Exogenous ascorbic acid mitigates flood stress damages of *Vigna angularis*

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*Received: 13 June 2017 / Accepted: 27 August 2017 / Published online: 1 September 2017*  
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**Abstract** Prolonged water stress adversely affects many aspects of plant physiology, resulting in severe damage to growth and productivity. In response to this and other environmental stresses, plants have evolved complex physiological and biochemical adaptations. To boost existing plant defense mechanisms, this study quantified the negative effects of waterlogging stress and how it may be mitigated by the addition of a natural protective agent. Adzuki beans (*Vigna angularis*) were grown in commercially available microbe-free soil and subjected to waterlogging stress for 2 weeks. Waterlogging significantly reduced all growth-related variables: shoot length, fresh and dry biomass, chlorophyll content in stressed versus unstressed plants. Waterlogging stress generated reactive oxygen species that heavily damaged plant tissues, causing electrolyte leakage and eliciting an antioxidative response. Specifically, stress-response phytohormone content altered, with a reduction in abscisic acid (ABA) and an increase in jasmonic acid (JA). Furthermore, antioxidants such as malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD), polyphenol oxidase (PPO), and peroxidase (POD) were significantly enhanced in waterlogged plants versus non-waterlogged plants. Supplementation of exogenous ascorbic acid (ASC) at 3, 5, and 7 mM revealed that the lowest concentration further reduced ABA and increased JA, enhancing water evaporation rates to raise water-stress tolerance. Moreover, 3 mM ASC also led to lower MDA, CAT, SOD, PPO, POD, and ascorbate peroxidase concentrations in waterlogged plants than in waterlogged plant not treated with ASC. Thus, ASC at a concentration of 3 mM was the most successful in relieving effects of waterlogging stress on plants.

**Keywords** Abscisic acid · Jasmonic acid · Reactive oxygen species · Waterlogging stress

**Introduction**

Adzuki bean (*Vigna angularis*) is among the most cultivated crops in East Asia and is also increasingly grown worldwide [1]. It is highly nutritious, rich with proteins, carbohydrates, and fats, while containing various therapeutic compounds including isoflavones, anthocyanins, dietary fiber, and saponin [2]. The protein content in adzuki beans is sufficient to fulfill the protein requirement of the human diet. To increase adzuki crop productivity, cultivation factors must be optimized [3]. Such factors include irrigation, fertilizer application, as well as protection from biotic and abiotic stress (e.g., pathogens, insect pests, extreme environmental conditions) [3, 4]. In particular, waterlogging restricts bean yield because *V. angularis* is cultivated during periods of high rainfall (~65% of annual precipitation), reducing yields by about 20–40% [5]. Waterlogged plants face extreme O₂ shortage due to decreased gas diffusion from the air into water [4]. Moreover, any remaining dissolved O₂ is depleted by respiration of the plant root and surrounding microbial respiration, resulting in complete inhibition of respiration in other plant parts [6]. Waterlogged plants thus cannot generate glucose...
and eventually suffer from metabolic dysfunction. Besides O₂ deficiency, waterlogging causes accumulation of toxic compounds, such as sulfides, metal ions (e.g., iron manganese), formic acid, acetaldehyde ethanol, and lactic acid [7].

Plants have developed a number of defensive mechanisms to cope with biotic and abiotic stresses, including waterlogging. These mechanisms involve sensitive detection systems and robust signal transduction pathways, resulting in a highly specific and tightly controlled response that ensures adequate defense without wasting resources [8].

Abscisic acid (ABA) is a major phytohormone involved in regulating whole-plant to cellular-level activity, including responses to environmental stress such as floods or drought [8]. Water stress significantly induces the expression of NCED, AAO, and ABA2 genes, responsible for ABA biosynthesis in the vascular tissues [9]. Rapid catalysis by genes such as OsABA8ox1 also serves to control ABA concentrations, which influence plant responses to water stress through regulating stomatal closure. Along with plant roots, stomata are key tissues involved in osmoregulation [10]. Under drought conditions, plants produce high ABA concentrations in the guard cells, inducing stomatal closure to conserve water. Under waterlogging, ABA is downregulated to keep the stomata open, allowing excessive water to evaporate [8].

Thousands of ABA-dependent genes are responsible for controlling secondary metabolism, senescence, protein synthesis, and osmoregulation in coordinating plant responses to water stress [11]. Within the past decade, the concept of hormonal cross talk has become widespread as researchers elucidated the connections between so-called stress hormones with “developmental” hormones such as jasmonic acid (JA), ethylene, and auxin, revealing a complex signaling network. Thus, the exact mechanism of a hormone during waterlogging stress involves both cross talk and resultant physiological responses [10].

Although JA is well known for its role plant defenses against biotic stress [12], its role against abiotic stresses is poorly understood. Under both forms of stress, of JA biosynthesis occurs through the octadecanoid pathway. The cross talk of JA with ABA appears to be important in signaling pathways activated during water stress [13]. Specifically, an increase in water influx leads to lower ABA concentrations; in turn, stomata remain open to evacuate excess water. In contrast, JA accumulates in waterlogged conditions to protect plants from osmotic stress [14] through the production of defense-related amino acids (e.g., proline) and proteins [12].

Waterlogging generates reactive oxygen species (ROS) that disturb photosynthetic machinery and thus decreases photosynthesis rates [15]. Specifically, superoxide (O₂⁻) and hydrogen peroxide (H₂O₂) accumulation damages chloroplast membranes and inhibits photosystem function [16]. Signs of oxidative stress include lipid compositional changes, lipid peroxidation, electrolyte leakage, and eventually cell death [17]. In response, the plant immune system produces antioxidants such as phenolic compounds, ascobic acid (ASC), and glutathione, as well as ROS-scavenging enzymes, to maintain redox homeostasis [12]. During ROS scavenging, superoxide dismutase (SOD) first catalyzes O₂⁻ into H₂O₂; next, peroxidase ( POD), catalase (CAT), glutathione reductase (GR), or ascorbate peroxidase (APX) can all act to reduce H₂O₂ into H₂O [13].

Ascobic acid is a water-soluble antioxidant that plays a major role in plant stress signaling (i.e., ROS scavenging) and other physiological processes, both enzymatic and non-enzymatic [18]. These processes include vital functions such as redox buffer, cofactor, signal transduction, and cell division [8]. Its general importance to plants has made ASC a common choice for exogenous application to mitigate biotic and abiotic stresses, including waterlogging. The application of exogenous ASC has been demonstrated to reduce symptoms of water stress (e.g., lipid peroxidation), improve leaf and root growth, as well as enhance nutrient uptake [19].

Materials and methods

Cultivation methods, growth conditions, and flooding treatment

Vigna angularis seeds were surface-sterilized with 75% ethanol for 2 min before being thoroughly washed with deionized distilled water. Sterilized seeds were sown in pots packed (9.5 × 9.5 × 8.5 cm) with commercially available horticultural soil (Sun Gro Horticulture Company, Agawam, USA). These pots were placed in a growth chamber maintained at 25 ± 5 °C, 60% relative humidity, a light intensity of 650 μmol/m²/s, and a 16/8-h light/dark cycle for 2 weeks without waterlogging stress. For ASC treatment, 100 mL of ASC solutions with concentrations of 0, 3, 5, and 7 mM was applied directly (just one time during the experiment) to the soil of the plants in the pots; then, the plants were allowed to absorb the solution for 24 h. Afterward, the plants were subjected to waterlogging stress by putting the pots in the water tank in submerged condition, up to the surface of soil for 2 weeks. Thus, one experiment was 4 week long (2 weeks without water stress and 2 weeks with water stress). On the other hand, a set of plants was grown in original growth conditions as described above without water stress for 4 weeks (control). Application of different concentrations of ASC in waterlogging stress was required to elaborate the role of specific amounts of ASC in waterlogging stress in comparison with
plants grown in normal conditions. The exogenous treatment of ASC under normal condition (without water stress) did not have a significant effect on plant growth (data not shown). Also, Xu et al. [20] showed similar results that exogenous treatment of ASC did not have a significant effect on the root elongation rate. Therefore, two sets of controls were used in the experiment: one set was without waterlogging stress and the other was 0 mM of ASC (mock treatment) in waterlogging stress to compare with other concentration of ASC mentioned above. Three replications of three plants each were used per treatment.

Measurement of plant growth

After 2 weeks of waterlogging stress, the following growth-related variables were measured: shoot length, root length, number of adventitious roots, chlorophyll content, leaf area, and biomass. Shoot and root lengths were measured separately. Plants were weighed immediately after harvesting to obtain fresh biomass, while dry biomass was measured after oven-drying at 80 °C overnight.

Endogenous abscisic acid (ABA) analysis

Plant samples were harvested and immediately ground in liquid nitrogen to extract hormones. Endogenous ABA was analyzed with gas chromatography–mass spectrometry (GC–MS) [21, 22]. Ground plant samples were mixed with 30 mL of extraction solution containing 95% isopropanol, 5% glacial acetic acid, and 20 ng of ABA internal standard. The suspension was passed through a silica cartridge (Sep-Pak; Waters Associates, Milford, Massachusetts, USA) pre-washed with 10 mL of diethylether:methanol (3:2, v/v) and 10 mL of dichloromethane. The column was eluted with 10 mL of diethylether: methanol (MeOH; 3:2, v/v) to collect ABA; the eluent was dried and methylated via the addition of diazomethane for GC/MS-SIM (selective ion monitoring) (6890 N Network GC system and the 5973 Network Mass Selective Detector; Agilent Technologies, Palo Alto, CA, USA), column equipped with DB-1 capillary column (30 m × 0.25 mm, i.d. 0.25 μm film thickness). The carrier gas, helium at a flow rate of 40 mL/min and oven temperature was kept at 60 °C for 1 min and then increased up to 200 °C at a rate of 15 °C/min, before being further increased to 250 °C at a rate of 5 °C/min with ionization voltage 70 eV. The Lab-Base (ThermoQuest, Manchester, UK) Data System was used to quantify Me-ABA under ions of m/z 162 and 190, as well as Me-[2H6]-ABA under ions of m/z 166 and 194.

Endogenous jasmonic acid (JA) analysis

Endogenous JA was extracted from plants crushed in liquid nitrogen. Crushed samples (100 ng) were dissolved in a solution of acetone and 50 mM citric acid (70:30 v/v); [9,10-2H2]-9,10-dihydro-JA (25 ng) was then added as an internal standard. The suspension was evaporated overnight at room temperature to prevent escape of volatile fatty acids. The remaining aqueous solution was filtered and extracted trice with 10 mL of diethyl ether. The resultant three fractions were combined and loaded on a solid-phase extraction cartridge filled with 500 mg of sorbent, aminopropyl. The column was washed with 7 mL of tri-chloromethane and 2-propanol (2:1, v/v) before endogenous JA elution with 10 mL of diethyl ether and acetic acid (98:2, v/v). The sample was then dried with a stream of O2 and free N2 gas; residues were esterified with diazomethane and dichloromethane was added until the final volume was 50 μL. Analysis of JA content in the samples was performed with GC/MS-SIM (6890 N Network GC system and 5973 Network Mass Selective Detector; Agilent Technologies). The oven temperature for JA was adjusted at 60 °C for initially 2 min and then increased to 140 °C at the rate of 10 °C/min and then increased to 170 °C at the rate of 3 °C/min and finally reached to 285 °C at the rate of 15 °C/min. Fragmentation was monitored at m/z 83, equivalent to the base peaks of JA and [9,10-2H2]-9,10-dihydro-JA. Endogenous JA concentration was calculated through a comparison of its peaks with the respective standards. Three replicates per treatment were used.

Measurement of oxidative damage and antioxidant response

Electrolyte leakage was measured to determine leaf integrity under waterlogging stress. One fresh, fully expanded leaf per sample from each ASC + waterlogging condition was placed in 50-mL falcon tubes with 30 mL of deionized distilled water and incubated at 25 °C for 2 h. Next, initial electrical conductivity (EC1) was measured with an electrical conductivity meter. Suspensions were then boiled at 100 °C for 20 min to denature leaf tissues.
and then cooled at room temperature for the measurement of final electrical conductivity (EC2). The percentage of electrolyte leakage is calculated with the following formula:

\[ E = \frac{EC1}{EC2} \times 100\% \]

**Lipid peroxidation analysis**

Lipid peroxidation of plants under ASC + waterlogging treatment and control was determined. First, 0.1 g fresh leaves was homogenized in 3 mL of (0.1%) trichloroacetic acid (TCA) using a mortar and pestle. The mixture was centrifuged at 4000 rpm for 10 min at 4 °C. The resultant supernatant (2 mL) was combined with a solution (2 mL) 20% TCA and 0.5% 2-thiobarbituric acid, then boiled for 30 min, cooled in ice, and centrifuged at 4000 rpm for 10 min. Optical density (OD) of the supernatant was measured at 532 nm. The lipid peroxidation level was expressed in micromoles of malondialdehyde (MDA) formed per g leaf tissue.

**Determination of reduced glutathione (GSH)**

Reduced glutathione (GSH) was extracted from fresh leaves of each treatment, which were first weighed before grinding 0.1 g in 3 mL of trichloroacetic acid (5%, v/v) using a chilled mortar and pestle. Homogenates were centrifuged at 12 000 rpm for 15 min at 4 °C. One milliliter of supernatant was mixed with 3 mL of 150 mM NaH2PO4 (pH 7.0) and 0.5 mL of Ellman’s reagent and then incubated at 30 °C for 5 min. Absorbance was measured at 412 nm, and GSH content was determined from comparison to a GSH standard curve.

**Catalase (CAT) activity assay**

Fresh leaves (0.5 g) from each sample were crushed in liquid nitrogen, then dissolved in extraction buffer [50 mM sodium phosphate (pH 7.0), 1% polyvinylpolypyrrolidone (PVP; w/v), 0.1 mM EDTA], and centrifuged (4000 rpm, 4 °C). Phosphate buffer (50 mM, pH 7.0) was then added to 100 µL of supernatant. H2O2 catalysis was initiated with the addition of 0.2 M H2O2 and monitored at 420 nm for 1-min intervals. To determine CAT activity, decrease in absorbance at 240 nm was monitored based on the decline in H2O2 extinction. The Bradford assay was used to determine total protein content in the plant extracts, with bovine serum albumin as standard. One unit of CAT activity was expressed as µg of H2O2 released per mg protein per min.

**Polyphenol oxidase (PPO) and peroxidase (POD) activity assay**

Samples were ground in liquid nitrogen, and 0.1 mL powder was suspended in extraction buffer (0.1 M phosphate buffer (pH 6.8), 50 µL pyrogallol). Next, 50 µL of H2O2 was added and the mixture was incubated at 30 °C for 5 min, resulting in the formation of purpurogallin. The reaction was stopped with the addition of 5% H2SO4 (v/v); PPO and POD activities were measured at 420 nm in a spectrophotometer. Activity was expressed as a 0.1 unit increase in enzyme absorbance.

**Estimation of total flavonoids**

Plant samples from all treatments were extracted with 10 mL of 80% methanol and then centrifuged at 4000 rpm and 4 °C for 10 min. Next, the supernatant (0.5 mL) was combined with 1.5 mL methanol, 0.1 mL potassium acetate, 0.1 mL aluminum chloride (AlCO3), and distilled water to yield a final volume of 5 mL; the solution was filtered (0.45 µM). The blank was prepared with all reagents except AlCO3. Sample absorbance was measured at 410 nm in a spectrophotometer, and flavonoid content was estimated via comparisons with a standard curve. The experiment was conducted in triplicate.

**Determination of proline content**

Proline content was determined using the ninhydrin method [23]. Proline was cold-extracted: fresh leaves (0.1 g) were ground in 2 mL of ethanol:water (40:60, v/v) and then refrigerated (4 °C) overnight before centrifuga-

**Determination of total phenolic content**

Total phenolic content was estimated following methods from Singleton et al. [23]. Waterlogged and control samples were first ground in liquid nitrogen. Next, plant powder (5 mg) was extracted with 5 mL of 70% methanol. Extracts (0.5 mL) were combined with 2.5 mL of 10% Folin–Ciocalteu’s reagent and 2.5 mL of 7.5% NaHCO3 to prepare the reaction mixture, which was incubated at 45 °C for 50 min. The blank comprised the same reagents but did
not include plant extracts. Sample absorbance was measured at 765 nm in a spectrophotometer. The experiment was performed in triplicate to obtain means for statistics. Phenol concentration was expressed as mg/mL and determined via comparisons with the standard curve of gallic acid (mg of GA/g of extract).

Photometric alcohol dehydrogenase (ADH) activity assay

The ADH assay is based on the enzyme’s conversion of ethanol to acetaldehyde in crude protein extracts, accompanied by NAD$^{+}$ reduction to NADH. Spectrophotometric analysis is then able to determine NADH formation. Plant samples were ground in liquid nitrogen and extracted with 5 mL of sodium phosphate buffer (pH 8). Including 1 mL extract, the reaction mixture contained 50 mM sodium phosphate buffer (pH 8), 150 mM ethanol, and 300 μM NAD$^{+}$. The blank contained all reagents except the extract. Sample absorbance was then measured at 340 nm in a spectrophotometer. Specific ADH activity was expressed as μmol NADH formed per min per mg total protein.

Superoxide dismutase (SOD) activity assay

Superoxide dismutases (SOD) are well-known metalloproteins that catalyze dismutation reactions of superoxide free radicals with O$_2$ and H$_2$O$_2$. Powder samples ground crushed in liquid nitrogen were extracted with 2 mL of 50 mM potassium phosphate buffer (pH 7.8). Because superoxide dismutation rates increase a thousand fold in the presence of SOD at slightly above neutral pH, SOD activity was measured based on superoxide inhibition of NBT reduction. The reaction mixture (2 mL) comprised 50 mM sodium phosphate buffer (pH 7.8), 2 mM EDTA, 9.9 mM methionine, 1.41 55 l M NBT hydrochloride, 0.025% w/v Triton-X100, and 0.044 g/mL riboflavin. An additional 20 μL of 1 mM riboflavin and 40 μL of extract were then added, followed by light exposure to initiate the reaction. Subsequently, reaction tubes were left in the dark for 30 min, and absorbance was measured at 560 nm. Enzymatic activity (g per fresh weight) was determined through comparisons to a standard curve obtained from pure SOD.

Results

Plant growth variables

The growth of waterlogged plants treated with ASC (0, 3, 5, and 7 mM) was analyzed to determine stress effects and the role ASC (Fig. 1). Shoot length decreased by 63% under waterlogging stress compared with unstressed control plants. However, 3 mM ASC significantly improved (p < 0.05) the shoot length of waterlogged plants, growing up to 25% longer than waterlogged plants 0 mM ASC. Although 5 and 7 mM ASC also significantly improved shoot length compared with 0 mM ASC plants, their effects were weaker than the 3 mM ASC treatment (Table 1). Similarly, root length in waterlogged plants was significantly reduced (p < 0.05) by 62% compared with unstressed plants. Treatment with 3 mM ASC reduced root lengths by only 36% compared with unstressed plants. Thus, 3 mM ASC was effective in improving growth under waterlogged conditions, as seen in the increased root lengths over stressed plants (0 mM ASC). Other ASC concentrations (5 and 7 mM) were ineffective; root lengths under these treatments did not differ significantly from waterlogged plants (0 mM ASC). Waterlogging also significantly reduced fresh biomass, dry biomass, and chlorophyll content (p < 0.05), but 3 mM ASC treatment improved all three variables compared with 0, 5, and 7 mM ASC treatments (Table 1).

Root anatomy under water stress and ASC treatment

Plants normally uptake soil O$_2$ through the roots for use in respiration, generating ATP via oxidative phosphorylation. Under waterlogged conditions, spaces between soil
particles are filled with water and plants cannot absorb O\(_2\), leading to hypoxia and reduced metabolism. Therefore, plant root cells have physiological adaptations to facilitate O\(_2\) uptake under these conditions. Waterlogging stress ruptured plant cells, causing redundancy in growth, development, and photosynthetic activities (Fig. 2). Furthermore, the 3 mM ASC treatment facilitated the development of aerenchyma to prevent cell rupture and improve O\(_2\) flux in the plant. Similarly, 5 and 7 mM ASC minimized cell rupture with aerenchyma formation in waterlogged plants, but these concentrations were less effective than 3 mM ASC.

**Lipid peroxidation and electrolyte leakage**

Waterlogging-related stress stimulates the production of ROS, which initiates biochemical reactions that are severely detrimental to plants. One such reaction is lipid peroxidation, which irreversibly damages membrane integrity and functionality. Malondialdehyde (MDA) is a by-product of lipid peroxidation and thus acts as an indicator of oxidative-stress-induced tissue damage. We showed that MDA content increased significantly \(p < 0.05\) in waterlogged versus unstressed plants (Fig. 3A). Additionally, 3 and 5 mM ASC application reduced MDA content down to unstressed levels. However, 7 mM ASC actually caused a significant increase in MDA content compared with waterlogged plants (0 mM ASC). Leaf electrolyte-leakage patterns were very similar: electrolyte leakage significantly increased in waterlogged plants, but was greatly reduced with 3 mM ASC treatment. In contrast, both 5 and 7 mM ASC were ineffective, causing greater electrolyte leakage (Fig. 3B).

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**Table 1** Plant growth variables of *Vigna angularis* plants exposed to waterlogging stress and treated with different concentrations of ascorbic acid (ASC)

| Treatment | S.L. (cm) | R.L. (cm) | F.B. (g) | D.B. (g) | C.C. (SPAD) |
|-----------|-----------|-----------|----------|----------|-------------|
| Control   | 55.46 ± 4.95\(^a\) | 40.1 ± 4.25\(^a\) | 30.2 ± 3.60\(^a\) | 11.46 ± 1.00\(^a\) | 56.1 ± 4.03\(^a\) |
| 0 mM      | 20.33 ± 2.15\(^b\) | 15.26 ± 3.09\(^b\) | 8.01 ± 3.82\(^b\) | 6.45 ± 0.78\(^b\) | 30.16 ± 4.71\(^b\) |
| 3 mM      | 41.36 ± 2.51\(^c\) | 25.53 ± 3.10\(^c\) | 20.63 ± 2.05\(^c\) | 10.01 ± 1.42\(^c\) | 46.36 ± 2.11\(^c\) |
| 5 mM      | 27.83 ± 2.60\(^d\) | 14.33 ± 1.43\(^d\) | 8.52 ± 1.49\(^d\) | 5.52 ± 1.49\(^d\) | 29.86 ± 4.54\(^d\) |
| 7 mM      | 30.4 ± 2.9\(^d\) | 16.46 ± 2.70\(^d\) | 8.34 ± 0.83\(^d\) | 4.61 ± 1.08\(^d\) | 29.66 ± 6.02\(^d\) |

Values in each column represent the mean ± SD. Those marked with different letters in each column are significantly different at \(p < 0.05\) as analyzed by Duncan’s multiple range test.

*S.L.* shoot length, *R.L.* root length, *F.B.* fresh biomass, *D.B.* dry biomass, *C.C.* chlorophyll contents, *SPAD* total chlorophyll per unit leaf area of leaf tissue (nmol/cm\(^2\))

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**Fig. 2** Root anatomy of *V. angularis* plants exposed to waterlogging stress and treated with different concentrations of ascorbic acid (ASC). Scale bars = 200 μm
Endogenous ABA levels

Through the regulation of stomatal closure, ABA is heavily involved in plant osmoregulation. Therefore, ABA is considered a major phytohormone under water stress. Moreover, ABA is responsible for parenchymatous cell formation in waterlogged roots [8]. This study analyzed endogenous ABA content in adzuki beans after 2 weeks of waterlogging in combination with ASC treatments at different concentrations (0, 3, 5, and 7 mM). We found that ABA was significantly \( p < 0.05 \) lower in waterlogged plants than in unstressed controls; this change presumably kept the stomata open to release excess water (Fig. 4A). Moreover, 3 mM ASC-treated plants had significantly \( p < 0.05 \) lower endogenous ABA concentrations than either unstressed plants or waterlogged plants (0 mM ASC). Both 5 and 7 mM ASC treatments increased endogenous ABA concentration in waterlogged plants. Overall, 3 mM ASC improved plant tolerance of waterlogging through reducing endogenous ABA concentration, resulting in evaporation that achieved some water balance.

Endogenous JA levels

Endogenous JA is a well-known stress hormone that accumulates under both abiotic and biotic stresses. Moreover, cross talk of ABA and JA was revealed to engage in cross talk with ABA, linking the two phytohormones during water stress. We found that JA content increased from 28.53 ng in unstressed control plants to 38.86 ng in

Fig. 3 Effects of different ascorbic acid (ASC) concentrations on plants under waterlogging stress. (A) Malondialdehyde (MDA) content indicating lipid peroxidation under waterlogging stress. (B) Electrolyte leakage from plant tissues during waterlogging stress. Values are presented as mean ± SD (error bars). Different letters over error bars indicate significant differences \( p < 0.05 \) according to Duncan’s multiple range tests. FW fresh weight of plants

Fig. 4 Variation of stress hormones in waterlogged plants under ASC treatment. Exogenous ASC application significantly regulated (A) abscisic acid (ABA) and (B) jasmonic acid concentrations. Values are presented as mean ± SD (error bars). Different letters over the error bars indicate significant differences \( p < 0.05 \) according to Duncan’s multiple range tests. g of plant gram of dry weight of plants.
waterlogged plants (Fig. 4B). When treated with 3 mM ASC, endogenous JA rose significantly to 103.4 ng in waterlogged plants. In contrast, JA concentration dropped in waterlogged plants to 35.14 and 18.55 ng, respectively, when treated with 5 and 7 mM ASC.

**Proline synthesis under waterlogged conditions**

Proline is an amino acid that plays a critical role in plant stress response. Under environmental stress, proline accumulation exerts a protective effect against stress-induced damage. Waterlogging significantly stimulated proline accumulation (p < 0.05) (Fig. 5A), suggesting the presence of stress-related damage in the measured plants. Proline accumulation was significantly reduced (p < 0.05) in waterlogged plants treated with 3 mM ASC compared to those of 0 mM ASC. While 5 and 7 mM ASC also significantly reduced proline compared with the 0 mM ASC condition, the reduction was less obvious than with 3 mM ASC. Our results demonstrated that 3 mM ASC was very effective as a preventive measure against waterlogging-related damage in plants.

**Reduced glutathione content**

Waterlogging and other stresses elicit ROS (e.g., H$_2$O$_2$, O$_2^-$) generation, thus disrupting cellular and organelle membrane integrity to cause ionic imbalance. However, plants have strong antioxidant systems in place to defend against oxidative damage. Exogenous antioxidants can bolster existing plant defenses during stressful conditions. Therefore, we treated waterlogged plants with different ASC concentrations to determine the effects of the exogenous enzyme on plant response to water stress. Waterlogging stress significantly reduced GSH content in plants (p < 0.05; Fig. 5B). Furthermore, 3 mM ASC treatment to waterlogged plants sharply reduced GSH content (p < 0.05) compared with waterlogged plants that were not treated with ASC. Although 5 and 7 mM ASC treatments also significantly (p < 0.05) decreased GSH compared with 0 mM ASC waterlogged plants, the effects of higher ASC concentrations were weaker than those of 3 mM ASC. Overall, ASC was effective in relieving waterlogging stress through the reduction in GSH content, which increases plant competence under stressful conditions.

**Analysis of CAT, PPO, POD, and SOD activities**

Antioxidative enzymes such as CAT, PPO, POD, and SOD are important to plant damage control under stress. In waterlogged plants, CAT activity was significantly higher than in unstressed controls (Fig. 6A). However, all ASC concentrations reduced CAT activity in waterlogged plants significantly compared with 0 mM ASC waterlogged plants, lowering the antioxidant to prestressed levels. Similarly, PPO activity was significantly (p < 0.05) higher in waterlogged plants than in unstressed plants (Fig. 6B). In addition, 3 and 7 mM ASC reduced PPO activity significantly (p < 0.05) compared to no ASC treatment in waterlogged plants. While 5 mM ASC also significantly reduced the PPO activity, it was less effective than 3 or 7 mM ASC. Waterlogging stress also significantly increased POD activity (p < 0.05) compared with activity in an unstressed plant, while 3 mM ASC treatment of waterlogged plants significantly reduced POD activity.

![Fig. 5](image-url) Antioxidant response in waterlogged plants treated with different ASC concentrations. (A) Proline content significantly increased in waterlogged plants compared with unstressed controls. However, 3 mM ASC reduced proline in waterlogged plants significantly more than no ASC treatment. (B) Reduced glutathione (GSH) content increased significantly in waterlogged plants. ASC treatments then significantly reduced GSH content. Values are presented as mean ± SD (error bars). Different letters over the error bars indicate significant differences (p < 0.05) according to Duncan’s multiple range tests. g of plant gram of dry weight of plants, FW fresh weight of plants.
compared with 0 mM ASC waterlogged plants ($p < 0.05$). However, 5 and 7 mM ASC addition did not significantly change POD activity (Fig. 6C). Finally, SOD activity in waterlogged plants was significantly higher ($p < 0.05$) than in unstressed controls, and all tested ASC concentrations reduced SOD activity in waterlogged plants compared with 0 mM ASC waterlogged plants (Fig. 6D).

**Determination of total polyphenolic and flavonoid content**

Phenolic compounds are natural antioxidants with strong redox potential and applications for human health in treatments against stress. The free radical scavenging capability of phenolic compounds make them useful for screening antioxidant activity. Flavonoids act as antioxidants in vitro as well as in vivo through shedding an OH group. Polyphenolic content was greater in waterlogged plants than in unstressed plants ($p < 0.05$; Fig. 7A). When treated with 3 mM ASC, phenolic content in stressed plants was significantly lower ($p < 0.05$) than in waterlogged plants not treated with ASC. Both 5 and 7 mM ASC were effective in reducing the phenolic content of waterlogged plants, but less so than 3 mM ASC.

Waterlogging significantly enhanced ($p < 0.05$) flavonoid content. The effect of ASC treatment on flavonoids in waterlogged plants was significant and dose dependent; 3 mM ASC was the most effective at reducing flavonoid content ($p < 0.05$) from the levels in 0 mM ASC waterlogged plants, followed by 5 mM ASC ($p < 0.05$) and finally by 7 mM ASC (Fig. 7B).

**Alcohol dehydrogenase activity**

To ensure survival, plants must evolve mechanisms to adjust cellular metabolism under O$_2$ deficiency. One such mechanism is the switch from aerobic respiration to lactic and alcohol fermentation, allowing glycolysis to continue for a longer period [24, 25]. Waterlogging stress significantly induced ($p < 0.05$) ADH activity compared with
unstressed plants (Fig. 8). All tested ASC concentrations reduced ADH activity in treated waterlogged plants versus 0 mM ASC waterlogged plants.

**Discussion**

Plant tissue development and cell growth are among the processes most sensitive to waterlogging stress, which triggers multiple responses [26]. Flooded conditions stimulate a chain of biochemical reactions, including excessive ROS production that inflicts damage, followed by upregulation of antioxidative enzymes and phytohormones to defend against stress [27]. Exogenous ASC application has beneficial effects on plant growth variables (e.g., survival rate, biomass, shoot and root growth) under water and heat stress [26, 28]. In the present study, we analyzed plant physiological and morphological responses to waterlogging stress. We demonstrated that waterlogging ruptured plant tissues, leading to high electrolyte leakage and increased ROS production; as a result, MDA content increased, reflecting the extent of oxidative damage on cellular membranes. Moreover, antioxidants such as GSH, MDA, CAT, PPO, POD, SOD, and APX were induced in waterlogged plants. Our data are in accordance with previous research in alfalfa (*Medicago sativa*) [29], thale cress (*Arabidopsis thaliana*) [30], and maize (*Zea mays*) [31] that also showed ROS accumulation under waterlogging stress. The degree of ROS accumulation depends on both their synthesis and the efficiency of ROS scavenging. The latter system operates through the synergistic activity of key enzymes centered around the ascorbate–glutathione cycle. Sustained SOD transcription and activity catalyzes the reaction of $\text{O}_2^-$ to $\text{H}_2\text{O}_2$, a crucial response in flooding tolerance [7]. Next, $\text{H}_2\text{O}_2$ is removed by the action of numerous enzymes, including CAT and APX. Ascorbic acid is also an important antioxidant, found at high concentrations in chloroplasts of leaf tissues and in various root cellular compartments [32]. Although extensive studies have documented ASC protecting leaves from oxidative damages, only a few reports have examined the effects of exogenous ASC on root tissues, in non-stressed or salt-stressed conditions [28]. Here, we showed that exogenous
ASC relieved waterlogging stress, although it could not completely reverse adverse effects. In addition, ASC treatment effectively suppressed ROS production and MDA accumulation in the root elongation zone, decreasing membrane lipid peroxidation. Thus, ASC functions directly as an antioxidant to mitigate waterlogging-related damage in plants. Besides compounds like ASC, plant ROS-scavenging systems also involve enzymatic pathways [33]. We showed that waterlogging stress significantly reduced antioxidant enzyme activity, indicating a weakening of these systems.

Abscisic acid and JA are among the most important phytohormones involved in stress defense. In particular, ABA regulates water content under both flood and drought conditions [26]. Here, waterlogging stress, along with 3 mM ASC treatment, reduced ABA content and enhanced JA content. This outcome is in line with ABA regulation of stomatal closure during osmeregulation [32]. Previous studies showed that plants produce high ABA concentrations in guard cells during drought stress, inducing stomatal closure to conserve water. However, ABA is downregulated under flooded conditions and the stomata remain open, leading to frequent evaporation of excess water [22].

In contrast, JA function in biotic stress responses is well elaborated [34], but its action in abiotic stress response is less explored. Both types of stress trigger JA biosynthesis through the octadecanoid pathway. Furthermore, research exists to show that the cross talk of JA and ABA is critical to the water-stress signaling pathway [14]. Higher water influx decreases ABA concentration to stimulate stomatal opening and evaporation, whereas the same conditions cause JA accumulation to defend against osmotic stress [10]. Overall, our results indicate that waterlogging triggers the expression of ABA and JA, while ASC scavenges waterlogging-induced ROS [10, 22].

We conclude that flooding severely affects the normal functioning of plants. To withstand waterlogging stress, plants exhibit a wide host of physiological changes controlled through complex genetic mechanisms, including hormonal and amino acid regulation, increased development of aerenchyma cells and adventitious roots, as well as antioxidant production against lipid peroxidation; these processes increase flood tolerance. Furthermore, we showed that exogenous ASC application can enhance plant tolerance of waterlogging stress.

Acknowledgments This work was carried out with the support of “Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ012286042017)” Rural Development Administration, Republic of Korea.

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