



## Research article

## *Pityrogramma calomelanos* (L.) Link: Silver fern as a copper excluder plant

Chin Hao Mah<sup>1#</sup>, Yamin Wang<sup>1#</sup>, Si Hui Lee<sup>1</sup>, Zhong Chen<sup>1</sup>,  
Jean Wan Hong Yong<sup>2</sup> and Swee Ngin Tan<sup>1\*</sup>

<sup>1</sup>Academic Group of Natural Sciences and Science Education, Nanyang Technological University, 1 Nanyang Walk, Singapore 637616

<sup>2</sup>Swedish University of Agricultural Sciences, Almas Allé 8, 750 07 Uppsala, Sweden

#The authors have equal contribution to the article.

\*Corresponding Author: [sweengin.tan@nie.edu.sg](mailto:sweengin.tan@nie.edu.sg)

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**Abstract:** Silver ferns, *Pityrogramma calomelanos*, were observed to be growing on soil highly polluted with copper (Cu), which indicated a possible Cu phytoremediation potential. In this study, the Cu accumulation pattern of *P. calomelanos* was determined by exposing the ferns to different Cu levels (0 to 2500 mg L<sup>-1</sup>) in the soil for about 3 weeks. The Cu content of the fronds analysed at 5-days interval showed that *P. calomelanos* exposed to Cu levels of up to 1000 mg L<sup>-1</sup> were able to maintain normal levels of Cu within their fronds throughout the experiment. At high level of 2500 mg L<sup>-1</sup> Cu soil content, *P. calomelanos* exhibited an initial toxicity phase with significantly higher Cu content in their green fronds, but recovery was observed within 10 days and Cu content recovered back to normal level. The bioconcentration factor (BCF) was in the range of 0.005–0.11, where the maximum copper concentration up to 2500 mg kg<sup>-1</sup>. The frond's nitrogen content had showed no significant differences between control and treated plants at the end of the experiment. Hence, the *P. calomelanos* has been identified as a good Cu excluder species which can potentially be used for phytostabilisation of Cu-polluted soils.

**Keywords:** Phytoremediation - Copper - Accumulation - Pteridaceae - Silver fern - Excluder.

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### INTRODUCTION

Copper (Cu) is an essential micronutrient to plants. It is involved in various physiological and cellular processes, as a key component in photosynthesis and oxidative stress protection. It also functions as a cofactor in enzymes involved in cell wall metabolism and the biogenesis of molybdenum cofactor (Yruela 2009). However, the redox cycling of Cu can also contribute to Cu toxicity in plants (Yruela 2005). Expose to excess environmental Cu can damage plant cells and impair cellular processes thereby affecting proteins and inactivating enzymes (Yruela 2009). Plants exposed to excess Cu eventually suffer visible physical symptoms such as leaf chlorosis, stunting, and retardation of root growth (Reichman 2002).

Soil Cu pollution can be caused by extensive use of Cu in the agricultural and industrial sectors. As Cu is a key component in fungicide, agricultural practices could contribute to Cu soil pollution, ranging from 400 to 1000 mg L<sup>-1</sup> in some vineyards due to the repeated usage of fungicide (Chaignon *et al.* 2003, Fernández-Calviño *et al.* 2008, Fernández-Calviño *et al.* 2010a, b). Inadequate processing of industrial waste such as electronics can result in high levels of environmental Cu pollution, ranging from 13000 to 47000 mg L<sup>-1</sup> (Hsiao *et al.* 2007). Furthermore, the rising world demand for Cu has also led to increasing mining and smelting activities, which in turn enhanced the soil Cu pollution (Gunn *et al.* 1995, Cassella *et al.* 2007, Aguilar *et al.* 2011). The residue from Cu mines can affect the environment within a 500 km radius (Hilson 2000), and the Cu bioaccumulation in plants and animals presents health hazards to humans (Wilson & Pyatt 2007).

Plants growing on Cu-polluted soils are able to use avoidance or tolerance mechanisms to defend themselves

from Cu toxicity (Tang *et al.* 1999). Those relying on the tolerance mechanisms will trigger sequestration in cells and intracellular compartments where the binding of Cu inside the cells is done through the aid of strong ligands and metallothioneins (Küpper *et al.* 2009). Such plants are known as Cu hyperaccumulators, where they transport the excess Cu to the aboveground biomass for storage. Other Cu-tolerant plants rely on avoidance mechanisms to grow on highly Cu-polluted soil. These Cu excluders may depend on efflux pumps to reduce Cu uptake, or root exudates to minimise Cu availability in the soil (Küpper *et al.* 2009). Such avoidance mechanisms potentially allow *Elsholtzia splendens* and *Silene vulgaris* to tolerate high soil Cu levels up to 8000 mg L<sup>-1</sup> (Song *et al.* 2004). Cu excluders may also immobilise the Cu within their roots without transporting to aboveground biomass, resulting in low shoot-to-root or shoot-to-soil Cu concentration ratio. These plants are also often found with high Cu concentrations in their roots, as seen in *Agrostis tenuis* and *Armeria maritima* ssp. *halleri* growing near a metal smelter in France (Dahmani-Muller *et al.* 2000).

Generally, Cu hyperaccumulators can be classified when the plant can accumulate more than 300 mg L<sup>-1</sup> of Cu (Baker & Brooks 1989, Reeves 2003, van der Ent *et al.* 2013) in the aboveground biomass. This classification indicator can also be achieved by excluder plants when the soil Cu content is beyond the classification limit and the limit of the plant's physiological mechanism can handle (van der Ent *et al.* 2013). Other important parameters that measure Cu tolerance are the bioconcentration factor (BCF) and the translocation factor (TF), which are determined by the ratio of shoot-to-soil Cu concentration ratio and shoot-to-root ratio, respectively (Baker & Whiting 2002, van der Ent *et al.* 2013). As each plant species may have a different defensive mechanism in reaction to excess Cu, plant tolerance to Cu is better compared across different plant species using BCF (van der Ent *et al.* 2013). Since Cu accumulation occurs mainly in the roots where only a small fraction is translocated to the shoots (Tsay *et al.* 1995), it is rare to observe Cu hyperaccumulators reported to have BCF of more than 1 (Küpper *et al.* 2009).

In addition, it is also important to observe the copper accumulation patterns by analysing the aboveground biomass Cu content of plants growing across a range of environmental soil concentrations. Due to the fact that the BCF is affected by the threshold limit of the individual plant species, it is important to observe the accumulation pattern across a range of soil Cu concentrations which will allow the plant to be correctly identified as a hyperaccumulator, indicator, excluder, or a non-tolerant plant (van der Ent *et al.* 2013, Hunt *et al.* 2014). Furthermore, other indicators on plant physiological status such as the nitrogen (N) content could be used to corroborate the results of Cu accumulation pattern (Ågren *et al.* 2012). Nitrogen partition has also been linked to leaf senescence (Gan & Amasino 1997), which is often observed in plants exposed to heavy metals (Ayeni *et al.* 2010). Exposure to heavy metals can affect nitrate assimilation in plants (Devriese *et al.* 2001), as the metal ions can disrupt proper functioning of nitrate reductase (NR) (Llorens *et al.* 2000). As such, the difference in the N content of plants growing on polluted and clean soil would indicate how well the plant species tolerates the excess heavy metals.

The silver fern, *Pityrogramma calomelanos* (L.) Link, was spotted on highly Cu-polluted soils in Sabah (Yong, pers. comm.), where it could be potentially a Cu-tolerant species. Although it has been previously reported as an arsenic hyperaccumulator (Francesconi *et al.* 2002, Yong *et al.* 2010) and was concluded to be tolerant of lead-polluted soils (Soongsombat *et al.* 2009), no work has been reported on its tolerance for Cu. As a fast-growing and abundant weed, this fern could potentially be used for the re-vegetation or remediation of Cu-polluted soil. This study aims to determine the accumulation pattern of Cu in *P. calomelanos* by exposing the ferns to different Cu levels in the range of 0~2500 mg L<sup>-1</sup> in a tropical greenhouse.

## MATERIALS AND METHODS

### Reagents and Standards

All chemicals utilized in our work were for analytical grade. Copper sulphate pentahydrate, CuSO<sub>4</sub>·5H<sub>2</sub>O (Riedel-De Haen AG, Seelze-Hannover, Germany), was used to prepare Cu stock solution for spiking experiments in the greenhouse. Nitric acid (65%, Merck, Germany), hydrogen peroxide (30% w/w, Scharlau, Spain), sulfuric acid (95–98%, Sigma, USA) and ultrapure water (18.2 MΩ·cm, Millipore Milli-Q, USA) were used for digestion of plant samples. Cu standard solution (single element, 1000 mg kg<sup>-1</sup>) used for Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) analysis was purchased from Perkin Elmer, USA. All glassware used were cleaned by soaking in 10% nitric acid overnight, ultrapure water for twelve hours and stored dry before use (Tow *et al.* 2016).

### Sampling and Sample Preparation

Plant Materials: *Pityrogramma calomelanos* of about 4 to 6 cm in frond length were collected from a

commercial plant nursery (Uvaria Tide, Singapore) and the Nanyang Technological University's (NTU) community herb garden. Each fern was transplanted into a 15 cm tall pot containing 1 kg of soil, and the pot was placed in a plastic container and maintained with 100 mL of water daily. HORTI foliar fertiliser with N:P:K ratio of 21:21:21 was applied daily, with the concentration increased from 1 to 3 g L<sup>-1</sup> over three weeks, and maintained at 3 g L<sup>-1</sup> thereafter. The ferns were left to acclimatise under full sun in the greenhouse for one month. The daily maximum photosynthetic photon flux density PPFD measured with a light meter Li-250A (LICOR, USA) was between 600 to 1200  $\mu\text{mol m}^{-2}\text{s}^{-1}$ , and the daily temperature ranged from 23 to 34°C.

**Plant Uptake:** The uptake of the heavy metal ions from the simulated contaminated soil to the fern was investigated and discussed in terms of bioconcentration factor (BCF) (Zayed *et al.* 1998);

$$BCF = \frac{[HM]_{fern}}{[HM]_{dry\ soil}}$$

Where,  $[HM]_{fern}$  is the concentration of heavy metal in the fern and  $[HM]_{dry\ soil}$  is the initial spiked concentrations (0, 200, 1000 and 2500 mg L<sup>-1</sup>).

**Copper Treatments:** At the end of the acclimatisation period, *P. calomelanos* were arranged into groups of four with similar frond lengths, and each group was subjected to the following Cu treatments: 0, 200, 1000 and 2500 mg L<sup>-1</sup>. The copper spike in the soil was done only once at the beginning of the experiment based on our earlier work on other pot experiments in a tropical greenhouse (Yong *et al.* 2010, Tow *et al.* 2016). The Cu stock solution was prepared from copper sulphate pentahydrate, CuSO<sub>4</sub>·5H<sub>2</sub>O (Riedel-De Haen AG, Seelze-Hannover, Germany). The groups were arranged randomly on the greenhouse bench. Each treatment had 16 individuals, and the experiment lasted for 20 days, with the same watering and fertiliser regime as the last week of the acclimatisation. Green fronds were randomly sampled across each treatment every five days. The plant samples were rinsed with ultrapure water, oven-dried for forty-eight hours and ground into fine powder for storage in a drying cabinet.

#### Sample Digestion and Analysis

The frond samples were then oven-dried at 80°C for 48 hours. Dried samples were ground into fine powder (A11 Basic Analytical Mill, IKA, Germany) prior to microwave (UltraWAVE Single Reaction Chamber (Milestone, Germany) digestion. Typically, 0.200 g of ground plant sample was accurately weighed into a quartz tube and 4 mL of 65% nitric acid was added. A water bath for the quartz tubes was prepared with 2 mL of 95–98% sulfuric acid, 5 mL of 30% hydrogen peroxide and 120 mL of ultrapure water. For every digestion, a quartz tube containing only 65% nitric acid was additionally included as the blank for Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) (Optima 8300, Perkin Elmer, USA) analysis. After cooling to room temperature, the digested samples were quantitatively transferred into 25 mL volumetric flasks and topped up with ultrapure water. The cloudy digested samples were filtered, using filter papers (No. 6 Whatman, Qualitative 70 mm), into falcon tubes and stored at 4°C prior to ICP-OES analysis within two weeks.

The N content of the fronds was analysed by the Dumas method described by Muñoz-Huerta *et al.* (2013) via an elemental analyser (Vario MICRO cube, Elementar, Germany).

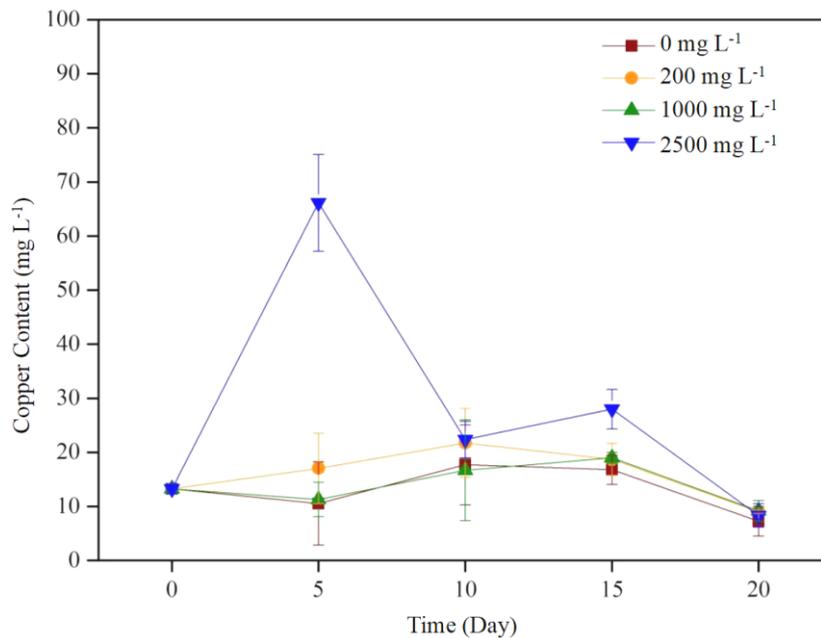
#### Statistical Analysis

Two-way analysis of variance (ANOVA) and Tukey's honest significance test were conducted to test for significant difference ( $p < 0.05$ ) between the measurements. All analyses were carried out using the statistical programming environment R, version 3.1.2 (R Core Team 2014).

## RESULTS AND DISCUSSION

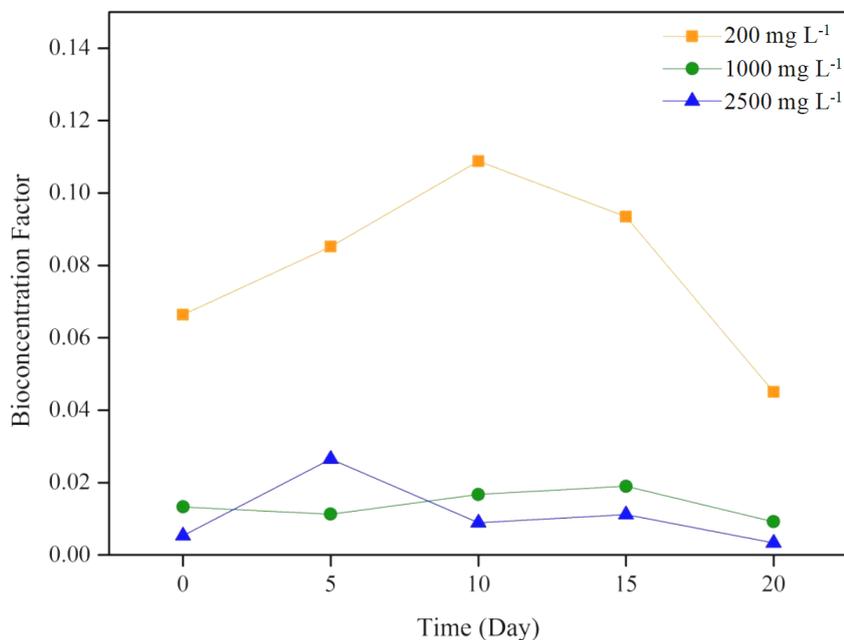
### Effect of Cu treatment on Cu Accumulation in *P. calomelanos*

In general, the Cu content in green fronds was less than 35 mg L<sup>-1</sup> even though the soil Cu levels ranged from 200 to 2500 mg L<sup>-1</sup> (Fig. 1). There was an initial increase in Cu content for plants treated with 2500 mg L<sup>-1</sup> Cu on the 5<sup>th</sup> day, but the Cu levels recovered to normal levels within 10 days. The Cu content of treated plants was also not significantly different ( $p > 0.05$ ) from the control plants, except for the significant peak on the 5<sup>th</sup> day for ferns treated with 2500 mg L<sup>-1</sup> Cu. These results indicated that *P. calomelanos* is potentially a Cu excluder species, where it does not show any significant physiological impact even when and soil Cu concentration increased to 2500 mg L<sup>-1</sup>. The ferns did not exhibit any toxicity phase in lower Cu treatments (0 to 1000 mg L<sup>-1</sup>), which indicated that the *P. calomelanos* has self-avoidance mechanism that was sufficient to cope with the sudden exposures of soil Cu content lower than 1000 mg L<sup>-1</sup>.



**Figure 1.** Effect of Cu treatment on Cu accumulation in green fronds of *Pityrogramma calomelanos* (L.) Link [Values are mean±S.E., n=3]

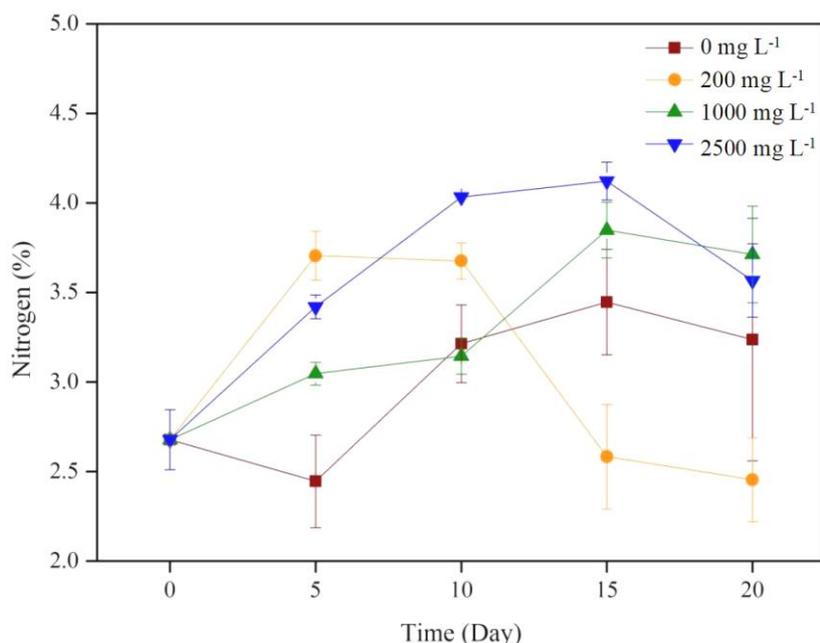
The Cu accumulation capability of *P. calomelanos* was assessed by the BCF and was compared against other Cu-tolerant plants (Zu *et al.* 2005, Boojar & Tavakkoli 2011). The BCF of less than 0.12 was observed for the green leaves in all Cu treatments throughout the experiment, and was further reduced to less than 0.06 by the 20<sup>th</sup> day (Fig. 2). This observation was comparable to other Cu excluder species such as *Ajuga chamaecistus* and *Cramb orientalis* L. that was found growing on soil with similar total Cu content (Boojar & Tavakkoli 2011), suggesting that *P. calomelanos* could be potentially used as a Cu excluder species.



**Figure 2.** Effect of Cu treatment on BCF in green fronds of *Pityrogramma calomelanos* (L.) Link.

#### Effect of Cu treatment on Nitrogen Content of *P. calomelanos*

Exposure to heavy metals can affect the nitrogen content of the plant (Llorens *et al.* 2000, Devriese *et al.* 2001). However, the nitrogen content does not show any significant difference between control and treated plants at each sampling point ( $p > 0.05$ ), which is expected for Cu-tolerant plants (Li *et al.* 2007). This observation further supports the use of *P. calomelanos* as a Cu excluder species. Although the N content exhibited variability during the experimental period, there was no correlation found between each treatment (Fig. 3). This could have been due to inter-plant variability and the small sample size used for N analysis (Watson & Gallier 2001, Muñoz-Huerta *et al.* 2013).



**Figure 3.** Effect of Cu treatment on N content in green fronds of *Pityrogramma calomelanos* (L.) Link. [Values are mean±S.E., n=3]

## CONCLUSIONS

The silver fern *P. calomelanos* was identified and concluded as a Cu excluder species as it contained much lower Cu content in fronds than the soil Cu levels where it could tolerate soil containing maximum Cu concentration of 2500 mg L<sup>-1</sup> without significant adverse physiological effects or changes to its N content. The BCF of this species is also comparable to other Cu excluder species grown on a similar range of soil Cu content, which evidently suggests that the fern adopts exclusion mechanisms to avoid metal influx into their above-ground biomass (Boojar & Tavakkoli 2011). Other plausible secondary mechanisms such as the leaf fall detoxification mechanism (Dahmani-Muller *et al.* 2000) and the chelation of excess Cu ions for storage in cells (Cobbett 2000) would explain the abundance of *P. calomelanos* thriving in the old Cu mine (Yong, pers. comm.).

Together with the capability of tolerating soil with high Cu content and the well-established arsenic hyperaccumulator (Francesconi *et al.* 2002, Yong *et al.* 2010), the *P. calomelanos* has the potential for multi-functional heavy metal phytoremediation applications. Future studies on this fern may focus on destructive sampling and root analysis, as well as the interactions between the tolerance and avoidance mechanisms of different heavy metal pollutants.

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