

EFFECT OF ENDOPHYTE INOCULATION ON THE ACCUMULATION OF MINERAL ELEMENTS AND ORGANIC ACIDS IN RICE (*ORYZA SATIVA* L.) UNDER OSMOTIC STRESS

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Abstract. This study was conducted to investigate the effects of endophyte inoculation on the growth, mineral element and organic acid content of non-endophyte inoculated seedlings (E-) and endophyte inoculated seedlings (E+) of rice (*Oryza sativa* L.) under 0, 5, 10, 15 and 20% PEG for 7 days. Osmotic stress significantly decreased plant height, shoot dry weight and chlorophyll content of the E- and E+, but root length and dry weight were first increased and then decreased. Endophyte inoculation significantly increased plant height, shoot dry weight and chlorophyll content, but decreased root length and had no effects on root dry weight. Endophyte inoculation significantly increased the K, Ca, Mg, and P contents, but reduced the Ni content of the leaves and roots under osmotic stress, while it increased the Mn content of the leaves and the Na content of the roots. Under osmotic stress the E+ accumulated more fumarate in the leaves in comparison with the E-, as well as more malate, acetate, fumarate and oxalate in the roots. These results suggest that endophyte inoculation improved osmotic stress tolerance of rice seedlings by enhancing mineral uptake and organic acid accumulation. The application of endophytes has a beneficial effect on plant tolerance to osmotic stress.

Keywords: plant-endophyte interaction, essential element, organic acid, tolerance

Introduction

Osmotic stresses include drought and salinity. Drought is the primary factor limiting global agricultural production (Sheshbahreh et al., 2019). Drought stress affects many physiological processes, including photosynthesis, assimilate transmission, cell expansion and mineral nutrient accumulation and transfer (Devnarain et al., 2016).

Essential elements have clear physiological roles, and plants cannot complete their life cycles without them. Generally, drought inhibits the uptake and transport of most mineral ions, resulting in nutrient deficiency (Salehi et al., 2016). Maintaining the uptake and homeostasis of mineral nutrients can enhance the resistance of plants to drought stress (Waraich et al., 2011). Water deficit conditions significantly diminish the P and K content of the shoots of *Matricaria chamomilla* (Salehi et al., 2016). In stressed apple plants, uptake of N, P, K, Ca, Mg, Fe, Mn, Cu, Zn, and B was decreased in comparison with that of well-watered plants (Liang et al., 2018).

In general, metal stress significantly increases the organic acid (OA) contents of various plant organs (Mahdavian et al., 2016), but the effects of drought stress on OAs vary. In response to water stress, the abundance of the majority of OAs decreased in wheat (Bowne et al., 2012), maize (Sicher and Barnaby, 2012) and creeping bentgrass (Jespersen et al., 2017). However, Timpa et al. (1986) reported that water-stressed cotton plants showed greater total amounts of organic acids (malate, citrate and oxalate) in comparison with irrigated plants. Under drought stress, citrate, fumarate and malate

accumulation was enhanced in the leaves of drought-tolerant wheat plants, but decreased in the roots (Kang et al., 2019). Moreover, drought stress increased the citrate content of potato leaflets (Barnaby et al., 2015) as well as the succinate content of maize leaves in a greenhouse study (Witt et al., 2012) and the malate content of thyme leaves (Ashrafi et al., 2018). Kim et al. (2017) found that acetate content is positively correlated with the survivability of crop plants such as wheat, rice, maize and canola. Moreover, OAs are intermediates involved in the assimilation of carbon and nitrogen, as well as osmotic regulation (Ashrafi et al., 2018).

The interaction between plants and endophytes is helpful for plants to deal with stress environment (Jung et al., 2012; Chinnaswamy et al., 2018). Plant growth promoting bacteria can improve the seed germination and enhance seedling growth of tomato under osmotic stress (Bhatt et al., 2015). Plant growth promoting rhizobacteria (PGPR) can alter the ion content of the leaves of tomato plants under salt stress (Van Oosten et al., 2018). Plant OAs such as oxalate and butyrate are by various stress (Ashraf and Harris, 2004). Endophytic fungus *Piriformospora indica* increased the amounts of malate, citrate and oxalate in the rhizosphere soil of *Brassica napus* seedlings (Wu et al., 2018). Soil, foliar, and soil + foliar applications of PGPR to promote strawberry yield also increased OA content under field conditions (Kitir et al., 2019).

Rice is very sensitive to osmotic stress at different growth stages (Swapna and Shylaraj, 2017; Nahar et al., 2018). In a previous study, we found that endophyte inoculation improves rice growth by enhancing the uptake of nutrient and altering the accumulation of OA under Na_2CO_3 or Pb stress (Li et al., 2017, 2019). There is little information on the use of endophytes to improve the accumulation of OAs and uptake of minerals in plants under osmotic stress. Therefore, this study was to investigate the potential beneficial effects of endophyte application on rice seedling under osmotic stress.

Materials and methods

Endophytic fungus and plant material

Endophytic strain EF0801 was isolated from the leaves of *Suaeda salsa* grown under saline zones across China and screened for Na_2CO_3 tolerance. Molecular identification of fungus EF0801 was based on internal transcribed spacer regions, which showed that it is congeneric to *Sordariomycetes* sp. (99% similarity). EF0801 was cultured on potato dextrose agar (PDA) plates at 4°C. To produce a 5% fungus culture, plates containing 75 mL of PDA solution were inoculated at the 3-day instar stage and cultured for 12 days at $24 \pm 1^\circ\text{C}$ with shaking at 180 rpm. The resulting fungal cultures were used for the infection treatments. The experimental materials, rice (*Oryza sativa* L.) seeds (Liaoxing1') were provided by Shenyang Agriculture University, China. Seeds were sterilized with NaOCl for 10 min, rinsed, germinated and grown on Hoagland's solution in an illumination chamber.

Experimental treatments

Three-day-old rice seedlings were divided into: non endophyte inoculated seedlings (E-) and endophyte inoculated seedlings (E+). E- were grown on Hoagland's solution, whereas E+ were grown on Hoagland's solution containing 5% fermentation broth, and endophyte EF0801 colonization was achieved via the rice roots. Each group (100 seedlings/pot) was exposed to 0 (control), 5, 10, 15 or 20% PEG-6000 (w/v in Hoagland

solution). Endophyte inoculation and PEG exposure were carried out simultaneously. According the method of Liu and Chen (2007) to determine the degree of endophyte inoculation. Roots were cleared in 20% KOH, acidified by 5% acetic acid and then stained. More than 90% of the E+ were colonized, whereas the E- were not colonized. The rice seedlings were cultured in an illumination chamber (28°C/22°C day/night, 14/10 h light/dark, 800 $\mu\text{mol m}^{-2}\text{s}^{-1}$ PPFD, and 80% air humidity). Fresh Hoagland's solution was added every day. After one week, the seedlings were collected and prepared for the analyses.

Estimation of growth parameters

Shoot height and root length of ten seedlings were recorded and then they were oven-dried at 80 °C for 48 h, after which the dry weight was measured.

Estimation of chlorophyll (Chl) content

Chlorophyll were extracted from fresh leaf with 80% acetone in the dark. Chl content was measured using the method reported by Lichtenthaler (1987).

Estimation of mineral elements

The leaves and roots of seedlings were oven-dried, ground and then pass through a 100-mesh sieve. Each sample (100 mg) was digested with $\text{HNO}_3/\text{HClO}_4$ (5:1 [v/v]) at 2600 kPa for 30 min in a microwave oven and brought to 50 mL with ultrapure water. The contents of macroelements (K, Ca, Mg and P) and microelements (Fe, Zn, Mn and Ni) were estimated using an inductively coupled plasma atomic emission spectrometer (ICP model Liberty 200, Varian Australia Pty. Ltd., Mulgrave Victoria, Australia) as reported by Filek et al. (2012).

$$\begin{aligned} &\text{The percentage changes in measured elemental content per seedling} \\ &(\%) = [(\text{measured elemental content in E+}) - (\text{measured elemental} \quad (\text{Eq.1}) \\ &\quad \text{content in E-})]/(\text{measured elemental content in E-}) \times 100 \end{aligned}$$

Estimation of OAs

Fresh sample was ground in deionized water, incubated at 70 °C for 15 min, and centrifuged at $10,000 \times g$ at 4 °C for 15 min. The supernatant was filtered, evaporated to dryness under reduced pressure at 40 °C, and then dissolved in ultrapure water to allow estimation of OAs by high performance liquid chromatography (Agilent 1200 HPLC System) according to the methods reported by Li et al. (2017).

Data analysis

The normality and homoscedasticity of the data were tested prior to statistical analysis. The effects of endophyte and osmotic stress were analyzed using two-way ANOVA. The threshold of significance for differences among the treatments was used LSD multiple comparisons ($p < 0.05$).

Results

Growth parameter and chlorophyll (Chl) content

Osmotic stress significantly decreased the plant height and Chl content of the E- and E+ (Fig. 1), but first increased and then decreased root length (Table 1). Endophyte inoculation significantly increased plant height and Chl content, but significantly decreased root length under osmotic stress.

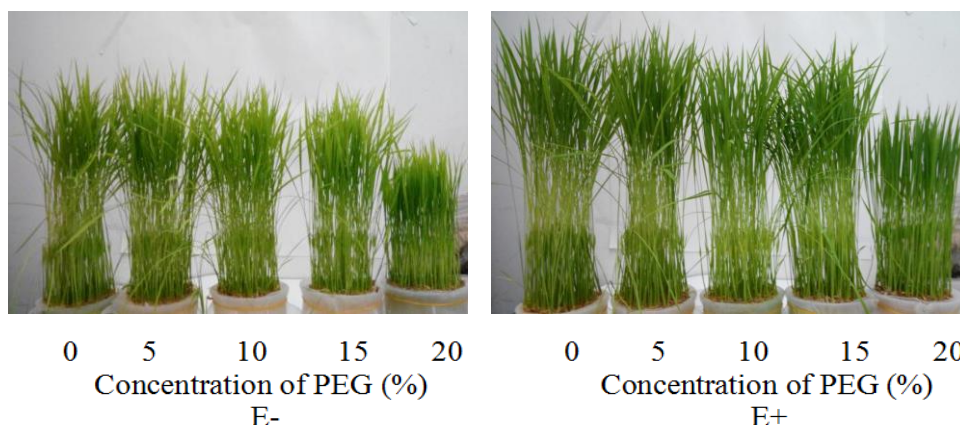


Figure 1. Effects of endophyte inoculation on the growth of rice seedlings subjected to osmotic stress

Table 1. Effects of endophyte inoculation on the growth, dry weight and Chl content of rice seedlings subjected to osmotic stress

Treatments	Plant height (cm)	Root length (cm)	Dry weight of shoots (mg/10 plant)	Dry weight of roots (mg/10 plant)	Chla+b content (mg/g·FW)
E-					
0	18.89±0.71cd	9.12±0.58b	70.68±4.29a	22.18±0.39bc	2.60±0.06c
5	17.75±0.95e	9.67±0.46a	54.76±3.09cd	24.77±0.36a	2.44±0.18cd
10	16.59±0.87f	7.94±0.56c	49.15±2.33de	22.31±0.23bc	2.30±0.15cd
15	14.94±0.90g	7.40±0.72cd	44.50±2.75ef	21.98±0.69c	2.13±0.18de
20	12.43±0.69i	6.90±0.73ed	39.37±2.67f	21.74±1.11c	1.82±0.13e
E+					
0	22.99±0.84a	8.03±0.73c	72.47±4.92a	22.34±0.65bc	3.74±0.22a
5	21.25±1.17b	8.80±0.66b	63.40±4.86b	25.09±0.66a	3.52±0.34ab
10	19.38±0.78c	7.22±0.41de	57.12±3.02bc	23.10±0.35b	3.36±0.26b
15	18.29±1.04de	7.06±0.86de	53.12±3.82cd	22.06±0.49c	2.58±0.18c
20	13.70±1.01h	6.36±0.56f	43.12±4.06ef	21.87±0.21c	2.23±0.20d

Values are the mean ± standard deviation of three replicates. Different letters indicate a significant difference at $P < 0.05$ (LSD test)

Osmotic stress significantly decreased the shoot dry weight of the E- and E+, but first increased and then decreased the root dry weight (Table 1). Endophyte inoculation significantly increased the shoot dry weight under 5–15% PEG, but showed no effects on the root dry weight.

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The elemental content of leaves

Osmotic stress significantly decreased the K and Fe contents of the E- (Fig. 2A,D). Endophyte inoculation significantly increased the K content of the leaves of plants subjected to 20% PEG, but it had no influence on Fe content.

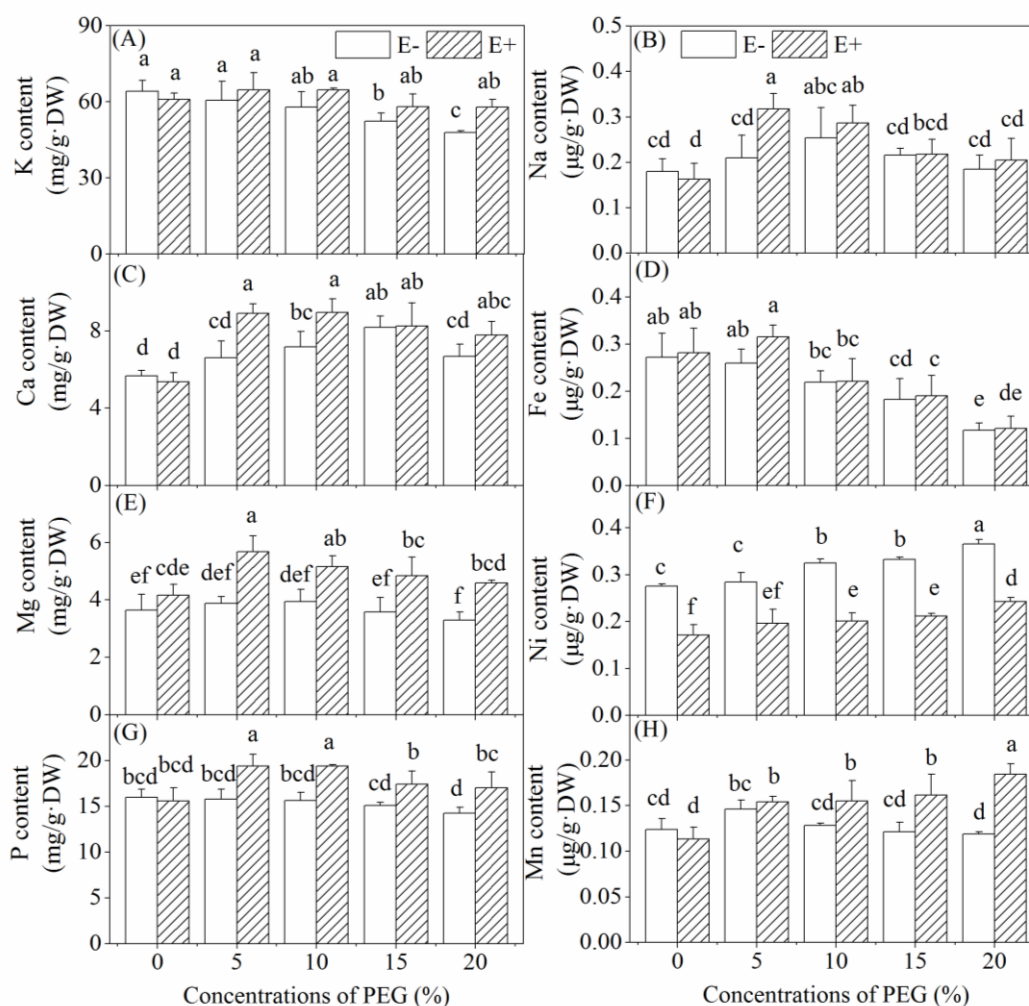


Figure 2. Effects of endophyte inoculation on the contents of macroelements (K, Ca, Mg and P) and microelements (Na, Fe, Ni and Mn) in the leaves of rice seedlings subjected to osmotic stress. The bars indicate the standard deviation ($n=3$). Different letters indicate a significant difference at $P < 0.05$ (LSD test)

Osmotic stress had no influence on the Na, Mg, P, or Mn contents in the leaves of the E- (Fig. 2B,E,G,H). In the E+ seedlings, osmotic stress increased Mn content, while it first increased and then decreased the Na, Mg and P contents. Endophyte inoculation significantly increased the Mg, P and Mn contents in plants subjected to osmotic stress

(except Mn in plants subjected to 5% PEG), while it only increased the Na content in plants subjected to 5% PEG.

Osmotic stress first increased and then decreased Ca content, while it increased Ni content in the leaves of E- and E+ (Fig. 2C,F). Endophyte inoculation significantly increased the Ca content of plants subjected to 5% and 10% PEG, but it significantly decreased Ni content under osmotic stress.

The elemental content of roots

Osmotic stress had no influence on the K and Fe contents in the roots of the E- (Fig. 3A,D). Endophyte inoculation significantly increased the K content under 10–20% PEG, but increased the Fe content under no PEG.

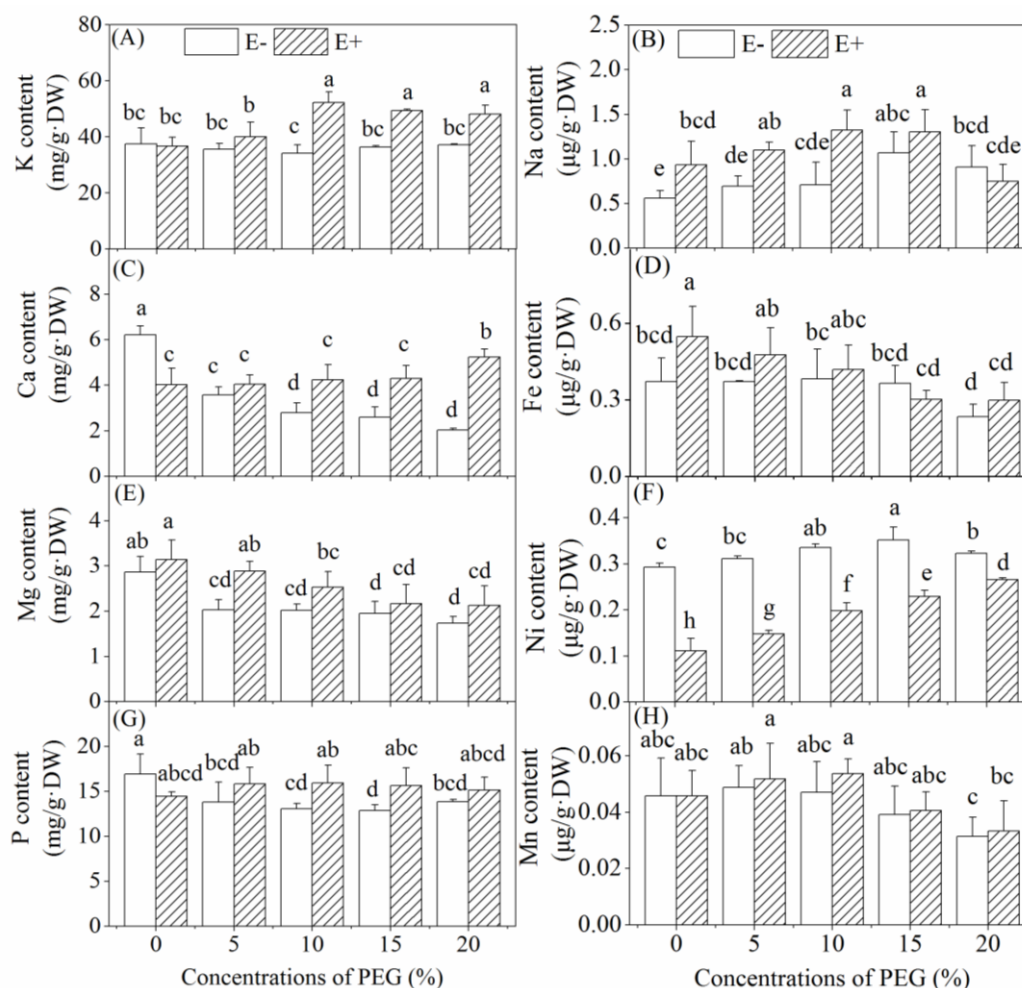


Figure 3. Effects of endophyte inoculation on the contents of macroelements (K, Ca, Mg and P) and microelements (Na, Fe, Ni and Mn) in the roots of rice seedlings subjected to osmotic stress. The bars indicate the standard deviation (n=3). Different letters indicate a significant difference at $P < 0.05$ (LSD test)

Osmotic stress first increased and then decreased the Na and Ni contents in the roots of the E- (Fig. 3B,F). Endophyte inoculation significantly increased the Na content of

plants subjected to 0–10% PEG, but it significantly decreased Ni content under osmotic stress.

Osmotic stress significantly decreased the Ca, Mg, P, Mn contents in the roots of the E- (Fig. 3C,E,G,H). Endophyte inoculation significantly decreased the Ca content of plants under no PEG, but it significantly increased that of plants subjected to 10–20% PEG. Endophyte inoculation significantly increased the P content of plants subjected to 10% and 15% PEG, while it had no influence on the Mg or Mn contents of plants subjected to PEG, with the exception of increased Mg content in plants subjected to 5% PEG.

The percentage changes of elemental content

For the percentage changes in the measured elemental content of the E+ relative to the E-, positive values show an increase, whereas negative values show a decrease according to Eq.1 (Table 2). Under osmotic stress, the percentage changes of most measured elements were positive values, which showed that the endophyte enhanced the total elemental content per seedling. However, the percentage change of Ni content was negative, which showed that the endophyte reduced the total Ni content per seedling.

Table 2. Effects of endophyte inoculation on the percentage changes in element contents per seedling subjected to osmotic stress

PEG (%)	K	Ca	Mg	P	Na	Fe	Ni	Mn
0	-2.28±0.51	-11.21±2.18	15.53±1.24	-3.64±0.61	29.98±3.47	18.92±3.14	-42.57±5.38	-5.33±1.13
5	21.62±6.14	48.08±4.44	64.73±7.15	34.78±2.63	66.42±5.89	36.33±4.55	-30.47±5.61	20.37±1.87
10	35.80±2.55	47.14±5.10	47.92±3.58	39.32±2.12	65.49±6.47	15.70±1.89	-31.61±3.16	37.42±3.61
15	33.28±3.74	26.60±1.74	51.11±6.02	32.99±3.41	21.83±2.28	3.69±1.37	-27.57±2.28	51.41±5.17
20	31.81±3.65	46.68±6.01	45.80±5.14	23.68±3.11	-6.92±3.16	20.87±4.47	-23.92±4.25	61.74±6.25

The OA content of leaves

Osmotic stress significantly decreased the tartrate, citrate and fumarate contents (Fig. 4A,E,G). Endophyte inoculation showed no significant effects on the tartrate or citrate contents of the leaves in plants under osmotic stress, with the exception of the tartrate content in plants under 20% PEG and the citrate content in plants under no PEG. However, endophyte inoculation significantly increased the fumarate content of the leaves in the E+ under osmotic stress.

In contrast, osmotic stress significantly increased the acetate and lactate contents of the E- (Fig. 4C,D). The lactate content of the E- and E+ did not differ significantly except in those subjected to 20% PEG, while the acetate content of the E+ was significantly greater than that of the E- subjected to 0 and 20% PEG.

Osmotic stress first increased and then decreased the malate and succinate contents of the E- (Fig. 4B,F). The malate content of the E+ was significantly greater than that of E- subjected to no PEG, and the succinate content of the E+ was significantly greater than that of E- subjected to 0 and 10% PEG.

Osmotic stress and endophyte inoculation had no significant effects on the oxalate content in leaves (Fig. 4H).

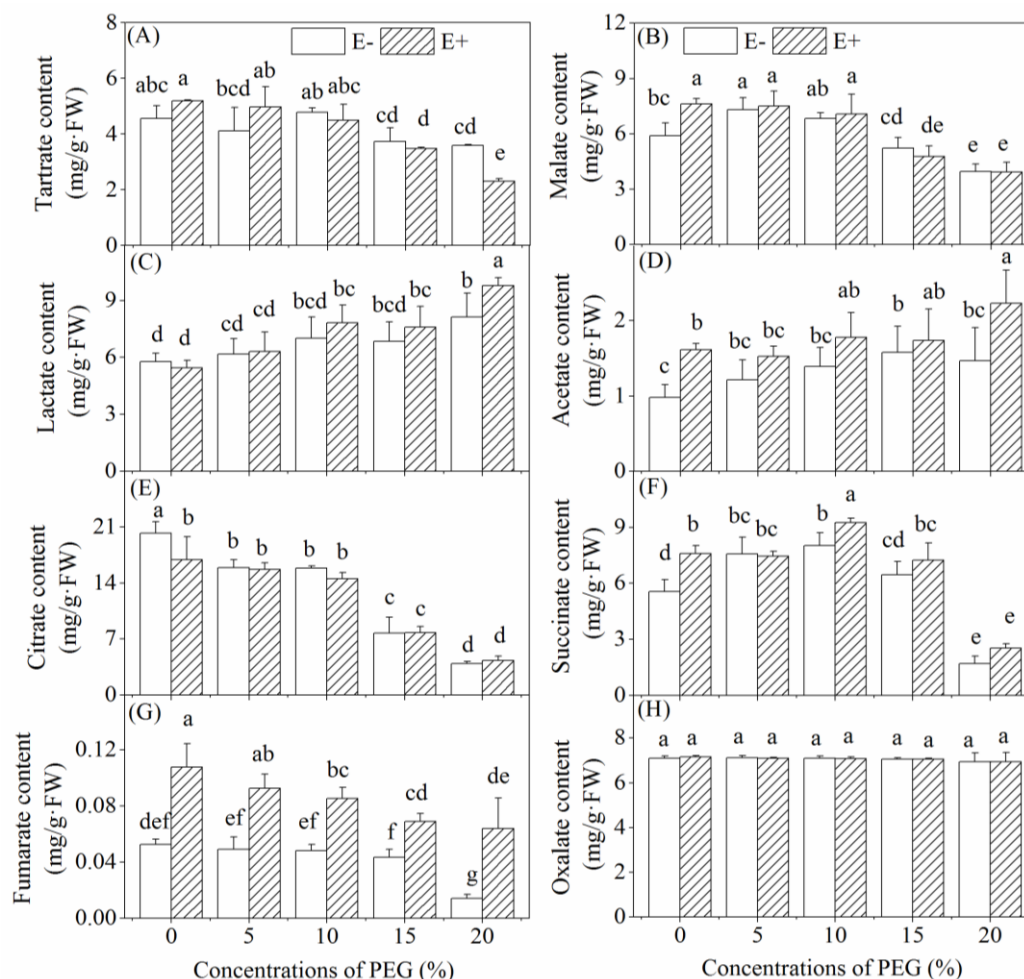


Figure 4. Effects of endophyte inoculation on the accumulation of eight OAs in the leaves of rice seedlings subjected to osmotic stress. The bars indicate the standard deviation ($n=3$). Different letters indicate a significant difference at $P < 0.05$ (LSD test)

The OA content of roots

Osmotic stress significantly decreased the tartrate, malate, citrate and oxalate contents of the E- (Fig. 5A,B,E,H). Compared to the E-, the tartrate content in the roots of the E+ subjected to 5% PEG was significantly reduced, but it was significantly increased in those subjected to 20% PEG. The malate content of the E+ was significantly greater than that of the E- subjected to 10% and 20% PEG, while the oxalate content of the E+ was significantly greater than that of the E- seedlings subjected to 15% and 20% PEG. Endophyte inoculation had no significant effect on the citrate content in roots.

Osmotic stress significantly increased the lactate and succinate contents of the E- (Fig. 5C,F), but endophyte inoculation had no significant effect on either of these OAs.

Osmotic stress first decreased and then increased the acetate content (Fig. 5D), whereas first increased and then decreased the fumarate content of the E- (Fig. 5G). The acetate content of the E+ was significantly greater than that of E- subjected to 5% and 10% PEG. The fumarate content of the E+ was significantly lower than that of E- subjected to 5% PEG, but it was significantly greater than that of E- subjected to 15% and 20% PEG.

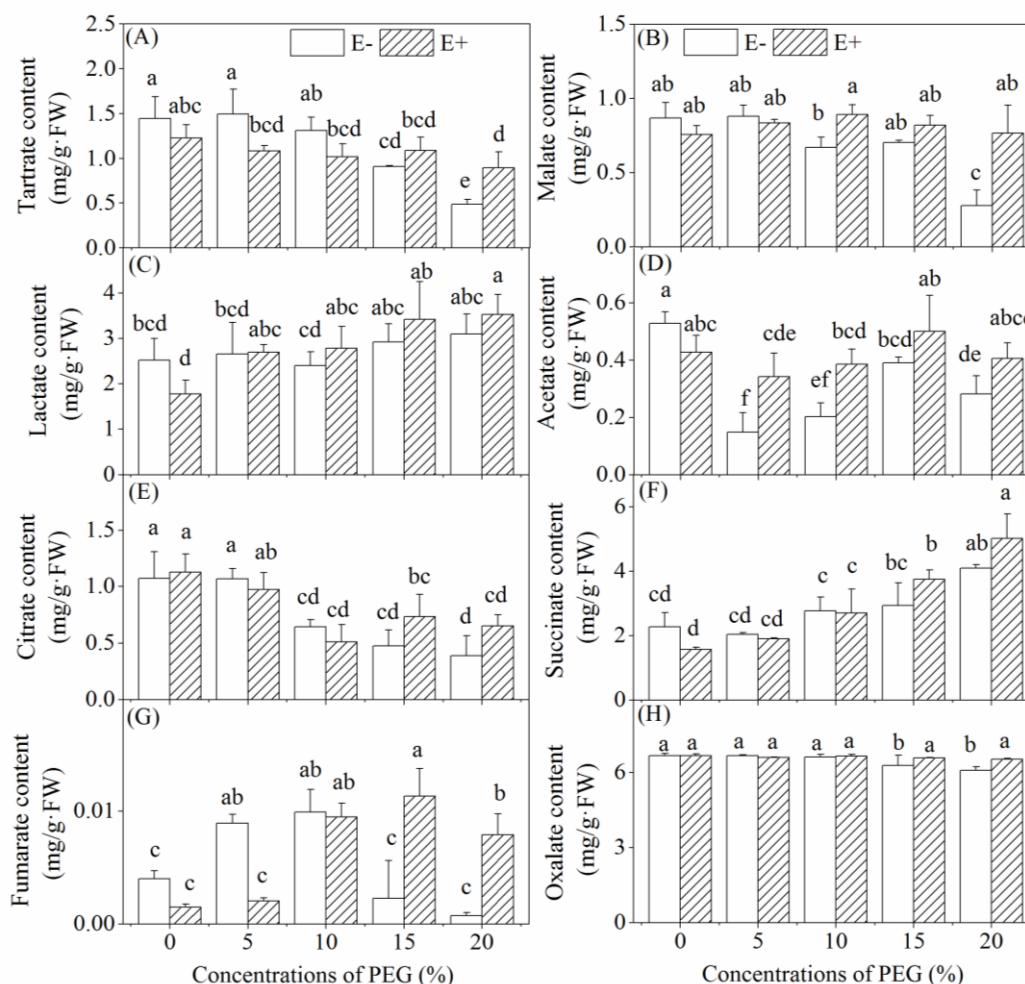


Figure 5. Effects of endophyte inoculation on the accumulation of eight OAs in the roots of rice seedlings subjected to osmotic stress. The bars indicate the standard deviation ($n=3$). Different letters indicate a significant difference at $P < 0.05$ (LSD test)

Discussion

It is well established that osmotic stress significantly inhibits growth by plants (Swapna and Shylaraj, 2017; Nahar et al., 2018). We found that osmotic stress produced significant depressive effect on the rice growth (Table 1). PGPR has been shown to increase plant resistance to osmotic stress (Ghosh et al., 2019). Our results suggest that endophyte inoculation alleviated decreases in plant height, the shoot dry weight and Chl content caused by osmotic stress.

Ion-mediated up-regulation of xylem hydraulics plays an important role in optimizing the translocation of water and nutrients, as well as in regulating plant tolerance (Oddo et al., 2011). The water potential in soil significantly affected the uptake of mineral nutrients (Salehi et al., 2016). Drought stress prevents the absorption of mineral nutrients by tomato (Sánchez-Rodríguez et al., 2010). Salt stress decreased Ca, Mg, Fe, and Zn concentrations in lucerne and white melilot (Yasar et al., 2014). In this research, we found that osmotic stress significantly decreased the uptake of K and Fe by the leaves, as well as the uptake of Ca, Mg, and P by the roots. Sucre and Suárez (2011) suggest that plants increase

absorption of Na^+ when they are subjected to water stress. Similar to their results, we noted that the Na content of rice seedlings was significantly increased under low-concentration PEG. The content of microelement Ni in rice seedlings was significantly increased by osmotic stress, which could lead to toxicity.

Endophytes can improve the uptake of many mineral nutrients in plants (Song et al., 2014). Reestablishing the ionic homeostasis of plants under osmotic stress can increase plant resistance, which can decrease the severity of injuries caused by water deficits and alleviate growth inhibition (Waraich et al., 2011). We observed that endophyte inoculation enhanced accumulation of most mineral nutrients which alleviated the detrimental effect of osmotic stress on rice seedlings (Table 2). Increased abundance of mineral nutrients, particularly K, Ca, Mg and P, can improve the cell water potential, stomatal conductance, Chl content and photosynthetic rate of plants (Ruiz-Sánchez et al., 2010). Furthermore, endophyte inoculation inhibited accumulation of Ni under osmotic stress, which may have alleviated the toxic effect of excessive Ni on the E^+ .

OAs play a vital role in protecting plants from stress by regulating osmotic potential, ionic balance, and other cellular processes (Ma et al., 2011). Accumulation of OAs is an important process involved in the development of increased drought tolerance (Jespersen et al., 2017; Kang et al., 2019). In *Phyllanthus*, drought stress increased the abundance of OAs such as malic, succinic, and citric acids (Filho et al., 2018). Moreover, the increased abundance of OA, such as malate, citrate and oxalate, can improve drought tolerance of *ipt* transgenic creeping bentgrass (Merewitz et al., 2012). In this study, the lactate and succinate contents of rice increased as osmotic stress increased. However, Dickinson et al. (2018) found that legumes suffering from abiotic stress showed decreased abundance of many OAs, including malate and citrate. We also found that the tartrate, malate and citrate contents of rice decreased as osmotic stress increased. Griesser et al. (2015) reported increased abundance of citrate, succinate and tartarate in rapevine leaves under drought stress, while that of malate was decreased. These results suggest that different OAs change in different manners in response to stress.

Endophytes can modulate OA metabolism in plants (Singh et al., 2018). Wu et al. (2018) showed that the accumulation of OAs (oxalate, malate and citrate) in the rhizosphere of *Brassica napus* was improved by endophytic fungus *Piriformospora indica*. The tolerance of *Nicotiana benthamiana* to water stress was enhanced due to increased accumulation of some OAs as a result of the presence of fungal endophytes (Dastogeer et al., 2017). In this study, endophyte inoculation significantly enhanced the accumulation of fumarate in the leaves of rice seedlings, and the accumulation of malate, oxalate, fumarate in the roots, which indicated that the leaves and roots trigger different changes in OA metabolism to increase tolerance to water stress.

Conclusions

Osmotic stress altered the accumulation of OAs and inhibited the uptake of nutrient element by rice seedlings, which alter plant growth and stress tolerance. The endophyte used in the present study is capable of enhancing accumulation of some OAs and improving nutrient absorption. The increased content of OAs and nutrient elements in E^+ promoted rice growth, facilitated osmotic adjustment, and induced osmotic stress tolerance. These findings suggest that endophytes could be used to improve plant resistance to abiotic stress in an eco-friendly way. There are intricate interactions between

plant metabolism changes and each stress, so future study will investigate the signaling pathways involved in the activation of these metabolism changes.

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