The Variation Tendency of Polyamines Forms and Components of Polyamine Metabolism in Zoysiagrass (Zoysia japonica Steud.) to Salt Stress with Exogenous Spermidine Application

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To understand dynamic changes in polyamines (PAs) forms and components of polyamine metabolism in zoysiagrass (Zoysia japonica Steud.) response to salt stress with exogenous spermidine (Spd) application, two Chinese zoysia cultivars, z081 and z057, were exposed to sodium chloride stress for 2, 4, 6, and 8 days. The z057 cultivar possesses higher salinity tolerance than the z081 cultivar. Salt stress decreased the zoysiagrass fresh weight (FW) and increased free Spd and spermine (Spm) levels and soluble and insoluble putrescine (Put), Spd and Spm levels in both cultivars. Moreover, salt stress enhanced the activities of arginine decarboxylase (ADC), ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase (SAMDC), and diamine oxidase (DAO). Exogenous Spd increased PA metabolism and ADC, SAMDC, and DAO activities and decreased free Put levels under salt stress conditions in both cultivars. In addition, structural equation modeling (SEM) showed that ODC, SAMDC, and DAO contributed to PA metabolism, and endogenous Spd levels also contributed to endogenous Spm levels. Free PAs may be the primary factor influencing the variation of other PA forms. SEM also indicated that ADC and polyamine oxidase (PAO) play a limited role in enhancing zoysia salt tolerance via PA metabolism under salt stress.

Keywords: zoysiagrass, components, polyamine metabolism, dynamic variation, exogenous spermidine, salinity stress

INTRODUCTION

Salinity stress is a major factor limiting plant growth and restricting the production of high-quality plants. Potential and actual plant yields differ considerably under salt stress. Salinity stress may cause a greater than 50% reduction in major perennial and annual crops worldwide (Wang et al., 2003). Plants have evolved highly coordinated and complex systems to adapt to salt stress using a variety of physiological and biochemical responses. A range of physiological, biochemical, morphological and molecular changes occur in response to salinity stress. Richards and Coleman (1952) determined that polyamine (PA) metabolism is involved in protecting and maintaining the
structure and function of cellular components under salt stress. Many studies have implicated PAs in plant growth and development (Pál et al., 2015).

PAs are low-molecular-weight aliphatic cations that are widely present among organisms (Hussain et al., 2011). In plants, PAs are mainly present as three types: the diamine putrescine (Put), the triamine spermidine (Spd), and the tetraamine spermine (Spm). All three major PAs are present in freely soluble forms or bound insoluble forms. The PA biosynthetic pathway has been extensively studied in plants (Kusano et al., 2008; Verasirera et al., 2010; Pegg and Casero, 2011; Gupta et al., 2013). In plants, ornithine decarboxylase (ODC) and arginine decarboxylase (ADC) catalyze Put production in three steps. Spd synthase (SPDS) catalyzes the formation of Spd from Put and an aminopropyl moiety donated from decarboxylated S-adenosylmethionine (dcSAM). Spd or thermospermine is synthesized from Spm by Spm synthase (SPMS) using dcSAM as an aminopropyl donor. In addition to the de novo synthesis of PAs, PA catabolism involves two classes of enzymes: diamine oxidases (DAOs) and FAD-containing polyamine oxidases (PAOs) (Cona et al., 2006; Alcázar et al., 2010; Moschou et al., 2012).

PAs are involved in various processes in plant growth and development, such as biofilm formation (Lee et al., 2009), xylem differentiation (Tisi et al., 2011), fruit ripening (Gil-Amado and Gomez-Jimenez, 2012), programmed cell death (Kim et al., 2013), and embryonic competence (Silveira et al., 2013). PAs are involved in salt tolerance, as evidenced by changes in PA concentrations in response to salt stress. The three major PAs (Put, Spd, and Spm) increase in abundance under salt stress (Yang et al., 2007). However, in most cases, only one of the three PAs increases significantly under salt stress (Liu et al., 2006). Wang and Liu (2009) reported that the Spd content increased significantly in response to salt stress, and Ikbal et al. (2014) observed the accumulation of Spd and Spm in response to salt stress. Similar results were observed in 18 rice species under drought stress (Do et al., 2014). In most cases, PA accumulation is higher in tolerant genotypes than in sensitive genotypes (Hatmi et al., 2015). The increased de novo synthesis of free PAs is primarily responsible for PA accumulation under salt stress. To understand the regulation of PAs at the transcriptional level, many studies have evaluated steady-state transcript levels of PA biosynthetic genes. The expression of PA biosynthetic genes such as ADC, SPDS, SPMS and SAMDC is increased upon exposure to salt stress (Uranse et al., 2004; Liu et al., 2006; Wang et al., 2011; Majumdar et al., 2013; Guo et al., 2014). These studies suggest that the accumulation of PAs is an adaptive mechanism in response to salt stress and that PA dynamics is complex.

The dynamic variation of PA content is considered a response to salt stress. To better understand the roles of PAs in the response to salinity stress, three approaches, including the application of exogenous PAs and PA synthesis inhibitors and the overexpression of biosynthetic genes, have been used. Exogenous Spd application has been shown to enhance the salt tolerance of different plants (Duan et al., 2008). A recent study by Li et al. (2016) demonstrated that application of 0.15 mM Spd alleviated the damage caused by salt stress in zoysia (Zoysia japonica Steud.). Exogenous Spd application also enhanced the salt tolerance of sorghum (Sorghum bicolor) seedlings (Yin et al., 2016). Hu et al. (2012) also found that exogenous application of 0.15 mM Spm reduced salt injury under salt stress in tomato. Several transgenic techniques for overexpressing genes encoding PA biosynthetic enzymes have been widely applied in rice and Arabidopsis thaliana (Roy and Wu, 2001; Kasukabe et al., 2004, 2006).

There is substantial evidence that PA (Put, Spd, Spm) levels undergo extensive changes under salinity stress with exogenous Spd application. However, these PAs (Put, Spd, Spm) are present in three forms (freely soluble, insoluble bound forms, and soluble forms). There have been few reports to date on dynamic variations in different forms of PA to salt stress with exogenous Spd application and components of polyamine metabolism. Our objective was to characterize the forms (freely soluble, insoluble bound forms, soluble forms) of PAs present under the salt stress and the components of PA metabolism in zoysiagrass.

MATERIALS AND METHODS

Plant Materials and Treatments
In this study, two zoysia (Zoysia japonica Steud.) cultivars (z057 and z081) were used. z057 is tolerant to salinity stress, whereas z081 is comparatively sensitive to salinity stress (Li et al., 2012). The cultivars were collected from China (Table 1) and cultivated under hydroponic conditions with 1/2 Hoagland solution (pH 6.6 ± 0.1, EC 1.8–2.0 dsm⁻¹) with pump aeration (China Agricultural University, Haidian, Beijing, China). The air temperatures at day and night in the greenhouse were 17–20°C and 25–28°C, respectively. The air relative humidity in the greenhouse was 60–70%.

Zoysia roots were pruned to 5 cm before treatment. Four treatments were then applied: (1) control, consisting of 1/2 Hoagland solution alone; (2) 1/2 Hoagland solution + 0.15 mM Spd; (3) 1/2 Hoagland solution + 150 mM NaCl; and (4) 1/2 Hoagland solution + 150 mM NaCl + 0.15 mM Spd.

Root samples were collected with three replicates on days 0, 2, 4, 6, and 8 after salinity treatment.

Root Growth
The dry weight was determined after drying at 75°C for 72 h.

PA Analysis
The PA extraction method is based on Sharma and Rajam (1995), with some modifications. Cold perchloric acid (PCA, 4 mL, 5% v/v) was added to the fresh root homogenates and incubated for 1 h at 4°C. 1,6-Hexanediamine was added to the homogenates as an internal standard. The homogenates were centrifuged at

| TABLE 1 | Zoysia japonica cultivars used in the study, growth conditions and plant sources. |
| --- | --- | --- | --- |
| Cultivar | Species | Source sponsor | Source location |
| z081 | Z. japonica | Qingdiao, Shandong | 36°05′N, 120°20′E |
| z057 | Z. japonica | HuaguoShan, Lianyungang | 34°36′N, 119°12′E |
12,000 × g at 4°C for 30 min. The supernatants were used to determine free and soluble conjugated PAs, and the residue was used to determine insoluble bound PAs. To determine soluble conjugated PAs, the PCA extract (1 mL) was mixed with 5 mL of 6 N HCl and hydrolyzed at 110°C for 18 h in a flame-sealed glass ampule. After acid hydrolysis, the HCl was evaporated at 70°C, and the residue was suspended in 2 mL of 5% PCA after centrifugation at 12,000 × g for 30 min at 4°C. The solution contained acid-soluble PAs, including those liberated from PA conjugates and free PAs. To determine insoluble bound PAs, the pellets were rinsed four times with 5% PCA to remove any traces of soluble PAs, followed by suspension in 5 mL of 6 N HCl. The same procedure above was used to hydrolyze this solution. PAs recovered from the hydrolyzed supernatants, nonhydrolyzed supernatants and pellets were benzoylated as follows. An aliquot of supernatant mixed with 2 mL of 2 N NaOH and 15 μL of benzoyl chloride was vortexed vigorously and incubated for 30 min at 37°C. Then, the reaction was terminated via the addition of 4 mL of saturated NaCl solution. Finally, 1.5 mL of the ether phase was dried and redissolved in 1 mL of methanol.

PAs were assayed by high-performance liquid chromatography (HPLC). Ten microliters of methanol solution containing benzoylated PAs was injected into a 20-μL loop and loaded onto a 5-μm particle-size C18 reverse-phase, 4.6-mm × 250-mm column (Eka Chemicals, Bohus, Sweden). The column temperature was maintained at 25°C. Samples were eluted with 64% methanol, and a flow rate of 0.8 mL min⁻¹ was maintained using a Dionex P680 Pump. PA peaks were detected with a UV detector at 254 nm. The concentrations of soluble conjugated PAs were calculated by subtracting free PA concentrations from acid-soluble PA concentrations.

**Analysis of PA Biosynthetic Enzyme Activity**

Fresh samples were homogenized in 100 mM potassium phosphate buffer (pH 8.0) containing 0.1 mM phenylmethylsulfonyl fluoride, 1 mM pyridoxal phosphate (PLP), 5 mM EDTA, 25 mM ascorbic acid and 0.1% polyvinylylpyrrolidone. After centrifugation at 12,000 × g for 40 min at 4°C, the supernatants were dialyzed at 4°C against 3 mL of 100 mM potassium phosphate buffer (pH 8.0) containing 1 mM pyridoxal phosphate (PLP), 0.05 mM PLP, 0.1 mM DTT, and 0.1 mM EDTA for 24 h in the dark. The dialyzed extracts were used for enzymatic assays.

The activities of ODC, ADC, and SAMDC were determined according to the procedure described by Zhao et al. (2003), with some modifications. The reaction mixtures were activated after adding 0.3 mL of the dialyzed enzyme extract and 100 mm Tris-HCl buffer (pH 8.0), 50 μM pyridoxal phosphate, 5 mM EDTA, and 5 mM DTT. Then, the reactions were incubated at 37°C for 2 min, followed by the addition of 0.2 mL of 25 mM L-ornithine, 0.2 mL of 25 mM L-arginine (pH 7.5) or 0.2 mL of 25 mM SAM. Then, the reaction mixtures were incubated at 37°C for 30 min, followed by the addition of PCA to a final concentration of 5%. Reaction mixtures were centrifuged at 3,000 × g for 10 min, and the supernatants (0.5 mL) were mixed with 1 mL of 2 mM NaOH and 10 μL of benzoyl chloride. The mixtures were stirred for 20 s. After incubation at 37°C for 30 min, 2 mL of NaCl solution and 3 mL of ether were added and stirred thoroughly, followed by centrifugation at 1,500 × g for 5 min and extraction with 3.0 mL of ether. Then, 1.5 mL of the ether phase was evaporated to dryness and redissolved in 3 mL of 60% methyl alcohol. Finally, the solutions were exposed to a UV light at a wavelength of 254 nm to measure enzymatic activity.

**Diamine and PA Oxidase Activity Assay**

PAO and DAO activities were determined by measuring the generation of H₂O₂, a PA oxidation product, according to the procedure of Su et al. (2005), with some modifications. Fresh samples were homogenized in 100 mM potassium phosphate buffer (pH 6.5). Then, the homogenates were centrifuged at 10,000 × g for 20 min at 4°C. The supernatants were used for the enzyme assay. Reaction mixtures contained 25 mL of potassium phosphate buffer (100 mM, pH 6.5), 0.2 mL of 4-aminoantipyrine/N,N-dimethylaniline reaction solutions, 0.1 mL of horseradish peroxidase (250 units mL⁻¹), and 0.2 mL of enzyme extract. The reactions were initiated by adding 15 μL of 20 mM Put to analyze DAO determination and 20 mM Spd+Spm to analyze PAO. One unit of enzyme activity was defined as 0.001 absorbance units of the change in the optical density.

**STATISTICAL ANALYSIS**

Growth measurements were performed with 10 replicates. The results are expressed as the mean ± standard error (SE). One-way analysis of variance (ANOVA) combined with an LSD test was used to determine the significance of the differences between treatments. Structural equation modeling (SEM) was used to explain the direct effects of related components and PA types on PA metabolism according to Grace (2006). Each arrow represents a causal relationship, i.e., a change in the variable at the tail of an arrow is a direct cause of the change in the variable at the head. Nonsignificant paths are indicated by dotted arrows. Larger standardized coefficients (listed beside each significant path) indicate that the variable at the tail has a stronger effect on the variable at the head. The original SEM was based on the complete theoretical knowledge. The X²-test was used to determine whether covariance structures suggested by the model adequately fit the actual covariance structures of the data. A nonsignificant X²-test (P > 0.05) indicates adequate model fit. The model modification indices provide a strong tool for data exploration and hypothesis generation if the initial model does not adequately fit.

**RESULTS**

**Plant Growth**

The root fresh weight (FW) decreased significantly in response to salinity stress in both zoysia cultivars. The addition of exogenous Spd alleviated salinity-mediated growth reduction to a certain extent. Losses in FW due to salt stress under the NaCl treatments
were 23.4% in z081 and 17.8% in z057, respectively, indicating the higher salt tolerance of z057 (Table 2).

Free PA Contents
Free PA levels showed a great difference in response to salinity stress with exogenous Spd application (Table 3). Spd treatment had almost no effect on free PAs in plants that were not exposed to salt stress. Free Put, Spd, and Spm exhibited similar trends in both cultivars, but certain differences were observed (Table 3). Free Put, Spd and Spm in the roots of z057 demonstrated an upward trend for 4 days after salt stress. In z081, free Spd, Spm, and Put levels increased for 6 days after salt stress, followed by a decline. However, the increase in PA levels was maintained longer in z057 than in z081. Exogenous Spd enhanced PA levels in both cultivars in response to salt stress except free Spd. The upward trend in free Put observed during salt stress was suppressed to a certain extent by the application of Spd, whereas the upward trends in free Spd and Spm upward were enhanced (Table 3).

Soluble Conjugated PA Contents
Soluble conjugated PA levels are reliable indexes of salt tolerance. In this study, we observed that only exogenous Spd application slightly reduced instead of enhanced conjugated PA levels under normal conditions (Table 4). Soluble conjugated PA contents increased under salt stress in both species but decreased as time progressed. However, the peaks occurred at different times in the two cultivars (Table 4). Exogenous Spd application enhanced PA levels to different extents under salt stress in both species, and root PA levels were higher in z081 than in z057 (Table 4).

Insoluble Bound PA Contents
As molecules involved in osmotic adjustment, the content of insoluble bound PAs is lower than those of other PA forms. However, many studies have indicated that insoluble bound PAs are important for plant salt tolerance. Exogenous Spd influenced insoluble bound PAs slightly under normal conditions (Table 5). Insoluble bound PAs showed a similar tendency as soluble conjugated PAs under salt stress. Exogenous Spd application

| TABLE 2 | Fresh weight of Zoysiagrass grown under salt stress with or without treatment with Spd for 8 days. |
|---------|---------------------------------|-------------------------------------------------|
| Cultivar | Treatment | Root fresh weight (g/cm²) |
| z057    | Control | 0.431 ± 0.007a |
|         | Spd | 0.434 ± 0.004a |
|         | NaCl | 0.354 ± 0.01c |
|         | NaCl+Spd | 0.409 ± 0.01b |
| z081    | Control | 0.397 ± 0.003a |
|         | Spd | 0.399 ± 0.002a |
|         | NaCl | 0.304 ± 0.004c |
|         | NaCl+Spd | 0.356 ± 0.008b |

The data represent the means ± SEs from three independent experiments. Values in a single column sharing the same letters are not significantly different (p < 0.05; Duncan’s multiple range test).

| TABLE 3 | Effects of Spd, salt, and salt+Spd on levels of free Put, Spd, and Spm in roots of zoysiagrass. |
|---------|---------------------------------|-------------------------------------------------|
| Species | Treatment | Free Put (nmol/g FW) |
| z057    | Control | n.a. |
|         | Spd | 1,512 ± 0.006a |
|         | NaCl | 1,354 ± 0.006a |
|         | NaCl+Spd | n.a. |
| z081    | Control | n.a. |
|         | Spd | 1,512 ± 0.006a |
|         | NaCl | 1,354 ± 0.006a |
|         | NaCl+Spd | n.a. |
TABLE 4 | Effects of Spd, salt, and salt+Spd on levels of soluble conjugated Put, Spd, and Spm in roots of zoysia grass.

| Species | Treatment | Soluble conjugated Put (nmol/g FW) | Soluble conjugated Spd (nmol/g FW) | Soluble conjugated Spm (nmol/g FW) |
|---------|-----------|-----------------------------------|-----------------------------------|-----------------------------------|
|         |           | Days 0 2 4 6 8                    | Days 0 2 4 6 8                    | Days 0 2 4 6 8                    |
| z057    | Salt      | 372 ± 7 1,152 ± 39a 1,762 ± 80b 1,429 ± 53a 840 ± 43b | 871 ± 32 2,341 ± 61b 2,364 ± 53b 1,747 ± 78b 2,151 ± 40b | 61 ± 2.1 73 ± 4.8b 135 ± 3.0b 139 ± 3.8b 83 ± 3.0b |
|         | Spd       | 369 ± 5 367 ± 7b 356 ± 7c 498 ± 22b 508 ± 14c | 851 ± 21 869 ± 15c 733 ± 12c 1116 ± 71c 734 ± 11c | 62 ± 2.6 61 ± 1.2b 73 ± 1.5c 67 ± 3.0c |
|         | Salt+Spd  | 383 ± 8 1,137 ± 49a 1,853 ± 72a 1,505 ± 47a 1,205 ± 39a | 852 ± 26 2,782 ± 30a 3,196 ± 73a 2,504 ± 91a 2,487 ± 55a | 63 ± 1.2 108 ± 4.2a 288 ± 6.7a 268 ± 5.2a 148 ± 3.8a |
| p       | n.s.      | ** ** ** **                     | ** ** ** **                     | ** ** ** **                     |

The values represent the means ± SEs of three replications per treatment. Different letters indicate significant differences (P < 0.05) with respect to treatment for each species. n.s., not significant; **P < 0.05 indicates the significance of the main effects determined by ANOVA. The values in the same column followed by the same letter are not significantly different at P < 0.05.

TABLE 5 | Effects of Spd, salt, and salt+Spd on levels of insoluble bound Put, Spd, and Spm in roots of zoysia grass.

| Species | Treatment | Insoluble bound Put (nmol/g FW) | Insoluble bound Spd (nmol/g FW) | Insoluble bound Spm (nmol/g FW) |
|---------|-----------|---------------------------------|---------------------------------|---------------------------------|
|         |           | Days 0 2 4 6 8                  | Days 0 2 4 6 8                  | Days 0 2 4 6 8                  |
| z057    | Control   | 113 ± 2.6 164 ± 4.0c 117 ± 3.1d 110 ± 3.2c 111 ± 2.6d | 111 ± 5.3 129 ± 6.0c 107 ± 2.0d 123 ± 4.0b 107 ± 3.8c | 35 ± 1.2 34 ± 1.5c 36 ± 1.5c 35 ± 3.0c 35 ± 1.2d |
|         | Salt      | 111 ± 2.6 354 ± 6.9b 357 ± 7.7b 259 ± 6.1a 257 ± 4.6b | 117 ± 2.8 642 ± 4.6a 1,168 ± 39a 760 ± 26a 736 ± 7.5a | 34 ± 1.0 96 ± 2.3b 72 ± 2.3b 52 ± 2.1b 55 ± 2.1b |
|         | Spd       | 116 ± 5.3 148 ± 2.6c 142 ± 1.7c 112 ± 2.1c 143 ± 3.1c | 106 ± 5.5 123 ± 1.5c 140 ± 4.7c 133 ± 2.5c 114 ± 2.0d | 36 ± 1.5 35 ± 1.5c 38 ± 0.6c 39 ± 1.0c 41 ± 1.0c |
|         | Salt+Spd  | 119 ± 8.1 573 ± 9.6a 576 ± 8.5a 396 ± 7.5a 368 ± 4.7a | 108 ± 6.1 533 ± 6.6b 688 ± 16b 731 ± 14a 662 ± 12.5b | 35 ± 1.5 159 ± 7.0a 87 ± 3.2a 70 ± 1.5a 66 ± 1.7a |
| p       | n.s.      | ** ** ** **                     | ** ** ** **                     | ** ** ** **                     |

The values represent the means ± SEs of three replications per treatment. Different letters indicate significant differences (P < 0.05) with respect to treatment for each species. n.s., not significant; **P < 0.05 indicates the significance of the main effects determined by ANOVA. The values in the same column followed by the same letter are not significantly different at P < 0.05.
increased PA levels significantly at different times in both cultivars exposed to salinity stress (Table 5).

**PA Biosynthetic Enzyme Activity**

ODC activity in the roots of both cultivars increased after 2 days of salinity and peaked on day 4 (Figure 1). The increase in ODC activity was elevated by the application of exogenous Spd during salt stress. ODC activity levels induced by exogenous Spd were greater in z057 than in z081 (Supplementary Table S2). ADC activity in the roots increased rapidly after 2 days and peaked at 4 and 6 days in z081 and z057 under saline conditions, respectively (Supplementary Figure S1). Furthermore, very high levels of ODC and ADC activity were maintained in the Spd+salt-treatment (Supplementary Figure S1). Exogenous Spd had almost no effects on the two cultivars under normal conditions except ADC activity on day 6 in z057 (Supplementary Table S1).

Salt stress caused a significant increase in SAMDC activity in the roots of both cultivars (Figure 1). This increase in SAMDC activity was enhanced and peaked on day 4 under salt stress, although the activity in z057 was higher than that in z081. Exogenous Spd led to higher and more persistent levels of SAMDC activity in z057 than in z081 under salt stress. In both cultivars, exogenous Spd had almost no effects on PA biosynthetic enzyme activity under nonsaline conditions (Figure 1).

![FIGURE 1](image) | Effects of Spd, salt and salt+Spd on activities of ADC, ODC, and SAMDC in roots of zoysia grass under 150 mM NaCl stress. The data represent the means ± SEs of three replicates. Values in a single column sharing the same letters were not significant difference (p < 0.05) (Duncan’s multiple range tests).
### PA Degradative Enzyme Activity

During salt stress, PAO activity increased rapidly in both cultivars (Figure 2). However, it decreased rapidly in z057 and gradually in z081. Exogenous Spd led to higher PAO activity in the roots of z081 under salt stress.

Salt stress induced a rapid increase in DAO activity in z081 roots but had little effect on z057 roots. Exogenous Spd enhanced DAO activity in both cultivars under salt stress (Figure 2).

### Pathway Analysis of Polyamine Metabolism

SEM was used to explain the direct effects of related components and PA types on PA metabolism. The original SEM was based on the complete theoretical knowledge. SEM showed that ODC was the main enzyme with a direct effect (0.691) on Put synthesis, and DAO showed direct effects (0.335) on Put catabolism (Figure 3). In addition, SAMDC and the endogenous Spd level showed direct effects of 0.532 and 0.213 on endogenous Spd and Spm levels, respectively. Furthermore, the endogenous Spm and Spm levels also affected the endogenous Spd and Put levels, respectively. As for the different PA forms, the free forms showed direct effects on the soluble conjugated forms among the major PAs (Figure 3).

### DISCUSSION

Salt stress involves a combination of osmotic stress and dehydration due to excess sodium ions and adversely limits plant growth and development (Rossetto et al., 2015). It has been widely reported that PA metabolism is one of the defense mechanisms that plants invoke in response to salt stress (Kusano et al., 2008). In this study, salt stress reduced the zoysia FW, and exogenous Spd application protected the FW from salt-induced injury (Table 1). This study found a similar pattern of results as previous research that exogenous PAs reduced the decline in plant FW upon exposure to salt stress (Hu et al., 2012).

Many studies have reported changes in PA levels under salt stress (Marco et al., 2011). PA contents differ following short-term and long-term exposure to salinity. Hu et al. (2012) reported the disturbance of PA homeostasis under short-term salt stress in tomato roots. In general, high Spd and Spm values are considered salt tolerance indices, as demonstrated by Li et al. (2016) in salt stress-sensitive and salt stress-resistant zoysiagrass varieties. In a previous study, PA levels changed under salt stress: Put levels decreased and Spd and Spm levels increased in all species examined (Zapata et al., 2004). In other species, an increase in the (Spd+Spm)/Put ratio was observed under...
FIGURE 3 | The structural equation model linking Zoysia japonica Steud. polyamine metabolism to related components. Each arrow represents a causal relationship, i.e., a change in the variable at the tail of an arrow is a direct cause of the change in the variable at the head. Nonsignificant paths are indicated by dotted arrows. Larger standardized coefficients (listed beside each significant path) indicate that the variable at the tail has a stronger effect on the variable at the head. F, free; S, soluble conjugated; i, insoluble bound; Put, diamine putrescine (nmol/g\(^{-1}\) FW); Spd, triamine spermidine (nmol g\(^{-1}\) FW); Spm, tetraamine spermine (nmol g\(^{-1}\) FW); ADC, arginine decarboxylase (nmol(Agm)·g\(^{-1}\) FW·h\(^{-1}\)); ODC, ornithine decarboxylase (nmol(Put)·g\(^{-1}\) FW·h\(^{-1}\)); SAMDC, S-adenosylmethionine decarboxylase (nmol(SAM)·g\(^{-1}\) FW·h\(^{-1}\)); PAO, polyamine oxidase (U g\(^{-1}\) FW); DAO, diamine oxidase (U g\(^{-1}\) FW).

Salt stress, and Spd and Spm contributed to osmotic stress tolerance in wheat seedlings (Liu et al., 2004). In addition, the concentrations of different PA forms (free, soluble, and insoluble) differ greatly under salt stress conditions with exogenous Spd application (Jia et al., 2010; Hu et al., 2012). In the present study, we determined the variation of the dynamic levels of different PA forms exposed to different conditions. Our results indicated that the three PA forms increased in the first stage and then decreased with increasing time following exposure to salt stress. Exogenous Spd application enhanced the levels of all PAs except free Put (Table 3). These data suggested that exogenous Spd might improve zoysiagrass growth and play a role in the regulation of PA forms in response to salinity stress.

ADC, ODC, and SAMDC activities change during environmental stress tolerance in most plant species, indicating that these enzymes are regulated by PA metabolism (Bagni and Tassoni, 2001; Liu et al., 2007). Diamine Put is synthesized by ADC or ODC, and triamine Spd is synthesized by SPDS from Put via the addition of an aminopropyl moiety donated by decarboxylated S-adenosylmethionine (dcSAM) formed by SAMDC (Hanfrey et al., 2002). The elevated activities of ADC, ODC, and SAMDC were a response to the enhancement of PA levels. In the present study, ODC and SAMDC activity were increased in both cultivars exposed to salt stress, and the pattern of change was consistent with the levels of certain PAs (Tables 3–5 and Figures 1, 2). Furthermore, exogenous Spd enhanced ADC activity, ODC activity as well as SAMDC activity in both cultivars (Figure 3).

DAO, which is localized to the plant cell wall, facilitates Put catabolism and is important for cross-linking reactions under stress conditions (Eller et al., 2006). Exogenous Spd increased DAO activity due to the concomitant decrease in free Put content. The application of exogenous Spd induced significant increases in Spd and Spm contents, which were attributable to increased SAMDC activity. Despite the large increases in ADC and ODC activities in both cultivars, little free Put accumulated in the roots due to the large increase in DAO and PAO activity and the conversion of free Put to conjugated and bound Put and free Spd and Spm (Ndairagije and Lutts, 2006).

PA metabolism is a very complex multistep process that is affected by many factors. PA levels are a quantitative characteristic of the salt-stress response. The effect of enzymes on Put levels depends on the activities of ODC and DAO, which are involved in Put catabolism (Figure 3). The SEM indicated that the activity of SAMDC contributed to Spd and Spm levels, whereas endogenous Spd contributed more to Spm than SAMDC activity (Figure 3). Many studies have reported that the three major PAs are present in three forms, and the three forms all showed dynamic changes to different extents under salt stress conditions with exogenous Spd application. Our current results indicate that the level of free PAs contributed to soluble and insoluble PA level among the major PAs, whereas soluble PAs (put and Spm) did not contribute to the levels of the corresponding PA forms (Figure 3).

In summary, salt stress decreased the zoysiagrass FW and increased free Spd and Spm and soluble and insoluble Put, Spd and Spm levels in both cultivars. Moreover, salt stress
enhanced the activity of ODC, SAMDC, and DAO. Exogenous Spd improved PA metabolism in response to salt stress. In addition, ODC, SAMDC, and DAO are the main enzymes of PA metabolism, and endogenous Spd levels also for endogenous Spm levels. Free PA forms may be the primary factor influencing the variations of other PA forms.

AUTHOR CONTRIBUTIONS

SL, PM, YW designed research; SL, PM, YW performed research; SL, PM, YW, YZ contributed new reagents/analytic tools; SL and LC analyzed data; and SL and YZ wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2017.00208/full#supplementary-material

REFERENCES

Alcázar, R., Altabella, T., Marco, F., Bortolotti, C., Reymond, M., Koncz, C., et al. (2010). Polyamines: molecules with regulatory functions in plant abiotic stress tolerance. *Planta* 231, 1237–1249. doi: 10.1007/s00726-010-1130-0

Bagui, N., and Tassoni, A. (2001). Biosynthesis, oxidation and conjugation of aliphatic polyamines in higher plants. *Amino Acids* 20, 301–317. doi: 10.1007/s007260170046

Cona, A., Rea, G., Angelini, R., Federico, R., and Tavladoraki, P. (2006). Functions of amine oxidases in plant development and defence. *Trends Plant Sci.* 11, 80–88. doi: 10.1016/j.tplants.2005.12.009

Do, P. T., Drechsel, O., Heyer, A. G., Hincha, D. K., and Zuther, E. (2014). Changes in free polyamine levels, expression of polyamine biosynthesis genes, and performance of rice cultivars under salt stress: a comparison with responses to drought. *Front. Plant Sci.* 5:182. doi: 10.3389/fpls.2014.00182

Duan, J., Li, J., Guo, S., and Kang, Y. (2008). Exogenous spermidine affects polyamine metabolism in salinity-stressed *Cucumis sativus* roots and enhances short-term salinity tolerance. *J. Plant Physiol.* 165, 1620–1635. doi: 10.1016/j.jplph.2007.11.006

Eller, M. H., Warner, A. L., and Knap, H. T. (2006). Genomic organization and expression analyses of putrescine pathway genes in soybean. *Plant Physiol. Biochem.* 44, 49–57. doi: 10.1016/j.plaphy.2006.01.006

Gil-Amado, J. A., and Gomez-Jimenez, M. C. (2012). Regulation of polyamine metabolism and biosynthetic gene expression during olive mature-fruit abscission. *Planta* 235, 1221–1237. doi: 10.1007/s00726-011-1570-1

Grace, J. B. (2006). *Structural Equation Modeling and Natural Systems*.

Hatmi, S., Gruau, C., Trotel-Aziz, P., Villaume, S., Rabenoelina, F., and Baillieul, M. (2009). An alternative polyamine biosynthetic pathway is widespread in bacteria and essential for biofilm formation in *Vibrio cholerae*. *J. Biol. Chem.* 284, 9899–9907. doi: 10.1074/jbc.M900110200

Hussain, S. S., Ali, M., Ahmad, M., and Siddique, K. H. (2011). Polyamines: natural and engineered abiotic and biotic stress tolerance in plants. *Biotechnol. Adv.* 29, 300–311. doi: 10.1016/j.biotechadv.2011.01.003

Ikbal, F. E., Hernández, J. A., Barba-Espin, G., Koussa, T., Aziz, A., Faize, M., et al. (2014). Enhanced salt-induced antioxidative responses involve a contribution of polyamine biosynthesis in grapevine plants. *J. Plant Physiol.* 171, 779–788. doi: 10.1016/j.jplph.2014.02.006

Jia, Y. X., Sun, J. S., Guo, S. R., Li, J., Hu, X. H., and Wang, S. P. (2010). Effect of root-applied spermidine on growth and respiratory metabolism in roots of cucumber (*Cucumis sativus*) seedlings under hypoxia. *Russ. J. Plant Physiol.* 57, 648–655. doi: 10.1134/S1021443710050079

Kasukabe, Y., He, L., Nada, K., Misawa, S., Ibara, I., and Tachibana, S. (2004). Overexpression of spermidine synthase enhances tolerance to multiple environmental stresses and up-regulates the expression of various stress-regulated genes in transgenic *Arabidopsis thaliana*. *Plant Cell Physiol.* 45, 712–722. doi: 10.1093/pcp/pcp083

Kasukabe, Y., He, L., Watakabe, Y., Otani, M., Shimada, T., and Tachibana, S. (2006). Improvement of environmental stress tolerance of sweet potato by introduction of genes for spermidine synthase. *Plant Biotechnol.* 23, 75–83. doi: 10.5511/plantbiotechnology.23.75

Kim, N. H., Kim, B. S., and Hwang, B. K. (2013). Pepper arginine decarboxylase is required for polyamine and γ-aminobutyric acid signaling in cell death and defense response. *Plant Physiol.* 162, 2067–2083. doi: 10.1104/pp.111.217372

Kusano, T., Berberich, T., Tateda, C., and Takahashi, Y. (2008). Polyamines: essential factors for growth and survival. *Planta* 228, 367–381. doi: 10.1007/s00425-008-0772-7

Lee, J., Spardio, V., Franti, D. E., Longgood, J., Camilli, A., Phillips, M. A., et al. (2009). An alternative polyamine biosynthetic pathway is widespread in bacteria and essential for biofilm formation in *Vibrio cholerae*. *J. Biol. Chem.* 284, 9899–9907. doi: 10.1074/jbc.M900110200

Li, S., Chen, J., Guo, H., Zong, J., Zhang, F., Chu, X., et al. (2012). Salinity tolerance evaluation of zoysia turfgrass germplasm. *Acta Pratacult.* 21, 43–51. doi: 10.11686/cxy20120406

Li, S., Jin, H., and Zhang, Q. (2016). The effect of exogenous spermidine concentration on polyamine metabolism and salt tolerance in Zoysia grass (*Zoysia japonica* Steud) subjected to short-term salinity stress. *Front. Plant Sci.* 7:1221. doi: 10.3389/fpls.2016.01221

Liu, H. P., Dong, B. H., Zhang, Y. Y., Liu, Z. P., and Liu, Y. L. (2004). Improvement of environmental stress tolerance of sweet potato by overexpression of spermidine synthase. *Plant Biotechnol.* 21, 601–612. doi: 10.1111/j.1395-3243.2004.00416.x

Liu, H. P., Dong, B. H., Zhang, Y. Y., Liu, Z. P., and Liu, Y. L. (2004). Overexpression of spermidine synthase enhances tolerance to multiple abiotic stresses and their ability to provide environmental stress tolerance to plants. *Biotechnol. Adv.* 22, 601–612. doi: 10.1016/j.biotechadv.2004.08.007

Liu, H. P., Dong, B. H., Zhang, Y. Y., Liu, Z. P., and Liu, Y. L. (2004). Overexpression of spermidine synthase enhances tolerance to multiple abiotic stresses and their ability to provide environmental stress tolerance to plants. *Biotechnol. Adv.* 22, 601–612. doi: 10.1016/j.biotechadv.2004.08.007

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Majumdar, R., Shao, L., Minocha, R., Long, S., and Minocha, S. C. (2013). Ornithine: the overlooked molecule in regulation of polyamine metabolism. *Plant Cell Physiol.* 54, 990–1004. doi: 10.1093/pcp/pct053

Marco, F., Alcázar, R., Tiburcio, A. F., and Carrasco, P. (2011). Interactions between polyamines and abiotic stress pathway responses unraveled by transcriptome analysis of polyamine overproducers. *omics* 15, 775–781. doi: 10.1089/omi.2011.0084

Moschou, P. N., Wu, J., Cona, A., Tavladoraki, P., Angelini, R., and Roubelakis-Angelakis, K. A. (2012). The polyamines and their catabolic products are significant players in the turnover of nitrogenous molecules in plants. *J. Exp. Bot.* 63, 5003–5015. doi: 10.1093/jxb/ers202

Ndaiyirage, J., and Lutts, S. (2006). Do exogenous polyamines have an impact on the response of a salt-sensitive rice cultivar to NaCl? *J. Plant Physiol.* 163, 506–516. doi: 10.1016/j.jplph.2005.04.034

Pil, M., and Szalai, G., and Janda, T. (2015). Speculation: polyamines are important in abiotic stress signaling. *Plant Sci.* 237, 16–23. doi: 10.1016/j.plantsci.2015.05.003

Pegg, A. E., and Casero, R. A. Jr. (2011). Current status of the polyamine research field. *Methods Mol. Biol.* 720, 3–35. doi: 10.1007/978-1-61779-034-8_1

Richards, F. J., and Coleman, R. G. (1952). Occurrence of putrescine in potassium-deficient barley. *Nature* 170, 479–481. doi: 10.1038/170460a

Rossetto, M. R., Vianello, F., Saeki, M. J., and Lima, G. P. (2015). Polyamines in conventional and organic vegetables exposed to exogenous ethylene. *Food Chem.* 188, 218–224. doi: 10.1016/j.foodchem.2015.04.125

Roy, M., and Wu, R. (2001). Arginine decarboxylase transgene expression and analysis of environmental stress tolerance in transgenic rice. *Plant Sci.* 160, 869–875. doi: 10.1016/S0168-9452(01)00337-5

Sharma, P., and Rajam, M. V. (1995). Spatial and temporal changes in endogenous polyamine levels associated with somatic embryogenesis from different hypocotyl segments of eggplant (*Solanum melongena* L.). *J. Plant Physiol.* 146, 658–664. doi: 10.1016/S0176-1617(11)81929-2

Silveira, V., de Vita, A. M., Macedo, A. F., Dias, M. F. R., Floh, E. I. S., and Santa-Catarina, C. (2013). Morphological and polyamine content changes in embryogenic and non-embryogenic callus of sugarcane. *Plant Cell Tiss. Organ Cult.* 114, 351–364. doi: 10.1007/s11240-013-0330-2

Su, G., An, Z., Zhang, W., and Liu, Y. (2005). Light promotes the synthesis of lignin through the production of H2O2 mediated by diamine oxidases in soybean hypocotyls. *J. Plant Physiol.* 162, 1297–1303. doi: 10.1016/j.jplph.2005.04.033

Tisi, A., Federico, R., Moreno, S., Lucretti, S., Moschou, P. N., Roubelakis-Angelakis, K. A., et al. (2011). Perturbation of polyamine catabolism can strongly affect root development and xylem differentiation. *Plant Physiol.* 157, 200–215. doi: 10.1104/pp.111.173153

Urao, K., Yoshida, Y., Nanjo, T., Ito, T., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2004). *Arabidopsis* stress-inducible gene for arginine decarboxylase *AtADC2* is required for accumulation of putrescine in salt tolerance. *Biochem. Biophys. Res. Commun.* 313, 369–375. doi: 10.1016/j.bbrc.2003.11.119

Vera-Sirera, F., Minguet, E. G., Singh, S. K., Ljung, K., Tuominen, H., Blázquez, M. A., et al. (2010). Role of polyamines in plant vascular development. *Plant Physiol. Biochem.* 48, 534–539. doi: 10.1016/j.plaphy.2010.01.011

Wang, J., and Liu, J.-H. (2009). Change in free polyamine contents and expression profiles of two polyamine biosynthetic genes in citrus embryogenic callus under abiotic stresses. *Biotecnol. Biotecnol. Equip.* 23, 1289–1293. doi: 10.1080/13102818.2009.10817655

Wang, J., Sun, P.-P., Chen, C.-L., Wang, Y., Fu, X.-Z., and Liu, J.-H. (2011). An arginine decarboxylase gene *PADC* from *Poncirus trifoliatea* confers abiotic stress tolerance and promotes primary root growth in *Arabidopsis*. *J. Exp. Bot.* 62, 2899–2914. doi: 10.1093/jxb/erq463

Wang, W., Vinocur, B., and Altman, A. (2003). Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta* 218, 1–14. doi: 10.1007/s00425-003-1105-5

Yang, J. C., Zhang, J. H., Liu, K., Wang, Z. Q., and Liu, L. J. (2007). Involvement of polyamines in the drought resistance of rice. *J. Exp. Bot.* 58, 1545–1555. doi: 10.1093/jxb/erm032

Yin, L., Wang, S., Tanaka, K., Fujihara, S., Itai, A., Den, X., et al. (2016). Silicon-mediated changes in polyamines participate in silicon-induced salt tolerance in *Sorghum bicolor*. *L. Plant Cell Envir.* 39, 245–258. doi: 10.1111/j.1365-3090.2015.02521

Zapata, P. I., Serrano, M., Pretel, M. T., Amorós, A., and Botella, M., Á. (2004). Polyamines and ethylene changes during germination of different plant species under salinity. *Plant Sci.* 167, 781–788. doi: 10.1016/j.plantsci.2004.05.014

Zhao, F. G., Sun, C., Liu, Y. L., and Zhang, W. H. (2003). Relationship between polyamine metabolism in roots and salt tolerance of barley seedlings. *Acta Bot. Sin.* 45, 295–300. doi: 10.1007/s11203-004-7380-9

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