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Full Length Research Paper

Structural and metabolic responses of *Ceratophyllum demersum* to eutrophic conditions

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Eutrophication in water bodies affects the growth of aquatic plants. In this study, we conducted static experiments to better understand the structural and metabolic responses of *Ceratophyllum demersum* under eutrophication conditions. The anatomical structure, nitrogen (N) and phosphorous (P) levels in tissue, malondiadehyde (MDA), and activities of three antioxidases (peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT)) of *C. dermersum* cultured at different nutrient levels (oligotropher, mesotropher, eutropher, and hypertrophics) were investigated. The results showed that with nutrient concentration increase, disordered anatomical structures and a cavity in stem of *C. demersum* existed; there was also an increase in the N and P contents of *C. demersum*. The MDA content improved with nutrient increase while POD and SOD activities initially increased and later decreased. CAT activity also increased during the experimental period. These finding suggested that changes in stem anatomical structure and the increase in antioxidant enzyme activity can be served as the indicators of the response of *C. demersum* to eutrophication conditions.

Key words: Submerged aquatic plants, anatomical structure, antioxidant enzymes, nitrogen and phosphorus eutrophication.

INTRODUCTION

Lake eutrophication has become a global concern among limnologists and environmental scientists (Shilla et al., 2006; Conley et al., 2009). Nitrogen (N)- and phosphorus (P)-caused eutro-phication are commonly found in lakes. As two essential nutrient elements for plant growth, both N and P levels in the environment influence the growth, metabolism, and community dynamics of plants. Elevated N and P levels in waters can significantly impact the physiology of sub-merged aquatic plants (Bremiga et al., 2005; Zhu et al., 2006; Ni, 2001; Xie et al., 2004; Thomaz et al., 2007; Willis and Mitsch, 1995). Nutrient enrichment affects plant photo-synthesis (Andrzej et al., 2005), biosynthesis (Wang et al, 2005), soluble sugar, biomass (Wang and Li, 2002; Cao and Li, 2004), and antioxidant enzyme active defense (Nimptsch, 2007; Cao et al., 2004; Cao et al., 2007). Moreover, increased ammonia concentration inhibits the growth of submerged aquatic plants, and can even lead to plant mortality (Yan et al., 2007).

The N and P contents of submerged aquatic plants are closely related to the environment (Shilla et al., 2006; Lauridsen et al., 2003; Lundberg, 1989). Baldy et al. (2007) found that P concentration in shoots and roots of *Berula erecta* was correlated to P level in waters, while Wang and Ji. (2006) found that *Ruppia maritima* could take up excess nutrients in eutrophic waters. Morphologic changes in plants reflect their adaptation to and are indicative of changes in environmental conditions. Thus, understanding the structural response of plants to different aquatic environments is important (Chen et al., 2005). However, the study of the structural changes of submerged aquatic plants under eutrophic conditions is lacking. Herein, in this study, we examined N and P nutrient uptake, antioxidant enzyme activity and structural

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Abbreviations: MDA, Malondiadehyde; **SOD,** superoxide dismutase; **POD,** peroxidise; **CAT,** catalase; **PBS,** phosphate buffer saline.

Table 1. N and P treatments.

Units	*Oligotropher (C1)*	*Mesotropher(C2)*	*Eutropher(C3)*	*Hypertrophic(C4)*
N (mg/L)	0.1	0.5	1.5	3.0
P (mg/L)	0.01	0.05	0.2	1.0

response of the shoot of *Ceratophyllum demersum* to illustrate the mechanism of degradation of contaminants in eutrophicated lakes. The results of the study could provide a theoretical basis for the restoration of the structure and function of aquatic ecosystems.

MATERIALS AND METHODS

Plant materials

C. demersum is a submerged perennial herb that grows in oligotrophic and eutrophic shallow waters. The plants used for testing were taken from the outdoor training breeding grounds of Wuhan Botanical Garden in July 2007. Plants were grown in a hydroponic nursery system 48 h before use.

Experimental design

Healthy plants with fresh weight of about 10 g were selected and cultivated in three-liter pots (diameter of bottom × high = 11 cm × 32 cm). Hydroponics with Hoaglands (1/40) was selected for the plant nursery. The N source was NH_4Cl and $KN0_3$, while P source was KH_2PO_4. Concentration gradients of N and P were arranged to mimic eutrophic conditions in lakes (Table 1).

Potted plants were placed in a growth chamber with light intensity of 3000 lux at 12 h per day at temperatures of 25°C. The experiment was replicated thrice and had duration of three weeks. To maintain the stability of N and P concentration in hydroponics, the nutrient solution (1/2 strength Hoaglands) was replaced every 2 days. Tested plants were sampled at 7, 14, and 21 day intervals. About 0.5g tissue (fresh weight) was collected for the analysis of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and malondiadehyde (MDA) activity. A group of plants was collected for the analysis of their total N and P content at 21 days from the onset of the experiment. For nutrient analysis, tissue samples were first rinsed with tap water followed by deionized water, and then dried with absorbent paper and kept in a drying oven at 80°C for 48 h to achieve constant dry weight. Plant stems were also collected via microslicing and fixed with 50% formalin-acetic acid-alcohol (FAA) solution. The stems were then paraffin-embedded and dyed with safranin and solid green dye. Slice thickness was about 8 - 10 um. Photographs were taken using an Olympus BX51 microscope.

Determination of nutrients and MDA

Plant samples were digested with a mixed solution of H_2S0_4 and H_20_2. The concentration of total N and P were analyzed by a nitrogen and phosphorus flow analyzer (ASTORIA-PACIFIC, INC, Clackamas, USA). To determine MDA, about 0.2 g of fresh leaves was added to 2 ml trichloroacetic acid (TCA) (10%) and ground into homogenate. Samples were centrifuged at 4,000 g for 10 min. Two milliliters of the supernatant was mixed with 2 ml of 0.6% thiobarbituric acid (TBA) and placed in a boiling water bath for 15 min. After rapid cooling, the samples were centrifuged at 4,000 g for 15 min. Absorbance was read at 450, 532, and 600 nm by a spectrophotometer (Li, 2000).

Determination of antioxidant enzyme activities

POD

About 0.5 g of fresh leaves mixed with 1 ml of 20 mmol L^{-1} KH_2PO_4 was ground into the homogenate. Consequently, 20 mmol L^{-1} KH_2PO_4 was added to obtain the final volume of 5 ml. The homogenate was poured into centrifuge tubes, centrifuged at 4,000 g for 15 min, and then stored at 4°C for POD analysis. A proper enzyme liquid-extraction was added to the 3 ml reaction system of 25 mmol L^{-1} in a pH 6.8 phosphate buffer saline (PBS) containing 10 mmol L^{-1} H_2O_2 and 0.1%v/v guaiacol. Change in absorbance at 470 nm was recorded by a spectrophotometer. Enzyme activity was calculated according to the molar absorptivity of guaiacol at 470 nm (26.6 mmol/L/cm).

CAT

About 0.2 g of fresh leaves was mixed with 4 ml PBS (pH 6.8) precooled in an ice bath, ground into a homogenate, and centrifuged at 10,000 g at 4°C for 20 min. Subsequently, the supernatant was collected. A proper enzyme liquid-extraction was added to the 3 ml reaction system of 25 mmol L^{-1} with a pH 6.8 PBS containing 20 mmol/L H_2O_2. The change of absorbance at 240 nm was recorded using a spectrophotometer. Enzyme activity was calculated according to the standard curve of the optical absorption of H_2O_2 at 240 nm.

SOD

About 0.2 g of fresh leaves was mixed with 4 ml PBS (pH 7.8), precooled in an ice bath, ground into a homogenate, and centrifuged at 10,000g at 4°C for 20 min. Next, the supernatant was collected. A proper enzyme liquid-extraction (inhibiting about 50% of the photocoloration) was added to the 3 ml reaction system of 50 mmol L^{-1} with a pH 7.8 PBS containing 75 µmol L^{-1} nitroblue tetrazolium (NBT), 13 mmol L^{-1} Met, and 2 µmol L^{-1} riboflavin. To obtain uniform reaction, they were placed in a light incubator (Philips 15W×4) for 20 min. The change of absorbance at 560 nm was recorded using a spectrophotometer. Enzyme activity was calculated according to standard curve of optical absorption at 560 nm.

Statistical data analysis

Analysis of variance (ANOVA) for antioxidant enzyme (SOD, POD, and CAT) activities, total N and P, and MDA was performed with the statistical package for the social sciences (SPSS) software. Significant differences between treatments were tested by one-way ANOVA.

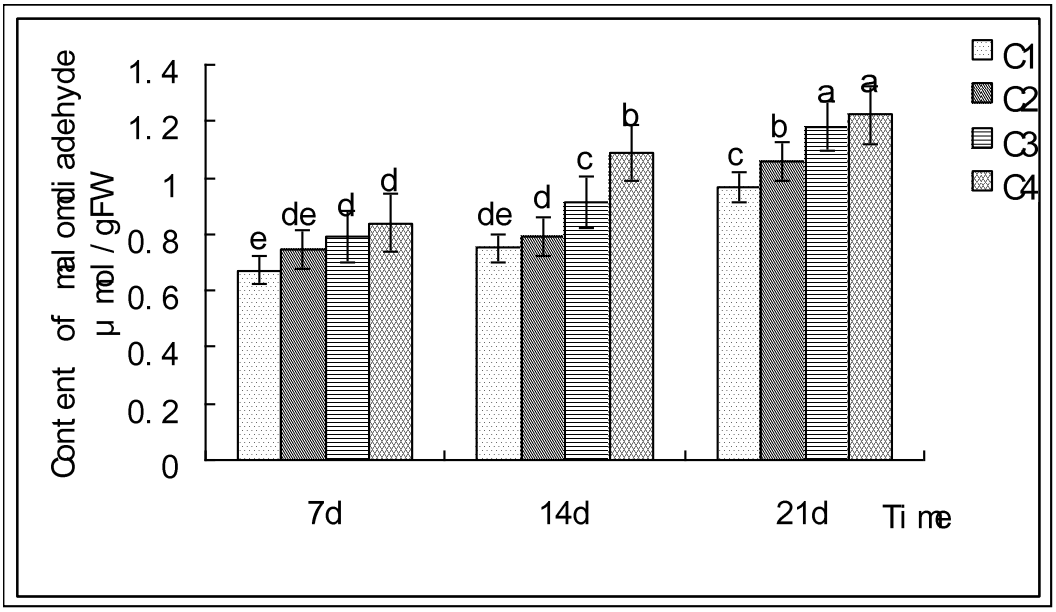

Figure 1. Malondiadehyde content of *C. demersum.* C1, Oligotropher; C2, mesotropher; C3, eutropher; C4, hypertrophic.

RESULTS

Effect of nutrient levels on the structure

As shown in Figure 6, the structure of the stem form of *C. demersum* markedly changed as nutrition levels increased. The epidermal stem formed and internal cells were neatly arranged under the oligotrophic condition. In contrast, the epidermis of the stem was rough and disordered under the mesotrophic condition. The epidermis of the stem ruptured under the hypertrophic condition. Moreover, the internal structures of the stem under eutrophic and hypertrophic conditions were empty.

Effect of nutrient levels on MDA content

The MDA content of *C. demersum* is presented in Figure 1. There was almost no difference in the MDA content of plants under oligotrophic, mesotrophic, eutrophic, and hypertrophic conditions on day 7. Meanwhile, under eutrophic and hypertrophic conditions, the MDA content was significantly higher compared with the oligotrophic condition on day 14. Meanwhile on day 21, the MDA was significantly higher under the mesotrophic, eutrophic, and hypertrophic conditions as compared to the oligotrophic condition ($df = 3$, $F = 47.99$, $P < 0.01$).

The MDA content was significantly higher for the four levels of nutrition on day 21 than on days 7 and 14 ($df = 2$, $F = 136.22$, $P < 0.01$). As observed during the experimental period, the MDA content of *C. demersum* increased with time.

Effect of nutrient levels on enzyme activities

Figure 2 shows the variations of *C. demersum* POD activity. POD activity of *C. demersum* under eutrophic and hypertrophic conditions was significantly higher compared with the oligotrophic condition ($df = 3$, $F = 33.97$, $P < 0.01$) on day 7. There was no statistically significant increase under the mesotrophic as opposed to the oligotrophic condition. On days 14 and 21, the results of POD showed that leaves exposed in mesotrophic, eutrophic, and hypertrophic conditions had significantly higher enzyme activities as compared to the oligotrophic condition ($df = 3$, $F = 10.9$, $P < 0.05$). POD activity did not differ statistically between mesotrophic and eutrophic controls on day 14. However, it significantly increased on day 14, as compared to day 7 (Figure 2), and was lowest on day 21 ($df = 2$, $F = 109.23$, $P < 0.01$).

Figure 3 shows variations in *C. demersum* SOD activity. SOD activity of *C. demersum* was significantly higher under mesotrophic, eutrophic, and hypertrophic than under oligotrophic condition (one-way ANOVA, $df = 3$, $F = 97.77$, $P < 0.01$). A significant increase was detected on days 14 and 21 as compared to day 7 (one-way ANOVA, $df = 2$, $F = 89.56$, $P < 0.01$). The variations in *C. demersum* SOD activity within the period of 21 days showed an initial inductive enhancement, which then turned to an inhibitive decline.

CAT activity of *C. demersum* showed almost no change under mesotrophic and oligotrophic conditions on days 7 and 21 (Figure 4). However, a statistically significant increase in CAT activity was detected in plants exposed to eutrophic and hypertrophic conditions ($P < 0.01$). CAT

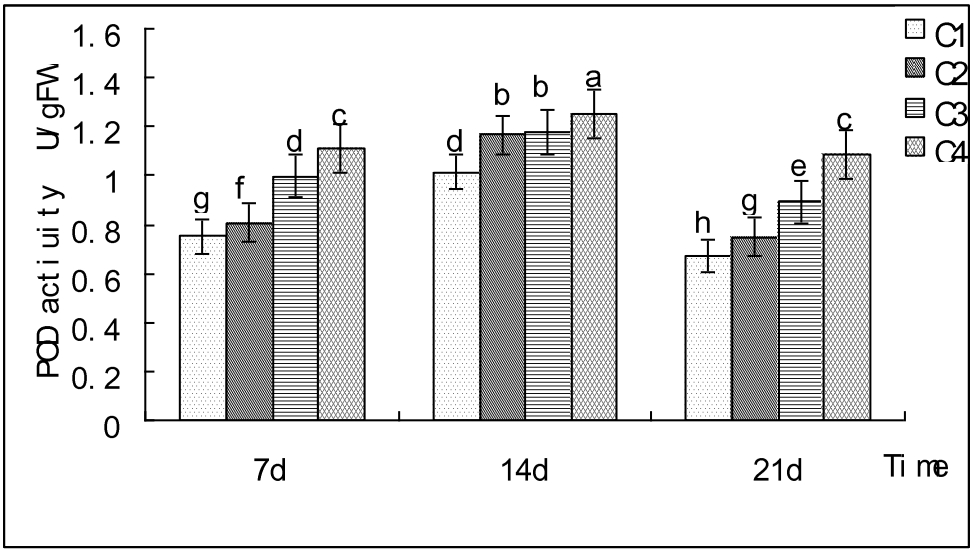

Figure 2. Peroxidase activity of *C. demersum.* C1, Oligotropher; C2, mesotropher; C3, eutropher; C4, hypertrophic.

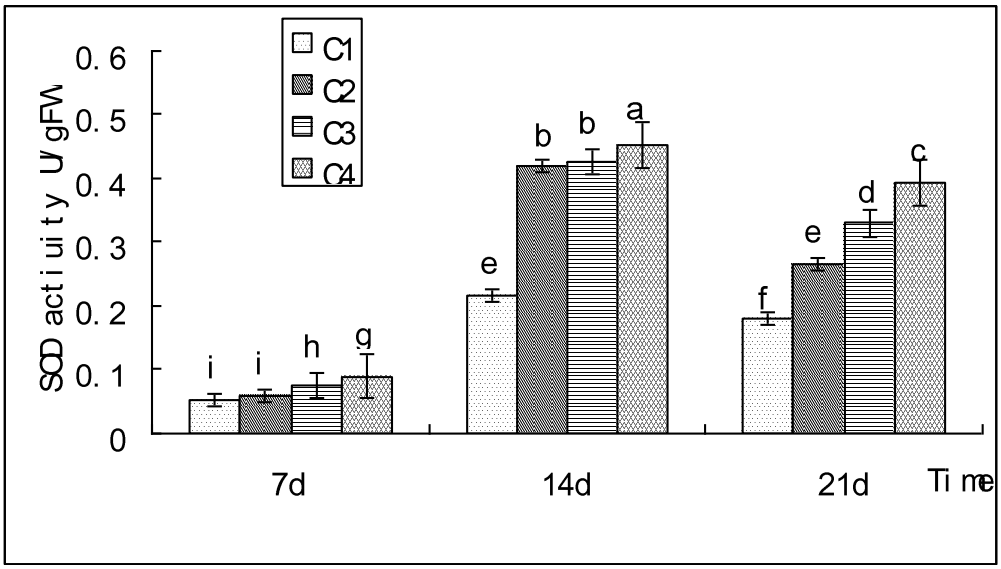

Figure 3. Superoxide dismutase activity of *C. demersum.* C1, Oligotropher; C2, mesotropher; C3, eutropher; C4, hypertrophic.

activity of *C. demersum* was significantly higher under mesotrophic, eutrophic, and hypertrophic conditions than in the oligotrophic condition on day 14 (Figure 4). However, CAT activity did not statistically differ under mesotrophic, eutrophic, and hypertrophic controls on day 14.

Effect of nutrient levels on total N and P

Figure 5 shows the total N and P content of *C. demersum* at different nutrition conditions. Nitrogen content of *C. demersum* ranged between 49.27 and 67.04 mg kg^{-1}. From Figure 5, it is evident that there was a statistical difference in N content of *C. demersum* at different nutrition conditions (one-way ANOVA, df = 3, F = 144.71, $P < 0.01$). Nitrogen content of *C. demersum* elevated with increase in nutrition levels. Phosphorus content of *C. demersum* ranged from 5.18 to 6.21 mg kg^{-1} (Figure 5). It was significantly higher under eutrophic and hypertrophic conditions than that under oligotrophic condition (one-way ANOVA, df = 3, F = 124.54, $P < 0.01$). Moreover, it had no statistically significant increase under the

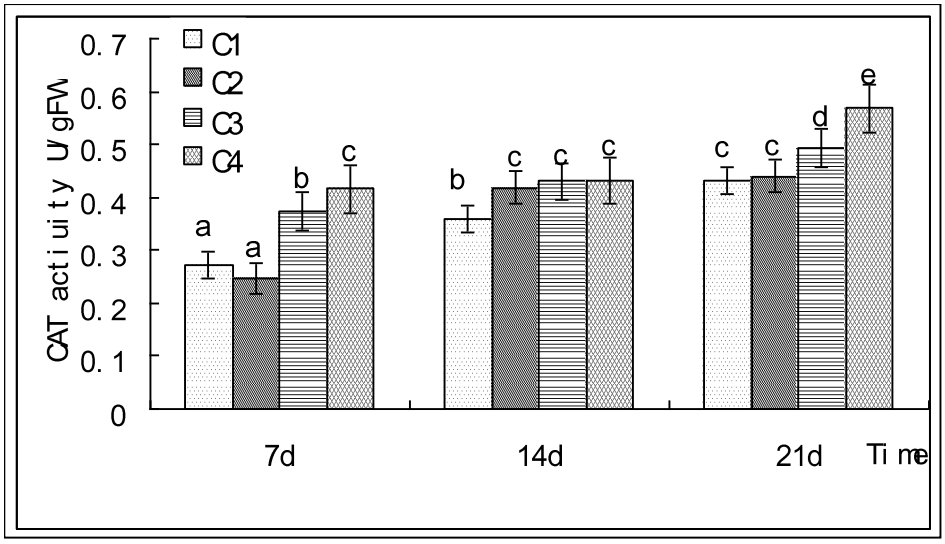

Figure 4. Catalase activity of *C. demersum.* C1, Oligotropher; C2, mesotropher; C3, eutropher; C4, hypertrophic.

mesotrophic conditions, as compared to that under the oligotrophic condition.

DISCUSSION

Environments exert tremendous influences on growth and morphologic and structural changes underlying plant tissues. Adverse environmental conditions can cause plant structural damage and dysfunction. In this study, we found that when the nutritional levels increased, aerenchyma in stems also increased to form a cavity, thus suggesting that there exists a possible response of submerged plants to eutrophication stresses. Puijalon et al. (2008) showed that eutrophication stresses contribute to the increase in the ability of aquatic plants to regenerate. Some plants have been found to either split under oxygen deficit environments or accelerate growth of aerenchyma (Watkin et al., 1998). In addition, the ventilation function of the stem played an important role in the process of adaptation by plants to the environment (Tao and Jing, 2004). Many aquatic plant cells form intercellular space and cavity through orderly separation and differentiation (Fan and Zhang, 2002). The pith compartments of large-scale cells in plant stem base were autolyzed to cause nearly all of its central cells to vanish (Della et al., 1999) and render the stem base hollow under flooded conditions. This will reduce the resistance to gas transportation. Similar changes in the stem cross section of *C. demersum* were exhibited in our investigations. Eutrophication in water body contributes to the increase in algae growth that will lead to the hypoxic conditions, even anoxia. This in turn worsens stress for *C. demersum*.

Lipid peroxidation in the cell membrane often occurs under adverse conditions. MDA, one of the end product of peroxidation, is usually used as an indicator of lipid peroxidation, indicating cell membrane peroxidation and reflecting adverse conditions under which plants grow (Li, 2000). Our results showed that there was a significant difference in MDA in *C. demersum* growing under different nutrient levels. The increase suggests that the antioxidants in *C. demersum* reacted properly to oxidative stress generated by increased nutrition. Fan et al. (2007) found that the MDA content in *Elodea nattalii* increased greatly under high N and P levels, which was similar to our results.

Studies (Li, 2000; Bowler et al., 1992) indicated that free radicals produced in adverse conditions were possibly related to membrane system injury and possibly, disturbed photosynthesis, respiration, and other metabolism process, as well as vegetable cell death. Plant bodies have free radicals, a type of protective system that eliminates, produces, and reduces the harm the environment pollutant may cause. SOD, POD, and CAT are essential enzymes for the protective system. It has been reported that antioxidant enzyme activities of submerged macrophytes were significantly influenced by N and P concentrations in waters (Wang and Li, 2002; Wang et al., 2005; Fan et al., 2007). The results of our experiment show that the changes in antioxidant enzyme activities of *C. demersum* were significantly influenced by its nutriational level. Under mesotrophic, eutrophic, and hypertrophic conditions, antioxidant enzyme activities (SOD, POD, CAT) increased, indicating that *C. demersum* experienced stress under these conditions. At the onset of the experiment, the resistance mechanism was also initiated, thereby increasing SOD, POD, and CAT

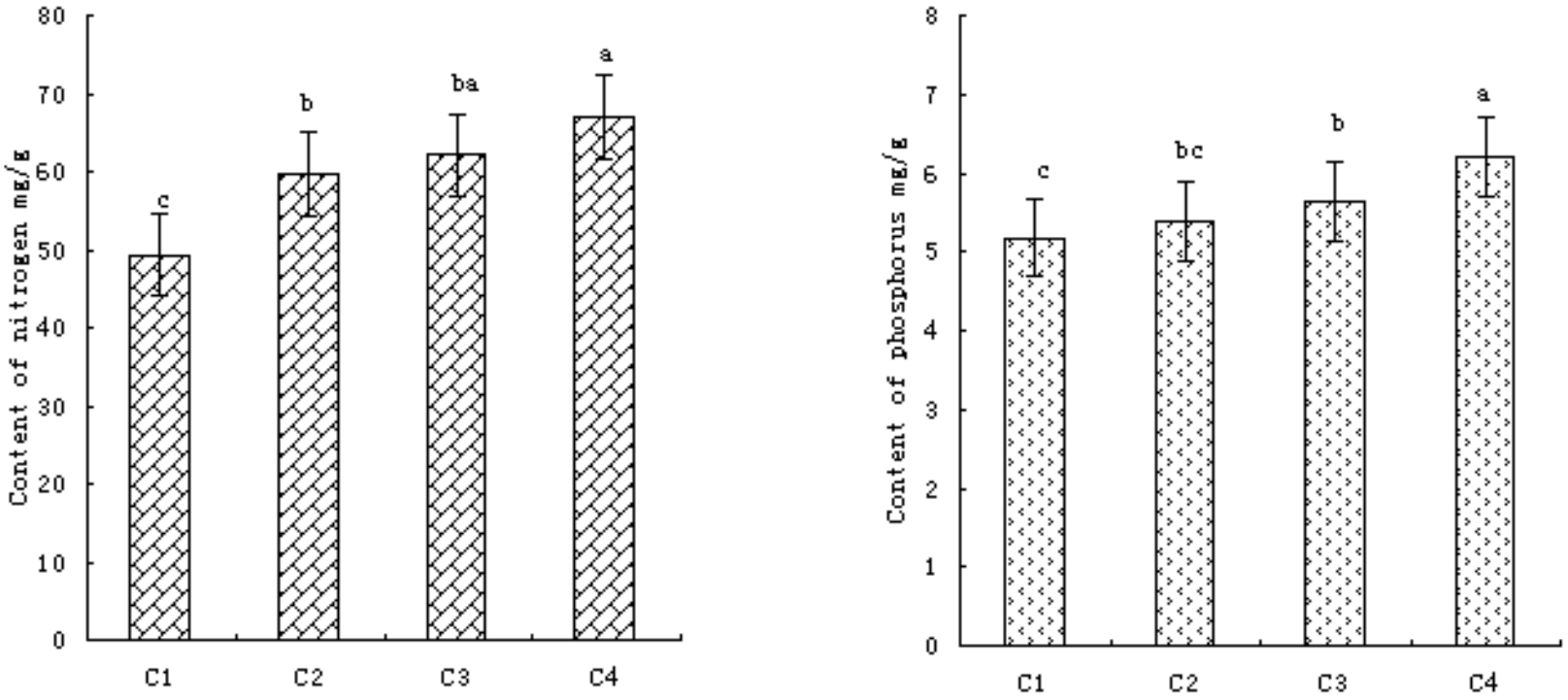

Figure 5. Nitrogen and phosphorus contents of *C. demersum.*

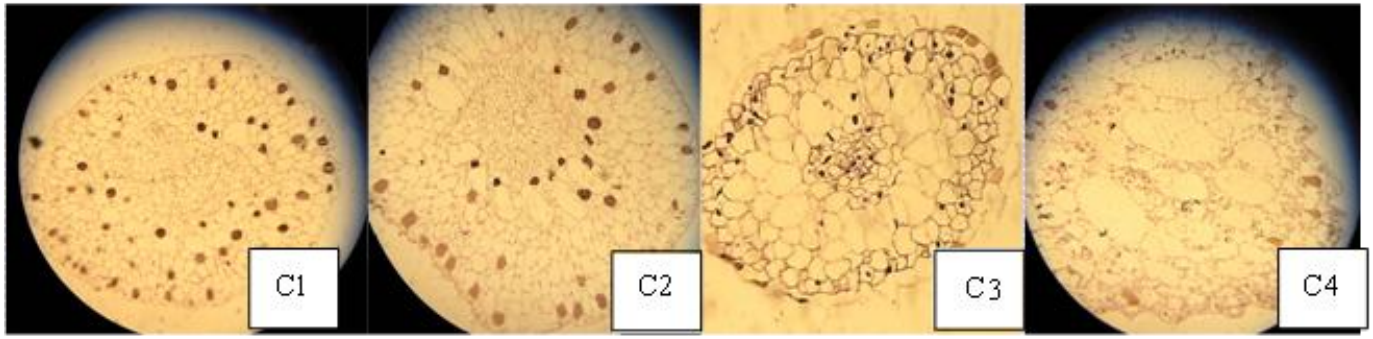

Figure 6. Anatomical structures of the stems of *C. demersum* at different nutritional levels (×400). C1, Oligotropher; C2, mesotropher; C3, eutropher; C4, hypertrophic.

activities as the nutritional level became elevated. This has the effect of eliminating active oxygen in the cell body, which then contributes to the enhancement of the protective film normal structure and the function, as well as resistance to stress. The CAT and POD activities of the submerged plants in the first sampling period remained at a relatively high level. The variations in POD and SOD activities within a period of 21 days can be summarized as an initial inductive enhancement and a subsequent inhibitive decline.

Some scholars claimed that carbohydrate consumption in plants influence enzyme response vigor (Ni, 2001). The rise in CAT activity of *C. demersum* extended with time, possibly due to the enzyme activity of the plant being activated gradually with the lapse of time. Some researchers hypothesized that the catalase of plants under adverse environmental conditions allows them to adjust their own enzyme mechanism, enhance their catalase activity, and construct a protective reaction to the adverse effect (Zhu et al., 2006). Although the oxidation resistance enzyme activities of *C. demersum* differ with time variation, a consistent increase was observed, which occurred along with the increase in nutritional levels. Antioxidant enzyme activity could serve as one of the indicators of submerged plants responding to stress of eutrophication.

On the other hand, plant nutrition element content is indicative of the ability of plants to absorb nutritive elements under certain site conditions. It is also an interactive result between the plant and environment. Results of this study indicate that the N and P contents of *C. demersum* increased along with the nutritional level. We assume that this increase may be related to the N and P absorption characteristic of the plant. When the concentrations of N and P are high in the water column, a large amount of N and P was absorbed by the leaves of plants. Barko and Smart (1981) discovered that when the N content in the water column was higher, the N content in the tissues of *Myriophyllum spicatum* was also higher. Wang and Ji, (2006) discovered that the nutrition accumulation of Ruppiaceae tissues increased along with the nutrition level of the water body. In nutrient-enriched habitats, Steinbachova-Vojtiskova et al. (2006) demonstrated that *Typha angustifolia* tended to store more nitrogen. Wang et al. (2008) reported that nitrogen accumulation in *Vallisneria spiraslis* increased in eutrophicated water. These reports are similar to the results obtained in our study.

In summary, the results of this study indicate that the elevation of nutrition levels in waters can change stem tissue structure, increase antioxidant enzyme activity, MDA content, and N and P contents in *C. demersum*.

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