

Exogenous ABA foliar spray treatment alleviates flooding stress in adzuki bean (*Vigna angularis*) during the seedling stage

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Abstract

Abscisic acid (ABA) plays an important role in regulating activities of antioxidant enzymes, hormone levels, accumulation of reactive oxygen species under stress. To understand the effects of exogenous ABA treatment on flooding tolerance at seedling stage in adzuki bean (*Vigna angularis*), the water-sensitive (WS) variety 'Tianjinhong' (TJH) and the water-tolerant (WT) variety 'Longxiaodou 4' (LXD 4) were foliar sprayed exogenous ABA with 20 μ M, followed by flooding stress for 5 days under pot conditions. Our results revealed that under flooding stress at seedling stage, the contents of H₂O₂ and MDA, proline and soluble protein in adzuki bean leaves significantly increased, the contents of ABA, IAA and SA significantly increased, and the activities of SOD, POD and CAT also significantly increased. Flooding treatment for 5 days resulted in a significant decrease in single pot yield of LXD 4 by 8.40% and TJH by 9.91%. Foliar spray of 20 μ M exogenous ABA could resist flooding stress, considerably increased the contents of proline and soluble protein, significantly decreased the contents of H₂O₂ and MDA, significantly increased the activities of SOD and POD, increased the contents of ABA and SA, and significantly inhibited the increase of IAA content in adzuki bean leaves. Spraying exogenous ABA significantly increased the yield of 'LXD' 4 by 6.95% after 4 days of flooding treatment, and 'TJH' by 4.46%. To sum up, there were significant differences in physiological stress among different varieties of adzuki bean under flooding stress, and spraying exogenous ABA could effectively alleviate the effects of flooding stress on physiology and yield of adzuki bean. The results of this study provided a theoretical basis for further studying the physiological mechanism of adzuki bean resisting flooding stress at seedling stage and improving the yield of adzuki bean under flooding stress. Thus, foliar spraying exogenous ABA effectively improved submergence tolerance by enhancing the activity of protective enzymes and osmoregulation. These results provided novel insights and were expected to aid in the development of more effective stress resistance cultivation methods in adzuki bean production.

Keywords: adzuki bean; exogenous ABA; flooding; seedling stage; yield

Abbreviation: LXD 4, Longxiaodou 4; TJH, Tianjinhong; ROS, reactive oxygen species; SOD, superoxide dismutase; POD, peroxidase; CAT, catalase; MDA, malondialdehyde; ABA, abscisic acid; H₂O₂

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hydrogen peroxide; O_2^- ; superoxide anion; MDA malondialdehyde; TCA Trichloroacetic Acid Solution; KI, Potassium iodide

Introduction

In recent years, global warming, extreme weather and flood disasters occur frequently, flooding stress has become the most common abiotic stress in agricultural production (Zhao *et al.*, 2018). According to statistics, 10% of irrigated land worldwide is affected by floods, which may reduce crop yields by 20% (Shabala, 2011). The agricultural flood disaster area in China accounts for about 19% of the total disaster area, and flooding is the second largest natural disaster in China (You *et al.*, 2016). Adzuki bean is one of the most important edible legume crops in China, and it is an important crop for planting structure adjustment, which is beneficial to the sustainable development of agriculture in China. Adzuki bean has the characteristics of strong adaptability and wide sowing range. Adzuki bean is resistant to barren, saline-alkali and drought, but it is sensitive to flooding stress. Spring flooding in spring sowing areas and heavy rain in summer sowing areas in China will cause flooding stress to adzuki bean seedling growth. It is very easy to form flooding hazards.

Flooding stress inhibits the seedling growth of plants, causes individual physiological differences of plants, and finally reduces crop yield and affects agricultural production (Normile, 2008). Flooding stress can induce cells to produce free radicals, which in turn leads to ion leakage and cell death. In order to resist the toxic effect of reactive oxygen species (ROS) under flooding stress, the activity of antioxidant enzymes in plants is increased, which can scavenge more free radicals. Studies have shown that high levels of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activities play an important role in the survival of many plants under long-term flooding conditions (Chen *et al.*, 2006). Under flooding stress, plant ROS metabolism is out of balance, resulting in excessive accumulation of hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-). And excessive ROS will cause membrane system damage and cell oxidation (Wu *et al.*, 2012), produce lipid peroxides and then gradually decompose into a series of complex compounds, including malondialdehyde (MDA), MDA can polymerize with enzyme proteins, causing membrane system denaturation and affecting normal physiological metabolism, and eventually lead to a decline in production. Related studies showed that the yield of maize decreased by 58.80%-69.80% after flooding stress at seedling stage (Yu *et al.*, 2015), the yield of mung bean could be reduced by 18.07% ~ 28.87% under flooding stress at the initial grain stage (Yu *et al.*, 2019), and the yield per plant decreased by 11.11% due to flooding in V3 stage of soybean (Zhang *et al.*, 2016).

In recent years, it has been widely recognized that chemical control technology can regulate crop physiological metabolism, enhance stress resistance and improve crop yield. Exogenous ABA can slow down the increase of ROS and MDA content under stress, maintain normal physical and chemical metabolic functions and pathways of crops, and alleviate stress damage. Exogenous ABA resists stress by regulating ROS accumulation and reducing MDA content (Xiang *et al.*, 2019), increasing the activity of antioxidant enzymes (Li *et al.*, 2017), increasing the content of osmotic regulators (Guo *et al.*, 2014), and regulating the dynamic balance of endogenous hormones (Xiang *et al.*, 2017). This has been confirmed in the study of stress such as low temperature (Xiang *et al.*, 2019), drought (Wang *et al.*, 2020), saline-alkali and heavy metal (Gao *et al.*, 2017; You *et al.*, 2019). However, there are few reports about exogenous ABA alleviating crop flooding stress, especially in adzuki bean seedling stage. When adzuki bean was subjected to flooding stress, the physiological metabolism of leaves was blocked and the establishment of seedlings was affected, resulting in a decrease in yield. Therefore, in this experiment, adzuki bean was flooded at seedling stage, and the effects of flooding stress and pre-spraying exogenous ABA on adzuki bean leaf stress and yield were studied in order to analyze the effect of exogenous ABA on adzuki bean flooding at seedling stage. The result of this paper can provide the theoretical support for flooding-resistant cultivation of adzuki bean.

Materials and Methods

Plant materials and treatments

The CT variety 'LXD 4' and the CS variety 'TJH' were subjected to flooding stress treatment and the detailed experimental design is shown in Table 1. Seeds were germinated on April 18, 2019 at an experimental farm in Harbin, Heilongjiang (45°75'27"N, 126°63'19"E). The average temperature and rainfall of this field ranged from 25-32 °C and 36-170 mm, respectively, with an average humidity of approximately 60% during the growing season. The pot experiment was conducted with a pot height of 23 cm and a diameter of 25 cm. The type of soil used in the experiment was meadow black soil. The natural air-dried loam was covered with 200g soil after sowing with 5.5 kg per pot, and 15 seedlings were preserved in each pot (5 holes × 3 plants). The plants were treated by foliar sprayed with 0 or 20 μM uniconazole at the seedling stage when the opposite leaves are fully unfolded and the first compound leaf is exposed on May 2, 2019. The exogenous ABA solution dried naturally on the leaves for 1 d for complete absorption. The treated plants were moved into a larger pot for flooding treatment for 1-5 d, and keep the water level above 2 cm in the soil during this treating period, which the opposite leaves of the plants were sampled at regular time intervals. The untreated plants (control) were grown at normal soil without flooding treatment. 3 pots of flooding stress were relieved by each treatment every day, which could be used for yield determination in the later stage. For the potted plants relieved of stress, the seedlings and weeds were observed and pulled one after another, and finally 5 seedlings were preserved in each barrel (5 holes × 1 plant). According to the actual air temperature and rainfall, the seedlings were watered until mature. All the plants were harvested on September 23-30, 2019.

Table 1. Experiment design

Varieties	Treatment number	Pesticide treatment	Temp. treatment
'LXD 4'	T1	Spray water	Normal water
	T3	Spray water	Waterlogging stress
	T3	Spray ABA	Waterlogging stress
'TJH'	T4	Spray water	Normal water
	T5	Spray water	Waterlogging stress
	T6	Spray ABA	Waterlogging stress

Measurement of Hydrogen peroxide (H₂O₂) contents

To measure H₂O₂ contents, taking 1.0 g plant samples, add 0.1% TCA, of 0.5 mL to grind under liquid nitrogen, and the homogenate obtained after grinding is centrifuged 20 min at 19000× g. Taking 0.5 mL of the supernatant, add 2 ml 1M KI solution and 0.5 ml 100 mM potassium sulfate buffer, dark reaction for 1 hour; after the reaction, take 0.1% TCA as a reference, determine the absorbance at 390 nm.

Determination of antioxidative enzyme activities

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT). For the total SOD assay, the reaction mixture consisted of 0.025% (w/v) Triton X-100, 0.1 mM EDTA, 2 μM riboflavin, 13 mM methionine, 50 mM HEPES (pH 7.6), 50 mM Na₂CO₃, 75 μM NBT, and an appropriate aliquot of the enzyme extract in a total volume of 5 mL. The reaction mixture was subjected to a light intensity of 350 μmol (photon) m⁻² s⁻¹ for 15 min. The amount of enzyme required to result in 50% inhibition of the reduction of NBT at 560 nm was designated as one unit of SOD activity.

Peroxidase (POD, EC 1.11.1.7) activity was estimated by guaiacol as the hydrogen donor, which has an extinction coefficient of 26.6 mM cm⁻¹ at 470 nm. The reaction mixture contained 0.25% (v/v) guaiacol, 0.1 M H₂O₂, and 10 mM sodium phosphate buffer (pH 6.0) in a total volume of 3 mL. A series of dilutions of the crude enzyme preparation (0.1 mL) was measured at an absorbance of 470 nm after 0, 1, 2, and 3 min.

Peroxidase activity was presented as $\mu\text{mol (product) min}^{-1} \text{g}^{-1}$ fresh mass (FM), as described by Wang *et al.* (2016).

Enzyme activities were estimated by a GE Ultrospec™ 2100 pro UV/Visible spectrophotometer (GE Healthcare, USA). Catalase (CAT, EC1.11.1.6) activity was measured by continuously monitoring the decomposition of H_2O_2 at an absorbance of 240 nm, with a minor modification. The reaction mixture consisted of 0.14 mM H_2O_2 , 100 mM potassium phosphate buffer (pH 7.0), and mitochondria in a total volume of 1 mL, and the absorbance at 240 nm was observed with a spectrophotometer. The molar extinction coefficient of H_2O_2 at 240 nm is 36 M cm^{-1} .

Measurement of free proline, soluble sugar, soluble protein, and malondialdehyde (MDA) contents

To measure free proline, 0.5 g of leaf sample was added to 10 mL of 3% sulfosalicylic acid and filtered through filter paper. A 2-mL aliquot of extract was added to 2 mL of glacial acetic acid and 2 mL of acid-ninhydrin, and the mixture was incubated in a heated water bath for 30 min. After cooling down, 4 mL of methylbenzene was added, and the mixture was mixed thoroughly before the absorbance at 520 nm was measured using a GE Ultrospec™ 2100 pro UV/Visible spectrophotometer (GE Healthcare, USA).

The soluble sugar content was estimated as described by Creelman *et al.* (1990). Leaf sample (0.1 g) was placed into a 10-mL centrifuge tube, and 5 mL of 80% alcohol was added. The mixture was incubated in a water bath set at 80 °C for 30 min. Then, the tube was cooled down and centrifuged at $1000 \times g$ for 10 min before the soluble sugar content was determined by the phenol-sulfuric acid method.

The soluble sugar content was estimated as described by Li *et al.* (2000). Leaf sample (0.5 g) was put into a mortar, grind with a little quartz sand and 5 mL distilled water, set the volume to 10 mL, centrifuge at $4000 \text{ r} \cdot \text{min}^{-1}$ for 10 min, take 1 mL of the supernatant, put it into the test tube with plug and add 5 mL Coomassie brilliant blue G250 solution, shake and place 2 min to fully react, colorimetric determination of absorbance and protein content under 595 nm.

The MDA content was determined by the thiobarbituric acid method. Leaf sample (0.1 g) was added to 5 mL of 0.05 M phosphate buffer (pH 7.8) at 4 °C, and the mixture was centrifuged at $12,000 \times g$ for 20 min. A 1.5-mL aliquot of the supernatant was transferred to a tube and mixed with 2.5 mL of 20% (w/v) trichloroacetic acid containing 0.5% (w/v) thiobarbituric acid. The mixture was heated in boiling water for 10 min and then quickly cooled in an ice bath before centrifugation at $1,800 \times g$ for 10 min. The absorbance of the supernatant was measured with a GE Ultrospec™ 2100 pro UV/Visible spectrophotometer (GE Healthcare, USA) at 532, 600, and 450 nm. The MDA concentration [μM] was expressed by the formula $6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}$.

Determination of hormones

Extract: 0.2 g plant tissue sample, add 10 mL methanol-water-formic acid solution to fully grind and centrifuge ($3000 \text{ r} \cdot \text{min}^{-1}$, 5 min), supernatant + Vc- ethanol solution concentrated to the aqueous phase, 2 purification: pass the Oasis HLB solid phase extraction column, clean the extraction column with 1 mL 20% methanol + 1 mL 80% methanol solution successively, The sample was passed through $0.22 \mu\text{m}$ organic filter membrane for HPLC-MS/MS analysis.

Statistical analysis

The measurements were performed six times and expressed as the mean \pm standard error by SPSS (V24.0, IBM Corporation, Armonk, NY, USA). The data were analyzed by one-way analysis of variance and Duncan's test using OriginPro2016 software (OriginLab Corporation, USA). A *P* value < 0.01 indicated a significant difference.

Results

Effect on membrane damage of adzuki bean seedlings

As shown in Figure 1A, the H_2O_2 content in adzuki bean leaves increased rapidly after flooding stress at seedling stage. Compared with CK (T1 or T4), the H_2O_2 content of 'LXD 4' and 'TJH' increased significantly under flooding stress (T2 and T5). After spraying ABA, the content of H_2O_2 under flooding stress was effectively reduced. When 'LXD 4' was treated from 3 d to 5 d, T3 decreased by 1.19%, 4.50% and 9.70% respectively compared with T2, and reached a significant difference on the 5th day. When 'TJH' was treated from 3 d to 5 d, T6 decreased significantly by 7.61%, 6.57% and 6.85% compared with T5, respectively. And it can be seen from Figure 1B, the content of MDA in adzuki bean leaves showed an upward trend. 'LXD 4' and TJH showed significantly higher flooding stress (T2 and T5) than CK (T1 or T4). Spraying ABA could obviously alleviate the increase of MDA content under flooding stress. When 'LXD 4' was treated from 2 days to 5 days, T3 decreased significantly by 14.62%, 8.75%, 8.86% and 13.76% compared with T2, respectively. After 'TJH' was treated for 2 days to 5 days, T6 decreased significantly by 15.18%, 19.25%, 15.08% and 9.15% compared with T5, respectively.

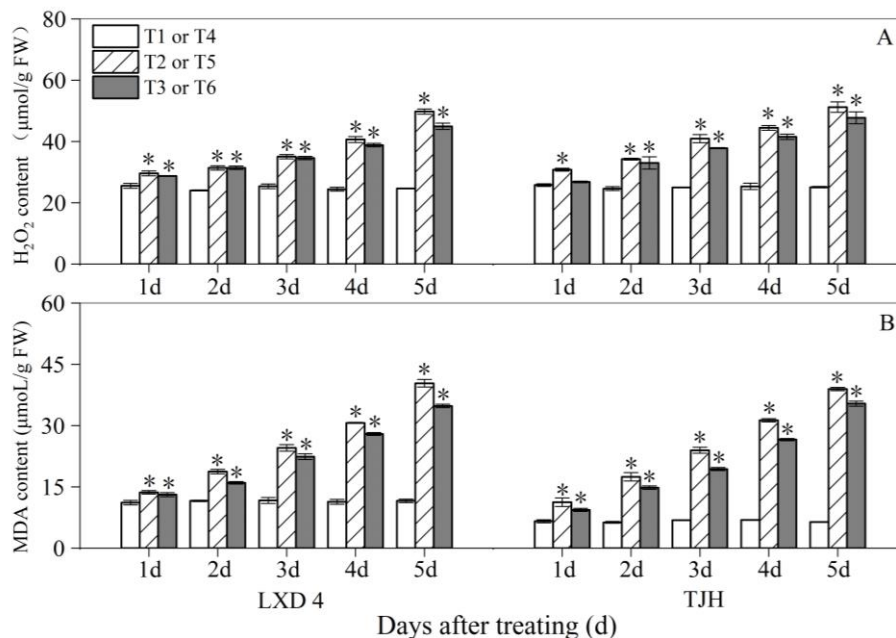


Figure 1. Effects of waterlogging stress and spraying ABA on H_2O_2 and MDA content in leaf of adzuki bean seedlings

Effect on protective enzyme system of adzuki bean seedlings

It can be seen from Figure 2A that after flooding and spraying ABA, the SOD activity of adzuki bean seedlings increased at first and then decreased. When 'LXD 4' was treated for 2 days and 3 days, T2 was significantly higher than T1 by 15.48% and 39.92%, respectively. Spraying ABA increased SOD activity compared with flooding stress, but did not reach a significant difference level. When 'TJH' was treated from 2 d to 4 d, T5 was significantly higher than T4 by 25.49%, 31.47% and 17.77%, respectively. Spraying ABA under flooding stress could effectively increase the SOD activity of 'TJH'. At 4 days and 5 days of treatment, T6 significantly increased 14.58% and 11.69% compared with T5, respectively.

It can be seen from Figure 2B that the activity of POD in adzuki bean increased at first and then decreased after flooding at seedling stage, and reached the highest value at the 4th days after treatment. Spraying ABA could further increase the activity of POD. 'LXD 4' showed $T3 > T2 > T1$ every day. During 5 days of treatment, T2 was significantly higher than T1 by 19.25%, 48.56%, 73.40%, 62.38% and 7.53%, respectively,

and T3 was significantly higher than T2 by 16.93%, 24.27%, 34.00%, 41.84% and 38.76%, respectively. 'TJH' showed T6 > T5 > T4 in each period. From 2 d to 5d of treatment, T5 was significantly higher than T4 by 54.14%, 73.90%, 53.73% and 21.41%, respectively. T6 was significantly higher than T5 by 24.44%, 13.55%, 20.85% and 37.26%, respectively.

As can be seen from Figure 2C, when LXD 4 was treated for 3 days, T2 was significantly higher than T1 by 40.96%, and on the 5th day, T2 was significantly lower than T1 by 24.61%. After 2 days of treatment, T3 was significantly higher than T2 by 20.75%, 25.64%, 36.78% and 22.22%, respectively. After 'TJH' was treated for 4 days, T5 was significantly higher than T4 by 11.36%, 27.59%, 41.94% and 22.47%, respectively. On the 5th day, T5 was significantly lower than T4 by 18.28%. At 4 d and 5 d of treatment, T6 was significantly higher than T5 by 16.51% and 37.83%, respectively.

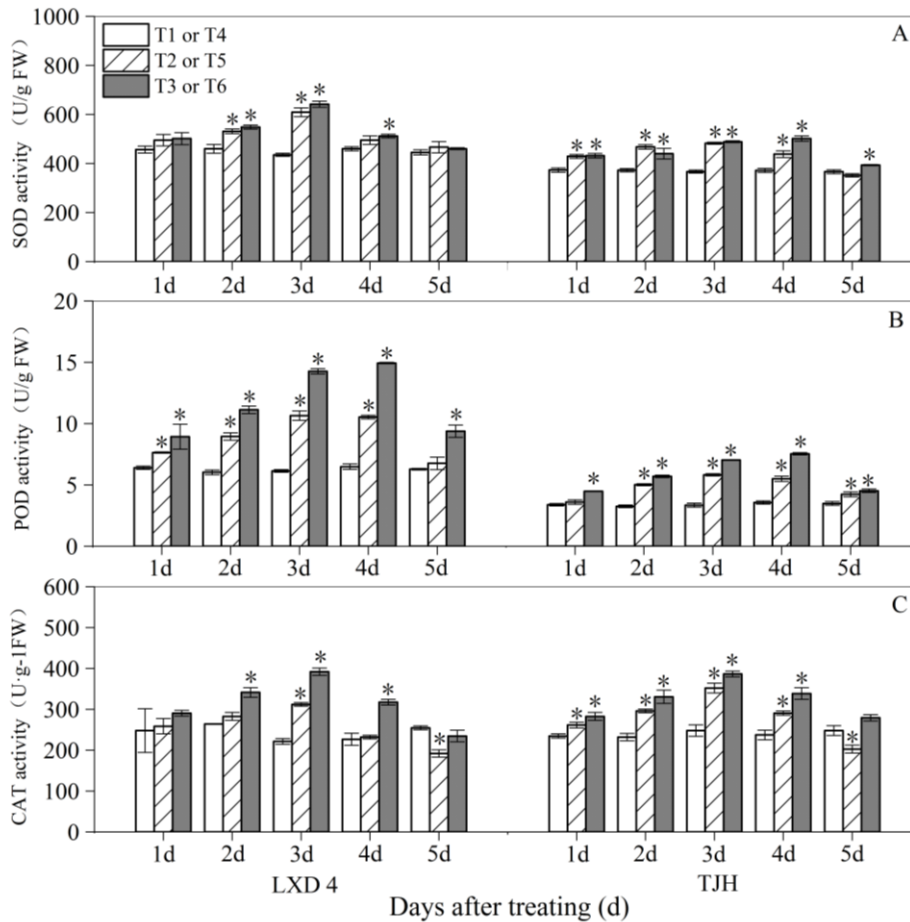


Figure 2. Effects of waterlogging stress and spraying ABA on SOD、POD and CAT activity in leaf of adzuki bean seedlings

Effect on the ratio of SOD/POD and SOD/CAT

The increase of the ratio of SOD/POD and SOD/CAT will further increase the oxidative stress of plants. Table 2 showed that SOD/POD in adzuki bean leaves decreased at first and then increased after flooding and spraying ABA at seedling stage. All treatments reached the valley value on the 4th day, and then began to increase. Spraying ABA could induce further decrease of SOD/POD, and LXD 4 showed T1 > T2 > T3 in each stage. Within 4 days of treatment, TJH showed T6 < T5. SOD/CAT decreased at first and then increased after flooding stress, and spraying exogenous ABA could significantly regulate the further decrease of SOD/CAT, which showed T3 < T2 and T6 < T5 in different sampling periods.

Table 2. Effect of waterlogging stress and spraying ABA on SOD/POD and SOD/CAT in leaf of adzuki bean seedlings

Varieties	Treatment	SOD/POD - Day after treating (d)					SOD/CAT - Day after treating (d)				
		1d	2d	3d	4d	5d	1d	2d	3d	4d	5d
'LXD 4'	T1	71.43	76.65	70.91	70.97	70.88	2.04	1.74	1.97	2.05	1.92
	T2	64.85	59.38	57.22	46.99	69.44	1.95	1.88	1.95	2.14	2.43
	T3	56.55	49.38	45.02	34.24	49.38	1.73	1.61	1.64	1.61	1.98
'TJH'	T4	110.40	114.73	110.45	104.55	105.39	1.59	1.61	1.49	1.57	1.49
	T5	119.88	93.21	82.96	80.13	83.41	1.64	1.58	1.37	1.51	1.75
	T6	96.36	77.27	69.49	66.56	87.20	1.53	1.34	1.26	1.49	1.41

Effect on osmotic regulating substance of adzuki bean seedlings

It can be seen from Figure 3A that after flooding and spraying ABA at seedling stage, the proline content in adzuki bean leaves increased at first and then decreased. The order of proline content in each treatment time of tested varieties was spraying ABA > flooding stress > CK. When LXD 4 treated from 1 d to 5 d, T2 was significantly higher than T1 by 45.73%, 64.86%, 100.81%, 89.84% and 63.86%, respectively. When treated for 3 days and 5 days, T3 was significantly higher than T2 by 14.39% and 16.38%. When TJH was treated from 1 d to 5 d, T5 was significantly higher than T4 by 62.07%, 57.32%, 70.82%, 120.94% and 71.59%. After 2 days and 3 days, T6 was significantly higher than T5 by 28.02% and 17.55%.

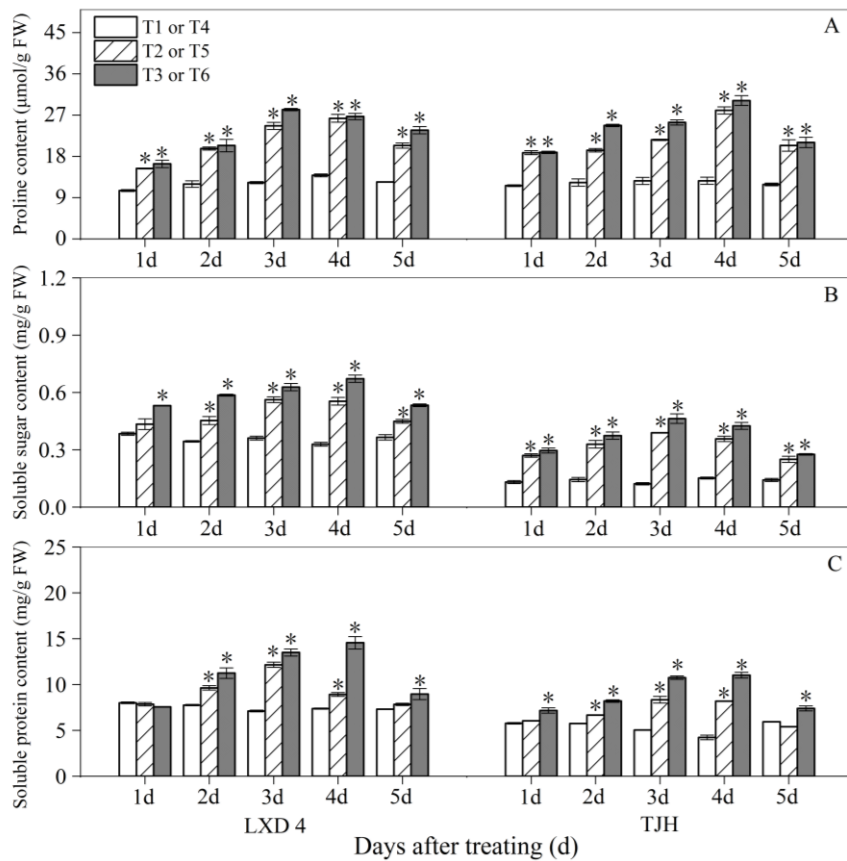


Figure 3. Effect of waterlogging stress and spraying ABA on proline soluble sugar and protein in leaf of adzuki bean seedlings

It can be seen from Figure 3B that the content of soluble sugar in adzuki bean leaves increased after flooding and spraying ABA. When LXD 4 was treated from 1 d to 5 d, it always was T2 > T1. from 2 d to 5 d of treatment, T2 was significantly higher than T1 by 31.56%, 55.59%, 68.42% and 22.82%, and T3 was significantly higher than T2 in each period. the increases were 22.41%, 29.38%, 11.89%, 21.24% and 18.83%, respectively. TJH was treated from 1 d to 5 d, T5 was significantly higher than T4, and at 3 d and 4 d, T6 was significantly higher than T5 by 19.26% and 19.18%.

As can be seen from figure 3C, when LXD 4 was treated from 2 d to 4 d, T2 was significantly higher than T1 by 24.18%, 70.88% and 20.84%, and T3 was significantly higher than T2 by 16.69%, 110.87% and 63.28%. When TJH was treated from 2 d to 4 d, T5 was significantly higher than T4 by 15.96%, 65.34% and 93.36%, and from 1 d to 5 d, T6 was significantly higher than T5 by 18.68%, 22.85%, 28.80%, 34.61% and 37.21%.

Effect on endogenous hormone of adzuki bean seedlings

The content of endogenous ABA in adzuki bean leaves increased after flooding at seedling stage, and spraying exogenous ABA could further increase the content of endogenous ABA (Figure 4A). LXD 4 showed T3 > T2 > T1 in each sampling period. after 1 d treatment, T2 was significantly higher than T1, with an increase of 76.51%, 84.46%, 106.01%, 107.57% and 28.66%, respectively. From 2 days of treatment, T3 was significantly higher than T2 by 11.18%, 11.02%, 55.73% and 15.64%, respectively. After TJH was treated for 5 days, T5 was significantly higher than T4, and after treatment from 1 d to 4 d, T6 was significantly higher than T5 by 22.00%, 22.13%, 1.05% and 6.58%.

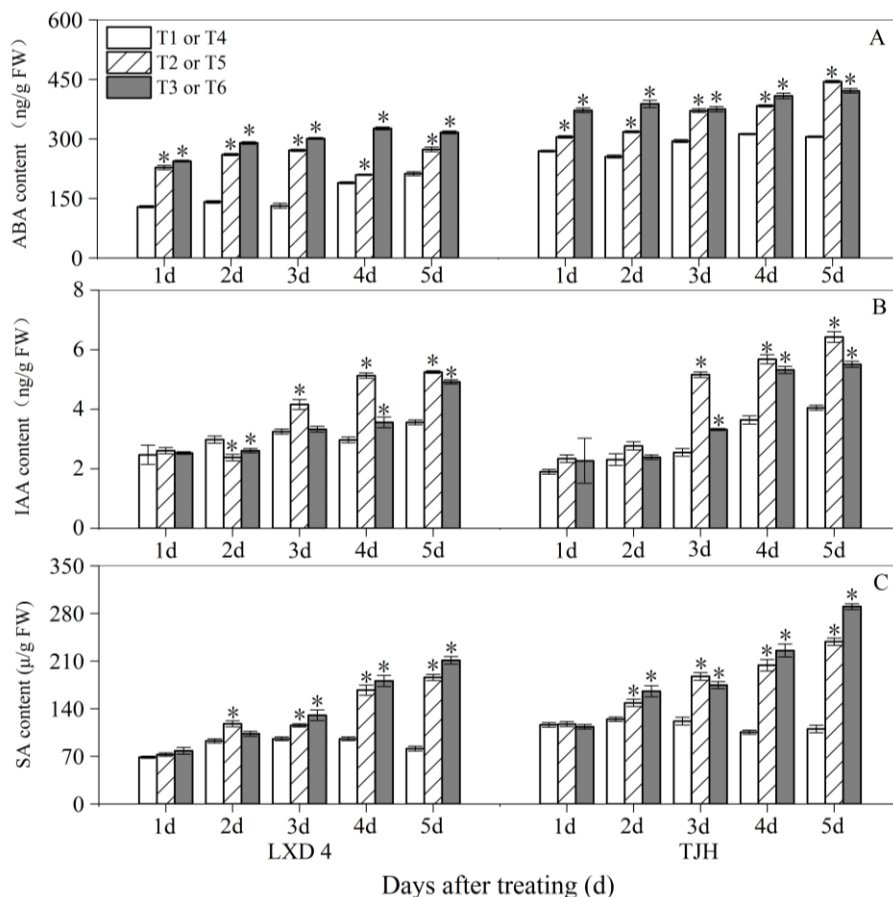


Figure 4. Effect of waterlogging stress and spraying ABA on ABA, IAA and SA content in leaf of adzuki bean seedlings

The IAA content in adzuki bean leaves increased after flooding at seedling stage, and the increase of IAA content was inhibited by spraying ABA (Figure 4B). When LXD 4 was treated at 2nd days, T2 was significantly lower than T1 by 20.13%. After treatment from 3 d to 5 d, T2 was significantly higher than T1 by 28.00%, 72.73% and 47.47%, respectively, while T3 was significantly lower than T2, with a decrease of 19.95%, 30.60% and 6.29%, respectively. When TJH was treated for 1 d and 2 d, there was no significant difference among T4, T5 and T6. When treated from 3 d to 5 d, T5 was significantly higher than T4 by 102.35%, 56.04% and 58.77%. Compared with T5, T6 decreased by 35.66%, 6.34% and 14.31%.

It can be seen that flooding at seedling stage caused an increase in SA content in adzuki bean leaves, and spraying exogenous ABA was beneficial to further increase SA content (Figure 4C). When LXD 4 was treated for 5 days, T2 was significantly higher than T1 by 27.01%, 20.54%, 74.80% and 128.56%, respectively; on the 5th day of treatment, the content of T3 was higher than T2 by 13.56%. After TJH was treated for 3 days to 5 days, T5 was significantly higher than T4 by 54.06%, 93.11% and 116.39%, respectively; T6 content was higher than T5 at 4 days and 5 days, and T6 was significantly higher than T5 by 21.60% at 5 d.

Effect on yield

When ‘LXD 4’ was treated for 1 d and 2 d, there was no significant difference among T1, T2 and T3; after 3 d treatment, T2 was significantly lower than T1 by 4.77%, 7.57% and 8.40%; at 3 d and 4 d treatment, T3 was significantly higher than T2 by 6.05% and 6.95%, and there was no significant difference between T3 and T2 at 5 d treatment. There was no significant difference among T4, T5 and T6 when ‘TJH’ was treated for 1 d and 2 d, T5 decreased significantly by 5.59%, 8.00% and 9.91% compared with T4 after 3 d treatment, and T6 was significantly higher than T5 by 1.84% and 4.46% at 3 d and 4 d treatment (Table 3).

Table 3. Effect of waterlogging stress and spraying ABA on yield per plant in leaf of adzuki bean seedlings (g)

Variety	Treatment number	Day after treating (d)				
		1d	2d	3d	4d	5d
‘LXD 4’	T1	30.37±0.22a	30.37±0.22a	30.37±0.22a	30.37±0.22a	30.37±0.22a
	T2	30.12±0.39a	29.85±0.39a	28.92±0.24b	28.07±0.25b	27.82±0.86b
	T3	31.00±0.18a	30.18±0.24a	30.67±0.38a	30.02±0.13a	27.94±0.44b
‘TJH’	T4	23.62±0.39a	23.62±0.39a	23.62±0.39a	23.62±0.39a	23.62±0.39a
	T5	22.57±1.07a	22.70±0.50a	22.30±0.20b	21.73±0.22c	21.28±0.15b
	T6	23.38±0.53a	22.76±0.80a	22.71±0.51a	22.70±0.13b	22.67±0.11b

Discussion

The production and clearance of H₂O₂ basically maintained a fine balance under normal conditions. Flooding stress caused the imbalance of ROS metabolism in plants, resulting in excessive accumulation of H₂O₂ and other substances. Because of its high redox activity, it can cause intracellular macromolecular oxidative damage, inhibit a variety of physiological and biochemical reactions in plants, and finally affect plant regulation and metabolism (SONG and SHE, 2010). MDA is the product of cell membrane peroxidation, which destroyed the structure and function of biofilm, caused serious damage of membrane and enzyme, decreased membrane resistance and membrane fluidity, and finally led to loss of selective absorption capacity of cell membrane and leakage of intracellular electrolytes (PAN and XUE, 2012). The results showed that flooding

increased the contents of H_2O_2 and MDA in adzuki bean, indicating that flooding stress caused cell membrane damage and affected cell metabolic function, which was similar to the results of Pan *et al.* (2020) and Qi *et al.* (2019) on different crops. Exogenous ABA had the physiological function of slowing down the increase of ROS and MDA content under stress. Pu *et al.* (2011) pointed out that exogenous ABA can effectively reduce the accumulation of MDA and maintain membrane integrity in watermelon under stress. Li *et al.* (2010) pointed out that exogenous ABA can effectively alleviate the accumulation of MDA and H_2O_2 in sugarcane under water stress, and ensure the normal physiological metabolism and biochemical process of crops under stress. This study also showed that spraying ABA could significantly inhibit the increase of H_2O_2 and MDA content in adzuki bean leaves under flooded conditions, reduce the damage of cell membrane fluidity and stability caused by flooding, and improve the physiological effect of crop resistance to flooding stress, which may be related to the physiological function of exogenous ABA to increase the activity of protective enzymes, because the balance system of intracellular free radical production and scavenging was destroyed when plants were under stress. There are many ways of scavenging free radicals in plants, the most important of which is antioxidant enzyme system. Plants maintained oxygen free radicals at a low level through the synergistic action of SOD, POD and CAT, which can slow down or defend against stress to some extent. This study showed that flooding stress significantly increased the activities of SOD, POD and CAT in different degrees, which was similar to the results of Liu *et al.* (2019) and Liu *et al.* (2020). This was because the plant self-protection mechanism was turned on, and the activities of SOD and POD were significantly increased to cope with the stress caused by adverse environment. Exogenous ABA can further increase the activities of SOD and POD in adzuki bean leaves, enhance the scavenging ability of ROS, and protect cells from injury as far as possible. Guo *et al.* (2014) pointed out that exogenous ABA treatment could increase SOD activity in rice leaves under water stress. Li *et al.* (2010) pointed out that exogenous ABA could effectively increase CAT activity in sugarcane leaves under water stress. The results showed that spraying exogenous ABA could further increase the activities of SOD, POD and CAT in sugarcane leaves under flooded conditions, and had a good promoting effect on adzuki bean resistance to flooding stress. The main function of SOD was to scavenge superoxide anion (O_2^-), but produce H_2O_2 at the same time, while POD and CAT degraded excess ROS, such as H_2O_2 by enzymatic action to avoid peroxidation damage caused by stress. Some studies have pointed out that the increase of the ratio of SOD/POD, SOD/CAT would further increase the oxidative stress of plants (Kanazawa *et al.*, 2010). The results of this study showed that spraying exogenous ABA could effectively reduce the ratio of SOD/POD, SOD/CAT in leaves under flooded conditions, indicating that the activities of POD and CAT increased faster than that of SOD after application of exogenous ABA, which could scavenge more H_2O_2 and effectively alleviate the damage of reactive oxygen species, which was one of the physiological reasons for exogenous ABA to enhance adzuki bean stress resistance.

Osmotic regulation was an important physiological mechanism for plants to induce protective response. Under stress, cells can actively accumulate organic osmotic regulators such as soluble sugar, soluble protein and proline to regulate cell osmotic potential, protect the normal function of various enzymes and cell membrane structures in plant tissues (You *et al.*, 2016). The results showed that flooding stress increased the contents of proline, soluble sugar and soluble protein in adzuki bean leaves, especially proline content, which could effectively alleviate the damage caused by flooding. Because proline can maintain cell expansion or osmotic balance to give plants stress tolerance, so as to stabilize the membrane structure and prevent electrolyte leakage. Spraying ABA can further increase the content of osmotic regulation substances, especially the content of soluble protein, increase the concentration of cell sap, increase the unfrozen water in water-holding tissue, and improve the stress resistance of plants.

The dynamic balance of endogenous hormones played an important role in the regulation of plant growth and development, and the change of hormone content played a key role in responding to abiotic stress (Duan *et al.*, 2015). The results showed that flooding stress inhibited the synthesis of IAA, gibberellin (GA) and cytokinin (CTK), while increased ABA and ethylene synthesis (Yan *et al.*, 2010). This study showed that flooding stress increased the contents of ABA, IAA and SA in adzuki bean, and showed a significant increase

trend with the prolongation of stress time, which was similar to the results of Chen *et al.* (2019). Exogenous ABA had a variety of regulatory effects on crop growth and development, including regulating endogenous hormones, enhancing resistance and so on. The results showed that spraying exogenous ABA could promote the further improvement of endogenous ABA and SA, and effectively regulate the increasing rate of IAA content, thus improving the resistance of adzuki bean. The reason was that the further increase of ABA content and effective regulation of IAA content can regulate and start the plant protection system, inhibit crop growth and development for a short time, reduce loss, maintain normal physiological activities, and resist stress damage.

Flooding stress led to the decrease of crop yield, and the effects were different in different periods. Yu *et al.* (2019) showed that flooding at flowering stage and podding stage could reduce mung bean yield, and flooding stress at flowering stage decreased yield more seriously. You *et al.* (2016) pointed out that flooding had different effects on maize yield in different periods, among which the seedling stage was the most sensitive to flooding. The results of this study showed that the yield of adzuki bean seedlings could be significantly reduced by more than 3% after 3 days of flooding stress. Spraying ABA can alleviate the yield loss caused by stress. Wang *et al.* (2020) pointed out that exogenous ABA could significantly increase sweet potato yield by 28.60% under water stress. Qu *et al.* (2017) pointed out that exogenous ABA significantly increased soybean yield under water stress. The same results were obtained in this study. Spraying exogenous ABA could effectively alleviate the effect of flooding on adzuki bean yield, especially for 'TJH' variety.

Conclusions

Flooding stress at seedling stage significantly increased the contents of H₂O₂ and MDA in adzuki bean leaves, increased the activities of SOD and POD, and increased proline, soluble protein and soluble sugar, resulting in an increase in the contents of ABA, IAA and SA, and finally led to a decrease in yield. Spraying exogenous ABA could slow down the stress damage of adzuki bean, relatively reduce the contents of H₂O₂ and MDA in adzuki bean leaves, enhance the activities of SOD, POD and CAT in adzuki bean leaves, and reduce the ratio of SOD/POD, SOD/CAT. Further increase the content of proline and soluble protein, increase the content of ABA and SA, effectively inhibit the increase of IAA content, improve the resistance of adzuki bean to flooding stress as a whole, and increase the yield of adzuki bean under flooding stress. Exogenous ABA had different regulation effects on different varieties of adzuki bean, but it can be used to resist flooding and alleviate flooding damage in adzuki bean.

Authors' Contributions

XHT conceived and devised the experimental design. LW, HN, WML, CDW and CLZ conducted the experiments. XHT, LW, and LJ performed the data analysis. LW and WXY wrote the manuscript. All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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