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Responses of *Lilium* hybrid 'Brindisi' to varying periods of waterlogging at vegetative stages

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Abstract

Lilium species with ornamental, edible, and medicinal values are distributed all over the world. Little is known about the responses of *Lilium* genotypes to waterlogging stress. *Lilium* hybrid 'Brindisi' was used to study the physiological responses of roots, bulbs, and leaves to 1, 2, 4, 8, and 13 d of waterlogging stress. Results showed that waterlogging stress seriously hindered the transport of nutrients from bulbs to stems and leaves. The physiological indicators could be divided into two categories. The first category was the physiological parameters indicating plant damage. The dry and fresh masses of stems and leaves, chlorophyll (Chl) *a*, Chl *b*, Chl *a*+*b*, and carotenoid content decreased, the dry and fresh masses of bulbs, malondialdehyde and H_2O_2 content increased under waterlogging stress. The other category was the physiological indicators that regulate the plant adaptability to waterlogging stress. Among them, superoxide dismutase and pyruvate decarboxylase activity changed little, proline content increased significantly, soluble sugar and protein content, and ascorbate peroxidase (APX), catalase (CAT), alcohol dehydrogenase, and lactate dehydrogenase activities increased in the early stage, and decreased in the later stage of waterlogging stress. The turning point of these physiological parameters was 4 - 8 d after waterlogging stress. Bulbs played an important role in alleviating flooding stress in the early stage of waterlogging. APX and CAT also played an important role in eliminating ROS in the early stage. This research lays foundation for the research on the mechanism of waterlogging tolerance and breeding of waterlogging-tolerant cultivars of *Lilium* spp.

Keywords: antioxidative enzymes, carotenoids, chlorophylls, *Lilium* spp., malondialdehyde, proline, waterlogging stress.

Introduction

Some environmental factors, such as abiotic stresses, have a serious impacts on crop yield and quality. Abiotic stresses include high or low temperature, drought, waterlogging, and salt-alkali stress. In recent years, waterlogging stress caused by rising groundwater levels, extreme climate, and unreasonable irrigation seriously threatened the yield and quality of crops (Muis *et al.* 2015). Long-term waterlogging stress has a negative impact on the entire growth cycle of plants, and may cause crop yield reduction. For example, waterlogging was the second major factor causing wheat yield loss, just behind the drought stress (Herzog *et al.* 2016). The Arkansas Rice Research and Extension Center simulated waterlogging to stress winter wheat, and resulted in the reduced yield by 34 % (Arguello *et al.* 2016). When cotton was subjected to waterlogging in different growth periods, the yield was reduced up to 63 % (Zhang *et al.* 2016). In addition to rice and cotton, many other plants such as forage legumes and peach were also affected by waterlogging (Striker and Colmer 2017, Penella *et al.* 2017). Therefore, researches

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Abbreviations: ABA - abscisic acid; ACO5 - 1-aminocyclopropane-1-carboxylic acid oxidase 5; ACS - 1-aminocyclopropane-1-carboxylic acid synthase; ADH - alcohol dehydrogenase; AMY3 - α-amylase 3; APX - ascorbate peroxidase; ARs - adventitious roots; BSA - bovine serum albumin; CAT - catalase; Chl - chlorophyll; GA - gibberellins; HRE2 - hypoxia response element 2; LDH - lactate dehydrogenase; LSD - least significant difference; MDA - malondialdehyde; PCA - principal components analysis; PDC - pyruvate decarboxylase; Pro - proline; ROS - reactive oxygen species; SK1 - SNORKEL1; SK2 - SNORKEL2; SOD - superoxide dismutase.
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on plant waterlogging tolerance have great significance for crop yield and quality safety.

The main reasons for the reduction of plant yield and quality caused by waterlogging are the low gas diffusion coefficient in water and the weak gas exchange capacity of plant tissues submerged in water, which may induce secondary stresses such as hypoxia or anaerobic stress (Pedersen *et al*. 2013). Waterlogging also has serious impact on plant photosynthesis, respiration, physiology, biochemistry, growth, as well as development of plants (Xu *et al*. 2006). In the long-term evolution processes, plants have formed a set of regulatory mechanisms at morphological, anatomical, physiological, and molecular levels to escape from waterlogging damage. Morphologically, plants can survive waterlogging through the formation of adventitious roots (ARs) to receive oxygen such as in *Arabidopsis* and *Solanum dulcamara* (Eysholdt-Derzsó and Sauter 2019, Yang *et al*. 2018). Physiologically, plants can adapt to waterlogging stress through phytohormones, antioxidant enzymes, and anaerobic respiration. Abscisic acid (ABA), gibberellins (GA), ethylene, and auxin are all involved in waterlogging or submergence responses (Xu *et al*. 2006, Eysholdt-Derzsó and Sauter 2017). Among these endogenous hormones, ethylene plays an important role and the accumulation of ethylene is a common phenomenon under waterlogging conditions (Hattori *et al*. 2009, Zhao *et al*. 2018). In addition, plants obtain their essential energy through glycolysis and ethanol fermentation when coping with the energy shortage, thus the pyruvate decarboxylase (PDC), lactate dehydrogenase (LDH), and alcohol dehydrogenase (ADH) activities increase significantly (Xu *et al*. 2014). Besides, the insufficient energy because of the shortage of oxygen leads to a burst of reactive oxygen species (ROS), which results in plant cell damage, increasing trend in lipid peroxidation and methylglyoxal content (Anee *et al*. 2019). ROS can be eliminated by antioxidant enzymes (Ahmed *et al*. 2002). In terms of molecular regulation, ethylene metabolism and anaerobic respiration are critical in the regulation of plants in response to waterlogging stress. Waterlogging activates the expression of *ACO5* and *ACS* genes (encoding 1-aminocyclopropane-1-carboxylic acid oxidase 5 and 1-aminocyclopropane-1-carboxylic acid synthase) directly or indirectly, which ultimately leads to the biosynthesis of ethylene and then promotes the expressions of the transcription factors such as *HRE2*, *SK1* and *SK2* (the group VII ethylene response factors - hypoxia response element 2, SNORKEL1, and SNORKEL2) (White *et al*. 2017, Schmidt *et al*. 2018). Among these transcription factors, overexpression of *HRE2* can significantly enhance the density of adventitious roots (Eysholdt-Derzsó and Sauter 2019), overexpression of *SK1* and *SK2* improve the submergence tolerance capability of rice (Hattori *et al*. 2009). The expression of *AMY3,* which encodes synthesis of α-amylase, is upregulated and finally mediates saccharide catabolism under hypoxic conditions (Park *et al*. 2010). The expressions of *PDC* and *ADH* genes in cotton are up-regulated under waterlogging conditions (Zhang *et al*. 2015). In summary, previous studies were mainly focused on crops such as sesame (Anee *et al*. 2019), vegetables or fruits such as *Solanum* species (Hartman *et al*. 2020), cucumber (Xu *et al*. 2014), citrus (Arbona *et al*. 2008) and peach (Penella *et al*. 2017) or herbaceous flowers such as chrysanthemum (Zhao *et al*. 2018) and petunia (Yin *et al*. 2019). There are few reports on the physiological responses of rhizome and bulb plants in response to waterlogging stress.

Lilium spp., a world-famous monocot bulbous plants with high ornamental, edible, and medicinal value, has been developed rapidly in the past 30 years (Chen *et al*. 2020). Until now, more than 10 000 cultivars are registered on international lily register website and China is one of the main consumer markets of ornamental *Lilium* spp. With the development of urban landscaping industry and home gardening, lily plants are gradually being planted in the open air which is more susceptible to the rainy weather. Therefore, it is particularly important to understand the physiological mechanism of lilies under waterlogging stress as well as breeding waterlogging-tolerant lily cultivars.

In this paper, *Lilium* hybrid 'Brindisi' with rather strong waterlogging tolerance was selected as the experimental material to study the physiological response of leaves, roots, and bulbs to different waterlogging periods. Researches on the physiological responses of *Lilium* spp. to waterlogging stress provides references on waterlogging tolerance mechanism, resistant germplasm evaluation and resistant breeding of bulbous plants.

Materials and methods

Plants and treatments: *Lilium* hybrid 'Brindisi', a bulbous plant with pink flower, was used as experimental material. Healthy bulbs with a girth diameter of 14 - 16 cm were selected and sterilized with 0.1 % (m/v) potassium permanganate for 10 min, then buried in peat and transferred to 2 - 6 °C cold storage for 45 d of vernalization. The vernalized bulbs were disinfected with 0.1 % potassium permanganate for 10 min again, and then planted in plastic pots (10 × 15 cm) with peat as the substrate. They were cultivated in the greenhouse of Shanghai Academy of Agricultural Sciences at Qingpu Base, Shanghai, China (31°14'N, 121°05'E) with 18/26 °C (min/max) temperatures at a 14-h photoperiod.

Waterlogging treatment was given by placing pots with 50 d old plants in large plastic vessels measuring 610 × 420 × 300 cm, filled with water to a height at least 2.0 cm above the level of the soil surface, whereas the control plants remained well-watered (60 % soil moisture) throughout the experiment. Treatments consisted of 1, 2, 4, 8, and 13 d of waterlogging with parallel control treatment for the same time. The experiment was carried out with three replications and each treatment involved 9 plants. At the end of each waterlogging treatment, roots, bulbs, and leaves were quickly frozen in liquid nitrogen for 2 min, and then stored at -80 °C.

Biomass measurements: All bulbs, stems, and leaves of each plant under waterlogging for 0 d (control), 13 d

(normal growth) and 13 d (waterlogging treatment) were sampled and their fresh masses (f.m.) were measured. Then they were dried in an oven at 60 °C until the mass did not decrease, which was determined dry masses (d.m.). The biomass changes were calculated as f.m. or d.m. at 13 d minus f.m. or d.m. at 0 d, respectively.

Pigment content measurement: The pigment content of leaves was detected according to Arnon *et al*. (1949). Samples of 0.5 g (9 samples of each treatment and 3 samples were mixed) were extracted with 80 % (v/v) acetone solution and then centrifuged at 12 000 *g* for 10 min. The supernatant was transferred into a microplate, and the absorbance was measured with *Multiskan Sky* spectrometer (*Thermo Scientific*, Finland). Three different wavelengths (663, 645, and 480 nm) were selected for measuring Chl *a*, Chl *b*, and carotenoid content.

Soluble sugar content measurement: Samples (0.1 g) were homogenized with 0.8 cm^3 of 80 % (v/v) ethanol in an ice bath, and the volume was adjusted to 1.5 cm^3. The extract was then heated in a water bath at 50 °C for 20 min, then centrifuged at 12 000 *g* for 10 min at room temperature. The supernatant was transferred to a microplate, and the absorbance was measured with *Multiskan Sky* at 620 nm. The soluble sugar content was calculated according to the standard curve of sucrose (Wei *et al*. 2014).

Protein content measurement: Samples (0.1 g) were homogenized with 0.1 M phosphate buffer in an ice bath. The extract was then centrifuged at 12 000 *g* and 4 °C for 10 min. The supernatant was transferred to a microplate and incubated at 37 °C for 30 min. The absorbance was measured with *Multiskan Sky* at 562 nm and the protein content was calculated according to the standard curve of bovine serum albumin (Samadi *et al*. 2020).

Proline content measurement: Proline content in roots, bulbs, and leaves were measured according to Bates *et al*. (1973). Samples (0.1 g) with 1 cm^3 of 3 % (m/v) sulfosalicylic acid solution was heated in a water bath at 90 °C for 10 min. The extract was then centrifuged at 12 000 *g* and 25 °C for 10 min. Then, 150 mm^3 of supernatant was taken, added 150 mm^3 glacial acetic acid and 150 mm^3 acidic ninhydrin solution, and heated in a water bath at 95 °C for 30 min. After cooling, 400 mm^3 of formaldehyde was added, then the mixture were shook for 1 min, and let them stand for 5 min. The mixture was transferred into a microplate, and the absorbance was measured with *Multiskan Sky* at 520 nm. The proline content was calculated according to the standard curve of 3 % sulfosalicylic acid (Bates *et al*. 1973).

Malondialdehyde (MDA) and H_2O_2 content measurement: MDA content in roots, bulbs, and leaves was measured according to Heath and Packer (1968). Samples (0.1 g) with 1 cm^3 of 0.05 M pre-cooled phosphate buffer (pH 7.8) was taken and homogenized in an ice bath. The extract was then centrifuged at 12 000 *g* and 4 °C for 10 min. 300 mm^3 of 0.5 % thiobarbituric acid solution was added in the 200 mm^3 supernatant, and kept in a water bath at 90 - 95 °C for 30 min. The solution was centrifuged at 12 000 g and 25 °C for 10 min, then transferred the supernatant into a microplate, and the absorbance was measured with *Multiskan Sky* at 532 and 600 nm.

For determination of H_2O_2 content, samples (0.1 g) with 1 cm^3 of acetone was taken and homogenized in an ice bath. The extract was then centrifuged at 12 000 *g* and 4 °C for 10 min. 250 mm^3 of supernatant was taken, added 25 mm^3 of 5 % (m/v) titanium sulfate and 50 mm^3 of concentrated ammonia, and mixed well. The mixed solution was centrifuged at 12 000 *g* and 25 °C for 10 min, the supernatant was discarded, and the precipitate was retained. 230 mm^3 of 2 M sulfuric acid was added to dissolve the precipitate, and the dissolving solution was transferred into the microplate. The absorbance was measured with *Multiskan Sky* at 410 nm and the H_2O_2 content was calculated according to the standard curve (Gutteridge and Halliwell 1990).

Detection of antioxidant enzyme and anaerobic respiratory enzyme activities: The activities of APX, CAT, SOD, PDC, ADH, and LDH were detected by kits purchased from *Suzhou Grace Biotechnology Co.* (Suzhou, China). The extraction and chromogenic reaction of enzyme solution were carried out according to the kit instructions. The absorbance values of APX, CAT, SOD, PDC, ADH and LDH were measured by *Multiskan Sky* at 290, 510, 450, 340, 340, and 340 nm, respectively (Jia *et al*. 2022).

Statistical analyses: Statistical analysis was conducted by *Excel 2010* and *SPSS 17.0* software. The data were expressed as means ± standard errors (SEs). In order to assess the statistical significance of the differences among sampling stages and between treatment and control data, a one-way analysis of variance (*ANOVA*) followed by the least significant difference (LSD) test when the *F*-test were done Principal component analysis (PCA) was carried out to display the variable distribution, the samples distribution, and the correlation analysis of physiological indicators under waterlogging stress using *R package* models (http://www.r-project.org/).

Results

After waterlogging stress, the root tips of hybrid 'Brindisi' began to rot and showed an empty tube after 2 d. Then the base of the bulb began to rot and the scales fell off after 4 d. The tip of the leaves began to turn yellow after 8 d (Fig. 1 Suppl.). Generally, the masses of stems and leaves were gradually increased but the mass of bulbs was gradually decreased during the vegetative growth of *Lilium* spp. In our research, the fresh and dry masses of leaves, stem and bulb were measured at 0 and 13 d under normal conditions or waterlogging treatments. Results showed that the fresh and dry masses of normally growing leaves were increased by 4.22 and 0.62 g, respectively;

Table 1. Biomass changes of *Lilium* hybrid 'Brindisi' under waterlogging for 13 d. Means ± SDs were calculated from three replicates for each treatment (* - $P \leq 0.05$; **- $P \leq 0.01$).

Tissue	Treatment	Fresh mass	Change of fresh mass	Dry mass	Change of dry mass	DM/FM [%]	Water [%]
Leaves	control	24.91±0.87	4.22±0.87	2.80±0.02	0.62±0.02	11.26±0.33	88.74±0.33
	waterlogging	13.52±0.63	-7.18±0.63	1.97±0.19	-0.21±0.0.19	14.58±0.94	85.42±0.94
	P-value	9.6536e-05**	9.6536e-05**	0.0169*	0.0169*	0.0167*	0.0167*
Stem	control	45.20±1.93	21.62±1.93	5.57±0.22	3.10±0.22	12.34±0.51	87.66±0.51
	waterlogging	33.89±2.00	10.31±2.00	4.35±0.18	1.87±0.18	12.84±0.31	87.17±0.31
	P-value	0.0021**	0.0021**	0.0018**	0.0018**	0.2352	0.2352
Bulb	control	12.11±0.11	-11.30±0.11	2.50±0.36	-2.19±0.36	20.61±2.76	79.39±2.76
	waterlogging	26.27±1.01	2.86±1.01	4.57±0.25	-0.11±0.25	17.41±0.87	82.59±0.87
	P-value	0.0015**	0.0015**	0.0019**	0.0019**	0.1745	0.1745

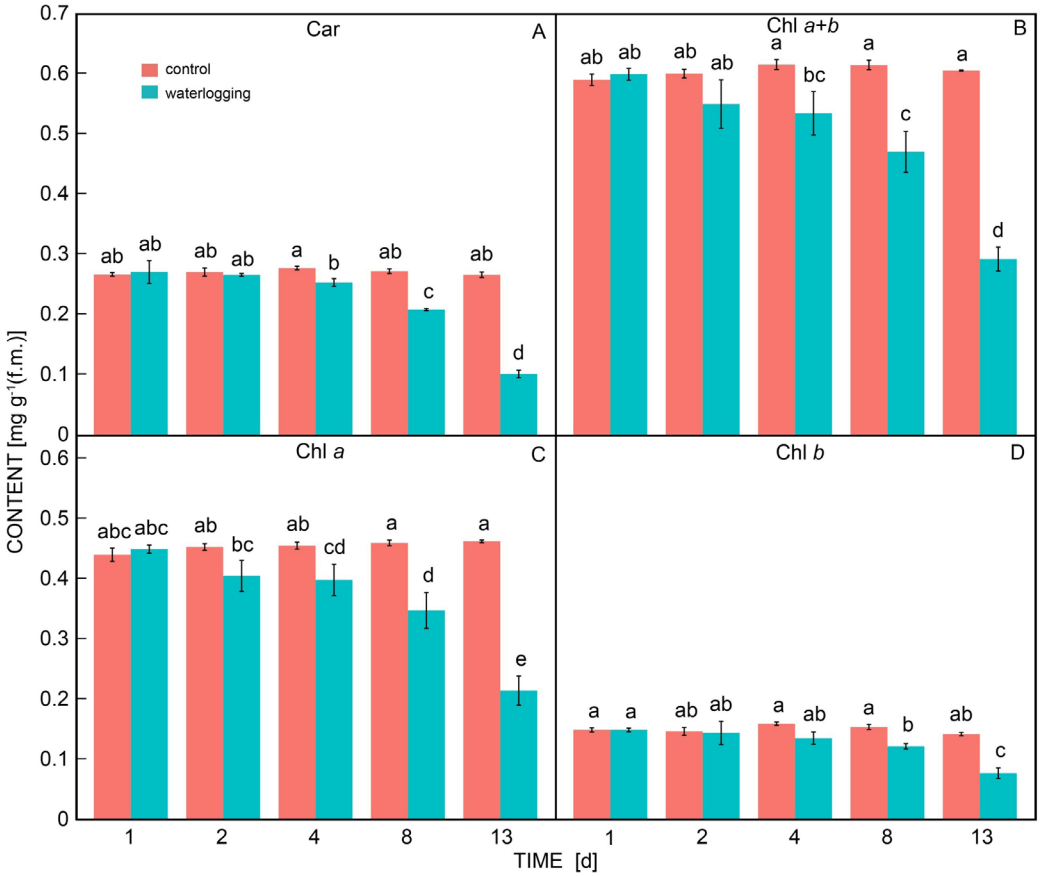

Fig. 1. Content of carotenoids (*A*), Chl *a*+*b* (*B*), Chl *a* (*C*), and Chl *b* (*D*) in leaves under different waterlogging duration. Means ± SDs were calculated from three replicates for each treatment. The significant differences were indicated by different letters ($P \leq 0.05$).

the fresh and dry masses of normally growing stem were increased by 21.62 and 3.10 g, respectively; the fresh and dry masses of the normally growing bulb were decreased by 11.30 and 2.19 g, respectively. Compared with 0 d, the dry and fresh masses of waterlogging leaves were decreased by 7.18 and 0.21 g, respectively, but the dry and fresh masses of waterlogging stem increased by 10.31 and 1.87 g, respectively. For waterlogging bulb, the fresh mass was significantly increased by 2.86 g but the dry mass was slightly decreased by 0.11 g (Table 1). We also compared the 13 d of waterlogging plants with contemporaneous control, the fresh and dry masses of leaves and stems were significantly decreased, but the fresh and dry masses of bulb were significantly increased. The dry-to-fresh biomass ratio in waterlogging leaves at 13 d was significantly higher than the contemporaneous control, and there was no significant differences in the bulb and stem. The water content in waterlogging-treated leaves at 13 d

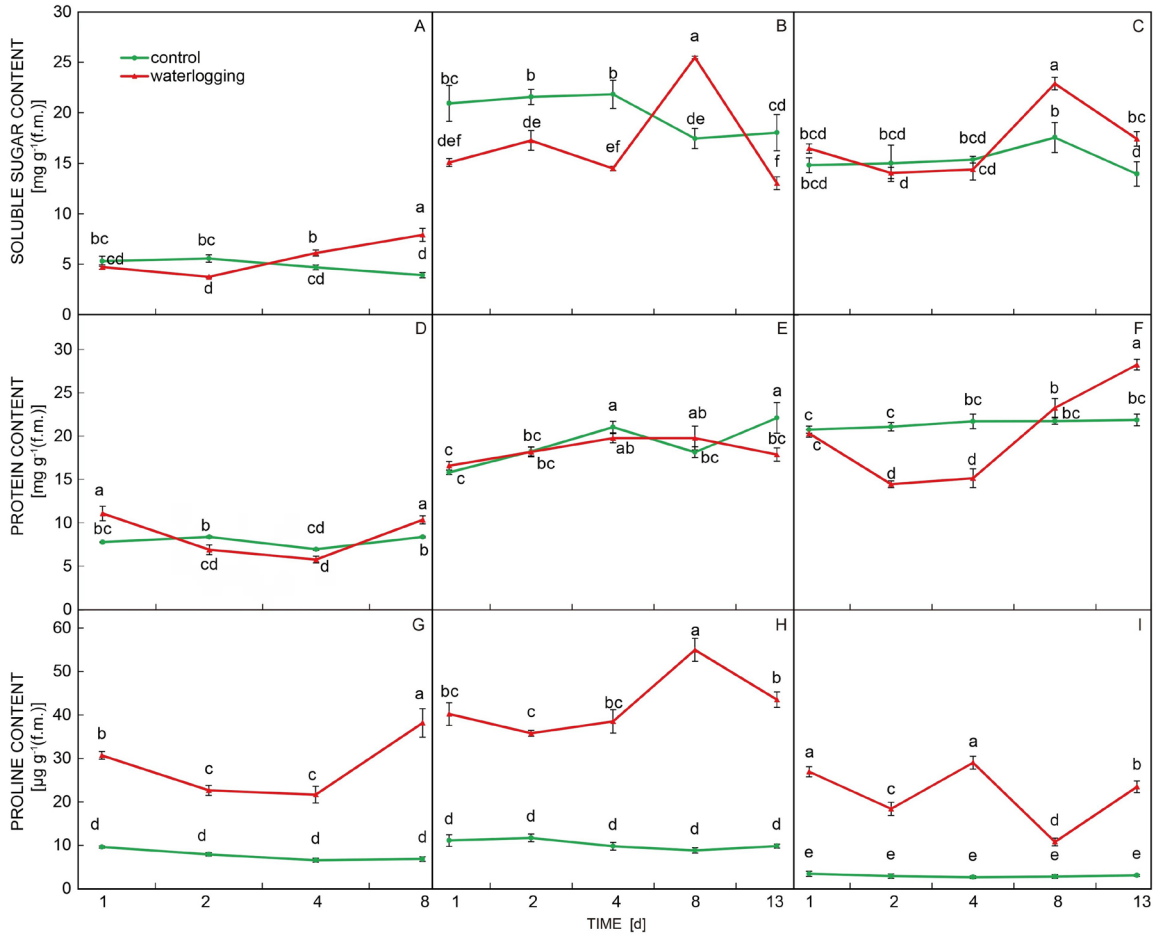

Fig. 2. Changes of soluble sugar content in roots (*A*), bulbs (*B*), and leaves (*C*) under different waterlogging duration. Changes of soluble protein content in roots (*D*), bulbs (*E*), and leaves (*F*) under different waterlogging duration. Changes of proline content in roots (*G*), bulbs (*H*), and leaves (*I*) under different waterlogging duration. Means ± SDs, $n = 3$; significant differences were indicated by different letters ($P \leq 0.05$).

was significantly lower than the contemporaneous control, and there was no significant differences in bulb and stem (Table 1).

The content of Chl *a*, Chl *b*, Chl *a*+*b*, and carotenoids in leaves gradually decreased with the duration of treatment and reached the lowest value at 13 d (Fig. 1). The content of carotenoid, Chl *a* and Chl *a*+*b* which were measured after 1 and 2 d had no significant differences compared with the contemporaneous control, but they were significantly decreased at 4, 8, and 13 d. Specifically, after 4, 8, and 13 d waterlogging treatment, the content of Chl *a* decreased by 12.47, 24.36 and 53.58 %, respectively; the content of Chl *a*+*b* decreased by 13.14, 23.46, and 51.69 %, respectively; and the content of carotenoids decreased by 8.61, 23.42, and 61.80 %, respectively, compared with 0 d. The content of Chl *b* significantly decreased by 20.75 and 45.53 %, respectively, at 8 and 13 d of waterlogging treatment.

In roots, the soluble sugar content did not change significantly after 1 d treatment, while it was significantly lower than the control by 32.72 % after 2 d of waterlogging. However, the soluble sugar content was significantly increased by 30.29 % at 4 d and 102.03 % at 8 d, respectively. The soluble sugar content in bulbs was reduced by 27.93 % (1 d), 19.99 % (2 d), and 33.65 % (4 d), respectively, compared with control, while it was significantly increased by 46.01 % at 8 d. At 13 d, the soluble sugar content decreased by 27.74 %. In leaves, the soluble sugar content did not change significantly at 1, 2, and 4 d, while it was significantly increased by 30.42 % (8 d) and 24.87 % (13 d), respectively. Totally, the soluble sugar content in bulbs was the highest, and in roots was the lowest. The soluble sugars in bulbs were sensitive to waterlogging stress (Fig. 2).

The protein content in leaves, roots, and bulbs under different waterlogging times was also determined (Fig. 2). The soluble protein content in roots declined at 1, 2, 4 d and started to rise at 8 d, while it declined at 1 and 2 d and started to rise at 4, 8, and 13 d in leaves. In bulbs, the content of soluble protein increased at 1, 2, 4 d and decreased at 8 and 13 d. In roots, compared with control, the soluble protein content significantly increased by 42.55 % after 1 d treatment, while it reduced at 2 and 4 d. After 8 d treatment, it increased significantly by 23.67 % compared to the control. In leaves, the protein content decreased significantly by 31.37 % (2 d) and 30.17 % (4 d), respectively, then increased significantly by 29.07 %

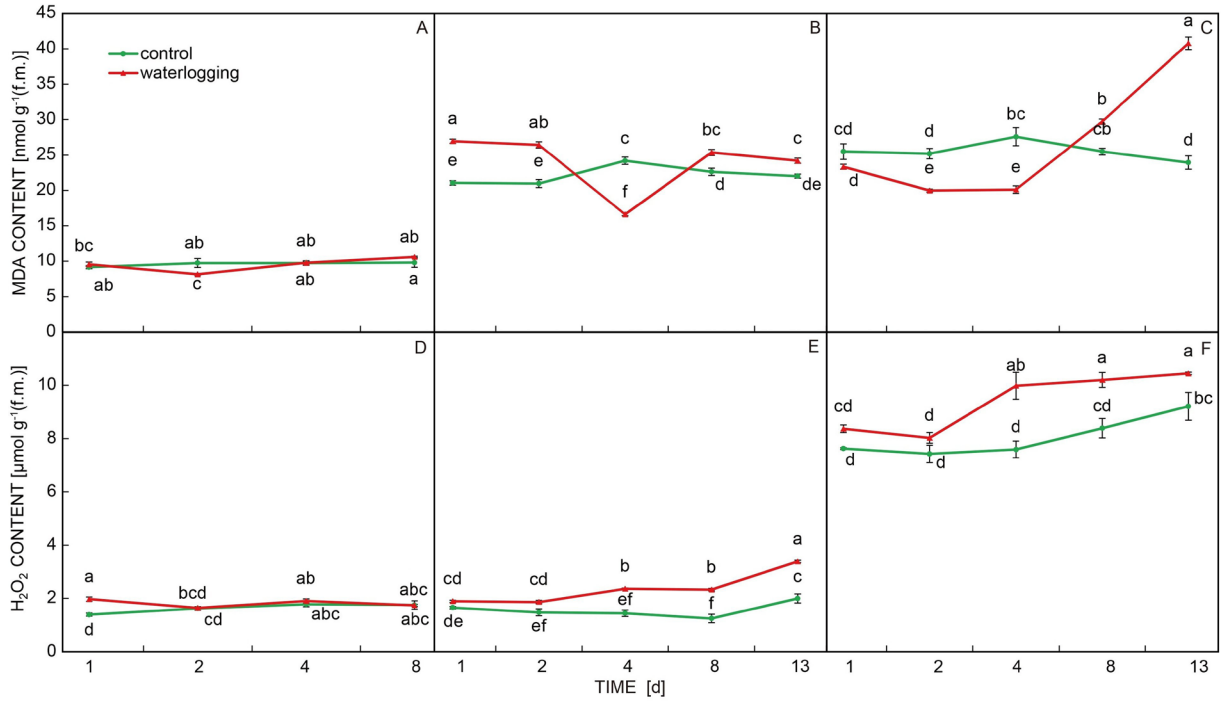

Fig. 3. Changes of MDA content in roots (*A*), bulbs (*B*), and leaves (*C*) and changes of H_2O_2 content in roots (*D*), bulbs (*E*), and leaves (*F*) under different waterlogging duration. Means ± SDs, $n = 3$; significant differences were indicated by different letters ($P \leq 0.05$).

at 13 d. The content of soluble protein in bulbs increased by 8.96 % at 8 d compared to control, but decreased significantly by 19.09 % at 13 d. Totally, the soluble protein content in bulbs and leaves was higher than in roots. Affected by waterlogging stress, the soluble protein content in bulbs had less change.

The results showed that the proline content in bulbs, leaves, and roots increased significantly after waterlogging treatment (Fig. 2). In roots, the proline content increased by 219.65 % (1 d), 186.13 % (2 d), 229.48 % (4 d), and 457.83 % (8 d), respectively, compared with the control. In bulbs, it increased by 262.21 % (1 d), 205.73 % (2 d), 293.50 % (4 d), 523.92 % (8 d), and 343.70 % (13 d), respectively, compared with the control. In leaves, it increased by 683.35 % (1 d), 530.69 % (2 d), 992.55 % (4 d), 282.58 % (8 d), and 664.48 % (13 d), respectively, compared with the control. The proline content in bulbs was the highest.

MDA, the end product of lipid peroxidation, is frequently used as an indicator of lipid peroxidation. Compared to the control group, the MDA content in bulbs (Fig. 3*B*) increased significantly by 27.96 % (1 d) and 25.98 % (2 d), respectively, then decreased by 31.32 % at 4 d. At 8 and 13 d, it increased again by 12.07 and 9.93 %, respectively. In leaves (Fig. 3*C*), the MDA content decreased by 20.72 % (2 d) and 27.09 % (4 d), then increased by 16.61 % (8 d) and 70.35 % (13 d), respectively, compared to the control. Waterlogging treatment did not change the MDA content significantly in roots (Fig. 3*A*) except the 16.38 % reduction after 2 d treatment. After waterlogging stress, the MDA content in bulbs changed earlier.

Affected by the waterlogging stress, the H_2O_2 content in bulbs (Fig. 3*E*) and leaves (Fig. 3*F*) gradually increased. In bulbs, the H_2O_2 content increased by 26.37 % (2 d), 63.62 % (4 d), 86.77 % (8 d), 69.29 % (13 d), respectively. In leaves, H_2O_2 content increased by 31.54 % (4 d), 21.61 % (8 d), and 13.43 % (13 d). The H_2O_2 content slightly changed in roots (Fig. 3*D*), it only increased by 41.88 % at 4 d of waterlogging.

The activities of antioxidant enzyme in roots (Fig. 4*A*), bulbs (Fig. 4*B*), and leaves (Fig. 4*C*) under different waterlogging times were determined. In bulbs, they decreased significantly by 51.98 % (2 d), 65.47 % (8 d), and 53.24 % (13 d) and increased by 19.41 % (4 d) compared to their controls. In leaves and roots, the APX activity increased first and then decreased. In leaves, it increased by 33.02 % (1 d), 18.36 % (2 d), and 25.40 % (4 d) and decreased by 15.01 % (8 d) and 42.49 % (13 d). In roots, it increased by 20.38 % (2 d) and 17.98 % (4 d) and decreased by 26.88 % (8 d) compared to controls. Totally, the APX activity in bulbs decreased. The APX activity in leaves and roots increased at the initial stages of waterlogging stress and began to decrease later at 8 d.

Plants waterlogged for 1, 2, and 8 d showed significantly higher CAT activity compared to their controls in roots (Fig. 4*D*) by 153.18, 20.41, and 22.90 %, respectively. In bulbs (Fig. 4*E*), the CAT activity increased by 53.57 % (1 d), 121.04 % (2 d), 106.00 % (4 d), and 73.50 % (8 d), then decreased by 13.58 % when they were waterlogged for 13 d. In leaves (Fig. 4*F*), plants waterlogged for 1, 2, 4, 8, and 13 d showed significantly higher CAT activity compared to their controls by 14.36, 81.02, 21.32, 157.66 and 81.26 %.

Compared to control, the SOD activity in roots (Fig. 4*G*) remained unchanged after waterlogging for 1, 2,

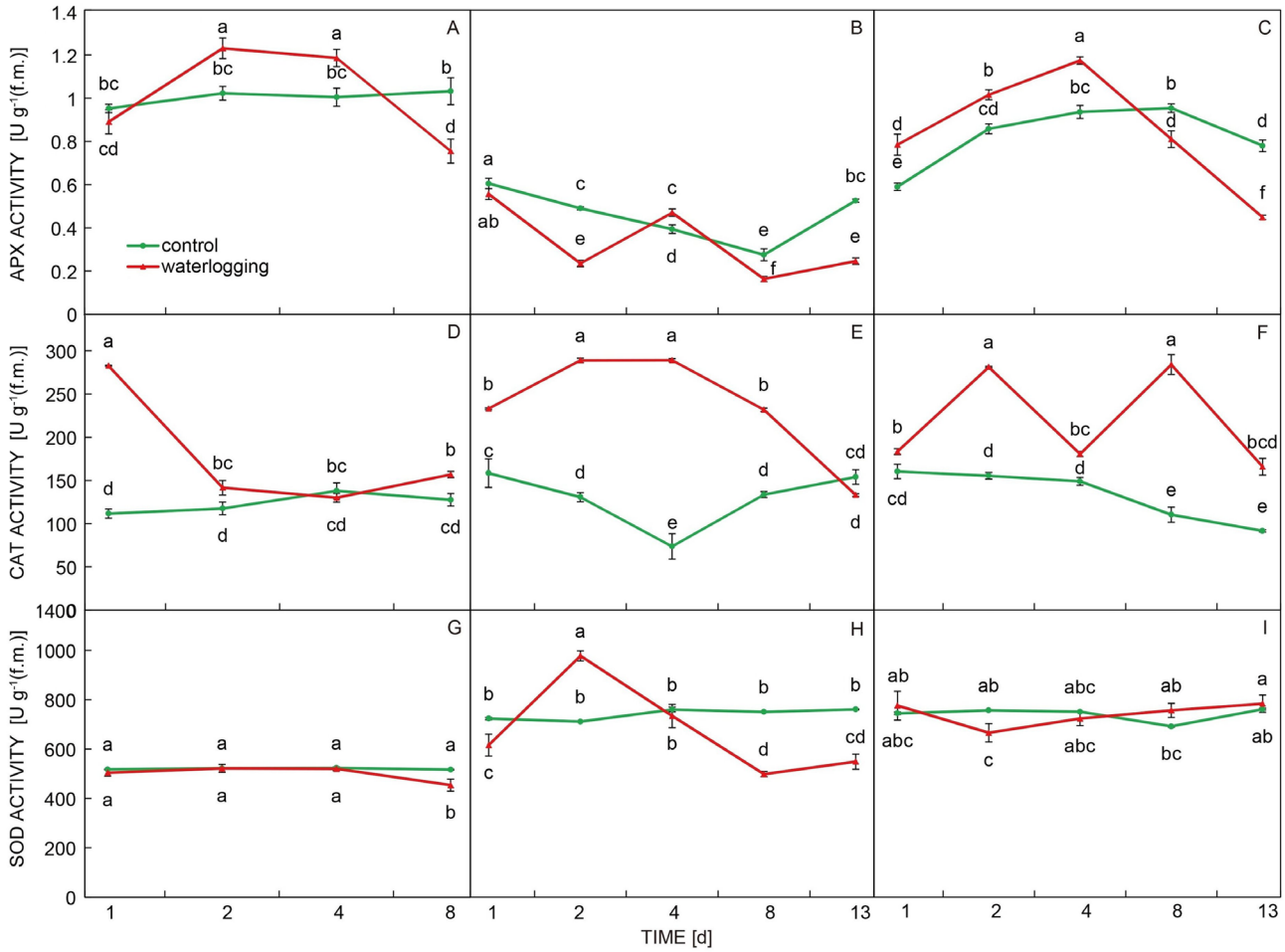

Fig. 4. Changes in APX activity in roots (*A*), bulbs (*B*), and leaves (*C*), CAT activity in roots (*D*), bulbs (*E*), and leaves (*F*), SOD activity in roots (*G*), bulbs (*H*), and leaves (*I*) under different waterlogging duration. Means ± SDs, $n = 3$; significant differences were indicated by different letters ($P \leq 0.05$) One unit (U) is defined as follows: APX: 1 μmol of AsA per gram of tissue oxidized per min at 25°C; CAT: 1 μmol of H_2O_2 per gram of tissue catalyzed decomposed per minute at 25°C; SOD: 50 % inhibition NBT reduction by xanthine-xanthine oxidase system.

and 4 d but it decreased at 8 d of waterlogging which was 12.15 % lower than control. In bulbs (Fig. 4*H*), the SOD activity decreased by 14.93 % (1 d), 33.61 % (8 d), and 27.82 % (13 d), but increased by 37.48 % when they were waterlogged for 2 d. The SOD activity in leaves (Fig. 4*I*) remained unchanged significantly after waterlogging for 1, 4, 8, and 13 d except for a reduction of 12.02 % for 2 d.

Waterlogging stress increased ADH activity by 220.85, 85.99, and 24.32 % in roots waterlogged for 1, 2 and 4 d, respectively, compared to their controls (Fig. 5*A*). Then it decreased significantly at 8 d when it was 40.64 % lower than control. In bulbs (Fig. 5*B*), the ADH enzyme activity was 19.42 % (2 d) and 18.45 % (8 d) lower compared to the control plants, and 297.67 % higher than the control plant after 13 d. In leaves (Fig. 5*C*), the ADH activity increased by 47.01 % (1 d) and 39.24 % (2 d), but decreased by 53.85 % (8 d) and 63.47 % (13 d), compared to their controls. The ADH activity in roots and leaves was sensitive to waterlogging stress.

The same trend was observed for LDH activity. Waterlogging stress increased LDH activity by 246.81 % (1 d), 107.04 % (2 d), and 50.54 % (4 d) in roots (Fig. 5*D*). Then it decreased significantly at 8 d which was 60.72 % lower than control. In bulbs (Fig. 5*E*), the LDH activity was 21.81 % (2 d), 9.46 % (4 d), and 37.42 % (8 d) lower compared to the control plants, and 229.20 % higher than the control after 13 d. In leaves (Fig. 5*F*), the LDH activity increased by 184.70 % (1 d) and 31.41 % (2 d), but decreased by 60.80 % (8 d) and 68.77 % (13 d), compared to their controls.

Upon exposure to waterlogging conditions, PDC activity in roots (Fig. 5*G*) increased by 8.25 % for 1 d, and decreased significantly by 31.72, 8.98, and 59.95 % after 2, 4, and 8 d. In bulbs (Fig. 5*H*), the PDC increased by 21.92, 30.30, and 24.98 %, respectively, for 2, 8, and 13 d. In leaves (Fig. 5*I*), reduced PDC activity was recorded when plants were waterlogged for 8 d (9 %).

Multivariate PCA analysis was conducted to identify potential combinations contributed to total variation in physiological indicators in response to waterlogging stress and a total of 11 principal components were identified. The first two principal components explained 42.1 and 18 %

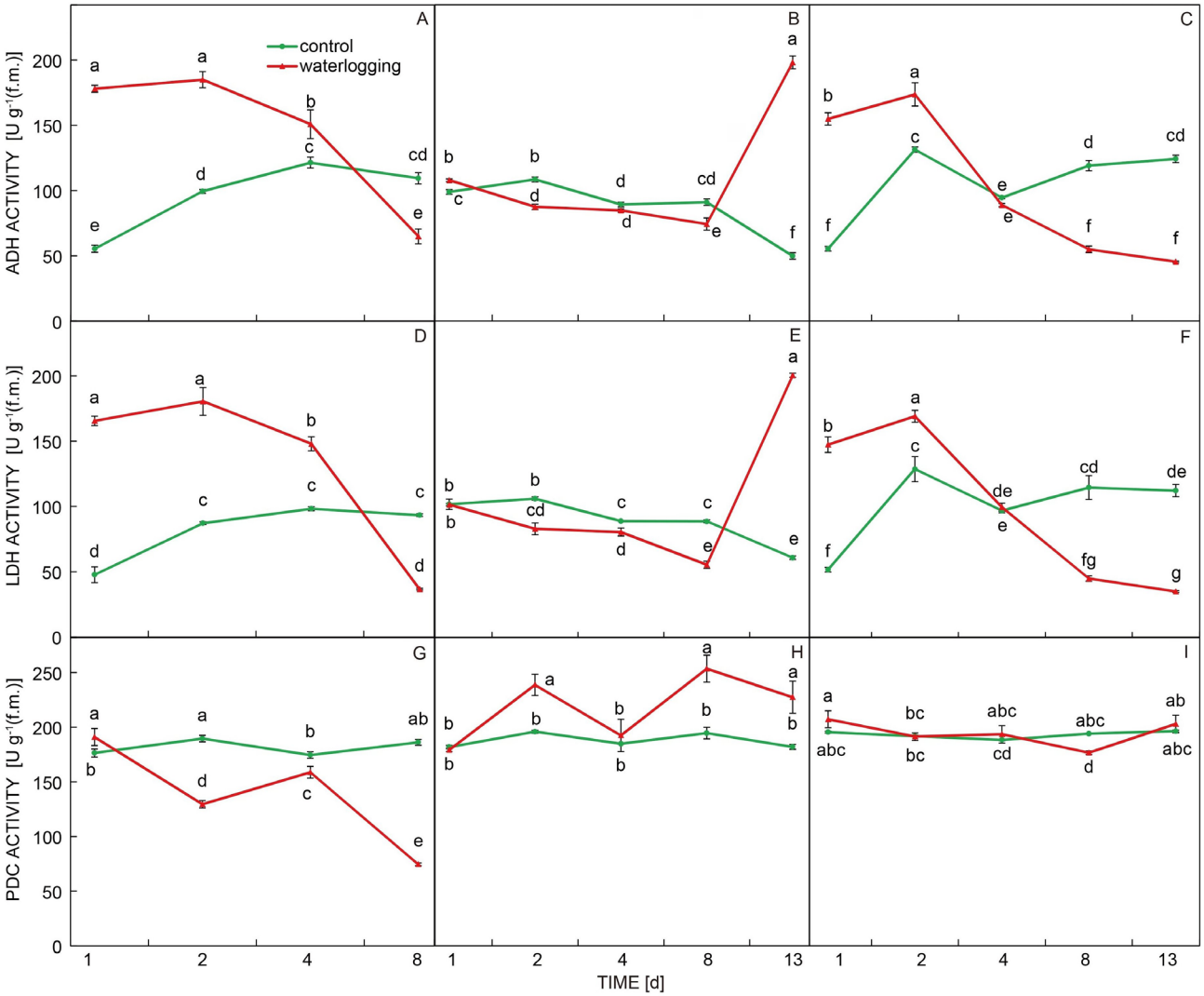

Fig. 5. Changes in ADH activity in roots (*A*), bulbs (*B*), and leaves (*C*), LDH activity in roots (*D*), bulbs (*E*), and leaves (*F*), and PDC activity in roots (*G*), bulbs (*H*), and leaves (*I*) under different waterlogging duration. Means ± SDs, $n = 3$; significant differences were indicated by different letters ($P \leq 0.05$. One unit (U) is defined as follows: ADH, LDH: 1 nmol of NADH per gram of tissue consumed per minute at 25°C; PDC: 1 nmol of NADH per gram of tissue oxidized per minute at 30°C.

of the total genetic variation. ADH, LDH, MDA, soluble sugar and total protein contributed significantly to the total variation (Fig. 2 Suppl.). The traits that contributed to the total variation in the first principal component were positively related to proline, CAT, PDC, ADH, LDH, MDA, soluble sugar, and negatively related to APX, H_2O_2, SOD as well as protein. The traits that contributed to the total variation in the second principal component were positively related to proline, CAT, PDC, MDA, SOD, H_2O_2, soluble sugar, protein, and negatively related to ADH, LDH, and APX. The PCA biplot results showed that all samples were spread in all four quadrants (Fig. 2 Suppl.). The samples of the control group were mainly concentrated in the third and fourth quadrants, and their distribution was relatively concentrated, while the samples of the waterlogging stress treatment group were scattered in the four quadrants with great changes. Fig. 2 Suppl. showed the high correlations among the eleven physiological indicators in response to waterlogging stress. Soluble sugar, soluble protein, and MDA had higher correlations with other physiological indexes, while CAT and proline had lower correlations with other physiological indexes. ADH, the anaerobic respiration enzyme, had the highest correlation (0.97) with LDH, but the correlation among PDC, ADH, and LDH was extremely low. Hydrogen peroxide was significantly related to MDA, indicating that the content of H_2O_2 was closely related to the degree of membrane lipid peroxidation.

Discussion

Waterlogging stress has a serious impact on *Lilium* hybrid 'Brindisi' growth and development, photosynthesis, osmosis, cell membrane, antioxidants, and energy supply. Stomata closure, transpiration reduction, as well

as photosynthesis inhibition are common responses that can occur in hours or days, depending on the tolerance of different species and cultivars to waterlogging stresses. Our results showed that waterlogging stress inhibited plant growth and development of *Lilium* hybrid 'Brindisi' and resulted in a significant reduction of the dry and fresh masses of stems and leaves which was consistent with the results in many plants such as watermelon (Yetisir *et al*. 2006) and alfalfa (Castonguay *et al*. 1993). However, we found that the dry and fresh masses of the waterlogged bulbs were significantly higher than the contemporaneous control. Plant photosynthetic parts of the plant were active only over a specific period of the year, thus plant utilized its unique underground organ to provide storage of energy (Lazare *et al.* 2018). We speculated that the transport of nutrients from bulb to shoot was inhibited under waterlogging stress, which might result in the higher bulb mass. In addition, the content of carotenoids, Chl *a*, Chl *b*, and Chl *a*+*b* in leaves were significantly lower than in the control. It showed that the pigment content in the leaves gradually decreased under continuous waterlogging stress, which was consistent with the results done on sesame (Wei *et al*. 2013) and cucumber (Xu *et al*. 2014).

Proline plays an important role in maintaining the metabolism and growth of plants under abiotic stress conditions, many results indicate a positive relationship between proline accumulation and tolerance of plants to various abiotic stresses (Ghosh *et al*. 2022). In our study, the proline content of roots, bulbs, and leaves increased significantly at the beginning of the waterlogging stress at 1 d. Wang *et al*. (2017) reported that the NaCl, mannitol, and ABA treatments significantly increases proline accumulation in leaves of *Lilium* cv. Sorbonne after 12 h stress treatment with values 3.39-, 1.81-, and 1.34-times higher than the control. Our results also indicated that flooding stress can promote the accumulation of a large amount of proline in lily plants in a short time.

MDA, the main product of membrane lipid peroxidation, is an important indicator to measure the degree of cell membrane damage. In our study, the MDA content showed a downward trend in the first 4 d, then gradually increased at 8 d, indicating that hybrid 'Brindisi' can reduce cytotoxicity and cell membrane damage through self-regulation in the first 4 d before serious cell membrane damage. The MDA content of cotton, sesame, and citrus was significantly higher than that of the control after waterlogging stress (Arbona *et al*. 2008, Zhang *et al*. 2016, Anee *et al*. 2019). Our results were different from these plants, probably because the bulbs had a stress-buffering effect in the first days of waterlogging.

Accumulation of ROS often occur under waterlogging stress (Blokhina *et al*. 2003). In this study, H_2O_2 content in lily bulbs and leaves gradually increased with the prolonged waterlogging time, which was consistent with previous studies done on cotton leaves and sesame seeds (Zhang *et al*. 2016, Anee *et al*. 2019), but the accumulation rate of H_2O_2 content was different because of the waterlogging tolerance of different species.

In order to reduce the damage of ROS, plants have evolved corresponding scavenging systems including antioxidant enzymes and antioxidant compounds (Lin *et al*. 2006). It has been reported that high activities of SOD, CAT, and APX play important roles in the waterlogging tolerance of mungbean (Ahmed *et al*. 2002), sunflower (Grassini *et al*. 2007), and wheat (Li *et al*. 2011). Yordanova *et al*. (2004) reported that APX and CAT activities increased, and SOD activity decreased under waterlogging stress in barley. APX and CAT activities were higher in strong waterlogging tolerant cultivars than in sensitive ones in citrus and seasame (Arbona *et al*. 2008, Wei *et al*. 2013). Our study showed that the activities of APX and CAT were higher in the early stages of waterlogging stress and lower in the later stage, which indicated that antioxidant enzyme played an important role in the early phases of waterlogging, but antioxidant enzymes had limited elimination ability, and their activity would decrease when oxidative stress exceeded their elimination ability.

The deprivation of plant oxygen changed the energy supply mode from aerobic respiration to anaerobic respiration under waterlogging stress (Ismail *et al*. 2009). Plants metabolize sugars and sucrose hydrolase activity played an important role in improving plant tolerance to waterlogging stress (Sairam *et al*. 2009). Therefore, sucrose synthase was up-regulated in plants under waterlogging stress, resulted in the increase of soluble sugar content more in the the tolerant genotypes than that in the sensitive genotypes. The same results had been reported in mung bean (Sairam *et al*. 2009), maize (Zeng *et al*. 1999), alfalfa (Castonguay *et al*. 1993, Zeng *et al*. 2019), and wheat (Albrecht *et al*. 2004, Huang and Johnson, 1995). Lily bulb scales contained a large amount of sugars, which were the main source of energy supply. The soluble sugar content decreased rapidly in lily during the 1 to 4 d of waterlogging stress, indicating that the soluble sugar was consumed to maintain the life activities under low oxygen or anaerobic conditions. The soluble sugar content in bulbs, roots, and leaves increased significantly on the 8th day of waterlogging stress, indicating that the process of sugar metabolism was initiated to maintain the supply of soluble sugar for anaerobic respiration.

Anaerobic respiration, such as glycolysis and fermentation (Ismail *et al*. 2009), was initiated under waterlogging stress and induced large amounts of anaerobic proteins (ANPs) including enzymes PDC, ADH, and LDH. Glycolysis generated pyruvate from glucose or glycogen, which was catalyzed by PDC to form acetaldehyde, and ADH further converted acetaldehyde into ethanol and produces NAD^+ (Ismond *et al*. 2003). The transcriptome and proteome analysis results of alfalfa (Zeng *et al*. 2019) and barley (Borrego-Benjumea *et al*. 2020) revealed that many anaerobic respiration related genes were up-regulated, and the activity of anaerobic respiration enzymes also increased under waterlogging stress. The ADH and PDC activities in sesame roots increased in the first 6 d of waterlogging stress and gradually decreased after 6 d, the LDH activity in sesame roots increased in the first 4 d of waterlogging stress and then decreased (Wei *et al*. 2013). Our results were mostly consistent with the results in sesame. But ADH and LDH activities in bulbs

changed later than those in roots and leaves, which may be due to the fact that bulbs required less energy than roots and leaves. The inconsistent trends of PDC activity in roots, bulbs, and leaves may be caused by the different sensitivity of different tissues to waterlogging stress.

Conclusions

Waterlogging stress seriously hindered the transport of nutrients from bulbs to stems and leaves. Under waterlogging stress, the dry and fresh masses of stems and leaves, Chl *a*, Chl *b*, Chl *a*+*b*, and carotenoid content in leaves decreased, while the dry and fresh masses of bulbs, MDA and H_2O_2 content increased. Proline content increased significantly, soluble sugar, protein, APX, CAT, ADH, and LDH activities increased in the early stage, and decreased in the later stage of waterlogging stress. The turning point was 4 - 8 d after waterlogging. Bulbs played an important role in alleviating flooding stress in the early stage of waterlogging. APX and CAT can eliminate ROS in the early stage. This research lays foundation for the research on the mechanism of waterlogging tolerance of *Lilium* spp.

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