EFFECT OF WATER LEVEL FLUCTUATIONS ON THE ANTIOXIDANT ENZYME SYSTEM AND THE DEGREE OF CELL MEMBRANE PEROXIDATION IN *VALLISNERIA NATANS*

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Abstract. In this study, by setting two different water level environments, rising and falling, we investigated the characteristics of changes in the antioxidant enzyme system and the degree of cell membrane peroxidation of *Vallisneria natans* under different intensities of water level fluctuations. The results showed that (1) Peroxidase (POD) and Superoxide dismutase (SOD) showed a trend of increasing first and then decreasing under the rising water level and a multi-peak decreasing trend under the falling water level. Catalase (CAT) generally showed a trend of increasing first and then decreasing during the change in water level. The activity of POD and SOD were higher in the water level with low fluctuation intensity, and CAT was higher in the water level with high fluctuation intensity. (2) Malondialdehyde (MDA) and the production rate of superoxide anion (O_2^-) showed a trend of rising first and then decreasing under the rising water level and a multi-peak decreasing trend under the falling water level. Overall, fluctuating water levels can produce growth stresses on *V. natans*, and in response to these stresses, the antioxidant enzyme system of *V. natans* produces a series of antioxidant protection mechanisms to maintain normal plant growth.
Keywords: *submerged plants, malondialdehyde, superoxide dismutase, water level changes, peroxidase*

Introduction

Submerged plants refer to the plant group whose whole plant body is submerged underwater (De Backer et al., 2012). They belong to large herbaceous plants and are the basis for water to maintain the biodiversity of the ecosystem (Zeng et al., 2017). As the main primary producers in lake ecosystems, submerged macrophytes play an important role in maintaining the stability of aquatic ecosystem structure and function by increasing spatial ecological niches (Nakamura et al., 2008), suppressing biotic and abiotic suspended matter, improving underwater light and dissolved oxygen conditions, and providing food (Irfanullah et al., 2004), shelter and other necessary conditions for other aquatic organisms (Havens et al., 2004). Periodic seasonal water level changes can cause changes in water level, light intensity, and other factors that can affect the ecological suitability of submerged macrophytes, thus affecting the spatial and temporal distribution of submerged macrophytes (Voesenek et al., 2016; Sasidharan et al., 2018), On the other hand, while non-periodic water level changes can destroy the long-term adaptation of aquatic vegetation to periodic changes in water level. The high water level in spring leads

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to insufficient underwater light, which is not conducive to the sprouting and seedling growth of submerged macrophytes, and low water level in summer leads to the exposure of aquatic plants, causing the crazy growth or decay of submerged macrophytes (Voesenek et al., 2004; Deegan et al., 2007). Therefore, water level fluctuations can produce stresses on submerged plants, and in response to these stresses, the antioxidant enzyme system of submerged plants generates a series of protective mechanisms as a way to improve plant tolerance and reduce damage to plants from water level fluctuations (Xin et al., 2018).

Vallisneria natans is a perennial submerged plant belonging to Hydrocharitaceae (Gao et al., 2020), which is widely distributed and has the characteristics of fast growth and strong regeneration (Xu et al., 2016). At the same time, *V. natans* plays an important role in maintaining and stabilizing the balance of the freshwater ecosystem, such as purifying water quality and providing food and habitat for aquatic organisms (Cao et al., 2016). *V. natans* is one of the dominant species of submerged macrophytes in the Poyang Lake wetland, and its growth, development, and distribution are closely related to the hydrological situation of Poyang Lake.

In this study, by setting two different water level environments, rising and falling, and simulating the microscopic experiment of water level fluctuation in Poyang Lake indoors, we investigated the effect of different water level fluctuation intensity on the change characteristics of the antioxidant enzyme system of *V. natans*. Based on the results of the study, the intrinsic limiting factors of the growth and development of the submerged plant *V. natans* in Poyang Lake under the change of natural water level were explored, and the adaptive mechanism of *V. natans* to the fluctuation of water level was revealed, to determine the suitable water level conditions for the growth of *V. natans*.

Materials and methods

Test material

In this study, *V. natans*, a typical submerged plant with wide distribution, large area, and important ecological significance in the Poyang Lake wetland, was selected as the research object. The seedlings of *V. natans* were pre-cultured for two weeks in a double-pot method (glass pots were placed in glass barrels with a height of 60-150 cm, an upper diameter of 57 cm, and a lower diameter of 45 cm) in March 2021 after winter bud propagation, and the water level was 50 cm. Each glass bucket has a small hole 4 cm in diameter, 10 cm from the bottom, usually sealed with a rubber stopper, which can be used to adjust the height of the water level in the glass bucket. The water level fluctuation control test was carried out at 19:00 on March 18, 2021. Two treatment modes of water level rising and falling were set up. Each treatment had six test groups, totaling 12 test groups and one treatment group with a water level of 50 cm. Each group was set up with 3 replicates. The *V. natans* with strong rhizomes, intact root activity, good growth and consistent health were selected and planted in cylindrical plastic test pots, with 3 pots in each group and 10 plants in each pot. The average height of *V. natans* reached 15.67 cm after pre-culture. The experiment was carried out in the plant sunroom of the Key Laboratory of Poyang Lake Wetland and Watershed Research of the Ministry of Education, Jiangxi Normal University, with the meadow swamp soil of Poyang Lake wetland as the substrate (pH 5.36, organic matter and total nitrogen content of 42 g/kg and 1.96 g/kg, respectively), and the average water temperature of 21.7 ± 5°C during the experiment. The average temperature was 24.4 ± 5°C, and the illumination was natural.

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The initial water level of rising fluctuation was 50 cm, and the water levels fluctuation intensity was 0.2 cm/d, 0.5 cm/d, 0.8 cm/d, 1.1 cm/d, 1.4 cm/d, 1.7 cm/d. After 50 d, the water level of each rising water level test group rose to 60 cm, 75 cm, 90 cm, 105 cm, 120 cm, and 135 cm, respectively. The initial fluctuating water levels were 60 cm, 75 cm, 90 cm, 105 cm, 120 cm, and 135 cm, i.e., the water level of each falling water level test group decreased to 50 cm after 50 d. (*Figure 1*).

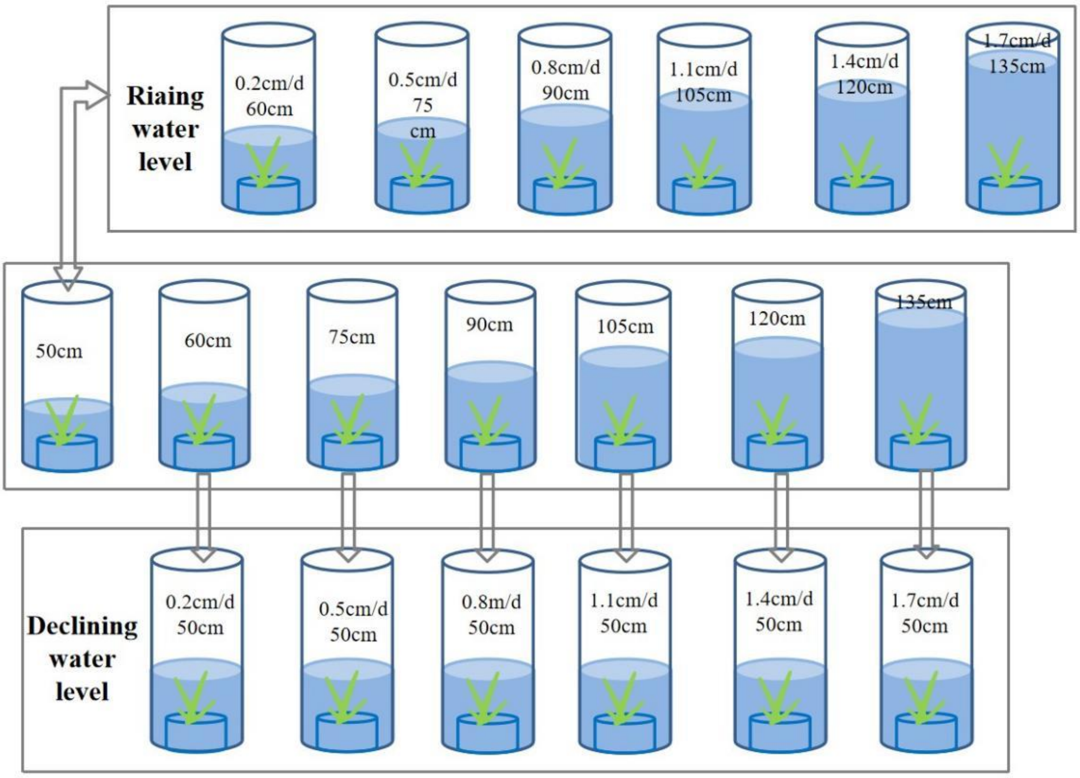

Figure 1. *Schematic diagram of experiment design*

This experiment lasted for 50 days, and the first experiment was started on March 18, 2021. The third leaf counted down from the top of the plant was used as the sample, and one leaf was collected from each plant to ensure the consistency of the sample. The antioxidant indexes of *V. natans* were tested every 10 days. The water level was controlled to reach the set value at 19:00 every day.

Test method and parameter determination

Each physiological index was measured once before the water level fluctuation control test as a starting reference value and was measured every 10 days. The following physiological indicators were measured: Superoxide dismutase (SOD) was determined by the nitrogen blue tetrazolium method (NBT). Peroxidase (POD) was determined by the guaiacol method. Catalase (CAT) was determined by the potassium permanganate titration method. Catalase (CAT) was determined by UV absorption metho. The production rate of superoxide anion (O_2^-) was determined by the hydroxylamine oxidation method. Malondialdehyde (MDA) was determined by the thiobarbituric acid method. The physiological index determination method is Principles and Techniques of Plant

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Physiological and Biochemical Experiments prepared by Wang Xuekui (Wang, 1990, 2015).

Data processing

Excel 2016 and Origin 2019 were used for data processing and chart making. SPSS 22.0 software was used to conduct one-way ANOVA for the sorted data, and two-factor ANOVA was conducted by integrating the test time and the two influencing factors of treatment intensity and test time under fluctuating water levels. LSD was used for multiple comparisons between groups to test the significance of differences. Pearson correlation coefficient was used to analyze the correlation between antioxidant indicators ($P<0.05$ significant correlation. $P<0.01$ very significant correlation). Test data are mean ± SD.

Results and analysis

Effects of water level fluctuation on antioxidant enzyme system of V. natans

Effect of water level fluctuation on superoxide dismutase

The POD was significantly ($P<0.01$) influenced by the intensity and duration of fluctuations under different fluctuation conditions. Under the rising fluctuation condition, *V. natans* POD activity was high in the fluctuating intensity range of 0-0.8 cm/d and was significantly higher ($P<0.01$) than the rest of the groups in the middle and late stages of the experiment (25-50 days), with the greatest variability among the groups at 30 days of the experiment ($P<0.001$). The within-group variability of the 1.1-1.7 cm/d test groups was significantly greater than that of the remaining groups throughout the test period ($P<0.05$).

The overall response to the length of the test showed a fluctuating trend of increasing and then decreasing, and a small increase in POD activity was observed in the 1.1-1.7 cm/d fluctuating intensity groups at 40-50 days of the test. The time point at which the POD activity of *V. natans* started to decline at different fluctuation intensities was inconsistent. Within the fluctuation intensities of 1.1-1.7 cm/d, it started to decline after 10 days of the test and the lowest content value appeared at 40 days of the test, which was earlier than the rest of the groups and the decline was greater than the rest of the groups. Under the downward fluctuation condition, the POD activity under the fluctuation intensities of 0.8-1.1 cm/d was less different than the rest of the groups during the test period. The overall trend of each group increased with the increase of the test time, and the difference between the groups was more significant in the early stage of the test ($P<0.01$), and the POD activity values were closer in the later stage of the test (*Fig. 2*).

Effect of water level fluctuation on peroxidase

Under different fluctuation conditions, influenced by the test duration and fluctuation intensity, the overall trend of *V. natans* SOD activity was ascending and then descending, and the low fluctuation intensity was higher than the high fluctuation intensity SOD activity. Treatment intensity, test duration, and the interaction between the two had a significant effect on SOD activity ($P<0.01$). Among the rising fluctuations, the 0-1.5 cm/d intensity fluctuation groups differed significantly ($P<0.01$) within the group throughout the test time, while the rest of the groups changed more moderately.

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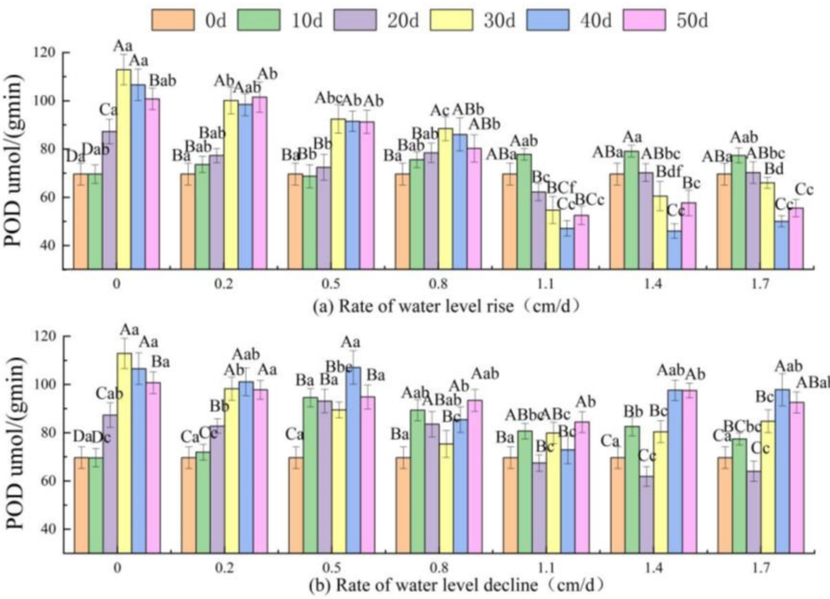

Figure 2. *Effect of fluctuating water level on Peroxidase (POD) of V. natans. Different capital letters indicate the difference between the same change rate and different test times, and different lowercase letters indicate the difference between the same test time and different change rates. Test data are mean ± SD*

At the late stage of the experiment (25-50 days), SOD activity showed greater variability among groups than in the earlier period (0-25 days) (P<0.01). During the period of 10-30 days of the experiment, the 0-0.5 cm/d experimental groups showed a significant increase, with a maximum increase of 19.93%. The 1.1-1.7 cm/d fluctuating intensity group showed an increase in SOD activity at 40-50 d of the experiment. In the falling water levels, the variability among different groups within the same test time was less than that of the rising fluctuation groups, and the variability among the same test group within different times was significant (P<0.05). In the late stage of the test (25-50 days), although all showed a decreasing trend, the decrease in each group was smaller than the increase in each group. In the rising variation, the SOD activity of 1.1-1.7 cm/d treatment intensities showed a rise in the late stage of the experiment (25-50 days), which was different from the falling fluctuation (*Fig. 3*).

Effect of water level fluctuation on catalase

The CAT activity of *V. natans* was significantly (P<0.05) affected by the length of the test, the intensity of fluctuations both of them, and the response to the length of the test all showed a rising and then falling trend (P<0.01). In the rising fluctuations, CAT activity at high fluctuation intensity was higher than that at low fluctuation intensity. 0.8-1.4 cm/d fluctuation intensity showed greater intra-group variability than the rest of the groups throughout the test period. The variability among groups was more pronounced at the end of the test period, with the largest difference of 3.82 μmol/(gmin) at 50 days. The 0.5-1.7 cm/d groups showed an overall decrease after 40 days, later than the rest of the groups. In the falling water level, the variability at different fluctuation intensities was less than that of the rising fluctuation groups in general. The decreasing trend of the test group in the range of 1.1-1.7 cm/d started at 30 days, earlier than the rising fluctuation experimental group, and the decreasing time of 0.5-0.8 cm/d was consistent with the

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rising fluctuation test groups. The difference among the groups was greater at the later stages of the experiment ($P<0.01$), but the CAT activity values were closer at 50 days of the experiment at 0.8-1.7 cm/d intensities (*Fig. 4*).

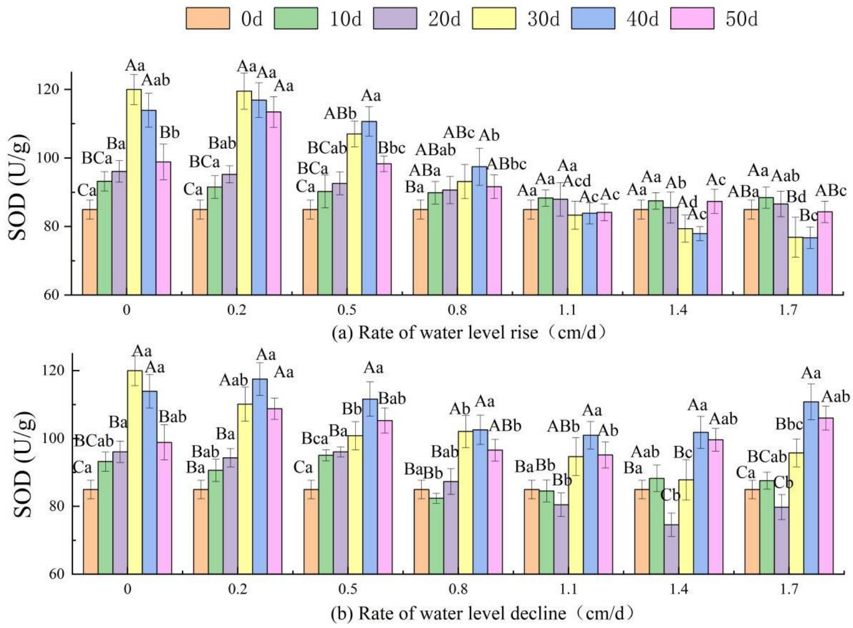

Figure 3. *Effect of fluctuating water level on Superoxide dismutase (SOD) of V. natans. Different capital letters indicate the difference between the same change rate and different test times, and different lowercase letters indicate the difference between the same test time and different change rates. Test data are mean ± SD*

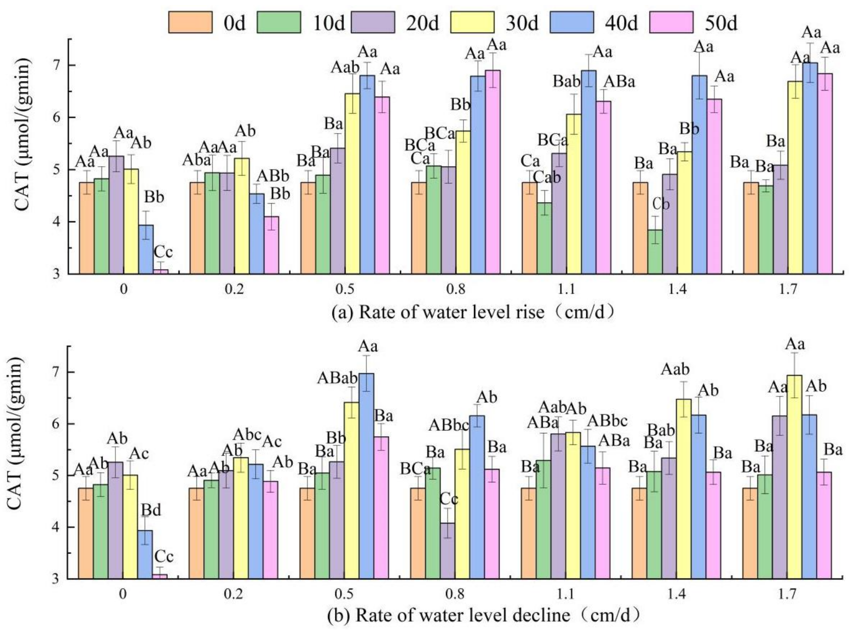

Figure 4. *Effect of fluctuating water level on Catalase (CAT) of V. natans. Different capital letters indicate the difference between the same change rate and different test times, and different lowercase letters indicate the difference between the same test time and different change rates. Test data are mean ± SD*

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Effect of water level fluctuations on the degree of membrane peroxidation in V. natans cells

Effect of water level fluctuation on the production rate of superoxide anion

The effect of *V. natans* O_2^- on test duration and fluctuation intensity was significant (P<0.01) under different water level changes and insignificant (P>0.05) by the combined effect of both. In general, the O_2^- content of *V. natans* was higher with less fluctuation intensity. In the upward fluctuations, the O_2^- content of *V. natans* produced at different fluctuation intensities was close in the 10-20 days test period. The 0.8-1.7 cm/d upward intensity groups showed more complex changes throughout the test cycle than the rest of the groups. The O_2^- content of *V. natans* generally increased and then decreased under the decreasing water level, but the decreasing intensity group at 0.8-1.7 cm/d showed a decreasing and then increasing trend of O_2^- content during 10 d of the test. 0.2-1.7 cm/d treatment intensities all showed another declining trend at 40 d of the test, with a maximum decline rate of 46.07%, and the decline started later than the 0 cm/d fluctuation group (*Fig. 5*).

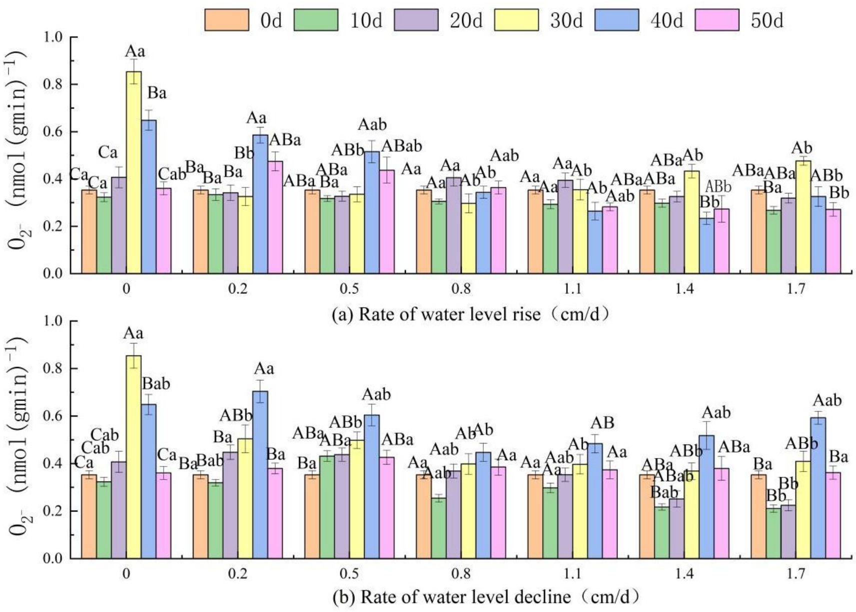

Figure 5. *Effect of fluctuating water level on Production rate of superoxide anion (O_2^-) of V. natans. Different capital letters indicate the difference between the same change rate and different test times, and different lowercase letters indicate the difference between the same test time and different change rates. Test data are mean ± SD*

Effect of water level fluctuation on malondialdehyde

The MDA content of *V. natans* under different water level fluctuations responded significantly (P<0.01) to the length of the test, fluctuation intensity, and the combined effect of both. The MDA content of *V. natans* under different fluctuation intensities showed a fluctuation trend of increasing and then decreasing with the increase of test time. Under the rising fluctuating water level condition, the MDA content was high at a low fluctuating intensity from an overall view. Significant intra-group variability (P<0.01) was observed for different fluctuation intensity test groups throughout the test

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time, and significant variability (P<0.05) was observed among different test groups during the same test time.

There was an overall trend of decreasing MDA content in all test groups from 30 days after the test, with less variability (P>0.05) in the 0.5-1.7 cm/d fluctuating intensity groups at the later stage (50 d). Overall, the intra-group variability was significantly higher than that of the rising fluctuation group throughout the test time for different falling fluctuation intensity test groups, and the intra-group variability was less than that of the 20-30 d falling experimental groups during the 40-50 days test time. 0-0.2 cm/d was a single peaked rising declining trend, and the rest of the groups showed a more complex double peaked rising declining trend. The remaining groups showed a more complex bimodal rising and falling trend. The 1.7 cm/d experimental group showed the greatest rise of 3.6 umol/g at a rate as high as 66.79% during the trial of 30-40 days. The intensity of 0.2-1.7 cm/d falling fluctuations decreased more sharply on trial 40 days, and at 50 days each falling fluctuation experimental group of *V. natans* maintained a higher MDA content, differing from the rising experimental groups (*Fig. 6*).

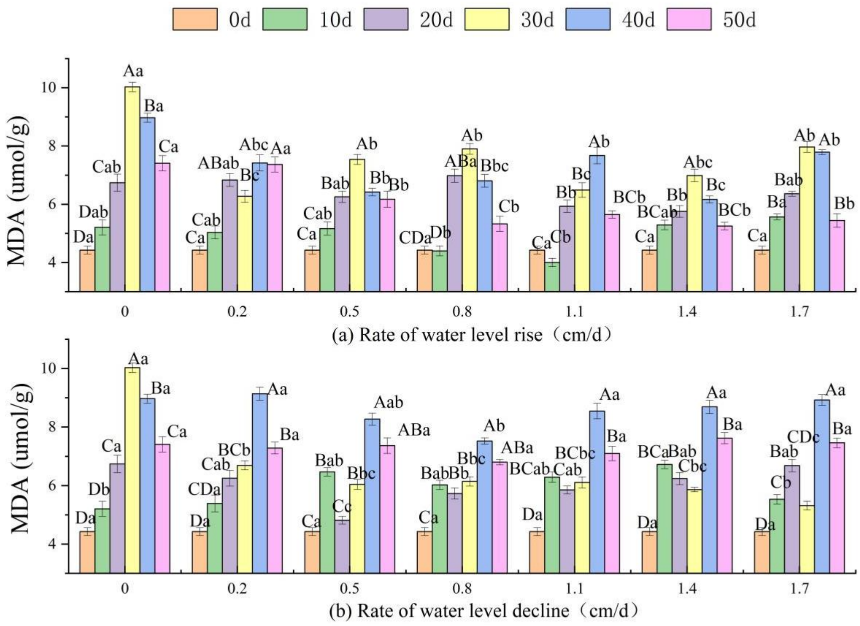

***Figure 6.** Effect of fluctuating water level on Malondialdehyde (MDA) of V. natans. Different capital letters indicate the difference between the same change rate and different test times, and different lowercase letters indicate the difference between the same test time and different change rates. Test data are mean ± SD*

Correlation analysis of change rate, test time and plant physiological indexes

A two-factor ANOVA was performed on the two influencing factors of water level fluctuation treatment intensity and test time in the case of combined rising and falling water level changes. It was found that SOD, O_2^-, and MDA responded significantly to the treatment intensity of water level fluctuation in rising water levels, and the test time had a significant effect on POD, SOD (P<0.001). In the case of falling water level fluctuation, the treatment intensity had a significant effect on SOD, POD, CAT, and MDA, and test time had a significant effect on MDA, and SOD, POD, MDA were significantly affected by both treatment intensity and test time (P<0.001). Under rising water level conditions,

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POD and CAT showed a highly significant correlation to fluctuating intensity, CAT, O_2^-, and MDA all responded significantly to test time, and SOD, POD, and MDA showed a highly significant response under the dual effect of treatment intensity and test time (P<0.01). In falling water levels, O_2^- showed a highly significant correlation to treatment intensity, SOD, POD, and O_2^- showed a highly significant correlation to test time, and CAT showed a highly significant correlation to treatment intensity and test time (P<0.01). There was no significant correlation between O_2^- and the interaction of treatment duration and intensity (P>0.05). At the descending water level, CAT had a significant response to the test duration. Under the combined effect of treatment intensity and test time, O_2^- had no significant correlation with it (P>0.05) (*Table 1*).

Table 1. *Result of two-way analysis of variance (ANOVA) testing for main effects of water level fluctuation, time, and their interactions on V. natans (F-value)*

Rising water level			
Type	**Processing intensity**	**Test time**	**Processing intensity×Test time**
SOD	59.095^{***}	19.655^{***}	10.094^{**}
CAT	45.32^{**}	63.264^{**}	14.17^{**}
POD	33.983^{**}	67.53^{***}	7.583^{**}
O_2^-	46.19^{***}	35.248^{**}	21.049^{ns}
MDA	65.449^{***}	582.071^{**}	36.633^{**}
Declining water level			
Type	**Processing intensity**	**Test time**	**Processing intensity×Test time**
SOD	20.766^{***}	91.356^{**}	3.707^{***}
CAT	25.175^{***}	35.686^{*}	6.48^{**}
POD	10.255^{***}	127.83^{**}	2.925^{***}
O_2^-	35.368^{**}	135.203^{**}	11.354^{ns}
MDA	35.803^{***}	8.873^{***}	8.504^{***}

The numbers in the table are F values, *** P<0.001, ** P<0.01, * P<0.05, ns P>0.05. SOD: superoxide dismutase, POD: peroxidase, CAT: catalase, O_2^-: production rate of superoxide anion, MDA: malondialdehyde

The correlation analysis of physiological indicators of *V. natans* showed that O_2^- was highly significantly negatively correlated with SOD (P<0.01) and highly significantly negatively correlated with CAT, POD, and MDA (P<0.05). CAT was highly significantly negatively correlated with POD and SOD (P<0.01) and did not correlate with MDA. POD was highly significantly and strongly positively correlated with SOD (P<0.01) and MDA (P<0.01). SOD showed a highly significant positive correlation with MDA (P<0.01) (*Table 2*).

Table 2. *Correlation Analysis of Morphological and Physiological Indexes of V. natans*

	O_2^-	CAT	POD	SOD	MDA
O_2^-	1				
CAT	-0.210^{*}	1			
POD	0.228^{*}	$-.377^{**}$	1		
SOD	0.292^{**}	$-.317^{**}$	0.783^{**}	1	
MDA	0.219^{*}	-0.069	0.513^{**}	0.568^{**}	1

* significant correlation at the 0.05 level (two-sided), * * significant correlation at the 0.01 level (two-sided). SOD: superoxide dismutase, POD: peroxidase, CAT: catalase, O_2^-: production rate of superoxide anion, MDA: malondialdehyde

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Discussion

Antioxidant enzyme system of V. natans

The enzyme of scavenging reactive oxygen species (ROS) in plants can directly reflect the stress status of plants (You et al., 2015). When plants are under stress, the excess ROS in the body can be removed by regulating the antioxidant enzyme system to improve the stress resistance of plants (Dou et al., 2013). In this study, we found that the overall response of POD and SOD to water level of *V. natans* showed high enzyme activity at low fluctuation intensity and low enzyme activity at high fluctuation intensity, while CAT showed low enzyme activity at low fluctuation intensity and high enzyme activity at high fluctuation intensity (Sofo et al., 2015). This may be because the overall rise in water level of *V. natans* in the range of low fluctuation intensity is small, and the water level is lower than other fluctuations to form growth stress (Dou et al., 2013), and the enzyme activities of POD and SOD increase with the degree of stress concentration to improve the resistance (Sousa et al., 2015). The stress generated by low fluctuation intensity resulted in H_2O_2 production exceeding CAT decomposition capacity, leading to low enzyme activity. It indicates that *V. natans* is more adaptable to higher water fluctuation intensity (Zang et al., 2015).

The response of POD and SOD activities of *V. natans* to the length of the test under upward fluctuation showed an overall trend of increasing and then decreasing, but in the 1.1-1.7 cm/d fluctuation intensity group, the POD and SOD activities increased at 40-50 days of the test, which indicated that *V. natans* was protected from stress by increasing the POD and SOD activities in the body to remove the excessive ROS in the body. The CAT in the upward fluctuation showed an overall trend of decreasing and then increasing at low fluctuation intensity, and a trend of decreasing, then increasing and then decreasing at high fluctuation intensity.

In the downward fluctuation, the POD and SOD activities of all groups of *V. natans* showed a fluctuating upward trend with the increase of test time, and showed a decreasing trend in the late test period (25-50 days) and the POD and SOD activity values among groups were close to each other. CAT showed a rising trend in the downward fluctuation and then decreasing trend. In the experiment of decreasing water level, the longer the time, the lower the water level of each test group, the more serious the *V. natans* suffered from light stress and needed more POD and SOD to resist the stress. When the stress exceeded the ability of defense system, the POD, SOD and CAT in *V. natans* could not maintain the high level and appeared to decline.

The degree of cell membrane peroxidation in V. natans

When plants suffer from stress, they produce O_2^-, -OH and other free radicals, which are converted into H_2O_2 and O_2^- under the action of SOD (Caverzan et al., 2016), while CAT can decompose H_2O_2 into H_2O and O_2^- (Ślesak et al., 2016), so that the cytoplasmic membrane of *V. natans* is protected from damage, with the continuous strengthening of adversity stress, the accumulation of H_2O_2 is increasing, exceeding the decomposition capacity of CAT, and the excess H_2O_2 in the body oxidizes the cytoplasmic (Li et al., 2017). Excess H_2O_2 in the body oxidizes the cytoplasmic membrane and produces MDA. In this study, we found that the response of *V. natans* MDA and O_2^- to water level generally showed a high content of low fluctuation intensity and low content of high fluctuation intensity. This is because the low fluctuation intensity of low water levels caused stress to the growth of *V. natans*.

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In the rising fluctuation, the MDA of *V. natans* showed a trend of rising first and then falling, and O_2^- showed a complex bimodal trend of increasing and then decreasing in the range of 0.5-1.7 cm/d rising and falling again. The fluctuation intensity of 0.8-1.7 cm/d showed a downward trend and the content of MDA and O_2^- was low in the later stage of the experiment, which may be because of the dynamic balance between the production and removal of ROS in the body of *V. natans* remained at a relatively good level.

In the declining water level, it was found that *V. natans* MDA showed a trend of rising, then falling and then rising again in the fluctuation range of 0.5-1.7 cm/d and declined after 40 days. O_2^- showed a trend of falling, then rising, and then falling again in the fluctuation range of 0.5-1.7 cm/d. The main reason was that the SOD enzyme activity of *V. natans* increased during this test time and cleared the O_2^- from the body.

Conclusion

In this study, the physiological and ecological response mechanism of the submerged plant *V. natans* under special hydrological conditions was studied by setting two different water level change conditions, rising and falling, and conducting indoor simulated water level change microscopic experiments, using water level and time as change factors, in order to reveal the response and adaptation mechanism of the submerged plant *V. natans* to water level change. The following conclusions were drawn.

Vallisneria natans showed adaptability to rising water level fluctuations in fluctuating water levels and showed some adaptability to falling water level fluctuations during the test period 0-40 days. Under both rising and falling water level fluctuation conditions, *V. natans* showed that growth stress would develop at low fluctuation intensity, leading to increasing antioxidant enzymes to improve resilience, while being more adaptive to high fluctuation intensity. This suggests that significant changes in water level affect *V. natans* plant height, but the study found that *V. natans* is temporal to high-intensity water level decline and growth is also inhibited in the later stages of the experiment. In terms of physiological indicators, *V. natans* elevated antioxidant enzyme activity and cytoplasmic peroxidation level in response to stress in decreasing water levels. Therefore, this experiment concluded that *V. natans* is more adaptive to rising water levels and high fluctuation intensity.

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Conflicts of competing interests. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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