij}	36.00^{hi}	7.33^{bcdef}	32.00^{hij}	7.53^{fg}	2.57^{efghij}	7.43^{ijk}	2.18^{jk}	5.03^{no}	1.9^{efg}	45.93^{bcd}	49.16^{abc}
	B3	7.66^{a}	71.33^{bcd}	8.66^{abcdef}	50.66^{bc}	12.14^{bcd}	3.42^{abc}	15.42^{cdef}	3.95^{abcd}	8.01^{ghij}	3.21^{d}	39.86^{cdefg}	46.5^{bcde}
	B1B2	6.06^{bcde}	64.00^{de}	9.66^{abc}	34.66^{fghi}	13.98^{ab}	3.51^{ab}	18.59^{b}	4.22^{ab}	8.76^{defghi}	4.08^{bc}	40.33^{cdefg}	44.5^{cde}
	B1B3	5.46^{fgh}	41.33^{gh}	6.00^{fg}	36.66^{efgh}	9.38^{ef}	2.73^{defgh}	11.55^{h}	2.37^{ijk}	5.98^{lmn}	1.71^{fgh}	49.83^{ab}	53.33^{a}
	B2B3	5.48^{fgh}	71.33^{bcd}	9.00^{abcde}	43.00^{de}	12.21^{bcd}	2.12^{hijk}	17.49^{bcd}	3.36^{defg}	7.8^{hij}	2.02^{ef}	35.73^{efgh}	53.00^{a}
S2000	N	3.58^{k}	13.00^{k}	2.66^{h}	17.33^{k}	4.12^{hi}	0.43^{no}	4.26^{lm}	0.91^{mn}	2.79^{p}	1.13^{hij}	26.66^{ij}	23.33^{jkl}
	B1	5.00^{hij}	51.33^{f}	7.00^{cdef}	30.00^{ij}	7.51^{fg}	1.17^{lm}	8.05^{ij}	1.94^{kl}	5.12^{mno}	1.54^{fghi}	38.66^{defg}	42.66^{def}
	B2	5.81^{cdef}	71.33^{bcd}	10.33^{a}	38.00^{efgh}	7.32^{fg}	1.66^{kl}	12.15^{gh}	3.13^{efgh}	7.57^{hijk}	3.07^{d}	34.33^{fgh}	50.00^{abc}
	B3	5.85^{cdef}	66.66^{cde}	9.00^{abcde}	51.33^{bc}	13.35^{abc}	3.22^{abcd}	16.52^{bcd}	3.41^{cdef}	9.58^{cde}	4.14^{bc}	35.00^{efgh}	42.66^{def}
	B1B2	6.14^{bcde}	60.00^{e}	6.33^{ef}	48.66^{bcd}	13.11^{abc}	3.26^{abcd}	17.79^{bc}	3.78^{abcde}	8.90^{defgh}	4.03^{bc}	33.5^{ghi}	42.83^{def}
	B1B3	5.46^{fgh}	75.00^{bc}	7.33^{bcdef}	32.66^{ghij}	11.41^{cde}	2.48^{efghij}	15.96^{bcde}	3.12^{efgh}	8.12^{fghij}	2.62^{de}	42.66^{bcde}	51.33^{ab}
	B2B3	5.78^{cdefg}	46.00^{fg}	6.66^{def}	37.33^{efgh}	10.04^{de}	2.21^{ghijk}	14.81^{cdefg}	2.92^{fghi}	9.39^{cdefg}	4.03^{bc}	34.66^{efgh}	50.73^{ab}
S4000	N	3.43^{kl}	14.00^{k}	3.00^{h}	19.33^{k}	3.69^{hi}	0.33^{no}	3.75^{lm}	0.79^{mn}	2.44^{p}	0.91^{hi}	23.83^{j}	22.66^{kl}
	B1	3.76^{k}	28.66^{ij}	9.33^{abcd}	27.33^{j}	5.82^{gh}	0.84^{mn}	6.41^{ijkl}	1.45^{lm}	4.92^{no}	1.24^{fghi}	40.16^{cdef}	26.9^{ijkl}
	B2	5.70^{defg}	76.66^{b}	9.66^{abc}	40.66^{ef}	13.8^{ab}	3.73^{a}	15.24^{cdef}	3.76^{abcde}	9.46^{cdef}	4.06^{bc}	40.83^{cdefg}	45.33^{bcde}
	B3	5.76^{cdefg}	65.33^{cde}	10.00^{ab}	40.00^{ef}	11.95^{bcd}	2.52^{efghij}	17.00^{bcd}	4.07^{abc}	7.45^{ijk}	3.16^{d}	40.83^{cdefg}	37.00^{fgh}
	B1B2	5.96^{cdef}	71.33^{bcd}	7.33^{bcdef}	39.66^{ef}	10.42^{de}	3.05^{bcde}	21.77^{a}	4.4^{a}	8.28^{efghij}	3.45^{cd}	37.33^{efgh}	38.16^{fg}
	B1B3	4.59^{ij}	63.33^{de}	6.66^{def}	30.00^{ij}	12.13^{bcd}	2.81^{cdefg}	12.73^{fgh}	2.69^{ghij}	6.43^{klm}	1.76^{fgh}	37.66^{efg}	48.16^{abcd}
	B2B3	6.24^{bcd}	87.66^{a}	9.33^{abcd}	40.00^{ef}	14.89^{a}	3.43^{abc}	17.54^{bc}	3.63^{bcde}	10.55^{bc}	4.83^{ab}	41.83^{cdef}	41.16^{ef}
S6000	N	2.96^{l}	12.00^{k}	3.66^{gh}	18.66^{k}	3.42^{hi}	0.17^{o}	2.32^{m}	0.41^{n}	2.20^{p}	0.84^{i}	22.23^{j}	22.00^{l}
	B1	4.86^{ij}	44.66^{fgh}	8.00^{abcdef}	36.66^{efgh}	5.74^{gh}	0.94^{mn}	5.18^{jklm}	1.44^{lm}	4.54^{o}	1.09^{hij}	37.26^{efgh}	32.00^{hi}
	B2	5.22^{ghi}	65.33^{cde}	7.33^{bcdef}	46.66^{cd}	10.18^{de}	2.04^{ijk}	13.4^{efgh}	3.13^{efgh}	7.19^{jkl}	2.79^{d}	46.83^{abc}	44.16^{cde}
	B3	6.35^{bc}	70.66^{bcd}	9.00^{abcde}	53.33^{b}	13.03^{abc}	2.66^{defgh}	12.04^{gh}	2.63^{hijk}	11.03^{ab}	5.29^{l}	34.83^{efgh}	28.16^{gh}
	B1B2	5.65^{efg}	62.66^{de}	7.66^{abcdef}	40.00^{ef}	10.44^{de}	2.25^{fghij}	14.6^{defg}	3.66^{bcde}	7.84^{hij}	3.05^{d}	41.00^{cdefg}	28.66^{ij}
	B1B3	7.22^{a}	78.33^{b}	9.66^{abc}	38.66^{efg}	13.85^{ab}	2.87^{cdef}	15.37^{cdef}	3.08^{efgh}	9.79^{bcd}	3.4^{cd}	36.83^{efgh}	47.66^{abcd}
	B2B3	6.26^{bcd}	50.66^{f}	8.00^{abcdef}	59.00^{a}	9.43^{ef}	1.98^{jk}	12.26^{gh}	1.96^{kl}	12.02^{a}	5.38^{a}	35.5^{efgh}	35.00^{gh}

TD, L no, B no, SL, LFW, LDW, RFW, RDW, SFW, SDW, RL and RW means; trunk diameter, leaf number, branch number, shoot length, leaf fresh weight, leaf dry weight, root fresh weight, root dry weight, shoot fresh weight, shoot dry weight, root length and root width respectively.

Fig. 1. Improvement in the morphological parameters of Mexican lime seedlings inoculated with endophytic bacteria, *B. megaterium* strain AM25 (B2) and *B. zhangzhouensis* strain Tv91C (B3) combination, under 4000 µs cm^{-1} water salinity. Control (N) was without endophytes inoculation in salinity condition.

Contents of chlorophyll and carotenoid

The ANOVA analysis of chlorophyll and carotenoid contents and antioxidant enzymes parameters were described in Table 3. As shown, most of the measured parameters were significant (P<0.001).

Table 3. The ANOVA analysis of interaction of salinity and endophytes on photosynthesis pigment and antioxidant enzymes parameters in inoculated Mexican lime seedling compared to the control

S.O.V	df	Mean of Squares									
		Photosynthesis Pigments						Antioxidant enzymes			Biochemical
		Chl a	Chl b	Chl T	Car	CAT	POD	SOD	GR	APX	MDA
Salinity	3	0.54^{***}	0.28^{***}	2.44^{***}	0.04^{***}	5761.18^{***}	2506.19^{***}	219976.83^{***}	5488.84^{***}	555846.75^{***}	93.70^{***}
Endophytes	6	0.16^{***}	0.06^{***}	0.09^{***}	0.02^{***}	1010.51^{***}	1625.3^{***}	34071.16^{***}	1442.12^{***}	146429.95^{***}	19.36^{***}
S*E	18	0.00^{***}	0.00^{***}	0.01^{***}	0.00^{***}	261.27^{***}	167.97^{***}	10280.58^{***}	173.64^{***}	17701.8^{***}	0.892^{***}
Error		0.00	0.00	0.00	0.00	2.50	1.9	0.01	2.97	491.57	0.124
***F*-value**		1012.12	478.37	531.06	77.76	416.27	394.82	4033636	351.39	215.84	123.05
C.V		1.14	1.69	1.72	3.5	3.13	3.4	0.04	4.78	3.07	7.96

Ch a, ch b, ch t and car means; chlorophyll a, chlorophyll b, chlorophyll total and carotenoids respectively.

Contents of chlorophylls and carotenoids decreased in response to higher salinity levels (Table 4). The chlorophyll content was increased in the endophyte-associated plants, whether or not salinity stress was applied (P<0.001) (Table 4). Plants inoculated with a combination of two bacterial endophytes (*B. aquimaris* strain OD14 and *B. zhangzhouensis* strain Tv91C) had more chlorophyll a content under 6000 µs cm^{-1} water salinity with 217% differences compared with the control (Table 4). Inoculation with *B. aquimaris* strain OD14 resulted in the highest increase in chlorophyll b content, compared to the control 6000 µs cm^{-1} water salinity with 61.9%. The plants inoculated with a combination of two bacterial endophytes *B. aquimaris* strain OD14 and *B. zhangzhouensis* strain Tv91C and B. megaterium strain AM25 individual had the

highest total chlorophyll content. Similar to the chlorophyll content, carotenoids decreased with higher salinity stress, and the plants with endophyte inoculation showed more carotenoid content compared with non-inoculated plants (Table 4). In response to the highest level of water salinity, a high carotenoid content was obtained in plants inoculated with *B. aquimaris* strain OD14 and *B. zhangzhouensis* strain Tv91C and B. aquimaris strain OD14 bacterial endophytes with 59.09% and 54.54% difference, compared to the control (Table 4).

Table 4. Changes in the chlorophylls and carotenoid contents in plants (mg g^{-1} FW), MDA content (nmol TBSRS g^{-1} FW) and antioxidant enzymes (U mg^{-1} FW) activity in Mexican lime inoculated with endophytic bacteria, B. aquimaris strain OD14, *B. megaterium* strain AM25 and *B. zhangzhouensis* strain Tv91C, inoculated individually and in combination with each other, under different salinity stress (0, 2000, 4000 and 6000 µs cm^{-1}). The control was treated with distilled water.

Salinity (ms cm^{-1})	Treats	Chl a (mg g^{-1} FW)	Chl b (mg g^{-1} FW)	Chl T (mg g^{-1} FW)	Cart (mg g^{-1} FW)	MDA (nmol TBSRS g^{-1} FW)	CAT (U mg^{-1} FW)	POD (U mg^{-1} FW)	SOD (U mg^{-1} FW)	GR (U mg^{-1} FW)	APX (U mg^{-1} FW)
S0	N	0.91^{h}	0.69^{gh}	1.6^{ef}	0.32j^{k}	4.91^{ef}	27.15^{mn}	9.83^{r}	66.22^{w}	10.48^{n}	324.39^{n}
	B1	1.08^{c}	0.81^{a}	1.68^{c}	0.44^{bc}	1.57^{k}	32.58^{l}	29.32^{o}	132.45^{t}	18.94^{kl}	490.00^{l}
	B2	1.15^{a}	0.8^{ab}	1.92^{a}	0.45^{b}	1.3^{k}	42.67^{jk}	36.82^{jk}	185.43^{n}	19.38^{kl}	530.00^{k}
	B3	1.06^{c}	0.74^{d}	1.63^{de}	0.38^{fg}	1.08^{k}	59.43^{g}	31.39^{no}	145.69^{q}	25.41^{j}	603.57^{j}
	B1 B2	1.09^{c}	0.77^{c}	1.92^{a}	0.48^{a}	1.65^{k}	40.38^{k}	32.22^{mn}	192.06^{m}	12.45^{n}	678.57^{hi}
	B1 B3	1.14^{ab}	0.79^{b}	1.82^{b}	0.41^{de}	1.64^{k}	57.75^{gh}	29.86^{no}	100.47^{v}	15.59^{m}	733.93^{g}
	B2 B3	0.94^{g}	0.7^{fg}	1.66^{cd}	0.39^{ef}	1.21^{k}	28.58^{m}	20.6^{p}	132.68^{s}	18.26^{klm}	408.93^{m}
S2000	N	0.72^{l}	0.65^{j}	1.52^{h}	0.28^{l}	6.94^{bc}	44.3^{j}	15.2^{q}	145.69^{q}	16.29^{lm}	492.86^{l}
	B1	1.01^{e}	0.77^{c}	1.65^{cd}	0.42^{cd}	2.57^{j}	68.05^{d}	50.15^{ef}	258.27^{g}	30.67^{i}	625.71^{j}
	B2	1.13^{b}	0.64^{jk}	1.58^{fg}	0.42^{cd}	3.34^{i}	73.00^{ab}	51.6^{de}	238.41^{i}	20.68^{k}	677.71^{hi}
	B3	0.91^{h}	0.63^{k}	1.55^{gh}	0.36^{ghi}	3.54^{hi}	75.05^{a}	49.47^{efg}	198.67^{l}	43.62^{fg}	618.57^{j}
	B1 B2	0.97^{f}	0.71^{ef}	1.67^{c}	0.44^{bc}	5.05^{ef}	71.14^{bc}	48.65^{fg}	218.54^{jk}	16.43^{lm}	750.00^{fg}
	B1 B3	1.02^{e}	0.67^{i}	1.66^{cd}	0.38^{fg}	3.57^{hi}	72.6^{ab}	42.19^{i}	284.76^{f}	31.69^{i}	686.31^{h}
	B2 B3	0.90^{h}	0.64^{jk}	1.51^{h}	0.38^{fg}	1.6^{k}	69.21^{cd}	38.65^{j}	165.56^{o}	34.74^{h}	642.14^{ij}
S4000	N	0.53^{o}	0.44^{p}	1.07^{l}	0.24^{mn}	7.32^{b}	41.5^{k}	21.92^{p}	198.67^{l}	19.38^{kl}	614.29^{j}
	B1	0.96^{f}	0.72^{e}	1.14^{k}	0.39^{ef}	4.43^{fg}	60.32^{fg}	55.79^{c}	317.88^{e}	37.73^{h}	681.43^{hi}
	B2	0.91^{h}	0.56^{m}	1.34^{i}	0.38^{fg}	4.43^{fg}	64.4^{e}	56.54^{c}	543.09^{a}	45.73^{f}	964.29^{c}
	B3	0.82^{i}	0.58^{l}	1.34^{i}	0.34^{ij}	4.03^{gh}	61.43^{f}	64.89^{b}	456.95^{b}	55.73^{e}	678.93^{hi}
	B1 B2	0.79^{jk}	0.54^{mn}	1.25^{j}	0.41^{de}	5.36^{e}	68.26^{d}	51.69^{de}	390.72^{d}	36.65^{h}	785.00^{ef}
	B1 B3	0.96^{f}	0.54^{mn}	1.23^{j}	0.37^{fgh}	4.77^{ef}	68.25^{d}	45.59^{h}	423.84^{c}	37.46^{h}	1031.07^{b}
	B2 B3	0.79^{jk}	0.55^{mn}	1.32^{i}	0.36^{ghi}	5.01^{ef}	61.75^{f}	79.3^{a}	258.27^{g}	59.03^{cd}	794.64^{e}
S6000	N	0.46^{p}	0.42^{q}	0.81^{o}	0.22^{n}	9.52^{a}	19.51^{o}	15.11^{q}	132.45^{t}	20.44^{k}	769.28^{efg}
	B1	0.72^{l}	0.68^{hi}	0.96^{m}	0.34^{ij}	6.06^{d}	15.02^{p}	42.37^{i}	251.65^{h}	60.76^{c}	770.00^{efg}
	B2	0.78^{k}	0.48^{o}	1.13^{k}	0.31^{k}	6.7^{bcd}	25.55^{n}	43.23^{i}	218.45^{k}	54.73^{e}	1053.57^{ab}
	B3	0.72^{l}	0.49^{o}	0.87^{n}	0.25^{m}	6.12^{d}	50.15^{i}	53.05^{d}	158.94^{p}	78.29^{a}	943.57^{c}
	B1 B2	0.68^{m}	0.42^{q}	1.11^{k}	0.31^{k}	6.87^{bc}	34.8^{l}	35.77^{kl}	218.66^{j}	56.68^{de}	885.00^{d}
	B1 B3	0.8^{j}	0.38^{r}	1.12^{k}	0.35^{hi}	6.64^{cd}	24.65^{n}	34.12^{lm}	105.95^{v}	41.56^{g}	1071.07^{a}
	B2 B3	0.6^{n}	0.41^{q}	0.94^{m}	0.32^{jk}	6.67^{cd}	55.36^{h}	47.64^{gh}	139.08^{r}	68.03^{b}	828.57^{d}

Chl a, chl b, chl t and car means; chlorophyll a, chlorophyll b, chlorophyll total and carotenoids respectively.

MDA content

Salinity stress caused a significant increase in contents of MDA in the Mexican lime seedlings (Fig. 2). However, a different response pattern was detected in the seedlings inoculated with bacterial endophytes when they experienced salinity stress. The highest amount of MDA content was obtained in 6000 µs cm^{-1} water salinity level and the endophyte-free plants (control). Inoculation with a combination of bacteria endophytes (B1B2, B1B3, B2B3) and individually applied of endophytes reduced the MDA content under 6000 µs cm^{-1} salinity, when compared with the control plants. The lowest lipid peroxidation was significantly ($P \leq 0.001$) observed in plants treated with *B. aquimaris* strain OD14 (57.09%), compared with the control.

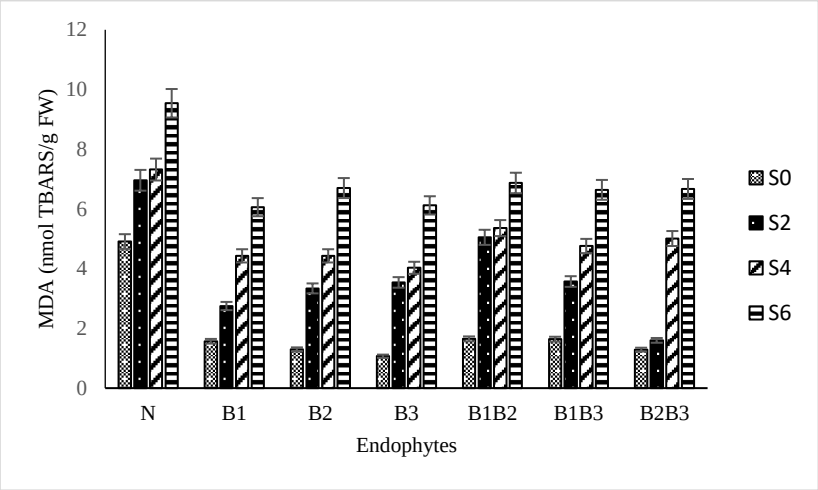
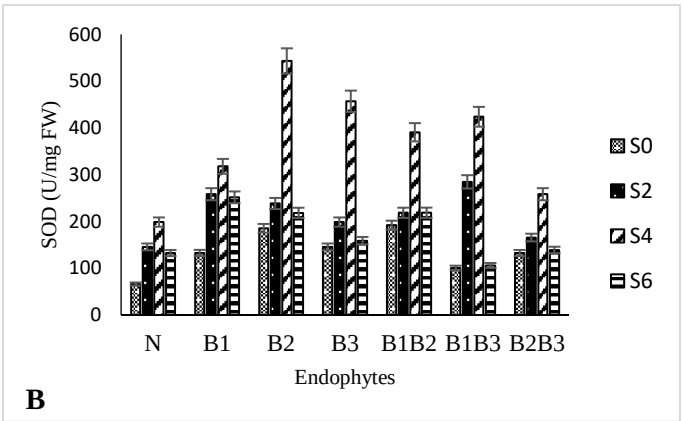
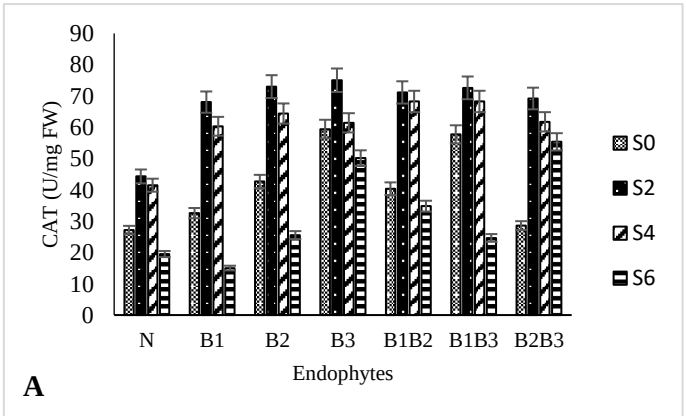


Fig. 2. Changes in the content of lipid peroxidation in leaves of Mexican lime inoculated with different endophytic bacteria including *B. aquimaris* strain OD14, *B. megaterium* strain AM25 and *B. zhangzhouensis* strain Tv91C, inoculation individually and in combination with each other under different salinity water stress (0, 2000, 4000 and 6000 µs cm^{-1}). Control plants were without endophytes inoculation in salinity condition.

Activities of antioxidant enzymes

Salinity stress caused different changes in the antioxidant enzyme activities. As shown in Fig. 3, seedlings under stress showed higher APX and GR activities, but lower CAT and SOD, activities, in highest level of water salinity. Activity of APX and GR had a positive correlation with increased salinity level of salinity. By increased salinity, activities of APX and GR were decreased (Fig. 3C and Fig. 3D). However, the salinity-stressed seedlings treated with bacteria endophytes showed an increase in CAT, SOD, APX, POD and GR activities. In the highest level of water salinity, the seedlings were colonized with all bacteria endophyte treatments. The most effective treatment significantly increased CAT activity by 183.75% (*B. megaterium* strain AM25 and *B. zhangzhouensis* strain Tv91C) (Fig. 3A), compared to that of SOD activity in Mexican lime leaves (89.99%) (*B. aquimaris* strain OD14) (Fig. 3B). It increased APX activity by 39.22% (by *B. megaterium* strain AM25 and *B. zhangzhouensis* strain Tv91C) (Fig. 3C), GR activity by 283.02% (by *B. zhangzhouensis* strain Tv91C) (Fig. 3D) and POD activity by 251.09% (by *B. zhangzhouensis* strain Tv91C) (Fig. 3E), under salinity stress conditions at 6000 µs cm^{-1} water salinity, compared to the non-colonized seedlings.





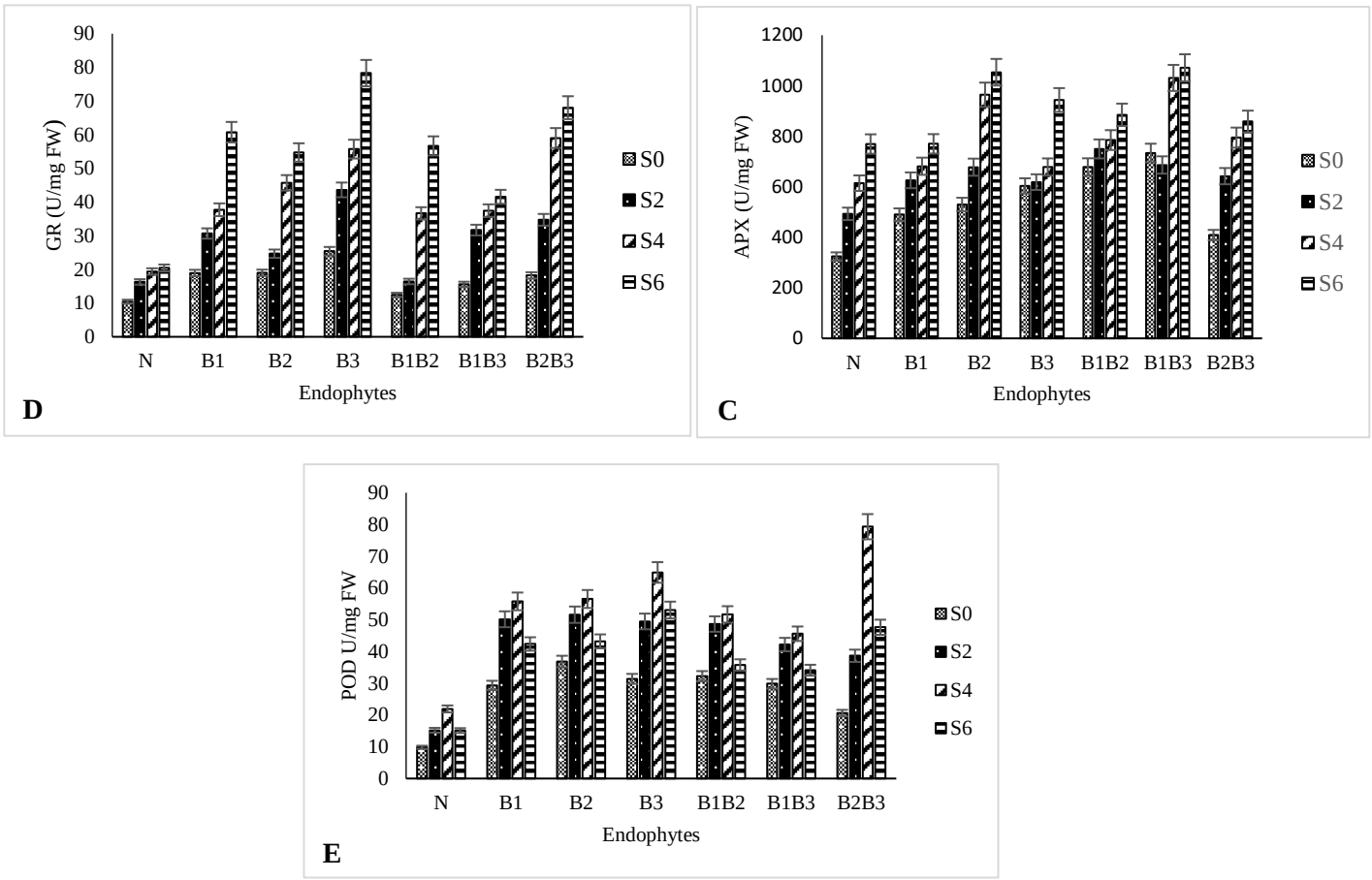


Fig. 3. Changes in the activities of catalase (CAT, A), superoxide dismutase (SOD, B), ascorbate peroxidase (APX, C) and glutathione reductase (GR, D), in leaves of Mexican lime inoculated with endophytic bacteria, including *B. aquimaris* strain OD14 (B1), *B. megaterium* strain AM25 (B2) and *B. zhangzhouensis* strain Tv91C (B3), inoculation individually and in combination with each other, under different salinity stress (0, 2000, 4000 and 6000 µs cm^{-1}). The control was without endophytes treatment in salinity condition.

Discussion

Salinity stress causes numerous physiological and biochemical changes in plants, such as plant growth and development, but decreases photosynthesis and respiration (Zhang and Blumwald, 2001; Sairam et al., 2002; Yang et al., 2014). The negative and harmful effects of salt stress on various plants have been stated by Wang et al. (2003), Hasegawa et al. (2000), Munns and Tester (2008) and Khan et al. (2011; 2012; 2013; 2014) in various crop plants such as cucumber, pepper, soybean, and rice. High NaCl concentrations negatively affected plant growth and development and a similar observation was found in Mexican lime seedlings subjected to salinity stress in this study.

Among several strategies that can improve plant tolerance under salinity stress, the use of biological agents like bacterial endophytes are more promising and easily available (Bailey et al., 2006). Macroalgae comprise a unique microbiome containing a variety of endophytes and are known to ameliorate various detrimental effects of numerous stresses. The endophytic bacterial community of this biome has been less explored. The potential role of the bacterial endophytes microbiome in countering abiotic stresses has recently been demonstrated (Brader et al., 2014). Therefore, the present study was conducted to explore the use of bacterial endophytes derived from macro algae to enhance salinity tolerance in Mexican lime seedlings.

Bacillus is a gram-positive, rod-shaped, endospore-forming genus of bacterium. Some industrially exploited *Bacillus* bacteria have the potential to act as an alternative host for the production of certain industrial enzymes, regarding salt stress and general resistance to abiotic stress (e.g. heat, UV radiation and oxidative stress during fermentation processes) (Stadtman and Levine, 2003; Schweder and

Hecker, 2004; Gioia et al., 2007).

The bacterial endophytes *B. aquimaris* strain OD14, B. megaterium strain JX285 and *B. zhangzhouensis* strain Tv91C were grown in NaCl-supplemented media to examine their responses to the presence of Na^+ and Cl^- ions in the medium. The isolates grew on 3 mol NaCl medium. The ability of particular bacterial strains to grow in high salinity is essential for plant adaptation in a stressful environment and can elucidate the survival mechanisms used by endophytes in natural environments with high salinity. These endophytes may extend their beneficial impacts to plants in similar environmental conditions and were selected based on their tolerance to salinity stress.

In the current study, we found that the inoculation of seedlings with bacterial endophytes improved growth, despite the exposure to salinity stress, resulting in better root and shoot biomass, leaves, branches and survival rate of the plants, compared to the control. It may be because endophytes can induce secondary metabolites such as auxin and gibberellin hormones in plants (Nanda et al., 2018). Similar plant growth-promoting and stress-mediatory responses of bacterial endophytes have also been noted by Ali et al. (2014), Blanco and Lugtenberg (2014) and Egamberdieva and Lugtenberg (2014).

Any reduction in chlorophyll content would influence photosynthetic capacity. In increased salinity stress levels, a reduction in chlorophyll a, b, total and carotenoids resulted from chlorophyll degradation and pigments photo-oxidation. Carotenoids are known for stabilizing and protecting the lipid phase of the thylakoid membranes while behaving as singlet-oxygen quenchers and radical scavengers (Bu et al., 2012). The higher carotenoids content in the endophyte-inoculated Mexican lime seedlings might protect the plants against salinity stress. Zhang and Nan (2007) found a higher accumulation of chlorophyll in endophyte-inoculated *Elymus dahuricus* under stress.

Salinity stress causes oxidative stress, which induces development and disturbs the role and function of cellular organs by the peroxidation of the lipid bilayer (Munns and Tester, 2008). Antioxidant activation can improve membrane stability and help scavenge ROS before they induce injury, whereas the MDA content could be assessed for the evaluation of stress injury (Rivero et al., 2014). In the current study, Mexican lime plants were grown under salinity conditions and the level of MDA (lipid peroxidation) production was significantly increased. Increased amounts of ROS auto catalyze the peroxidation of lipid membranes and affect membrane semi-permeability under severe stress conditions (Khan et al., 2013). While colonization with bacterial endophytes prevented lipid peroxidation, which was the lowest compared to the control plants. Individual applications of *B. zhangzhouensis* strain Tv91C and *B. aquimaris* strain OD14 reduced the level of lipid peroxidation in inoculated seedlings compared with the control, possibly because membrane damage is prevented and antioxidant responses are induced by endophytes. This protects the plants from oxidative damage. Bacterial endophytes reportedly mitigated stress by lowering the lipid oxidation level (Kang et al., 2014; Lastochkina et al., 2017; Lastochkina et al., 2021; Maslennikova and Lastochkina, 2021), which is consistent with the findings of the present study. Thus, our results indicated less membrane injury in plants treated with endophytes than in plants treated only with NaCl. Similar results with endophytes incubation on *Elymus dahuricus* and *Oryza sativa* were reported in the literature (Zhang et al., 2010; Shukla et al., 2012).

During salinity stress, a high rate of antioxidant enzymatic activities can contribute to mitigating the negative effects of stress (Halverson et al., 2000; Ali et al., 2014). Colonization with bacterial endophytes helped seedlings under salinity stress, making the endophytes mitigate oxidative damage by producing either extra or intracellular scavenging enzymes (White and Torres, 2010) such as CAT and SOD. In scavenging pathways, H_2O_2 is converted into water with the help of oxidative-stress enzymes such as CAT (Jincy et al., 2017). The results of the present study also supported the regulatory activation of GR, POD, SOD, APX and CAT enzymes, thereby confirming similar cases in other reports (Naveed et al., 2014).

SOD is the first line of defense against ROS by converting superoxide radicals (Alscher et al., 2002) and catalases (CATs) while safeguarding cells from oxidative stress (Quan et al., 2008; Halo et al., 2015). In current study, CAT and SOD activity decreased in response to salinity. However, when plants were treated with the endophyte, the CAT and SOD activity were significantly increased, showing a protective role of endophytes in scavenging ROSs in Mexican lime seedlings under salinity stress conditions.

Under salinity conditions, glutathione levels were significantly higher in endophyte-treated plants compared with those treated only with NaCl. This is in agreement with recent findings by Khan et al. (2015) that the level of glutathione increased during cadmium stress. We showed that GR and APX contents increased in inoculated seedlings

because of salinity. Individual applications of *B. zhangzhouensis* strain Tv91C bacterial endophyte had the highest activity of GR. The combination of B. megaterium strain JX285 and *B. zhangzhouensis* strain Tv91C generated the highest activity of APX to counter the toxic effects of salinity. A similar finding by Gill et al. (2013) showed that glutathione can help plants in tolerating abiotic stress.

Here, we evaluated the potential of inoculating lime seedlings with three Macroalgae bacterial endophytes. The performance of seedlings significantly ($P<0.001$) improved by treatments with the *B. aquimaris* strain OD14 (Accession number of MT278260), *B. megaterium* strain JX285 (Accession number of MN626631) and *B. zhangzhouensis* strain Tv91C (Accession number of MN611359) individually and in combination with each other. Their applications improved the growth of lime plants under salinity stress. The inoculation of this bacterial endophyte significantly increased plant growth attributes, i.e. length of shoot and root, leaves and root biomass, shoot diameter, root width, leaf and branch count, chlorophyll and carotenoid contents, and antioxidant activity, compared to the salinity treatment alone. This shows that bacterial endophytes played a regulatory role in mitigating salinity stress. The *Bacillus zhangzhouensis* strain Tv91C had the highest level of seedling growth and resistance against salt stress.

Conclusion

ROS removal from cells occurs either directly (by CAT, APX, SOD and POD) or indirectly (by redox antioxidants such as glutathione). Isolated endophytes showed the ability to grow effectively under saline conditions without compromising their potent cellular machinery for antioxidant production, as observed from the activities of GR, POD, APX, SOD, and CAT. The results also suggest that bacterial endophytes may stabilize ROS-based adverse effects by regulating antioxidants and related enzymes. The inoculation of bacterial endophyte strains may improve plant growth-promoting effects, similar to the well-known effects of PGPR and osmoprotectants in crop plants. Our results suggested that bacterial endophytes mitigated the adverse effects of salinity stress in Mexican lime plants and can be used in horticulture as an environmentally-friendly method.

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Conflict of Interest

The authors indicate no conflict of interest for this work.

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