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Valuable alkaloids content is preserved in *Camptotheca acuminata* and *Morus alba* grown in trace elements contaminated soil

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ABSTRACT

Phytoextraction of trace elements (TE) using woody species is an economically challenging soil remediation approach because of the long time needed. Yet, some trees contain alkaloids that can be exploited along structural components to enhance biomass value. As alkaloids are thought to be involved in plant defence mechanisms, we hypothesized that potentially hostile phytoremediation conditions could increase their level. Camptothecin in *Camptotheca acuminata* and 1-deoxynojirimycin in *Morus alba* were measured from trees grown in a field in presence of Cu, Pb and Zn all together, and from *M. alba* grown in a greenhouse in presence of Cd or other abiotic stressors (NaCl and bending). The trees did not extract TE in the field, but *M. alba* stems accumulated Cd in the greenhouse experiment, with no consequence on stomatal conductance and leaves pigments concentration. Camptothecin and 1-deoxynojirimycin concentrations were preserved under all experimental conditions, as was biomass yield, and phenolics were slightly increased in *M. alba* exposed to TE. This study provides evidence that valuable and persistent alkaloids and phenolics can be extracted from trees facing phytoremediation-associated stresses, without a negative impact on their quantity and on biomass yield. Such products could generate a sustainable stream of revenues during phytoremediation.

NOVELTY STATEMENT

There is scarce data on tree alkaloid content and scarcer data on how it is affected by exposure to trace elements in a phytoremediation context. We provide evidence that the content of two specific alkaloids is not altered in *Morus alba* and *Camptotheca acuminata* exposed to moderate to elevated levels of contaminating trace elements. The manuscript introduces the use of *M. alba* for phytoremediation in the Americas and is the first to propose the use of *C. acuminata* on trace element contaminated sites to produce camptothecin, a valuable anticancer alkaloid.

KEYWORDS

Soil contamination; phytoextraction; wood extractives; alkaloids; *Morus alba*; *Camptotheca acuminata*

Introduction

Soil contamination is a threat to human health and to many ecosystems worldwide (Rodríguez Eugenio *et al.* 2018; Shikha and Singh 2021). Traditional rehabilitation techniques are either based on excavation and disposal or use physicochemical methods to extract and wash the contaminants (Guemiza *et al.* 2017). Phytoextraction is a promising phytoremediation approach to remove trace elements (TE) from contaminated soils by concentrating them in plant biomass (Pilon-Smits 2005; Suman *et al.* 2018; Shikha and Singh 2021). Despite having numerous advantages when compared to traditional techniques, phytoextraction involves slow processes and is hardly economically attractive (Thewys and Kuppens 2008). Yet, phytoremediation using woody species generates a redeemable output in lignocellulosic biomass, for which demand is expected to grow with economy decarbonization and the need for renewable energy and green sources of chemicals (Londo *et al.* 2018). Along with calories ready to be burned, trees produce amazing libraries of chemical entities, making up 2 to 14% of their dry mass (Telmo and Lousada 2011). Extracting chemicals from wood, before it is used as an energy crop, could increase biomass value and phytoremediation attractiveness (Sas *et al.* 2021).

Wood phytochemicals include alkaloids, a broad and diverse group of nitrogen-containing molecules that are specific to genera or few species (Desgagné-Penix 2017). Alkaloids comprise very potent modulators of biological activities, like caffeine and quinine (Desgagné-Penix 2017). Less known alkaloids are the alpha-glucosidase inhibitor 1-deoxynojirimycin (DNJ), found in *Morus alba*, and the type I topoisomerase inhibitor camptothecin (CPT), isolated from *Camptotheca acuminata*. The former has antidiabetic properties (Desgagné-Penix 2017), while the latter is used in the synthesis of an anticancer drug (Isah 2016).

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Not much is currently known about how growth on degraded (*e.g.,* elevated salinity) or TE-contaminated lands could alter alkaloid levels in trees. Alkaloids likely appeared in plants as a protective trait against biotic stresses (Waller and Nowacki 1978). As biotic and abiotic stress responses share similar metabolic pathways in plants (Wasternack 2014), it is conceivable that alkaloids are increased in trees grown in potentially hostile phytoremediation conditions. Supporting this idea, jasmonic acid, a phytohormone associated with stress response, has been shown to increase CPT production in *C. acuminata* cultured cells (Deepthi and Satheeshkumar 2017). Examples of TE-induced alkaloid accumulation have also been documented in other plants (Srivastava and Srivastava 2010; Soleimani *et al.* 2020).

To be suitable for TE phytoextraction, a tree species must accumulate the contaminants of interest in its harvestable parts and should generate an important volume of biomass (Pulford and Watson 2003). *M. alba* possesses many appealing qualities for phytotechnologies: it accumulates TE in its stems, grows fast, develops an extensive root system, resists drought and tolerates poor soil and air quality (Bunger and Thomson 1938; Jiang *et al.* 2017). Its ability to extract Cd is well described (Wang 2002; Wang *et al.* 2004; Zhao *et al.* 2013; Nikolova 2015; Huang *et al.* 2018; Zeng, Guo, *et al.* 2020; Zeng, Huang *et al.* 2020). The hardiness of the *M. alba* cultivar 'Tatarica' is compatible with southern Canadian climates. *C. acuminata* is also a fast-growing species, but not much is currently known about its potential for phytoremediation.

We hypothesized that trees grown under phytoremediation-associated abiotic stresses would increase their content in alkaloids which can be extracted to enhance biomass value. In order to test this hypothesis, *M. alba* and *C. acuminata* were grown in a field containing Cu, Pb and Zn all together and *M. alba* was grown in a greenhouse in presence of Cd or NaCl, or facing mechanical stress.

Materials and methods

Field experiment – material, treatments, sampling, and biomass preparation

Two-year-old seedlings of *M. alba* 'Nongsang No. 14′ and *C. acuminata*, with a diameter measured at 20 cm from the ground of 1.3–1.5 cm and 1.5–1.8 cm, respectively, were purchased from the Hangzhou plant nursery (Hangzhou, Zhejiang, China). Trees were planted in spring 2017 in 4 replication blocks of 3 individuals per block (12 trees per treatment), at a density of 1 tree per 1.375 m^2, according to a split-plot experimental design at the Shanghai Chenshan Botanical Garden (31°04'39"N, 121°11'12"E) in China. Details about the experimental site are provided elsewhere (Kou *et al.* 2018; Vincent *et al.* 2018; Shang *et al.* 2020). Briefly, the treatments included control not contaminated soil and soil spiked with a mix of $CuCl_2$ (0.342 kg/m^2), $PbCl_2$ (0.654 kg/m^2) and $ZnCl_2$ (1.018 kg/m^2) powders. In November 2018, after two growing seasons, one tree per control plot ($n = 4$) and one tree per plot spiked with the three TE (a treatment referred to as "3 M"; $n = 4$) were harvested, along with one composite soil sample per 3 M plot ($n = 4$). Stems were air-dried to constant weight and water content was calculated to determine biomass dry weight. Subsamples of *M. alba* ($n = 4$ for each condition) and *C. acuminata* ($n = 4$ for control and $n = 3$ for 3 M) were sent to Canada for phytochemical analysis. These stems were milled (<2 mm) and sieved to keep particles of 180–850 µm.

Greenhouse experiment – material, treatments, sampling, and biomass preparation

A complementary experiment was conducted in a controlled greenhouse environment with *M. alba*, tolerant to southern Canadian climates. Two-year-old seedlings of *M. alba* 'Tatarica' were purchased from Arbo-Quebecium (Ste-Catherine-de-Hatley, Canada) and transferred into 2.5 L pots filled with BM6 peat-and-perlite medium (Berger, Ste-Modeste, Canada). Plants were grown in a polyethylene grow tunnel at the Montréal Botanical Garden, Canada (45°33′43″N, 73°34′18″W) from June 9, 2020 (week 0) to September 16, 2020 (week 14; 100 days after transplantation). They were watered when the soil surface appeared dry (every 1 to 3 days) and fertilized (20-20-20) on weeks 7, 9, 10 and 14. After two weeks of initial growth, 24 plants were distributed into six replication blocks of four plants, each plant of a block receiving one of the four treatments: control, salt stress, Cd exposure and mechanical stress. To avoid osmotic shock linked to salt stress, NaCl was added on three occasions during watering: to reach 1 mmol per liter of soil in week 3, 2 mmol/L in week 7 and 5 mmol/L in week 11. $CdSO_4$ (Alfa Aesar, Ward Hill, MA) was added once during watering in week 4, at 50 mg Cd/kg of soil (dry weight basis). For mechanical stress, the trees' main stem was gradually bent to 35° from the vertical axis. A plate was placed under each pot to collect and pour back runoff water into the pots.

On harvest day, plant aerial parts were weighed fresh. On every specimen, a 2.5 cm stem section (at 10 cm from the soil) was collected and flash-frozen for phytochemistry assays after lyophilization and grinding using a TissueLyzer II (Qiagen) with stainless-steel beads. For bent trees, an additional 5 cm stem section was collected, split longitudinally into tension wood (TW, facing up) and opposite wood (OW, facing down), and treated as above. Two topmost fully developed leaves were collected. One was flash-frozen in liquid nitrogen prior to lyophilization and powdering for phytochemistry assay and the other was kept on ice prior to photosynthetic pigments measurement. The remaining stems and leaves were separated, weighed again and air-dried indoor to constant weight for biomass dry weight determination and complementary phytochemistry assays. For the latter, air-dried stems were milled (< 2 mm) and sieved to keep particles of 180–850 µm, while air-dried leaves were pulverized using a pestle. Composite soil samples were collected for total Cd determination.

Greenhouse experiment – soil characterization

Soil electrical conductivity was measured in control and salt stress groups just before plants harvest using a soil moisture

meter FieldScout TDR 150 with 12 cm probes (Spectrum Technologies, Aurora, IL). Three determinations were performed in each pot.

Greenhouse experiment – plant physiology

Stomatal conductance was measured on the topmost fully developed leaf of each plant, two determinations per leaf, using an SC-1 leaf porometer (Meter, Pullman, WA) on two different days between 9:30 and 11:00 in week 14. Values were similar on both days and only data recorded on the second day are shown. Photosynthetic pigments were assayed in dimethyl sulfoxide as previously described (Hiscox and Israelstam 1979), recording absorbance at 665, 649 and 480 nm on a single sample of 100 mg per plant. Chlorophyll a and b were quantified based on Garg formulas (Garg 2012): Chl a (mg/L) = $12.19\,A_{665} - 3.45\,A_{649}$; Chl b (mg/L) = $21.99\,A_{649} - 5.32\,A_{665}$ and carotenoids based on Wellburn formula (Wellburn 1994): Carotenoids (µg/mL) = $(1000\,A_{480} - 2.14$ Chl a $- 70.16$ Chl b$)/220$.

Phytochemistry from M. alba samples

Water-soluble compounds were extracted from both powdered freeze-dried stems (greenhouse experiment) and milled air-dried stems (field and greenhouse experiments) following a procedure modified from Kim *et al.* (2003): 1.5 mL of HCl 0.1% in water was added to 30 mg of material. Suspensions were incubated for 45 min in a water bath at 50 °C with agitation (100 rpm) and centrifuged at 12,000 × *g*, 5 min. Supernatants were transferred to new tubes, and pellets were extracted a second time using the same conditions. Supernatants were pooled, filtered (0.2 µm) and stored at 4 °C until analysis. 1-deoxynojirimycin (DNJ) and glutathione, reduced and oxidized forms, were quantified by LC-MS/MS with duplicate injections. Separation was achieved on a SeQuant ZIC-pHILIC 150 × 4.6 mm, 5 µm particles HPLC column (MilliporeSigma) with a linear gradient from 65% to 40% of mobile phase B where mobile phase A is 5 mM ammonium formate in water, pH 3.0 and B is 0.2% formic acid in acetonitrile. The following transitions were used to quantify the analytes in positive ions mode based on a calibration curve prepared with pure standards: DNJ: 164.2/69.0; oxidized glutathione (GSSG): 613.2/355.1; reduced glutathione (GSH): 308.1/179.0. Total phenolic content was determined with the Folin-Ciocâlteu method (Kupina *et al.* 2018) with gallic acid as a standard. Values obtained from air-dried material were corrected for moisture content. For the field experiment, analyses were performed on four technical replicates. As values were identical between technical replicates, only single determinations were performed on the greenhouse samples.

Phytochemistry from C. acuminata samples

Camptothecin (CPT) was extracted from milled air-dried stems following a procedure modified from Zhao *et al.* (2010): 10 mL of 60% ethanol in water were added to 150 mg of material. Suspensions were incubated 1 h in a water bath at 60 °C with agitation (100 rpm). Extraction solutions were transferred to new tubes, and pellets were extracted two more times using the same conditions. Supernatants were pooled, filtered (0.2 µm) and stored at 4 °C until analysis. CPT was quantified by LC-MS/MS with duplicate injections. Separation was achieved on a Zorbax Eclipse Plus C18, 150 × 3 mm, 3.5 µm particles HPLC column (Agilent Technologies) with a linear gradient from 0% to 100% of mobile phase B in 9 min at 0.5 mL/min, where mobile phase A was 5% acetonitrile in water, 0.1% formic acid and B was 95% acetonitrile in water, 0.1% formic acid. Transition 349.1/305.5 was used to semi-quantify CPT in positive ions mode based on a calibration curve prepared with a pure standard. Values were corrected for samples' moisture content. Analyses were performed on three technical replicates per sample.

Trace element content

For the field experiment, total recoverable (HNO_3-soluble) Cu, Pb and Zn content was determined in stems and soil by ICP-MS, one determination per sample, as described previously (Vincent *et al.* 2018). Quality assurance and quality control for soil and plant tissues were assessed using technical duplicates, method blanks and standard reference materials from the Chinese Academy of Measurement Sciences for each batch of samples. For the greenhouse experiment, total recoverable (HNO_3-soluble) Cd, Zn, Fe and Cu were determined in leaves, stems and soil by ICP-MS as described elsewhere (Desjardins *et al.* 2018). Single determinations were performed. Assay accuracy was validated using SRM 1573a (tomato leaves; NIST) as a reference.

Statistical analysis

Unless otherwise stated, values in the text are expressed as mean ± standard error of the mean. Box plots include minimum to maximum value spreading (whiskers), quartiles, median (lines) and mean ("+"). Two-tailed paired *t*-tests were performed using GraphPad Prism v9.1.0, assuming Gaussian distribution, pairing data according to blocks. Differences were considered statistically significant at $p < 0.05$ and as a trend when p values were between 0.05 and 0.10.

Results

Biomass yield

Two complementary experiments were conducted to test the hypothesis that trees growing under abiotic stresses increase their alkaloids content. In a field experiment, testing the effect of TE, survival rates were 58% (7/12) for *C. acuminata* and 75% (9/12) for *M. alba*, regardless of the treatment. Harvested *C. acuminata* trees from control plots weighted 0.31 ± 0.06 kg DW/tree (Figure 1A). Those from 3 M plots

were not statistically different, weighing 0.39 ± 0.17 kg DW/tree. Similar observations were done with *M. alba* samples. Specimens from the control group reached 0.75 ± 0.30 kg dry weight (DW)/tree (Figure 1B), while those harvested on 3 M plots weighted 0.39 ± 0.16 kg DW/tree, not statistically different.

M. alba was further studied in a greenhouse pot experiment (Figure 1C). At harvest date, *M. alba* aboveground parts had reached a total biomass weight of 13.3 ± 1.0 g DW/tree in the control group (Figure 1D). Similar biomass weights were measured for the three other groups, with values of 13.7 ± 1.3, 12.8 ± 1.7 and 12.7 ± 1.3 g DW/tree for the Cd, NaCl and bending treatment, respectively (Figure 1D).

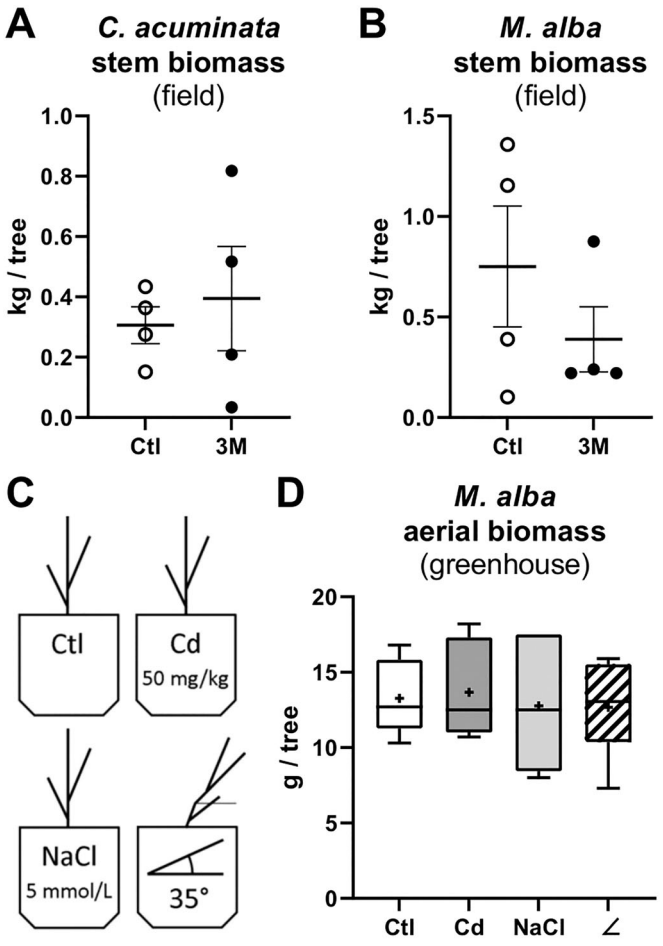

Figure 1. Exposure to trace elements and other abiotic stresses does not impair *Camptotheca acuminata* and *Morus alba* growth. (A,B) Stem biomass of *C. acuminata* (panel A) and *M. alba* (panel B) measured after two years of growth in a field soil spiked (3 M) or not (Ctl) with salts of Cu, Pb and Zn all together (see Table I for actual concentrations). Values are expressed as air dry weight per tree, shown with mean and SEM, $n = 4$. (C) Schematic representation of the greenhouse experimental conditions. For mechanical stress, trees were bent to an angle of 35° from the vertical axis, measured at plant half-height. (D) Air dry biomass of aboveground parts for control (Ctl) plants and those treated with Cd, NaCl or bent (∠). Mean values are shown as a "+" in box and whiskers plots, $n = 6$.

Plant physiology

To document the physiological consequences of the treatments applied in the greenhouse, stomatal conductance and photosynthetic pigments content were measured. The stomatal conductance was 350 ± 14 mmol/m^{2}s for leaves of the control plants. This parameter was not different when measured in treated trees, with values of 344 ± 24, 334 ± 9 and 338 ± 17 mmol/m^{2}s for NaCl, Cd and bending treatments, respectively (Figure 2A). Chlorophyll (Chl) a and b were measured in *M. alba* fresh leaves. In samples from untreated trees, Chl a and b contents were 1.45 ± 0.11 and 0.46 ± 0.04 µmol/g, respectively. Chlorophyll content was similar in the other treatment groups (Figure 2B). The concentration of carotenoids was also very similar in all samples, making approximately 0.037% of leaves' fresh weight (Figure 2C).

Trace elements phytoextraction and soil characteristics

In the field trial, despite elevated soil TE concentration in 3 M plots, *M. alba* and *C. acuminata* stems accumulated the same level of Cu, Pb or Zn, were they grown on control or 3 M plots (Table 1). In the greenhouse experiment, the total soil Cd concentration was 3.1 ± 1.3 mg per kg of soil at harvest date in the Cd group (Figure 3A). Leaves and stems of Cd-treated *M. alba* accumulated respectively 0.28 ± 0.08 and 0.61 ± 0.12 mg of Cd per kg DW (Figure 3B), while 0.020 ± 0.002 and 0.030 ± 0.002 mg/kg DW was found in control trees. Soil electrical conductivity was 0.15 ± 0.01 dS/m in the control group and 0.18 ± 0.02 dS/m in the NaCl

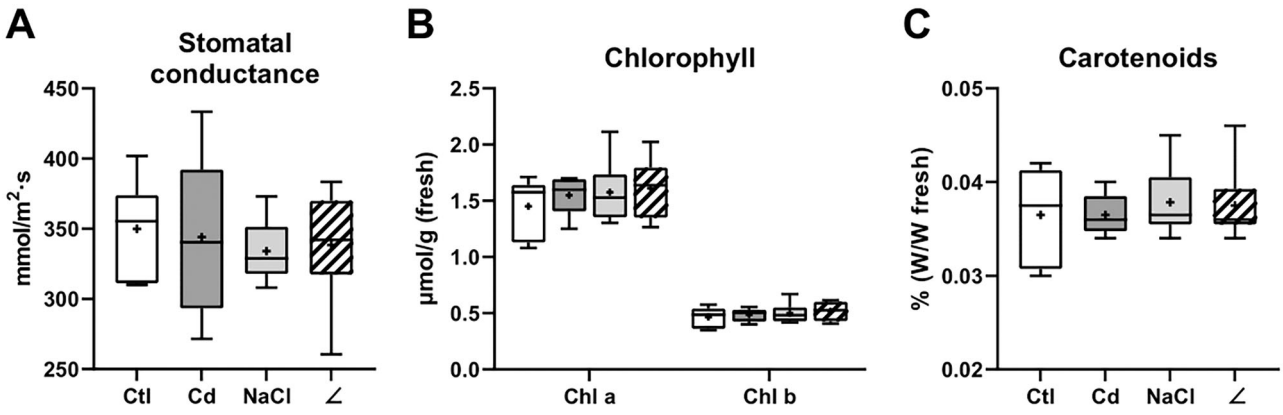

Figure 2. Treatments did not alter *Morus alba* leaves stomatal conductance and photosynthetic pigments concentration in the greenhouse experiment. (A) Stomatal conductance of topmost fully developed leaves for control (Ctl) plants and those treated with Cd, NaCl or bent (∠). (B,C) Chlorophyll (Chl) a and b (panel B) and carotenoids (panel C) assayed in dimethyl sulfoxide extracts. Mean values are shown as a "+" in box and whiskers plots, $n = 6$.

group (Figure 3C), a difference not statistically significant. Soil electrical conductivity has not been measured for the other groups.

Phytochemistry

Specific alkaloids of interest were extracted from samples collected in both experiments. In the field trial, CPT concentrations were identical in extracts from control and 3 M-treated trees, at 0.42 ± 0.03 g/kg DW (Figure 4A), and DNJ concentrations were similar, reaching 0.80 ± 0.21 g/kg DW in control and 0.65 ± 0.07 g/kg DW in 3 M-treated trees (Figure 4B).

In the greenhouse trial, glutathione was measured along DNJ in freeze-dried (fresh) *M. alba* stems. In control plants, 1.21 ± 0.13 g of DNJ/kg DW were extracted (Figure 4C). In extracts from other groups, DNJ concentrations were 1.43 ± 0.16, 1.27 ± 0.14 and 1.23 ± 0.17 g/kg DW for Cd, NaCl and bending, respectively, not statistically different from what was found in the control group. No oxidized glutathione (GSSG) could be measured in the extracts, but 42 ± 4 mg of reduced glutathione (GSH)/kg DW were measured in samples from control plants (Figure 4D). Similar values were measured in samples from the other groups: 28 ± 7, 37 ± 7 and 35 ± 6 mg/kg DW in Cd-treated, NaCl-treated and bent trees, respectively.

Finally, total phenolic content was also measured in *M. alba* extracts (Figure 4E). Water-soluble phenolics accounted for 5.9 ± 0.3 g/kg DW in control fresh samples. In the Cd group, phenolics content was more elevated, reaching 6.9 ± 0.5 g/kg DW ($p < 0.05$), whereas it was not statistically different in the NaCl group, at 6.8 ± 0.4 g/kg DW, and was only trending higher in the bent group, at 7.3 ± 0.5 g/kg DW ($p = 0.0655$). In bent trees, DNJ, GSH and phenolics content was similar in tension wood and opposite wood (Figure 4C–E).

Phenolics were also measured in air-dried stems from *M. alba*. In extracts prepared from samples collected in the field experiment, we measured a total phenolic content of 6.1 ± 0.5 g/kg DW in control samples and 6.5 ± 0.5 g/kg DW in 3 M samples (Figure 4F). When extracts were prepared from greenhouse experiment's air-dried samples, phenolics were more abundant, making 24.5 ± 1.4 g/kg DW in the control plants and 25.8 ± 1.7 g/kg DW in Cd-treated plants (Figure 4F).

Table 1. Soil and biomass total trace element content for the field experiment.

	Cu (mg/kg)	Pb (mg/kg)	Zn (mg/kg)
Control soil[a]	28 ± 2	22 ± 1	83 ± 7
TE-spiked soil (3 M)	609 ± 96***	1270 ± 85***	1158 ± 52***
M. alba stems, Ctl	3.9 ± 1.2	n.d.	52 ± 14
M. alba stems, 3 M	4.4 ± 0.2	n.d.	46 ± 8
C. acuminata stems, Ctl	7.0 ± 0.8	n.d.	73 ± 9
C. acuminata stems, 3 M	9.0 ± 0.5	n.d.	81 ± 16

Values expressed as mean ± SEM, $n = 4$.
[a]Values collected in November 2016 (last data available) – all other values are from harvest date, November 2018; n.d., not detected; ***$p < 0.001$ versus control soil.

Distribution of metallic cofactors in M. alba

Zn, Fe and Cu were measured along with Cd in control and Cd-treated samples. Leaves had identical or similar Zn, Fe and Cu concentrations in control and Cd-treated trees (Figure 5A). In stems, on the contrary, Zn concentration was significantly more elevated in Cd-treated samples, at 49 ± 4 mg/kg DW for the control group and 65 ± 7 mg/kg DW for the Cd treatment group ($p < 0.05$), and Fe concentration trended higher, with control at 7.4 ± 0.5 mg/kg DW and Cd-treated at 9.8 ± 0.9 mg/kg DW ($p = 0.065$; Figure 5B). Cu concentration in stems was unaffected by Cd exposure, measured at 3.0 ± 0.1 mg/kg DW for both treatments. Therefore, considering values at the level of the organism, Cd treatment induced a shift in Zn and Fe leaf/stem concentration ratios (Figure 5C), with no change in total aboveground concentration (Figure 5D). For Zn, leaf/stem ratio was 1.81 ± 0.15 in control trees and 1.39 ± 0.16 in Cd-treated ones ($p < 0.05$), while for Fe it was 3.6 ± 0.4 in the control group and 2.2 ± 0.3 in the Cd group ($p < 0.01$).

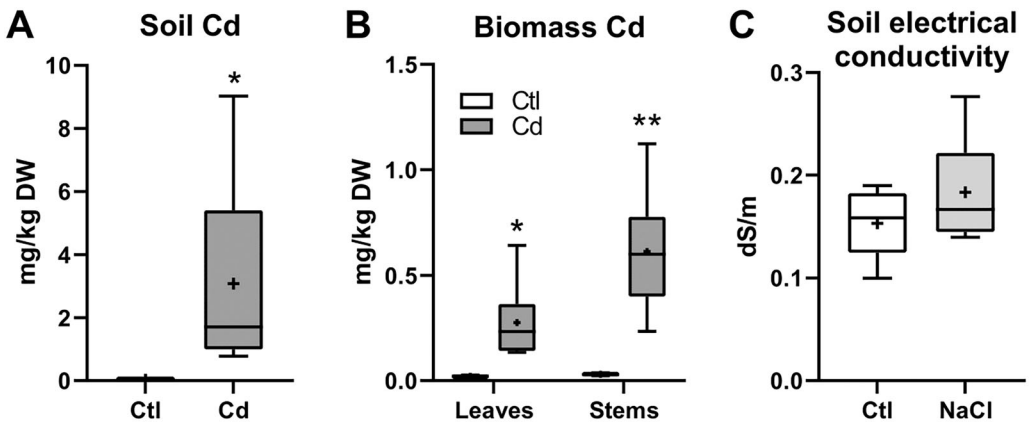

Figure 3. *Morus alba* aboveground parts accumulated Cd in a greenhouse experiment designed to expose trees to various abiotic stresses. (A,B) Total soil Cd content (panel A) and total leaf and stem Cd content (panel B) measured in Cd and control (Ctl) groups at harvest time. Cd was added as Cd sulfate, 50 mg/kg air dry weight. Cd contents are normalized per sample's oven dry weight. (C) Soil electrical conductivity measured in the NaCl and Ctl groups. Salt concentration was increased gradually to reach 5 mmol per L of soil. Mean values are shown as a "+" in box and whiskers plots, $n = 6$. Two-tailed paired t test: *$p < 0.05$; **$p < 0.01$.

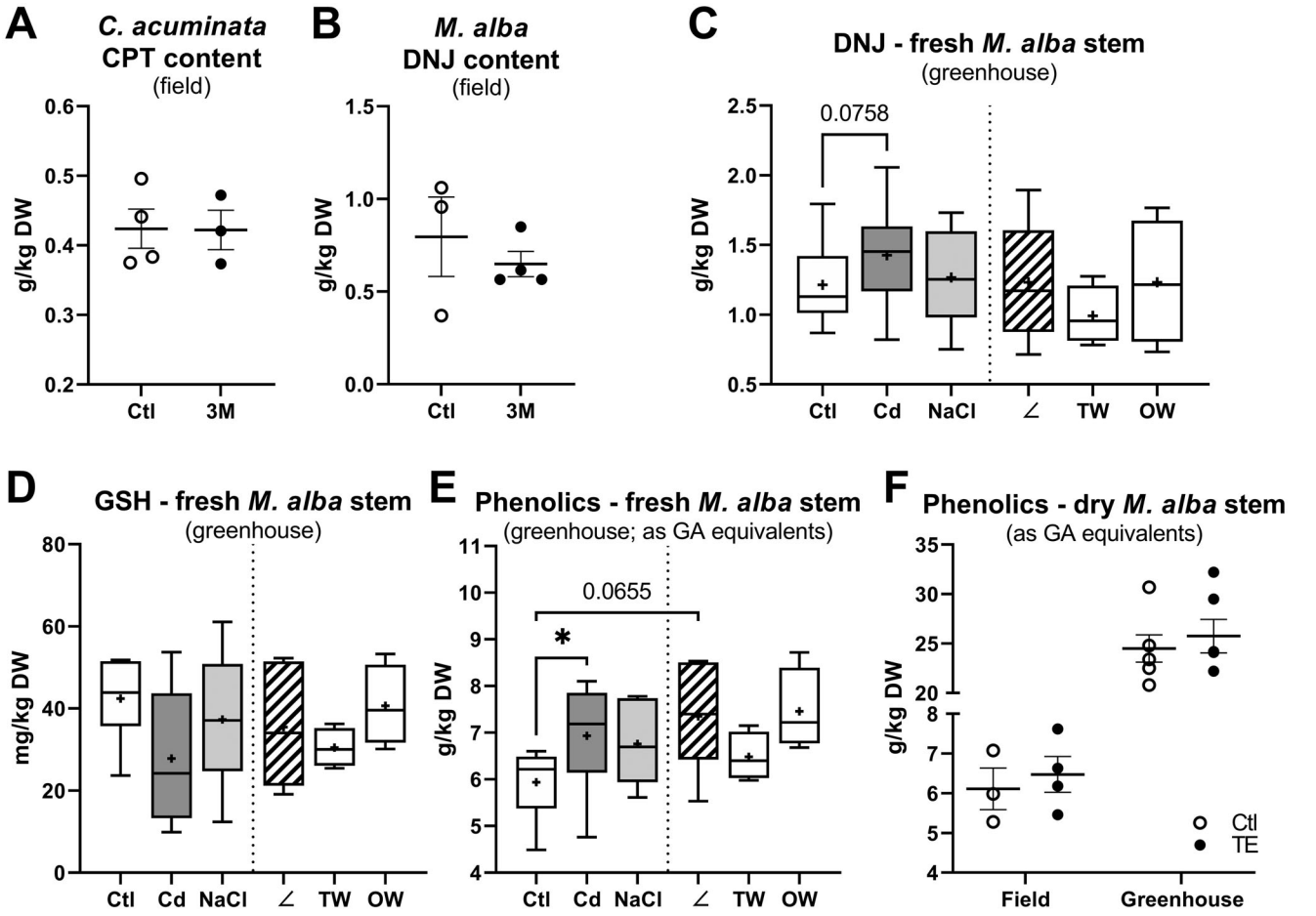

Figure 4. Treatments did not alter trees' specific alkaloid content nor glutathione (GSH) content, but Cd treatment increased total phenolic content in the greenhouse experiment. (A,B) camptothecin (CPT, panel A) and 1-deoxynojirimycin (DNJ, panel B) content in stems of *M. alba* and *C. acuminata*, respectively, grown in the field experiment under control condition (Ctl) or exposed to trace elements (3 M). Quantities are normalized per oven dry weight, shown with mean and SEM, $n = 3$ or 4. (C–E) DNJ (panel C), GSH (panel D) and total phenolic content (panel E) in extracts obtained from *M. alba* freeze-dried stem samples of control (Ctl) plants and those treated with Cd, NaCl or bent (∠). Stem sections from bent trees were separated longitudinally in tension wood (TW; facing up, against the bending force) and opposite wood (OW; facing down). Mean values are shown as a "+" in box and whiskers plots, $n = 6$. Two-tailed paired t test: $^*p < 0.05$. (F) Phenolics were also measured in extracts prepared from dry *M. alba* stems. Quantities are normalized per oven dry weight (DW), shown with mean and SEM, $n = 3$ or 4 for the field experiment samples and $n = 6$ for the greenhouse experiment. TE: Trace elements, either Cu, Pb and Zn all together for the field experiment or Cd for the greenhouse experiment; GA: gallic acid.

Discussion

We hypothesized that trees grown under stressful phytoremediation-associated conditions would increase their content in valuable alkaloids. To test this hypothesis, we first studied specific alkaloids from *C. acuminata* and *M. alba* planted in a field, in plots contaminated or not with Cu, Pb and Zn all together. In this experiment, TE did not alter CPT content in *C. acuminata* stems. Whether trees were stressed is not clear. The treatment had no impact on biomass production and stems from trees exposed to TE failed to accumulate more metals than control ones. We do not know yet if the absence of TE bioaccumulation in *C. acuminata* stems is the result of a successful avoidance strategy (Sanità Di Toppi and Gabbrielli 1999) or the consequence of a very low bioavailability (Kabata-Pendias 2004) in the specific context of our study. To our knowledge, this study is the first to consider the use of *C. acuminata* for TE phytoextraction in a phytoremediation context.

CPT level was similar to those reported elsewhere for dry shoots of young trees (Liu and Adams 1996; Liu *et al.* 1997). Should future studies conclude that *C. acuminata* is of low interest for phytotechnologies, our data still suggests it is an attractive species for agroforestry on TE-contaminated land: *C. acuminata* is a fast-growing species, appears tolerant to elevated levels of Cu, Pb and Zn in the soil, and contain CPT as a persistent extractable phytochemicals. CPT high economic value could enhance the profitability of a biorefinery producing biofuel from *C. acuminata* grown on marginal TE-contaminated lands. Moreover, stressful conditions able to elicit an increase in jasmonic acid could stimulate CPT biosynthesis as reported *in vitro* (Deepthi and Satheeshkumar 2017).

Similar results were obtained with *M. alba* and its specific alkaloid DNJ in the field experiment. Trees grown on contaminated soils had the same level of DNJ as had control trees. As for *C. acuminata*, conditions did not appear stressful to the trees. Treated *M. alba* did not accumulate more TE in their stems than control trees, they had a normal survival rate and a normal growth. *M. alba* had previously been shown to accumulate Cu (Nikolova 2015), Pb (Zhao *et al.* 2013; Nikolova 2015; Zhou *et al.* 2015) and Zn (Nikolova

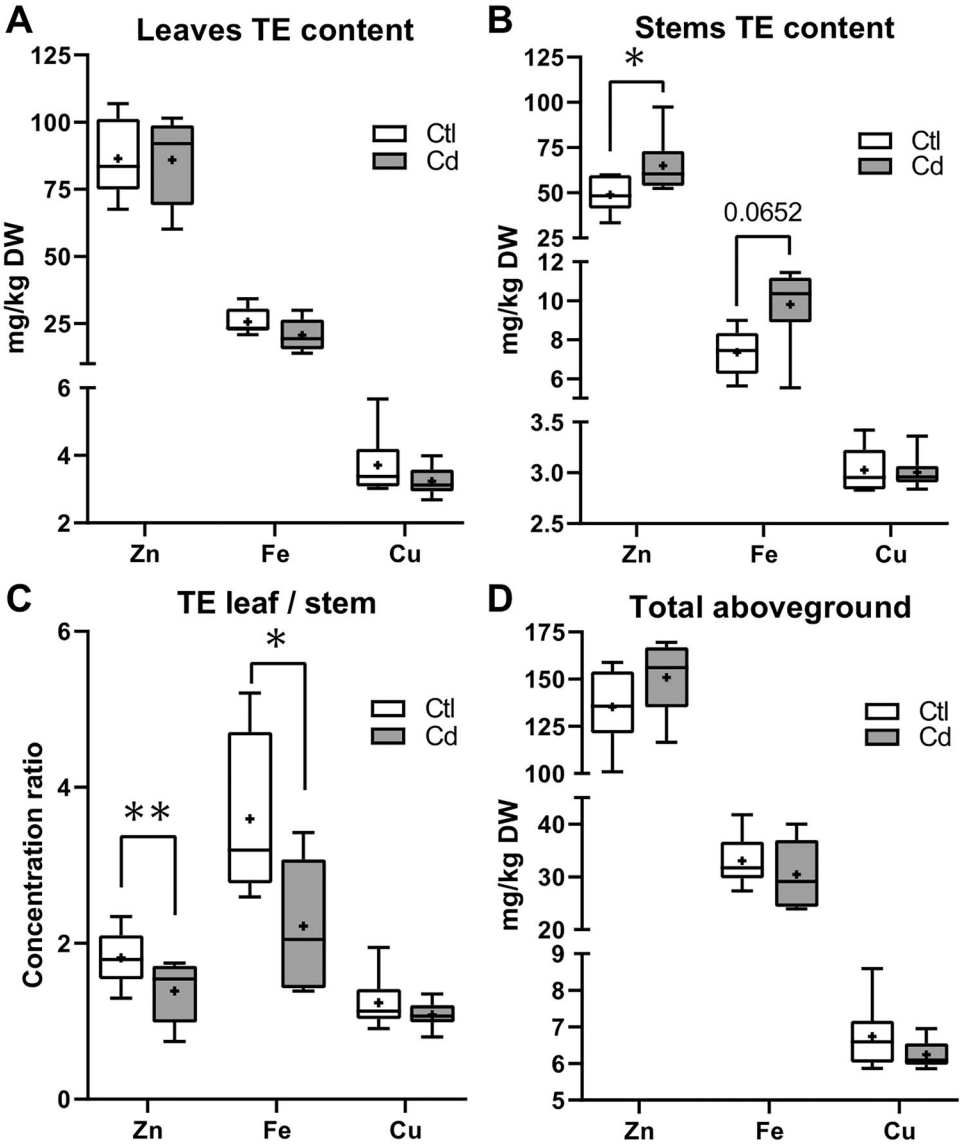

Figure 5. Exposition to Cd modifies Zn and Fe, but not Cu distribution in *Morus alba* aboveground parts. (A,B) Trace elements (TE) Zn, Fe and Cu were measured in air dry leaves (panel A) and stems (panel B). (C) Concentration ratios were obtained by dividing leaf by stem concentration for each individual tree. (D) Sum of individuals TE in each tree. Mean values are shown as a "+" in box and whiskers plots, $n = 6$. Two-tailed paired t test: $^{*}p < 0.05$; $^{**}p < 0.01$.

2015) in their stems in controlled experiments. Growing the species in a soil bearing approximately 760 mg of Pb per kg (less than 3 M soils here), Zhou *et al.* reported a considerably hindered growth with Pb accumulation in stems and leaves (Zhou *et al.* 2015). The soil pH is among the numerous variables influencing TE bioavailability and plants uptake (Kabata-Pendias 2004). Ashfaq, Afzal and Hanif noted a strong dependency on soil pH for Zn accumulation in *M. alba* leaves, with accumulation decreasing with increasing soil pH, and only a modest Zn accumulation at pH 6.0 (Ashfaq *et al.* 2010). The soil pH was 5.0 in Zhou *et al.* experiment (Zhou *et al.* 2015) and 8.0 at our experimental site (Shang *et al.* 2020), influencing negatively TE bioavailability.

In the greenhouse experiment, however, *M. alba* did accumulate Cd in their aboveground parts. When measured after 100 days of cultivation, soil Cd concentration was 3 mg/kg DW, at the limit to be considered hazardous to human health (de Vries *et al.* 2007), and stem content reached 0.6 mg/kg DW – giving a bioconcentration factor of 0.2. This bioconcentration factor is well below the full potential of the species for Cd bioconcentration: Zhao *et al.* reported factors above 1 for Cd in *M. alba* stems (Zhao *et al.* 2013). Cd bioaccumulation had no impact on aboveground biomass, on leaves' pigments content and on stomatal conductance, suggesting very minor stress to the plants, if any. Here again, DNJ stems content remained unaltered. Therefore, studying two unrelated species and two unrelated specific alkaloids, we observed no change in alkaloid content in dry stems collected from trees grown on TE-contaminated soils.

Contrarily to DNJ, total phenolic content slightly increased in *M. alba* stems exposed to Cd. Such a Cd effect had already been reported in *Phaseolus vulgaris* (Fuhrer 1982) and *Matricaria chamomilla* (Kováčik and Bačkor 2007) leaves, and in *Salix viminalis* leaves and stems (Vollenweider *et al.* 2006). That said, samples' origin and preparation had a larger impact on phenolics content than

treatments. In the greenhouse experiment, extracting phytochemicals from air-dried samples instead of fresh (cryo-preserved) ones yielded roughly four times more phenolics. An increase in phytochemicals extractability in dry samples could explain the difference (Havelt *et al.* 2020). This observation is important as air-dry stems are a more convenient input for a biorefinery. Plant phenolics are known for their antioxidant activity and are thought to prevent TE-induced oxidative stress in plant tissues (Bartwal *et al.* 2013). Extracted wood phenolics have numerous commercial applications (Devappa *et al.* 2015). An abundant water-soluble phenol in *M. alba* is resorcinol (benzene-1,3-diol) (Rowe and Conner 1979; Golpayegani *et al.* 2014) employed among other applications as an additive to rubber and polymers, and as a reagent in the synthesis of resorcinol-formaldehyde resins (Dressler 2013).

The extraction of valuable alkaloids and phenolics from harvested wood could enhance the economic viability and overall appeal of phytoremediation projects. An interesting concept in that view is the integration of the extractive valorization in refineries producing biofuels from phytoremediation-generated biomass (Devappa *et al.* 2015; Sas *et al.* 2021). Three of our observations are promising in this context: 1) wood alkaloid content was unresponsive to TE; 2) exposition to TE increased wood phenolic content; 3) biomass yields were normal despite TE phytoextraction and accumulation in *M. alba* stems.

Insights into M. alba *TE handling*

Zeng, Guo, *et al.* (2020) reported Cd accumulation in *M. alba* stems roughly 30 times more elevated than what is reported here, with no or minor effects on aboveground biomass. An increase in phenolics, as reported here, may participate in *M. alba* ability to handle Cd toxicity. Vollenweider *et al.* observed Cd accumulation in thickened cell walls of *Salix viminalis*, in structures containing proanthocyanidins (Vollenweider *et al.* 2006). Cd has also been shown to induce "lignin-like" insoluble phenolics in *Phaseolus vulgaris* stems (Fuhrer 1982). Only soluble phenolics were measured in this work, but lignin and proanthocyanidins are biosynthesized from water-soluble phenolic intermediates, which our assay could have detected.

The activation of Cd-sequestrating pathways involving phenolics or other molecules like phytochelatins may alter the distribution of other heavy metallic cations (Zeng, Guo *et al.* 2020). We observed a clear decrease in Fe and Zn leaf/stem ratios in Cd-treated plants, suggesting disturbed transportation mechanisms for these metals in treated *M. alba*. Fe and Zn, along Mn and Cu are essential cofactors for superoxide dismutase (either Cu/Zn or Fe/Mn) and catalase (Fe) activities, regulating redox status. By hampering the translocation of Fe, Zn and other heavy cations, exposition to Cd could interfere with photosynthetic processes and redox equilibrium (Prince *et al.* 2002). Glutathione is another key molecule for redox homeostasis and a precursor of phytochelatins. Only reduced glutathione could be measured in extracts and none of the treatments influenced its concentration. We did not quantify phytochelatins, but if their synthesis has been increased in Cd-treated plants, it did not alter the overall glutathione pool at harvest date.

Exposure to other stresses

The phytoremediation approaches are also applied to soils with elevated salinity or sodicity (Jesus *et al.* 2015; Jiang *et al.* 2017). We included a condition to study the impact of elevated soil NaCl concentration on *M. alba* DNJ level in our greenhouse experiment. NaCl has been added to a theoretical 5 mmol per liter of soil based on a previous pilot experiment using willows (E. Sas, unpublished work). This addition was insufficient to significantly increase soil electrical conductivity and alter leaf stomatal conductance. While saline soils have electrical conductivity values above 4 dS/m (Jesus *et al.* 2015), they remained below 0.3 dS/m in our soils. More aggressive experimental conditions will be needed to study the effect of salinity stress on *M. alba*.

The rationale to include a mechanical stress condition originated from observations made during method development. We noticed the presence of cotton-wool-like aggregates in milled *M. alba* wood which we thought could be composed of gelatinous fibers linked to tension wood (Fisher and Stevenson 1981). In angiosperms, tension wood containing gelatinous fibers will develop in a bent stem to reorient its growth toward the vertical (Brereton *et al.* 2011). During DNJ assay validation, we noted that water extracts from samples enriched in these cotton-wool-like aggregates were also enriched in DNJ (J. Lamontagne personal observations). We, therefore, hypothesized that an increase in tension wood could lead to an increase in DNJ content. Against this hypothesis, DNJ content was normal in bent stems, otherwise enriched in gelatinous fibers (Figure S1).

M. Alba *utilization in phytotechnologies*

M. alba cultivation on TE-contaminated lands is mainly studied in the context of sericulture, where leaves are harvested for the rearing of silkworms (Prince *et al.* 2002; Wang *et al.* 2004; Łochyńska and Oleszak 2013; Lei *et al.* 2019). Sericulture offers a safe way to use TE-contaminated arable lands (Wang 2002; Wang *et al.* 2004). In countries with no sericulture industry, drought resistance could still make *M. alba* an interesting species for phytoremediation, reducing the need for irrigation. *M. alba* has deep and widely spread roots (Bunger and Thomson 1938), an advantage both for drought resistance and phytoremediation. Biomass production and cost of implementation remain, however, to be assessed under temperate climates.

Conclusion

Our observations are in accordance with the view that phytoremediation-generated biomass could be used to produce both biofuels and valuable persistent alkaloids and phenolics in biorefineries, generating a sustainable stream of revenue during phytoremediation and enhancing the economic

viability of phytotechnology. The data also encourage the exploratory introduction of *M. alba* in phytoremediation projects in North America, where it may offer an alternative to *Salix* and *Populus* species.

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Disclosure statement

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