Plant growth and nutrient composition in shrub and arbor willows grown in Cu contaminated soil as affected by flooding

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Abstract

Flooding can adversely worsen the metal contaminated soil and plant growth thus, it is crucial to explore the ecophysiological responses of plants upon co-exposure to heavy metals and flooding. Here, the plant growth, photosynthesis, and nutrient elements composition in arbor willow (*Salix jiangsuensis* 'J172') and shrub willow (*Salix integra* 'Yizhibi') were studied using a pot experiment with Cu contaminated soil (239.51 mg•kg^{-1}) under flooded versus non flooded condition. *Salix integra* showed larger BCF_S than *Salix jiangsuensis* in both treatments, soil flooding significantly decreased the Cu contents and BCF while obviously increased TF values in both willow species ($p < 0.05$). Soil flooding markedly enhanced the leaf C:P and N:P ratios, while significantly decreased root C:P and N:P ratios, as compared to non flooded condition. The shrub willow exhibited better tolerance to soil flooding with little alteration in biomass and photosynthetic rate, and showed greater potential of Cu accumulation capacity, even though its total biomass was significantly lower than arbor willow. Our study also helps further understanding the nutrient balance and stoichiometry of willows in Cu contaminated soil and their response to soil flooding, helping the management of Cu-contaminated flooded soils.

Introduction

Heavy metal contamination in soil primarily results from the anthropogenic sources such as mining, industrial sludge, farming, and disposal of waste (Ali et al., 2013) and has become one of the most serious issues in the world. Copper (Cu) is an essential micronutrient required by important physiological and biochemical processes in most organisms (Adrees et al., 2015). However, industrial and agricultural activities (Cu-based fungicides, bactericides and pesticides, waste water discharge, sewage sludge application etc.) have led to the soil Cu contamination (Li et al., 2014; Rehman et al., 2019). Exceeded Cu levels in soil could cause toxicity to organism by interfering the normal functions and subsequently cause severe problems to natural ecosystem and human health (Brunetto et al., 2016). Thus, in order to minimize the impacts of Cu on ecosystems, it is urgent to remediate Cu-contaminated soils. Phytoremediation, as an *in situ*, green alternative, cost-effective and environmentally friendly approach, has been widely used for soil remediation in recent decades (Luo et al., 2016). Previously, much interest concentrated on identifying hyperaccumulator plants, whose above-ground tissues can accumulate heavy metals at higher concentrations (Pulford and Watson, 2003). But limitations of herbaceous hyperaccumulator plants for phytoremediation have been also appeared, therefore considerable interest in the potential use of fast-growing woody species for phytoremediation have drawn attention (Cao et al., 2018; Pilipović et al., 2019). For example, the leaves of *Salix dasyclado* accumulated as high as 230–315 mg kg^{-1} Cd dry biomass (Utmazian et al., 2007) and *Salix integra* accumulated 90–288 mg kg^{-1} Cd in leaves under hydroponic condition (Wang et al., 2014). Other studies have also proved that fast-growing woody plants are superior to hyperaccumulators for phytoremediation of heavy metal contaminated soils (Luo et al., 2016; Marmiroli et al., 2011). Furthermore, the willows can be used in short rotation coppice allowing the formation of high biomass energy production and carbon sequestration (Algreen et al., 2013; Sylvain et al., 2016). Using willows in phytoremediation is of highly potential to reduce the environmental risks of metal contaminants.

In addition to the heavy metal contamination, flooding is another severe issue worldwide because of the climate changes, and it has a strong influence on the vegetations or crops (Bourgeade et al., 2018; Valliyodan et al., 2016). Meanwhile, flooding is one of the major abiotic stress factors that markedly affect the plants in riparian zone (Rodríguez-Gamir et al., 2011). Diffusion of air in water is greatly reduced by flooding, causing a decrease of oxygen availability for the root zone. Furthermore, soil flooding could lead to the root dysfunction, decline of photosynthesis and respiration, nutrient uptake and translocation (Colmer and Flowers, 2008; Pierce et al., 2010). To cope with anoxic environment yielded by soil flooding, plants develop adventitious roots/aerenchyma tissues to avoid tissue anoxia (Yang et al., 2017). In recent decades, many floodplain soils have been heavily contaminated by heavy metals (Du Laing et al., 2009; Rennert and Rinklebe, 2010; Rinklebe et al., 2007), and the mobility of metals in soil is greatly affected by the frequent flooding events that triggers significant changes (Rinklebe et al., 2016; Shaheen et al., 2016). Importantly, willow (*Salix* spp) species has become a significant promising candidate to minimize adverse effects from global climate change, and most willows are well adapted to soil flooding and have high capacity for heavy metal phytoremediation (Cao et al., 2018; Kuzovkina and Volk, 2009). Thus, it is essential to study the performance of willow trees in metal contaminated soil as affected by flooding.

Plants need both macro- and micro-elements to maintain their normal growth and development (Han et al., 2011; Wen et al., 2018). Among these essential elements, carbon (C), nitrogen (N) and phosphorus (P) are three major nutrients affecting plant growth, and their stoichiometric ratios could indicate the nutrient dynamics in response to the changing environments (Högberg et al. 2017; Hu et al., 2018; Huang et al., 2019). Under flooded conditions, plants exhibit different strategies for nutrient uptake and utilization, and the alteration could lead to the differences in elemental stoichiometry (Li et al., 2013; Yuan et al., 2013). Previous studies mainly focused on the nutrient composition and stoichiometric response to abiotic stresses such as water, drought, heavy metal stress (Li et al., 2013; Viciedo et al., 2019), whereas investigation on variations of nutrient stoichiometric ratios of organ comparisons in response to flooding metal-contaminated soil still lags behind.

Here, we aimed to compare the responses of arbor willow (*Salix jiangsuensis* ‘J172’) and shrub willow (*Salix integra* ‘Yizhibi’) as affected by flooding in a Cu-contaminated soil. We examined the growth and photosynthetic efficiency of two willows upon exposure to Cu and flooding; explored the effects of flooding and Cu stresses on nutrients alteration and their stoichiometric characteristics; compared the phytoremediation capacity of two willow to Cu under flooded versus non-flooded conditions. Our findings will provide a valuable information for phyto-management of Cu-contaminated wetlands using fast growing willows.

Materials And Methods

Soil preparation and plant cultivation

The Cu-contaminated soil was collected from the surface horizon (0–20 cm) of a local field nearby Hangzhou City, Zhejiang Province. The physicochemical properties of the soil were presented in Table S1, with a relatively high total Cu concentration of 239.51 mg kg^{-1}. Air-dried soil (1.5 kg) was weighed into polyvinylchloride (PVC) tubes with 11 cm in inner diameter and 25 cm in height.

Two willow species, *Salix jiangsuensis* ‘J172’, an arbor hybrid willow with broad leaves, and *Salix integra* ‘Yizhibi’, a shrub willow with narrow and long leaves, were selected in the current study. One-year-old willow branches approximately 0.8 cm in diameter were cut into 15 cm length, and then planted in each PVC tube as described above. The PVC tubes were randomly placed in the greenhouse, and each treatment consisted of four replicates and each replicate contained three seedlings. The plants were first cultivated for 4 months under the greenhouse conditions (temperature: 23–28 °C; relative humidity: 60–65%) in Research Institute of Subtropical Forestry, Chinese Academy of Forestry. Soil moisture content was maintained at 70% water holding capacity by adding tap water every day. After 4 months growth, non-flooded (NF) seedlings were still daily watered. For flooded (F) treatment, plants were grown in a depth of 10 cm water level from the soil surface, lasting for 3 months. The whole experiment was lasted for 7 months before harvest.

Measurement leaf gas exchange

Leaf gas exchange was measured on day 7, 14, 28, 56, 90 after flooded treatment started. Three expanded mature leaves of each plant were selected for leaf gas exchange measurement. Photosynthesis parameters, including net photosynthetic rate (P_n), stomatal conductance (g_s), intercellular CO_2 concentration (C_i) and transpiration rate (T_r) were determined by a portable photosynthesis system (LiCor 6400; Lincoln, NE, USA) as described in Cao et al. (2018). The instrumental parameters were set the intensity of 1000 μmol photon $m^{-2}s^{-1}$ from 9:00 to 14:00; the air flow through the sample chamber were set at 500 μmol s^{-1}, and the CO_2 concentration in the sample chamber was 400 μmol mol^{-1}.

After the last time point of photosynthesis reading (90 d of flooding), the root and above-ground tissues (cuttings, stems and leaves) of each plant were sampled and washed thoroughly with deionized water. Plant tissues were dried in an oven at 75 °C for 72 h. Sample dry weights were recorded and oven-dried tissues were ground into fine powder using a ball miller (propeller mill, IKA, Staufen, Germany) for further analysis. Fresh roots (1 g) across all the treatments were extracted using the cold dithionite-citrate-bicarbonate (DCB) method (Cao et al., 2017) to determine the Cu, iron (Fe), manganese (Mn), and sulfur (S) content in Fe plaque on the root surfaces.

Determination of Cu and other nutrients

The fine powder (about 50 mg) of different tissues was digested in a mixture (4 mL concentrated HNO_3 and 1 mL concentrated $HClO_4$) at 200 °C for 120 min in a hot block system (ED36, Lab Tech, Germany). The digests were cooled down to the ambient temperature and were made up to 25 mL with 2% (*v/v*) diluted HNO_3 solution. Subsequently, Cu and other nutrients such as P, S, potassium (K), calcium (Ca) and magnesium (Mg) , were determined using inductively coupled plasma atomic emission spectrometry (ICP-AES; PerkinElmer Optima 8000, Waltham, MA, USA). To ensure the quality of analyses, a certified reference material of poplar leaves (GBW 07604, National Research Center for Certified Reference Materials, China) was utilized through the process of plant digestion and element analysis. Good agreement was obtained between our method and certified values (Table S2). C content was determined using an elemental analyzer (Vario Macro cube, Elementar, Germany) after sample combustion in an oxygen atmosphere. For N content, plant tissues were digested using H_2SO_4-H_2O_2 method according to Bai et al. (2012), and the digests were determined by an automated Kjeldahl analyzer (Kjeltec 8400, FOSS, Copenhagen, Denmark).

Calculations and statistical analysis

Bioconcentration factor (BCF) indicates the efficiency of metal accumulation in plant tissues from the surrounding environment, and is calculated as the ratio of target metal content in plant tissues to that in the soils (Ali et al., 2013). The translocation factor (TF) indicates the efficiency of the plants in translocating the metals from the roots to shoots, and is calculated as the ratio of target metal content in above-ground tissues to that in plant roots (Padmavathiamma and Li, 2007).

All statistical analyses were performed with Data Processing System Version software (DPS13.01, Zhejiang University, Hangzhou, China). Mean and standard deviation (SD) values of four replicates were calculated. Two-way analysis of variance (ANOVAs) with the flooded treatment and willow species as the factors were employed to test any difference among flooded and non-flooded conditions, different willow species, and their interactions. Differences were considered significant when the *p* value of analysis of variance F-test was 0.05. The pearson's correlation analysis and principal component analysis (PCA) were performed in R software. The figures were plotted using Origin 2018 (Origin Lab, USA).

Results

Plant growth and biomass production

Regardless of non-flooded or flooded treatment, the phenotypes of both *Salix integra* 'Yizhibi' and *Salix jiangsuensis* 'J172' were healthy without any visual damages observed (e.g. leaf necrosis and abscission), and visible hypertrophied lenticels adventitious occurred on the submerged portions of cuttings under flooded conditions. Soil flooding suppressed the willow growth as determined by the plant height, which was decreased by 11.35 and 10.01% in *Salix integra* 'Yizhibi' and *Salix jiangsuensis* 'J172', respectively. Soil flooding markedly decreased the root biomass by 29.87 and 24.74% in *Salix integra* 'Yizhibi' and *S. jiangsuensis* 'J172', respectively (Table 1). In addition, the decrease extents of the root biomass were notably greater than that of leaf and cutting biomass. Notably, the leaf biomass of *Salix jiangsuensis* 'J172' was significantly higher than that of *Salix integra* 'Yizhibi' under flooded condition. Moreover,soil flooding slightly decreased theleaf biomass of *Salix jiangsuensis* 'J172' by 3.75% and the reduction degree was markedly lower than *Salix integra* 'Yizhibi'. Additionally, soil flooding also reduced the total biomass (7.80 and 10.69%) of both two willow species as compared to their respective non-flooded treatment.

Leaf gas exchange

As shown in Fig 1, soil flooding significantly decreased the P_n of *Salix integra* 'Yizhibi' and *Salix jiangsuensis* 'J172' (*p* 0.05) as compared to non-flooded condition, and the decreases at different flooding stages in *Salix integra* 'Yizhibi' were generally higher than *Salix jiangsuensis* 'J172'. Additionally, the P_n first elevated as the duration of flooding increased to 28 d in both willow species, and then decreased after 56 d exposure of flooding under non-flooded/flooded conditions. Similarly, the highest g_s and T_r were both observed on the 28 d after flooding under both conditions. However, the C_i was obviously high on the 56 d after flooding, and the interaction of flooding treatment and flooding time had no significant impact on *Salix jiangsuensis* 'J172' (*p* 0.05). After 28 d of flooding, the g_s and C_i values were decreased by 28.90 and 10.04% in *Salix jiangsuensis* 'J172', respectively, but these two parameters in *Salix integra* 'Yizhibi' increased (13.11 and 11.08% for g_s and C_i) as affected by soil flooding compared to the respective non-flooded condition. The largest reductions in T_r, which were 36.22% in *Salix jiangsuensis* 'J172' and 25.86% in *Salix integra* 'Yizhibi', caused by flooding were observed at 90 d after flooding.

Accumulation and distribution of Cu in willows

The Cu concentrations in willows under non-flooded/flooded conditions are shown in Fig 2. The highest Cu concentration was found in roots regardless of treatments or willow species. Generally, the Cu accumulation in different tissues was in a descending order of root > cutting > stem > leaf. *Salix integra* 'Yizhibi' accumulated more Cu in roots (418.10 and 648.85 mg kg^{-1}) than that in *Salix jiangsuensis* 'J172' (159.32 and 563.56 mg kg^{-1}) in the flooded and non-flooded treatment, respectively. Moreover, the decrease

(71.73%) in the root Cu concentrations of *Salix jiangsuensis* ‘J172’ were greater than that in *Salix integra* ‘Yizhibi’ (35.56%) as affected by soil flooding.

Additionally, the root BCF values of *Salix integra* ‘Yizhibi’ were higher than 1 (Table 2) and were also markedly higher than that of *Salix jiangsuensis* ‘J172’ under flooded andnon-flooded conditions. The BCF values of roots in two willow species were all significantly higher than the above-ground BCF under non-flooded and flooded conditions. Moreover, soil flooding decreased the BCF of roots (35.56 and 71.91%) and above-ground tissues (29.51 and 21.57%) in *Salix integra* ‘Yizhibi’ and *Salix jiangsuensis* ‘J172’, respectively. Conversely, soil flooding increased the TF values, and the increment of TF in *Salix jiangsuensis* ‘J172’ was 172.73%, which was significant higher than that in *S. integra* ‘Yizhibi’ (8.70%). Additionally, we observed that the contents of Fe and Mn in both roots and the Fe plaque on the root surfaces were significantly higher in the flooded treatment in comparison with the non-flooded treatment (Table 3). Significantly negative correlations were found between DCB-Cu and DCB-Fe/DCB-Mn in root surfaces of both willow species, which are consistent with the correlations between root Cu, Fe and Mn contents (Table S3 and Fig 4).

Plant C, N, P and corresponding stoichiometry

The plant C concentration was slightly altered and no significant difference was observed between non-flooded and flooded conditions (p 0.05). Soil flooding significantly decreased the root N by 11.35% in *Salix jiangsuensis* ‘J172’ compared to the corresponding non-flooded treatment. For leaves and stems, the P concentrations of both willow species sharply decreased by 21.25 40.47% as affected by soil flooding, whereas the root P concentrations significantly increased under flooded condition compared to non-flooded condition (Table 4). As a result, the C:P and N:P ratios in leaves and stems were markedly enhanced by soil flooding, while soil flooding significantly decreased the root C:P and N:P ratios as compared to non-flooded condition. Moreover, significant differences in C, N and P stoichiometry among different tissues were also observed, regardless of the presence of flooding (Table 4). The lowest C:N ratio (32.74–43.18) was observed in leaves, but the lowest C:P (75.30–111.01) and N:P (1.13–1.32) ratio were both found in roots.

Other nutrients and multi-element:Cu stoichiometry

The concentrations of Fe and Mn in both willow roots were significantly increased under flooded condition as compared to non-flooded condition, which were 2.30–3.51 and 4.12–5.73 folds higher than the corresponding non-flooded condition, respectively (Fig 2). In stems, the P and K concentrations exhibited a decreasing trend, which significantly reduced by 16.74–21.89% (Table S4) by soil flooding. Interestingly, the concentrations of Ca, Mg and S all increased in roots of both willow species as affected by soil flooding, but generally reduced in leaves, stems and cuttings. Both willow species had higher mineral element: Cu ratio in leaves than in other tissues, regardless of the presence of flooding. Additionally, soil flooding had significant effect on the multi-element: Cu stoichiometry in different tissues (p 0.05, Table 5). For *Salix integra* ‘Yizhibi’, there were significant elevation for flooded condition in all element: Cu ratios of leaves, except for P: Cu ratio. Moreover, other element: Cu were decreased by soil flooding in *Salix jiangsuensis* ‘J172’, but Fe: Cu ratio increased.

Discussion

Effects of flooding on willow growth and photosynthesis

Soil flooding results in water saturation and insufficient oxygen supply, and has adverse impact on plant, including growth inhibition and photosynthesis decrease (Du et al., 2012). In the current study, *Salix integra* ‘Yizhibi’ and *Salix jiangsuensis* ‘J172’ exhibited sharp reductions in root biomass as affected by soil flooding, indicating that roots were sensitive to flooding stress. Other studies have reported that Cuinduced morphological alterations could cause growth inhibition and reduce the root biomass (Adrees et al., 2015; Benimali et al., 2010). Moreover, flooding could induce poor soil aeration and hypoxia in the rhizosphere, and the root growth was stunted, which can adversely influence the uptake of water and mineral elements (Chen et al., 2002). In accordance with our earlier study (Cao et al., 2017), the co-exposure of high Cu contamination and flooding affected the root morphology, resulting in the reduction of the root biomass. Additionally, the biomass of *Salix jiangsuensis* ‘J172’ were all significantly higher than *Salix integra* ‘Yizhibi’ under both non-flooded and flooded conditions, but the plant height of *Salix jiangsuensis* ‘J172’ were slightly lower than that of *Salix integra* ‘Yizhibi’ during the experimental period (Table 1). The reduction extent of the biomass in response to Cu stress were largely determined by plant species or clones (Borghi et al., 2007). The total Cu content of the tested soil obviously exceeded the phytotoxic range of 60–125 mg kg^{-1} (Kabata-Pendias and Pendias, 1984), which could retard the plant growth. Although the inhibition extent to plant biomass varied, both willow species could survive and grow well in a co-existing scenario of high Cu level and flooding for 90 d. Previous study reported that the certain plants that could withstand flooding stress for more than 50 d were proposed to be a relative high flooding tolerance species (Yu et al., 2015). Therefore, both the shrub and arbor willows could be tolerant to flooding and Cu.

Flooding stress generally caused the alteration in leaf photosynthesis, which can be contributed to the difference in flood tolerance of woody species (Du et al., 2012). The photosynthetic responses of *Salix jiangsuensis* 'J172' and *Salix integra* 'Yizhibi' were consistent with the previous studies that soil flooding generally induced decreases in P_n and g_s in flood tolerant woody species (Li et al., 2011; Yu et al., 2015). Additionally, photosynthesis was markedly decreased within a few days after flooding, and the P_n could be slowly elevated as a result of the gradually reopened stomata with the prolonged flooding duration (Kozlowski, 1997; Mielke et al., 2003). In the current study, both willow species exhibited significant decrease in P_n and T_r on different days during flooding (p 0.05). After flooding for 90 d, all the photosynthesis parameters were further decreased in *Salix integra* 'Yizhibi' and *Salix jiangsuensis* 'J172', while P_n could maintain at a relatively high level, and there exhibited significantly positive correlation among P_n, g_s and T_r (p 0.05). It was also demonstrated that the early decrease of P_n in flooded plants was associated with stomatal closure, leading to a reduced CO_2 uptake by leaves (Chen et al., 2005a; Li et al., 2011). Furthermore, the decrease of g_s could cause the reduction of C_i concentration and photosynthetic substrate, leading to the decline of P_n (Farquhar and Sharkey, 1982; Kozlowski, 1997).

Effect of flooding on Cu accumulation and distribution

Generally, willows showed good potentials in Cu phytoremediation under suitable environmental conditions (Cao et al., 2018). Moreover, the remediation efficiency relies on a variety of factors, such as soil properties, metal type, plant species and environmental conditions (Vandecasteele et al., 2005; Zimmer et al., 2011). The arbor willow (*Salix jiangsuensis* 'J172') and shrub willow (*Salix integra* 'Yizhibi') showed significant variations in Cu accumulation and distribution under flooded condition versus non-flooded condition. We observed that large amount of Cu accumulated in roots of both willow species (Fig 2), and the Cu BCFs of roots were significantly higher than those of above-ground tissues (Table 2). Similar results were also evident in other wetland plants, in which the shoot tissues had lower capacity to accumulate heavy metals (Yang et al., 2017; Ye et al., 1997), suggesting the detoxification strategy by restricting most of the heavy metals in roots (Kuzovkina et al., 2004) rather than in shoots. In the current study, soil flooding significantly decreased the Cu contents in roots of two willow species (p 0.05). This result is similar with a previous study,that flooding treated soil decreased the Cd accumulation of *Salix cinerea* (Vandecasteele et al., 2010). However, Kissoon et al. (2011) showed that flooding treatment of *Typha angustifolia* accumulated more metals (Al, Fe, Mn and Zn) in roots as compared to non-flooded treatment. We speculated that the discrepancy might be because of the variation in absorbing capacity to metals in different wetland plant species, metal types, and the total level of metals in soils.

Although the total biomass of arbor willow (*Salix jiangsuensis* 'J172') was markedly higher than shrub willow (*Salix integra* 'Yizhibi'), the Cu uptake capacity of *Salix jiangsuensis* 'J172' (2.94 and 0.74 mg per plant) was much lower than that of *Salix integra* 'Yizhibi' (2.99 and 1.39 mg per plant) under non-flooded and flooded conditions. These results were accompanied with the higher BCF values in roots (2.74 and 1.74) and root/shoot ratio (0.38 and 0.27) of *Salix integra* 'Yizhibi' than that of *Salix jiangsuensis* 'J172' under non-flooded and flooded conditions. Therefore, the metal tolerance and accumulation capacity were not only due to the total biomass production but also the root/shoot ratio could modulate ion absorption from the soil (Ekvall and Greger, 2003). In terms of Cu accumulation capacity and plant biomass, our results imply that shurb willow (*Salix integra* 'Yizhibi') exhibit potential for Cu phytostabilization with relative high Cu content in roots regardless of flooded or non-flooded condition. It also worth pointing out that the markedly high biomass of the arbor willow (*Salix jiangsuensis* 'J172') could make the process of phytoextraction quite effectively.

Here, the Cu accumulation capacity expressed as content per plant in both willow species markedly decreased by soil flooding (p 0.05). Additionally, our results imply that soil flooding significantly enhanced the Fe and Mn accumulation in roots or root surfaces as compared to non-flooded condition, but significantly decreased the root Cu content (Table 3). Furthermore, markedly negative correlations were also observed between DCB-Cu and DCB-Fe, DCB-Cu and DCB-Mn on the root of both willow species (Table S3). Similar results were reported by Du Laing et al. (2009), the metal mobility could be influenced by Fe/Mn oxide reduction/oxidation. For example, the formation of Fe plaque in rice resulted in the decline of As, Cd and Pb uptake and translocation (Cheng et al., 2014). Several studies also reported that Fe plaque formed on the root surface decreased metal accumulation, and further influenced the metal sequestration and translocation (Chen et al., 2005b; Zimmer et al., 2011). Consequently, the lower Cu accumulation in roots may be attributed to the presence of Fe/Mn plaque. Furthermore, the DCB-Cu was positively correlated with DCB-S (r 0.74, p

0.01) (Table S3), but the root Cu concentration was negatively correlated with the root S concentration (r -0.97, *p* 0.01) (Fig 4). Another explanation for the decreased Cu concentrations in roots could be related to the sulfide formation. Soil flooding could lead to oxygen depletion in the rhizosphere, and further caused soil aerobic conditions (Kozlowski, 1997). Previous studies also found the reduction process of Cu (Ⅱ) to Cu (I), subsequently resulting in Cu_2S precipitation (Du Laing et al., 2009; Simpson et al., 2000).

Effects of flooding on stoichiometry patterns of plant C, N and P

The variations in nutrient elements contents of wetland plants might reflect their available content in waterlogged soils. In this study, soil flooding induced alteration in the contents of C, N, P and their corresponding stoichiometry in *Salix integra* 'Yizhibi' and *Salix jiangsuensis* 'J172' (Table 4). Here, the plant C contents were less variable than nutrient stoichiometry traits, and exhibited no significant changes under flooded condition in comparison with non-flooded condition (*p* 0.05). It could be ascribed as that C in plants was not directly involved in activities of plant production, instead, mainly providing the structural basis as a relatively stable plant skeleton (Qiu et al., 2020; Zhang et al., 2019). It was also suggested that the C contents were stable and not affected by water supply/water depths in shrub *Zygophyllum xanthoxylum* and macrophyte species such as *Potamogeton malaianus*, *Potamogeton maackianus*, *Myriophyllum spicatum*, *Ceratophyllum demersum* and *Hydrilla verticillata* (Niu et al., 2019; Li et al., 2013).

Both willow species exhibited lower N and P concentrations in leaves under flooded condition than non-flooded condition, and the patterns of the N and P response to soil flooding were similar to *Zea mays* (Lizaso et al., 2001), *Lepidium latifolium* (Chen et al., 2005) and *Triticum aestivum* (Trought and Drew, 1980). According to our analysis, significantly positive correlation between leaf N and leaf P concentrations were observed, regardless of flooding. N and P are both foundational elements and participate in multi physiological processes, including plant growth (Penuelas et al., 2013), nutrient availability (Collins et al., 2016) and environmental adaption (Li et al., 2014). Thus, changes in leaf N are usually consistent with leaf P under the same environmental condition (Huang et al., 2019). Furthermore, soil flooding significantly enhanced the ratio of C:P in leaves of *Salix integra* 'Yizhibi' (69.66%) and *Salix jiangsuensis* 'J172' (44.42%) compared to non-flooded condition, indicating that soil flooding led to an elevated demand for assimilating more C to maintain the normal metabolisms in plants (Herbert et al., 2003; Huang et al., 2019). The present study also found that soil flooding significantly reduced the ratio of C:P in roots of both willow species, and the variation in *Salix integra* 'Yizhibi' (28.71%) was higher than that of *Salix jiangsuensis* 'J172' (10.37%). In line with previous observations in wetland plants, the ratios of C:P was markedly declined by waterlogging (Güsewell and Koerselman, 2002; Li et al., 2013). Under flooded conditions, the pronounced increases of root P were observed in *Salix integra* 'Yizhibi' and *Salix jiangsuensis* 'J172', but root C in both willows were relative stable, might be a possible reason for the reduced root C:P ratio. Additionally, in agreement with other studies, significant higher N contents and N:P ratio were observed in leaves than other tissues (stems, cuttings, and roots) (Hu et al., 2018; Li et al., 2013; Sardans and Peñuelas, 2015). The N and P contents and their corresponding stoichiometry differed significantly among the willow tissues because of the structures and functions/activities of different organs (Hu et al., 2018). Generally, leaves contained many chloroplasts which were highly active in metabolic and photosynthetic processes, then higher N contents and N:P ratio than other organs were required (Minden and Kleyer, 2014).

Effects of flooding on other nutrients and their correlation with Cu

The nutrient contents among tissues displays significant differences (Fig 2). Generally, leaves accumulated higher concentrations of macroelements (N, P, K and Mg) than those in roots of *Salix integra* 'Yizhibi' across both treatments. The differences possibly resulted from the mobility of nutrient elements in different tissues (Jiang et al., 2018). For instance, elements related to photosynthesis, encompassing N, P and K, were translocated and accumulated in leaves to promote plant growth (Huang et al., 2019). However, trace element such as Fe and Mn accumulated more in roots than in leaves (Cao et al., 2017), and it could tightly bound within the root cells, contributing to greater accumulation in the belowground tissues (Jiang et al., 2018). Indeed, the elements required for high concentrations are considered less sensitive and less variable to environmental variations during the plant growth and development (Han et al., 2011).

The Cu toxicity could influence the nutrient uptake and distribution within plant tissues due to disruption of water homeostasis, cellular permeability barrier and changes of physiological function (Cao et al., 2017; Chrysargyris et al., 2019). The macro- and micro-elements were significantly affected by co-exposure of flooding and Cu stress, and they exhibited different correlation with the Cu levels. Among all element: Cu ratios, Fe:Cu, Mn:Cu and K:Cu ratios were the top three contributors for different tissues of the two willow species under two treatments (Fig 3 and Table S5), probably due to the physiological roles of these trace elements in plants and their correlations with Cu in ecological processes under flooded and non-flooded conditions. Excessive Cu levels in soils could cause phytotoxicity, and further alter the uptake, transport, and utilization of the mineral elements (e.g. Fe, Ca and Mg) (Cao et al., 2017; Ducic and Polle, 2005). Additionally, elemental composition and nutrient stoichiometry for homoeostatic regulation are vital physiological mechanisms to maintain the normal growth for plants suffered from environmental stresses (Lei et al., 2015; Peñuelas et al., 2010; Yu et al., 2010). Karimi and Folt (2006) revealed that homeostatic capacities were highest for macronutrients, intermediate for essential trace elements and lowest for non-essential metals. Relevant study also suggested that macronutrients such as N, P and Ca were strongly stable, whereas trace elements were weak in equilibrium capacity (Jiang et al., 2018).

The decreased P and Ca in leaves are likely due to the enhanced Fe and Mn contents in roots, which could inhibit their uptake and immobilize them in roots, further interfering their translocation to shoots (McKevlin et al., 1987; Chen et al., 2005a). This is partially ascribed to Fe/Mn plaque formation in root surfaces under soil flooding (Cao et al., 2017). Another possible explanation is that these elements could compete with Cu in transport pathways, because most metal transporters could work with various metal ions (Cao et al., 2017; Solti et al., 2011). The enhanced accumulation of Ca, Mg and S in roots under flooding conditions could be explained by the rhizosphere oxidation processes, which might further stimulate nutrient uptake, affect the nutrient sequestration, and create concentration gradients of nutrients that promote the nutrient element movement in roots (Kissoon et al., 2011; Moore et al., 1994). It was demonstrated that more metabolically active organs could likely accumulate more nutrients (e.g. N, Ca, K, P) to maintain high photosynthesis in leaves and uptake capacity in root (Zhang et al., 2020). Therefore, the results observed in our study indicated that increased uptake of nutrients is helpful for improving photosynthetic capacity and is also crucial to willow survival during flooding.

Conclusion

Both willow species were able to grow well and showed relatively high tolerance to the combined stresses of Cu and flooding. Under flooded condition, *Salix integra* 'Yizhibi' showed greater potential of Cu accumulation capacity, even though its total biomass was significantly lower than *Salix jiangsuensis* 'J172' (arbor willow). Moreover, *Salix integra* 'Yizhibi' was noted to be suitable for Cu phytostabilization with relative high Cu accumulation in roots regardless of flooding. Although characteristics of Cu accumulation differed between two willows, both species presented suitable tolerance mechanisms (regulating photosynthesis, nutrient uptake) to cope with flooding stress. Our study also helps further understanding the nutrient balance and stoichiometry of willows in Cu contaminated soil and their responses to soil flooding.

Declarations

Author contribution

Yini Cao: Conception and experiment design, Methodology, Formal analysis, Writing -original draft, Writing-review and editing; Chuanxin Ma: Critical revision of the article for important intellectual content, Obtaining of funding; Jie Chen & Jiang Xiao: Methodology, Analysis and interpretation of the data; Jiuxi Shi: Statistical expertise, Validation; Guangcai Chen: Conceptualization, Writing-review and editing, Project administration.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethical approval

This paper has not been submitted to any other journal for publication, and all authors have no actual or potential conflicts of interest to this manuscript.

Consent for participate

All authors have read and agree to the contents of the manuscript and consent to its publication.

Consent to publish

All authors consent to the publication of this paper.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Tables

Due to technical limitations, Table 1, 4 and 5 are only available as a download in the Supplemental Files section.

Table 2 Bioconcentration factor (BCF) and translocation factor (TF) in *S. integra* and *S. jiangsuensis* after 90 d of non-flooded/flooding treatments

	S.integra 'Yizhbi'		*S.jiangsuensis* 'J172'		Significance		
	Non-flooded	Flooded	Non-flooded	Flooded	Treatments	Species	Treatments Species
BCF-root	2.70 0.08a	1.74 0.04c	2.35 0.05b	0.66 0.02d	****	****	****
BCF-aboveground tissues	0.061 0.001a	0.043 0.001c	0.051 0.001b	0.040 0.002d	****	****	***
TF	0.023 0.000bc	0.025 0.000b	0.022 0.000c	0.060 0.003a	****	****	****

Each value represents the mean of four replicates Stand Deviation

Different letters indicated significant difference among the 4 treatments (2 willow species under non-fooded and flooded conditions) at 0.05 level by Fisher's

LSD test. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001.

Table 3 Fe, Mn, Cu and S concentration (DW) in the plaque on the root surface of *S. integra* 'Yizhbi' and *S. jiangsuensis* 'J172' exposed to Cu contaminated soil under non-flooded/flooding conditions for 90 d.

Willow species	Treatments	DCB Fe (mg kg^{-1})	DCB Mn (mg kg^{-1})	DCB Cu (mg kg^{-1})	DCB S (g kg^{-1})
S.integra 'Yizhbi'	Non-flooded	32.49 2.81d	6.67 0.22d	16.19 1.27a	28.20 1.30b
	Flooded	1286.90 74.77a	46.54 2.33a	3.08 0.25d	19.03 0.35c
S.jiangsuensis 'J172'	Non-flooded	563.76 33.07c	16.22 0.72c	12.54 0.82b	7.84 0.16c
	Flooded	1065.10 19.89b	28.68 2.29b	32.05 1.07a	14.96 1.12d
Significance	Treatments	****	ns	****	****
	Species	****	ns	ns	ns
	Treatments Species	****	****	****	***

Each value represents the mean of four replicates Stand Deviation

Different letters indicated significant difference among the 4 treatments (2 willow species under non-fooded and flooded conditions) at 0.05 level by Fisher's

LSD test. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

Figures

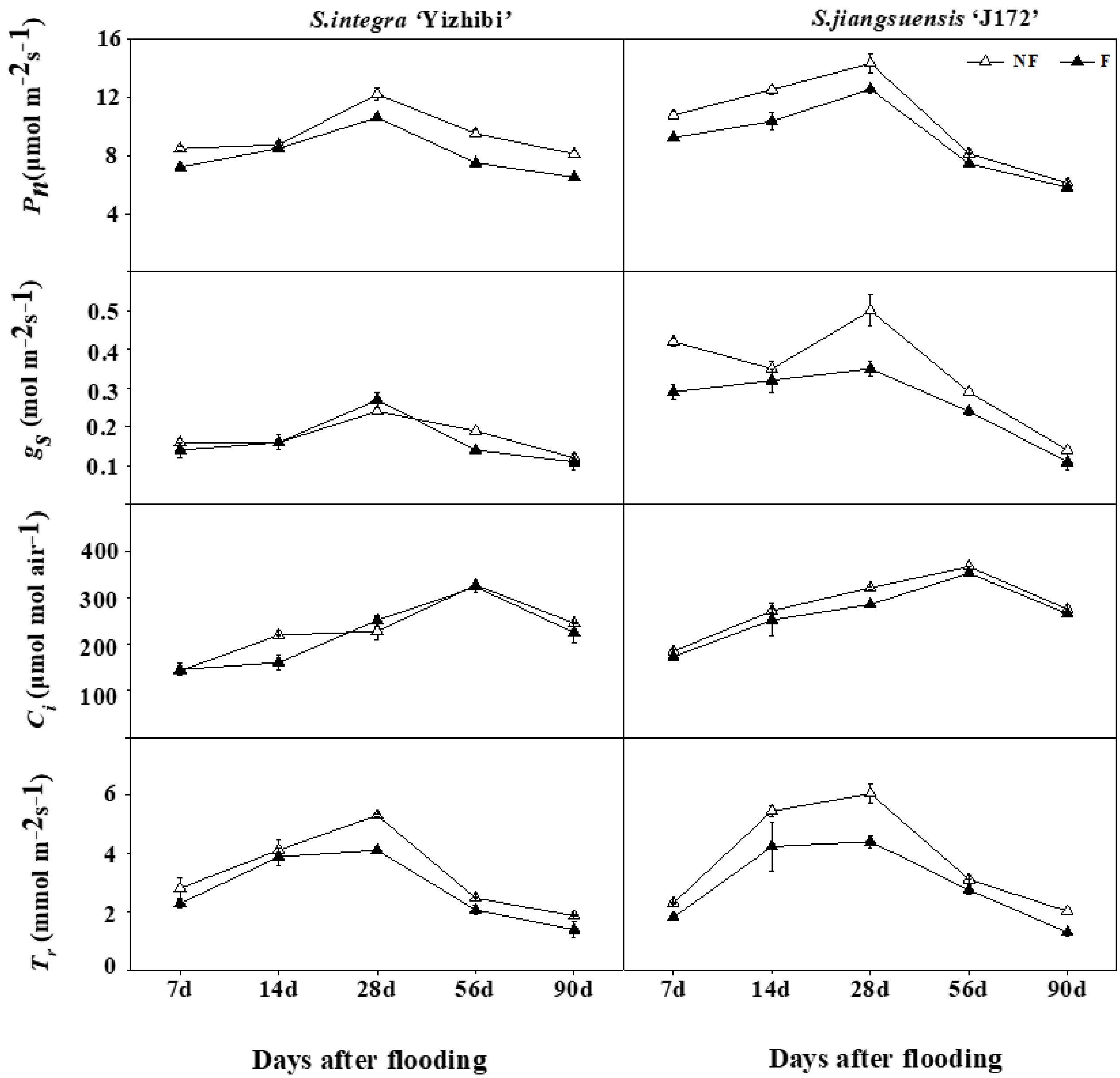

Figure 1

Temporal changes in photosynthetic rate (Pn, μmol.m-2s-1), stomatal conductance (gs, mol H2O m-2s-1), intercellular CO2 concentration (Ci, μmol CO2 mol air-1) and transpiration rate (Tr, mmol H2O m-2s-1) in leaves of Salix integra (S. integra) 'Yizhbi' and Salix jiangsuensis (S. jiangsuensis) exposed to Cu contaminated soil under non-flooded andflooded conditions for 90 d.

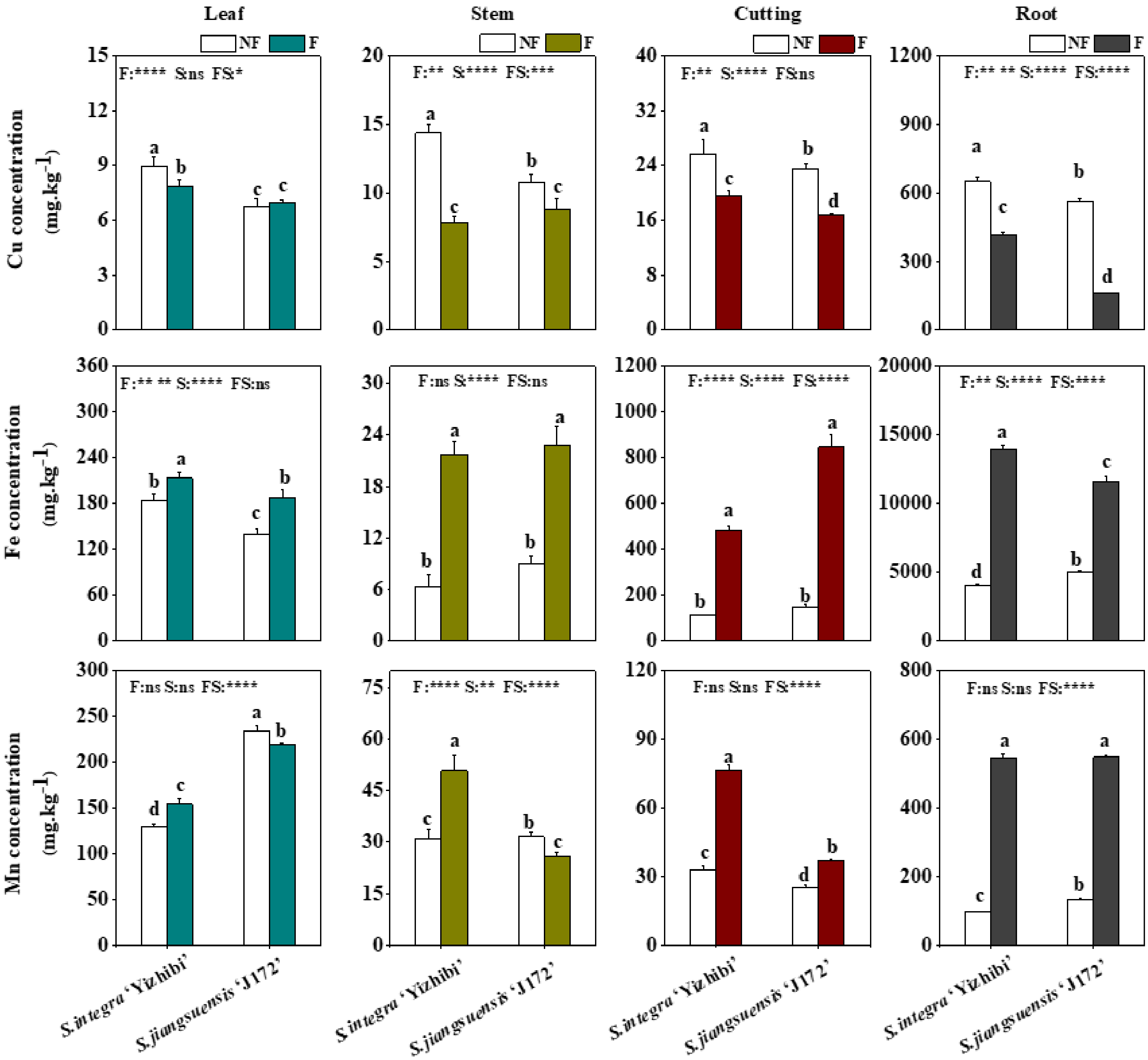

Figure 2

Trace elements (Cu, Fe, Mn in mg.kg-1 DW) in plant tissues of S. integra 'Yizhbi' and S. jiangsuensis 'J172' exposed to Cu contaminated soil under non-flooded andflooded conditions for 90 d. The data indicate the means±SD (n=4). Different letters indicated significant difference among the 4 treatments (2 willow species under non-fooded and flooded conditions) at 0.05 level by Fisher's. P values of ANOVA of willow species(S), soil flooding (F), and their interactions (FS) are also shown. (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001, ns: not significant).

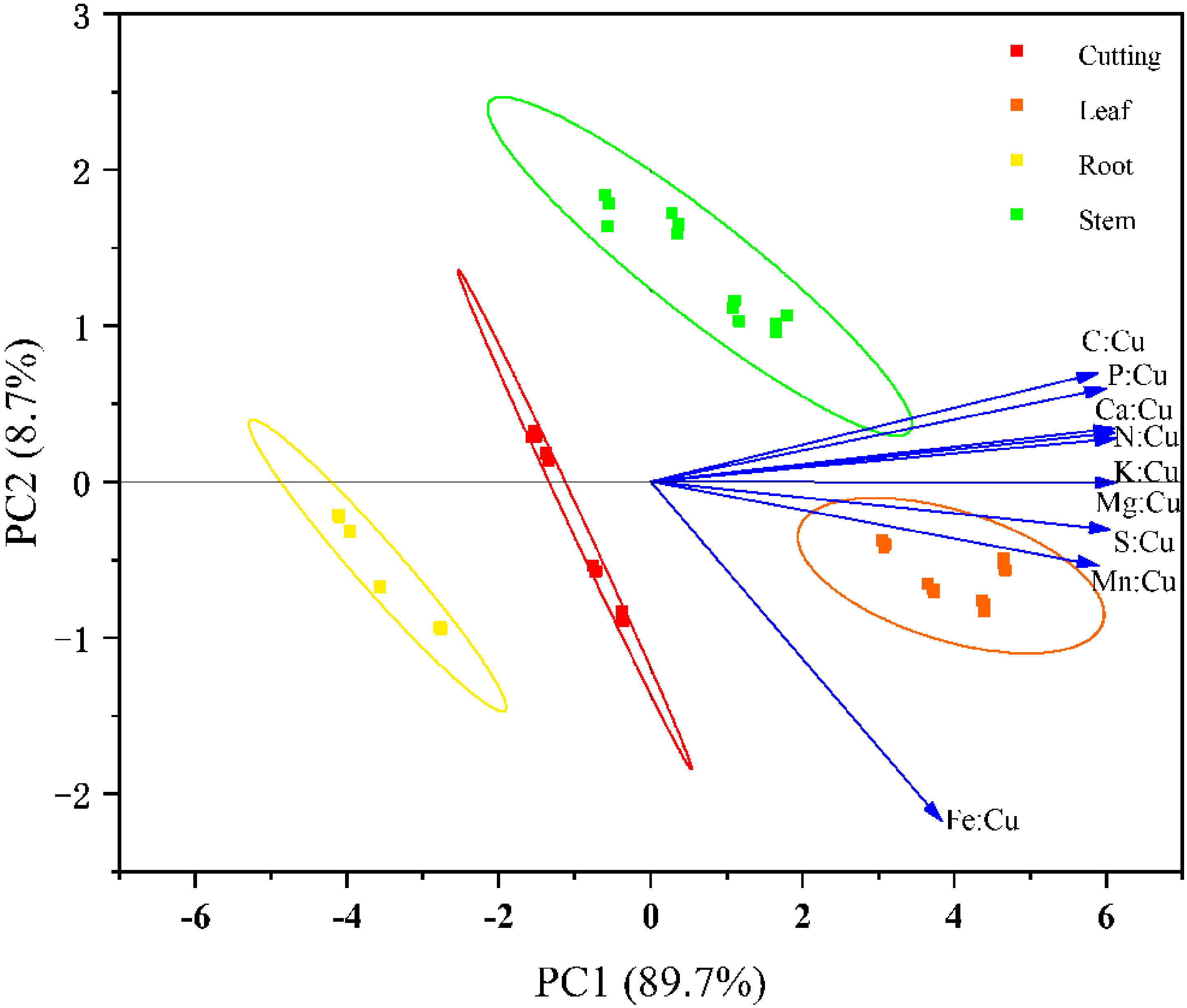

Figure 3

Principal component analysis (PCA) of different element:Cu ratios of two willows in response to non-flooded andflooded treatments with Cu contamination. The first two principal component of scores was indicated.

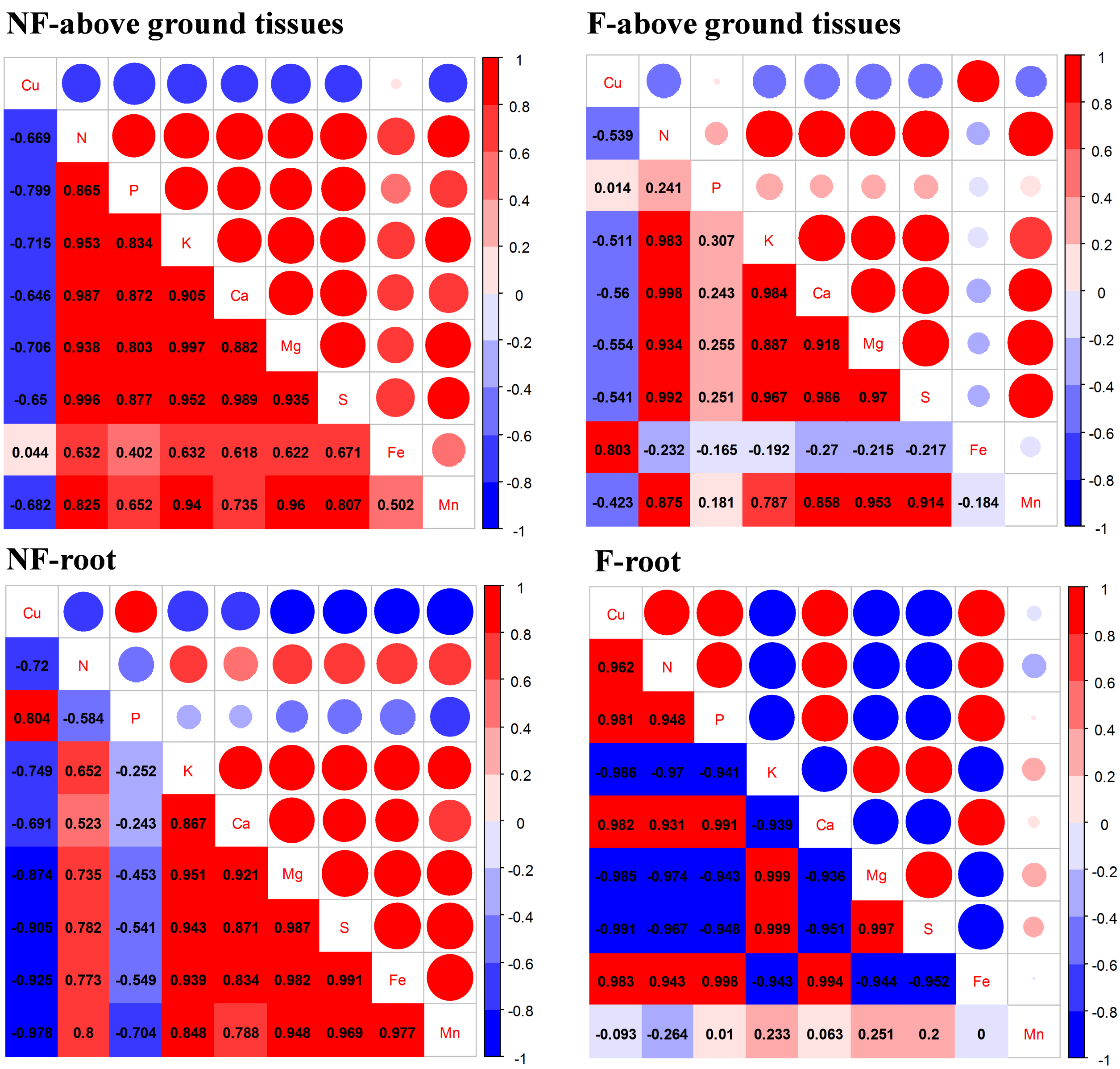

Figure 4

Pearson's correlation analysis performed on element concentrations measured in above-ground parts and roots in of S. integra 'Yizhbi' and S. jiangsuensis 'J172' exposed to Cu contaminated soil under non-flooded andflooded conditions for 90 d. Positive correlations are displayed in red and negative correlations in blue color. Color intensity and circle size are proportional to the correlation coefficients. The significant level of correlation test which is less than 0.05 are shown in the figure.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1and4and5.docx
- TwowillowspeciesSI.docx