

Research paper

Effects of waterlogging on some chemicals and antioxidant responses in *Zanthoxylum armatum* DC. seedlings

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Abstract. *Zanthoxylum armatum* DC. (*Z. armatum*), known as the prickly ash or toothache tree, shows higher productivity and interesting agronomic features for spices, oilseeds and medicinal values. Waterlogging is one of the major stresses restricting the growth, development and production of *Z. armatum* in Southwest China due to flash rainfall and floods. However, the effects of waterlogging stress on the growth and antioxidant responses in *Z. armatum* seedlings have not been reported. In this study, the physiological responses in *Z. armatum* seedlings were investigated under waterlogging with different depths (2.7, 5.5, 11 cm above the ground level) and durations (2, 4 and 6 days). No significant differences in the averages of height, stem diameter and number of leaves were observed. However, the apical leaflets of seedlings that were submerged to a depth of 11 cm above the ground become soft on day 6. Chlorophyll and malondialdehyde (MDA) content rose significantly with the waterlogging levels on different treatment days compared to the control. There were no significant differences in the relative electrical conductivity between the control and treatments on day 2, but the values of the treatment groups were higher than those of in the control on days 4 and 6. The generally increased content of soluble sugar and soluble proteins within a small range with prolonged duration of the stress showed little differences between waterlogging stress and the controls. Superoxide dismutase (SOD) and peroxidase (POD) activity showed different changes in different tested groups at different treatment times, as compared with controls. The morphological and physiological characteristics had different responses to stress duration and waterlogging degree as well as their interactions. These findings were strong evidence that these plants may develop strategies and have a certain tolerance to short-term waterlogging, especially mild waterlogging.

Key words: waterlogging stress, physiological responses, ROS-scavenging enzymes, defensive mechanism.

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Introduction

Waterlogging, as a consequence of excess rainfall, tides, floods, and lack of proper drainage facilities, is a major environmental stress restricting plant growth, development and production, and it affects approximately 10% of land area on the earth (Tian *et al.*, 2021). Waterlogging decreases oxygen content in soil and often depletes the oxygen in the root zone either completely (anoxia) or partially (hypoxia), thus affecting plant abilities in the uptake and transport of nutrients. Normally, chlorosis, necrosis, defoliation, growth reduction, reduced N fixation, yield loss, and

plant death are affected by waterlogging stress during plant growth, which often occur at various vegetative and reproductive stages (Zhou *et al.*, 2020; Pan *et al.*, 2021). Moreover, alterations in root hydraulic conductivity, light interception, stomatal conductance, CO₂ assimilation, drastic reduction of photosynthesis, and generation of secondary metabolites are responsible for reducing plants' potential to grow, and show their productivity under waterlogging stress (Loreti *et al.*, 2016; Manghwar *et al.*, 2024). To alleviate the consequences of oxygen deprivation, plants subjected to waterlogging stress tend to survive through a range of biochemical,

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physiological, anatomical and morphological adaptations. Ethylene production (aerenchyma formation) and the induction of barriers to radial O₂ loss formation) and the induction of barriers to radial O₂ loss strengthen plants' surface roots and adventitious roots, which is some of the major adaptive responses under waterlogging stress (Pan *et al.*, 2021). The mechanisms of damage and adaptation under waterlogging stress have been studied in some plant species, such as *Poa pratensis* L. (Puyang *et al.*, 2015), *Zea mays* L. (Tian *et al.*, 2019), and *Elaeagnus angustifolia* L. (Liu *et al.*, 2020), etc. These findings lead us to understand that the effects of waterlogging on plants are complex phenomena and vary with plant species, developmental stages, waterlogging depth, waterlogging duration, nutrient availability, environmental temperature and soil types, etc.

Plants exposed to waterlogging stress inevitably undergo the production of reactive oxygen species (ROS), which rapidly attack leaf chloroplasts and ultimately lead to leaf chlorosis and senescence, and further oxidative stress (Pan *et al.*, 2021). To cope with oxidative stress, plants develop an antioxidant defense system, including both enzymatic and non-enzymatic antioxidants which scavenge excess ROS. The enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), peroxidases (POD), etc., have been reported to play a key role in coping with biotic and abiotic stresses, including waterlogging (Loreti *et al.*, 2016; Wang *et al.*, 2018). Significant changes in SOD, POD and CAT activities were observed in *Cajanus cajan* (L.) Millsp. subjected to 2–8 days of waterlogging, while the activities were variable according to the duration of stress (Sairam *et al.*, 2009). Puyang *et al.* (2015) observed higher SOD and APX activity, isozymes intensity and gene expression level in *Poa pratensis*, which may play crucial roles in Kentucky bluegrass's tolerance to waterlogging stress. Similarly, the up-regulation of SOD, POD, and CAT activity, as well as increased proline, soluble protein content and relative chlorophyll content were recorded in *Z. mays* seedlings when subjected to varying degrees of waterlogging stress (Tian *et al.*, 2019). In cherry rootstocks, the responses and tolerance to short-term waterlogging positively correlated with the enhanced CAT, POD, and glutathione reductase (GR) activity (Jia *et al.*, 2019). In contrast, Chugh *et al.* (2011) reported that waterlogging stress may cause a decrease in SOD, CAT and ascorbate peroxidase (APX) activities in maize. Similarly, Candan & Tarhan (2012) also suggested that, in *Mentha pulegium* L., the SOD, CAT and POD activities were lower than those of the control under waterlogged

stress conditions. These findings suggest that these antioxidant enzymes may be involved in modulating the resistance of plants exposed to waterlogging.

Zanthoxylum armatum (*Z. armatum*), a deciduous tree that belongs to the Rutaceae family, has significant economic value as an important food condiment, spice, and medicine. The leaves, fruits, stem, bark and seeds possess valuable biological and pharmacological properties such as anti-bacterial, anti-fungal, anti-viral, anti-inflammatory, and anti-oxidant qualities. This plant has been used in several indigenous medicinal practices to cure stomach issues, toothache, chest infections, dental problems, digestive problems, and scabies, etc. (Phuyal *et al.*, 2019; Singh *et al.*, 2021). It is usually cultivated and grown at altitudes up to 2,200 m through Southwest China, India, Pakistan, Nepal, etc. Moreover, it can be grown on less fertile soil, marginal lands and can be harvested after 2–3 years of planting (Hu *et al.*, 2023). Its green fruit (namely Qinghuajiao) is usually harvested at its immature state in Southwest China and directly used as a typical seasoning spice due to its special flavor and pungent taste in Sichuan Cuisine for more than 2000 years (Zhang *et al.*, 2021). In Southwest China, flooding in rainfed lowland areas during the rainy season is a very common phenomenon. Although most of those cultivated areas are subjected to low incidence of waterlogging stress, the growth and development of *Z. armatum* seedlings are susceptible to waterlogging stress, especially at the early seedling growth stage (Xu *et al.*, 2019; Wu *et al.*, 2024). Some reports have focused on the effects of waterlogging stress on the growth, development and yield of plants (Wiraguna *et al.*, 2020; Tian *et al.*, 2021). However, few studies have focused on the effects of waterlogging stress on the morphological and physiological responses in economic trees at different treatment times. Waterlogging puts significant abiotic production constraints on *Z. armatum* in low-lying districts and high rainfall areas. Most of the field studies reported were conducted with long-term waterlogging, but in practice, short-term flooding is also more common (Xu *et al.*, 2019; Wu *et al.*, 2024). The aim of this study is to assess the effects of waterlogging for 2, 4, and 6 days on the morphological and antioxidant responses of *Z. armatum* seedlings. The results will help to get a better theoretical basis for rational water management for seasonal waterlogging of *Z. armatum* seedlings, and how waterlogging stress influences the morphological and physiological responses in plants.

Material and Methods

Materials and chemicals

Soil samples were collected from District Wenjiang, Chengdu, China (N30° 70'E103° 83'), and crushed, screened and tilled for 3 days. Soil pH was determined using an electrode pH meter in a soil: water (1:2) suspension (Yang *et al.*, 2023). The total nitrogen and P content was determined by the Kjeldahl method and Mo-Sb colorimetric method (Zhi *et al.*, 2023). The K content was determined by atomic absorption spectrophotometry (Zhi *et al.*, 2022). The organic matter content was determined by the loss on ignition method (Ball, 1964). The soil was classified as alluvial soil, and its physical-chemical characteristics were pH 5.13, organic matter 35.51 g/kg, total nitrogen 1.67 g/kg, total phosphorus (P) 2.487 g/kg, and total potassium 0.832 g/kg. The soil was air-dried and sieved through a 2 mm-mesh screen. All chemical and reagents used were of analytical grade.

Plant material and pot experiment

The pot experiment was carried out in the greenhouse of teaching building No. 5 of Sichuan Agricultural University, Chengdu, China (latitude 30°42'32.166"N, longitude 103°51'38.239"E). *Z. armatum* seedlings with averages of plant height (13.34 cm) and stem diameter (0.27 cm) were obtained from Danling, Sichuan, China. These seedlings were placed into plastic pots (20 cm×23 cm, height vs. diameter) filled with a mixture of alluvial soil, sandy loam and coconut husk (25:2:1, 15 cm). During the seedling transplantation, dead seedlings were replaced with healthy ones and watered a single time to ensure that there was a single healthy seedling per pot. These seedlings were cultured for 1 month, and the healthy seedlings reached a height of 44.14 cm and stem diameter of 0.78 cm, odd-pinnate compound leaves of 8–9, and the treatments were initiated. In order to create the flooding treatment, seedlings cultured in plastic pots were placed in larger plastic containers with different water depth of 2.7, 5.5 and 11 cm above the ground level, respectively. The water depth in each pot was adjusted twice per day during the experimental period. The waterlogging experiments were arranged in a completely randomized design consisting of four groups, replicated thrice. The pots were separated into four groups, and each group consisted of twenty seedlings. One group was allowed to grow normally and had the relative water content (75%) without waterlogging stress to serve as the control group (CK). The remaining three groups were considered

waterlogged groups. Group A seedlings were flooded at 2.7 cm above the ground level. Group B seedlings were flooded at 5.5 cm above the ground level. Group C seedlings were flooded at 11 cm above the ground level. During the treatment process, the morphological changes and survival of seedlings were observed and monitored on days 2, 4 and 6. Moreover, the leaves were collected randomly in different treatment groups and stored at -80°C for further analysis.

Estimation of chlorophyll content

Chlorophyll content was measured according to the Yordanova & Popova (2001) method. In brief, leaf mass (100 mg) was extracted using 10 ml ethanol-acetone (1:1, V/V), and the extractions were transferred to a 15 ml tube, and then placed in the dark for 24 h. The absorbance was measured at 645 nm and 663 nm using a UV-vis spectrophotometer. The chlorophyll content was calculated according to the following formulae:

$$\text{Chlorophyll a content (mg/g)} = \frac{(12.7 \times D_{663} - 2.69 \times D_{645}) \times V}{1000 \times W}, \quad (1)$$

$$\text{Chlorophyll b content (mg/g)} = \frac{(22.7 \times D_{645} - 4.68 \times D_{663}) \times V}{1000 \times W}, \quad (2)$$

$$\text{Total chlorophyll (mg/g)} = \text{Chla} + \text{Chlb}, \quad (3)$$

where D663 and D645 represent the corresponding wavelengths of the light density value, respectively. V represents the extracting liquid volume, and W represents leaf fresh weight.

Estimation of relative electrical conductivity

Relative electrical conductivity was measured according to the method of Isidoro & Aragües (2006). 0.1 g of leaves were immersed in 10 ml distilled water for 12 h, and the conductivity of the solution was measured using a conductivity meter (R_1). The conductivity (R_2) was measured again after the samples were boiled for 30 min. The relative electrical conductivity was calculated as $R_1/R_2 \times 100\%$.

Estimation of malordialdehyde (MDA) and soluble sugar

MDA and soluble sugar content were determined using trichloroacetic acid (TCA) method (Yang *et al.*, 2023). 0.1 g of leaf was homogenized using 8 ml TCA of 10% (w/v), and the supernatants were harvested by centrifuging at 12,000 rpm for 15 min at 4°C. The reaction mixtures contained 2 ml of extraction, and 2 ml of 0.6% (w/v) thio-

barbituric acid (TBA), and were incubated in boiling water for 10 min. The absorbance of the reaction solution was measured at 450, 532, and 600 nm, respectively. MDA content was calculated using the following formula:

$$C_{MDA} (\mu\text{mol/g FW}) = \frac{[(6.45 \times A_{532}) - (6.45 \times A_{600}) - (0.56 \times A_{450})] \times V}{(1000 \times W)}, \quad (4)$$

$$\text{The sugar content } (\mu\text{mol/g FW}) = \frac{(11.71 \times A_{450} \times V \times 2)}{(1000 \times W)}, \quad (5)$$

where V represents the extracting liquid volume, and W represents leaf fresh weight.

Protein extraction and assay of SOD and POD activity

Leaves (0.2 g) were homogenized using liquid nitrogen and extracted with 2 ml phosphate buffer (50 mM, pH 7.0) and 1 mM EDTA. The supernatant was harvested by centrifuging at 12,000 rpm for 10 min at 4 °C, and used for determination of SOD, POD and protein content. The soluble protein content was determined using the Bradford method (Hammond & Kruger, 1988), and expressed in mg/g. SOD activity was assayed according to the nitroblue tetrazolium (NBT) method (Gao *et al.*, 2010). The reaction mixture contained 2.9 ml sodium phosphate buffer (pH 7.8, 50 mM) with 13 mM methionine, 75 μM NBT, 2 μM riboflavin, and 100 μl enzyme extract, and exposed to light for 15 min. The absorbance was read at 560 nm using a UV-vis spectrophotometer. One unit of SOD activity was defined as the amount of enzyme that causes 50% inhibition in the reduction of NBT, and was expressed in units of the enzyme (U/g FW). POD activity was determined by the method of Gao *et al.* (2010). The reaction mixture (3 ml) consisted of 2.8 ml of 3% guaiacol in 50 mM Tris-HCl (pH 7.0) and 100 μl 2% H_2O_2 . The reaction was started by adding 100 μl of enzyme extract, and the absorbance was measured at 470 nm. One unit of enzyme activity was defined as the amount of enzyme which produces 1.0 absorbance change at 470 nm per min. The activity was expressed as enzyme units per gram fresh weight (U/g FW).

Statistical analysis

Data are presented as mean values ($n = 3$, $\pm$ standard error). Three replicates were measured for each treatment. One-way analysis of variance (ANOVA) and Tukey's test was performed using Waller-Duncan multiinterval test using SPSS20.0 software (IBM® Corporation, USA). Two-way analysis of variance of 3×3 factorial design was conducted to determine the effects of waterlogging duration and depth

on the activity of some chemicals and antioxidant enzymes. Moreover, the minimum significant difference (LSD) in ANOVA test was used to analyze the significant difference. Statistical significance was set at 95% confidence level ($p < 0.05$).

Results

Effects of waterlogging stress on morphological characteristics

In the present study, all tested *Z. armatum* seedlings grew well, and survived for 6 days in Group A and B under waterlogging stress. Moreover, no significant morphological differences in the averages of height, stem diameter and number of leaves were observed. However, in Group C, the apical leaflets became soft on day 6, and they showed a slight drooping posture, and the basal leaflets began to turn yellow (data not shown). Moreover, with the prolonged waterlogging time up to day 9, the leaf drooped at the top of the seedling and became soft and prolapsed, and the leaf margin was not curved. Moreover, the leaf drooped at the middle and lower of seedling showed wilting symptoms, and the bottom leaves began to become yellow and the leaflets with 2~3 pieces fell off when touched (data not shown). The morphology indexes showed that *Z. armatum* seedlings are affected by waterlogging duration and depth in tested conditions.

Effects of waterlogging stress on chlorophyll content

As shown in Figure 1, the chlorophyll content showed different changes depending on the varying depth and duration of waterlogging, and the values were significantly affected by different waterlogging stress on days 2, 4 and 6. On day 2, the chlorophyll content in Group A was significantly higher than those of in the control. On day 4, the chlorophyll content in Group A, B and C were 12.88%, 31.38%, 17.21% higher than compared to the control, respectively. The values in Group B are higher than those in Group A and C. On day 6, the chlorophyll content in Group A, B and C was increased to 125.25%, 110.76%, and 106.81% of the control, respectively ($p < 0.05$). During the stress period, the chlorophyll content in Group A reached its highest value on the sixth day. However, the chlorophyll content in Group B and C firstly increased on day 4, and then decreased on day 6. The different responses in the chlorophyll content were affected by stress duration and waterlogging degree as well as their interactions.

Effects of waterlogging on relative electrical conductivity in leaves

As shown in Figure 2, data revealed significant differences in relative electrical conductivity between the different depth and duration of waterlogging and their interactions. On day 2, the relative electrical conductivity of the treatment groups was significantly less than those of in the control, and the decreases were recorded at B and C, only representing 85.51%, and 86.22% of the control values, respectively.

On days 4 and 6, the relative electrical conductivity in Group A reached a higher level of 125.84% and 131.86% than those of in the control, indicating an elevated amount of membrane damage. In Group B, the relative electrical conductivity increased by 10.11% on day 4, but the values decreased by 14.49% and 7.79% on days 2 and 6 in relation to the control groups. Similarly, in Group C, the relative electrical conductivity decreased by 13.78% and 8.14% on days 2 and 6 compared to the control,

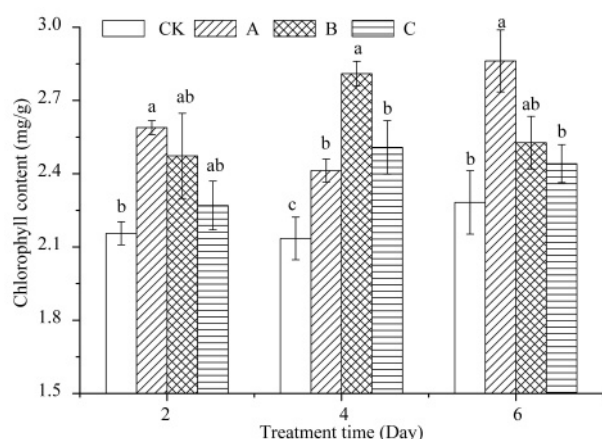


Figure 1. Effects of waterlogging stress on chlorophyll content of *Z. armatum* leaves. The vertical bars represent the standard errors, $n=3$. Bars followed by different letter(s) are statistically significant at $p < 0.05$. CK: Seedlings were not flooded. A: Seedlings were flooded at 2.7 cm above the ground level. B: Seedlings were flooded at 5.5 cm above the ground level. C: Seedlings were flooded at 11 cm above the ground level.

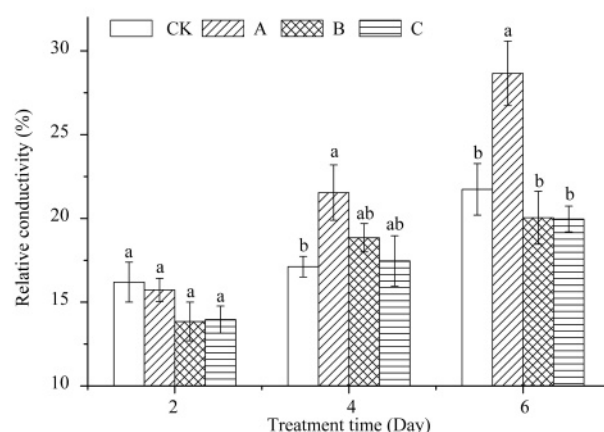


Figure 2. Effects of waterlogging stress on the relative electrical conductivity of *Z. armatum* leaves. The vertical bars represent the standard errors, $n=3$. Bars followed by different letter(s) are statistically significant at $p < 0.05$. CK: Seedlings were not flooded. A: Seedlings were flooded at 2.7 cm above the ground level. B: Seedlings were flooded at 5.5 cm above the ground level. C: Seedlings were flooded at 11 cm above the ground level.

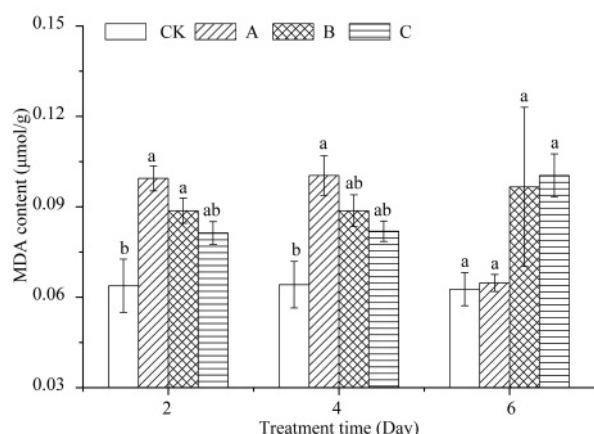


Figure 3. Effects of waterlogging stress on the malondialdehyde (MDA) content of *Z. armatum* leaves. The vertical bars represent the standard errors, $n=3$. Bars followed by different letter(s) are statistically significant at $p < 0.05$. CK: Seedlings were not flooded. A: Seedlings were flooded at 2.7 cm above the ground level. B: Seedlings were flooded at 5.5 cm above the ground level. C: Seedlings were flooded at 11 cm above the ground level.

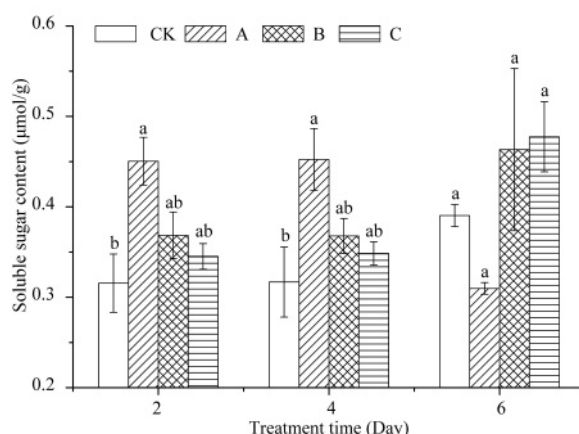


Figure 4. Effects of waterlogging stress on the soluble sugar content of *Z. armatum* leaves. The vertical bars represent the standard errors, $n=3$. Bars followed by different letter(s) are statistically significant at $p < 0.05$. CK: Seedlings were not flooded. A: Seedlings were flooded at 2.7 cm above the ground level. B: Seedlings were flooded at 5.5 cm above the ground level. C: Seedlings were flooded at 11 cm above the ground level.

respectively, but no significant changes were found on day 4. The changes were affected by stress duration and waterlogging degree as well as their interactions.

Effects of waterlogging stress on the MDA content

As shown in Figure 3, during the stress treatment period, the MDA content of each treatment group was higher than that of the control group. On day 2, the MDA content in Group A, B and C was 55.91%, 39.05% and 27.51% higher than those observed in the control ($p < 0.05$), respectively. On day 4, the MDA content in the three groups showed no significant difference compared to those on day 2. On day 6, the MDA content in Group B and C showed a 54.2% and 60.2% higher rate than those observed in the control, respectively. However, no significant differences were found between the control and Group A. These data indicated that membrane lipids in leaves are affected by the depth and duration of waterlogging and their interactions. These seedlings experienced the greatest amount of cell membrane damage and were the most sensitive to the waterlogging environment.

Effects of waterlogging stress on the sugar content

As shown in Figure 4, waterlogging stress resulted in a generally increased of soluble sugar content within a small range with prolonged duration of the stress, except for Group A on day 6. On day 2 of stress, the soluble sugar content in Group A, B and C was higher than those in the control ($p < 0.05$), reaching 142.74%, 116.77% and 109.41% of the control, respectively. On day 4, the soluble sugar content in Group A, B and C showed similar changes compared to those of day 2. On day 6, the soluble sugar content in Group B and C increased by 18.7% and 22.3% compared to those of the control values, respectively. However, the values in Group A decreased by 20.7% compared to the control. Collectively, these data indicated that the depth and duration of waterlogging and their interactions may affect the accumulation of soluble sugars, and a reduction of cellular osmotic potential in *Z. armatum* seedlings.

Effects of waterlogging stress on the soluble protein content

As shown in Figure 5, little differences were observed in the leaf soluble proteins between waterlogging stress and the controls at different treatment times. On day 2, the soluble protein content in Group A, B and C increased to 148.51%, 136.64% and 128.71% compared to

the controls, respectively. However, on day 4, the soluble protein content showed a significant decrease among the tested groups, and the values in Group A, B and C significantly decreased to 72.68%, 51.96% and 90.89% compared to those in the controls ($p < 0.05$), respectively. On day 6, the values in Group B and C increased by 1.056 and 2.022-fold compared with the control, respectively. No significant differences were observed between the control and Group A. The present results suggested that the changes in soluble proteins are significantly affected by the depth and duration of waterlogging and their interactions. This may be due to that protein synthesis might be inhibited or activated under the waterlogging stress.

Effects of waterlogging stress on SOD activity

As shown in Figure 6, SOD activity showed different changes in different tested groups, as compared with controls. On day 2, SOD activity in Group C increased by 14.7% compared with the controls, but the values in Group A and B decreased to 63.25% and 86.19% compared to the controls, respectively. On day 4, SOD activity in Group A, B and C increased by 40.24%, 32.11% and 57.32% compared to the control, respectively. On day 6, SOD activity showed different increases among the tested groups. The values in Group A, B and C significantly increased to 32.97%, 21.54% and 27.91% compared to those in the controls ($p < 0.05$), respectively. These data indicated that the depth and duration of waterlogging and their interactions had significant effects on SOD activity in *Z. armatum* seedlings.

Effects of waterlogging stress on POD activity

The effects of waterlogging stress on POD activity in *Z. armatum* leaves are displayed in Figure 7. On day 2, POD activity in Group A, B and C increased by 66.91%, 148.31% and 83.74% compared with the controls, respectively. On day 4, POD activity in Group B increased by 19.29% compared to the control, but the values in Group A and C were lower than those in the control group. After 6 days of waterlogging stress, the POD activity of Group B and C was 2.71% and 20.48% greater than what was observed in the control groups, respectively. However, the values in Group A significantly decreased to 84.78% compared to the control. These results indicated that the depth and duration of waterlogging and their interactions had significant effects on POD activity in *Z. armatum* seedlings.

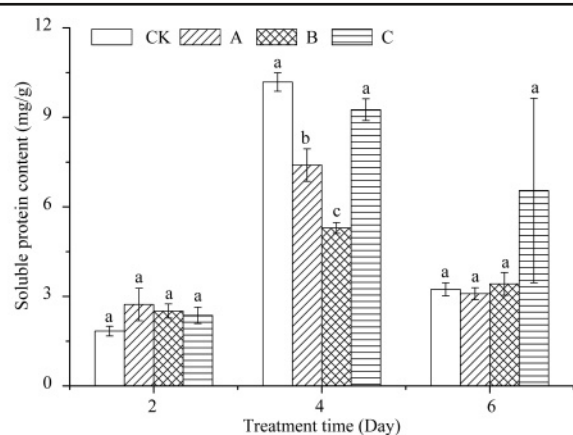


Figure 5. Effects of waterlogging stress on the soluble protein content of *Z. armatum* leaves. The vertical bars represent the standard errors, $n=3$. Bars followed by different letter(s) are statistically significant at $p < 0.05$. CK: Seedlings were not flooded. A: Seedlings were flooded at 2.7 cm above the ground level. B: Seedlings were flooded at 5.5 cm above the ground level. C: Seedlings were flooded at 11 cm above the ground level.

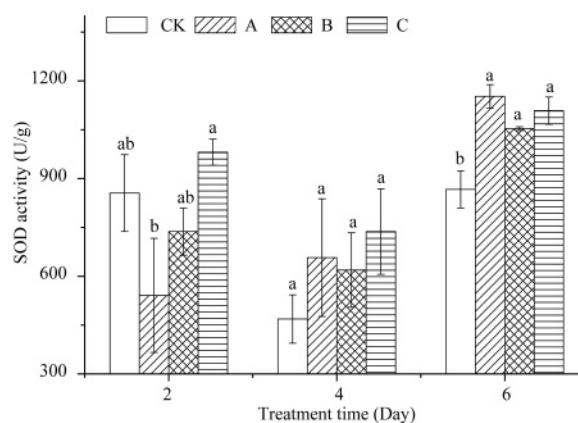


Figure 6. Effects of waterlogging stress on the superoxide dismutase (SOD) activity of *Z. armatum* leaves. The vertical bars represent the standard errors, $n=3$. Bars followed by different letter(s) are statistically significant at $p < 0.05$. CK: Seedlings were not flooded. A: Seedlings were flooded at 2.7 cm above the ground level. B: Seedlings were flooded at 5.5 cm above the ground level. C: Seedlings were flooded at 11 cm above the ground level.

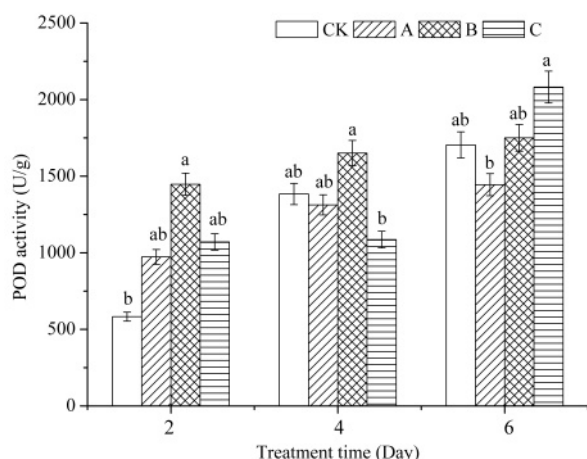


Figure 7. Effects of waterlogging stress on the peroxidase (POD) activity of *Z. armatum* leaves. The vertical bars represent the standard errors, $n=3$. Bars followed by different letter(s) are statistically significant at $p < 0.05$. CK: Seedlings were not flooded. A: Seedlings were flooded at 2.7 cm above the ground level. B: Seedlings were flooded at 5.5 cm above the ground level. C: Seedlings were flooded at 11 cm above the ground level.

Discussion

Waterlogging is a natural and environmental stress factor worldwide, and many plants suffer severe growth inhibition when the root is waterlogged at different times (Loreti *et al.*, 2016). Exposure of plants to waterlogging stress results in the generation of excessive ROS balance disruption (Pan *et al.*, 2021; Manghwar *et al.*, 2024). Thus, oxidative stress would arise if

the balance between ROS generation and removal was broken. To prevent oxidative damage due to ROS accumulation induced by waterlogging stress and ensure normal plant growth, many enzymatic and non-enzymatic antioxidants are capable of removing ROSs and their products and/or repairing oxidative damage (Manghwar *et al.*, 2024).

In several plants, the negative effects of waterlogging on their growth and yield have been studied (Tian *et al.*, 2021; Pan *et al.*, 2021; Manghwar *et al.*, 2024). Therefore, it is imperative to compare enzymatic and non-enzymatic antioxidants among plant species under waterlogging stress, which will help us to understand the role of these molecules against short-term (2, 4 and 6 days) waterlogging stress. In the present study, the results showed that no significant changes in morphological characteristics were observed in Group A and B. However, in Group C, the apical leaflets became soft, and showed a slight drooping posture, and the basal leaflets began to turn yellow on day 6. The wilting and chlorosis of leaves was often regarded as early symptoms of waterlogging stress, and visual symptoms of waterlogging stress were the yellowing of the foliage and leaf wilting (Loreti *et al.*, 2016; Zhou *et al.*, 2020; Pan *et al.*, 2021). *Z. armatum* seedlings showed accelerated senescence of the bottom leaves as compared to the upper leaves, which might be reflected by the loss of chlorophyll of plants under waterlogging stress conditions for more than 6 days. Our results were consistent with recent reports that waterlogging stress might affect

growth and development in *Mentha aquatica* L., poplar, *Sedum spectabile* Boreau “Carl” and *Sedum spectabile* “Rosenteller” (Haddadi *et al.*, 2016; Zhou *et al.*, 2019; Zhang *et al.*, 2019). These findings indicated that waterlogging stress associated with the reduction in seedling growth and biomass might be due to alterations in various physiochemical mechanisms.

Photosynthetic machinery is considered as one of the essential factors for the detection of waterlogging stress. Chlorophyll plays a key role in seedling growth and adaptation to different environmental conditions (Loreti *et al.*, 2016; Pan *et al.*, 2021). The present results showed that chlorophyll content is significantly affected by alternating waterlogging stress on days 2, 4 and 6, and showed different changes depending on the varying depth and duration of waterlogging (Figure 1). The overall decrease in chlorophyll content during different periods of waterlogging has also been reported in pigeonpea (Bansal & Srivastava, 2015). This finding in this study seems to contradict previous findings, and further studies are therefore required.

When plants are stressed by environmental factors, their membrane permeability will be increased due to the damage of function or the destruction of the structure. Salts or organic substances in the cell will be exuded to varying degrees, which results in a change in the relative electrical conductivity of plant tissues (Loreti *et al.*, 2016; Pan *et al.*, 2021). Relative electrical conductivity, which is a measure of cell integrity and cell membrane leakiness, has been considered a screening criterion for water tolerance. MDA is one of the products of lipid peroxidation in plant cells and is usually used as an indicator of membrane injury (Gao *et al.*, 2010; Pan *et al.*, 2021). Its levels may reflect the degree of plant damage under stress (Loreti *et al.*, 2016). In the present study, waterlogging stress exhibited a significantly higher concentration of MDA content in *Z. armatum* leaves than in the control (Figure 3). A strong correlation was found between MDA content and relative electrical conductivity, indicating that short-term stress damaged cell membranes in *Z. armatum* leaves. This accumulation of MDA was in good accordance with previous results, as described by Candan & Tarhan (2012) and Haddadi *et al.* (2016).

Under waterlogging stress, in order to minimize lipid peroxidation and ensure osmotic adjustment, many compatible solutes accumulate to relatively high levels in plants with enhanced stress resistance by accumulating osmolytes, such as soluble

sugar and protein, among others (Loreti *et al.*, 2016; Pan *et al.*, 2021). The change in soluble sugar content may reflect its adaptability to an adverse environment to a certain extent (Yu *et al.*, 2017; Zhou *et al.*, 2020). Zhang *et al.* (2019) studied the effect of waterlogging on soluble carbohydrate concentration in *Sedum spectabile* “Carl” leaves and observed a significant increase. However, Sairam *et al.* (2009) reported a continuous decline in the total sugar content and maximum decline was observed on day 6 of waterlogging treatment in four pigeonpea genotypes. Our results showed that waterlogging stress may result in generally increased soluble sugar content within a small range with the prolonged duration of waterlogging (Figure 4).

A major energy source used in plants are carbohydrates, and the decline or increase in carbohydrate level plays a major role in waterlogging tolerance mechanisms in plants (Saddhe *et al.*, 2021). In plant cells, soluble protein plays an important role in regulating the osmotic potential, and is known to respond to a wide variety of stressors (Yu *et al.*, 2017). In this study, our results suggested that the observed decline or increase in soluble proteins in *Z. armatum* seedlings are significantly affected by the depth and duration of waterlogging and their interactions (Figure 5). This may be due to the inhibition and/or accumulation of protein synthesis in stressed leaves in order to improve osmotic pressure and to consequently avoid water loss. Previous reports showed that waterlogging stress may induce the accumulation of soluble protein in *Mentha aquatica* and *Zea mays* plants (Haddadi *et al.*, 2016; Tian *et al.*, 2019). Moreover, changes in the soluble protein content and protein pattern induced by proteolytic degradation in white clover were found (Stoychev *et al.*, 2013). The mechanism by which waterlogging stress affects soluble sugar and protein content in *Z. armatum* leaves is complex and needs further study.

Waterlogging stress may cause cellular damage in plants due to the increased rate of ROS. SOD serves as the first critical defense antioxidative enzyme to scavenge the excess ROS under waterlogging stress (Duhan *et al.*, 2018). The present study showed that SOD activity in the treated groups is significantly higher than that in the control on days 4 and 6. An increased level of SOD enzyme activity was noticed in seedlings grown under waterlogging stress over the control plants (Figure 6). The present results are in agreement with the results of other plant species, including Kentucky bluegrass, *Mentha aquatica* and *Zea mays* (Puyang *et al.*, 2015; Haddadi *et al.*, 2016;

Tian *et al.*, 2019). Decreased SOD enzyme activity was also observed, and this might be due to the production of excess ROS and induced cell toxicity in waterlogging-treated plant cells (Chugh *et al.*, 2011). These results showed that SOD may be capable of controlling the damage caused by waterlogging stress within a certain range. However, the capability of plants to combat oxidative stress failed as the stress treatment progressed. POD is considered an important ROS scavenger to protect plant cells from waterlogging stress-induced oxidative stress (Loreti *et al.*, 2016; Pan *et al.*, 2021). Increased POD activity indicates that waterlogging stress might be involved in suppression of H₂O₂ occurrence in *Mentha aquatica* and *Zea mays* plants (Haddadi *et al.*, 2016; Tian *et al.*, 2019). Arbona *et al.* (2008) also examined the effect of waterlogging on citrus and observed increased POD activity depending on the plant genotypes. For illustration, stress-sensitive cultivars showed lower activity than tolerant cultivars (Arbona *et al.*, 2008). Waterlogging stress increased SOD and POD activity depending on genotypes, organs, stress duration and waterlogging degree as well as their interactions (Haddadi *et al.*, 2016; Tian *et al.*, 2019; Pan *et al.*, 2021). In the present study, short-term stress, especially on day 2, may induce the activity of POD in all tested groups. However, with the prolonged waterlogging time up to days 4 and 6, the activity was inhibited (Figure 7). This might be due to its weak ability to minimize membrane lipid peroxidation under waterlogging stress. Moreover, SOD and POD activity were positively correlated with chlorophyll, MDA, relative electrical conductivity, sugar and protein content. These data support the hypothesis that more free radicals accumulated in seedlings resulted in oxidative stress and

perturbation of membrane integrity, consequently leading to a slightly drooping posture, and finally the wilting of seedlings.

Conclusion

This study emphasized the dynamic variations in chemical parameters and antioxidant enzyme activity in *Z. armatum* seedlings subjected to waterlogging stress. In summary, our findings allow us to conclude that morphology characteristics, chlorophyll, MDA, relative electrical conductivity, sugar, and protein content as well as antioxidant enzymes in *Z. armatum* seedlings exposed to waterlogging stress show significant differences between stress degree and treatment times. Higher sugar and protein content as well as content as well as SOD and POD activity, to a certain extent, indicated that the tolerance capacity of *Z. armatum* seedlings can protect this plant from oxidative damage caused by waterlogging stress. These variations may be attributed to a better understanding of the response mechanisms of *Z. armatum* to waterlogging stress, which will help provide basic data for the waterlogging management of *Z. armatum* plants. However, the biochemical networks involved in antioxidant responses to waterlogging stress during the cultivation and management of *Z. armatum* plants are complex and should be further investigated.

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