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Highlighted article

Assimilation and physiological effects of ferrocyanide on weeping willows

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ABSTRACT

Uptake, assimilation, and toxicity of exogenous iron cyanide complexes in plants were investigated. Pre-rooted young weeping willows (*Salix babylonica* L.) were exposed to hydroponic solutions spiked with potassium ferrocyanide at 24.0±1 °C for 192 h. Transpiration rates, chlorophyll contents, soluble protein, and activities of superoxide dismutases (SOD), catalase (CAT), and peroxidase (POD) of the plants were monitored to determine toxicity to the cuttings. Of all selected parameters, POD activity in leaves was the most sensitive bioindicator to the increase of ferrocyanide concentrations. Between 11% and 19% of applied ferrocyanide in the solutions was removed by willows at the end of the incubation period. Only small amounts of ferrocyanide were recovered in different parts of the plant materials. Mass balance analysis showed that more than 90% of the ferrocyanide taken up from the hydroponic solutions was assimilated by plants. The assimilation of ferrocyanide by plants showed a dose-dependent manner. These findings suggest that phytoremediation of ferrocyanide-contaminating wastewater and soils can be possible for the environmental cleaning up.

1. Introduction

It is evident that all vascular plants, numerous fungi and algae, naturally produce cyanide as by-products in the synthesis of the plant hormone and pheromone ethylene (Peiser et al., 1984). The majority of cyanide in the environment, however, originates from anthropogenic activities. It is estimated that more than 100,000 tons of cyanide enters the environment annually (Mudder and Botz, 2001). Free cyanide in the environment reacts quickly with available metal ions to form stable complexes, e.g., ferrocyanide $Fe^{II}(CN)_6^{4-}$ and ferricyanide $Fe^{III}(CN)_6^{3-}$ (Theis and West, 1986; Meeussen et al., 1992). The complexity of the chemistry of CN and its complexes makes it very difficult to predict the fate of CN in the environment. Indeed, iron cyanide complexes can be the dominant chemical forms in soil and groundwater, and may be present at concentrations >1% on dry weight (DW) basis in contaminated media (Samiotakis and Ebbs, 2004).

Toxic effects of chemicals to plants can be measured in different ways in which metabolic responses of vascular plants to chemicals have been grouped in three categories: unique features, common features, and growth parameters (Nellessen and Fletcher, 1993). In plants, adverse environmental conditions often lead to the elevated generation of reactive oxygen species and, consequently, superoxide dismutases (SOD), catalase (CAT), and peroxidase (POD) have been proposed to be important biochemical indicators in plant stress response and tolerance (Tsang et al., 1991). The primary source of toxicity observed from CN and its complexes is free cyanide to organisms (Shifrin et al., 1996; Larsen et al., 2004, 2005; Yu et al., 2005a, b, c), while no toxic signs on basket willows (*Salix viminalis*) exposed to ferro- or ferricyanide at 10 mg CN L^{-1} has been found (Larsen and Trapp, 2006). A similar result has been reported in the study by Samiotakis and Ebbs (2004), where barley, oat, and wild cane exposed to 50 mg ferrocyanide L^{-1} without any adverse effects. Additionally, Prussian blue, an iron complexed form of cyanide, was not lethal at levels up to 2500 mg L^{-1} (Larsen et al., 2005).

Degradation of ferrocyanide by microorganisms was confined to a limited number of bacterial and fungal strains (Barclay et al., 1998a, b; Dursun et al., 1999). Unlike botanical assimilation of free cyanide through the cyanoalanine pathway (Miller and Conn, 1980; Maruyama et al., 2001), direct evidence of the plant-mediated degradation of ferrocyanide is still under investigation, although several studies have been conducted to clarify the possible mechanisms involved (Ebbs et al., 2003; Samiotakis and Ebbs, 2004; Larsen and Trapp, 2006; Yu et al., 2006b). However, uptake of ferrocyanide by plants is possible, and is probably followed by metabolism inside plants (Ebbs et al., 2003; Larsen and Trapp, 2006). Ebbs et al. (2006) also proposed that phytophotolysis (light-mediated dissociation within plant tissues) as it was observed for some organic chemicals and might play a role in the assimilation and transport of iron cyanide complexes.

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In a recent work by Kang et al. (2007), approximately 19% of the iron cyanides (Prussian blue) in the cyanogenic plant species, sorghum (*Sorghum bicolor* var P 721), and flax (*Linum usitassimum* var Omega-Gold), was transformed, but 7% of the ^{14}C-labeled cyanide was converted to $^{14}CO_2$ by sorghum and 6% by flax and a small amount of non-altered cyanide was shown to be associated with the plants. Thus, if plants can metabolize free cyanide and take up iron cyanide complexes, they could be used more effectively for phytoremediation of cyanide polluted soils. The objectives of this investigation were to ascertain the extent of ferrocyanide uptake, assimilation and accumulation in weeping willows, and to determine the effects of iron cyanide complex on metabolic activities of weeping willows.

2. Materials and methods

2.1. Trees specimens and exposure regimes

Weeping willows (*Salix babylonica* L.) were sampled from those grown on the campus of Hunan Agricultural University, PR China. Tree cuttings of 40 cm in length were removed from a single mature tree and all cuttings used in this study were obtained from a single tree. They were placed in buckets of tap water maintained at room temperature of 15–18 °C under natural sunlight until new roots and leaves appeared. After a 2-month of growth, each young rooted cutting was transferred to a 250 mL Erlenmeyer flask filled with approximately 200 mL modified ISO 8692 nutrient solution (Table 1), which was prepared from reagent grade chemicals. The flasks were all sealed with cork stoppers around the tree and silicon sealant (Dow Chemical Co., Midland, MI) between the tree stem and the flask to prevent escape of water or chemicals, and wrapped with aluminum foil to inhibit potential growth of algae in flask. All flasks were housed in a climate control chamber kept at a constant temperature of 24.0±1 °C under natural sunlight (light:dark cycle 14:10 h). The plants were first conditioned for 48 h to allow adaptation to the new environmental conditions. Then, the weight of the plant–flask system was measured and recorded individually. The flasks including the tree cuttings were weighed again after 24 h. By doing this way, the transpiration rate was calculated for the seedling growing in each flask. Tree seedlings with a similar transpiration rate were selected and grouped for the tests. The nutrient solution in each flask was replaced by spiked solution, except for the controls. Ferrocyanide used was in the chemical form of potassium ferrocyanide [$K_4Fe(CN)_6$] of analytical grade with ⩾95% purity. It should be noted that 1 mg $K_4Fe(CN)_6$ equals to 0.4239 mg CN. For each treatment, nine replicates were prepared. Different concentrations of potassium ferrocyanide, between 0 (control) and 423.9 mg CN L^{-1}, were prepared in nine replicates of willow cuttings. Additionally, two sets of controls were made: one control was with ferrocyanide, but without plant cuttings to quantify the effects of loss during handling, volatilization, hydrolysis and/or degradation by microorganism; the other was with trees in the nutrient solution without addition of ferrocyanide to quantify the transpiration rate of the non-exposed control trees. To prevent precipitation of cyanide complexes, the only iron source in the nutrient solution was the ferrocyanide.

2.2. Normalized relative transpiration

The transpiration rate of plants is coupled to the photosynthesis, and an inhibition of transpiration is a reliable and quick measure of toxic effects (Trapp et al., 2000). The weight loss, compared to initial loss, was the toxicity criteria. To compare the toxic effect on tree cuttings with different initial transpiration (before the toxicant is added), the weight loss is expressed as relative transpiration. The transpiration was normalized with respect to the initial transpiration (to eliminate the necessity of finding cuttings with similar initial transpiration) and with respect to the transpiration of non-contaminated control cuttings (to include the effect of normal growth of the tree cuttings during the test). The mean normalized relative transpiration (NRT) was calculated by the following equation:

$$\mathrm{NRT} = \frac{(1/n)\sum_{i=1}^{n} T_i(C,t)/T_i(C,0)}{(1/m)\sum_{j=1}^{n} T_j(0,t)/T_j(0,0)},$$

where C is concentration (mg L^{-1}), t is time period (d), T is absolute transpiration (g d^{-1}), i is replicate 1, 2, …, n and j is control 1, 2, …, m. The NRT of controls is always set at 100%; NRT <100% shows inhibition of the trees' transpiration, which is usually connected with other effects (reduced growth, and, in severe cases, death).

Table 1
Composition of the modified ISO 8692 nutrient solution used in this study

Macronutrients (μmol L^{-1})		Micronutrients (nmol L^{-1})	
$NaNO_3$	2823.9	H_3BO_3	2992.1
$MgCl_2 \cdot 6H_2O$	59.0	$MnCl_2 \cdot 4H_2O$	2097.0
$CaCl_2 \cdot 2H_2O$	122.4	$ZnCl_2$	22.0
$MgSO_4 \cdot 7H_2O$	60.9	$CoCl_2 \cdot 6H_2O$	6.3
KH_2PO_4	246.0	$CuCl_2 \cdot 2H_2O$	0.1
$NaHCO_3$	1785.5	$NaMoO_4 \cdot 2H_2O$	28.9

2.3. Chlorophyll measurement

The chlorophyll content in leaves was determined spectrophotometrically at the end of the experiments (192 h). Plant leaves were cut into small pieces, precisely weighed (0.5 g fresh weight (FW)) and placed in 25 mL flasks. Then, 80% acetone was filled to the mark of 25 mL. Three separate flasks were conducted for each treatment group. All flasks were placed in the dark for 24 h. During this period, flasks were shaken twice. The absorption of light at 645 and 663 nm was measured in a cuvette with an optical path of 10 mm against 80% acetone as a blank. The amount of chlorophyll *a* and chlorophyll *b* in plant leaves was calculated following the formulae below according to Maclachalam and Zalik (1963):

$$C_a = \frac{(12.3D_{663} - 0.86D_{645}) \times V}{d \times 1000 \times W},$$

$$C_b = \frac{(19.3D_{645} - 3.60D_{663}) \times V}{d \times 1000 \times W},$$

where C_a is the concentration of chlorophyll *a* (mg g^{-1} FW), C_b is the concentration of chlorophyll *b* (mg g^{-1} FW), D is the optical density (OD) at the specific wave length indicated, V is the final volume (mL), W is the fresh weight of leaf materials (g), and d is the length of the light path (cm).

2.4. Enzyme activity measurement

The activities of three antioxidant enzymes, namely SOD, CAT, and POD, were measured in fresh leaves of the seedlings at the end of the experiment. Fresh leaves were taken from the shoot and 0.3 g of leaves (fresh weight) was precisely weighted and placed in a grinder and 1.4 mL of phosphate buffer solution (pH 7.8, containing NaH_2PO_4, Na_2HPO_4, PVPP, EDTA, and mercapto-ethanol) was added before grinding. The grinding was performed in an ice-bath and then centrifuged at 8000 rpm for 15 min, the supernatant was collected and stored at 4 °C and used in the subsequent enzyme assays. Each enzyme was measured independently. SOD, POD, and CAT activities in leaves of plants were determined spectrophotometrically as described by Yu et al. (2006a) previously.

2.4.1. Assay of SOD activity

The reaction mixture (3 mL) contained 13 mM methionine, 0.075 mM NBT, 0.1 mM EDTA, 0.002 mM riboflavin, and 0.1 mL of enzyme extract in 50 mM phosphate buffer solution (pH 7.8). The mixture in test tube was placed on a rotating tube holder at 25 °C for 10 min for thorough mixing. The absorbance was measured spectrophotometrically at 550 nm in a cuvette with an optical path length of 10 mm against the reaction mixture without enzyme extract. One unit of SOD activity (U g^{-1} FW) was defined as the amount of enzyme, which caused 50% inhibition of the initial rate of reaction in the absence of the enzyme.

2.4.2. Assay of CAT activity

The enzyme extract (0.1 mL) was first added into 2 mL assay mixture (50 mM Tris–HCl buffer, pH 6.8, containing 5 mM H_2O_2). The reaction was terminated by adding 0.1 mL of 20% titanic tetrachloride after incubation for 1 min at 25 °C. The absorbance of the reaction solutions was measured at 405 nm spectrophotometrically against the reaction mixture without enzyme extract. One unit of CAT activity (U g^{-1} FW) was defined as the amount of CAT, which decomposed 1 μmol H_2O_2 in 1 min at 25 °C.

2.4.3. Assay of POD activity

The reaction mixture (3 mL) was composed of 100 mM potassium phosphate buffer solution (pH 7.0), 20 mM guaiacol, 65 mM H_2O_2, and 0.1 mL enzyme extract. Changes in absorbance were recorded spectrophotometrically at 470 nm against the reaction mixture without any enzyme extract after incubation for 3 min at 25 °C. One unit of POD activity (U g^{-1} FW) was defined as the amount of enzyme that caused an increase of 0.001 absorbance in 1 min.

2.5. Soluble protein measurement

The soluble protein content was determined spectrophotometrically in fresh leaves from the top shoot as described by Jin and Ding (1981). Three separate