Research Article
The Absorption and Distribution Characteristics of Willow Clones to Copper and Its Detoxification Mechanism

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Phytoremediation technology is a measure to purify pollutants in soil or water through the absorption, volatilization, root filtration, degradation, and stabilization of plants, and its core is to find plants with large biomass and high enrichment. The wild willow tree found in Tonglushan is a fast-growing Euphorbiaceae plant, which has strong tolerance to the heavy metal copper (Cu) and has the characteristics of developed root system, barren resistance, and high economic value. Taking willow as the research object, this paper studied the composition, copper enrichment sites, morphology, subcellular distribution characteristics and absorption, transportation, and enrichment mechanism of willow root exudates under copper stress through hydroponic and pot experiments. By adding phosphate fertilizers, inducers, and other agronomic control measures, the strengthening technology and mechanism of Cu-contaminated soil phytoremediation were studied. The main research contents and results are as follows: the results of hydroponics and pot experiments show that the willow tree has a certain tolerance to Cu, and Cu mainly accumulates in its roots. Oxalic acid, succinic acid, tartaric acid, citric acid, and malic acid are the main organic acids in willow root exudates. Root exudation activity acidified the rhizosphere soil, increased acid exchangeability, and reducible Cu content, while other forms of Cu content decreased. Root exudates affect the uptake and accumulation of copper by willow trees by altering the bioavailability of copper in soil.

1. Introduction

How to effectively and completely remove heavy metals from the environment is a hot research topic at present [1]. In recent years, the development and application of phytoremediation technologies have provided new methods for solving or mitigating soil pollution problems. Phytoremediation can be divided into the following categories according to its principles and implementation methods: plant extraction, plant volatilization, plant filtration, plant degradation, plant fixation, and combined repair [2]. Plant extraction is the transfer and concentration of pollutants (metals or organic matter) from the soil to the aboveground parts of plants using plants with strong contaminant-enriching ability, and the contaminants are removed by harvesting the aboveground parts of plants; in some cases, root can also be harvested [3]. Plant stability refers to the use of plant roots to change the soil environment or root exudates to precipitate heavy metals to reduce the bioavailability of heavy metals and reduce the pollution of heavy metals to the environment. Its advantage is that it does not need to deal with plant tissues loaded with heavy metals. Plant volatilization is a phytoremediation method that converts specific heavy metals in soil into volatile state through plant roots or converts them into gaseous substances and releases them to the atmosphere through plants. Phytoremediation technology is considered as a very promising technology for contaminated soil remediation. Compared with traditional physical repair, chemical repair (rinsing method, passivation method and redox method, etc.), and electric repair, phytoremediation has the characteristics of low cost, easy operation, in situ repair, and less disturbance to the environment: simultaneous planting. The plants can improve the local ecological environment, purify the air, reduce soil erosion, etc. [4, 5]. Therefore, plant extraction technology has huge market prospects and economic value.

Willow is the general name of Salix plants in the Salix family. It has rich germplasm resources, wide distribution,
high survival rate of cutting propagation, high economic value, and high ornamental value. It is widely used in landscaping. However, there are few studies on willow clones in the remediation of heavy metal pollution. The key to phytoremediation is to find the right plant species. Among them, ideal plants for plant extraction should have the following characteristics: can tolerate high concentrations of metals, accumulate high concentrations of metals at harvest sites, grow fast, have large biomass, and have a more developed root system. Plants that can absorb heavy metals in excess and transport them to the shoots of plants for storage, and the concentration of heavy metals accumulated on the shoots is more than 100 times that of ordinary plants, which are defined as hyperaccumulators of heavy metals. The distribution and abundance of different types of heavy metal elements in the crust are different, and their background values in the soil environment and plants are also quite different, so the metal contents in the hyperaccumulators of different types of heavy metals are also different [6]. At present, the reference value proposed by Feng et al. is widely referenced; that is, the Cd in the leaves or shoots of plants (both calculated by dry weight) exceeds 100 mg/kg, and the Co, Cu, Ni, and Pb exceed 1000 mg/kg [7]. In fact, this defined value is only used as a reference for the characteristic threshold of hyperenriched plant concentration, not absolute.

2. Materials and Methods

China is an early country in the use of bioenergy plants, but it is still limited to the primary stage of direct combustion and carbon production. During the “Sixth Five-Year Plan” period, a special topic was listed in the national scientific and technological key project “Breeding of Main Fast-Growing and High-yielding Tree Species”, and “Research on the Selection and Introduction of Best Fuelwood Species” was carried out [8]. During the “Seventh Five-Year Plan” period, the country has launched the key project “Introduction and selection of fine fuelwood species for the development and research of rural renewable energy technology, research on fuelwood forest cultivation technology and various benefits” as the main topic, which is set up nationwide. 13 test areas, 26 test stations, and 206 species were introduced and tested, and 60 species of energy forest trees suitable for cultivation in different regions were screened. For more than 10 years, these selected tree species have played an important role in local ecological construction and alleviation of the energy crisis [9]. Regarding the research on willow energy forest, during the “Seventh Five-Year Plan” period, the original area of Ningxia and the Zhuangdian area of Henan carried out the “Introduction Experiment of New Willow Varieties” and the “Introduction Experiment of Excellent Willow Clones” in different regions of the country, and new varieties were also selected. Heilongjiang province has also carried out the “Species Selection and Creation Technology of Willow Fuelwood Forest.” The Jiangsu Academy of Forestry Sciences has successively bred 7 excellent clones of wood arbor and willow, including Sullu 172 [10]. For these 7 clones, studies on the adaptability of growth, phenology, and freezing damage were carried out; after more than 20 years of introduction, cultivation, and artificial cross-breeding, Heilongjiang Shelter Forest Research Institute identified and launched 2 new varieties of fast-growing arboretum willow trees in 1985: Pop 109 willow and Hanshi 329 willow [11]. Farzana et al. [12] studied the growth of new clones of willow and the activity of soil enzymes under salt stress and concluded that willow can absorb Hg in coal ash soil and Pb, which have a purifying effect on the soil. There are more than 500 species of willows in the world, and there are 256 species, 120 varieties, and 33 forms in China. Willow has the characteristics of rich germplasm resources, wide distribution, and high survival rate of cutting propagation. Willow has strong adaptability in heavy metal polluted areas. The study found that Artemisia willow enriched more zinc in leaves, while white willow enriched more zinc in roots. Willow has more copper in its roots than other willows, but less copper in its leaves. From the above results, it can be seen that different willow clones have different enrichment characteristics of heavy metals.

Compared with foreign energy forest plants, there are several prominent problems in the excellent energy plants that have been screened in China: first, there are not many excellent varieties or clones [13]. There are many tree species such as Quercus japonica, Robinia pseudoacacia, Amorpha japonica, Sand jujube, sea buckthorn, and willow tree selected in northern China, but there are not many varieties with obvious advantages. The growth rate is only 1/10~1/2 of the annual biomass of the Swedish willow energy forest, and the harvest cycle of the energy forest is more than 4 to 6 years and some even more than 10 years; the third is Sweden. The willow energy forest has formed a model management system from seedling breeding, land preparation, soil fertilization, afforestation design, field management, and harvesting. However, most of the energy forest management in China is extensive, and energy forest management measures and technologies need to be improved [14].

It can be seen from this that energy willow is indeed a very promising energy plant with strong adaptability, wide cultivable area, and great commercial potential; countries around the world are very concerned about the introduction of new clones of energy forest plants. The new clone of energy willow has become an energy forest material that many experts are eager to introduce, and breakthroughs have also been made in the utilization technology, and the development potential is huge [15].

3. Results and Discussion

3.1. Experimental Method of Copper Absorption and Distribution in Willow. Research shows that it is difficult to remove too many heavy metals into agricultural soil, and they can enter the food chain through agricultural activities to enrich and enlarge, endangering ecosystem and human health. Among the common heavy metals, Cu is the most common and harmful. Although Cu is an essential element for crops, excess will also produce obvious biological effects. Select willow seeds with uniform size and full grains, soak them in 75% alcohol for 30 s, then rinse with sterilized distilled water for 3 times, and then soak them in sterile water
for 24 h, and sow them in the holes of the seedling board filled with sterilized river sand. After the seeds germinate, select willow seedlings with uniform growth, rinse them with tap water and distilled water for 3 to 4 times in turn, and transfer them to 1/4 Hoagland nutrient solution for pre-cultivation for 2 weeks before use [16].

The willow plants are gently removed from the nutrient solution without damaging the roots. The willow roots were then rinsed with deionized water for 5 min, and the whole plant was incubated in sterilized deionized water (CO-free) for 6 hours. The container is wrapped in a black plastic bag to protect from light, and the collection time of root exudates is 9:00-15:00. The collected root exudates were immediately filtered through a 0.45 μm filter membrane and freeze-dried [17]. The concentrated root exudates were dissolved in deionized water and passed through cation and anion exchange resins in turn, and then, the resin was rinsed with 1 mol/L HCl to obtain purified root exudates, which were freeze-dried again and stored at -20°C for subsequent use [18].

Before harvesting, tap water and distilled water were used to wash the roots and shoots of the plants in turn, and the fresh weight was weighed after absorbing the water with absorbent paper. Put it in an oven, fix it at 105°C for 30 min, and bake at 65°C to constant weight and weigh. The shoots and root samples were pulverized by a small pulverizer and passed through a 0.25 mm sieve. Weigh 0.5 g of plant samples into the digestive tube, add 10 mL of mixed acid HNO and -HCl-HClO4, and place it in a temperature-controlled far-infrared digestion furnace (LWY-84 type) for digestion. Boil, the digestion is completed, and dilute to a 50 mL volumetric flask with distilled water and filter. The relationship between the properties and components of willow root exudates and plant biomass and Cu accumulation was analyzed using a linear regression equation and a fitting optimization method.

3.2. Impact Assessment of Heavy Metal Pollution. Heavy metal pollution in soil refers to the damage of high content of heavy metals in soil to organisms and the deterioration of ecological environment quality, which has the characteristics of concealment, long term, and irreversibility. The harm of soil heavy metal pollution to the ecological environment is mainly manifested in reducing soil fertility, reducing crop yield, and quality and can cause harm to human health and life through the food chain. With the development of industry, the usage and application quota of heavy metals are constantly expanding, and the resulting environmental pollution problem is becoming more and more prominent. Therefore, the environmental quality of soil has also become one of the focuses of people’s attention [19]. The residual accumulation of pollutants in soil is limited by the fact that it will not cause the growth obstacle of crops and excessive accumulation in grains or edible parts (not exceeding food hygiene standards) or affect the environmental quality of soil, water, and so on. The soil environmental quality standard stipulates the maximum allowable concentration index value of pollutants in the soil and the corresponding monitoring methods according to the application function of the soil, the protection objectives, and the main properties of the soil.

The geoaccumulation index is commonly referred to as the Muller index. It not only considers the impact of background values caused by natural geological processes but also pays full attention to the impact of human activities on heavy metal pollution. Therefore, the index not only reflects the natural change characteristics of heavy metal distribution but also can judge the impact of other activities on the environment. It is an important parameter to distinguish the impact of human activities. Its expression formula is as follows:

\[
I_{geo} = \log_{2} \left( \frac{C_n}{1.5BE_{n,i}} \right).
\]

Contamination degree is one of the most intuitive and commonly used parameters for evaluating heavy metal pollution [20–24]. This parameter represents the excess of the monitored heavy metal elements and can be expressed as

\[
C_d = \sum_{i=1}^{n} C_{f,i},
\]

\[
C_{f,i} = \frac{C_{A,i}}{C_{N,i}} - 1.
\]

\(C_x\) indicates the analytical value of the \(i\)-th element (concentration in the sample); \(C_u\) indicates the allowable upper limit of the element concentration in the environment, which is generally the soil environmental quality standard. It is not difficult to see that the \(C_d\) value represents a comprehensive index of heavy metal pollution in a certain study area.

The formula for calculating the single factor pollution index is

\[
P_i = \frac{C_i}{S_i}.
\]

Common formulas for calculating the comprehensive pollution index are

\[
P = \sum_{i=1}^{n} P_i W_i.
\]

In the formula, \(P\) refers to the weighted comprehensive pollution index of a sampling point; \(P_i\) refers to the single-factor quality index of pollutant \(i\); \(W_i\) refers to the weight coefficient of pollutant \(i\); \(n\) refers to the number of evaluation parameters.

\[
P = \left[ \frac{P_{\text{imax}}^2 + P_{\text{ave}}^2}{2} \right]^{1/2}.
\]
4. Result Analysis and Discussion

4.1. Experimental Results and Analysis. Figure 1 shows the dry weight of different organs of willow under Cu stress treatment under hydroponic conditions. The dry weight of willows treated with T1 increased by 4% compared with CK. With the increase of exogenous Cu concentration (≥100 μmol/L), the biomass of willow trees decreased. Compared with T1, the root and plant dry weight in T2 treatment (250 μmol/L Cu) decreased by 25% and 14%, respectively, both reaching significant levels (P ≤ 0.05). However, the difference in dry weight of willow shoots among all treatments was not significant (P > 0.05).

Figure 1 shows the uptake and accumulation of Cu in shoots and roots of willow under exogenous Cu stress. With the increase of exogenous Cu concentration, the Cu content in shoots and roots increased rapidly. In the control treatment (CK), the Cu contents in the shoots and roots were 27.3 and 749.7 mg/kg (dry weight), respectively, but in the T4 treatment (750 μmol/L Cu), the Cu contents in the shoots and roots, respectively, reached 177.1 and 14586.7 mg/kg (dry weight), which were 6.48 and 19.5 times the Cu concentration of the control treatment, respectively. The root Cu content reached a significant difference between the treatments (P ≤ 0.05), while the shoot Cu content was not significantly different among the low-concentration Cu treatments (T1, T2, and T3) (P > 0.05).

Figure 2 shows the pH and EC values of willow root exudates under different Cu treatments. The EC value of willow root exudates gradually increased with the increase of exogenous Cu. Compared with the control group (CK), the EC value in the T1 treatment (100 mmol/L Cu) increased by 2.64 times. Similarly, the root exudate EC values in the T2, T3, and T4 treatments were increased by 2.68, 3.79, and 2.64 compared with the control treatment, respectively. 4.13 times, which means that with the increase of Cu addition, willow root exudates increase, and the permeability of root cell membrane increases.

The pH of root exudates in the control (CK) treatment was 6.53, which was slightly lower than that of deionized water. The pH of root exudates decreased gradually with the increase of Cu concentration, and the T4 treatment decreased by 0.4 units compared with the CK group, which may be caused by the presence of amino acids or organic acids in the root exudates. Compared with CK, pH values
in other treatments (100, 250, and 500 μmol/L) were decreased by 0.11, 0.26, and 0.32 units, respectively, as shown in Figure 3.

4.2. Low Molecular Weight Organic Acid and Amino Acid Content. Found that the mobility of metals increased significantly with decreasing rhizosphere pH. Changes in rhizosphere pH may involve the following processes: CO2 released by respiration, release of root exudates, efflux or reabsorption of H+ or HCO3, and organic acids generated by microbial metabolism. In the present study, the pH of the root exudate solution decreased with increasing Cu dose by up to 0.4 units. Studies have shown that root exudates in rhizosphere environments lower pH by 0.2-0.5 units compared to nonrhizosphere soils. There was a significant linear relationship between the pH value of root exudates and the concentrations of succinic acid ($R = -0.96, P < 0.01$), tartaric acid ($R = -0.98, P < 0.01$), and citric acid ($R = -0.90, P < 0.05$). There was a negative correlation with the total organic acid concentration ($R = -0.98, P < 0.01$). Although there was a linear positive correlation between pH and oxalate concentration ($R = 0.86, P < 0.05$), oxalate content accounted for up to 0.52% of the total LMWOA. The decrease in pH of root exudates may be due to the increase in the content of low molecular weight organic acids and amino acids, which contain carboxyl, sulfonic acid, and amide groups that contribute to the increase of H in solution. Reported that the carboxylate concentration in root
Exudation reached the highest at pH 4.8. The conductivity of root exudates increased with increasing doses of Cu in solution, laterally supporting that pH changes were the result of increased root exudates. Different plants produce root exudates under metal stress, and low molecular weight organic acids are the most important organic components in root exudates as shown in Figure 4.

4.3. Effects of Cu Treatment on Cu Accumulation in Different Organs and Tissues of Willow. The content of Cu in different organs of willow increased gradually with the increase of exogenous Cu treatment concentration, and the content of Cu in different organs of willow after Cu treatment was significantly different from that of the control. The Cu contents in roots, stems, and leaves of the control treatments were 13, 83, and 21 mg/kg, respectively, and Cu contents in shoots were higher than those in roots. It can be seen from Figure 5 that the Cu content in the roots of willow trees after Cu treatment is higher than that in the shoots. When 24 mg/L was added, the Cu contents of willow roots, stems, and leaves were 920, 11, and 24 times that of the control treatment, respectively.

Figure 6 shows the CuK edge spectra of samples from different parts of willow and related standards treated with CuSO4. Use the linear fitting function in the Athena software to perform linear fitting on the spectral lines obtained by the test. Through fitting, it was found that the morphology of Cu in the roots, stems, and leaves of willow was as follows: citrate-Cu, arginine-Cu, and CuO were the main components in the roots, and their proportions in the total Cu were 34%, 35.8%, and 18.1%, respectively. In the stem of willow, Cu mainly exists in the form of relatively stable CuO and GSH-Cu, which account for 30.9% and 39.5% of the total Cu, respectively. In willow leaves, Cu exists in relatively scattered forms, mainly in the form of citrate-Cu, arginine-Cu, CuO, and GSH-Cu, which account for 24.9%, 25.6%, 15.1%, and 13.5% of the total Cu, respectively. There is a certain amount of Cu3(PO4)2 and CuCl2 (ionic Cu) in the roots.
stems, and leaves of willow, and the proportion of 
$\text{Cu}_3(\text{PO}_4)_2$ in the willow roots and leaves in the total Cu is higher than that of CuCl$_2$.

The concentration of Cu accumulated in willow gradually decreased in the order of roots, stems, and leaves, which was consistent with the previous research results: roots are the main accumulation organ of Cu, and the efficiency of Cu transfer from roots to shoots is not high. Amino acid homeostasis is critical for plant growth, development, and defense against environmental stresses. The balance of amino acid content in plants is regulated by neobiosynthesis, accumulation/transfer, and protein synthesis/degradation (Zoghlami et al. 2011). Cu stress promoted the accumulation of free amino acids in willow, and its total content in different parts of willow reached the maximum in Cu. This is consistent with previous studies: exogenous cadmium treatment promoted the accumulation of free amino acids in wheat seedlings, and its total content reached a maximum at the highest concentration of cadmium (Lesko et al. 2002). Total amino acid content was also increased by 39.73% compared to control plants after 7 days of exposure to copper stress in Brassica napus seedlings (Yadav et al. 2017). The content of free amino acids in different parts of willow under heavy metal Cu stress showed a significant linear relationship with the concentration of Cu treatment, and the linear fitting results are shown in Figure 7. The total amount of amino acids in roots and leaves had a good linear relationship with the concentration of Cu treatment, and the correlation coefficient $R$ value was 0.98.

The total amount of free amino acids in the shoots was higher than that in the roots, which may be due to the higher degree of lignification in the roots. The total content of free amino acids in shoots and roots of wheat after cadmium stress ranged from 1260 to 2530 μg g$^{-1}$ and 2050 to 3140 μg g$^{-1}$, respectively (Lesko et al. 2002). Similarly, after exposure of Brassica napus seedlings to copper stress for 7 days, cysteine content increased significantly with the increase of copper ion content in the growth medium, and its content and ratio reached the maximum at the highest concentration of copper ion treatment (0.75 μmol/L).

Under the treatment of different concentrations of copper, the main amino acids in willow remained unchanged, but the contents differed several times. Among them are aspartic acid (Asp), threonine (Thr), arginine (Arg), amino acid (Glu), and histidine (His). When cadmium was not added (control group), the main free amino acids in wheat were aspartic acid, alanine, valine, lysine, histidine, and arginine (Lesko et al., 2002). The main free amino acids in willow callus after copper stress treatment were threonine (Thr), arginine (Arg), and alanine (Ala) (Huang et al.

Figure 5: Cu content in different parts of willow after Cu treatment.

Figure 6: Cu-XANES spectra of different tissues and standard samples of willow treated with 24 mg/L CuSO$_4$.
After Cd stress treatment, the levels of asparagine, methionine, and lysine increased in lettuce (Costa and Morel 1994). Methionine can help the transfer of copper ions in plants; the contents of asparagine, glutamine, and branched-chain amino acids (valine, isoleucine, phenylalanine, and tryptophan) in its roots increased significantly (Zoghlami et al. 2011). Bhatia et al. (2005) found that the contents of aspartic acid, alanine, and glutamine were significantly increased under Ni stress, which had a complexing effect with Ni in the xylem.

5. Conclusion

How to effectively and completely remove heavy metals from the environment is a hot research topic at present. In recent years, the development and application of phytoremediation technologies have provided new methods for solving or mitigating soil pollution problems. This paper studied the absorption and distribution characteristics of copper in willow clones and its detoxification mechanism. High concentration of exogenous Cu caused stress to willow, inhibiting the growth of willow, destroying photosynthetic pigments in leaves, and increasing the level of lipid film peroxidation in willow leaves. Under hydroponic conditions, most of the Cu accumulated in the roots of willow, and the order of Cu content in each subcellular component in the root system was cell wall > organelle > cytoplasmic soluble components, while leaves were all cytoplasmic soluble components in the order of point > cell wall > organelles. It can be seen that vacuolar compartmentalization and cell wall retention are important tolerance mechanisms for willow to cope with Cu stress. With the increase of Cu treatment concentration, the total amount of free amino acids and free ammonia in roots, leaves, and phloem gradually increased: aspartic acid, threonine, arginine, glutamic acid, and alanine were the most abundant, and amino acids, such as valine, may be involved in the detoxification and transport of Cu. Using synchrotron radiation XANES technology to analyze the cumulative binding forms of Cu in different parts of willow, it was found that complex forms such as citrate-Cu and arginine-Cu and precipitated forms such as Cu(PO4)2 and CuO existed in the roots, stems, and leaves of willow. This suggests that intracellular deposition is another pathway for willow resistance to Cu.

Data Availability

The figures used to support the findings of this study are included in the article.

Conflicts of Interest

The author declare that there are no conflicts of interest.

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