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Possible Application of the Medicinal Plant *Hyoscyamus albus* in Phytoremediation: Excess Copper Compensates for Iron Deficiency, Depending on the Light Conditions

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Abstract

Seedlings of the medicinal plant *Hyoscyamus albus* were supplied with an excess of Cu to examine the possible application in phytoremediation. The seedlings were cultured in B5 medium supplied with basal 0.1 μM Cu and 200 μM Cu under various light conditions: short day (SD); long day (LD); and continuous light (CL). In addition, the effect of supplying 200 μM Cu under Fe deficiency was determined, in order to elucidate the interaction between Cu and Fe. Interestingly, Fe-deficiency symptoms that developed in plants grown with basic levels of Cu under LD almost disappeared when excess Cu was supplied. Plant growth mainly depended on the photo irradiation period (SD < LD~CL); and 200 μM Cu did not inhibit growth at all when Fe was available, whereas in the absence of Fe, CL caused damage to growth. Analysis of the Cu and Fe contents of the plants revealed that Cu was distributed equally in both the aerial parts and roots, whereas most of the Fe was found in the roots; under Fe deficiency, Cu accumulation in the roots apparently increased. Cu was mainly distributed in the soluble fraction, which included vacuoles and the cell-wall fraction. These results provide evidence indicating that *H. albus* seedlings are tolerant of Cu present in excess. Furthermore, excess Cu was able to compensate for Fe deficiency, depending on the light conditions. Continuous light inhibited this effect, probably as a result of the induction of Mn defi-

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ciency. The possible applications of this newly discovered cuprophyte are discussed.

Keywords

Copper Accumulator, Medicinal Plant, *Hyoscyamus albus*

1. Introduction

Hyoscyamus albus (Solanaceae, white henbane) is an annual or biennial plant growing on the Continent, particularly in France and in Indian subcontinent, and used as a source of hyoscyamine, which is an important anticholinergic drug of plant origin [1]. Aside from its medicinal applications, however, the other properties of this plant have attracted little attention. Recently, we found that roots of *H. albus* continued to grow under conditions of iron (Fe) deficiency, during which they secreted riboflavin into the rhizosphere [2] [3]. Fe is an essential element for plants; however, plants face Fe deficiency in neutral and alkaline soils because ferric iron, despite being abundant in the Earth's crust, is almost insoluble and therefore unavailable to plant roots. The area affected by Fe deficiency amounts to about 30% of global total land area [4] and the prevailing situation has deteriorated due to recent climatic changes and human activities. Therefore, tolerance to Fe deficiency is an agronomically-useful trait.

A potentially important aspect of Fe deficiency is its relationship to the availability and uptake of other metal ions. Cu is also an essential element for plants, being involved in various redox reactions, including electron transfer; however, the toxicity of excess Cu to organisms has been widely recognized because Cu produces reactive oxygen species such as hydroxyl radicals, which cause oxidative damage to lipids, proteins and DNA, resulting in cellular deterioration [5]. Some plants survive high concentrations of Cu by limiting their uptake and accumulation of Cu (denoted as excluders); whereas others, which can actively uptake and accumulate Cu, are defined as accumulators/hyperaccumulators [6]. Plants face Cu excess close to Cu mines and in Cu-polluted areas resulting from industrial activities. It is recognized that Cu excess can induce Fe deficiency, depending on the form of Fe [5]. In addition, the tight linkage between Cu and Fe homeostasis has been well recognized [7]-[9].

In this paper, we report on the effects of Cu excess, in combination with Fe availability, on growth and metal accumulation in the seedlings of medicinal *H. albus*. Previously, the evaluation and comparison of Cu- and Cd-scavenging capabilities of *in vivo*- and *in vitro*-grown *Lycopersicon esculentum* revealed that the concentrations of both metals in *in vivo*-grown leaves were much higher than in *in vitro*-grown leaves [10]. In addition, plants previously reported to be Cu-hyperaccumulators have been found to require reassessment, because most of the Cu detected in the aerial parts of these plants was found to be the result of Cu adsorption at the leaf surface [11] and could be washed out by water and detergent [12]. For this reason, before carrying out experiments on plants in the field, we have here undertaken experiments with *in vitro* cultures, using culture media containing known concentrations of minerals, including Cu and Fe, carried out under sterile conditions to avoid indirect effects caused by microorganisms.

2. Materials and Methods

2.1. Plant Materials

Seeds of *Hyoscyamus albus* L. (Solanaceae) were provided by Dr. Laiq ur Rahman (CIMAP, India) and then collected from mature plants after cultivation at Nagasaki University, Japan. *H. albus* seedlings were germinated under sterile conditions. Seeds were treated with 70% EtOH for 1 min, followed by 1% sodium hydrochloride for 15 min, and were then washed three times with sterile water. The seeds were placed on Petri dishes containing 0.8% agar medium and kept at 25˚C in the dark until germination. Germinated seedlings were first placed under continuous dim light (22 μmol·m^{-2}·s^{-1}) and then, when the cotyledons had developed fully, were transferred to tubes containing Murashige and Skoog basal medium [13] (containing 100 μM Fe and 0.1 μM Cu) supplemented with 1% sucrose and solidified with 0.1% gellan gum. After two leaves had appeared, the base (between the roots and the aerial part) of each plantlet was secured with a silicone sponge and put into a wide-

mouth (ϕ 35 mm) 100 ml conical flask containing 20 mL of liquid B5 medium [14] and three combinations of Fe/Cu concentrations as follows: control (100 μM Fe and 0.1 μM Cu) (denoted as +Fe/Cu1); excess Cu (100 μM Fe and 200 μM Cu) (+Fe/Cu2000); and excess Cu under Fe deficiency (0 μM Fe and 200 μM Cu) (−Fe/Cu2000). Fe was supplied as Fe(III)-EDTA (Doujin Co., Japan). When excess Cu was supplied, as $CuSO_4$ (Wako Chemical, Japan), $EDTANa_2$ (Doujin Co., Japan) was also added at the equivalent molarity in order to avoid precipitation. Media were autoclaved at 121˚C for 15 min before use. Additionally, a Mn deficiency experiment was also carried out, by omitting $MnSO_4$ (Wako Chemical, Japan) from B5 medium as follows: control (6 μM Mn and 0.1 μM Cu); Mn deficiency (0 μM Mn and 0.1 μM Cu); excess Cu under Mn deficiency (0 μM Mn and 200 μM Cu).

Four seedlings were selected for each treatment. The plantlets in the flasks were then incubated for 2 - 4 weeks at 25˚C on a rotary shaker at 50 rpm in the culture room under three different light conditions (121 $\mu mol \cdot m^{-2} \cdot s^{-1}$): short day (SD), 10 h light - 24 h dark; long day (LD), 16 h light - 8 h dark; continuous light (CL), 24 h light. In addition, continuous dim light (22 $\mu mol \cdot m^{-2} \cdot s^{-1}$) was also used. The cultured plants were harvested after thorough washing with distilled water, followed by blotting with filter papers.

2.2. Growth Measurement

Harvested plants were divided into rosette leaves (leaf blades and petioles), stems and roots. Leaf numbers were counted and leaf blade sizes (widths and lengths) and petiole and root lengths (in the case of roots, the length of the longest root) were measured using micrometer calipers. The samples were weighed (FW, fresh weight) and then dried at 50˚C to constant weight, in order to measure dry weight (DW) and to calculate water content (%). Since stem development was negligible during culture, stems were weighed together with petioles.

2.3. Fractionation

Fresh tissues (roots and aerial parts) of *H. albus* seedlings were fractionated according to the reported method [15], with some modifications. Harvested fresh materials were homogenized in pre-chilled extraction buffer containing 50 mM Tri-HCl (pH 7.5), 250 mM sucrose, and 10 mM DTT. The cell wall was collected by nylon mesh filtration (82 μm) under vacuum and then the filtrate was centrifuged at 880 × g for 15 min, followed by 21,880 × g for 30 min. The respective sedimentations were denoted as nucleus/plastid and mitochondrial fractions, according to staining with acetocarmine, iodine and TTC solutions, respectively.

2.4. Analysis of Metal Contents

Tissues and fractions were digested with 60% (w/v) HNO_3 in a microwave oven (Perkin Elmer, Multiwave) at 160˚C for 20 min. Heavy metal contents were determined by inductively coupled plasma-optical emission spectrometry (ICP-OES) (Horiba, Ultima 2).

2.5. Statistical Analyses

All results were expressed as means and sd. Statistical significance was assessed by ANOVA, followed by Dunnett's multiple comparison tests (Excel Statistics, SSRI, Tokyo, Japan).

3. Results and Discussion

3.1. Effects of Cu Excess and Fe Deficiency on Seedling Growth

Previously, we observed that *H. albus* roots were able to grow under Fe deficiency [2] [3]. In addition, the roots grew well under a range of Cu concentrations (from 0 to 200 μM) (unpublished data). Although an excess of Cu seemed to favor growth of the roots, and the roots were tolerant of Fe deficiency, no information was available concerning the behavior of whole plants. Cultured roots are usually incubated in the dark, but in order to grow intact plants, photoirradiation is necessary. Therefore, we initially investigated the responses of *H. albus* seedlings cultured in liquid nutrient medium supplied with 0.1 μM Cu (control) and 200 μM Cu (Cu excess), respectively, in the presence and absence of Fe, under long day conditions (LD) for 2 weeks (**Figure 1**). We found that under Fe deficiency the seedlings grew, but that in comparison to control plants (**Figure 1(A)**), their roots showed morphological changes, such as swollen root tips and shorter branches that were of a yellow color (**Figure 1(B)**). In the aerial parts, the leaves showed a chlorosis-like pale yellow coloration under Fe deficiency.

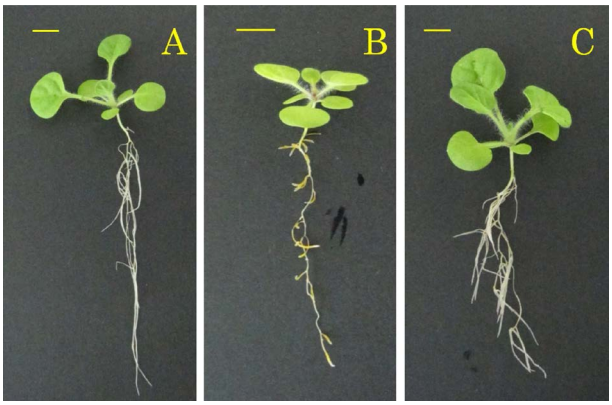

Figure 1. *H. albus* seedlings treated with three different Cu/Fe concentrations. Seedlings were incubated for 2 weeks at 25˚C on a rotary shaker at 50 rpm in the incubator under long day (LD) conditions, 16 h light (121 μmol·m^{-2}·s^{-1}) - 8 h dark. Photos: (A) A control plant treated with $CuSO_4$ (0.1 μM) and Fe(III)-EDTA (100 μM) in B5 basal medium; (B) A plant subjected to Fe deficiency by treatment with B5 Cu (0.1 μM) without Fe (−Fe); (C) A plant subjected to Cu excess (200 μM) in combination with Fe deficiency (−Fe). Bars indicate 1 cm.

On the other hand, *H. albus* seedlings were able to survive—and even grew better—under conditions of Cu excess. Furthermore, when the seedlings were cultured under both Cu excess and Fe deficiency, surprisingly the leaf color was restored and the root tips grew normally (**Figure 1(C)**). In comparison to control plants (**Figure 1(A)**), both roots and leaves developed well under Cu excess combined with Fe deficiency, although the primary root length seemed to be shorter (**Figure 1(C)**).

In considering the growth of intact plants, photo irradiation is one of the most important factors. To determine the effects of Cu excess on plant growth under Fe-replete and -deplete conditions, we cultured the seedlings under different light regimes, such as short day (SD), long day (LD) and continuous light (CL). After 2 and 4 weeks, the sizes (numbers of leaves, leaf sizes, and the lengths of petioles, stems and roots) and the weights (fresh weights, FW; dry weights, DW; water contents) of the harvested plants were measured (**Table 1**, **Table 2**). Under the same conditions of illumination and heavy metal availability, the values for leaf number, leaf size, petiole length, and root length all increased after 4 weeks, compared to after 2 weeks. Under Fe-replete conditions, an excess of Cu had no effect on plant size (**Table 1**). Illumination was the most important factor affecting plant size: seedlings treated with LD and CL developed better than those treated with SD for 4 weeks, although no significant difference was found between LD and CL (SD < CL~LD). FW and DW showed the same response to illumination (SD < CL~LD), both at the whole plant level (**Table 2**) and at the organ level (data not shown). The water content of *in vitro*-grown *H. albus* plants was around 95%, when Fe was supplied. On the other hand, in the absence of Fe and under Cu excess, CL caused some detrimental effects to the primary root length and leaf blade size, although leaf number was not affected (**Table 1**). In addition, partial chlorosis of the leaves and swelling of the root tips were observed (data not shown). Furthermore, CL caused some FW reduction, but interestingly, no reduction was found on a DW basis, indicating a significant decrease in water content (to *ca*. 91%) at the whole plant level (**Table 2**). This phenomenon was especially apparent in roots, in which the water content decreased to less than 90% (data not shown).

In general, Fe deficiency causes typical morphological changes, such as root tip swelling [16] [17] and leaf chlorosis [18] [19]; we observed such symptoms in *H. albus* seedlings cultured under Fe deficiency (**Figure 1(B)**). Although Cu and Fe homeostasis have been studied extensively, and diminished Fe accumulation under conditions of Cu excess has been reported [5] [20] [21], our observation in *H. albus* seedlings that typical Fe deficiency symptoms are abated under conditions of Cu excess (**Figure 1(C)**) is, to the best of our knowledge, novel. It suggests that Cu is able to compensate for Fe deficiency in *H. albus* plants.

Previous reports have indicated that Cu excess inhibits root elongation [22] [23] and causes chlorosis in leaves [5] [20]. Our study using different light regimes revealed that under Fe-replete conditions, Cu excess (200 mM) appeared not to affect growth, whereas under conditions of Fe deficiency together with Cu excess, damage appeared only under CL (**Table 1**). When *H. albus* seedlings were cultured without Fe under continuous dim light (22 μmol·m^{-2}·s^{-1}), no damage was found either in the roots or in the aerial parts, although plant growth was poor in comparison to that under higher irradiation (121 μmol·m^{-2}·s^{-1}) (data not shown). This suggests that it is

Table 1. Plant development of *H. albus* seedlings after culturing under various Cu/Fe supply and light conditions: changes in number, size and length.

Culture period	Light condition	Cu/Fe condition	Leaf No.	Leaf blade size		Petiole length	Root length
				Length (mm)	Width (mm)	(mm)	(mm)
2 wks	SD	+Fe Cu1	7.5 ± 0.6^{a}	17.6 ± 1.8^{a}	18.2 ± 1.1^{a}	13.2 ± 1.6^{b}	207.2 ± 66.7^{c}
		+Fe Cu2000	7.8 ± 0.5^{a}	17.3 ± 1.0^{a}	17.1 ± 1.6^{a}	13.3 ± 1.5^{b}	180.9 ± 38.5^{c}
		−Fe Cu2000	7.8 ± 0.5^{a}	17.2 ± 2.9^{a}	18.2 ± 2.5^{a}	16.5 ± 4.1^{b}	120.4 ± 27.9^{b}
	LD	+Fe Cu1	8.3 ± 0.6^{b}	21.1 ± 2.9^{b}	19.0 ± 2.8^{ab}	15.1 ± 4.7^{b}	264.4 ± 8.7^{d}
		+Fe Cu2000	8.8 ± 1.0^{b}	22.2 ± 4.2^{b}	19.9 ± 2.0^{ab}	17.7 ± 4.4^{bc}	195.7 ± 64.4^{c}
		−Fe Cu2000	9.2 ± 0.8^{b}	24.3 ± 4.1^{bc}	22.8 ± 3.5^{b}	22.6 ± 7.8^{c}	164.0 ± 84.4^{c}
	CL	+Fe Cu2000	7.7 ± 0.6^{a}	20.5 ± 1.8^{b}	22.8 ± 0.8^{b}	12.6 ± 3.8^{ab}	200.0 ± 40.5^{c}
		−Fe Cu2000	8.3 ± 0.6^{b}	15.9 ± 2.1^{a}	15.8 ± 3.0^{a}	9.7 ± 3.3^{a}	52.8 ± 18.0^{a}
4 wks	SD	+Fe Cu1	9.3 ± 0.5^{a}	22.0 ± 0.3^{a}	22.3 ± 1.2^{b}	18.8 ± 2.0^{b}	209.2 ± 47.4^{b}
		+Fe Cu2000	9.8 ± 0.5^{a}	21.7 ± 3.1^{a}	22.4 ± 1.7^{b}	18.5 ± 4.6^{b}	204.3 ± 39.3^{b}
		−Fe Cu2000	9.8 ± 0.5^{a}	24.2 ± 2.3^{ab}	23.9 ± 2.5^{b}	21.3 ± 4.0^{b}	219.2 ± 40.2^{b}
	LD	+Fe Cu1	10.3 ± 1.0^{ab}	31.9 ± 4.2^{c}	30.3 ± 3.7^{c}	27.1 ± 9.6^{bc}	301.4 ± 48.2^{c}
		+Fe Cu2000	11.5 ± 0.6^{b}	29.5 ± 6.6^{bc}	29.9 ± 4.4^{c}	25.3 ± 5.3^{bc}	284.3 ± 55.0^{c}
		−Fe Cu2000	12.3 ± 1.0^{bc}	34.0 ± 5.0^{c}	31.4 ± 1.4^{c}	35.4 ± 9.3^{c}	182.2 ± 63.8^{b}
	CL	+Fe Cu2000	12.0 ± 0.0^{bc}	35.9 ± 5.3^{c}	32.3 ± 2.5^{c}	22.2 ± 5.9^{b}	208.2 ± 73.8^{b}
		−Fe Cu2000	11.3 ± 0.6^{b}	21.1 ± 4.9^{a}	20.6 ± 6.6^{ab}	14.6 ± 7.4^{a}	90.3 ± 6.4^{a}

Seedlings were treated with control Cu1 (0.1 μM) and excess Cu2000 (200 μM) in combination with/without Fe for 2 and 4 weeks at 25˚C on a rotary shaker at 50 rpm in the incubator, under three different light regimes (121 μmol·m^{-2}·s^{-1}): short day (SD), 10 h light - 14 h dark; long day (LD), 16 h light - 8 h dark: continuous light (CL), 24 h light. –Fe, no addition of Fe; +Fe, with addition of 100 μM Fe(III)-EDTA. Data are means and SD of 4 plants. Different letters indicate significant differences between the treatments ($P < 0.05$) after 2-way ANOVA.

Table 2. Plant development of *H. albus* seedlings after culturing under various Cu/Fe supply and light conditions: changes in fresh weight (FW), dry weight (DW) and water content.

Culture period	Light condition	Cu/Fe condition	Whole plant		
			mg FW	mg DW	Water content (%)
2 wks	SD	+Fe Cu1	280 ± 47^{a}	14.5 ± 3.1^{a}	94.8 ± 0.3^{a}
		+Fe Cu2000	303 ± 79^{a}	14.9 ± 3.4^{a}	95.1 ± 0.5^{a}
		−Fe Cu2000	326 ± 131^{a}	15.9 ± 5.0^{a}	95.1 ± 0.7^{a}
	LD	+Fe Cu1	461 ± 149^{b}	26.9 ± 5.3^{b}	94.2 ± 0.8^{a}
		+Fe Cu2000	668 ± 330^{b}	33.5 ± 11.6^{b}	95.0 ± 0.7^{a}
		−Fe Cu2000	895 ± 404^{b}	45.4 ± 20.4^{b}	94.9 ± 0.4^{a}
	CL	+Fe Cu2000	576 ± 102^{b}	32.5 ± 4.3^{b}	94.4 ± 0.2^{a}
		−Fe Cu2000	371 ± 135^{ab}	32.0 ± 8.6^{b}	91.1 ± 1.2^{b}
4 wks	SD	+Fe Cu1	635 ± 95^{a}	26.9 ± 4.3^{a}	95.8 ± 0.1^{a}
		+Fe Cu2000	730 ± 262^{a}	32.8 ± 1.0^{a}	95.5 ± 0.2^{a}
		−Fe Cu2000	781 ± 189^{a}	34.5 ± 7.1^{a}	95.6 ± 0.3^{a}
	LD	+Fe Cu1	1644 ±702^{b}	73.9 ± 25.5^{b}	95.5 ± 0.5^{a}
		+Fe Cu2000	1978 ± 794^{b}	85.4 ± 29.1^{b}	95.7 ± 0.4^{a}
		−Fe Cu2000	2573 ± 792^{b}	121.2 ± 41.3^{b}	95.3 ± 0.1^{a}
	CL	+Fe Cu2000	2562 ± 310^{b}	114.1 ±11.3^{b}	95.5 ± 0.1^{a}
		−Fe Cu2000	1747 ± 1099^{ab}	143.7 ± 67.4^{b}	91.8 ±1 .8^{b}

Seedlings were treated with control Cu1 (0.1 μM) and excess Cu2000 (200 μM) in combination with/without Fe for 2 and 4 weeks at 25˚C on a rotary shaker at 50 rpm in the incubator, under three different light regimes (121 μmol·m^{-2}·s^{-1}): short day (SD), 10 h light - 14 h dark; long day (LD), 16 h light - 8 h dark: continuous light (CL), 24 h light. –Fe, no addition of Fe; +Fe, with addition of 100 μM Fe(III)-EDTA. Data are means and SD of 4 plants. Different letters indicate significant differences between the treatments ($P < 0.05$) after 2-way ANOVA

the overall exposure to light energy (*i.e.* the product of light intensity and duration) that is critical for the appearance of damage under Fe deficiency. Foyer and Noctor have commented that when light is in excess, or when the electron transport capacity in the chloroplast is limited, electrons can directly reduce molecular oxygen [24]. The continuous production of reactive oxygen species (ROS) in chloroplasts during photosynthesis, in the absence of a fully adequate scavenging mechanism such as Fe superoxide dismutase (FeSOD), must be a potential cause of damage by continuous light [7]. Our results showed that growth inhibition was apparent not only in the leaves but also in the roots, suggesting that the radical scavenging system associated with mitochondrial respiration might be impaired also.

3.2. Cu Accumulation in Plants under Cu Excess

The Cu contents of the *H. albus* plants cultured under Cu excess, with or without Fe, in combination with the various illumination conditions described above were analyzed by ICP-OES. Since the Cu contents of plants supplied with 0.1 μM Cu were below the limit of detection of our assay system (detection limit, 0.2 $mg \cdot L^{-1}$), only data for plants grown under Cu excess are shown (**Figure 2**). Expressed on a whole-plant basis, the values for Cu accumulation in seedlings cultured for 4 weeks were very much greater than in those cultured for 2 weeks. The illumination conditions strongly affected Cu accumulation. The amounts of Cu in whole plants cultured under LD were more than 6 times greater than in those cultured under SD. Furthermore, under the same illumination conditions, appreciably more Cu was accumulated in plants cultured under Fe deficiency than in plants cultured under Fe-replete conditions. Thus, when Fe was deficient, the amount of Cu reached 740 ± 92 $nmol \cdot plant^{-1}$ under LD, declining by a quarter under CL (560 ± 110 $nmol \cdot plant^{-1}$). Under Fe-replete conditions, less Cu was accumulated and no difference was found between LD and CL (419 ± 122 and 452 ± 73 $nmol \cdot plant^{-1}$, respectively). Cu was accumulated in both aerial parts and roots; and under Fe-replete conditions, more than half of the Cu was recovered from the aerial parts (leaf blade, petiole and stem), and the leaf blade was the organ in which the largest proportion of the Cu was accumulated. Under Fe deficiency, however, Cu accumulation increased in the roots (**Figure 2**). Thus, the Cu contents in the leaves and roots of seedlings cultured for 4 weeks under LD were as follows: under Fe deficiency, 223 ± 126 $\mu g \cdot g^{-1}$ DW and 1220 ± 184 $\mu g \cdot g^{-1}$ DW, respectively, and under Fe-replete conditions, 208 ± 86 $\mu g \cdot g^{-1}$ DW and 415 ± 121 $\mu g \cdot g^{-1}$ DW, respectively. Fe availability therefore had little or no effect on leaf Cu content; in contrast, under Fe deficiency, root Cu content was about five times higher than leaf Cu content, whereas under Fe-replete conditions it was only about twice as high.

These results indicated that the amounts of Cu translocated from the roots to the aerial parts were similar between the LD and the CL conditions, regardless of Fe availability; however, when Fe was deficient, and under given illumination conditions, more Cu was accumulated in the roots than in the aerial parts. Since most of the Fe was accumulated in the roots, as discussed below (**Figure 3**), Cu might presumably occupy sites that are vacated under conditions of Fe deficiency. The Cu concentration in the leaves of seedlings treated for 4 weeks under LD was greater than 200 $\mu g \cdot g^{-1}$ DW, and this value is toxic to Cu non-tolerant plants: typically, Cu toxicity begins to be observed above 20 $\mu g \cdot g^{-1}$ DW [5]. Although concentrations of Cu higher than 200 μM have been used in some previously reported studies [25]-[28], in the work reported here we did not treat seedlings with more than 200 μM Cu so as to avoid precipitation by interaction with other ions, and for not more than 4 weeks because of the limitations of the medium and space in a seedling growth container. Because we used only rosette-stage seedlings, which could be grown on to flowering stage, it might be possible to achieve higher levels of Cu accumulation if *H. albus* plants were treated with much higher levels of Cu in the soil, and for longer periods. *Lycopersicon esculentum* plants accumulated more Cu in *in vivo*-grown leaves than *in vitro*-grown leaves [10]. In *Brassica napus* plants, older leaves accumulated Cu to a greater extent than younger leaves [29]. We have also found a similar tendency in *H. albus* seedlings (data not shown).

3.3. Cu and Fe Allocations at the Organ and Cellular Levels

The high levels of incorporation of Cu into *H. albus* seedlings raised the question of the sites of accumulation of Cu at the subcellular level. Seedlings exposed to excess Cu and Fe-replete conditions under LD for 4 weeks were therefore first divided into aerial parts and roots, and then their homogenates were divided into four fractions by filtration and centrifugation, according to a previous report [15], with some modifications; the four fractions comprised a cell-wall fraction, a fraction containing nuclei and plastids, a mitochondrial fraction and, lastly, a soluble fraction including vacuoles and cytosol. Both the Cu contents and, for comparison, the Fe contents

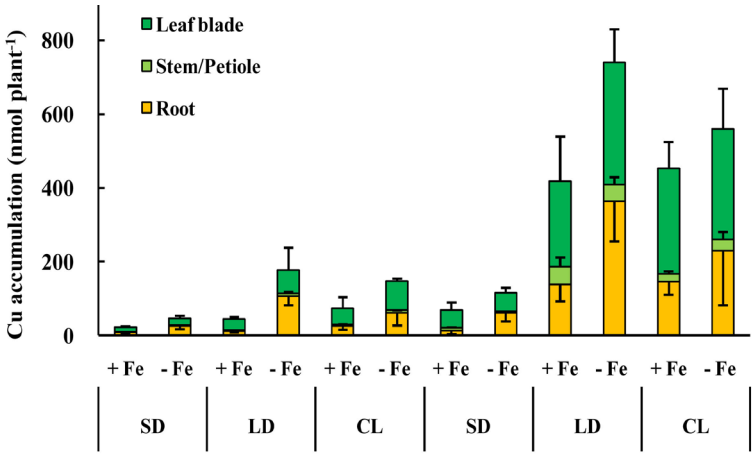

Figure 2. Cu distribution in various organs of *H. albus* plants. Seedlings were treated with Cu excess (200 μM) in combination with/without Fe for 2 and 4 weeks at 25˚C on a rotary shaker at 50 rpm in the incubator, under three different light regimes (121 μmol·m^{-2}·s^{-1}): short day (SD), 10 h light - 14 h dark; long day (LD), 16 h light - 8 h dark: continuous light (CL), 24 h light. –Fe, no addition of Fe; +Fe, with addition of 100 μM Fe(III)-EDTA. Data are means and sd of 4 samples.

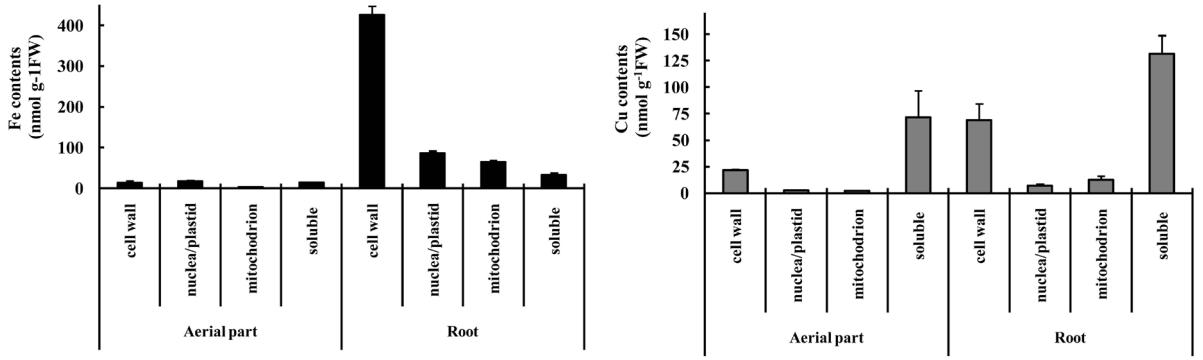

Figure 3. Cu and Fe allocations in various fractions from aerial parts and roots. Seedlings supplied with Cu excess (200 μM) together with Fe (100 μM) were cultured under long day (LD) condition for 4 weeks. Freshly harvested tissues (*ca.* 2 g) were ground in 50 mM Tris-HCl buffer (pH 7.5) containing 250 mM sucrose and 10 mM DTT at 4˚C and then filtered through nylon mesh (84 mm) *in vacuo*. Cell wall was recovered on the filter. The filtrate was centrifuged at 880 × g, followed by 21,880 × g: the pellets were denoted as "nucleus and plastids fraction" and "mitochondrial fraction", respectively, and the rest of the solute as "soluble fraction". Experiments were repeated three times and the average and standard deviation are given.

of fractions were analyzed. The results confirmed that Cu was present both in the aerial parts and in the roots (**Figure 3**); in both parts of the plants, the soluble fraction contained the largest proportion of the Cu (73% and 60%, respectively), followed by the cell-wall fraction (22% and 31%, respectively). The remaining Cu was found in the nuclei-plus-plastids and mitochondrial fractions. In the case of Fe, the roots contained 13 times more Fe than the aerial parts (on a nmol·g^{-1} FW basis); thus, in the roots, the cell-wall fraction contained the largest proportion (70%), followed by the nuclei-plus-plastids fraction (14%) and the mitochondrial fraction (11%) (**Figure 3**).

The most common cellular mechanisms of Cu tolerance are binding of the metal ion to the cell wall, allocation into vacuoles and incorporation into cellular components such as proteins and phenolics [30]. In *Daucus carota* [31], most of the Cu was found to be sequestered in the cell wall. Wang and Liu [32] also concluded from their results that plants lessen the toxicity of heavy metals by accumulating them in the cell wall. Similarly, *Bechmeria nivea* root cells accumulated around 50% of their total cadmium (Cd) in the cell wall, followed by 37% in the soluble fraction [15]. Our results that Cu accumulation occurred mainly in the soluble and cell-wall fractions in both the aerial parts and roots of *H. albus* are consistent with the above-mentioned reports. In the case of the soluble fraction, the vacuoles must be the main site of sequestration acting to prevent cytosolic Cu toxicity [33].

3.4. Effects of Cu and Fe Levels on the Accumulation of Other Minerals

Treatment of *H. albus* seedlings with excess Cu resulted in high levels of Cu accumulation and, under Fe deficiency, more Cu was accumulated in the roots (**Figure 2**, **Figure 3**). To understand the effects of Cu/Fe levels on the accumulation of other minerals, divalent mineral cations such as Ca, Mg, Mn and Zn were also analyzed in seedlings cultured for 4 weeks under LD (**Figure 4**). Under Fe-replete conditions, Cu excess did not affect the accumulation of any of these ions. On the other hand, under Fe deficiency, the Ca content was apparently increased, whereas the Mn content was significantly decreased, though only in the roots. To examine whether the decrease in Mn was an important cause of the damage resulting from the combination of Cu excess and Fe deficiency, we also determined (in place of Fe deficiency) the effects on growth and Cu accumulation of Mn deficiency (removal of Mn completely from the culture medium) in combination with Cu excess (200 μM) for 3 weeks under LD. The results showed that leaf chlorosis was observed, although Cu accumulation was also promoted (data not shown).

A range of effects on mineral accumulation in Cu-treated plants have been reported. In bean plants, excess Cu (4 and 15 μM) induced Fe deficiency, resulting in decreased leaf chlorophyll [21], and in Cu-tolerant *Commelina communis*, 100 μM Cu inhibited Fe, Mn and Zn accumulation significantly [34]. On the other hand, in roots of *Elsholtzia argyi*, the concentrations of K, Ca, Mg, Mn, and Zn were not affected, or were even increased, in a Cu-tolerant ecotype [35]. In the work reported here, *H. albus* plants were presumably able to thrive under excess Cu because the accumulation of minerals, including Ca, Mg, Mn and Zn, was not affected significantly, provided that sufficient Fe was available (**Figure 3**). However, Mn contents decreased in the roots subjected to Cu excess and Fe deficiency in combination, even under LD. These results suggested that Mn deficiency induced by Cu excess in the presence of Fe deficiency was a possible contributory cause of the observed growth damage and reduced water content that occurred under CL (**Table 1**, **Table 2**).

4. Conclusions

Hyoscyamus spp. have a folkloric history as poisonous plants and they contain hyoscyamine as a major tropane alkaloid used in medicine [36]. In this paper, we have presented evidence indicating that *H. albus* seedlings are tolerant of Cu present in excess. Furthermore, excess Cu was able to compensate for Fe deficiency, depending on the light conditions. Continuous light inhibited this effect, probably as a result of the induction of Mn deficiency.

Although many plants displaying Cu tolerance have been reported [25] [37]-[39], this is the first report relating to the medicinal plant, *H. albus*. These interesting findings suggest the novel possibility of utilizing this plant both as a source of medicinal compounds and as a Cu accumulator. Since a high accumulation of Cu renders

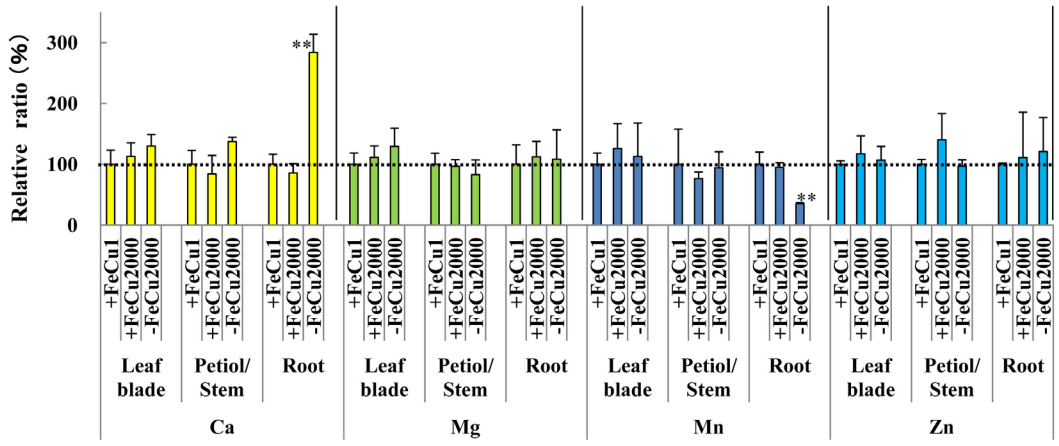

Figure 4. Minerals (Ca, Mg, Mn and Zn) contents in the various organs. Seedlings were subjected to different Cu/Fe concentrations and cultured under long day (LD) conditions for 4 weeks. +Fe, with 100 μM Fe(III)-EDTA; −Fe, without Fe; Cu1, 0.1 μM $CuSO_4$; Cu2000, 200 μM $CuSO_4$. Control (+Fe Cu1) is expressed as 100%. Others are expressed as relative values. Data are means and sd of 4 samples. Data are analyzed by ANOVA and Student *t*-test. **Reveals significant differences at the levels of $P < 0.01$ compared with control.

plants toxic and non-edible, *H. albus*, which is toxic on account of its alkaloid content, may become additionally toxic when it absorbs Cu. Whereas hyoscyamine is a feeding deterrent for vertebrates as well as several insects [40], Cu has a fungicidal effect [41]; therefore, by virtue of complementary mechanisms, *H. albus* plants may be equipped to protect themselves from attack by a variety of organisms and to thrive on Cu-polluted soils. In such areas, the cultivation of edible crop plants such as vegetables and cereals is not feasible even for phytoremediation, due to accidental and intentional distribution as foods. If *H. albus* plants were to be cultivated in a Cu-polluted area, both hyoscyamine as a drug and Cu as a useful metal could be recovered from the harvested plants by extraction: alkaloids would be extracted by organic solvent and Cu would remain in the mineral fraction. Recovery of Cu in this way from heavily-polluted areas could provide an additional source of income for farmers. Further study is now needed of the cultivation of medicinal *H. albus* plants in the field as both Cu scavengers and alkaloid producers.

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