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Responses in root physiological characteristics of *Vallisneria natans* (Hydrocharitaceae) to increasing nutrient loadings

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Abstract – We selected the submerged macrophyte *Vallisneria natans* (Lour.) Hara for investigating the effects of nutrient loadings (nitrogen (N)-phosphorus (P) in mg·L^{-1}: (1) 0.5, 0.05; (2) 1.0, 0.1; (3) 5.0, 0.5; (4) 10.0, 1.0) on root physiological characteristics using sand culture during the growth season (June to October). Results showed that the best root growth was in macrophytes exposed to moderate nutrient conditions (N-P 2, in 1.0 and 0.10 mg· L^{-1}), and high nutrient loadings induced declines in root growth. Analysis of root antioxidant enzyme (superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT)) activities, protein content and cell ultrastructure revealed that the root of *V. natans* was subject to the stress of senescence during the end of the growth season. Moreover, high nutrient loadings increased oxidative stress in aging roots of *V. natans*, and made the cell ultrastructure of roots vulnerable to damage during senescence. The results for the changes in CAT activity suggest that CAT can serve as an important component of antioxidant defense mechanism in aging roots of *V. natans* to protect against nutrient loading induced oxidative injury in the early period.

Key-words: Nutrient loading / oxidative stress / physiological response / root / Vallisneria natans

Résumé – Réponses dans les caractéristiques physiologiques des racines de *Vallisneria natans* (Hydrocharitaceae) à l'augmentation des charges en éléments nutritifs. Nous avons choisi le macrophyte submergé *Vallisneria natans* (Lour.) Hara pour étudier les effets des charges d'éléments nutritifs (azote (N)-phosphore (P) en mg·L^{-1} : (1) de 0,5, 0,05 ; (2) 1,0 0,1 ; (3) 5.0, 0.5 ; (4) 10.0, 1.0) sur les caractéristiques physiologiques des racines en utilisant une culture sur sable pendant la saison de croissance (juin à octobre). Les résultats ont montré que la croissance des racines était la meilleure pour les macrophytes exposées à des conditions modérées de nutriments (NP (2), 1,0 et 0,10 mg·L^{-1}), et que des charges élevées en éléments nutritifs induisaient une baisse de la croissance des racines. L'analyse de l'activité des enzymes antioxydantes des racines (superoxyde dismutase (SOD), peroxydase (POD) et catalase (CAT)), de la teneur en protéines et de l'ultrastructure cellulaire ont révélé que la racine de *V. natans* était soumise à la contrainte de la sénescence au cours de la fin de la saison de croissance. En outre, des charges élevées en nutriments augmentaient le stress oxydatif dans le vieillissement des racines de *V. natans*, et rendaient l'ultrastructure cellulaire des racines vulnérable aux dommages pendant la sénescence. Les résultats sur les changements dans l'activité CAT suggéraient que CAT peut servir comme une composante importante du mécanisme de défense antioxydante dans le vieillissement des racines de *V. natans* pour se protéger contre la charge en nutriments induisant des lésions oxydatives dans la période initiale.

Mots-clés : Charge en éléments nutritifs / stress oxydatif / réponse physiologique / racine / Vallisneria natans

1 Introduction

Growth and reproduction in plants rely upon inorganic nutrients in the environment, among which nitrogen (N) and phosphorus (P) are often the primary limiting nutrients (Duarte, 1990; Vitousek and Howarth, 1991; Romero *et al.*, 2006; Harpole *et al.*, 2011). Most studies suggest that increased availability of N and P nutrients promotes growth of submerged macrophytes at nutrient limitation (Bulthuis *et al.*, 1992; Agawin *et al.*, 1996; Udy and Dennison, 1997;

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Terrados *et al*., 1999; Lee and Dunton, 2000). The mechanism may be that increased nutrients induce photosynthetic carbon fixation and metabolism (Turpin *et al*., 1990; Turpin, 1991; Lee and Dunton, 1999). However, in aquatic ecosystem, the enrichment of N and P can also adversely influence the growth of submerged macrophytes (Touchette *et al*., 2003; Olsen and Valiela, 2010). Many studies have shown a close relationship between eutrophication of the water and a decrease in submerged plants (Balls *et al*., 1989; Hosper and Jagtman, 1990; Jeppesen *et al*., 1990; Klein, 1993; Sand-Jensen *et al*., 2000). The mechanisms causing the loss of submerged plants during eutrophication are not well understood.

Plants have a series of mechanisms for responding to changes in the environment (Wu *et al*., 2007; Fox *et al*., 2013, 2014), and developing a thorough understanding of the responses of submerged macrophytes to eutrophication is an important foundation for understanding their decline. The root system of submerged macrophyte is very important to macrophyte growth because it provides physical support and anchorage, absorbs nutrients, stores carbohydrates, and synthesizes growth regulators (Maitai and Newton, 1982; Wang *et al*., 2009). The physiological basis of submerged macrophyte root growth in response to nutrient loadings is poorly understood. Therefore, if we had a sound physiological understanding of these processes, we could have a better understanding of the mechanisms that lead to the loss of macrophytes during eutrophication. In the present work, we studied the perennial, submerged macrophyte, *Vallisneria natans* (Lour.) Hara, which is widely distributed in China. We investigated the response of the physiological characteristics of the plant root, to increasing nutrient loadings. Our objectives were to: (i) determine how the physiological characteristics of *V. natans* were affected by increasing nutrient loadings, and (ii) increase understanding of the decline of this submerged macrophyte during eutrophication.

2 Materials and methods

2.1 Plant materials

On March 7 2010, winter buds of *V. natans* (fresh weight 1.02 ± 0.37 g) were planted in ten plastic containers (length 50 cm, width 38 cm, depth 25 cm) with 20 cm of tap water and 5 cm of sand for culture, and maintained under greenhouse conditions. These winter buds subsequently produced more than 1000 plants. Approximately two months after the winter buds were planted, young, *V. natans* plants of similar sizes (about 20 cm of height) were transplanted into sand in individual plastic pots (diameter 7 cm, depth 10 cm). Twenty-one pots (each planted with a single plant) were then transferred to each of 16 high-density polyethylene containers (volume 100 L, top diameter 51 cm, bottom diameter 40 cm, depth 63 cm) containing full tap water (as experimental medium). During the first 2 weeks after potting, all plants were maintained under the same conditions in the greenhouse for acclimation prior to the experiment.

2.2 Experimental design

The experimental treatment, conducted in 16 high-density polyethylene containers in the greenhouse, was started on May 29, 2010 and continued for 4 months (June to October). Considering the nutrient levels of freshwater lakes in eastern China's coastal areas, we manipulated the nutrient loadings in the water column as the experimental factor, with four increasing levels of N and P, as follows (in $mg{\cdot}L^{-1}$): NP-1, 0.5, 0.05; NP-2, 1.0, 0.1; NP-3, 5.0, 0.5; and NP-4, 10.0, 1.0. This experiment was a completely randomized design with 4 replications per treatment. Water lost to evaporation was replenished with tap water (total N 2.274 ± 0.012 $mg{\cdot}L^{-1}$, total P 0.032 ± 0.001 $mg{\cdot}L^{-1}$) to maintain the original water volume during the experiment. Considering the total water volume in each container, the nutrients contained in the tap water were considered negligible. Additionally, to maintain a constant concentration of nutrients throughout the experiment, the total N and total P in the water were determined every 2 weeks (Jin and Tu, 1990), and additional nutrients were added as concentrated solutions of potassium nitrate and potassium dihydrogen phosphate as needed.

Starting one week after the nutrient treatments began, the first sample of plant material was collected, and subsequent samples were collected every 2 weeks. In total, sampling took place 9 times during a period of 4 months. On each sampling day, 2 pots were taken from each of the 16 containers. One pot was used to measure the physiological parameters of the plant, and the other pot was used to determine plant biomass. The plants were gently washed with water, and put directly into sterile storage bags, then refrigerated and transported to the laboratory within 0.5 h. The root biomass was determined gravimetrically after drying at 80 °C to achieve a constant weight. To determine four physiological parameters (superoxide dismutase (SOD) activity, peroxidase (POD) activity, catalase (CAT) activity, and protein content), the fresh roots were weighed and then stored at −80 °C until analyzed. The removed plants were replaced with similar plants from the ten plastic containers to maintain the same plant density in the 16 high-density polyethylene containers. The newly positioned plants were marked and excluded from subsequent sampling. At the end of the experiment, we collected additional roots on September 30 for ultrastructural observation.

2.3 Measurement of protective enzyme activity and protein content

The roots of *V. natans* were homogenized on ice with mortar and pestle in 50 mM phosphate buffer. The homogenate was separated centrifuging it at 10 000 rpm for 20 min, and the supernatant liquid was analyzed (Ding *et al*., 2007). SOD activity, POD activity, CAT activity and soluble protein content were assayed according to Chen and Wang (2002).

The reaction mixture for determination of SOD activity contained 2.5 mL of 13 μM methionine, 0.25 mL of 63 μM nitroblue tetrazolium (NBT: an indicator of superoxide radical production), 0.1 mL of 13 μM riboflavin, 0.1 mL of 50 mM phosphate buffer (pH 7.8), and 0.1 mL of the enzyme solution.

One unit of SOD was defined as the amount of enzyme that inhibits 50% NBT reduction. The reaction mixture for determination of POD activity contained 1 mL of 0.2% guaiacol, 2 mL of 0.3% H_2O_2, 0.9 mL of 50 mM phosphate buffer (pH 7.0), and 0.1 mL of the enzyme solution. Activity of POD was determined by the increase in absorbance at 470 nm due to guaiacol oxidation, and one unit of the enzyme activity was defined as an increase in absorbance of 0.01 per min. The reaction mixture for determination of CAT activity contained 2 mL of 0.3% H_2O_2, 1.9 mL of H_2O, and 0.1 mL of the enzyme solution. The activity of CAT was measured by ultraviolet spectroscopy, monitoring the rate of decomposition of H_2O_2 at 240 nm. One unit of CAT activity was defined as a decrease in absorbance of 0.01 per min. The Coomassie Brilliant Blue G-250 assay was used to estimate protein content in plant tissues. The activity of the three enzymes studied was expressed in units per protein content.

2.4 Transmission electron microscopy investigation of root cell

At about 2 cm from the tip, the root was cut into small pieces, which were then fixed in 4% glutaraldehyde. Afterwards, the roots were rinsed in 0.1 M phosphate buffer, post-fixed with 1% osmium tetroxide and subject to a dehydration series (30%, 50%, 70%, 90%, 100%, 100% acetone). Pieces were then infiltrated with 812 resin and allowed to polymerize for 60 h (24 h at 35 °C, 24 h at 45 °C and 12 h at 60 °C). Ultra-thin sections were cut on an ultramicrotome (LKB2088 Ultramicotome, Sweden), stained with double staining of uranium and lead (acetic acid uranium-lead citrate), and examined using a transmission electron microscope (JEM 1010, Japan).

2.5 Statistical analysis

We used repeated measures ANOVA, with treatment as the main factor and sampling date 'time' as the repeated measure. To correct for violations of sphericity, the Greenhouse-Geisser adjustment was used. Significant differences among treatment means ($p < 0.05$) were determined by Bonferroni test. These data were analyzed using SPSS for Windows version 11.5 (Chicago, IL, USA).

3 Results

The different nutrient loadings had significant effects on the root growth and physiology of *V. natans* as evidenced by root biomass, protein content, and CAT activity (Table 1). The protein content, SOD activity, POD activity and CAT activity showed significant differences among the different sampling times (Table 1), which demonstrate that there were significant temporal variations in the root physiological characteristics of *V. natans*.

The differences for mean root parameters from different treatments are displayed in Figure 1. The analysis of mean root biomass per plant under different nutrient treatments revealed that plants grown under the NP-1 and NP-2 treatments had significantly higher mean root biomass per plant than did those grown under the NP-4 treatment (Figure 1A). Multiple comparison (repeated measures ANOVA, and the Bonferroni test) showed that the mean protein content per gram root fresh weight was significantly higher in the NP-4 treatment than in the other three treatments (Figure 1B); however, the mean CAT activity per milligram protein was significantly higher in NP-3 than in NP-1 and NP-4 (Figure 1E). Furthermore, the NP-3 plants have higher mean SOD activity per milligram protein and lower mean POD activity per milligram protein than the plants in other treatments, although differences among these treatments were not statistically significant (Figures 1C and 1D).

Table 1. Results of repeated measures ANOVA testing the effects of different nutrient loadings and treatment time on 5 parameters of *Vallisneria natans* roots.

Parameters	Factors and interactions	*F* value	*P* value
Biomass	Treatment	8.149	0.003**
	Time	2.680	0.091
	Treatment × Time	0.971	0.465
Protein content	Treatment	6.789	0.006**
	Time	19.687	<0.001***
	Treatment × Time	1.661	0.044*
SOD activity	Treatment	2.812	0.085
	Time	15.414	<0.001***
	Treatment × Time	3.011	0.022*
POD activity	Treatment	0.590	0.633
	Time	11.807	<0.001***
	Treatment × Time	2.142	0.005**
CAT activity	Treatment	6.719	0.007**
	Time	12.884	<0.001***
	Treatment × Time	2.047	0.044*

*: $p < 0.05$; **: $p < 0.01$; and ***: $p < 0.001$.

The changes of different physiological parameters under different nutrient loadings with time are presented in Figure 2. It showed a marked increase in root biomass with time under NP-1 and NP-2 treatments relative to other treatments, although the increase in differences among plant individuals was associated with plant growth and development (Figure 2A). Protein content per gram root fresh weight slightly increased and then gradually declined after 5 weeks of treatment (Figure 2B), while SOD, POD and CAT activity per milligram protein all sharply rose after week 13 and peaked at week 15 (Figures 2C–2E).

Root cell ultrastructure of *V. natans* in different treatments is displayed in Figure 3. There was a noticeable phenomenon in root ultrastructure. The root cells contained more prominent vacuoles, some of which partly or fully encircled the nucleus. Under the NP-1, NP-2 and NP-3 treatments, the nuclear envelope clearly revealed the double membrane structure, but it was discontinuous under NP-4 treatment (Figure 3).

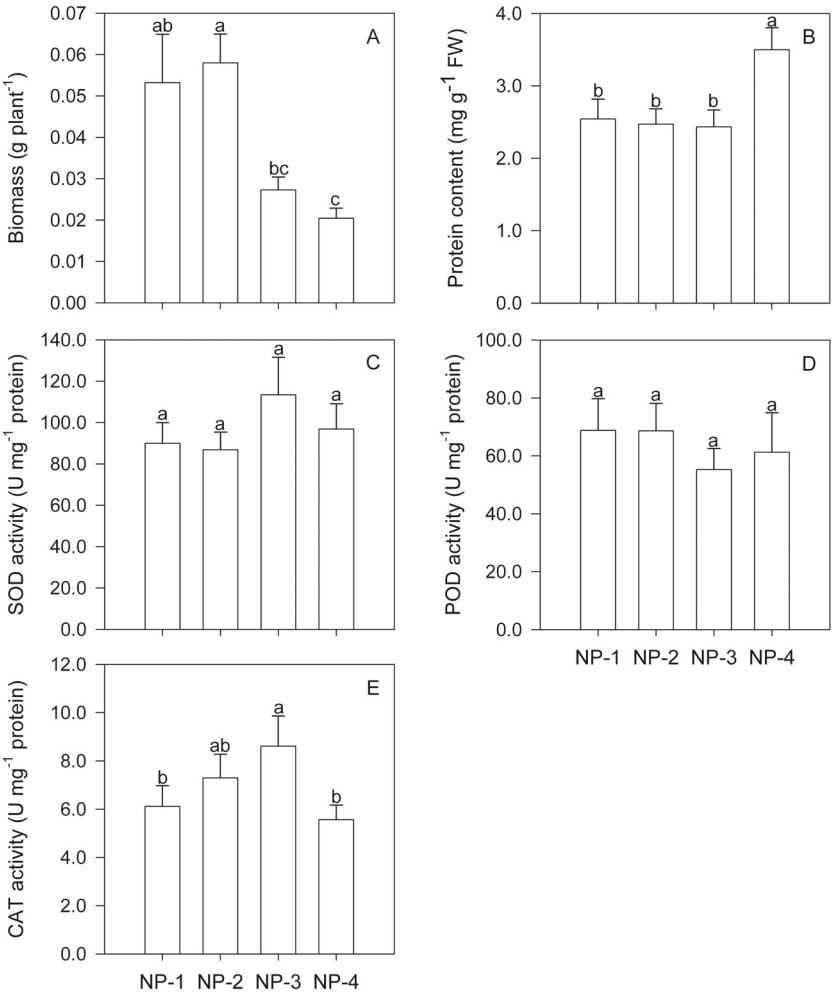

Fig. 1. Mean over the 17 week trial for each treatment of 5 parameters of *Vallisneria natans* roots (means ± SE). (A) biomass, (B) protein content, (C) SOD activity, (D) POD activity and (E) CAT activity. Different letters indicate significant difference at $p < 0.05$ (determined by mean separation with Bonferroni test). Increasing levels of N-P (in mg·L^{-1}): NP-1, 0.5, 0.05; NP-2, 1.0, 0.1; NP-3, 5.0, 0.5; and NP-4, 10.0, 1.0.

4 Discussion

The best root growth occurred in macrophytes exposed to NP-2 treatment. When comparing NP-1 and NP-2 treatments, we found that increased availability of N and P nutrients promoted root growth of *V. natans* (Figure 1A). The mechanism may be that increased nutrients induce plant photosynthetic carbon fixation and metabolism under the nutrient limitation (Turpin *et al.*, 1990; Turpin, 1991; Lee and Dunton, 1999). However, when comparing NP-3 and NP-4 treatments with the NP-2 treatment, it was clear that high nutrient loadings also induced declines in the growth of root (Figures 1A and 2A). These findings were consistent with Olsen and Valiela (2010).

Developing an understanding of the responses in root physiological characteristics of submerged macrophytes to increasing nutrient loadings is an important foundation for understanding the mechanisms underlying the poor growth of roots under high nutrient conditions. The degradation of proteins with time is one of the main symptoms of plant senescence (Lohman *et al.*, 1994; Criado *et al.*, 2007; Rolny *et al.*, 2011). Plant senescence is often associated with increased oxidative damage (Procházková and Wilhelmová, 2007). SOD is a critical component of antioxidative defense system in plants (Scandalios, 1993). CAT is another major antioxidant enzyme that protects plant cells from hydrogen peroxide (Witlekens *et al.*, 1995). POD is a multifunctional and ubiquitous enzyme found in plants, and is involved in numerous cellular processes such as development and stress responses (Jouili *et al.*, 2011). These antioxidant enzyme activities change markedly in response to oxidative stress. Hence, protein content per gram root fresh weight of all treatments gradually declined close to the end of the experiment and the huge fluctuation in root antioxidant enzyme activity after week 13 (Figures 2B–2E), implied that the root was subject to the stress of senescence at the end of the experiment. Additionally, extensive cell vacuolation is commonly seen in old or stressed cells (Visviki and Rachlin, 1994). The phenomenon of the root cells containing more prominent vacuoles also indicated the root was in the senescence stage.

When roots of *V. natans* were subjected to high nutrient loadings compared with NP-1 and NP-2 treatments, different responses in root physiological parameters were observed. The mean CAT activity per milligram protein was significantly

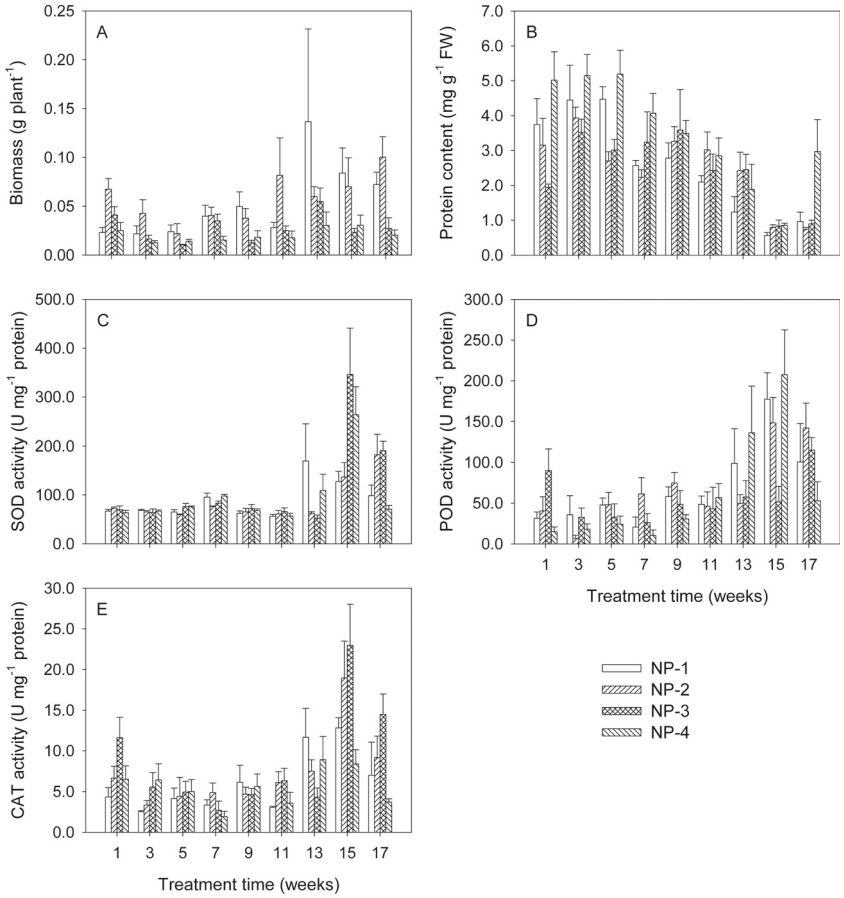

Fig. 2. Temporal variation in 5 parameters (means ± SE, n = 4) for *Vallisneria natans* roots under 4 different nutrient loadings. (A) biomass, (B) protein content, (C) SOD activity, (D) POD activity and (E) CAT activity. Increasing levels of N-P (in mg·L^{-1}): NP-1, 0.5, 0.05; NP-2, 1.0, 0.1; NP-3, 5.0, 0.5; and NP-4, 10.0, 1.0.

stimulated when the plants were exposed to NP-3 treatment (Figure 1E). It is possible that the increased mean CAT activity observed in the roots of NP-3 treatments plants contributes to a reduction in the oxidative damage. The NP-4 treatment considerably inhibited mean CAT activity of roots compared with the NP-3 treatment (Figure 1E), and significantly increased mean protein content (Figure 1B). Considering that a number of nitrogen-containing compounds accumulate in plants in response to environmental stress conditions (Rabe, 1990), these results suggested that NP-4 treatment enhances oxidative stress and impairs the antioxidant enzymic system in aging root of *V. natans*. Root cell ultrastructure was also altered with increasing nutrient loadings, and provided an evidence for oxidative stress. Transmission electron microscopy showed that under NP-4 treatment, the nuclear envelope was discontinuous, in contrast to the intact nuclear envelope of the 3 other treatments (Figure 3). When the reactive oxygen species levels exceed cellular antioxidant capacity, cellular structural and functional damage may occur (Yu, 1994; Fridovich, 1998; Choudhury and Panda, 2005). The alterations observed in the aging roots of plants exposed to high nutrient loadings might be due to an increase in the production of reactive oxygen species. Additionally, the poor root growth of submerged macrophyte at high nutrient loadings can reduce the anchorage strength of the plant, and can cause the sediment to become loose and unstructured, which may result in easy dislodgement of submerged macrophyte and resuspension of sediment by waves and currents. Our results may be of importance for the management of aquatic ecosystems, and may provide new insight into some of the mechanisms underlying the sudden switch from a clear macrophyte-dominated state to turbid phytoplankton-dominated state that occurs with eutrophication.

In conclusion, increased availability of N and P promoted root growth under low nutrient conditions, however, high nutrient loadings were unfavorable for root growth. Our results suggest that CAT could serve as an important component of antioxidant defense mechanism in aging root of *V. natans*, to protect against nutrient loading induced oxidative injury in the early period. Furthermore, our results showed that high nutrient loading enhanced oxidative stress in aging roots of the submerged macrophytes, impaired root function, and made the cell ultrastructure of the roots vulnerable to damage during senescence.

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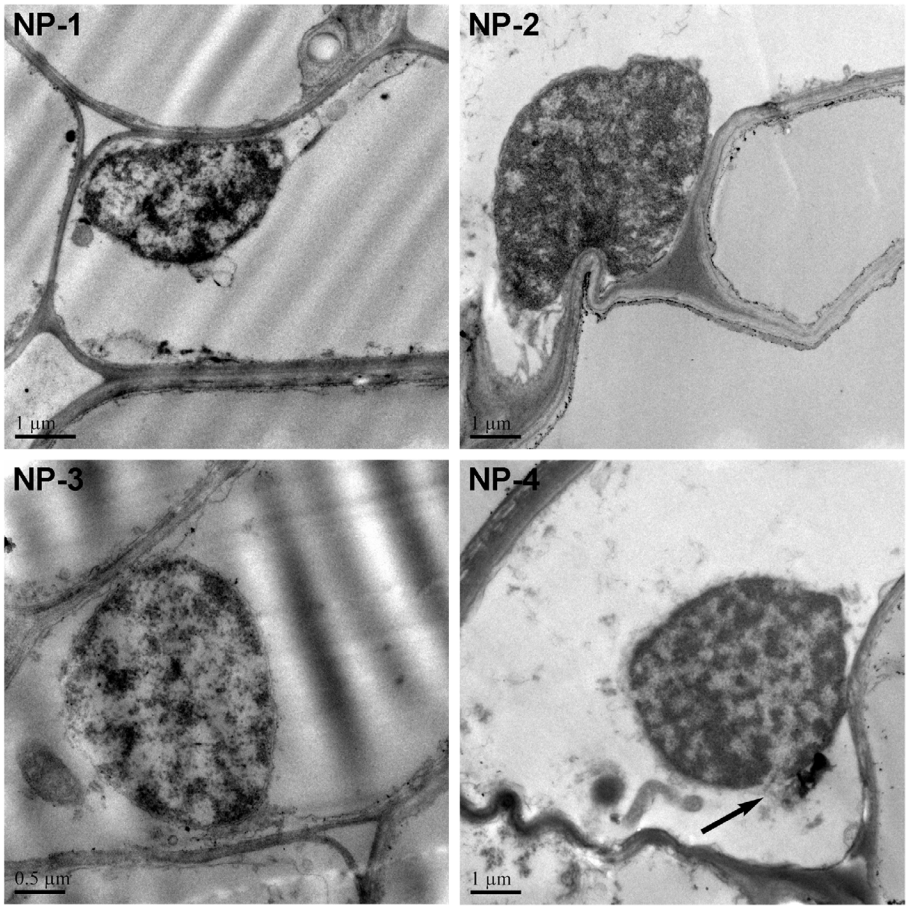

Fig. 3. Changes in root cell ultrastructure of *Vallisneria natans* under different nutrient loading treatments. Increasing levels of N-P (in $mg \cdot L^{-1}$): NP-1, 0.5, 0.05; NP-2, 1.0, 0.1; NP-3, 5.0, 0.5; and NP-4, 10.0, 1.0.

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