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柳树对亚铁氰化物的吸收、代谢及其毒性研究

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摘要: 为探明亚铁氰化物在植物体内的迁移、转化及对植物的毒性作用, 以长出新根须和嫩叶的垂柳(*Salix babylonica* L.)枝条为材料, 在自行设计的 250 ml 生物反应器中生长 192 h, 培养温度为 24.0±1℃, 亚铁氰化物水溶液的浓度分别为 52.99, 105.98, 211.95 和 317.93 mg CN L^{-1}。结果表明: (1) 低浓度实验组(52.99 mg CN L^{-1})水溶液中 10.85%的亚铁氰化物被植物吸收, 随着浓度的升高吸收到植物体内的亚铁氰化物的比例(%)依次递减, 但是统计学分析显示各实验组单位体重(湿重)的植物吸收亚铁氰化物的量无显著性差异; (2) 在植物的各个部位都能检测到微量的亚铁氰化物, 表明亚铁氰化物通过植物的蒸腾作用在植物体内的迁移。由于没有检测到在气态下的总氰化物, 表明植物的蒸腾作用没有将亚铁氰化物释放到大气中; (3) 尽管植物吸收到体内的亚铁氰化物是有限的, 但物质平衡实验证明其在植物体内迁移的过程中超过 96%的都能被植物有效转化; (4) 所用的 4 种亚铁氰化物浓度在 192 h 内没有对柳树产生毒性作用。因此认为: 依据亚铁氰化物在水溶液→植物→空气系统内的迁移和转化, 亚铁氰化物的植物修复是可能的。

关键词: 氰化物; 亚铁氰化物; 代谢; 植物修复; 毒性; 迁移; 柳树
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Uptake, Metabolism, and Toxicity of Iron Cyanide Complex in Weeping Willows

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Abstract: Uptake, metabolism and toxicity of iron cyanide complex in trees were investigated. Pre-rooted weeping willows (*Salix babylonica* L.) were exposed to hydroponic solution spiked with ferrocyanide at 24.0±1℃ for 192 h. Four different treatment concentrations of ferrocyanide were used (52.99, 105.98, 211.95 and 317.93 mg CN L^{-1}). Cyanide in water, in tissues of aerial part of plants and in air was analyzed spectrophotometrically. Results from this study indicated less than 10.85% reduction of the applied iron cyanide complex was detected in hydroponic solution in the presence of plants. Little amounts of cyanide were found in all parts of plant tissues, indicating the passage of ferrocyanide through the plants. Mass balance studies showed that iron cyanide complex moving into plants from hydroponic solution can be metabolized during transport. Phytotoxic effects were not found in all treatment groups, even at high doses of ferrocyanide within a 192-h exposure period. In conclusion, transport and metabolism of ferrocyanide in plants is most likely to happen and phytoremediation of this iron cyanide complex in field application may be possible.

Key words: Cyanide; Ferrocyanide; Metabolism; Phytoremediation; Toxicity; Transport; Willows

Cyanide is the commonly used reagent for gold and silver extraction. The annual production of cyanide hydrogen is about 1.4 million tons; more than 100 000 tons of cyanide disperse to the environment

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annually[1]. It is therefore not surprising that problems and catastrophic accidents occur repeatedly, mainly associated with gold mining. Although free cyanide (CN^-, HCN) is one of the most toxic chemicals to wildlife and human health, a number of plants investigated so far, were found to posses enzymes that can detoxify cyanide[2, 3]. Cyanide in plants is rapidly metabolized by the enzyme β-cyanoalanine synthase (CAS)[4, 5]. Asparagine was the only metabolic product detected in experiments with ^{14}C-labeled CN^- for both cyanogenic and non-cyanogenic plants[6]. Cyanide in environment can be present as free or simple cyanide or as cyanates and thiocyanates[7]. Most reacts with metal cations, forming a variety of metal cyanide complexes. Among them, iron cyanides ($Fe(CN)_6^{4-}$) are the most common and stable species, which are frequently found in contaminated aqueous environmental matrices[7]. Degradation by microorganisms of these compounds was confined to a limited number of bacterial and fungal strains[8, 9]. The decomposition of complexed cyanide to free cyanide under the exposure of light was reported by Kjeldsen[10]. Phytoremediation of cyanide has been carefully studied in a number of plants from three different continents and climate zones[2, 3, 7, 11-13]. No work has been found concerning the phytoremediation of ferrocyanide to terrestrial plants from China. Beavis and Vercesi[14] described a mitochondrial anion channel capable of transporting ferrocyanide. Therefore, we assume that plants may utilize iron cyanide complexes as a substrate during the plant metabolism. In this study, the uptake, transport, metabolism and toxicity of iron cyanide complex were determined for weeping willows growing in hydroponic solution. These results are suggestive of phytoremediation of ferrocyanide in field application.

1 Materials and Methods

1.1 Tree and exposure regimes

Weeping willow (*Salix babylonica* L.) was sampled from nature at the campus of the Hunan Agricultural University, China. Forty-cm long tree cuttings were removed from mature specimens of a single tree. After two-months of growth in buckets with tap water, pre-rooted cuttings were transferred to a 250 ml Erlenmeyer flask filled with approximately 200 ml modified ISO 8692 nutrient solution (Table 1). The flasks were all sealed with cork stoppers and play dough to prevent the escape of water or chemicals, and wrapped with aluminum foil to inhibit algae growth. The flasks were put in a climate chamber with a constant temperature of 24.0 ±1℃ under continuous artificial light. The plants remained there 48 h to allow them to adapt to their new living environment. Then, the weight of the plant system was measured. Twenty-four hours later, the flasks with the trees were weighed again. By this, the transpiration was determined. Trees with similar transpiration were selected for the tests. For each treatment concentration, six replicates were measured. The nutrient solution of these trees was exchanged to ferrocyanide-spiked solution, except for controls. Five different treatment concentrations of ferrocyanide were used (0, 52.99, 105.98, 211.95 and 317.93 mg CN L^{-1}). Note that 1 mg $K_4Fe(CN)_6$ equals to 0.423 9 mg CN. At the end of the experiment (192 h), the water, roots, leaves and stems were all analyzed for ferrocyanide or the total cyanide. A mass

Table 1 Composition of nutrient solution used in plant uptake experiments

Ingredient	Concentration (μmol/L)	Ingredient	Concentration (μmol/L)
$NaNO_3$	2823.9	HBO_3	2992.1
$MgCl_2·6H_2O$	59.0	$MnCl_2·4H_2O$	2097.0
$CaCl_2·2H_2O$	122.4	$ZnCl_2$	22.0
$MgSO_4·7H_2O$	60.9	$CoCl_2·6H_2O$	6.3
KH_2PO_4	246.0	$CuCl_2·2H_2O$	0.1
$NaHCO_3$	1785.5	$NaMoO_4·2H_2O$	28.9

balance was computed to assess whether ferrocyanide lost from water could be recovered in plant biomass or whether metabolism of this iron cyanide complex had occurred.

1.2 Chemical analysis

The concentration of ferrocyanide in water was analyzed by a standard method provided by State Environmental Protection Administration of China[15]. Results were expressed as mg CN L^{-1}. The detection limit of this method was determined from blank plus three standard deviation of 10 replicates to be 0.08 mg CN L^{-1}.

Total cyanide is the sum of easy liberatable cyanide and complexed cyanide. Sample pretreatment was prepared in the following way. Ten milliliters of sodium hydroxide of 1% was poured in the absorption vessel of the distillation unit. Fresh plant biomass (2.0 to 10 g FW, depending on the harvested weight of plant materials) was cut into pieces and placed in a 500 ml round bottom flask, and then 200 ml of distilled water was added. Then 10 ml of ethylenediamine tetraacetic acid disodium salt with the concentration of 10% (V/M) and 10 ml of phosphoric acid (*per analysis*, in China: ≥85% purity) were added before heating and mixing. Approximately 100 ml distilled solution containing cyanide from plant tissues were collected, quantitatively transferred to a 100 ml volumetric flask and made up to volume with water. The solution was stored at below 6℃ until the concentration of cyanide was determined. The samples were all analyzed with a maximum hold time of 4 hours. Cyanide in distilled solution was also analyzed by a standard method provided by State Environmental Protection Administration of China[15]. The detection limit of this method was determined to be between 0.004 and 0.25 mg CN L^{-1}.

1.3 Cyanide transpired by plants

Ferrocyanide transpired was measured using a refined test chamber (Fig. 1). Treated plants were prepared as described above and placed into a glass chamber (20 cm × 20 cm × 50 cm) with air flowing through at 24℃. Tubing from the outflow of the vessel is connected to a gas trap tube containing 5 ml sodium hydroxide of 1% that would trap any airborne cyanide. The gas trap tube was wrapped with aluminum foil and changed daily, after which all gas tubes were analyzed for the total cyanide and free cyanide. The duration of this test was 192 h.

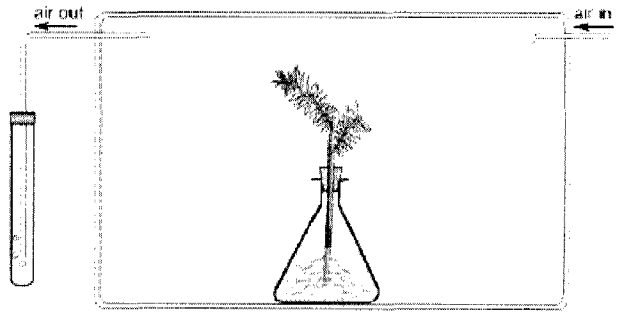

Fig. 1 Test chamber system for measuring ferrocyanide transpired in air

2 Results and Discussion

2.1 Iron cyanide complex uptake from hydroponic solution by willows

Fig. 2 shows the mass reduction (%) of ferrocyanide in hydroponic solution with different treatment concentrations after 192 h exposure. In the controls in the absence of plants, no change of ferrocyanide concentrations was detected over the entire period of exposure (data not shown), indicating the disappearance of ferrocyanide in water can be accounted to the uptake by willows. Amounts of ferrocyanide in hydroponic solution were reduced in all treatments, ranging from 1.21% to 10.85% of initial mass. Results showed amounts of applied ferrocyanide moving into plant tissues, but predominantly remaining in the hydroponic solutions. Ebbs et al.[7] used willows (*Salix eriocephala* L. var. *michaux*) to quantify the plant uptake, transport and metabolism of potassium cyanide and potassium ferrocyanide labelled with ^{15}N. Approximately 8% of the initial ferrocyanide was reduced in water during the presence of plants. Results from the study of Samiotakis and Ebbs[16] also imply barely (*Hordeum vulgare* L.), oat (*Avena sativa* L.) and wild cane (*Sorghum bicolor* L.) can extract ferrocyanide from the contaminated compartments.

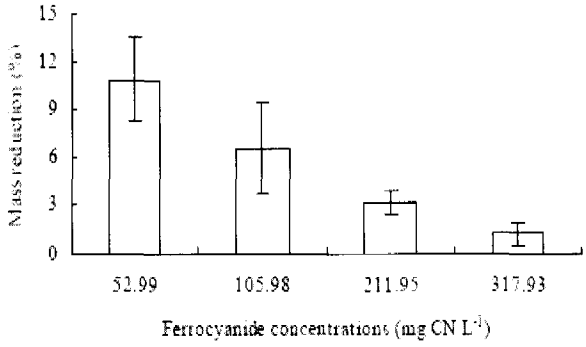

Fig. 2 Ferrocyanide reduction (%) in hydroponic solutions with different treatment concentrations
Bars = Standard errors of the means (n = 6).

2.2 The mass balance of cyanide

The water, roots, leaves and stems were all analyzed for ferrocyanide or total cyanide after 192 h exposure. Fig. 3 shows the concentration of total cyanide in plant tissues (mg CN kg^{-1} FW). Cyanide was found in all parts of plant materials in all treatment concentrations, confirming passage of ferrocyanide through the plants. Significant concentrations of cyanide in roots were found in all treatment groups. The same results were also found in the studies of Ebbs et al.[7] and Samiotakis and Ebbs[16]. It is also shown from Fig. 3 that the measured concentrations in plant materials are increased with the exposed doses of ferrocyanide.

Due to the leafy portion being exposed to the air, ferrocyanide in water may have transpired through the

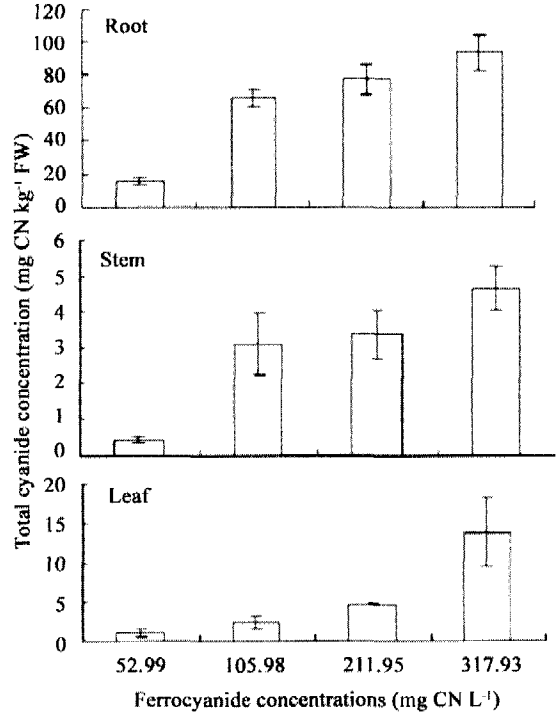

Fig. 3 Measured total cyanide concentrations (mg CN kg^{-1} FW) in the tissues of plants exposed to different concentrations of ferrocyanide
Values are means of six replicates. Bars = Standard errors of the means (n = 6).

leaves without metabolism. In this study, no cyanide (the concentration may below the limit of detection) transpired by plant leaves was trapped over a 192-h test period using the test chamber. The same was also reported in the study of Ebbs et al.[7]. Therefore, a mass balance for ferrocyanide within the planted system was calculated using total cyanide in plant tissues and

Table 2 Mass balance of ferrocyanide

Exposed concentration (mg CN L^{-1})	Ferrocyanide in solution (mg CN)		Ferrocyanide in tissue (μg CN)			Ferrocyanide metabolized (mg CN)	Metabolism rate (mg CN $kg^{-1}h^{-1}$)
	Initial	Final	Root	Leaf	Stem		
52.99	13.25	11.81	22.23	4.76	12.76	1.27	0.16
		(±0.347)	(±1.796)	(±1.202)	(±4.964)	(±0.374)	(±0.022)
105.98	26.49	24.70	70.94	12.92	94.14	1.41	0.23
		(±0.549)	(±1.748)	(±2.076)	(±21.837)	(±0.342)	(±0.079)
211.95	52.99	51.55	73.81	21.08	100.04	1.24	0.22
		(±0.378)	(±11.753)	(±7.055)	(±16.400)	(±0.382)	(±0.054)
317.93	79.84	77.65	74.09	64.48	126.71	1.86	0.23
		(±1.039)	(±35.652)	(±22.288)	(±30.507)	(±0.789)	(±0.071)

* Values are means of six replicates.

the solution total cyanide data. Table 1 gives the amounts of recovered mass from plant tissues and from the solution. When willows were exposed to a low dose of ferrocyanide (52.99 mg CN L^{-1}), only 3.33±0.489% (n=6) of the total reduction ferrocyanide in hydroponic solution was detected in the plant materials. With increasing the exposed doses of ferrocyanide, the initial mass remaining in the plant tissues increased in a range from 10.46 ±3.087% to 14.31±3.908%. The mass balance studies (data shown in Table 2) showed that the majority of the total reduction ferrocyanide in water was metabolized during transport through willows. Results from the mass balance studies also indicated the difference between the metabolism rates was comparably small. The mean of the four values of metabolism rates was 0.21 mg CN kg^{-1} h^{-1} and the standard deviation was 0.034 mg CN kg^{-1} h^{-1}, implying the velocity of ferrocyanide metabolism in willows was independent of the substrate concentrations and probably limited by enzyme capacity.

Results presented here and additional data [7,16] suggest that uptake, transport and metabolism of ferrocyanide for plants are most likely to happen. Then, it is of interest to discuss the mechanism of ferrocyanide transport from hydroponic solution to plants. The structure that enables plants to extract water and nutrient from different environmental compartments allows chemical to enter the plants and to be subsequently transported inside. However, plant cuticles are the limiting barrier in the uptake of a wide range of chemicals. Probably there are two pathways for water soluble compounds moving into the plant tissues, passive transport (uptake with water and translocation upwards in the xylem, and the diffusion in water phase into roots) and active transport (via a special carrier). So which pathway causes the ferrocyanide moving into plant tissues? Ferrocyanide has long been considered membrane impermeable [17]. In this case, weeping willows almost extracted the same amount of applied ferrocyanide from the hydroponic solution in all treatment groups. This implies that active transport might be the pathway causing ferrocyanide moving into root symplasm. Therefore, we postulate that there may have a special carrier or anion channel in the root membrane responsible for the transport of ferrocyanide. If this iron cyanide complex can be transported across one membrane, then there may be mechanisms that allow for its transport across other membranes as well. Since trace amounts of initial mass was also found in all parts of plant tissues, mass balance studies provided additional evidence to confirm the transport of ferrocyanide through the different tissues of plants. The assimilation of free cyanide into asparagine through cyanoalanine pathway has been widely found in a number of plants [2-6], yet it is not clear whether cyanoalanine synthase can use ferrocyanide directly as a substrate in plants. Further comprehensive studies are needed to determine the detail mechanism of this iron cyanide complex transport and metabolism.

2.3 Phytotoxicity of iron cyanide complex in weeping willows

The toxic effect was quantified by measuring the transpiration of the trees. The transpiration of plants is coupled to the photosynthesis, and an inhibition of transpiration is a reliable and fast measurement of toxic effects [18]. Fig. 4 shows the relative transpiration of willows exposed to different doses of ferrocyanide. The relative transpiration was expressed by the ratio of the transpiration of plants exposed to toxicant to the transpiration of plants exposed to nutrient solution without toxicant. A higher relative transpiration (1.26±0.105, n= 6) was observed in treated plants exposed to ferrocyanide of 52.99 mg CN L^{-1} in comparison with untreated plants (1.08±0.165, n= 6), implying this dose may stimulate the growth of plants. A slight difference in the relative transpiration between treated and untreated plants was found, probably stemming from plants (No attempt was made to select homogeneous plant materials in this study). The mean of the relative transpiration of four treatment groups is 1.09 ±0.132. The relative transpiration of control is 1.08±0.165 (n= 6), indicating the doses of ferrocyanide to be used in this study did not cause toxic effects on weeping willows. Symptoms of

chlorosis in leaves were not found in all plants over the entire period of exposure. There are also no significances in the growth of plants between treated and untreated plants (data not shown), giving the conclusion that the plant can keep up their physiological functioning within the plant systems spiked with ferrocyanide over the entire period of exposure.

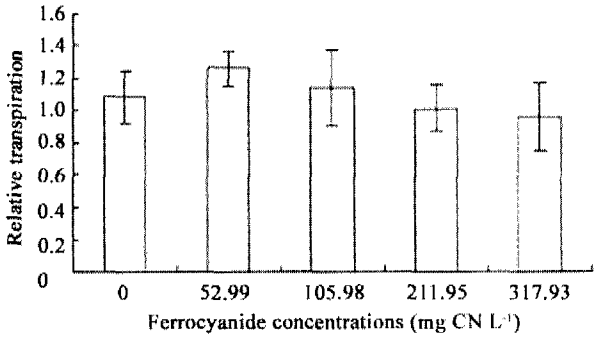

Fig. 4 Relative transpiration of plants exposed to different treatment concentrations of ferrocyanide
Bars = Standard errors of the means (n = 6).

3 Conclusions

It was found in this study that weeping willows can extract, transport and metabolize ferrocyanide without phytotoxicity. Less than 10.85% of the applied iron cyanide complex was detected to move into plant materials from hydroponic solution. Amounts of ferrocyanide were found in all parts of plant tissues, indicating passage of ferrocyanide through the plants. Mass balance studies showed that initial ferrocyanide moving into plant materials can be metabolized during transport. Phytotoxic effects were not found in all treatment groups, even at high doses of ferrocyanide. This gives the conclusion that phytoremediation of this iron cyanide complex is most likely to happen.

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