Chemosphere 77 (2009) 1019–1025

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Photosynthetic responses to heavy metal terbium stress in horseradish leaves

Lihong Wang [c], Qing Zhou [a,c,*], Xiaohua Huang [b,*]

[a] The Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, China
[b] Jiangsu Key Laboratory of Biofunctional Materials, College of Chemistry and Environment Science, Nanjing Normal University, Nanjing 210097, China
[c] School of Environmental and Civil Engineering, Jiangnan University, Wuxi 214122, China

ARTICLE INFO

Article history:
Received 24 August 2008
Received in revised form 20 June 2009
Accepted 29 July 2009
Available online 26 August 2009

Keywords:
Terbium (Tb)
Rare earth elements
Horseradish
Armoracia rusticana (Lam.) Gaerth.
Toxic effect

ABSTRACT

In order to understand the toxic effect of terbium (Tb(III)) on the plant photosynthesis, we investigated the photosynthesis, the ultrastructural structure of chloroplast, the subcellular distribution of horseradish peroxidase (HRP), the activity of HRP, and the content of malondialdehyde in horseradish by a portable gas exchange system, transmission electron microscopy and the other biochemical technologies. The results indicated that after horseradish treated with 5 mg L^{-1} Tb(III), the subcellular distribution of HRP was not obviously changed. However, the activities of guaiacol and ascorbate HRP were decreased comparing with that of horseradish treated without Tb(III). It could cause the peroxidation of membrane lipid, the damage of chloroplast ultrastructure and the decrease in the photosynthesis and the content of chlorophyll. Moreover, after horseradish treated with 60 mg L^{-1} of Tb(III), the distribution of HRP on the plasma membrane and tonoplast was decreased, while the distribution of HRP on the cell wall was increased comparing with that of horseradish treated without Tb(III). The change in the subcellular distribution of HRP could induce the excess accumulation of the reactive oxygen, leading to the damage of the chloroplast ultrastructure and then the decrease in the photosynthesis. Furthermore, the effects of Tb(III) on the indexes mentioned above were increased with prolonging the treating time of Tb(III). These results demonstrated that the function of HRP in horseradish treated with Tb(III) was decreased, leading to the damage of chloroplast ultrastructure and then the inhibition of photosynthesis. It was a possible toxic effect of Tb(III) on the plant photosynthesis.

1. Introduction

It has been reported that fertilizer containing rare earth elements (REEs) can improve the metabolism, yield and quality of crop since the 1970s (Hu et al., 2004). REEs have been also widely used in functional materials, catalysts, and additives due to their specially physical and chemical properties (Chen et al., 2006; Kakushima et al., 2007; Rodewald et al., 2007). The application of REEs has accelerated the transport and accumulation of REEs in the environment (Sun et al., 1996, 1998; Yang et al., 1999; Hu et al., 2002). The transport and accumulation of REEs inevitably influence the environmental safety and the human health (Zhu et al., 1996). Therefore, REEs have already been classified as the main environmental pollutants in China since 1990s (National Natural Science Foundation of China, 1996).

At the high concentration of REEs, they could inhibit the growth and development of plant (Diatloff et al., 1995, 1999; Zeng et al., 2006; Kobayashi et al., 2007). However, the inhibition mechanism is still unclear despite the efforts of many investigators (Hong et al., 1999; Hu et al., 2002; Zeng et al., 2006; Wang et al., 2008). As we known, plant photosynthesis is essential for the growth of plant. It has been reported that REEs can change the plant photosynthesis and then regulate the growth of plant (Hu et al., 2004; Yan et al., 2005). At the high concentration of REEs, they can inhibit the plant photosynthesis (Chen et al., 2001; Yan et al., 2005). However, until now, the inhibition mechanism of REEs, especially heavy REEs, on the plant photosynthesis is unknown. The biochemical behavior of the heavy REEs in plant is different from that of the light REEs (Ding et al., 2006; Ye et al., 2008). The heavy REEs exist in the environment and REEs fertilizer (Ni, 1995). Therefore, it is important to study the toxic effect of heavy REEs on the plant photosynthesis.

Terbium (Tb) is one of the heavy REEs and heavy metal elements (Babula et al., 2008). In China, the content of REEs in soil is about 76–629 mg kg^{-1}, while the maximum content of the soluble or available REEs is about 200 mg kg^{-1} (Xiong, 1995), in which they can be utilized by plant. Meanwhile, the accumulation content of REEs in plant is about 4–168 mg kg^{-1} (Xiong, 1995). The distribution of REEs in the tissues of many plants is in the order of leaf > root > stem (Hu et al., 2004). Therefore, 5 and 60 mg kg^{-1} Tb(III) were selected to treat horseradish leaves in order to

* Corresponding authors. Address: The Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University, Wuxi 214122, China (Q. Zhou). Tel.: +86 510 85326581; fax: +86 25 85891651.
E-mail addresses: qingzhou510@yahoo.com (Q. Zhou), wxxhhuang@yahoo.com (X. Huang).

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doi:10.1016/j.chemosphere.2009.07.065

investigate the toxic mechanism of Tb(III) on the plant photosynthesis. Here, the toxic effects of Tb(III) on the photosynthesis, the ultrastructure of chloroplast, the subcellular distribution of horseradish peroxidase (HRP), the activities of guaiacol and ascorbate HRP and the content of malondialdehyde (MDA) in horseradish were investigated by a portable gas exchange system, transmission electron microscopy (TEM) and the other biochemical technologies. Our results could provide some references to understand the toxic mechanism of REEs on plant. We suggested that the accumulation of REEs in the environment is the potential threat to the environmental safety and human health.

2. Materials and methods

2.1. Materials and treatment

The 1 g L^{-1} of $TbCl_3$ stock solution was prepared with dissolving appropriate quantities of terbium (III) chloride hexahydrate ($TbCl_3{\cdot}6H_20$, Aldrich Chemical Co.) in the distilled water. Using the stock solution, 5 mg L^{-1} and 60 mg L^{-1} $TbCl_3$ solutions with pH 6.5–6.6 were prepared. All the other reagents used were of analytical grade.

Horseradish (*Armoracia rusticana* (Lam.) Gaerth.) is a perennial herb of the brassicaceae family. It is widely used as a flavouring agent across the world (Veitch, 2004). In addition, horseradish is rich sources of horseradish peroxidase (HRP) (Veitch, 2004), which is an important industrial product.

Horseradishes were obtained from Nanyang Science and Technology Extension Station (Dafeng, China). They were planted in plastic pots in the middle of March (2006) with soil culture. The soil was sandy with pH 7.95. Its capacity of cation exchange was 16.2 me $(100\ g)^{-1}$. Its content of the organic matter was 0.83%. Its content of N, P_2O_5, K_2O and Tb was 75, 10, 68 and 0.033 μg g^{-1} soil, respectively. The soil was prepared with mixing soil, farmyard manure and P, K in accordance to the normal agricultural practice. The recommended content of P and K for horseradish was 3 and 1 g kg^{-1} soil, respectively. In the middle of August, 2006, the supplement of N was applied for horseradish, and the content of N was 1 g kg^{-1} soil. N, P and K were given in the form of urea, superphosphate and potassium sulfate, respectively. Each plastic pot (40 cm in diameter) was filled with 8 kg soil and three plants were grown in each pot. Horseradishes were grown in a glasshouse at 20–25 °C and a 16 h photoperiod with the photosynthetically active photon flux density of 300 μmol m^{-2} s^{-1}.

A 150 mL solution containing 5 or 60 mg L^{-1} Tb(III) solution with pH 6.5–6.6 was evenly sprayed on the leaves of a single 7-month-old horseradish until the drops began to fall. The same amount of distilled water was applied to another set as the control. All treatments were performed in triplicate. The horseradish leaves treated with and without Tb(III) for 24 h and 48 h were sampled and prepared for the measurement of the content of chlorophyll (Chl), the net photosynthetic rate (Pn), the concentration of intracellular CO_2 (Ci), the stomatic conductance (Gs), the transpiration rate (En), and the water use efficiency (WUE), the activities of guaiacol and ascorbate HRP and the content of malondialdehyde (MDA), and the observation of the chloroplast ultrastructure and the subcellular distribution of HRP in horseradish.

2.2. Measurements of the net photosynthesis rate and the content of chlorophyll

The Pn, En, Gs, and Ci were measured with a portable gas exchange system (CIRAS-1, PP Systems International Ltd., UK) under the culture condition of horseradish at 25 ± 2 °C. The photosynthetically active photon flux density was 300 μmol m^{-2} s^{-1}. The 320 μL L^{-1} CO_2 was provided by the CIRAS-1 photosynthesis system. The WUE was expressed with the ratio of Pn to En.

Chlorophyll was extracted with 80% acetone. The extract was centrifuged at 5300*g* for 10 min. Then the absorbance of the supernatant was read at 645 and 663 nm, respectively. The content of chlorophyll was calculated according to the equation $20.2A_{645} + 8.02A_{663}$ (Lichtenthaler, 1987).

2.3. Measurements of the activities of guaiacol and ascorbate HRP and the content of MDA

Frozen fresh leaves were ground to fine powders with a mortar and pestle under liquid nitrogen. The proteins were then extracted at 4 °C with a cold 50 mM potassium phosphate (pH 7.0). The homogenate was centrifuged at 4 °C for 20 min at 12 000*g*. The supernatant was the extract of the crude enzyme. The activity of guaiacol HRP was measured according to the literature (Hammerschmidt et al., 1982). The reaction mixture contained phosphate buffer (25 mM, pH 7.0), guaiacol (0.05%), 10 mM H_2O_2, and the extract. Activity was determined by the increase in absorbance at 470 nm due to the oxidation of guaiacol (E = 26.6 mM^{-1} cm^{-1}). Ascorbate peroxidase was determined according to the literature (Garcia-Limones et al., 2002). The reaction mixture consisted of 100 μL ascorbate (5 mM), 100 μL H_2O_2 (100 mM), 100 μL EDTA (1 mM), 100 μL of the extract and was completed to a final volume of 1 mL with 125 mM potassium phosphate buffer (pH 7.0). The oxidation of ascorbate was determined by the increase in absorbance at 290 nm (E = 2.8 mM^{-1} cm^{-1}).

The level of the membrane lipid peroxidation was expressed as the content of MDA. The content of MDA was determined according to the literature (Shah et al., 2001). About 1 g fresh leaves were ground in 0.25% 2-thiobarbituric acid (TBA) in 10% trichloroacetic acid (TCA) using a mortar and pestle. Following 15 min heating at 95 °C, the mixture was quickly cooled in an ice bath and centrifuged at 10 000*g* for 10 min. The absorbance of the supernatant was read at 532 nm and corrected for unspecific turbidity by deducting the absorbance of the supernatant at 600 nm and 440 nm. The blank was 0.25% TBA in 10% TCA. The concentrations of lipid peroxides together with oxidative modified proteins of plants were thus quantified in terms of MDA level. The content of MDA was expressed as equation:

$$\mathrm{MDA(nmol/mL)} = 10^6 \times (A - B/157\,000) \quad (1)$$

$$A = Abs_{532+\mathrm{TBA}} - Abs_{600+\mathrm{TBA}} - (Abs_{532-\mathrm{TBA}} - Abs_{600-\mathrm{TBA}}) \quad (2)$$

$$B = (Abs_{440+\mathrm{TBA}} - Abs_{600+\mathrm{TBA}}) \times 0.0571 \quad (3)$$

Here 157 000 was the molar extinction coefficient for MDA. The molar absorbance of 1–10 mM sucrose at 532 nm and 440 nm was 8.4 and 147, respectively, giving a ratio of 0.0571.

2.4. TEM observation of the chloroplast ultrastructure and the subcelluar distribution of HRP in horseradish

To establish the subcellular distribution of HRP in horseradish leaf, 3,3,-diamino benzidine (DAB) an electron-dense peroxidase stain was employed (Bestwick et al., 1998; Andrews et al., 2002). Fresh leaves treated without and with Tb(III) were sliced into 1.5 × 2 mm and placed in 50 mM sodium cacodylate buffer solution (Buffer A) (pH 7.0) with 1% (v/v) glutaraldehyde and 1% (v/v) paraformaldehyde for 2 h at 4 °C. It was washed with buffer A solution for three times, and each for 1 h. Then the sample was rinsed for 30 min with Buffer B solution (50 mM potassium phosphate buffer solution with 20 mM 3-amino-1,2,4-triazole (ATZ)). ATZ in the Buffer B solution can prevent from peroxidative artefacts