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Enhanced phytoremediation capacity of a mixed-species plantation of *Eucalyptus globulus* and *Chickpeas*

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ABSTRACT

The objective of this research was to determine the phytoremediation efficiency for a mixed plantation of non-nitrogen-fixing *Eucalyptus globulus* with nitrogen-fixing *Chickpeas* in soil contaminated by heavy metals including Cd, Cu, Hg and Pb. Plants in the mixed-species system produced more biomass, circulated more nutrients and water and absorbed more toxic materials due to the nitrogen fixation of *Chickpeas* and the generation of root and canopy stratification, which reduced nutrients and light competition.

It would take, respectively, 40, 68, 4225 and 127 years to reduce the concentration of Cd, Cu, Hg and Pb in the soil to safe levels, which is approximately half the time needed to achieve the same goal for *Eucalyptus globulus* or *Chickpeas* monocultures. The mixed cultivation enhances the phytoremediation efficiency of Cd and Cu contaminated soil significantly, but it is ineffective in reducing the Hg level in soil because of its low bioavailability.

1. Introduction

The continuous industrialization of countries and growth of the world's population have led to the dramatically elevated release of a wide variety of chemicals into the environment, which leads to severe public health problems. Soil, water, and air can be contaminated with heavy metals that cannot be degraded by microbial or chemical processes.

Techniques used to remove heavy metal ions include vitrification, soil excavation, soil flushing, solidification, chemical precipitation, electrokinetics, and adsorption among others (Polechońska and Klink, 2014). Most of the conventional methods are either extremely expensive and labor intensive or have deleterious effects, such as causing irreversible changes in soil structure, the destruction of native soil microflora and secondary pollution. Therefore, after using traditional techniques, agricultural activities would no longer be possible. Alternatively, phytoremediation has long been recognized as a cost-effective and environmentally friendly method that utilizes plants that are capable of extracting heavy metals from the soil. Furthermore, phytoremediation can prevent soil erosion by both wind and water, replace the use of fossil fuels as an energy supply and decrease greenhouse gas emissions by storing carbon in non-harvesting parts. Additionally, this process can be cultivated on marginal land and does not compete for agricultural soils. Species that can be utilized for the remediation of contaminated soil are those with high biomass production, which are resistant to extreme weather, have a high tolerance to heavy metal accumulation and are easy to establish. Some non-hyperaccumulators can extract comparative quantity of pollutants as hyperaccumulators due to greater biomass production despite the fact that the target heavy metals in tissues of them does not conform to the criteria of a hyperaccumulator (Bech et al., 2012).

In recent years, there have been numerous studies on heavy metal tolerance and their uptake by plants. However, almost all of these studies focused on single species and neglected the phytoremediation potential of mixed species either as part of an in-situ or ex-situ experiment.

Eucalyptus globulus is the most adaptive species used for the phytoremediation of heavy metals owing to its high aboveground biomass, fast growth and high tolerance to heavy metals. This species stores a mass of heavy metals in its roots (Mughini et al., 2013) and, therefore, the elements in the trunks are not high enough to impact the market value of its wood. Forrester et al. (2010) found that mixed-

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species plantations containing non-nitrogen-fixing and nitrogen-fixing species can increase the productivity of each species by increasing the N availability and decreasing intra-specific competition. In the present study, the contents of heavy metals in different plant organs of two species planted in monoculture and together are discussed and the remediation efficiency of using mixed plantations of *Eucalyptus globulus* with *Chickpeas* for phytoremediation were investigated.

2. Materials and methods

2.1. Study area description and growth conditions

The experimental site, Guiyu, locates in southeast Guangdong Province, China, with a subtropical continental climate. Its mean annual air temperature is approximately 21.5 °C, and annual rainfall varies from 980 to 1550 mm. Guiyu has a local population of approximately 150,000 and has been involved in e-waste dismantling activities for >30 years. It has accepted massive amounts of imported e-waste annually and approximately 6000 family artisanal enterprises participate in this high-polluting industry, using primitive techniques including incineration, sorting, employing strong acid to recover rare metals, burning cables to recycle copper wires and melting circuit boards on a coal stove to separate precious elements. In the process of recycling, fly ash and effluent laden with toxic materials are usually discharged, resulting in contaminated air, soil, surface water and crops. Although uncontrolled open burning and strong acid leaching of e-waste are considered illegal in Guiyu and have been banned for many years, the pollution can still be detected in farmland soils and streams.

Three experimental contaminated soil disposal sites (20 × 24 m) were established on otherwise valuable arable land in Guiyu, a size above the threshold of 100–150 m^2 established for real remediation situations. The experimental field was divided into six 10 × 8 m subplots including a surrounding row of buffer trees to prevent the transfer of heavy metals and nutrients between plots by rain. The experimental sites were plowed three times using normal agricultural machinery to drastically homogenize the soil after the e-waste was manually removed.

Plant transpiration was counted based on the Penman–Monteith equation and the modified Jarvis–Stewart model (Estévez et al., 2009; Whitley et al., 2009).

2.2. Soil characterization

The soil in this experiment is a typical Ferric Acrisol, according to previous regional geochemical surveys. 60 soil samples were collected from the surface soil (20 cm) before air dried and sieved (2 mm) for analysis. The pH of the soil was measured in situ with water at 1:2 (w/v) ratio using a pH meter. The CEC of the soil was determined using the ammonium acetate saturation method and the total organic carbon (TOC) was determined using ferrous ammonium sulfate titration after the oxidation of the organic matter in soil through potassium permanganate. Table 1 presents the physical and chemical properties of the soil.

Table 1
Physical and chemical properties of the soil.

Soil type		Ferric Acrisols
pH		6.4 ± 1.1
CEC	cmol kg^{-1}	12.7 ± 3.3
TOC	g kg^{-1}	42 ± 6
Cd	mg kg^{-1}	0.61 ± 0.17
Hg	mg kg^{-1}	0.44 ± 0.18
Pb	mg kg^{-1}	69.5 ± 19.4
Cu	mg kg^{-1}	56.2 ± 21.7
Cr	mg kg^{-1}	57.1 ± 13.5
Zn	mg kg^{-1}	111.27 ± 35.08

2.3. Experimental design

Three experiments were performed, E1, E2 and E3. In early April 2010, healthy, three-year-old *Eucalyptus globulus* were transplanted by hand into E1 from another experiment site that served similar purposes. Planted at a density of 2500 per ha with a low level application of phosphate fertilizer (200 kg per ha of calcium superphosphate), the trees were spaced at 4 m^2 intervals, which is the recommended planting density when phytoremediation is the main plantation objective (Guoa et al., 2002). For the E2 experiment, *Chickpea* seeds were obtained commercially from Guangzhou and planted at the same time at a planting density of 45 × 5 cm, for a total of 445 thousand crops per ha, which results in the best comprehensive performance (Wu et al., 2008). *Eucalyptus globulus* and *Chickpeas* were planted together (50% *Eucalyptus globulus* and 50% *Chickpeas*) in the E3 experiment. All plants were harvested manually in late August.

2.4. Laboratory analyses

Five plants of each subplot were collected randomly and separated into roots and shoots to determine the biomass and concentrations of heavy metals. Plant parts were washed with tap water to remove any adhering sediments and dried to constant weight in an oven at 80 °C. Dried plant samples were pulverized for digestion. The powder samples were pressed into pellets after being packaged with boric acid and the Pb, Cu, Cr and Zn content in the pellets was estimated using an X-ray fluorescence spectrometer. Soil or plant samples were digested with a solution containing 75% concentrated HCl and 25% concentrated HNO_3 (v/v) in a 50-mL Teflon crucible. A 1-mL aliquot of solution was then placed into a 10-mL colorimetric tube and diluted with 3% nitric acid. The concentrations of Cd were analysed using inductively coupled plasma-mass spectrometry (ICP-MS). The Hg in soil or plant samples was determined by cold vapor generation atomic fluorescence spectrometry and the samples were digested with a mixed medium of potassium permanganate, sulfuric acid and potassium persulfate.

2.5. Statistical analyses

The data were processed for two-way analysis of variance (ANOVA) using SPSS 15.0. All results were expressed as the mean ± S.D. of the six replicates. Mean separation was conducted based on Duncan's multiple range tests. Significant differences were assessed at the level $P < 0.05$.

To study heavy metals uptake and bio-concentration behavior, the following indices were used: BCF = the element in the plant tissue / the element in the soil. A BCF > 1 elucidates the plant is suitable for phytoremediation purposes. TF = the element in shoot / the element in root. The TF value is used to determine the internal transport of metals from root to shoot. The total metal extraction by roots/shoots (TE) = the metal in part of the plant × the dry biomass yield of the part. The TE value represents the total heavy metals absorbed from the soil by the plant.

3. Results

3.1. Impact of heavy metals in the soil on plant growth

As shown in Table 1, the Cd, Hg, Pb, and Cu contents in the top 20 cm of soil exceeded the upper limits set by the US Environmental Protection Agency and the Grade II environmental quality standard for soils used for agricultural purposes in China, which signifies that the soil was moderately polluted by these heavy metals. The concentrations of Cr and Zn in the surface soil were below the safe limit.

There were no visual symptoms of phytotoxicity such as yellowing, chlorosis, leaf necrosis or reduced growth on *Eucalyptus globulus* during the experiment, which is in agreement with the findings of Arriagada et al. (2007), who found that *Eucalyptus* is one of the most promising heavy metal phytoremediation plants with a large capacity for absorbing different types of metals from polluted soils. The low amount of *Chickpeas* dry biomass produced in the experiment was ascribed to the plants sensitivity to heavy metals (Gupta et al., 2006) compared to the production of *Chickpeas* in uncontaminated soil (Kashiwagi et al., 2006; Soltani and Sinclair, 2011). The mature leaves of the *Chickpeas* monoculture showed slight etiolation and some leaves were crinkled, which indicated signs of chlorosis and early senescence. There was also competition with *Eucalyptus globulus* for nutrients such as phosphorus (P), calcium (Ca), magnesium (Mg), copper (Cu) and zinc (Zn) that induced more overt phytotoxic symptoms when the two species were planted together.

3.2. Biomass production and transpiration of the plants in different experiment sites

Both belowground and aboveground biomass production of *Eucalyptus globulus* was significantly higher when grown with *Chickpeas* than when in the monoculture despite having only half the density of the plants. The *Chickpea* augments were slightly lower when grown with *Eucalyptus globulus* than when in the monoculture, demonstrating the species' relatively lower stocking densities. However, *Chickpea* roots and aboveground biomass in mixture amounted to 78% and 83% of those in the monoculture, respectively, indicating their excellent growth as trees in mixture only have half of the number of trees in the monoculture (Table 2).

Eucalyptus globulus in the mixture have the highest transpiration and *Chickpeas* in the monoculture have the lowest transpiration. The monthly transpiration of *Eucalyptus globulus* in the monoculture was highest in May (627 mm) and that of *Chickpeas* was highest in August (37 mm). The average transpiration amount of *Eucalyptus globulus* was 542 and 496 mm in the mixture and monoculture, whereas that of *Chickpeas* was 29 and 20 mm, respectively.

3.3. Phytoremediation potential of the plants under different cultivation protocols

In all experiments, the concentration of heavy metals in *Eucalyptus globulus* and *Chickpeas* that exceeded the toxicity threshold for soil was significantly higher in roots than in shoots. The shoots of *Eucalyptus globulus* in the mixture exhibited a significantly higher ($P < 0.05$) concentration of Cd and Cu than in the monoculture and the shoot's Hg and Pb concentrations were nearly the same in each experiment. All heavy metals in the roots of *Eucalyptus globulus* were significantly higher ($P < 0.05$) in the mixture than in the monoculture. The Cd, Hg and Cu concentrations in the shoots of *Chickpeas* were relatively higher in the mixture than in the monoculture and the roots of the plants accumulated significantly more ($P < 0.05$) Cd and Cu in the mixture. The root's Hg and Pb concentrations were higher in the mixture than in the monoculture but not significantly so (Table 2).

According to Sandhya and Tarun (2013), a high BCF at low concentrations of toxic metals is important for phytoremediation. A $BCF > 1$ elucidates that the plant are suitable for phytoremediation. A $TF < 1$ indicates that more heavy metals are confined to roots and the uptake of shoots is lower. The remediation effect depends on the concentrations of heavy metals in the plant tissue and the biomass of the species. Although the concentration of all heavy metals in *Eucalyptus globulus* does not achieve the threshold of a hyperaccumulator, it can still be considered suitable for phytoremediation due to its higher biomass production. *Eucalyptus globulus* in the mixture accumulates the greatest amount of the target contaminants per plant. *Chickpeas* are not considered to be an effective phytoremediator due to their small biomass and low concentration of heavy metals.

In this analysis we assumed TE, which was used to calculate how much time was needed to reduce heavy metals in the soil to their recommended limits, remains constant in the overall age of the plants. We also assume that metal pollution occurs only in the top 20 cm of the soil surface layer, which gives a total soil mass of 2700 t ha^{-1} (Ferric Acrisols density of 1.35 g cm^{-3}).

It would take 59, 6895, 120 and 93 years for *Eucalyptus globulus* in monoculture to reduce the initial concentration of soil Cd, Hg, Pb and Cu from 0.61 mg kg^{-1}, 0.44 mg kg^{-1}, 69.5 mg kg^{-1} and 56.2 mg kg^{-1} to 0.3 mg kg^{-1}, 0.3 mg kg^{-1}, 50 mg kg^{-1}, and 50 mg kg^{-1}, respectively. Moreover, 94, 8484, 390 and 221 years would be needed for *Chickpeas* in monoculture to remediate the soils that are contaminated with heavy metals, respectively. Mixed-species plantations enhance the phytoremediation efficiency and take approximately half the time to achieve the same purpose. It would take 40, 4225, 127 and 68 years to extract superfluous heavy metals in mixture, respectively (Table 3).

4. Discussion

Compared to engineering technologies, phytoremediation has been demonstrated as a non-invasive, environmentally friendly and more cost-effective alternative for the remediation of contaminated soils (Ali et al., 2013). The cultivation method directly influences the plants' susceptibility and translocation as well as tolerance to and accumulation of heavy metals (Pandey, 2012). Thus, identification of suitable cultivation protocols has become an important aspect of phytoremediation.

4.1. Increased production and extraction of heavy metals in mixtures

Competition, competitive reduction and facilitation are the three most significant factors in mixtures of non-nitrogen-fixing and nitrogen-fixing species (Jose et al., 2006). Plants should impose an adverse impact on other plants when there are several interactions occurring in these mixed stands. Competitive reduction arises if the inter-specific competition for nutrient elements in mixed-species plantations is less than the intra-specific competition in the monocultures. Facilitation occurs in mixtures where one type of plant promotes the growth of other species, such as a nitrogen-fixing species increasing the total productivity of mixed-species and the utilization levels for nutrients and water.

Much of the published research has reported that the production of biomass in mixtures of *Eucalyptus globulus* and a nitrogen-fixing species such as *Chickpeas* increased significantly compared with *Eucalyptus* monocultures (Bauhus et al., 2004; Kaye et al., 2000) due to the increased supply of nutrients from nitrogen fixation, facilitated rates of

Table 2
Mean dry biomass and average concentrations of heavy metals in parts of the plants under different cultivation protocols.

	Dry weight (kg)		Concentration (mg kg^{-1})							
			Cd		Hg		Pb		Cu	
	Roots	Shoots	Roots	Shoots	Roots	Shoots	Roots	Shoots	Roots	Shoots
E1	1.3	2.9	3.37	0.46	0.0104	0.0029	85.4	22.3	40.13	6.75
E2	0.0063	0.0171	2.59	0.07	0.0078	0.0031	43.5	1.7	21.69	2.08
$E3_E$	1.7	3.8	5.21	0.77	0.0188	0.0034	110.7	24.1	52.1	17.31
$E3_C$	0.0049	0.0142	4.79	0.29	0.0086	0.0079	53.9	2.1	37.78	3.52

$E3_E$ is *Eucalyptus globulus* in the mixture and $E3_C$ is *Chickpeas* in the mixture.

Table 3
Phytoremediation potential of the plant.

	BCF							
	Cd		Hg		Pb		Cu	
	Roots	Shoots	Roots	Shoots	Roots	Shoots	Roots	Shoots
E1	5.52	0.75	0.02	0.007	1.23	0.32	0.71	0.12
E2	4.25	0.11	0.02	0.007	0.63	0.02	0.39	0.04
E3(E)	8.54	1.26	0.04	0.008	1.59	0.35	0.93	0.31
E3(C)	7.85	0.48	0.02	0.018	0.78	0.03	0.67	0.06

	TF				TE (mg/plant)			
	Cd	Hg	Pb	Cu	Cd	Hg	Pb	Cu
E1	0.14	0.28	0.26	0.17	5.72	0.02	175.69	71.74
E2	0.03	0.40	0.04	0.10	0.02	0.00010	0.30	0.17
E3(E)	0.15	0.18	0.22	0.33	11.78	0.04	279.77	154.35
E3(C)	0.06	0.92	0.04	0.09	0.03	0.00015	0.29	0.24

nutrient cycling and enhanced water use efficiency (Binkley et al., 1992; Forrester et al., 2006).

Eucalyptus globulus are observably taller (average 8.7 m) than *Chickpeas* (average 0.45 m), thus canopy stratification is developed in mixture and fierce competition for light from companion species is avoided (Hubbard et al., 2004). As a less shade tolerant species (du Toit and Dovey, 2005), *Eucalyptus globulus* in mixture could intercept a greater amount of light from a smaller planting density, compared to a monoculture. Easier to obtain light in mixture was a key factor for the production increases of *Eucalyptus globulus*. The growth of nitrogen-fixing species in this research, which were grown in a lower canopy position, has been reduced. Because *Eucalyptus globulus* maintains deeper roots and greater fine root biomass in the deeper layers, competition for resources has been reduced with root stratification; the associated species can obtain nutrients from different soil layers, which corresponds well with Neave and Florence (1994) who researched the vertical differentiation in rooting structures of various plants.

4.2. Enhanced phytoremediation potential

Lotfy and Mostafa (2014) considered plant transpiration a key process for the success of phytoremediation in soil because plants must transpire enough water to take up contaminants. The translocation of toxic metals occurs when plants accumulate soluble metals in the rhizosphere by absorbing water through their root systems (Macci et al., 2013). Root exudates, which are influenced by the transpiration rate and water use efficiency of plants (McGrath et al., 2001), might affect the translocation, transformation, concentration and remobilization of nutrients and heavy metals, and improve the efficiency of soil remediation (Bianchi et al., 2010). The transpiration rate of plants may vary due to changing management techniques and unpredictable, comprehensive situations among other variables (Rothe and Binkley, 2001).

The incremental remediation efficiency of the individual plants in mixture has a relationship with the annual input of nitrogen fixation of approximately 90 kg ha year^{-1} from *Chickpeas* (Singh and Virmani, 1996). The accelerated rates of water and nitrogen cycling and the reduced competition for nutrients and light are due to the formation of root and canopy stratification in the mixtures.

By synthesizing the analytical results, the more the plants transpire water and utilize nutrients, the better the phytoremediation efficiency. Increased production, transpiration and accumulation could be a typical response of non-nitrogen-fixing species to the rational planting methods. Other similar studies have indicated that aboveground respiration can be an essential part of the gross primary production (Giardina et al., 2003); there is a positive correlation between the aboveground respiration and the biomass production, plant nitrogen content (Abreu et al., 2012) and sapwood volume (Vertessy et al., 1995), which were higher in 50% *Eucalyptus globulus* + 50% *Acacia mearnsii* than in 100% *Eucalyptus globulus* or 100% *Acacia mearnsii*, thus indicating that the gross primary production was highest in mixtures. Giardina et al. (2003) observed that in fertilized *Eucalyptus saligna* and *Pinus radiata* plantations, the increasing gross primary production was related to increases in water use efficiency and nutrient utilization.

4.3. Phytoremediation efficiency for different heavy metals

Cadmium is relatively mobile in soil (Zhao et al., 2003) and Cu is an essential micronutrient for most plants whose phytotoxicity depends on its concentration (Chigbo et al., 2013). Both metals are liable to be taken up through membrane transporters in plants as specific essential nutrients (Tyerman and Skerrett, 1999), similar to Ca, Mg and Zn. Thus the phytoremediation efficiency of Cd and Cu was significant in the mixed-species plantations. Pb is a non-essential element and is known to be hazardous to plants. Plants are typically considered unlikely to generate transporters specific for Pb, but Tomsig and Suszkiw (1991) observed permeation of Pb through Ca-channels. The phytoremediation of Pb was observed, but was not as significant as Cd and Cu. Most of the Hg was present as an insoluble sulfide that was cinnabar. Because of its low solubility, the bioavailability of Hg in various media is commonly low compared to other heavy metals; thus, phytoremediation techniques are generally ineffective for Hg contaminated soil.

5. Conclusion

We can conclude that mixed cultivation is generally an efficient way to enhance phytoremediation efficiency and reduce the time required for phytoremediation due to increased production, transpiration and heavy metal accumulation of plants in a mixture compared to monoculture plants. The phytoremediation efficiency of Cd and Cu was most significant in the mixed-species plantations, but there was almost no scavenging effect against Hg because it is comparatively stable in soil and its phytoextraction rate is limited by low solubility. Another experiment has been designed to overcome this problem.

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