Exogenously Applied Spermidine Alleviates Hypoxia Stress in *Phyllostachys Praecox* Seedlings Via Changes in Endogenous Hormones and Gene Expression

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Abstract

Purpose

Hypoxia stress is thought to be one of the major abiotic stresses that inhibits the growth and development of higher plants. *Phyllostachys pracecox* is sensitive to oxygen and suffers soil hypoxia during cultivation; however, the corresponding measures to mitigate this stress are still limited in practice. In this study, a simulated hypoxia stress with flooding was conducted to investigate the regulatory effect of Spermidine (Spd) on *P. praecox* seedlings.

Methods

Indicators including growth, membrane lipid peroxidation, S-adenosylmethionine decarboxylase (SAMDC), ACC oxidase (ACO) and ACC synthetase (ACS) activities, indole-3-acetic acid (IAA) and abscisic acid (ABA) content, and expression of hormone-related genes in *P. praecox* were measured.

Results

Application of 1 mM and 2 mM Spd could alleviate plant growth inhibition and reduce oxidative damage from hypoxia stress. Exogenous Spd significantly (P<0.05) increased SAMDC activity, enhanced ABA and IAA content, and reduced ACO and ACS activities to protect membranes from lipid peroxidation. Moreover, exogenous Spd up-regulated the expression of auxin-related genes *auxin responsive factor1* (*ARF1*), *auxin1 protein (AUX1)*, *auxin2 protein (AUX2)*, *auxin3 protein (AUX3)* and *auxin4 protein (AUX4)*, and down-regulated the expression of ethylene-related *ACO* and *ACS* genes during flooding.

Conclusion

The results indicated that exogenous Spd altered hormone concentrations and the expression of hormone-related genes, thereby protecting the bamboo growth. Our data suggest that Spd can be used to reduce hypoxia-induced cell damage and improve the adaptability of *P. praecox* to flooding stress.

Introduction

Hypoxia is a serious impeding factor for plant growth, and results in significant yield losses (Bailey-Serres and Voesenek 2008). Hypoxia mainly includes flooded hypoxia and non-flooded hypoxia, such as soil compaction and mulching (Xu et al. 2017). Plants are obligate aerobes, requiring oxygen for mitochondrial respiration and energy production. An unanticipated decline in oxygen availability (hypoxia), as caused by roots becoming flooded, or foliage submergence, triggers physiological changes and gene transcription (Gibbs et al. 2011).

Polyamines (PAs) are a class of low-molecular-weight aliphatic nitrogenous bases with strong biological activity, that are widely present in bacteria, animals, plants and other living organisms (Alcázar et al. 2007). PAs mainly include diamine putrescine (Put), triamine spermidine (Spd) and tetraamine spermine (Spm), among which Put and Spd exist in all organisms, while Spm only exists in prokaryotic bacteria and eukaryotes (Tavladoraki et al. 2012; Alcázar et al. 2011). Studies have shown that polyamines can participate in a variety of metabolic processes related to plant growth and development, such as cell division and differentiation, root elongation, leaf senescence, programmed cell death, DNA synthesis, and transcription of genes (Wimalasekera et al, 2011; Chen

et al. 2019). The mechanism of the involvement of PAs in plant stress resistance and senescence prevention is that polyamines are closely related to hormones, such as auxin, abscisic acid (ABA) and ethylene which could adjust cell senescence (Xu et al. 2011; Guo et al. 2018; Wang et al. 2019). Spd is closely associated with stress tolerance. Previous studies have shown that the application of Spd can enhance plant tolerance to abiotic stresses, such as heavy metals, drought, waterlogging and salt stress (Wimalasekera et al. 2011; Xu et al. 2011; Saha 2015; Li et al. 2016). Yiu et al. (2009) reported that Spd and Spm can maintain the water balance of plant leaves and roots under flooding stress. It also significantly delayed the loss of chlorophyll, enhanced photosynthesis, reduced ROS content, and prevented lipid peroxidation caused by flooding. Spd and Spm help maintain the activities of antioxidant enzymes under flooding. The protective effect of Spd was found to be greater than that of Spm (Yiu et al. 2009). However, the mechanism by which Spd regulates antioxidants under flooding conditions is not yet clear, and it is worthy of further study.

PA is related to many growth and development processes regulated by hormones. Applied with endogenous indole-3-acetic acid (IAA), the content of PAs and key enzymes changed significantly, indicating that there is a synergistic effect of PAs and auxin on plant growth (Singhal et al. 2010; Pieruzzi et al. 2011). On the contrary, PAs and ethylene have antagonistic effects, in that PAs inhibit cell senescence and ethylene promotes senescence (Takahashi et al. 2010), because PAs and ethylene compete for the common substrate S-adenosyl-L-methionine (SAM). SAM is converted to ethylene by ACC synthase (ACS) and ACC oxidase (ACO; Wang et al. 2002). PAs regulate ethylene biosynthesis by inhibiting the accumulation of ACS transcription, and ethylene is an effective inhibitor of arginine decarboxylase (ADC) and S-adenosylmethionine decarboxylase (SAMDC; Ning et al. 1992). SAMDC is a key enzyme in the synthesis of Spd and Spm (Mehta et al. 2002).

There is a complex network of cross-talk between PAs, ABA and nitric oxide (NO; Wimalasekera et al. 2011). For instance, large amounts of ABA are induced to activate downstream gene expression and other physiological responses under flooding (Klingler et al. 2010). A previous study showed that ABA increased the content of PAs (Put, Spd, and Spm) in grapes and activated the polyamine oxidation pathway, leading to stomatal closure (Toumi et al. 2010). On the other hand, some studies have shown that ABA has a certain inhibitory effect on the amount of PAs in plant tissues (Mahajan and Sharma, 2009). Alcázar et al. (2010) found that PAs regulate stomatal responses by inducing closure and reducing aperture, partly via interaction with ABA and NO.

Phyllostachys praecox f. is a monoaxial scattered bamboo species of the genus *Phyllostachys* sub-family of *Gramineae*. It has the characteristics of fast growth, early shoots, fresh taste and high yield. It has been widely promoted and cultivated in most Southern provinces of China (An et al. 1994). Previous studies have shown that the bamboo rhizomes of *P. praecox* often suffered soil hypoxia with mulching cultivation (Xu et al. 2017). *P. praecox* is susceptible to flooding in the wild environment. However, the adaptive molecular mechanism of *P. praecox* to respond to hypoxia, and the regulatory effects of Spd on hypoxia in *P. praecox* have not been identified. Therefore, the objectives of this study were to test the following hypotheses: (1) exogenous Spd can alleviate growth inhibition and oxidative damage of *P. praecox* under soil hypoxia stress; and (2) the cross-talk between Spd and hormones triggers the expression of related genes, and initiates downstream protective mechanisms. This study could provide a reference for illustrating the stress resistance mechanism of *P. praecox* under soil hypoxia, and help develop more stress-tolerant varieties to meet a sustainable production.

Materials And Methods

Plant material and experimental design

The annual seedlings of *P. praecox* were selected as experiment materials. In May 2020, the rhizome of *P. praecox* was completely taken out from the Panmugang base of Zhejiang Agriculture and Forestry University, China. The selected bamboo seedlings were same in size and height, and had a complete structure. *P. praecox* has three to four young shoots and healthy roots. The seedlings were washed with tap water, and then rinsed twice with distilled water. Each seedling was transplanted into a plastic flowerpot with a height of 22 cm, diameter of 23 cm, and a hole at the bottom. A tray was placed at the bottom of the flowerpot. The soil used in the plastic pot was a mixture of 75% garden soil and 25% nutrient substrate. The flowerpots were transferred to the greenhouse (N 30°23', E 119°72') of Zhejiang Agriculture and Forestry University with controlled conditions. The temperature in the greenhouse was controlled at 25–30°C/15–18°C for day/night, and the humidity was controlled at 60–75%. Half-strength nutrient solution was applied at the recovering stage. After one month, all bamboo leaves expanded and were prepared for further tests.

Uniformly-grown bamboo seedlings were subjected to stress treatment, with the experiments adopting a completely random design combination. There were four treatments (1) CK (control), (2) flooding, (3) flooding + 1 mM Spd, (4) flooding + 2 mM Spd. Each treatment had five replicates. The flooding treatment ensured that the water depth was 5 cm on the soil surface. From the first day after flooding, Spd was sprayed every day. The spraying time was between 09:00–10:00 to guarantee the leaves were wet without dripping. The control treatment was sprayed with distilled water under the same conditions. Seedling samples in five replicates were collected on day 0, and on the second, fourth, sixth and eighth day after the experiments were performed. The collected samples were immediately frozen in liquid nitrogen and stored at –80 °C for further use. Leaf length and area were measured after eight days of experimentation.

Sample analysis

Malondialdehyde (MDA) content measurement

Lipid peroxidation is frequently expressed as MDA content. In brief, 0.5 g of fresh leaves were homogenized in 10 ml of 10% trichloroacetic acid (TCA), and centrifuged at 5000 g for 10 min. 2 ml of 0.6% thiobarbituric acid (TBA) in 10% TCA was added to an aliquot of 2 ml of supernatant. The mixture was heated in boiling water for 15 min, and then quickly cooled in an ice bath. The absorbance of the solution was measured at 440 nm, 532 nm, and 600 nm in a spectrophotometer, 3 biological replicates. MDA content was calculated with the OD absorbance. The MDA content was determined as described by Hodges et al. (1999).

Enzyme activity assay

Fresh leaves (1 g) were ground with liquid nitrogen and suspended in 9 ml physiological saline in a pre-chilled mortar and pestle, placed in an ice bath. The homogenate was centrifuged at 12 000 × *g* for 10 min at 4°C, and the supernatant was collected. Nitrate reductase (NR) activity was determined using the assay kits supplied by the manufacturer [NR assay kit (A096-1-2), Nanjing Jiancheng Bioengineering Institute, China)].

ACC, ACS and SAMDC activities were determined using the ELISA plant assay kit following the manufacturer's instructions (Shanghai Enzyme-linked Biotechnology Co., Ltd., China). The kit uses double-ant ibody one-step sandwich enzyme-linked immunosorbent assay (ELISA). Purified plant ACC antibody, ACS antibody, SAMDC

antibody with HRP enzyme-catalyzed label and the corresponding enzymes were used to form an antibody-antigen-enzyme-antibody complex which produced a blue substance after reacting with the tetramethylbenzidine (TMB) substrate solution. The OD value was measured at a wavelength of 450 nm with a microplate reader to calculate the sample activity.

Hormone measurement

The endogenous hormones including auxin (IAA) and abscisic acid (ABA) concentrations were determined using the manufacturer's instructions (ELISA plant hormones assay kit, Shanghai Enzyme-linked Biotechnology Co., Ltd.). Purified plant IAA antibody, ABA antibody with horseradish peroxidase (HRP) enzyme-catalyzed label and hormones were used to form an antibody-antigen-enzyme-antibody complex, which produced a blue substance after reacting with the TMB substrate solution. The OD value was measured at a wavelength of 450 nm with a microplate reader for calculation of the concentrations.

RNA extraction and quantitative RT–PCR (qRT–PCR) analyses

Gene expression was measured by using quantitative reverse transcriptase polymerase chain reaction (qRT–PCR). Total RNAs in leaves were extracted using OminiPlant RNA Kit (CWBIO, CW2598, China). The concentration of mRNA was determined by a nucleic acid analyzer (Nano Drop 2000c, Thermo Scientific, USA) and the RNA quality was assessed using agarose gel electrophoresis. The extracted RNA was reverse transcribed into cDNA using the PrimeScript^{TM} RT reagent Kit with gDNA Eraser (Takara, RR047A, China) and the synthesized cDNA was subjected to PCR. Primers were designed for the sequences of *Actin*, *ARF1*, *ACS*, *ACO*, *AUX1*, *AUX2*, *AUX3* and *AUX4* by Primer 5.0. *Actin* was used as a control, and used for qRT–PCR (Table 1). The primer length was set to between 18 bp and 26 bp, and the expected length of the amplified product was between 80 bp and 250 bp. The primers were synthesized by Sangon Biotech (Shanghai, China). The fluorescent dye used for qRT–PCR (Roche Light Cycler 480II, Switzerland) was Ultra SYBR Mixture (Takara, RR820A, China). Relative gene expression was measured by the $2^{-\Delta\Delta CT}$ method, relative to the house-keeping gene *Actin* (Livak et al. 2001).

Statistical analysis

The physiological parameters were analyzed statistically via two-way analysis of repeat measurements (ANOVA) according to Duncan's Multiple Range test. The F value was obtained from ANOVA. In the text, ns, *, ** and *** represent not significant, and significant difference at $P<0.05$, $P<0.01$, and $P<0.001$, respectively. The sample variability was presented in a line diagram with SD. The significant differences among treatments were marked by different superscripts (a-d) at the level of $P<0.05$ by Duncan's method. All data were analyzed using the SPSS software package (SPSS 20.0).

Results

Plant growth effects following flooding

The bamboo leaf length (LL) and area (LA) following treatment with Spd are shown in Fig. 1. After 8 d of incubation, the LL and LA were obviously reduced in comparison with the control. Exogenous Spd application alleviated the LL and LA reduction under flooding, and increased the leaf size to some extent (Fig. 1).

Membrane damage affected by flooding

With the extension of flooding time, the concentration of MDA increased and was higher than that of the control. After 8 d of flooding, the MDA concentrations increased by 123.5% (Fig. 2), compared with control (CK) plants. However, MDA content was also found to be significantly (P<0.05) decreased in flooding +1 mM Spd (52%) and flooding + 2 mM Spd treatments (18.6%), compared with flooding treatment alone (Fig. 2). There was no significant difference (P<0.05) between Spd treatment groups and CK. These results indicate that exogenous Spd caused a significant (P<0.05) decrease in the concentration of MDA under flooding treatment.

Effect of flooding on activity of stress-related enzymes

After 2 d, flooding significantly (P<0.05) increased SAMDC activity (Fig. 3a). During the 4-8 days of flooding, SAMDC activity in leaves decreased significantly (P<0.05) under flooding, whereas exogenous Spd application increased the SAMDC activity under flooding (Table 2). There was no significant (P<0.05) difference between flooding + 1 mM Spd and flooding + 2 mM Spd treatments.

NR activity in leaves first increased and then decreased gradually throughout the experimental period following flooding. Compared with the flooded group, NR activity of 1 mM Spd treatment decreased significantly by 33% on the 2nd, but the decrease rate decreased with the time to 4% on the 8th day. Furthermore, with increased Spd concentrations, NR activity decreased throughout the experimental period (Fig. 3b).

ACO and ACS activities of leaves in *P. praecox* under flooding significantly (P<0.05) improved (Fig. 4). After 4 d flooding treatment, the ACO and ACS activities were increased by 44.3% and 40.4%, respectively, compared with control plants. Furthermore, as the concentration of Spd increased, the activity of ACS decreased (Fig. 4b). However, no significant difference (P<0.05) in ACO activity was observed in the plants with 1 mM Spd + flooding and 2 mM Spd + flooding treatments (Fig. 4a).

Effect of flooding on hormone content

Flooding increased ABA content during the 2–6 days of the experiment and exogenous Spd increased ABA content compared to the flooding (Fig. 5a). With the increase in Spd concentration, the ABA content increased as well. After 8 d flooding treatment, the ABA content was significantly (P<0.05) decreased compared with that of control, and Spd improved ABA content to the control level.

After two days of flooding stress, IAA concentrations increased compared with the control (Fig. 5b). Exogenous Spd increased IAA content under flooding. After 8 d flooding treatment, there was an obvious decrease in IAA content compared with the control. However, the IAA concentration was increased by Spd treatment, and it increased with the increase in Spd concentration.

Effect of flooding on relative gene expression

Expression of *ACS* and *ACO* first increased and then decreased during the course of the experiment (Fig. 6). After 4 d of flooding treatment, expression of *ACS* and *ACO* in leaves under flooding were significantly (P<0.05) up-regulated to 87.3% and 29.6% of the controls, respectively. On the other hand, exogenous Spd treatment down-regulated *ACO* and *ACS* gene expression after 4 d and 8 d flooding treatment.

Expression of *AUX2*, *AUX3* and *AUX4* increased and then decreased during the experiment. After 4 d flooding treatment, expression of *AUX1*, *AUX2*, *AUX3,* and *AUX4* was significantly (P<0.05) reduced to 76.9%, 59.1%,

88.3%, and 90.6% compared with the controls, respectively (Fig. 7a-d). Treatment with flooding + 2 mM Spd significantly (P<0.05) up-regulated expression of *AUX1* and *AUX2,* and 1 mM Spd did not alter the expression under flooding at 4 d (Fig. 7a, b). After 8 d, with the increase in Spd concentration, *AUX1* expression was also up-regulated (Fig. 7a). There was no significant (P<0.05) difference in *AUX2* expression between flooding + 1 mM and flooding + 2 mM Spd plants (Fig. 7b). Exogenous Spd at 2 mM up-regulated the expression of *AUX3* and *AUX4* under flooding. Treatment with 1 mM Spd did not significantly (P<0.05) alter the expression of *AUX3* and *AUX4* under flooding after 8 d (Fig. 7c, d).

In the control treatments, expression of *ARF1* first increased and then decreased. Flooding caused a sharp down-regulation in *ARF1* expression, when compared with the control. After 4 d flooding treatment, the expression of *ARF1* was down-regulated by 44.8% compared with control (Fig. 7e). After 8 d, *ARF1* expression decreased overall. However, exogenous Spd up-regulated the expression of *ARF1* under flooding and reached the control levels. There was no significant (P<0.05) difference between expression of *ARF1* in flooding + 1 mM Spd and flooding + 2 mM Spd treatments.

Discussion

As aerobic organisms, plants need oxygen (O_2) to support respiration, metabolism and growth. Plants frequently suffer from hypoxic stress due to the low O_2 concentration that is caused by long-term flooding, waterlogging, soil compaction or soil cover management (Bailey-Serres and Voesenek, 2008, Xu et al. 2017). Hypoxia triggers plant physiological changes and gene expression (Gibbs et al. 2011). In our study, flooding was shown to inhibit the leaf length and leaf area in *Phyllostachys praecox,* as expected. Exogenous Spd alleviated bamboo growth inhibition with flooding (Fig. 1). This was consistent with the result that Spd alleviated the inhibition of soybean seedling growth under excess soil moisture (Sidhu et al. 2020).

Generally, membrane lipid peroxidation increases significantly (P<0.05) with time under flooding (Yiu et al. 2009). In this study, the MDA content increased with time, suggesting that the flooding treatment enhanced lipid peroxidation. The application of Spd reduced the MDA content, indicating that Spd can reduce the oxidative stress caused by flooding (Fig. 2, Table 2). This is in accordance with that reported by Hussain et al. (2019), where Spd reduced MDA content and ROS concentrations in *Brassica juncea* leaves.

SAMDC is the rate-limiting enzyme in the synthesis of Spd and Spm in plants (Mehta et al. 2002). Studies have reported that after 6 h of drought stress, the PA content in *CaSAMDC*-overexpressing transgenic Arabidopsis increased, and the accumulation of ROS in cells decreased significantly (Wi et al. 2014). In the present study, flooding induced an increase in SAMDC activity after 2 d. In the middle and late stages of the experiment, SAMDC activity decreased significantly (P<0.05) under flooding (Fig. 3a, Table 2). The transient increase in SAMDC activity may lead to an increase in PA content to protect bamboo from flooding stress. However, as the degree of stress increases, a large amount of ethylene is synthesized in plants. Therefore, it is possible that the concentrations of Spd and Spm that share a common precursor (SAM) with ethylene decreased, and the activity of SAMDC therefore decreased. We found that after exogenous Spd application, the SAMDC activity increased significantly (P<0.05) and the ethylene biosynthesis rate-limiting enzyme activities and gene expression decreased. This may be because exogenous Spd led to an increase in the content of endogenous Spd and Spm in bamboo. The increase in SAMDC activity may accelerate the conversion of free Put to Spd and Spm, and then the conversion of free Spd to Spm, while reducing the ethylene content. This hypothesis is consistent with

previous studies, where Hu et al. (2012) found that exogenous Spd significantly improved Spd and Spm content, and enhanced SAMDC activity under salt stress.

Nitric oxide plays a key role as an intra- and intercellular messenger, inducing various processes in plants, including the expression of related genes and programmed cell death, stomatal closure, seed germination, cadmium toxicity and root development (Wendehenne et al. 2001, Angélique Besson-Bard and Wendehenne, 2009). The source of NO in plants is very rich, and it is mainly produced through the activities of NO synthase (NOS) and nitrate reductase (NR). NR is a cytosolic enzyme that catalyzes NADH-dependent nitrate reduction into nitrite. Nitrate reduction may contribute to cellular acclimation to low oxygen deprivation by regenerating NAD^+ from NADH. Accordingly, species tolerant to oxygen deprivation exhibit higher NR activity than sensitive ones (Bailey-Serres and Voesenek, 2008). In the present study, we found that flooding significantly (P<0.05) improved the NR activity of leaves (Fig. 3b, Table 2). Both in tobacco and in tomato, it has been shown that the absence or the decrease in NR activity in transgenic plants or the addition of tungstate (a potent inhibitor of NR activity) enhances the symptoms of hypoxia. These symptoms are accompanied by a reduction in plant growth (Stoimenova et al. 2003, Horchani et al. 2010). We also found that exogenous Spd decreased NR activity under flooding conditions. Furthermore, with the increase of Spd concentration, NR activity decreased significantly (P<0.05) (Fig. 3b, Table 2). It might be possible that PAs promote the interaction between NO and 14-3-3 proteins to inhibit NR (Rosales et al. 2012).

Polyamines are often regarded as second messengers of plant growth regulators or plant hormones (Tassoni et al. 2000). S-adenosylmethionine (SAM) is a common precursor in the biosynthesis pathway of polyamines and ethylene. Therefore, there may be different interactions between polyamines and ethylene in cells (Ning et al. 1992). One possible interaction is the mutually antagonistic relationship, and the other is that there is no antagonistic relationship between the two. ACS and ACO activities are generally the rate-limiting step in the ethylene biosynthetic pathway (Xu and Zhang, 2015). In the present study, we found that flooding significantly (P<0.05) improved enzyme activities and expression of *ACS* and *ACO,* which were significantly (P<0.05) down-regulated by Spd (Fig. 6, Table 3). Moreover, ACS is more sensitive to flooding stress than ACO (Table 3). It indicates that ethylene and polyamines may have an antagonistic effect under flooding. These results are similar to those of previous studies (Rieu et al., Yu et al. 2014). Polyamines are involved in the regulation of the expression of *ACS* (Pathak et al. 2014). The production of ethylene can be affected by the regulation of ACC synthase and ACC oxidase, at the same time, ethylene can also affect the amount of polyamines in tissues by inhibiting the activity of polyamine synthases such as ADC (Ning et al. 1992).

With ABA, studies have shown that ABA has a certain inhibitory effect on the content of polyamines in plant tissues (Mahajan and Sharma, 2009, Guo et al. 2018). Under water-deficient conditions, the endogenous ABA content from roots to leaves increased and induced stomatal closure (Matías et al. 2015). On the other hand, Luo et al. (2019) suggested that the promoting effect of external Spd on grain filling of wheat was significantly related to the increasing concentration of ABA in grains. The ABA response to flooding may differ and depend on the plant species and duration of flooding. Under normal circumstances, ABA mainly exists in the chloroplast membrane of mesophyll cells by binding to proteins. When the plant is under stress, the bound ABA is quickly released and changes into a free state, which increases the content of ABA in the plant (Sembdner et al. 1980). The results of the present study showed that ABA content increased in the early stages of flooding. This showed that ABA responds quickly in the initial stage of flooding stress, and mainly protects the cell structure and function of bamboo. Exogenous Spd increased ABA content under flooding stress in the initial stages. With the

increase of exogenous Spd concentration, the ABA content also increased (Fig. 5a, Table 2). This is consistent with previous studies. Tajti et al. (2019) suggested that PAs induced ABA accumulation in Spd-treated plants. In our study, after 8 d flooding, ABA content of flooded plants significantly (P<0.05) decreased compared with controls (Fig. 5a, Table 2). This is probably due to the increase in membrane permeability and cell function damage by the prolonged flooding time. The rate of ABA catabolism was higher than its rate of synthesis, which led to a decrease in the ABA content. Wang et al. (2010) also found that after 15 d of flooding, the ABA content decreased in squash trees.

It is well known that IAA can promote plant growth. At the beginning of flooding, the content of IAA increased in bamboo tissues. The increase of auxin content at the early stage of flooding is beneficial to the initiation of stem elongation, leaf growth and oxygen transport (Spanu et al. 1994, Eysholdt-Derzso and Sauter, 2017). Similarly, we found that IAA content increased during 2-4 d of flooding. However, we also noted a significant (P<0.05) decrease in IAA content on the sixth and eighth day after flooding (Fig. 5b, Table 2). This is probably because in the late stages of flooding, photosynthesis of *P. praecox* was severely blocked, membrane lipid peroxidation was severe, and cellular structures were destroyed, thus, the bamboo could not provide energy and substances to meet the needs of IAA synthesis. In our study, exogenous Spd significantly (P<0.05) increased IAA content of bamboo under flooding (Fig. 5b, Table 2). This may be a protection mechanism for plants to adapt to the flooded environment. Li et al. (2018) suggested that drought stress significantly increased the ABA, methyl jasmonate (MeJA) and salicylic acid (SA) concentrations, and notably decreased the IAA, gibberellins (GA3) and zeatin-riboside (ZR) concentrations in maize seedlings.

Many auxin-related genes participate in plant development by regulating the auxin balance in processes such as cell division and elongation, morphogenesis of roots and stems, apical dominance, and plant leaf bud and fruit development (Chandler and William, 2016). Some genes, such as auxin/indole-3-Acetic Acid (*Aux/IAA*) are responsive to auxin stimulation in the early stage of auxin signal transduction (Chapman and Estelle, 2009). Auxin signaling involves the regulation of gene expression by Auxin Response Factors (ARFs) and their inhibition by Aux/IAA proteins (Ori, 2019). ARFs can initiate or inhibit the expression of primary early auxin response genes by specifically binding to the auxin response elements (AuxREs) in the promoter part (Shin et al. 2007), and participate in different growth processes of plants. In Arabidopsis, the ARF protein family is divided into two categories: transcription activator and transcription repressor (Tiwari and Guilfoyle, 2003). So far, only ARF2, ARF3, ARF4 and ARF9 proteins have been proved to have transcriptional inhibition through plant protoplast transformation experiments (Ulmasov, 1997).

There is evidence that the expression of *ARF* is affected by environmental or hormonal signals. For example, as the degree of leaf senescence deepens, the expression of *ARF2* increased, while that of *ARF1* decreased in Arabidopsis leaves (Ellis et al. 2005). Similarly, we found that flooding significantly (P<0.05) decreased *ARF1* expression of leaves and Spd application up-regulated *ARF1* gene expression under flooding (Fig. 7e, Table 3). It was suggested that *ARF1* was likely to be a transcription activator. It has been reported that *AUX1* is an auxin uptake carrier (Marchant, 2014). *AUX1* could directly be involved in induction of ROS signaling via the H_2O_2-mediated pathway, which prevents further increase in oxidative damage. Alternatively, AUX1 could indirectly influence cell elongation and cell division by regulating auxin levels and the auxin signaling network, in turn controlling the root growth under stress (Krishnamurthy and Rathinasabapathi, 2013). The present study showed that the expression of *AUX1*, *AUX2*, *AUX3* and *AUX4* genes of bamboo under flooding was significantly (P<0.05) reduced, and *AUX1*, *AUX2* and *AUX4* were more sensitive to flooding (Table 3), while exogenous Spd up-regulated

the expression of these genes (Fig. 7a-d, Table 3). This may be due to the synergistic effect of spermidine and IAA to alleviate the damage caused by flooding stress.

Conclusions

Soil hypoxia induced the growth and membrane lipid injury in *P. praecox* leaves. Exogenous application of Spd enhanced the tolerance against hypoxia by increasing SAMDC activity and IAA and ABA concentrations, up-regulating expression of auxin-related genes (*ARF1*, *AUX1*, *AUX2*, *AUX3* and *AUX4*), reducing the activity of ethylene-related enzymes and genes (*ACS* and *ACO*), and decreasing NR activity, thereby enhancing the ability of *P. praecox* to maintain the stability of cell membrane structure. These results supported our hypothesis that exogenous Spd can alleviate growth inhibition and oxidative damage of *P. praecox* under soil hypoxia stress and the cross-talk between Spd and hormones triggers the expression of related genes, and initiates downstream protective mechanisms. Overall, Spd could increase *P. praecox* adaptability to flooding stress that may be useful for the sustainable production of bamboo in practice.

Declarations

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Tables

Table 1 qRT-PCR specific primers for examined genes

Gene name	Primer sequence (5'-3')	Accession (http://www.ncbi.nl.nih.gov/)
Actin	F: CGTCAAAGCCCCAAGAACAC	FJ601918.1
	R: GCTAGGAAAGACAGCCCTGG	
ARF1	F: ATGCACCTGGTATGGCGAAT	KU721918.1
	R: TCCAAGTGACCATGCCCAAA	
ACS	F: TCAGCTCGTTCGTCCATCAC	AB085172.1
	R: TTAGCTACGCGTTGGTCGTC	
ACO	F: GATCACCAACGGCAGGTACA	AB044747.1
	R: TCCTCGAACACGAACTTGGG	
AUX1	F: GTTCGTGAAGGTGAGCATGG	KU721904.1
	R: CGTTCATGCCGTTCATCCCT	
AUX2	F: TCTGAGGATGTACGGAGGGT	KU721905.1
	R: GCATCAGATCGCCGTCCTTG	
AUX3	F: AAGGGCATGAACGAGAGCAA	KU721906.1
	R: CGACTCGACGAACATCTCCC	
AUX4	F: TGACCAGCCGATGACGAAG	KU721907.1
	R: GCTGCTTGGAAGGTGTTCCT	

Table 2 The F value was obtained from the analysis of variance (ANOVA) on the data of the hormone concentration and enzyme activities in the leaves when different concentrations of exogenous Spd were applied under flooding

Source of variation	df	MDA	SAMDC	IAA	ABA	ACS	ACO
Flooding	1	388.13***	2.31*	4.46*	1.65*	289.46***	49.04***
Time	4	162.02***	6.89**	4.68**	41.09***	58.53***	12.26***
1 mM Spd	1	233.26***	4.72*	75.70***	51.68***	177.78***	8.93**
2 mM Spd	1	501.57***	20.34***	157.95***	170.23***	420.54***	4.82*
Flooding× Time	4	80.12***	6.33**	19.34***	11.50***	31.59***	6.52**
1 mM Spd × Time	4	44.34***	8.54***	18.26***	8.08**	16.55***	3.28*
2 mM Spd× Time	4	81.56***	16.15***	24.57***	16.56***	39.63***	4.48**

MDA-malondialdehyde content; SAMDC-S-adenosylmethionine decarboxylase activity; IAA-auxin content; ABA-abscisic acid; ACS-ACC synthetase activity; ACO-ACC oxidase activity; Spd-spermidine; ns, non-significant; *, **,

*** significant at 0.05, 0.01 and 0.001 probability, respectively.

Table 3 The F value is obtained from the analysis of variance (ANOVA) on the data of the relative gene expressions in the leaves when different levels of exogenous spd were applied under flooding.

Source of variation	df	*ACS*	*ACO*	*ARF1*	*AUX1*	*AUX2*	*AUX3*	*AUX4*
water	1	1583.22***	49.40***	16.67**	344.76***	319.37***	113.11***	352.88***
time	2	240.66***	343.05***	245.44***	189.58***	452.16***	81.59***	343.17***
1mM Spd	1	1092.56***	253.07***	37.40***	104.64***	11.71**	206.75***	129.63***
2mM Spd	1	320.34***	397.77***	87.60***	97.97***	40.37***	145.08***	229.10***
water × time	2	438.82***	24.52***	5.83*	90.95***	84.45***	81.05***	228.03***
1mM Spd × time	2	315.96***	199.93***	16.50***	55.89***	2.96**	129.04***	99.35***
2mM Spd × time	2	101.06***	183.31***	44.06***	24.60***	11.98**	43.26***	84.34***

ACS-ACC synthetase expression; *ACO-ACC oxidase* expression; *ARF1-auxin responsive factor1* expression; *AUX1-auxin1 protein* expression, *AUX2-auxin2 protein* expression, *AUX3-auxin3 protein* expression and *AUX4-auxin4 protein* expression; Spd-spermidine; ns, non-significant; *, **, *** significant at 0.05, 0.01 and 0.001 probability, respectively.

Figures

Treatments	Leaf length (cm)	leaf area (cm^2)
CK	8.90±0.20c	12.74±0.36c
Flooding	7.63±0.06d	10.46±0.30d
Flooding+1mM Spd	10.6±0.20b	15.39±0.30b
Flooding+2mM Spd	11.80±0.10a	22.54±0.22a

Figure 1

Effects of exogenous Spd application on leaf length and area of P. praecox after 8 d of flooding. Vertical bars represent ± the standard error of the mean (n = 3, n represents the biological replicates). Values for the same day followed by different letters are significantly different (P<0.05).

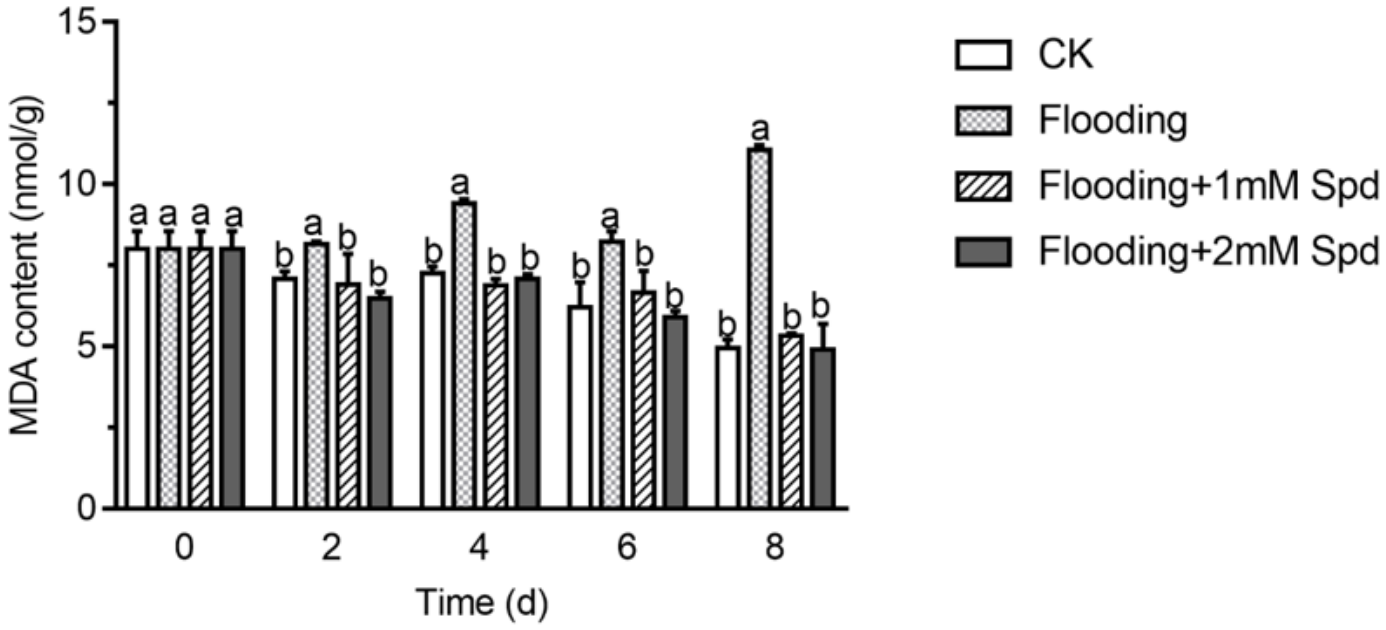

Figure 2

Effects of exogenous Spd application on MDA content of leaves in P. praecox under flooding for the indicated duration. Vertical bars represent ± the standard error of the mean (n = 3, n represents the biological replicates). Values for the same day followed by different letters are significantly different (P<0.05).

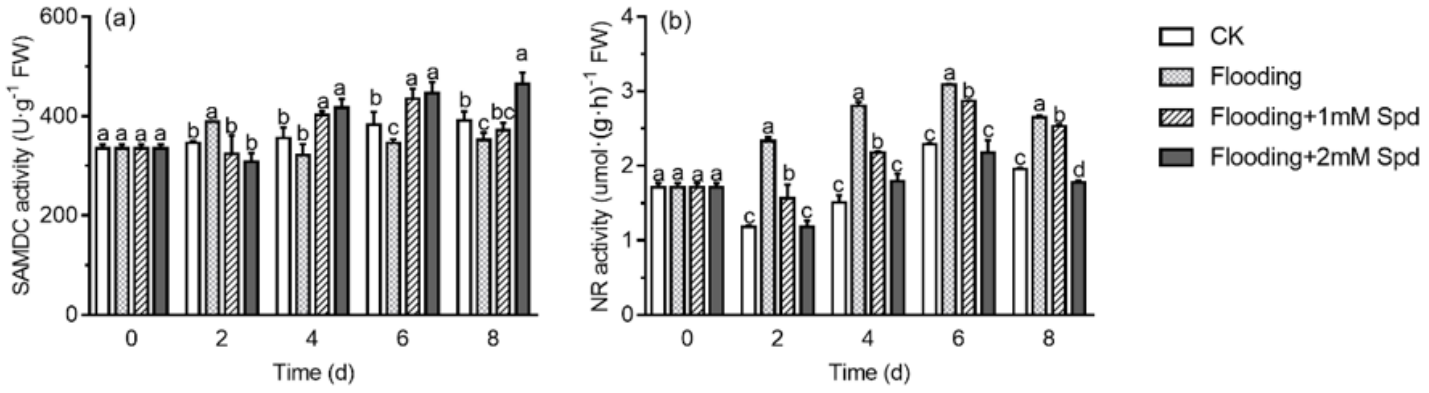

Figure 3

Effects of exogenous Spd application on SAMDC (a) and NR (b) activities of leaves in P. praecox under flooding for the indicated duration. Vertical bars represent ± the standard error of the mean (n = 3, n represents the biological replicates). Values for the same day followed by different letters are significantly different (P<0.05).

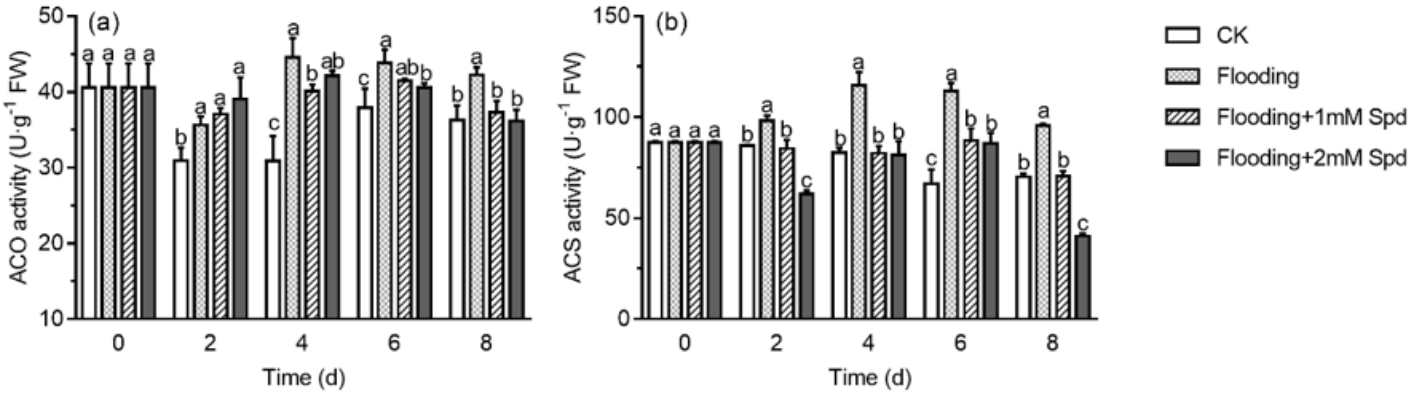

Figure 4

Effects of exogenous Spd application on ACO (a) and ACS (b) activities of leaves in P. praecox under flooding for the indicated duration. Vertical bars represent ± the standard error of the mean (n = 3, n represents the biological replicates). Values for the same day followed by different letters are significantly different (P<0.05).

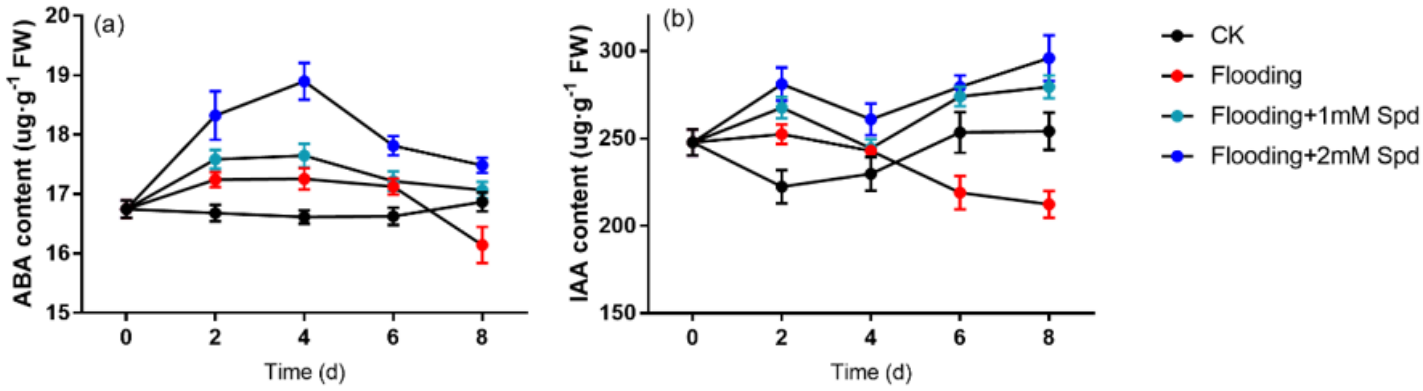

Figure 5

Effects of exogenous Spd application on ABA (a) and IAA (b) content of leaves in P. praecox under flooding for the indicated duration. Vertical bars represent ± the standard error of the mean (n = 3, n represents the biological replicates). Values for the same day followed by different letters are significantly different (P<0.05).

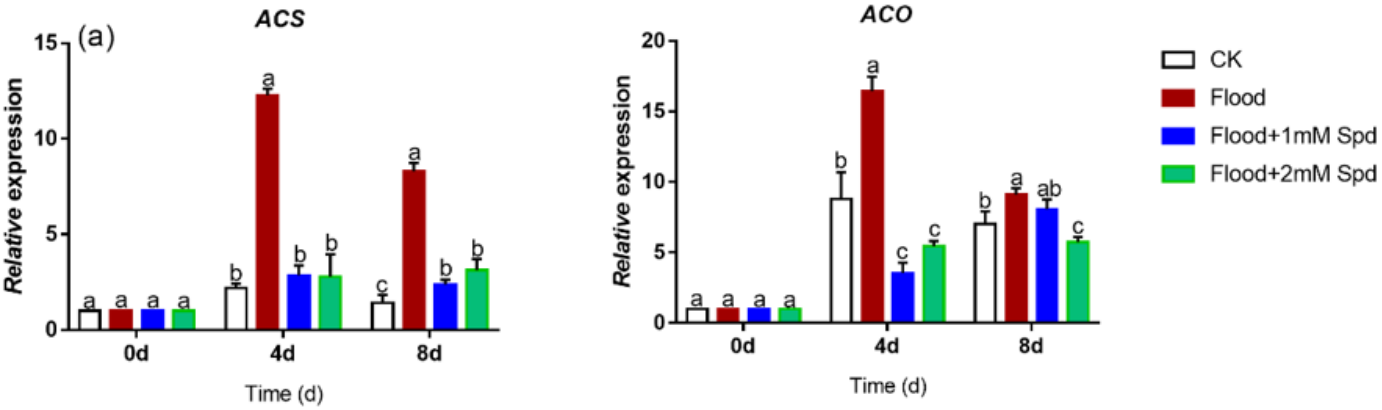

Figure 6

Effects of exogenous Spd application on the relative expression of ACS (a) and ACO (b) in leaves of P. praecox under flooding for 0, 4 d and 8 d. Vertical bars represent ± the standard error of the mean (n = 3, n represents the biological replicates). Values for the same day followed by different letters are significantly different (P<0.05).

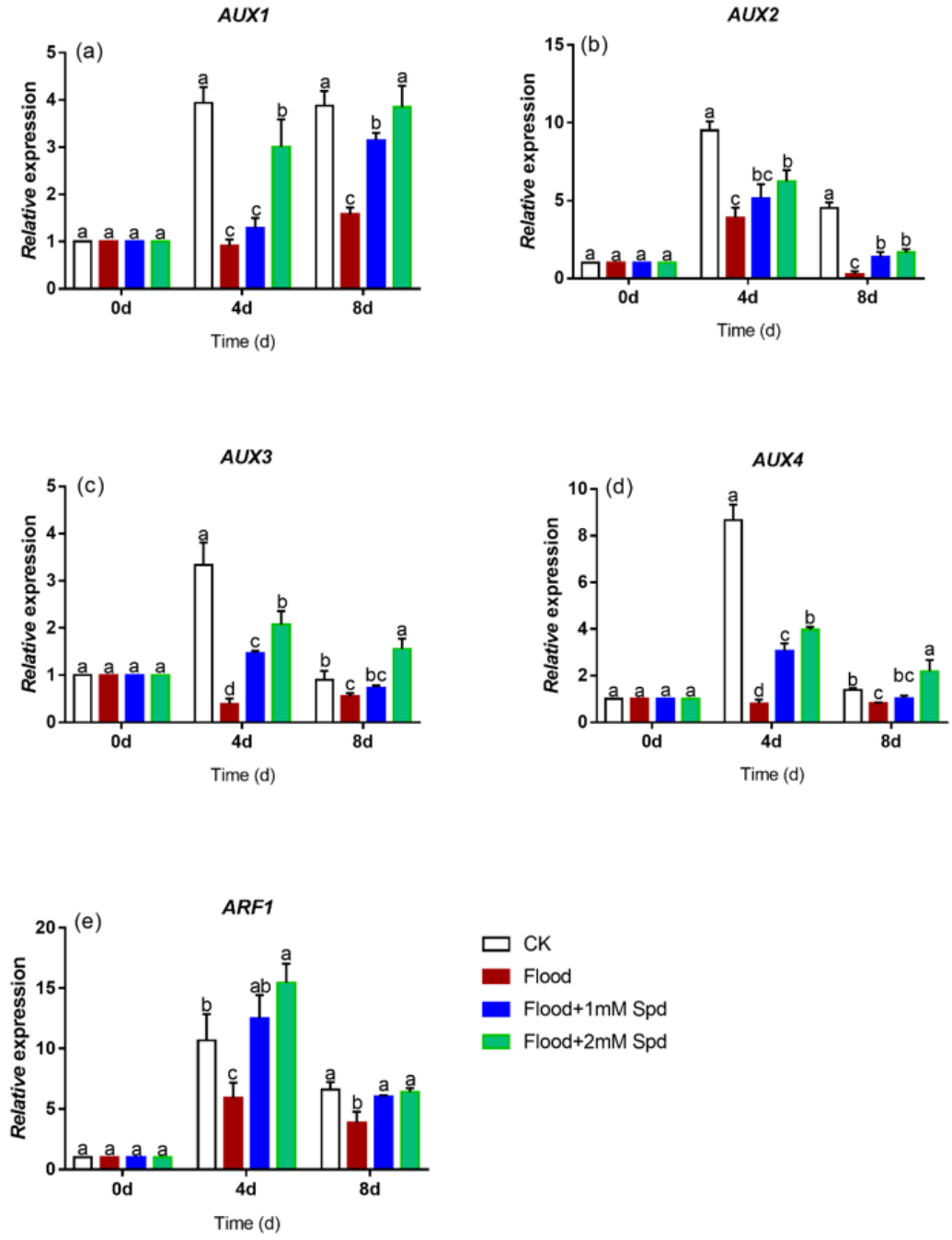

Figure 7

Effects of exogenous Spd application on the relative expression of AUX1 (a), AUX2 (b), AUX3 (c), AUX4 (d), and ARF1 (e) in leaves of P. praecox under flooding for 0, 4 d and 8 d. Vertical bars represent ± the standard error of the mean (n = 3, n represents the biological replicates). Values for the same day followed by different letters are significantly different (P<0.05).