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REGULAR ARTICLE

Effect of the fungal endophyte *Epichloë bromicola* on polyamines in wild barley (*Hordeum brevisubulatum*) under salt stress

Taixiang Chen · Chunjie Li · James F. White · Zhibiao Nan

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Abstract
Background and aims The endophytic fungus *Epichloë bromicola* forms mutualistic symbiotic associations with wild barley (*Hordeum brevisubulatum*) in the saline-alkali areas of northwestern China. *E. bromicola* enhances the tolerance of *H. brevisubulatum* to salt stress. Because plant polyamine metabolism is closely related to microbial infection and tolerance to diverse abiotic stresses, we hypothesized that in symbiotic plants polyamine modification may result from *E. bromicola* infection, and that improved tolerance to abiotic stress by the presence of this endophyte might be related to polyamine modification. Our focus in this study was to investigate whether *E. bromicola* affects polyamine metabolism in host plants under salt stress.

Methods E. bromicola infected (E+) and endophyte free (E-) wild barley plants were subjected to NaCl treatments (0, 100, 200 and 300 mM). Dry weight, diamine putrescine (Put), triamine spermidine (Spd) and tetramine spermine (Spm) content and the content of their free, soluble conjugated and insoluble bound forms were measured after 21 d exposure to stress.

Results E. bromicola infection led to significant amelioration of salt stress in *H. brevisubulatum*. The presence of the endophyte significantly increased dry weight, spermidine and spermine content, but decreased putrescine content and the putrescine:(spermidine + spermine) ratio. *E. bromicola* infection also lowered the proportion of putrescine in total polyamines, but increased the proportion of spermidine and spermine in total polyamines. Furthermore, *E. bromicola* infection significantly increased the proportion of insoluble bound forms of polyamines, and decreased the proportion of free forms of polyamines and soluble conjugated forms of polyamines.

Conclusions H. brevisubulatum salinity stress tolerance induced by *E. bromicola* infection correlated with enhanced conversion of putrescine to spermidine and spermine, as well as improved shift ability from free forms and soluble conjugated forms of polyamines to insoluble bound forms of polyamines.

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Keywords *Hordeum brevisubulatum* · *Epichloë* endophyte · Salt stress · Polyamines · Free form polyamines · Soluble conjugated form polyamines · Insoluble bound form polyamines

Introduction

Salinity is a most persistent and ever-increasing problem in many arid and semi-arid ecosystems of the world (Omar et al. 2009). Salinity stress reduces growth and changes performance and morphology of plants by a multitude of mechanisms including water deficiency, ion toxicity, hormonal imbalance, reactive oxygen toxicity, and nutritional imbalance which may cause membrane destabilization (Flowers 2004; Omar et al. 2009). To combat this toxicity, plant cells synthesize some metabolites, and activate some salt stress related genes and proteins (Roy et al. 2014). Among these classes of metabolites, polyamines are one of the most effective against salinity stress (Groppa and Benavides 2008).

Polyamines are small aliphatic nitrogen compounds that are present in free, soluble conjugated (covalently conjugated with small molecules such as phenolic acids) or insoluble bound forms (bound with macromolecules such as proteins, DNA and RNA). Diamine putrescine (Put), triamine spermidine (Spd) and tetramine spermine (Spm) are three of the most abundant and widespread naturally occurring polyamines. These polyamines are ubiquitous in a wide range of organisms from animals to plants and microbes (Alcázar et al. 2006; Groppa and Benavides 2008). In plants, polyamines influence several physiological processes, including ion channels, antioxidant systems, photosynthesis and nutrient transport systems (Ioannidis et al. 2012; Saha et al. 2015), but not always with the same specificity and efficiency. Changes in polyamine concentrations are essential for regulation of polyamine signaling systems during abiotic stress, including balance between polyamine synthesis and polyamine catabolism, balance with plant hormones, and balance between antioxidant functions and reactive oxygen species (ROS) production (Pál et al. 2015). The biosynthesis and degradation pathways and key enzymes of polyamine metabolism have been well characterized (Duan et al. 2008; Fu et al. 2016; Groppa and Benavides 2008; Kusano et al. 2008; Pál et al. 2015), and polyamines in plants are dynamic and undergo rapid inter-conversion in the polyamine cycle (Fig. 1).

Many studies have shown that plant polyamine metabolism is implicated in inducing tolerance under a wide range of abiotic stresses, including salinity, drought, extreme environmental temperature, heavy metals, ultraviolet - B radiation, mechanical damage and herbicide treatment (Groppa and Benavides 2008; Kasukabe et al. 2004). For example, salt stress increases the free, soluble conjugated, and total spermine content in sunflower leaf tissues (Mutlu and Bozcuk 2005). Salinity stress caused a significant increase in spermine and spermidine content but lowered putrescine content in seedlings of tomato, pepper, lettuce and broccoli (Zapata et al. 2004). In numerous plants, accumulation of polyamine content resulting from various abiotic stresses often correlates with improvement of plant tolerance. It has also long been known that polyamine metabolism changes in plant cells interacting with pathogenic fungi (Asthir et al. 2004), virus infection (Walters 2003), and plant-mycorrhizal associations (Kytöviita and Sarjala 1997; Walters 2000). For example, brown rust (*Puccinia hordei*) and powdery mildew (*Blumeria graminis* f. sp. *hordei*) infection increased polyamine accumulation in barley (*Hordeum vulgare*) (Greenland and Lewis 1984; Walters and Wylie 1986). Infection by the fungal pathogens *Erysiphe cichoracearum* and *Alternaria tenuis,* and the bacterial pathogen *Pseudomonas tabaci* resulted in a decreased free putrescine and spermidine content in tobacco leaves, with the greatest reduction in infection by pathogens causing the most severe tissue damage (Edreva 1997). Higher contents of free polyamines were detected in association with *Lotus glaber* and arbuscular mycorrhiza (*Glomus intraradices*) compared to non-mycorrhizal *Lotus glaber* (Sannazzaro et al. 2007). However, research on polyamine metabolism in interactions between microorganisms and plants lags behind other research regarding plant polyamines. The available data demonstrates that the nature of polyamine changes depends upon the microbe, plant part involved, and the nature of the plant-microorganism interaction with the host (i.e., mutualistic or parasitic).

Species of the fungal genus *Epichloë* (formerly *Neotyphodium*) are a group of biotrophic endophyte that form symbioses with temperate grasses in the subfamily Pooideae (Hettiarachchige et al. 2015; Schardl et al. 2013); they live asymptomatically and intercellularly within the above-ground parts of the host, and roots are not colonized by the fungal

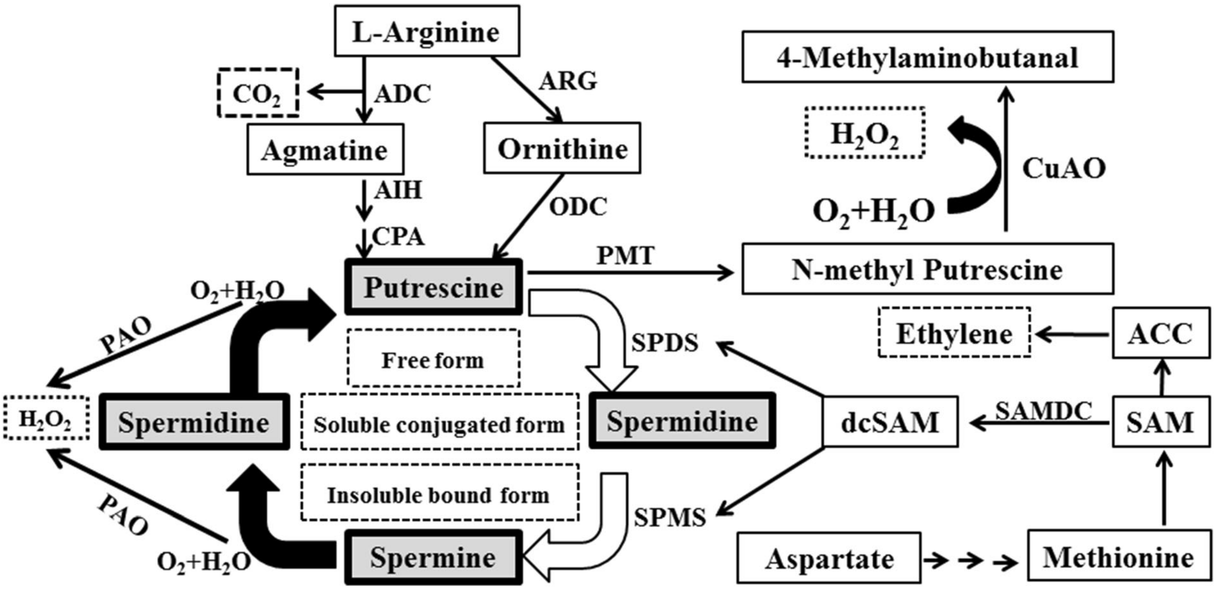

Fig. 1 Pathways of synthesis and catabolism of the main polyamines (putrescine, spermidine and spermine) in plants. (Based on Gupta et al. 2013; Pál et al. 2015; Kuznetsov and Shevyakova 2007). ADC: arginine decarboxylase; ODC: ornithine decarboxylase; ARG: arginase; AIH: agmatine iminohydrolase; CPA: *N*-carbamoyl putrescine amidohydrolase; SPDS: spermidine synthase; SPMS: spermine synthase; PAO: polyamine oxidase; PMT: putrescine *N*-methyltransferase; CuAO: copper-dependent amine oxidase; SAMDC: *S*-adenosylmethionine decarboxylase; ACC: 1-aminocyclopropane-1-carboxylic acid; SAM: *S*-adenosylmethionine; dcSAM: decarboxylated *S*-adenosylmethionine; White arrows: synthesis, black arrows: catabolism

endophyte. *Epichloë* endophyte are present in embryos of seeds by virtue of their colonization of reproductive tissues of the parent plant. The growth of the fungal endophyte is fully synchronized with that of host grasses, beginning with growth of the fungus into embryos upon seed germination, and hyphal colonization of leaves as leaves are developing. When leaf growth stops, hyphal growth ceases but hyphae remain metabolically active for the life of the leaf (Christensen et al. 2008). Some species of *Epichloë* are vertically transmitted via the mother plant lineage to the seeds. This type of spread is clonal and asexual, resulting in the same endophyte genotype being transmitted to seeds. Other *Epichloë* species are sexual, developing an external stroma that encloses and inhibits inflorescence development, causing the condition referred to as “choke disease”. Reproductive spores (ascospores) released from stromata can infect other healthy grasses of the same species, and thus are horizontally transmitted (Christensen et al. 2008; Leuchtmann et al. 2014; Saikkonen et al. 2013; Saikkonen et al. 2004; Soto-Barajas et al. 2016). Most *Epichloë* species are host specific and most of the asexual species are confined to a single host grass (Ekanayake et al. 2012; Gundel et al. 2012).

Many studies have shown that *Epichloë* endophyte play a role in enhancing tolerance of the grass host to stresses such as high soil saline content (Reza and Mirlohi 2010; Song et al. 2015a), drought (Malinowski and Belesky 2000; Oberhofer et al. 2014), high soil heavy metal content (Monnet et al. 2001; Zhang et al. 2010), insects (Kuldau and Bacon 2008) and disease (Iannone et al. 2017; Ma et al. 2015; Xia et al. 2015). *Epichloë* endophyte-mediated stress protection is often associated with increased nutrient absorption and secondary metabolites. For example, specific fungal secondary metabolites, such as loline alkaloids produced by some *Epichloë* endophyte, affect osmotic potential and reduce the effects of drought stress (Kuldau and Bacon 2008). *Epichloë* endophyte involved in nutrient absorption and ionic balance adjustment increase the competitive ability of the host plant under salinity stress (Reza and Mirlohi 2010; Song et al. 2015a). Although improvements in stress tolerance of host grasses infected by *Epichloë* endophyte are well known, there has been limited study of the effects of *Epichloë* infection on host-produced secondary metabolites. It seems likely that presence and metabolic activity of *Epichloë* endophyte may substantially contribute to or alter metabolic activities of the host.

To our knowledge, there is a paucity of research on involvement of polyamine metabolism in *Epichloë* endophyte-host plant interactions subjected to abiotic stress. We hypothesized that improved tolerance to abiotic stress by *Epichloë* infection might be related to polyamine modification, and this may be a remediating mechanism of *Epichloë* endophyte. Therefore, in this work we aimed to investigate whether the presence of an *Epichloë* endophyte affects polyamine metabolism in host plants under salt stress, and whether the protective role to host plants was associated with polyamine metabolism.

Wild barley (*Hordeum brevisubulatum*) is an important forage grass that is widely adaptable to a variety of stress conditions, including salt, alkali and water stresses, and for this reason, *H. brevisubulatum* is often used as a model plant in studies on salinity (Wang et al. 2016). In addition, the asexual *Epichloë* endophyte *E. bromicola* is present in the majority of *H. brevisubulatum* plants with infection frequencies ranging from 80% to 90% in the saline-alkali areas of Linze county, Gansu province, China (Song et al. 2015b; Chen et al. 2018). It has been reported that *E. bromicola* plays an important role in improving wild barley salt stress by adjusting ionic homeostasis and modifying nutrient stoichiometry (Song et al. 2015a). Furthermore, many studies have shown the existence of links between polyamine metabolism and redox homeostasis (Gupta et al. 2013), ion homeostasis (Yamasaki and Cohen 2006) and nutrient uptake and transport (Tytti and Seppo 2002). For this reason, *H. brevisubulatum* was selected as test material for this study. In this study we examined changes in content of three kinds of polyamines (putrescine (Put), spermidine (Spd) and spermine (Spm)) and their presence in various forms (free, soluble conjugated or insoluble bound forms) to gain insights as to whether polyamines are the mechanism by which the systemic *Epichloë* endophyte enhances growth of *H. brevisubulatum* in high salinity environments.

Materials and methods

Plant material

Mature reproductive tillers of *H. brevisubulatum* plants were collected from natural populations on property of the Linze Experimental Station (Lanzhou University, Gansu province, China). *Epichloë* endophyte infection status was then determined by microscopic examination using the aniline blue staining method and PCR using *Epichloë* specific primers (tub2-exon 4u-2: GTTTCGTCCGAGTTCTCGAC, tub2-exon 1d-1: GAGAAAATGCGTGAGATTGT) (Moon et al. 2002). The PCR analyses were performed in a total volume of 25 μl (12 μl 2 × Taq MasterMix (ComWin Biotech Corp., Ltd., Beijing, China), 10 μl double-distilled H_2O, 1 μl DNA (50 ng/μl), 1 μl target-specific forward primer (10 μM) and 1 μl target-specific reverse primer (10 μM)). The cycling condition used to amplify *tub2* were an initial denaturation step for 5 min at 94 °C, 30 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 1 min, extension at 72 °C for 1 min, followed by a final synthesis step at 72 °C for 10 min. Products were analysed by gel electrophoresis on a 1.5% agarose gel in 1 × TAE buffer. The DNA fragments were stained with Gold View (Solarbio Corp., Beijing, China) and viewed by UV transillumination. As positive controls, we used *E. bromicola* WBE1 isolated from *H. brevisubulatum*. *Epichloë*-free *H. brevisubulatum* were served as the negative control.

In order to acquire genetically comparable endophyte infected (E+) and endophyte free (E-) *H. brevisubulatum* seeds, seeds from one *E. bromicola* infected mature ear were separated into two equal parts: one part was treated for 1.5 h with a 100 times dilution of 70% Thiophanate-Methyl (Jiangsu Rotam Chemistry Co. Ltd., Jiangsu, China) to kill the *Epichloë* endophyte, and then was rinsed with water to remove attached fungicide; the other part was treated with distilled water. Fungicide treated (E-) and untreated (E+) seeds were then planted in vermiculite that had been sterilized in an oven at 150 °C for 24 h. All plants were irrigated with water and 1/2 strength Hoagland nutrient solution. Clones of E+ and E- plants were clipped to 5 cm above the soil line; each plant was clonally propagated resulting in numerous plantlets. Seeds were collected from these E+ and E- tillers and the endophyte infection status was monitored again by microscopic examination and PCR using *Epichloë* specific primers as described above. By this means, we obtained two genetically similar populations of *H. brevisubulatum* that differed only in the absence (E-) or presence (E+) of the *E. bromicola*. All acquired seeds were stored at 4 °C to maintain endophyte viability. During the experiment, plants were examined several times using the aniline blue staining method of the absence or presence of *E. bromicola*.

Experimental design

Seeds harvested from E+ and E- plants were sown into two trays (one tray for E+ and one tray for E-) containing heat-sterilized (150 ° C for 24 h) vermiculite and irrigated with water as required. After 2 weeks, seedlings were transplanted into black plastic rectangular containers (15 cm × 8 cm × 5 cm) with six seedlings per container, with each container filled with 2 L of 1/2 strength Hoagland nutrient solution. The nutrient solution was renewed every three days to avoid ion nutrient imbalance.

After 7 d, NaCl (0 mM, 100 mM, 200 mM and 300 mM) was gradually applied in nutrient solutions and replicated three times (3 containers) in a randomized complete block design. In order to avoid salt shock, plants were treated with 50 mM salt for the first day and 100 mM salt for the second day, and the full concentration of each treatment was applied from day 3 onwards. During the stress experiment, solutions in each container were renewed every three days to ensure that a constant NaCl concentration was maintained. In addition, the position of each container in the block was changed randomly every 3 days to reduce positional effects. After 21 d exposure to NaCl stress, plants were harvested for analysis, and carefully rinsed with distilled water to remove the NaCl and nutrient solution from the surface of the plants. Some of plants were frozen in liquid nitrogen in order to measure polyamines, and some plants were oven-dried at 80 ° C to a constant weight to determine dry weight. Plant material was also collected before the salt stress for polyamines analyses to assess pre-experiment levels (show as W0 in the figure legend). During the experiment, the nutrient solutions in the container were aerated by an air pump for 30 min at 6-h intervals. Greenhouse conditions were maintained at a temperature of 28 ° C/25 ° C (day/night) and a photoperiod of 16/8 h (light/dark; the flux density was about 800 μmol m^{-2} s^{-1}), with an approximate relative humidity of 65%.

Polyamine (PA) analyses

Three kinds of polyamine: diamine putrescine, triamine spermidine and tetramine spermine contents and their free, soluble conjugated and insoluble bound forms contents were measured (Fig. 1).

Polyamines were extracted and measured using the methods of Hu et al. (2012): briefly, approximately 1.0 g of fresh leaf blade material was homogenized with 4 mL of 5% (*v*/v) cold perchloric acid with a mortar and pestle, and the ground material was incubated at 4 ° C for 60 min, then 1,6-hexanediamine (an internal standard) was added to the homogenate. The mixture was then centrifuged at 12,000×g for 30 min at 4 ° C for clarification. The supernatant was either used to measure free polyamine concentrations or further hydrolyzed to determine soluble conjugated polyamines. For supernatant hydrolysis, 1 ml of perchloric acid extract (supernatant) was mixed with 5 ml of 6 N hydrochloric acid and hydrolyzed at 110 ° C for 18 h in a reagent bottle. After acid hydrolysis, the hydrochloric acid was evaporated at 70 ° C, and the residue dissolved with 2 ml of 5% (*v*/v) perchloric acid. The mixture (acid-soluble polyamines fraction containing free and soluble conjugated polyamines) was then centrifuged at 12,000×g for 30 min at 4 ° C. To acquire insoluble bound polyamines forms, the precipitate was washed several times with 5% (*v*/v) perchloric acid to remove any residue of soluble polyamines and then the precipitate was suspended in 5 ml of 6 N hydrochloric acid. This solution was then hydrolyzed using the method described above.

Polyamines acquired from the non-hydrolyzed supernatant (free form), hydrolyzed supernatant (free and soluble conjugated form), and the precipitate (insoluble bound form) were then benzoylated as follows: 1 ml of the supernatant was treated with 2 ml of 2 N sodium hydroxide and 15 μl of benzoyl chloride, incubated for 30 min at 37 ° C after mixing vigorously, then the reaction was terminated by adding 4 ml of saturated sodium chloride solution. Thereafter, the benzoyl polyamines were extracted with 3 ml cold diethyl ether, then 1.5 ml of the diethyl ether phase was evaporated, finally the residuum was redissolved in 1 ml methanol.

Polyamines were quantified using Agilent 1100 high-performance liquid chromatography, with an Agilent 250 mm × 4.6 mm reverse-phase C18 column. Eluents used were 64% (*v*/v) methanol at a flow rate of 1 ml min^{-1}, and column temperature was maintained at 25 ° C. A fixed-wavelength UV detector (G1314, VWD, Agilent) at 254 nm was used to detect polyamine peaks, and 20 μl of benzoyl samples were identified and quantified by comparison with external standard curves. The polyamine standards used were purchased from Sigma-Aldrich Company (China).

Soluble conjugated polyamine contents were calculated by the subtraction of the free polyamine concentration from the acid-soluble polyamine concentration, and polyamine concentrations were expressed as nmol/g fresh weight.

Statistical analysis

Data were analyzed by SPSS statistical software (Ver. 19.0, SPSS, Inc., Chicago, IL, USA). Two-way analysis of variance was used to determine the effects of endophyte (E) and salt stress (S) on three kinds of polyamines, the proportion of putrescine, spermidine and spermine in the total polyamines, the proportion of free form, soluble conjugated form and insoluble bound form polyamines in the total polyamines and putrescine: (spermidine + spermine) ratios. Significance of difference tests between E+ and E- plants under the same salt concentration stress in all of the parameters were carried out by independent *T*-test, and significant difference between different salt concentrations in all of the parameters were carried out using Duncan's test at $P < 0.05$. All data were presented as mean ± standard errors.

Results

Plant dry weights

After treatment with three concentrations of NaCl for 21 d, the differences between E+ and E- plants under the high NaCl concentration (300 mM) were evident. The results showed that compared with the control treatment (0 mM), the dry weight of plants decreased as the salt concentrations increased (Fig. 2), and endophyte infection significantly positively influenced plant dry weight especially under the higher salt concentration but not under the control (Fig. 2). We can conclude that the presence of endophyte on *H. brevisubulatum* led to amelioration of salt stress.

Putrescine content

Salt stress positively affected the free form, insoluble bound form and total putrescine content of *H. brevisubulatum* plants (Fig. 3a, c, d, Table 1), while having no effects on the soluble conjugated form of putrescine (Fig. 3b, Table 1). The presence of endophyte significantly decreased each form and total putrescine content at higher salt concentration treatments. The difference between the E+ and E- plants became larger as the salt concentrations increased, while they showed no significant difference under the control and low salinity conditions (100 mM) (Fig. 3).

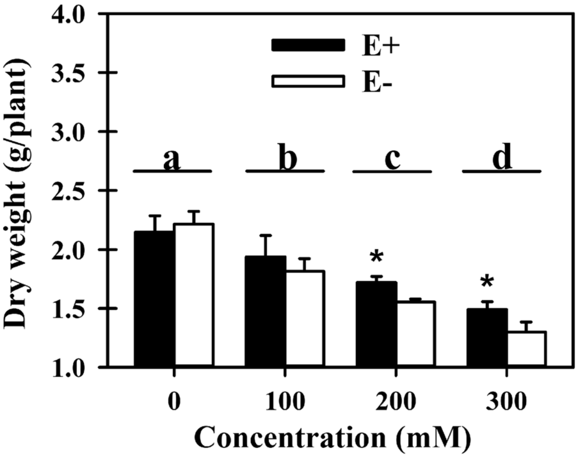

Fig. 2 Dry weight of *Hordeum brevisubulatum* with (E+) and without (E-) endophyte under salt stress. Values are means ± standard error (SE). An * on the top of bars indicate significant difference at $P < 0.05$ (independent *t*-test) between E+ and E- plants under the same salt concentration. Different lower-case letters above lines means significant difference between different salt concentration treatments using Duncan's test at $P < 0.05$

Spermidine content

Irrespective of endophyte infection, the insoluble bound form and total spermidine content of plants increased at first and then declined as the salt concentrations increased (Fig. 4c, d), while soluble conjugated spermidine content increased gradually with increasing stress levels (Fig. 4b), however, the effects of salt stress on free form spermidine content was not significant (Fig. 4a, Table 1). Endophyte infection strengthened the increase of the insoluble bound form and the total spermidine content under 100 and 200 mM stress, but alleviated the decrease under the highest salt concentration (300 mM) (Fig. 4c, d). The presence of the endophyte also enhanced the increase of the soluble conjugated form of spermidine (Fig. 4b) but alleviated the decrease of the free form of spermidine under salt stress (Fig. 4a). The E+ plants had higher levels of total and each form of spermidine than E- plants under salt stress (but not under the control) (Fig. 4).

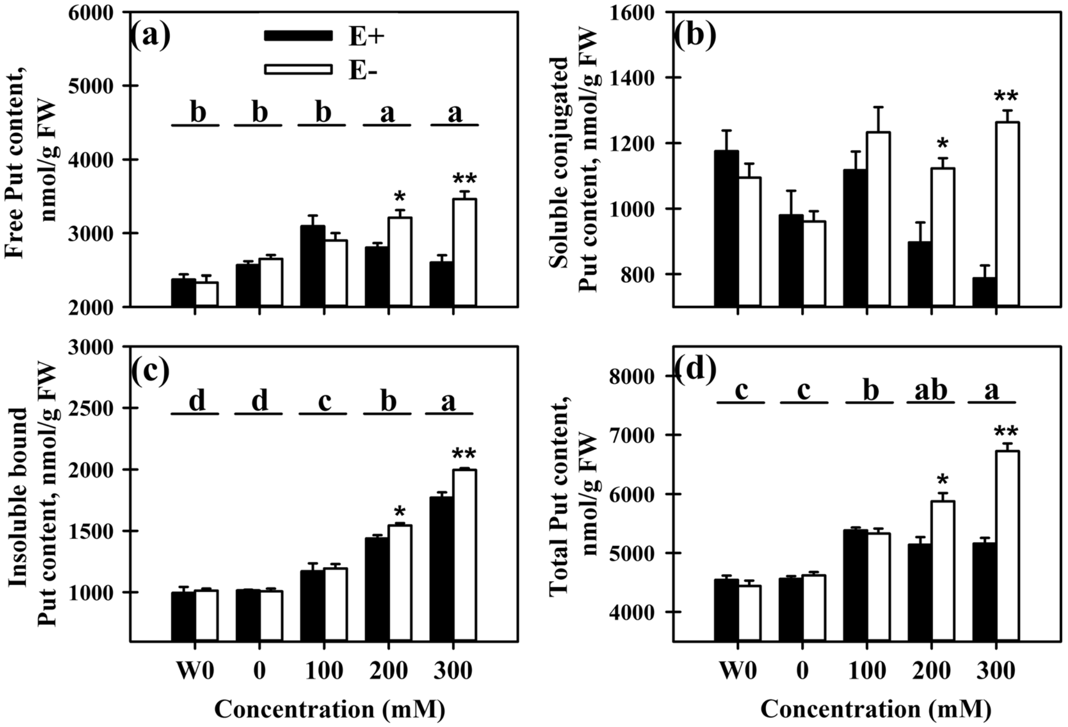

Fig. 3 Free form (a), soluble conjugated form (b), insoluble bound form (c) and total (d) putrescine (Put) content of *Hordeum brevisubulatum* with (E+) and without (E-) endophyte under salt stress. W0 means prior to salt stress. Values are means ± standard error (SE). * and ** on the top of bars indicate significant difference at $P < 0.05$ and $P < 0.01$, respectively (independent *t*-test), between E+ and E- plants under the same salt concentration. Different lower-case letters above lines means significant difference between different salt concentration treatments using Duncan's test at $P < 0.05$

Spermine content

Insoluble bound form and total spermine content showed a gradually increasing trend with increasing salt concentrations (Fig. 5c, d), but free form spermine content decreased at 100 and 200 mM salt stress and then showed a sharp increase under the 300 mM salt stress (Fig. 5a). Soluble conjugated spermine content showed an increasing trend under 100 and 200 mM salt condition compared to control

Table 1 Two-way ANOVA for the effects of endophyte (E) and salt concentrations (S) on putrescine, spermidine and spermine content of *Hordeum brevisubulatum*

	Treatments	df	Free form content		Soluble conjugated form content		Insoluble bound form content		Total content	
			F-value	*P*	F-value	*P*	F-value	*P*	F-value	*P*
Putrescine	E	1	6646.13	<0.001	27.59	<0.001	13.33	0.002	69.10	<0.001
	S	3	6744.07	<0.001	5.59	0.008	260.63	<0.001	66.07	<0.001
	E × S	3	7051.15	<0.001	7.58	0.002	4.82	0.014	28.90	<0.001
Spermidine	E	1	6.15	0.025	60.60	<0.001	44.84	<0.001	40.57	<0.001
	S	3	0.53	0.670	26.00	<0.001	59.25	<0.001	10.52	<0.001
	E × S	3	4.57	0.017	8.34	0.001	10.04	0.001	11.76	<0.001
Spermine	E	1	0.41	0.531	92.68	<0.001	41.57	<0.001	10.47	0.005
	S	3	40.10	<0.001	33.67	<0.001	85.51	<0.001	31.50	<0.001
	E × S	3	0.07	0.974	28.24	<0.001	13.16	<0.001	2.48	0.098

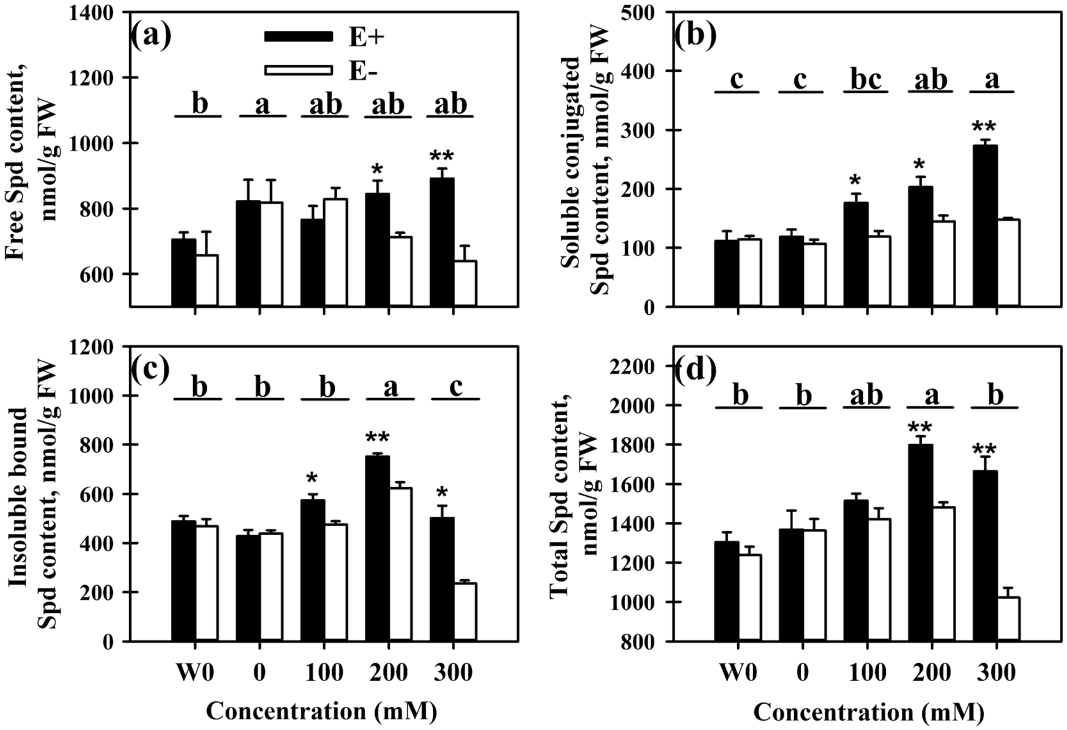

Fig. 4 Free form (a), soluble conjugated form (b), insoluble bound form (c) and total (d) spermidine (Spd) content of *Hordeum brevisubulatum* with (E+) and without (E-) endophyte under salt stress. W0 means prior to salt stress. Values are means ± standard error (SE). * and ** on the top of bars indicate significant difference at $P < 0.05$ and $P < 0.01$, respectively (independent *t*-test), between E+ and E- plants under the same salt concentration. Different lower-case letters above lines means significant difference between different salt concentration treatments using Duncan's test at $P < 0.05$

and then sharply decreased under the 300 mM salt stress (Fig. 5b). Endophyte infection significantly increased the soluble conjugated form and the total spermine content under the salinity treatment, and E+ plants had higher conjugated form and higher total spermine content than E- plants within the measured range of salt stress (Fig. 5b, d, Table 1). The insoluble bound form of spermine showed a different pattern, it was significantly lower in E+ plants than in E- plants when exposed to salt stress treatments (Fig. 5c).

The proportion of putrescine, spermidine and spermine in the total polyamines

Total polyamine content including putrescine, spermidine and spermine was analyzed, and found to decrease as a consequence of endophyte infection. Differences between E+ and E- plants became larger with increasing salt concentration. E- plants had higher total polyamine content than E+ plants at 200 and 300 mM salt (Fig. 6a). However, due to significantly higher levels of putrescine than spermidine or spermine in *H. brevisubulatum* plants (Fig. 3d, 4d and 5d), even though E+ plants had higher spermidine and spermine content under salt stress, total polyamine content was higher in E- plants (Fig. 6a). The results showed that the proportion of putrescine content in total polyamines of E+ plants was significantly lower than of E- plants at the higher stress levels (Fig. 6b); the proportion of spermidine and the proportion of spermine in total polyamine content showed an opposite pattern, they were higher in E+ plants than in E- plants under salt stress treatment (Fig. 6c, d). From these results, we can conclude that endophyte infection enhanced the conversion ability of *H. brevisubulatum* from putrescine to spermidine and spermine in the saline environment (Fig. 6, Table 2).

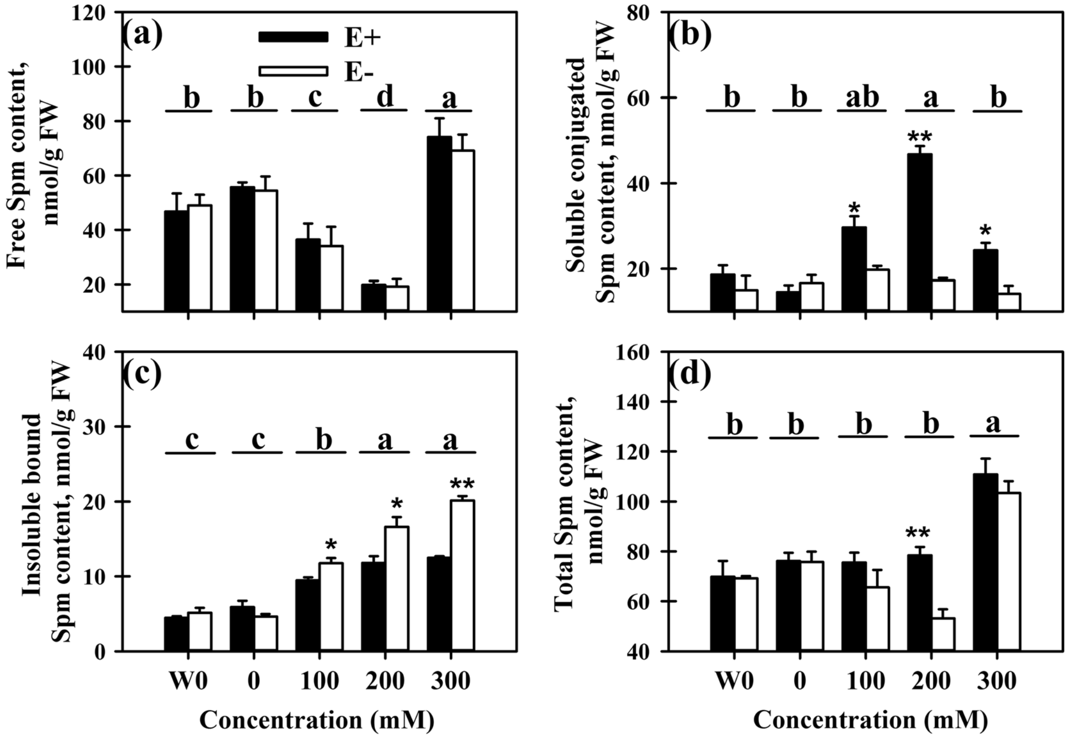

Fig. 5 Free form (a), soluble conjugated form (b), insoluble bound form (c) and total (d) spermine (Spm) content of *Hordeum brevisubulatum* with (E+) and without (E-) endophyte under salt stress. W0 means prior to salt stress. Values are means ± standard error (SE). * and ** on the top of bars indicate significant difference at $P < 0.05$ and $P < 0.01$, respectively (independent *t*-test), between E+ and E- plants under the same salt concentration. Different lower-case letters above lines means significant difference between different salt concentration treatments using Duncan's test at $P < 0.05$

The proportion of free form, soluble conjugated form and insoluble bound form polyamines in the total polyamines

Irrespective of the presence of endophyte, the proportion of free form and the proportion of soluble conjugated form polyamines in total polyamines showed a decreasing trend under salt stress (Fig. 7a, b), but the proportion of insoluble bound form polyamines in total polyamines showed a different pattern, being significantly increased in the 200 and 300 mM salt treatments (Fig. 7c). Endophyte infection reduced the proportion of free form and the proportion of soluble conjugated form polyamines in total polyamines; polyamines were lower in E+ plants than in E- plants under higher salt concentration treatments (Fig. 7a, b), but the presence of endophyte strengthened the increase of the proportion of insoluble bound polyamines in total polyamines when exposed to salt treatments, with the difference between E+ and E- plants being significant ($P < 0.05$) at 200 and 300 mM salt (Fig. 7c). These results indicate that endophyte infection improved the shift ability of *H. brevisubulatum* from free form and soluble conjugated form polyamines to insoluble bound form polyamines under salt stress (Fig. 7, Table 3).

Putrescine: (spermidine + spermine) ratio

The insoluble bound form and total putrescine: (spermidine + spermine) ratio showed a sharp increase under the 300 mM salt stress (Fig. 8c, d), while the soluble conjugated form putrescine: (spermidine + spermine) ratio declined as the salt stress increased (Fig. 8b). However, salt stress had no effects on the free form putrescine: (spermidine + spermine) ratio (Fig. 8a, Table 4). Endophyte infection lowered each form and total putrescine: (spermidine + spermine) ratios; they were significantly higher in E- plants than in E+ plants especially under higher salt stress concentrations (Fig. 8). Endophyte infection increased spermidine and spermine content but lowered putrescine content in the high salt treatment.

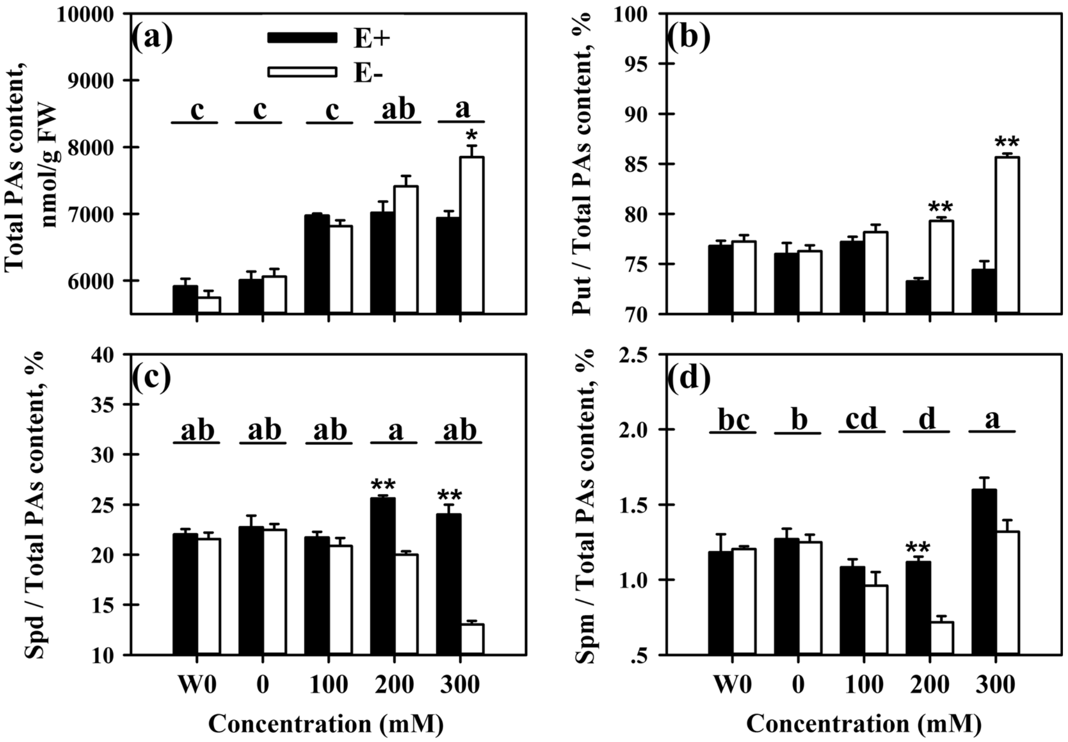

Fig. 6 Total polyamine (putrescine + spermidine + spermine) content, proportion of putrescine, spermidine and spermine in the total polyamines of *Hordeum brevisubulatum* with (E+) and without (E-) endophyte under salt stress. W0 means prior to salt stress. Values are means ± standard error (SE). * and ** on the top of bars indicate significant difference at $P < 0.05$ and $P < 0.01$, respectively (independent *t*-test), between E+ and E- plants under the same salt concentration. Different lower-case letters above lines means significant difference between different salt concentration treatments using Duncan's test at $P < 0.05$

Discussion

Understanding the mechanism by which infection by the *Epichloë* endophyte enhance the tolerance of grass plants to biotic and abiotic stresses is essential for improvement and persistence of host plants for a range of applications, e.g., breeding of new varieties for arid areas and cultivating plants in high salinity areas for soil conservation and improvement. High salinity poses threats to agricultural sustainability, and causes adverse damage to plant growth and productivity. Our results indicate that *E. bromicola*-infected *H. brevisubulatum* plants are better adapted to high salt soils than the genetically identical endophyte-free plants. *E. bromicola* infected *H. brevisubulatum* outperformed uninfected plants only under salt stress, and the differences were usually more apparent at the higher stress conditions, whereas no significant differences were observed in non-stressed plants. Similar results were also found

Table 2 Two-way ANOVA for the effects of endophyte (E) and salt concentrations (S) on total polyamine content, and the proportion of putrescine, spermidine and spermine in the total polyamines of *Hordeum brevisubulatum*

Treatments	df	Total polyamine content		Putrescine content / total polyamine content		Spermidine content / total polyamine content		Spermine content/ total polyamine content	
		F-value	*P*	F-value	*P*	F-value	*P*	F-value	*P*
E	1	10.97	0.004	97.08	<0.001	80.53	<0.001	20.05	<0.001
S	3	44.14	<0.001	14.69	<0.001	16.01	<0.001	28.06	<0.001
E × S	3	6.60	0.004	29.45	<0.001	25.51	<0.001	3.33	0.046

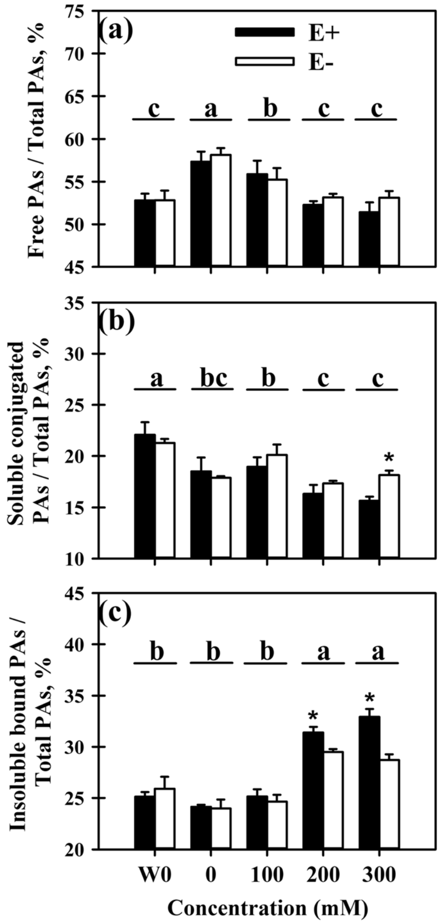

Fig. 7 Proportion of free form, soluble conjugated and insoluble bound form polyamines in the total polyamines of *Hordeum brevisubulatum* with (E+) and without (E-) endophyte under salt stress. W0 means prior to salt stress. Values are means ± standard error (SE). * and ** on the top of bars indicate significant difference at $P < 0.05$ and $P < 0.01$, respectively (independent *t*-test), between E+ and E- plants under the same salt concentration. Different lower-case letters above lines means significant difference between different salt concentration treatments using Duncan's test at $P < 0.05$

by Saikkonen et al. (2006), Song et al. (2015a) and Xia et al. (2015). We infer that adverse conditions (e.g., excessive Na^+ injury, osmotic stress) may increase the benefit of *Epichloë* endophyte to the grass.

Polyamine changes due to microbe infection (biotic stress)

Plant polyamine metabolism undergoes changes in response to microbe infection, and the change pattern depends on the relationship (mutualistic or antagonistic) of the microbe to its host plant. Our results with *H. brevisubulatum* plants containing *E. bromicola* suggest that metabolism of polyamines is correlated with mutualistic *Epichloë* endophyte infection and abiotic stress. Endophyte-originated and/or host-originated polyamines may have positive effects on growth of *H. brevisubulatum* exposed to salt stress. In antagonistic host-pathogen interactions, polyamines relate to pathogen recognition and defense activation, and are accompanied by hypersensitive responses. Conjugated polyamines exhibit antimicrobial properties and contribute to the defensive systems of plants against pathogenic microorganisms (Pál et al. 2011). Hydrogen peroxide derived from polyamine catabolism contributed to reactive oxygen species generation resulting in hypersensitive cell death during the hypersensitive response (Yamaguchi and Sano 2003). There are also several studies that have shown that polyamines act as regulatory factors in plant-arbuscular mycorrhizal fungus interactions; they act in the molecular signaling events between the symbiosis partners (Ghachtouli et al. 1996; Parádi et al. 2003; Smith et al. 1998). Although it is the roots of host grasses that are exposed to the salinity conditions, there are no hyphae in roots of *Epichloë* endophyte infected plants. The changes in root functions of nutrient uptake in *Epichloë* endophyte-grass associations are probably regulated through signals induced by the fungus. Polyamines have been shown to interconnect with hormones (namely auxins, abscisic acid, gibberellins and cytokinins), and reactive oxygen species (ROS) and nitric oxide (NO) to form a complex signaling network (Tiburcio et al. 2014). For example, polyamines can induce NO production, which serves as a signal that induces salt tolerance by decreasing the Na^+ to K^+ ratio through stimulation of gene expression of tonoplast Na^+/H^+ antiporter and plasma membrane H^+-ATPase (Yamasaki and Cohen 2006). It has been also suggested that polyamine induced NO production could be remediated by the polyamine oxidation reaction product H_2O_2 (Wimalasekera et al. 2011). This may indicate that *E. bromicola* -induced polyamine metabolism may mediate the action of plant signaling molecules that regulate mechanisms and adaptation processes of salt stress tolerance in *H. brevisubulatum* and ensure a better survival than endophyte-free conspecifics. However, to better understand the intrinsic mechanisms underlying crosstalk between polyamines, plant hormones and signal molecules, further detailed study will be needed in this area.

Table 3 Two-way ANOVA for the effects of endophyte (E) and salt concentrations (S) on the proportion of free form, soluble conjugated form and insoluble bound form polyamines in the total polyamines of *Hordeum brevisubulatum*

Treatments	df	Free form content / total polyamine content		Soluble conjugated form content / total polyamine content		Insoluble bound form content / total polyamine content	
		F-value	*P*	F-value	*P*	F-value	*P*
E	1	0.87	0.365	3.38	0.085	15.14	0.001
S	3	12.13	<0.001	5.43	0.009	67.40	<0.001
E × S	3	0.44	0.731	1.37	0.287	4.48	0.018

Polyamine changes due to abiotic stress

Rapid change in levels of polyamines in response to abiotic stress suggests that polyamines function in alleviation of stress. In our experiment, irrespective of endophyte infection, stress-induced changes in putrescine, spermidine and spermine content occurred under salinity conditions despite being followed by a decrease after its peak value in spermidine content. E+ plants, which showed more salt-tolerance, accumulated higher spermidine and spermine content, but lower putrescine content, than E- plants. Similar results were found in other studies. For example, sensitivity to salt-stress *Solanum* species was also reported to be associated with an increase in putrescine content and an apparent impairment in the capacity to shift putrescine to spermidine (Santa-Cruz et al. 1997). And according to Krishnamurthy and Bhagwat (1989), salt-tolerant rice

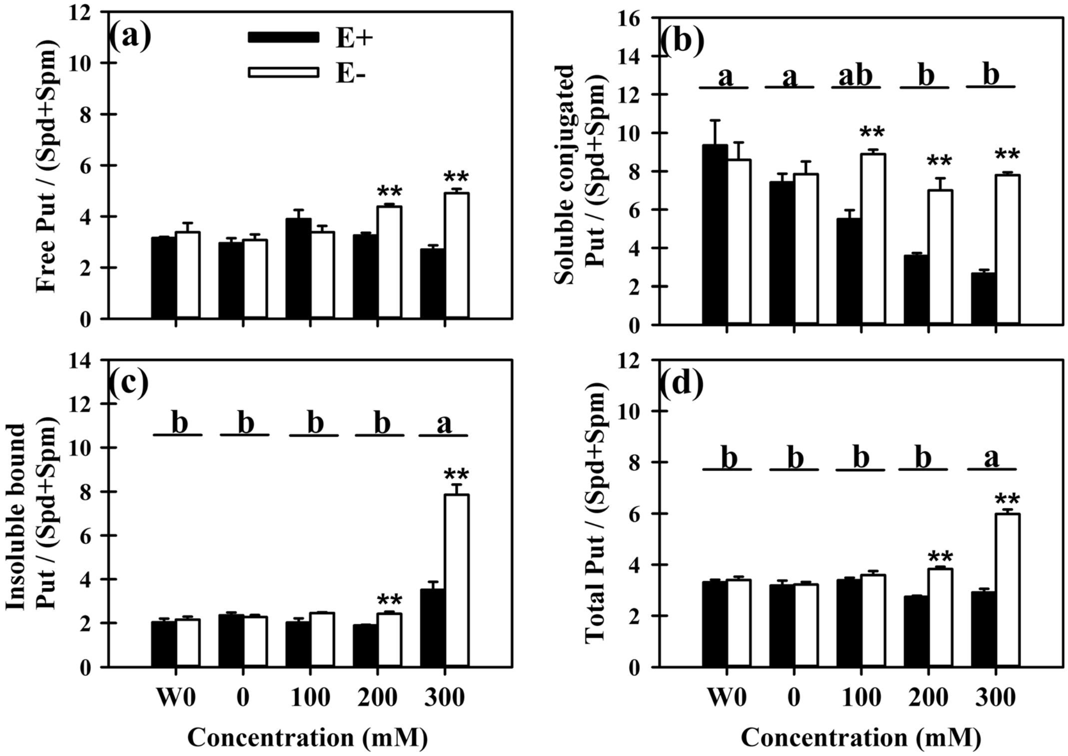

Fig. 8 Free form, soluble conjugated form and insoluble bound form and total putrescine: (spermidine + spermine) of *Hordeum brevisubulatum* with (E+) and without (E-) endophyte under salt stress. W0 means prior to salt stress. Values are means ± standard error (SE). * and ** on the top of bars indicate significant difference at $P < 0.05$ and $P < 0.01$, respectively (independent *t*-test), between E+ and E- plants under the same salt concentration. Different lower-case letters above lines means significant difference between different salt concentration treatments using Duncan's test at $P < 0.05$

Table 4 Two-way ANOVA for the effects of endophyte (E) and salt concentrations (S) on the free form, soluble conjugated form, insoluble bound form and total putrescine: (spermidine + spermine) of *Hordeum brevisubulatum*

Treatments	df	Free form put: (spd + spm)		Soluble conjugated form put: (spd + spm)		Insoluble bound form put: (spd + spm)		Total put: (spd + spm)	
		F-value	*P*	F-value	*P*	F-value	*P*	F-value	*P*
E	1	25.07	<0.001	109.98	<0.001	65.00	<0.001	135.45	<0.001
S	3	6.67	0.004	18.12	<0.001	113.40	<0.001	36.93	<0.001
E × S	3	16.41	<0.001	10.95	<0.001	39.59	<0.001	54.26	<0.001

cultivars accumulated more spermidine and spermine resulting in improved levels of total polyamines, with a relative decrease of putrescine content. However, differences in polyamine responses to salinity stress have been observed among and within species. For example, Prakash and Prathapasenan (1988) reported that endogenous content of polyamines decreased in rice seedlings under salinity stress. In tomato, salinity treatment decreased the content of putrescine and spermidine in both salt-tolerant and salt-sensitive lines. The spermine content greatly decreased in salt-sensitive lines over the salinization period, however, it did not decrease with salt stress in salt-tolerant lines (Santa-Cruz et al. 1997). Owing to the reduction of chlorophyll breakdown and the stabilization of membrane systems either by inhibiting phospholipid trans-bilayer movement (Bratton 1994) or by stabilizing molecular complexes of thylakoid membranes (Besford et al. 1993), spermidine and spermine may serve as protectants from stress conditions in plants. Spermidine and spermine also may significantly reduce the leakage of amino acids and electrolytes induced by salinity stress (Chattopadhayay et al. 2002). Putrescine may have different effects than those reported for spermidine and/or spermine since it causes membrane depolarization and increases potassium leakage. Increased levels of putrescine may correlate with reduced plant growth and may increase adverse effects of salinity stress. It has been reported that some plants accumulate higher amounts of endogenous spermidine and spermine as a way to cope with excessive salt stress (Duan et al. 2008; Roychoudhury et al. 2011). Results presented in our study show that *Epichloë* endophyte infection reduced the accumulation of putrescine and enhanced the increase of spermidine and spermine under salt stress, suggesting a positive role of the *Epichloë* endophyte in host plant response to salt stress.

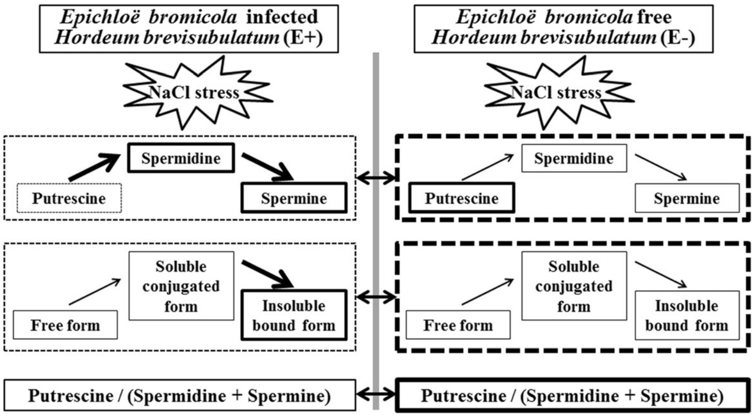

Fig. 9 Proposed polyamine modification mechanism for endophyte-mediated salt tolerance in *Hordeum brevisubulatum*. The thickness of the arrows indicate differences in the intensity of shift ability in the polyamine cycle in *Hordeum brevisubulatum*. The thickness of the textboxes indicate differences in the content of different polyamines and different form polyamines between *Epichloë bromicola* infected and *Epichloë bromicola* free *Hordeum brevisubulatum*. The thickness of the dotted line textboxes indicate differences in the content of total polyamines (putrescine + spermidine + spermine)

Relationship between polyamine and proline under salt stress

Proline is associated with the activation of reactive oxygen species scavenging enzymes during salinity stress; there was a strong correlation between increased level of proline and the capacity to survive under high salinity conditions (Gupta and Huang 2014). Song (2015) reported that salt stress results in accumulation of proline in the same material as in our study; the presence of the *Epichloë* endophyte enhanced the increase in proline, especially under the higher stress conditions. Previous studies have shown that proline levels are tightly correlated to spermidine content in stressed plants (Smith et al. 1998). Moreover, putrescine catabolism may also contribute to proline; proline and putrescine are connected via the activity of relevant enzyme metabolism as a precursor-product relationship (Aziz et al. 1998). We infer that the impact of the *Epichloë* endophyte on salt tolerance of host plants is also associated with the interaction between polyamine metabolism and proline content.

Polyamines regulate redox homeostasis under salt stress

Under salt stress, plant cells rapidly accumulate reactive oxygen species (ROS), a response widely known “oxidative burst”. Low levels of ROS (H_2O_2) function in signal transduction; however, at higher concentrations ROS can lead to nucleic acid damage, membrane lipid peroxidation, protein denaturation, and cell death (Miller et al. 2008). On the other hand, plants produce a diverse set of antioxidants to counteract the potentially detrimental effects of the oxidative burst, such as polyamines, that have antioxidative properties. Polyamines function in redox homeostasis management during plant abiotic stress response by: 1) regulating cellular responses through modulation of ROS homeostasis, 2) activating enzymes and scavenging free radicals, and 3) promoting ROS degradation (Fiscaletti et al. 2013; Gupta et al. 2013). Based on our results, we infer that endophyte-infection modulates polyamine change to promote ROS homeostasis and enhance salt stress tolerance. Our study showed that E+ plants had higher free and soluble conjugated forms of spermidine and spermine. Because free polyamines have the ability to detoxify hydrogen peroxide and superoxide anions, while the soluble conjugated forms are responsible for scavenging other ROS (Kubi 2005), soluble conjugated forms also show higher antioxidant ability than free forms of polyamines (Kuznetsov and Shevyakova 2007); it thus may be inferred that E+ plants had more antioxidant capacity than E- plants. Similar results were found in other studies, for example, previous studies have suggested that *Epichloë* endophyte can prevent ROS production or induce hosts to more efficiently scavenge ROS to mitigate the adverse effects of abiotic stress (Rodriguez et al. 2008). White Jr. and Torres (2010) suggested that ROS produced by endophyte may enhance antioxidants (for example: phenolics, mannitol, proline) production of symbiotic plants, and thus may protect plants from oxidative stress produced by various biotic and abiotic stresses. Tanaka et al. (2006) demonstrated that for the endophyte *E. festucae*, ROS production is critical in the mutualistic relationship between fungus and plants.

Polyamines related to mechanisms controlling ion balance and photosynthesis, and regulating nutrient uptake of plants under salt stress

Plant salt stress adaptive responses are also closely related to the ability to control ion transport and ion homeostasis. Studies on the same materials as in our study have shown that *Epichloë* endophyte infected plants have higher N, P and K^+ content, but lower Na^+ content, and lower ratios of C:N, C:P, Na^+:K^+ than in endophyte-free plants (Song et al. 2015a). Together with our results, we can infer that the response of polyamine changes in *H. brevisubulatum* to *E. bromicola* infection may be related to mechanisms controlling ion balance and regulating nutrient uptake of plants. Polyamine accumulation in plants requires an osmotic signal and/or the presence of permeable ions, such as sodium and potassium (Erdei et al. 1996; Tytti and Seppo 2002; Zhao et al. 2007). Polyamines are reported to improve salt tolerance through their effects on ion transport, ROS, and affecting intracellular Ca^{2+} homeostasis by adjusting Ca^{2+} transport systems in the plasma membranes of root cells (Zepeda-Jazo et al. 2011). For example, polyamines play a role in the improvement of K^+/Na^+ homeostasis by restricting Na^+ influx into roots and by suppressing K^+ efflux from shoots (Zhao et al. 2007). Spermine limits Na^+ influx and K^+ efflux by blocking non-selective cation channels and inward-rectifying K^+ channels. Putrescine and spermine reduce hydroxyl radical-induced K^+ efflux (Shabala et al. 2007; Zepeda-Jazo et al. 2008). Polyamines enhance membrane stability and interact with plasma membrane phospholipids to affect cation-anion transport (for example: H^+- and Ca^{2+}-ATPase transporters) in salinity environment (Pottosin and Shabala 2014). The cumulative effects of hydrogen peroxide and polyamines in response to salinity stress leads to a rapid rise in intracellular Ca^{2+} content, and causes a positive feedback to ROS production through NADPH-oxidase localized in plasma membranes (Bose et al. 2014). On the other hand, increased intracellular Ca^{2+} activates non-selective cation channels and inhibits Na^+-induced K^+ efflux, thus ameliorating the toxicity of Na^+ (Shabala et al. 2006). Several studies have also shown the importance of the interaction between polyamines and nutrient uptake of plants, for example, putrescine increases occur under mineral nutrient deficiency. Nitrogen deficiencies accelerated changes in polyamine content, especially in soluble conjugated and insoluble bound forms (Tytti and Seppo 2002). Polyamines also play roles in phosphorus nutrition-dependent regulation of AM symbiosis (Smith et al. 1998), and could also be considered as nitrogen storage to meet the needs of energy during salinity stress (Pál et al. 2015). It has been demonstrated that *Epichloë* endophyte infection improved photosynthetic rates and transpiration rates under biotic or abiotic stress (Rozpądek et al. 2015; Song 2015; Xia et al. 2016). There are also several lines of evidence supporting the relationship between photosynthesis and polyamines. For example, higher transpiration rate could promote long-distance transport of polyamines from roots to shoots via xylem vessels (Smith et al. 1998). Transglutaminase catalyzes the conjugation of polyamine to photosynthetic compounds, and this may lead to improved photosynthetic ability under stress conditions (Ioannidis et al. 2012). Thus, in *E. bromicola* infected *H. brevisubulatum*, the mechanism by which the presence of the endophyte can ameliorate the effects of salt on plants appears to involve changes in polyamines. Modified polyamine metabolism is also related to mechanisms that control photosynthesis, ion homeostasis, nutrient uptake and transport.

How soluble conjugated and insoluble bound polyamines regulate stress tolerance

In our study, E+ plants, which showed more salt tolerance than E- plants, accumulated more soluble conjugated and insoluble bound spermidine, and soluble conjugated spermine, but lower soluble conjugated and insoluble bound putrescine, and lower insoluble bound spermine. A key role for soluble conjugated and insoluble bound polyamines in the regulation of stress tolerance has been proposed; polyamine function is regulated by conjugation reactions through binding and interaction with nucleic acids, proteins or phospholipids (Bouchereau et al. 1999). Conjugated polyamines are preferred substrates for peroxidase; they react with peroxidases and may remove hydrogen peroxide in the apoplast (Negrel and Lherminier 1987); they also regulate interactions between polyamines and inorganic cations such as Ca^{2+}, which relates to the proposed role of polyamines on membrane stabilization (Bouchereau et al. 1999). Enhanced levels of membrane-bound polyamines have been associated with stabilization and maintenance of plasma membranes, mitochondria and tonoplasts, and these appear to be involved in the acclimatization of plants to salinity environments (Hu et al. 2012). Protein-bound polyamines are also involved in photosynthetic functions (Margosiak et al. 1990). However, high levels of putrescine conjugates can contribute to cell multiplication inhibition and cell differentiation suppression, and lead to abnormal floral development in several species (Martin-Tanguy 1997). Previous studies demonstrated that overexpressing the *S*-adenosylmethioine decarboxylase (*SAMDC*) gene in tobacco plants lead to an increase in conjugated form polyamines, and transgenic plants exhibited enhanced tolerance to drought and salinity (Waie and Rajam 2003). From above mentioned, it is likely to indicate that enhanced conversion of free form and soluble conjugated form polyamines to insoluble bound form polyamines are related to mechanisms of salt tolerance induced by *Epichloë* endophyte infection.

The relationship between putrescine: (spermidine + spermine) ratio and stress tolerance

Salinity stress leads to the change in the putrescine: (spermidine + spermine) ratio in plants. Our study revealed that the insoluble bound form and the total putrescine: (spermidine + spermine) ratio increased with salinity, which is consistent with the idea of a protective role of spermidine and spermine against saline stress (Pál et al. 2015). Endophyte infection significantly lowered the putrescine: (spermidine + spermine) ratio, especially under the higher stress levels, which could alleviate salinity damage to host plants. This result suggests a positive role for the endophyte in host plant responses to salt stress. In several systems, an accumulation of spermidine and spermine, but not putrescine, may confer stress tolerance (Zapata et al. 2004). Increased accumulation of putrescine, leading to a higher putrescine: (spermidine + spermine) ratio, may even injure plants. It was found that salt-sensitive plant species accumulate more putrescine than salt-tolerant species. The putrescine: (spermidine + spermine) ratio decreased with salinity increase in salt tolerant tomato species, but not in salt-sensitive species (Santa-Cruz et al. 1997). Furthermore, the primary factors responsible for salinity tolerance are not only elevated levels of spermidine and spermine but also the enhanced turnover, and the ability to accumulate high levels of insoluble bound polyamine.

Conclusions

Our study is the first indication of the possible role of polyamines in the symbiosis between host plant and mutualistic *Epichloë* species under abiotic stress. Our primary experiments showed that *Epichloë* endophyte infection resulted in an increase in each form and total spermidine, soluble conjugated form and total spermine, but decreased total putrescine, and insoluble bound form spermine. *Epichloë* endophyte infection also decreased the putrescine: (spermidine + spermine) ratios. Furthermore, *Epichloë* endophyte infection significantly increased the proportion of the insoluble bound form polyamine, while lowering the proportion of the free form polyamine and soluble conjugated form polyamine. Based on these results, we conclude that salinity stress tolerance induced by *Epichloë* endophyte infection correlated with the enhanced conversion of putrescine to spermidine and spermine, as well as an improved shift ability from the free form and the soluble conjugated form polyamine to the insoluble bound form polyamine (Fig. 9).

Based on our observations and reports from Song et al. (2015a), we propose that the change of polyamine metabolism *in planta* produced by *Epichloë* endophyte infection positively regulates photosynthesis, ion balance, nutrient stoichiometry and signal transduction. However, the situation may be reverse where polyamine metabolism is affected by some of these physiological characters, thereby preventing excessive adverse damage to *H. brevisubulatum* growth induced by NaCl. While crosstalk and interplay between polyamine turnover and other physiological systems during salinity stress is complex, it is essential to understand how this system works for its potential in enhancing plant salinity stress tolerance. Besides direct protective roles, polyamines serve as signaling molecules to regulate polyamine-mediated signaling pathways. It is tempting to speculate that improved abiotic stress tolerance induced by *Epichloë* endophyte is not only related to changed polyamine metabolism but also by signaling processes. In order to fully understand *Epichloë* endophyte induced polyamine metabolism and the possible mechanisms by which the presence of *Epichloë* endophyte enhances growth of *H. brevisubulatum* exposed to salt stress, further research is needed on polyamine-related signaling pathways in *H. brevisubulatum*, together with other physiological traits including photosynthesis and nutrient transport.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Disclosures The article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent This study did not involve human participants, and so informed consent was not required.

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