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Original Research Paper

Effects of Paper Mill Wastewater on Seedling Growth and Antioxidant System of Reeds

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ABSTRACT

Effects of different concentrations of wastewater (chemical oxygen demands of 300, 175 and 50 mg·L^{-1}) from a paper mill on seedling growth and antioxidant system in reeds were tested in experimental pools that simulated the wetland ecosystem. Root length, biomass and moisture content, but not shoot moisture content and plant height, significantly increased with increases in wastewater concentration. At 300 mg·L^{-1}, shoot biomass increased by 52.5% and root biomass increased by 73.05% over the control. Malondialdehyde content, production rate of superoxide anions and hydrogen peroxide content all decreased with increasing concentration. At 300 mg·L^{-1}, malondialdehyde content, production rate of superoxide anions and hydrogen peroxide content were 0.34, 0.24 and 0.16 times, respectively, those of the control in leaves and were 0.25, 0.19 and 0.17 times, respectively, those of the control in roots. Superoxide dismutase, peroxidase and catalase activities and ascorbic acid and glutathione contents in reeds significantly increased with increasing concentrations of wastewater. These results suggest that a concentration of 300 mg·L^{-1} could improve the activities of antioxidant enzymes, inhibit the generation of reactive oxygen species and reduce the generation of malondialdehyde, thus effectively alleviating the damage caused by salinity in wetland soil.

INTRODUCTION

The Panjin Shuangtai estuarine wetland, a transit point on the western Pacific bird migration route, lies in the north of the Bohai Sea in northeastern China. The wetland's ecosystem has been preserved intact, but increased industrialization, global warming, rising sea levels and intrusion of saltwater are seriously increasing the salinity of the soil (Wang et al. 2012). High levels of soluble salts in soils harm plants. Plants, can reduce the damage by altering their morphological characteristics, osmotic properties and levels of antioxidants (Liu et al. 2004, Gomez et al. 2004).

Phragmites communis, the common reed, is one of the major types of vegetation in the Shuangtai estuarine wetland and is a pioneer species that restores moderately saline-alkaline wetland and removes impurities released by a local paper mill. Many paper mills in China discharge large quantities of wastewater into wetland, mostly distributed along rivers. With proper treatment, however, this wastewater can be used for irrigation, alleviating the shortage of irrigation water from wetland, and can promote plant growth and improve soils (Kiziloglu et al. 2008, Ding et al. 2005). The effects on plant growth of wastewater used for irrigation have been studied (Li et al. 2008, Chung et al. 2008), but the influence on antioxidants in reeds has rarely been reported. In this study, we monitored seedling growth, membrane lipid peroxidation, reactive oxygen species and antioxidants in reeds by irrigating experimental pools, simulating the Liaoning Shuangtai ecosystem, with different concentrations of paper mill wastewater. The studies may serve to provide reference point for the ecological restoration of salinized wetland.

MATERIALS AND METHODS

Study area: The Shuangtai estuarine national nature reserve (40°452' N-41°102' N, 121°302' E-122°002' E), established in 1988 with the approval of the State Council, is one of the largest wetland nature reserve in China and is an important international wader reserve. The reserve has a warm temperate monsoon climate with an annual average temperature of 8.3°C and an annual rainfall of 623.2 mm. Soil types in the region are mainly coastal saline soil and swampy soil, which are composed of seawater and river alluvium. In recent years, though, global warming, decreased precipitation and increased inputs of industrial and agricultural water are reducing the reserves of clean water, leading to the degeneration of the reed wetland and the red beaches common in the area. *Phragmites communis*, *Suaeda heteroptera* and

Tamarix dominate the vegetation in the reserve, with *P. communis* being a pioneer species.

Materials: Selected rhizomes for transplanting were cut from ungerminated reeds from the experimental areas of Shuangtai estuarine wetland. These academic research activities are freely allowed in the experimental areas of nature reserve without the approval of related government management department under the management system of China. Soil samples were also collected from same places. The soil type was coastal saline soil with a bulk density of 1.03 g/cm^3, pH of 8.5 and organic matter content of 1.046%. The experiment began in 2012 in the comprehensive test base of the College of Water Conservancy of Shenyang Agricultural University.

Wastewater was collected from the effluent of the Jincheng paper mill in Liaoning province. The wastewater had a pH of 8.3, chemical oxygen demand (COD) of 300 mg/L, total nitrogen content of 29.5 mg/L and total phosphorus content of 3.28 mg/L.

Concrete test pools were constructed for simulating natural wetland. Each pool was 2m × 1m × 0.8m (length × width × internal depth) on a foundation embedded 0.8m in the ground. The rhizomes were transplanted into the soil collected from the estuary and were irrigated with wastewater as shown in Fig. 1.

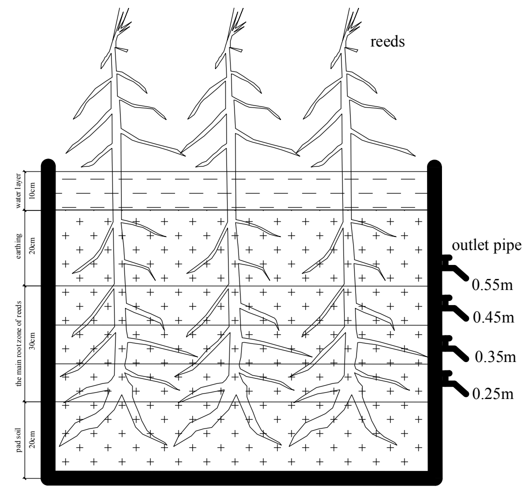

Fig. 1: Cross-section diagram of test device.

Experimental design: The collected wastewater had a COD concentration of 300 mg/L and was diluted with tap water to produce CODs of 175 and 50 mg/L. One control pool (CK, COD = 0 mg/L) was irrigated with tap water. At germination, each experimental pool was irrigated with 0.2 m^3 of water, and the reeds were harvested after three weeks. The roots were separated from the shoots and then washed with tap water and deionized water. The roots and shoots were weighed to obtain the fresh weight (FW), dried for 20 min at 105°C and finally dried at 80°C. The differences between the fresh and dry weights gave the moisture contents of the roots and shoots. At least two independent experiments with three replicates were performed.

Physiological indices: Malondialdehyde (MDA) content was measured with a thiobarbituric acid colorimetric assay (Li et al. 2000). The production rate of superoxide anions ($O_2^{\bullet-}$) in μmol/g FW/min was determined by the hydroxylamine deionized water hydrochloride method (Wang et al. 1990). Hydrogen peroxide (H_2O_2) content was measured with a spectrophotometer (Zou et al. 2000). Superoxide dismutase activity was determined by the nitro blue tetrazolium (NBT) method (Dhinds 1981), where one unit of activity is defined as 50 percent inhibition of NBT photochemical reduction per unit time. Peroxidase (POD) activity was measured with the guaiacol colorimetric assay (Zhang et al. 1990) and represented as changes of optical density per minute at a wavelength of 470 nm. Catalase (CAT) activity was determined by ultraviolet absorption spectrometry (Carmak & Marschner 1992), where one unit of activity was defined as a 0.1 reduction in optical density per minute at a wavelength of 240 nm. Ascorbic acid (AsA) content was determined by spectrophotometry (Law et al. 1983), and glutathione (GSH) content was determined by ultraviolet spectrophotometry (Nagalakshmi & Prasad 2001).

Data processing and analysis: Data are presented as means ± standard deviations. A one-way ANOVA was tested for differences among plant samples irrigated with different concentrations of wastewater. Ducan's test was used for multiple comparisons between treatments at a significance level of 0.05. Significant differences at $P < 0.05$ are indicated by different letters in the figures.

RESULTS AND DISCUSSION

Effects of wastewater on reed growth: The effects of different wastewater concentrations on reed growth are given in Table 1. Root length, FW and moisture content increased significantly with increasing concentration. Plant heights and the moisture contents of shoots were not significantly different from the control at concentrations of 50 and 175 mg/L. Plant height and shoot moisture content were significantly higher at a concentration of 300 mg/L than at concentrations of 175 mg/L or lower. Compared to CK, concentrations of 300 and 175 mg/L produced respective increases of 26.88% and 61.59% in plant height, 21.70% and 31.25% in root length, 49.38% and 52.50% in shoot FW, 73.05% and 106.7% in root FW, 1.45% and 3.04% in shoot moisture content and 3.12% and 4.60% in root moisture

Table 1: Effects of paper mill wastewater on reed growth. * indicates a significance level of 0.05, ** indicates a significance level of 0.01, *** indicates a significance level of 0.001. Sig. indicates the significance level.

COD (mg/L)	Plant height (cm)	Root length (cm)	Fresh weight (g/plant)		Moisture content (%)	
			Shoot	Root	Shoot	Root
300	14.43±0.97a	2.52±0.04a	7.01±0.14a	1.94±0.12a	80.36±1.62a	94.28±0.59a
175	11.33±1.49b	2.34±0.06b	6.87±0.08a	1.63±0.11b	79.12±0.25ab	92.94±0.43b
50	11.24±0.89b	2.12±0.06c	6.27±0.08b	1.34±0.12c	78.57±0.63ab	91.16±0.22c
CK	8.93±0.63b	1.92±0.04d	4.60±0.12c	0.94±0.14d	77.99±0.41b	90.13±0.66d
Sig.	**	***	***	***	ns	***

content. The changes in the moisture contents of the shoots were not significantly different from those of the roots. The wastewater promoted the growth of reeds to some extent, and the effects were generally greater on roots than on shoots.

Effects of wastewater on MDA: Fig. 2 indicates that MDA content was affected significantly ($P< 0.01$) at different concentrations of wastewater, decreasing with increasing concentration. At concentrations of 0 (CK) and 50 mg/L, MDA content was higher in the roots than in the leaves. MDA content in the roots was 1.17 and 1.09 times higher than in the leaves at concentrations of 0 and 50 mg/L, respectively. MDA contents were considerably lower at concentrations of 175 mg/L and 300 mg/L. Compared to CK, MDA contents at these concentrations respectively decreased by 39.44% and 39.44% in leaves and by 49.35% and 49.35% in roots. MDA contents were slightly higher in leaves than in roots. Leaves accumulated more MDA at higher concentrations, perhaps due to lower levels of soluble salt ions, higher soil organic matter contents or an improved growth environment for reeds after irrigation with wastewater.

Effects of wastewater on the generation of reactive oxygen species: The production rate of $O_2^{\bullet -}$ in reed leaves decreased with increasing wastewater concentrations (Fig. 3A), which at all concentrations were significantly lower than in CK ($P< 0.01$). At concentrations of 50, 175 and 300 mg/L, the production rates of $O_2^{\bullet -}$ were 0.70, 0.39 and 0.24 times, respectively, that of CK. The changes in the production rate of $O_2^{\bullet -}$ in roots were similar to those in leaves,which at all concentrations were significantly lower than that in CK ($P< 0.01$). The production rate of $O_2^{\bullet -}$ was significantly lower at 175 than at 300 mg/L, with values of 0.319 and 0.332 μmol/g FW/min, respectively. These values were 0.35 and 0.19 times lower, respectively, than that of CK. The production rate of $O_2^{\bullet -}$ was higher in leaves than in roots, especially at a concentration of 300 mg/L, where the rate was 50.68% higher.

The H_2O_2 contents in leaves decreased with increasing wastewater concentration (Fig. 3B), which at all concentrations were significantly lower than in CK ($P< 0.01$). At concentrations of 50, 175 and 300 mg/L, the H_2O_2 contents were 0.48, 0.33 and 0.16 times, respectively, that of CK and differed very significantly at different concentrations ($P< 0.01$). The changes in H_2O_2 contents were similar in roots and leaves,which at all concentrations were significantly lower than in CK ($P< 0.01$). At 50, 175 and 300 mg/L, the H_2O_2 contents were 0.40, 0.34 and 0.19 times, respectively, that of CK and differed very significantly at different concentrations ($P< 0.01$).

Effects of wastewater on the activity of antioxidant enzymes: SOD activity in the reeds increased with increasing wastewater concentration and was higher in roots than in leaves. Activities at all concentrations differed significantly from that of CK ($P< 0.01$) (Fig. 4A). At concentrations of 50, 175 and 300 mg/L, the SOD activities in leaves were 1.62, 2.49 and 3.41 times, respectively, that of the control and differed very significantly at different concentrations ($P< 0.01$). The SOD activities in roots were 1.55, 2.11 and 2.64 times, respectively that of the control and differed very significantly at different concentrations ($P< 0.01$).

POD activity in the reeds increased with increasing wastewater concentration, which at all concentrations differed significantly from that of CK ($P< 0.01$) (Fig. 4B). At concentrations of 50, 175 and 300 mg/L, the POD activities in leaves were 1.37, 2.00 and 2.36 times, respectively, that of CK. The POD activities in roots were 1.12, 1.31 and 1.54 times, respectively, that of CK and differed very significantly at different concentrations ($P< 0.01$). POD activity was higher in roots than in leaves.

CAT activity in leaves increased modestly with increasing wastewater concentration, which at all concentrations differed significantly from that of CK ($P< 0.01$) (Fig. 4C). At concentrations of 50, 175 and 300 mg/L, the CAT activities were 13.64%, 26.04% and 40.47%, respectively, higher than that of CK. The CAT activities were higher in roots than in leaves. At concentrations of 50, 175 and 300 mg/L, the CAT activities were 14.75%, 41% and 71.31%, respectively, higher than that of CK. The CAT activities

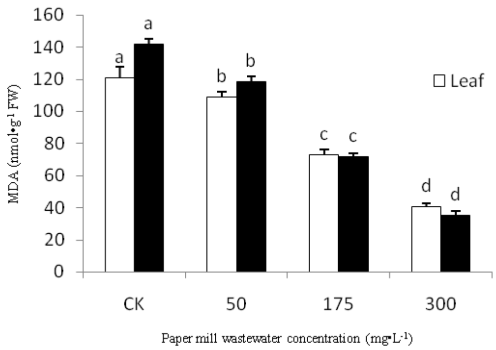

Fig. 2: Effects of paper mill wastewater on MDA content.

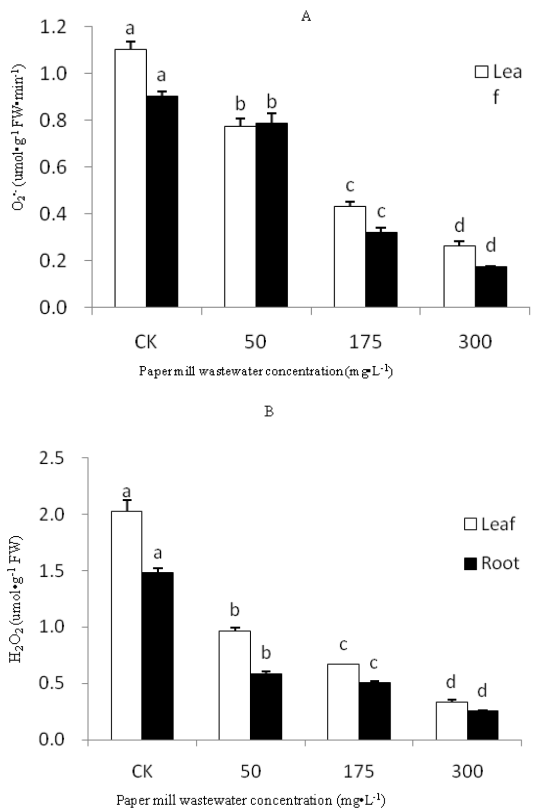

Fig. 3: Effects of paper mill wastewater on production rate of O_2^{-} (A) and H_2O_2 content (B).

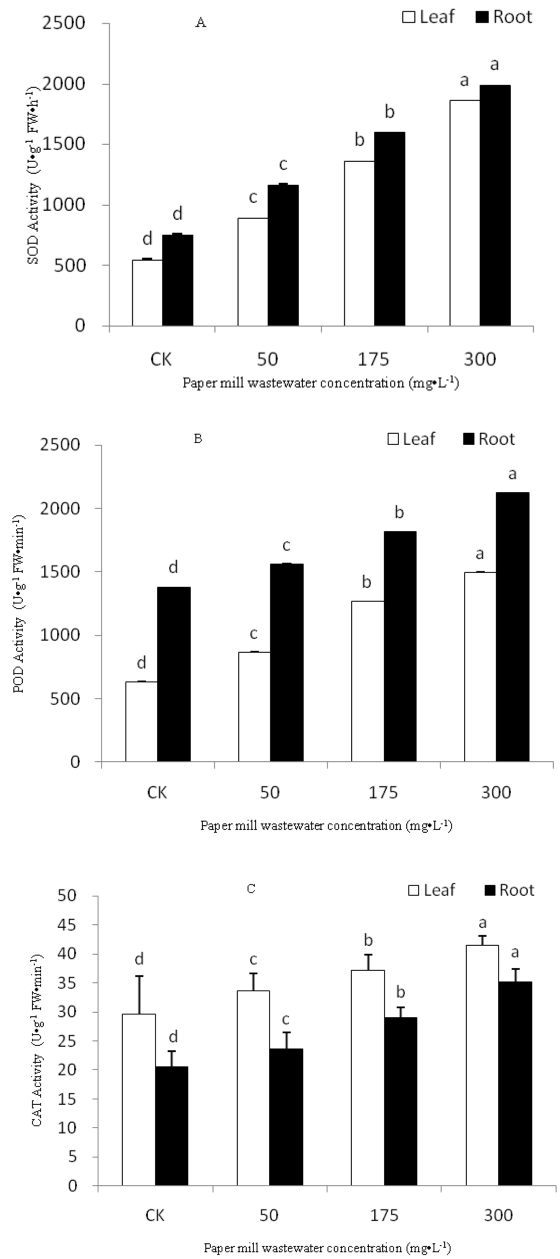

Fig. 4: Effects of paper mill wastewater on the activity of the antioxidant enzymes SOD (A), POD (B) and CAT (C).

differed very significantly at different concentrations ($P< 0.01$).

Effects of wastewater on antioxidants: The AsA content in leaves increased with increasing wastewater concentration (Fig. 5A) and reached a maximum of 10.52 mg/100g FW at 300 mg/L. At concentrations of 50, 175 and 300 mg/L, the AsA contents were 1.03, 1.12 and 1.24 times, respectively, that of CK and differed significantly from each other at all concentrations ($P< 0.01$). The AsA contents were higher in roots than in leaves. At concentrations of 50, 175 and 300 mg/L, the AsA contents were 1.04, 1.18 and 1.35 times, respectively, that of CK.

The GSH content in reeds increased with increasing wastewater concentration, and was higher in roots than in leaves and at all concentrations differed significantly from that of CK ($P< 0.01$) (Fig. 5B). At concentrations of 50, 175

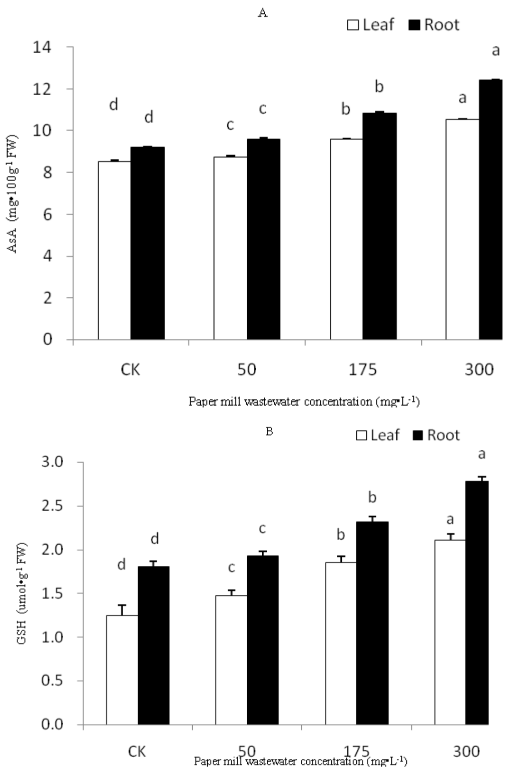

Fig. 5: Effects of paper mill wastewater on antioxidants AsA (A) and GSH (B).

and 300 mg/L, the GSH contents in leaves were 1.18, 1.48 and 1.68 times, respectively, that of CK and differed significantly from each other at all concentrations ($P< 0.01$). The GSH contents in roots were 1.07, 1.28 and 1.54 times, respectively, that of CK. It was represented that the GSH content in reed root was higher than the leaf from the figure.

Under normal physiological conditions, the antioxidant system of plants can provide sufficient protection against reactive oxygen damage, thereby avoiding physical disorders caused by reactive oxygen species. Under conditions of stress, plants will produce more reactive oxygen species, leading to the peroxidation of membrane lipids and the accumulation of peroxidation products such as MDA, thereby damaging the membrane system and affecting plant metabolism (Rina & Guilin 2013). The experimental results indicated that MDA content, production rate of $O_2^{\cdot-}$ and H_2O_2 content were higher in the control than in the treatments of paper mill wastewater. Salinization produces free radicals and causes membrane lipid peroxidation in reeds, but irrigation with wastewater can relieve these reactions, improve the chemical properties of the soil of saline wetland and prevent the accumulation of salt in the soil (Ma et al. 2010). MDA content in roots was higher than in leaves at 50 mg/L but was lower at higher concentrations of wastewater (Fig. 2), similar to the response of Indian mustard to wastewater from a brewery (Bharagava et al. 2008). In addition, high concentrations of wastewater decreased the ratio of the shoot/roots FW (Table 1), which indicated that shoots were more sensitive to the wastewater than roots (Singh et al. 2009), as were MDA content, production rate of $O_2^{\cdot-}$ and H_2O_2 content.

Antioxidant enzymes such as SOD, POD and CAT are synergistic and reduce excessive levels of reactive oxygen species to maintain the balance of reactive oxygen metabolism, protect membrane structure, and allow plants to survive and relieve or resist damage from stress to some extent (Liang et al. 2003). SOD is the first line of defence against reactive oxygen species, neutralizing superoxide and converting O_2^- into H_2O_2. CAT and POD then convert H_2O_2 into H_2O, preventing the accumulation of H_2O_2 and $O_2^{\cdot-}$ (Jin et al. 2010). In this paper, SOD, POD and CAT activities in reeds increased with increasing wastewater concentration, perhaps due to heavy metal ions in the wastewater inducing the expression of genes encoding these antioxidant enzymes (Nayek et al. 2010). In addition, SOD and POD activities were higher in roots than in leaves. Roots absorb most of the minerals and water and are first to experience stress and to receive direct damage (Somka et al. 2008), so plants can best adapt to stress by increasing the activities of SOD and POD in roots. Antioxidants such as AsA and GSH are also responsible for the removal of H_2O_2 (Mallick & Mohn 2000) and play an important role in preventing damage to plants by reactive oxygen, usually by a two-phase coupling effect (Nagalakshmi & Prasad 2001). The research results indicated that AsA and GSH contents in reeds increased with increasing concentrations of wastewater, consistent with the response of plants to tannery sludge (Sinha et al. 2009).

CONCLUSIONS

1. As a pioneer species of the Liaoning Shuangtai estuarine wetland, reeds were able to adapt to the paper mill wastewater, growing differently under different concentrations of wastewater. Plant height, root length, biomass and moisture content were maximal at 300 mg/L, where plants had an average height of 14.43 cm, root length of 2.52 cm, shoot biomass of 7.01 g/plant, root biomass of 1.94 g/plant, shoot moisture content of 80.36% and root moisture content of 94.28%. Irrigation with paper mill wastewater promoted the growth of the reeds.
2. MDA content, the production rate of $O_2^{\cdot-}$ and H_2O_2 content differed significantly at different concentrations of wastewater, decreasing with increasing wastewater concentration, i.e. were minimal at a concentration of 300 mg/L. We propose that irrigation with paper mill

wastewater can improve the physical and chemical properties of the soil, alleviate salinization and reduce reactive oxygen content, thereby promoting the growth of plants.

3. SOD, POD and CAT activities and AsA and GSH contents significantly increased in reed leaves and roots with increases in wastewater concentration. Wastewater can promote the activity of antioxidant enzymes and the content of antioxidants in reeds.
4. The research results suggest that paper mill wastewater can significantly promote the activity of antioxidant enzymes and the content of antioxidants and inhibit the generation of reactive oxygen species, thus promoting plant growth.

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