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Maastricht University

Faculty of Medicine and Life Sciences
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Master of Biomedical Sciences

Master's thesis

The influence of woody biochar on the root system architecture of Arabidopsis thaliana exposed to cadmium

Wim Kuypers

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Environmental Health Sciences

SUPERVISOR :

Prof. dr. Ann CUYPERS

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The influence of woody biochar on the root system architecture of *Arabidopsis thaliana* exposed to cadmium

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*Running title: *Root charchitecture*

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ABSTRACT

The application of biochar to cadmium (Cd) contaminated soils received particular attention due to its ability to stabilize Cd and thereby limit uptake by the plant. However, the influence of biochar on root system architecture (RSA) has received little attention. Nonetheless, the extent and the position of roots can influence metal uptake. The present study evaluated the influence of woody biochar on the RSA of *Arabidopsis thaliana* exposed to Cd. Root growth parameters of *A. thaliana* seedlings grown in vertical agar plates with biochar and Cd in single or combined application were quantified. Cadmium concentrations in seedlings were measured and expression of genes involved in glutathione metabolism, ethylene biosynthesis, and oxidative stress were quantified. Our results show that the addition of biochar resulted in shorter lateral roots and the formation of dense root hairs. Furthermore, biochar combined with Cd resulted in four times lower Cd accumulations than seedlings exposed to Cd without biochar. The addition of biochar to Cd-exposed roots resulted in significantly lower expression levels of genes involved in ethylene biosynthesis, glutathione metabolism and oxidative stress compared to expression levels in Cd-exposed roots without biochar. In conclusion, the application of woody biochar effectively reduces the bioavailable Cd in the medium of VAPs, and consequently, alleviates Cd-induced stress, resulting in lower expression of genes involved in the Cd-induced stress response. Despite the reduced Cd concentrations in seedlings, no increased root growth was observed.

INTRODUCTION

Industrial activities coupled with the agricultural application of metal-containing pesticides, fertilizers and sewage sludge have contributed to the worldwide dispersion of toxic metals in our soils, such as cadmium (Cd) **[**1**]**. Cadmium contamination of soils can increase uptake by crops grown for animal or human consumption. Because of the immobile nature of Cd, its accumulation in soils can be hazardous to a variety of organisms **[**2**]**. Plants' excess Cd uptake can induce toxic effects, disrupt nutrient homeostasis, and interfere with numerous physiological processes [3]. For instance, Cd can negatively affect plants by disturbing the oxidative balance, resulting in elevated levels of reactive oxygen species (ROS) [4-7]. Cuypers *et al*. (2010) observed that Cd stress indirectly caused the generation of ROS and affected the oxidative stress-related responses in *Arabidopsis thaliana* **[**7**]**. The binding of Cd to specific proteins can lead to activity inhibition or structural distortions of these proteins. Ultimately, Cd stress can affect the phytohormonal functioning of the plant. Schellingen *et al.* (2014) observed a Cd-induced increase in levels of the plant stress hormone ethylene and its molecular precursor 1-aminocyclopropane-1-carboxylic acid (ACC) in *A. thaliana* [8]. Plants evolved different defensive mechanisms to cope with metal toxicity, such as metal compartmentalization or sequestration **[**9**]**. Glutathione (GSH), for example, plays a crucial role in plant responses to Cd. It acts as an antioxidant and precursor of metal-chelating phytochelatins (PCs) [10]. However, when pollution levels are high, competition of metals with key micronutrients can lead to severe toxicity

and growth reduction of the plant [11]. Ultimately, this can harm crop production and food safety [12, 13]. High concentrations of Cd do not only lead to toxicity in plants. Animals and humans that consume these contaminated plants have a high risk of bioaccumulation of these toxic elements [14]. Due to its exceptionally long half-life, once absorbed, Cd irreversibly accumulates in vital tissues, primarily in the kidney, but also in other vital organs such as the liver and lungs [15]. Bioaccumulation of Cd can lead to a higher prevalence of hypertension, renal dysfunction, bone demineralization and urinary stones [1, 16].

In plants, the root system is the primary place where metals gain access. Plants can accumulate Cd through metal transporters in the membrane of their root system [17]. However, plants do not encode specific transporter proteins for Cd^{2+} ion transport into roots nor the translocation to the shoot [18]. The physicochemical similarities between Cd and iron (Fe), zinc (Zn), calcium (Ca) and copper (Cu) ions allow Cd to enter root cells through various types of transporters [19]. Important examples of transporter families are the ZIP family of Zn/Fe transport and heavy metal ATPases (HMAs) [20]. Wu et al. (2021) observed increased expression of the *ZIP2* gene in roots of *A. thaliana* exposed to 15 µM Cd combined with 2 µM Zn in a hydroponic culture [21]. Furthermore, multiple studies observed significant increases in *HMA2* expression in *A. thaliana* roots after exposure to 50 µM Cd [22, 23]. The root cell wall is the first part to interact with metals and is the plant part that contains most metals when grown on contaminated soils [24, 25]. Consequently, the root is also the organ that will be affected first. The root system architecture (RSA) plays an essential role in the uptake and translocation of water and nutrients and mechanical support. In general, the dicotyl RSA consists of two predominant root types: the primary root, which anchors the plant into the soil by growing downwards, and the secondary or lateral roots, which explore the soil through radial growth that is responsive to environmental stimuli [26]. On a microscale, root hairs are also included. They increase the surface area, aiding with water and nutrient uptake [27]. The environmental factors affecting root growth (*e.g.* nutrient/water availability, soil density, temperature, contaminants) are variable and complex [28]. Consequently, roots are plastic in their architecture, depending on their environment. Changes in RSA occur when plants modify the growth of primary and lateral roots to adjust to their environment. For example, Khare *et al.* (2017) observed that localized Cd treatment inhibited primary root growth and enhanced lateral root formation in a split root agar system and thereby promoted the exploration of noncontaminated areas [29].

Exposure to environmental stressors such as Cd can affect the structure of the root system, influencing the water and nutrient uptake of the plant. Kohanova *et al.* (2018) observed that Cd inhibited plant growth and reduced primary root length, number of lateral roots and root hairs per plant [30]. Furthermore, Cd was reported to induce lateral root density [31]. Vitti *et al.* (2013) showed that the addition of Cd to the media of 2-week-old plants caused near 50% primary root length reduction and an increase in root branching, root hair density, and root mean diameter [32]. Remans *et al.* (2012) observed a Cd dose-dependent inhibition of *A. thaliana* primary root growth and increased density of short lateral roots but indicated that further research into the molecular mechanisms behind these changes should be conducted in root systems [33]. Multiple studies described general stress-induced morphogenic responses (SIMRs) caused by different contaminants and determined by gradients of plant hormones (*e.g.* ethylene and auxin), ROS and antioxidants, which can result in altered elongation and differentiation of cells [33, 34]. Ultimately, these SIMRs lead to reduced primary root growth and increased lateral root density.

Accumulation of Cd in plants can be reduced by limiting the bioavailability [35]. Therefore, controlling Cd bioavailability and optimizing plant growth in polluted soils is imperative in order to minimize the impact on plants and ultimately on animals and humans, as well as to increase crop yield. Additionally, optimizing plant growth on contaminated soils is essential to guarantee future feed supply and meet with the increasing demand for biomass for renewable energy production without contravening on food supplies [36]. One effective way of limiting the bioavailability of Cd is the application of biochar.

Due to its porous structure, its high cation exchange capacity (CEC), and the presence of various functional groups (*e.g.*, hydroxyl and carboxyl groups), biochar can immobilize certain metals and limit their bioavailability [37]. It is an organic, carbon-rich amendment produced from the thermochemical conversion of biomass in an oxygen-limited condition (pyrolysis) [38]. Examples of biomasses are agricultural waste, plants, fermentation residue and manure. The physical and chemical characteristics of biochar differ based on the pyrolysis temperature, pyrolysis duration and the type of biomass [39]. Additionally, biochar has other interesting properties such as high carbon content, water holding capacity, and the ability to maintain certain soil parameters (*e.g.* pH) stable. Besides, it increases soil porosity and provides essential nutrients, increasing the overall soil quality [40]. Due to these unique properties and its ability to adsorb contaminants (*e.g.* Cd) from the environment, biochar application is often used to research the remediation of contaminated soils and water. Nevertheless, it has to be mentioned that biochar can also have disadvantages. For example, toxic metals and polycyclic aromatic hydrocarbons (PAHs) can accumulate in biochar based on the type of biomass and pyrolysis process [39]. Multiple studies investigated the influence of different biochar types on the RSA, but the results are variable. Varela *et al*. (2013) observed an increase in root size and biomass in water spinach (*Ipomoea aquatica*) after the addition of woody biochar [41]. Nevertheless, van de Voorde et al. (2014) observed opposite results when using biochar derived from grass for *Jacobaea vulgaris* [42]. A meta-analysis by Xiang *et al*. (2017) showed that over 136 articles, biochar application increased root biomass (+32%), root volume (+29%) and surface area (+39%). Additionally, biochar induced increases in root length (+52%) and the number of root tips (+17%). Interestingly, they stated that the biochar production process (pyrolysis temperature and duration) plays a more important role in regulating RSA changes than the biochar type [43]. The soil environment may be affected by the specific characteristics of biochar, as well as the application rate and the amount used. Consequently, this could alter root traits.

Biochar research mainly involves chemical studies, but biological research is still in its infancy. Over the past years, the number of studies into the effects of biochar on plant stress and growth of plants exposed to Cd has been increasing. A study by Abbas et al. (2017) found that biochar application in saline soils contaminated with Cd improved plant growth and reduced the Cd uptake in wheat (*Triticum aestivum L.*). Furthermore, biochar application reduced the oxidative stress in plants and reduced the bioavailability of Cd [44]. Ren et al. (2021) observed that the addition of peanut shell biochar resulted in an increased root length and root biomass in Tobacco plants exposed to Cd. Moreover, biochar alleviated Cd stress by reducing Cd uptake [45]. Most of these studies show promising results regarding the implementation of biochar [44-48]. However, most research has focused mainly on the plants' above-ground parts. Only a few studies have dealt with the influence of biochar on the RSA of plants exposed to Cd, and the underlying mechanisms remain largely unexplored. Nevertheless, the morphology and extent of roots can significantly influence contaminant uptake. The RSA plays a crucial role in plant fitness and crop performance. The roots are in direct contact with Cd and biochar and are responsible for translocating nutrients to the shoot. Biochar could, for example, lead to a lower concentration of bioavailable Cd, leading to enhanced root growth and an increase in biomass and, ultimately, crop yield. Moreover, the specific characteristics of different types of biochar could influence the RSA differently.

These results display the complex interactions between the plants' root system, Cd, and biochar and suggest that additional investigations are needed to obtain insights into the combined effects of biochar and Cd on the plants' RSA. The present study aims to increase the understanding of the influence of woody biochar on the RSA and the molecular mechanisms involved in the roots of *A. thaliana* seedlings exposed to Cd. Moreover, we aim to confirm, in a new way, that biochar has positive effects at the molecular and phenotypic level of roots and can play a role in using Cd-contaminated soils for growing crops. Accordingly, we evaluated the effects of two different types of woody biochar on the RSA of *A. thaliana* exposed to Cd in a vertical agar plate (VAP) cultivation system. Biochar derived from the tree bark of *Pinus sylvestris* (TB), and biochar derived from Medium-

Density Fibreboard plates (MDF). Moreover, we compared root growth parameters (*e.g.* primary root length, lateral root length and lateral root density) of plants grown on Cd-polluted agar in combination with or without woody biochar. Additionally, metal extractions were conducted to quantify the accumulation of Cd and other elements. Finally, real-time quantitative PCR (RT-qPCR) was carried out to gain more insight into the influence of biochar and Cd on root gene expression levels of genes involved in metal transport, cell differentiation, oxidative stress, ethylene biosynthesis and glutathione metabolism.

EXPERIMENTAL PROCEDURES

Biochar assembly – The following biomasses were used to create TB and MDF biochar, respectively: wood from the tree bark of *Pinus sylvestris* (100 % *Pinus Sylvestris*, Agrofino, Belgium) and waste from Medium-Density Fibreboard plates (Act&Sorb, Belgium). Before pyrolysis, all materials were shredded to a particle size < 1 cm using a Retsch SM100 shredder (Retsch, Haan, Germany). Wood biomass was carbonized through conventional pyrolysis at a temperature of 450 °C at an inclination angle of 2°, which resulted in a 15 min duration. Biochar were grinded using a ball mill (Retsch, Haan, Germany) and outgassed at 105 °C for 24 hours to remove excess PAHs. Additionally, the biochar were sieved using a 63 µm sieve. Particles smaller than 63 µm were used for plant experiments.

Vertical agar plates – Arabidopsis thaliana wild-type (Columbia ecotype) *A. thaliana* seeds were surface sterilized with 70% ethanol, followed by several washing steps with sterile dH_2O. Seeds were sown on 12 x 12-cm agar plates containing ¼ MS medium. All media contained 2.2 g L^{-1} MS basal salt mixture and 8 g L^{-1} plant tissue culture agar (Lab-M, Bury, UK) and were adjusted to pH 5.7-5.8 with KOH. Additionally, germination plates contained 5 g L^{-1} sucrose. After stratification at 4 °C in the dark for 48h, germination plates were placed vertically in a growth chamber. Established growth conditions for cultivation were 12 h photoperiod with day/night temperatures of respectively 22/18°C and 65% relative humidity. A combination of blue, red and far-red led modules was used to simulate a photosynthetic active radiation (PAR) spectrum of 176 µmol/m²s. After seven days of growth, seedlings were transferred to treatment plates where 0.5% TB or 0.5% MDF biochar were mixed into the medium. The appropriate amounts of filter-sterilised $CdSO_4/K_2SO_4$ solution were added to the plates to achieve a Cd concentration of 0 µM, 10 µM, 25 µM or 60 µM. K_2SO_4 was added to complement the SO_4^{2-} concentration in media with different $CdSO_4$ concentrations. The top 2 cm agar of all treatment plates was removed using a scalpel to create an air gap for the shoots. After one week on treatment plates, plates were scanned using a Canon 250D camera in a Nippon Genetics FAS-Digi pro image chamber (settings: ISO 400, exp. time 30s, aperture 14) and harvested for either element determination or gene expression analysis. Root growth parameters of the images (*e.g.* primary root length, lateral root length and lateral root density) were quantified using the RootNav software v1.8.1.

Quantification of Cd concentrations – Whole seedlings were harvested. Seedlings were weighed, washed in ice-cold 10 mM $Pb(NO_3)_2$ for 15 min and rinsed twice with dH_2O to exchange surface-bound elements. For every condition, six samples were prepared (±50 mg/sample). All samples were oven-dried (at 60 °C for three weeks) and weighed to determine the dry weight (DW). After drying, samples were digested in heating blocks by applying 1 mL HNO_3 suprapur to each sample. As references, three blanks (only HNO_3) and three certified reference samples (±50 mg Spinach) were included for quality assurance. The temperature was raised to 110 °C until the HNO_3 content was dried out. After repeating HNO_3 digestion three times, 1 mL 37% HCl was applied to each sample, and the temperature was raised again to 110 °C until HCl content was evaporated. Afterwards, 500 µL of 20% HCl and 4.5 mL Millipore H_2O were added. The dissolved elements in the resulting 5 mL 2% HCl were poured into 15 mL Sterilin tubes. Cadmium and nutrient concentrations were determined using inductively coupled plasma-optical emission spectrometry (ICP-OES). Element concentrations were calculated per kg DW (mg/kg DW).

Gene expression analysis – After seven days on treatment plates, roots were harvested for gene expression analysis. Six root samples per condition were transferred into Safe-Lock 2 mL Eppendorf

containing two stainless steel beads and placed in liquid nitrogen. After harvest, samples were stored at -70 °C. First, samples were shredded using the Retsch Mixer Mill MM400. RNA was extracted using the RNAqueous™ Micro kit (Thermo Fisher Scientific), according to the manufacturer's instructions. The concentration and purity of RNA were evaluated using the Nanodrop 1000 spectrophotometer. Agarose gel electrophoresis was performed to assess whether RNA was degraded. Four samples per condition were selected for cDNA synthesis based on the RNA concentration and purity. Treated RNA was converted to single-stranded cDNA using the TURBO DNA-free kit to eliminate DNA and the PrimeScript™ RT reagent Kit to reverse transcribe RNA into cDNA, according to the manufacturer's instructions. To accomplish equal cDNA concentrations, samples were diluted to the same concentration using RNase-free water. A ten-fold dilution of cDNA was prepared using a 1/10 TE buffer. Prepared samples were stored at - 20 °C. Real-Time Quantitative PCR (RT-qPCR) was performed with the QuantStudio™ 3 system using SYBR® Green chemistry, and 300 mM primer concentrations were used. Gene expression was calculated relatively as 2-ΔCq and was normalized with a normalization factor based on the expression of three reference genes selected out of ten by the GrayNorm algorithm (Supplementary Table 1). Before statistical analysis, outliers were determined using GraphPad and removed.

Zinc-regulated transporter protein 2 (*ZIP2*), heavy metal ATPase 2 (*HMA2*), CAPRICE-LIKE MYB 3 (*CPL3*), glutamate-cysteine ligase (*GSH1*), glutathione reductase 1 (*GR1*), respiratory burst oxidase homologue C (*RBOHC*), Oxidative signal-inducible kinase 1 (*OXI1*) and ACC synthase 2 (*ACS2*) were the genes of interest.

Statistical analysis – Datasets of RSA parameters, Cd-concentrations and gene expression were statistically analyzed using RStudio statistical software version 4.0.5. Normality (Shapiro-Wilk test) and homoscedasticity (Bartlett test) were tested. Transformations (*i.e.* sqrt, inv, exp, log) were applied when datasets did not meet normality or homoscedasticity. Calculated (non)normalized data from RT-qPCR analysis were logarithmically transformed to improve normal distribution. Normally distributed data were analyzed using a two-way ANOVA and a Tukey HSD test for multiple comparison. If datasets did not meet the requirements for normality or homoscedasticity, the non-parametrical Kruskal-Wallis test was used in combination with a Pairwise Wilcoxon rank-sum test for two-by-two comparison.

RESULTS

Parameters of the root system architecture – To determine the morphological influence of TB and MDF biochar on the RSA of *A. thaliana* exposed to Cd, RSA parameters (*i.e.* primary root length, lateral root length and lateral root density) were analyzed after one week of growth on treatment plates containing reference medium (RM), 0,5% TB biochar (TB) or 0,5% MDF biochar (MDF) combined with 0, 10 or 25 µM $CdSO_4$. Exposure to Cd with or without biochar affected *A. thaliana* primary root length (PRL) similarly while causing different effects on the lateral root length (LRL) and the lateral root density (LRD) (**Fig. 1A-C**). We observed a gradual Cd concentration dependent decline in PRL of seedlings grown on RM, TB and MDF plates (**Fig. 1A**). Primary root lengths of seedlings grown on RM were inhibited by 25 µM $CdSO_4$, showing a 23% decline in length compared to the control (**Fig. 1A**). The same effect was visible in seedlings grown on TB and MDF biochar with 25 µM $CdSO_4$ showing a decrease in PRL of 27% and 28%, respectively, compared to their controls. This indicates a growth-inhibiting effect on primary roots by 25 µM $CdSO_4$. Exposure to 10 µM $CdSO_4$ in RM and MDF plates resulted in a slight non-significant decrease in PRL compared to their control. However, in TB plates, the presence of 10 µM $CdSO_4$ inhibited PR growth by 18% compared to plants grown on TB 0 µM $CdSO_4$ (**Fig. 1A**).

In contrast with the similar PRL of seedlings grown on RM, TB and MDF, the LRL differed significantly when RM plates were compared to TB and MDF conditions (**Fig 1B**). The LRL of seedlings grown on TB and MDF without $CdSO_4$ were reduced by 36% and 16%, respectively, compared to RM. The same trend was observed for TB and MDF conditions with 10 µM and 25 µM $CdSO_4$. This observation indicates that the application of 0,5% TB and MDF biochar reduces

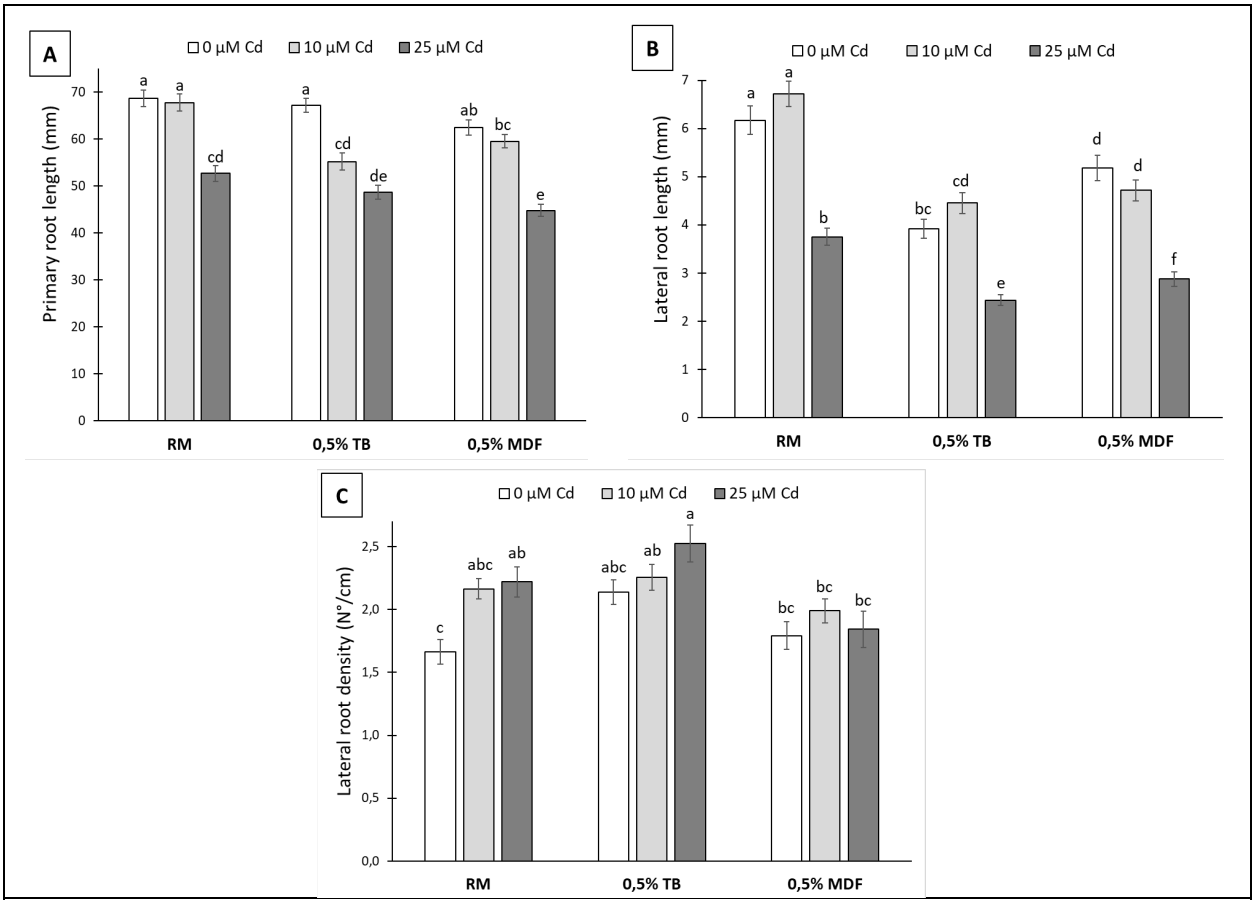

Fig. 1 – The influence of TB and MDF biochar on RSA parameters of seedlings exposed to Cd. Seven-day-old seedlings were transferred to treatment plates with reference medium (RM), 0,5% TB or MDF biochar combined with 0 µM, 10 µM or 25 µM $CdSO_4$. (A) Primary root lengths of seedlings after being transferred to treatment plates for seven days, (B) lateral root lengths and (C) lateral root density depicted as the number of lateral roots per cm primary root. Values represent the mean ± SE of 25 individual seedlings. Statistical significance was defined at $P < 0.05$ determined using analysis of variance (Two-way ANOVA). Different letters indicate significant differences between conditions.

LRL (**Fig. 1B**). Despite the overall decrease of LRL in both biochar conditions, a trend was visible between the RM, TB and MDF groups, showing a Cd dependent decrease when seedlings were grown on plates with 25 µM $CdSO_4$. The LRL of seedlings grown on RM and TB with 10 µM $CdSO_4$ slightly increased compared to their control (**Fig. 1B**). In seedlings grown on RM, the LRD increased with increasing $CdSO_4$ concentrations (**Fig. 1C**). This indicates that $CdSO_4$ stress resulted in more lateral roots per cm primary root. The same was observed for the LRD of seedlings grown on TB. However, increasing concentrations of $CdSO_4$. did not affect the LRD of seedlings grown on MDF plates. In addition to the previously mentioned RSA differences, we observed that roots of seedlings grown on TB 10 µM and 25 µM $CdSO_4$ showed high densities of root hairs on both PR and LR compared to RM conditions who showed no visible formation of root hairs (**Supplementary Fig. S1**).

Fresh and dry weights – To determine if biochar had a beneficial effect on the biomass of seedlings exposed to $CdSO_4$, fresh weight (FW) and dry weight (DW) of seedlings were determined after seven days on treatment plates (**Fig. 2A-C**). Fresh weight was increased by 20% in the presence of 10 µM $CdSO_4$ for seedlings grown on RM compared to the control (**Fig. 2A**). Furthermore, the application of TB biochar without Cd resulted in a 30% increase in FW compared to

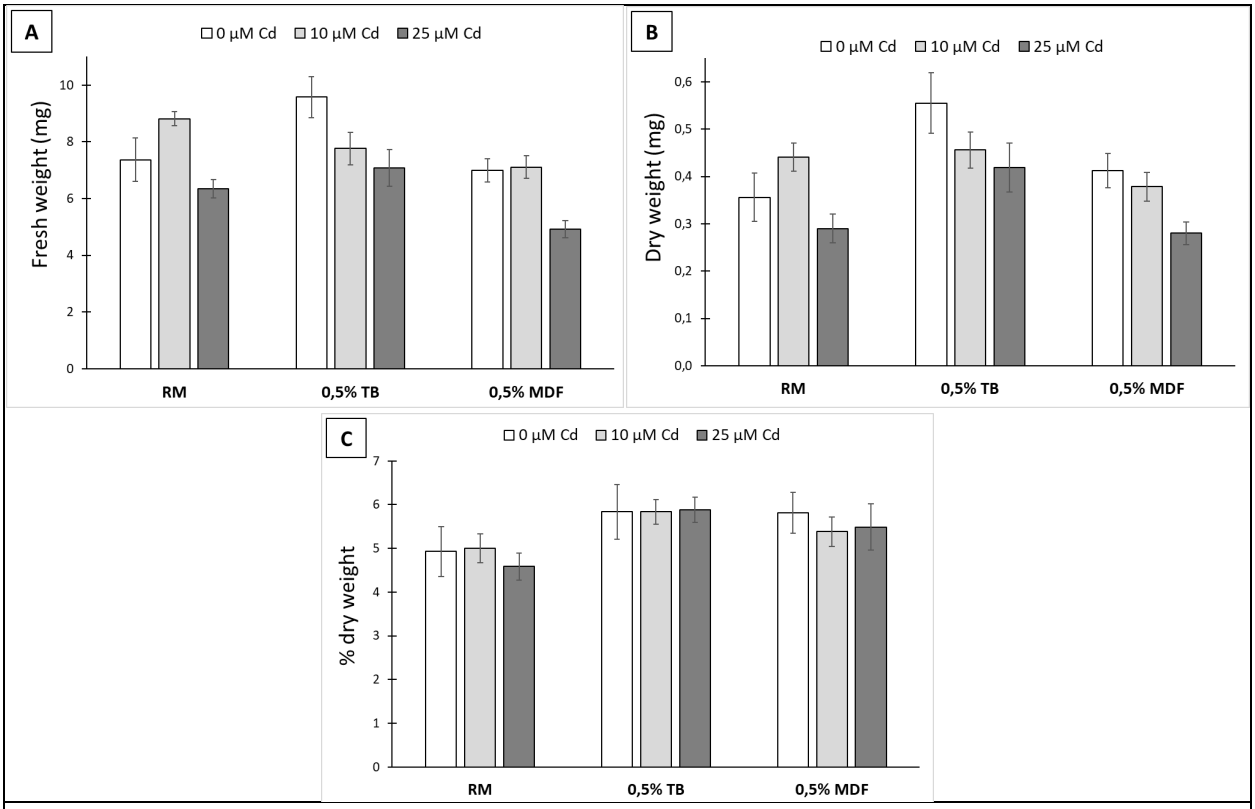

Fig. 2 – Effect of Cd and/or biochar on fresh and dry weights of seedlings. Seven-day-old seedlings were transferred to treatment plates with reference medium (RM), 0,5% TB or MDF biochar in combination with 0 µM, 10 µM or 25 µM $CdSO_4$. **(A)** Fresh weights of seedlings after being transferred to treatment plates for seven days, **(B)** percentage of dry weight compared to fresh weight. Values represent the mean ± SE of six biological replicates containing ±50 mg seedlings per sample.

the RM control. However, the presence of 25 µM $CdSO_4$ reduced FW markedly in RM, TB and MDF compared with the control and 10 µM $CdSO_4$ treatment. Overall, FWs of TB conditions were higher than RM and MDF conditions. The same trends were observed when comparing DW of seedlings grown under RM, TB and MDF conditions (**Fig. 2B**). When comparing the DW percentages, we observed that the %DW of biochar conditions were between 17% and 28% higher than RM conditions (**Fig. 2C**).

Cadmium concentrations – To evaluate whether seedlings accumulated Cd and if the addition of biochar could reduce the Cd concentration, seedlings were harvested for ICP-OES analysis after one week on treatment plates containing RM, TB or MDF combined with 0, 10 and 25 µM $CdSO_4$. We observed a significant accumulation of Cd concentration in seedlings grown on RM with 10 µM and 25 µM $CdSO_4$ compared to the control, which showed no Cd accumulation (**Fig. 3**). Seedlings grown on RM 25 µM $CdSO_4$ accumulated 73% more Cd (827 ± 85 mg/kg DW) in comparison with seedlings grown on RM 10 µM $CdSO_4$ (478 ± 34 mg/kg DW). In RM, TB and MDF-grown seedlings, the Cd concentration was highest-level after exposure to 25 µM $CdSO_4$. However, the Cd concentration in seedlings grown on TB with 25 µM $CdSO_4$ was four times lower (209 ± 13 mg/kg DW) than in seedlings grown on RM 25 µM $CdSO_4$. A similar three-fold reduction in Cd concentration was observed for seedlings grown on TB 25 µM (304 ± 47 mg/kg DW) compared to RM 25 µM (**Fig. 3**). This observation indicates that the addition of biochar reduces the Cd accumulation in seedlings. Other measured element concentrations can be consulted in the supplementals (**Supplementary Table S2**).

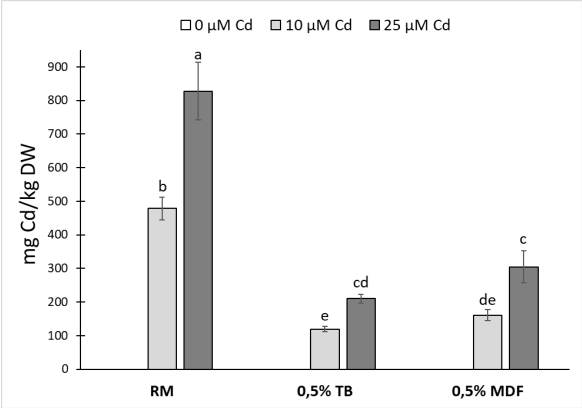

Fig. 3 – Cadmium concentrations, expressed on a dry weight basis, in *A. thaliana* seedlings grown for seven days on RM, TB or MDF agar plates combined with 0, 10 or 25 µM $CdSO_4$. Data are expressed as means ± SE of six biological replicates per condition. Statistical significance was defined at $P < 0.05$ determined using analysis of variance (Two-way ANOVA). Different letters indicate significant differences between conditions.

Gene expression analysis – To gain more insight into the molecular influence of biochar and Cd on the root system, gene expression levels of genes involved in metal transport, cell differentiation, oxidative stress, ethylene biosynthesis and glutathione metabolism were analyzed in roots of seedlings grown on RM and TB treatment plates with 0, 10, 25 and 60 µM $CdSO_4$ for seven days (**Fig. 4A-H**). Due to a new batch of MDF biochar and subsequent reduced growth of seedlings grown on MDF, MDF conditions were not taken into account. The expression level of each gene at RM 0 µM $CdSO_4$ was used as the internal control to demonstrate whether the gene expression had been induced or suppressed.

Plants do not encode specific transporter proteins for Cd^{2+} ion transport into roots nor the translocation to the shoot. However, the physicochemical similarities between Cd and Fe, Zn and Cu ions allow Cd to enter root cells and its subsequent translocation through the xylem by metal transporters like zinc-regulated transporter protein 2 (*ZIP2*) and heavy metal ATPase 2 (*HMA2*). In our experiment, the expression of *ZIP2* remained stable in both RM and TB conditions, although TB conditions showed slightly lower expression levels (**Fig. 4A**). The expression of *HMA2* in RM grown roots was upregulated in a Cd dose-dependent manner (**Fig. 4B**). Moreover, exposure to 60 µM $CdSO_4$ resulted in a four-fold increase in root *HMA2* transcript levels compared to the control. A similar trend was observed in the *HMA2* transcript levels of roots grown on TB biochar, with a more than five-fold increase in the 60 µM $CdSO_4$ exposure group. These data suggest an increased Cd efflux from the roots to shoot with increasing Cd concentrations.

The CAPRICE-LIKE MYB 3 (*CPL3*) gene encodes for a MYB-related protein. These proteins are involved in various processes, including control of cellular morphogenesis, circadian rhythm and secondary metabolism. Furthermore, they play a role in several of abiotic stress responses. In RM conditions, a Cd-dependent decrease of *CPL3* expression was observed, in contrast to the expression levels in the TB 10 µM and 25 µM $CdSO_4$ conditions, which showed a 1,5-fold increase compared to the control (**Fig. 4C**). Yet, in the TB 60 µM $CdSO_4$ condition, *CPL3* transcript levels decreased significantly to a similar level as RM 60 µM $CdSO_4$.

We investigated the Cd-induced effects on the antioxidant GSH metabolism by analyzing the expression of genes encoding for enzymes that are involved in GSH biosynthesis (*GSH1*) and the recycling of GSSG (oxidized form of GSH) to its reduced form GSH (*GR1*) (**Fig. 4D-E**). In RM conditions, the expression of glutamate-cysteine ligase (*GSH1*), which encodes for the initial enzyme in GSH biosynthesis, was downregulated in a Cd-dose-dependent manner (**Fig. 4D**). A similar Cd-dependent downward trend was observed for the expression of *GSH1* in TB conditions, although expression levels were slightly higher than in RM conditions. The expression of glutathione reductase 1 (*GR1*) in RM conditions increased with increasing Cd concentrations, with the highest expression level at 60 µM $CdSO_4$ being two-fold higher than the control (**Fig. 4E**). In contrast, *GR1* expression remained stable in the TB 0, 10 and 25 µM $CdSO_4$ conditions. Nevertheless, a 1.37-fold

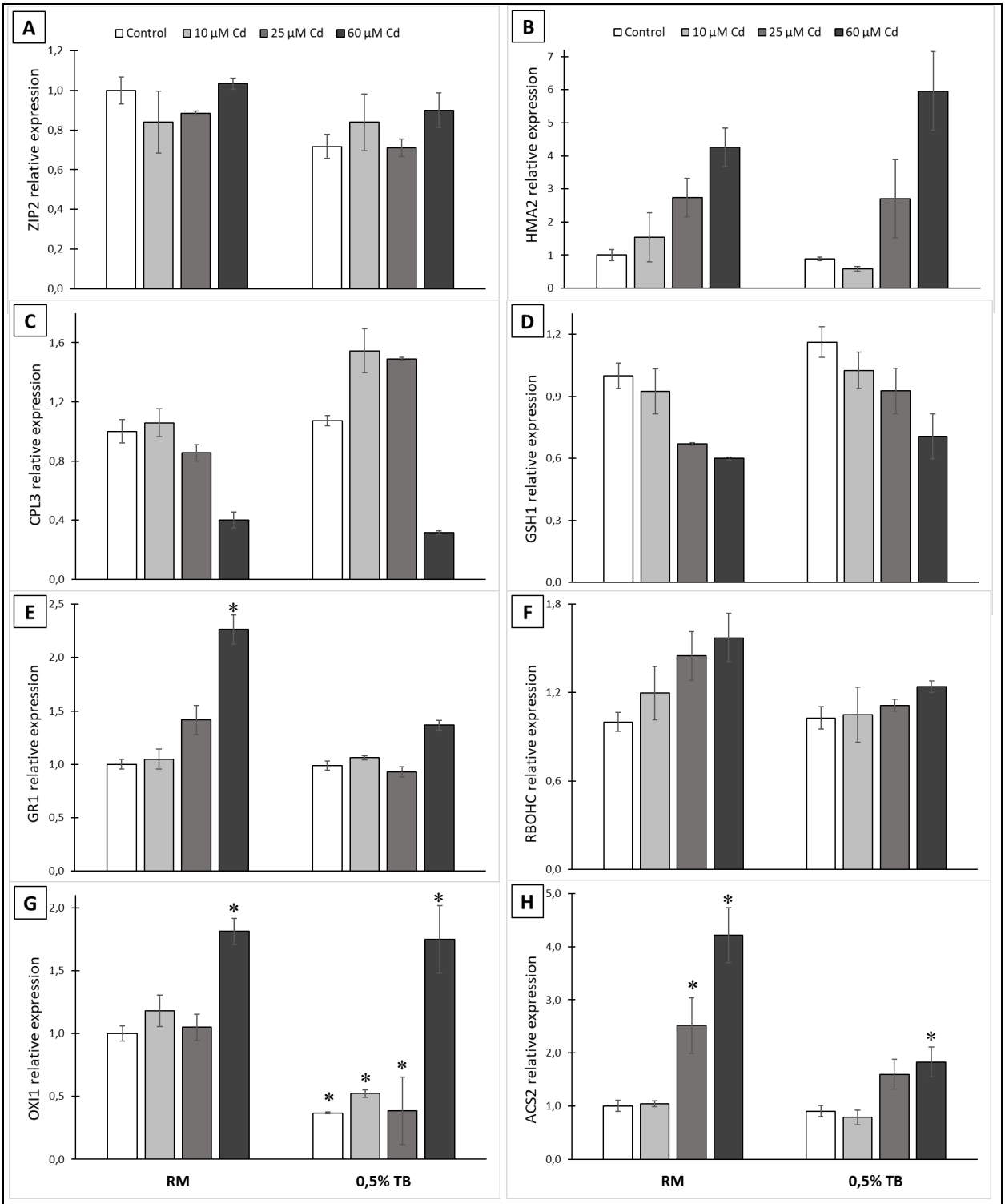

Fig. 4 – Comparison of the relative expression of genes involved in metal transport (*ZIP2, HMA2*), cell differentiation (*CPL3*), glutathione metabolism (*GSH1, GR1*), ROS (*RBOHC, OXI1*) and ethylene biosynthesis (*ACS2*) in roots of seedlings grown on TB biochar with 0, 10 or 25 µM $CdSO_4$ or grown on RM with 0, 10, 25, 60 µM $CdSO_4$ in a VAP cultivation system. Data are given as the mean ± SE relative expression of four biological replicates relative to the RM control. Statistical significance was defined at $P < 0.05$ determined using analysis of variance (Two-way ANOVA) or non-parametrical Kruskal-Wallis. (*) indicates significant differences ($P<0.05$) compared to the RM control.

increase was observed in TB 60 µM $CdSO_4$ compared to the control. These results suggest that less GSSG needs to be reduced back to GSH when biochar is applied with Cd compared to when seedlings are exposed to Cd alone.

The respiratory burst oxidase homologue C (*RBOHC*) gene encodes for a Cd-induced NADPH oxidase that plays an important role in ROS production. The *RBOHC* transcript levels in RM conditions were upregulated in a Cd-dependent manner, with expression being highest in roots of seedlings exposed to 60 µM $CdSO_4$ (**Fig. 4F**). Similarly, the expression of *RBOHC* in TB conditions showed an increasing trend depending on the Cd concentration. However, this increase was to a minor extent compared to the RM plants, indicating that the addition of TB biochar lowered *RBOHC* expression in roots. Oxidative signal-inducible kinase 1 (*OXI1*) is plays a crucial role in Cd-induced ROS detection by activating the MPK3-6 pathway. In RM 10 and 25 µM $CdSO_4$ conditions, *OXI1* expression remained stable compared to the control (**Fig. 4G**). Exposure to RM 60 µM $CdSO_4$ upregulated *OXI* expression significantly, showing a 1.8-fold increase. When we compare TB conditions to RM conditions, a similar trend was visible, although the expression of *OXI1* in TB 0, 10 and 25 µM $CdSO_4$ was approximately 50% lower than RM 0, 10 and 25 µM $CdSO_4$, indicating impaired signal transduction in these TB conditions. Nevertheless, roots of seedlings grown on TB 60 µM $CdSO_4$ showed a significant increase in *OXI1* expression similar to the expression in RM 60 µM $CdSO_4$.

In ethylene biosynthesis, ACC synthesis is the rate-limiting step. ACC synthase 2 (*ACS2*) is one of the enzymes responsible for the formation of ACC. Transcript levels of *ACS2* increased in a Cd dose-dependent manner in RM conditions (**Fig. 4H**). In RM 60 µM Cd, *ACS2* expression was upregulated four-fold compared to the control. Transcript levels were significantly lower in all TB conditions compared to the same Cd conditions in RM, and the Cd dose-dependent increase was less pronounced. These results suggest that the addition of biochar reduces ethylene biosynthesis in the presence of Cd.

DISCUSSION

Understanding the influence of biochar in combination with contaminants on root growth could yield useful information for improving plant growth and yield. The present study aimed to gain more insight into the phenotypic and molecular influence of woody biochar on the RSA of *A. thaliana* exposed to Cd.

Our results suggest that Cd inhibits primary and lateral root growth and induces an increase in ROS and ethylene in *A. thaliana* roots. Furthermore, the application of woody biochar has beneficial effects on the root system of *A. thaliana* by alleviating Cd stress. However, it is not yet clear whether biochar alone has a beneficial effect on the RSA phenotype. We showed that the addition of biochar resulted in shorter lateral roots and the formation of dense root hairs. Furthermore, TB and MDF biochar combined with Cd resulted in remarkable lower Cd accumulations compared to seedlings exposed to Cd without biochar. The addition of TB biochar to Cd-exposed roots resulted in significantly lower expression levels of genes involved in ethylene biosynthesis, glutathione metabolism and oxidative stress compared to expression levels in Cd-exposed roots without biochar.

Influence of Cd on RSA – We observed a Cd dose-dependent decrease in PRL and LRL followed by an increase in LRD (**Fig. 1A-C**). Additionally, Cd concentrations in seedlings increased with increasing Cd exposure (**Fig. 3**). These results reflect the toxic effect of increasing Cd concentrations in seedlings resulting in reduced elongation of primary and lateral roots. The increasing LRD could indicate a SIMR where plants increase the root surface area by the formation of lateral roots or root hairs as a stress avoidance mechanism [32, 49]. Similar results were observed by multiple studies that investigated the influence of Cd exposure on the RSA of *A. thaliana* grown on VAPs [30, 33, 50]. However, Remans *et al.* (2012) observed increasing LRLs and numbers of LRs when seedlings were grown on split agar plates with Cd-contaminated and Cd-noncontaminated zones, indicating avoidance of Cd and colonization of noncontaminated areas when plants have access to less/non-contaminated areas [33]. Our gene expression results suggest that high exposure to Cd leads to enhanced ROS production,

changes in the GSH metabolism, and increased ethylene biosynthesis (**Fig. 4D-H**). Glutathione (GSH) is an essential compound in the plants' defense against Cd as it can chelate Cd^{2+} ions and reduce hydrogen peroxide (H_2O_2), resulting in glutathione disulfide (GSSG). Furthermore, GSH is the precursor of phytochelatins (PCs), which have a higher affinity for Cd. We observed a Cd-dose-dependent decrease in *GSH1* expression levels (**Fig. 4D**). In contrast to our observation, a Cd dose-dependent increase of *GSH1* and *GSH2* in roots after 72h has been previously reported in hydroponically grown *A. thaliana* [51]. On the contrary, Schellingen *et al.* (2015) also observed a decrease in *GSH1* expression, but a significant increase in *GSH2* expression in *A. thaliana* leaves exposed to 5 µM Cd in hydroponically grown plants [52]. A possible explanation for the difference in observation is the different time points when measurements were taken or the use of different cultivation systems. Additionally, there is a difference between responses in different cultivation systems. However, we hypothesize that *GSH2* levels are also upregulated in VAP cultivation after exposure to Cd since GSH biosynthesis usually increases to cope with elevating Cd stress [53]. The Cd dose-dependent increase in expression of *GR1* (**Fig. 4E**) could indicate an increase in the recycling process of GSSG back to GSH to maintain GSH levels in order to respond to Cd stress. A similar Cd dose-dependent increase in *GR1* expression in roots was observed by Jozefczak *et al.* (2014), who investigated the differential GSH response of hydroponically grown *A. thaliana* leaves and roots to Cd [51]. We observed a Cd-dependent increase in expression levels of *RBOHC* (**Fig 4F**). The NADPH oxidase enzyme (*RBOHC*) is involved in Cd stress and the consequent production of ROS [54]. This could indicate that ROS levels in the roots increase with increasing Cd exposure. Furthermore, Oxidative signal-inducible kinase 1 (*OXI1*) expression increased markedly in roots exposed to 60 µM Cd (**Fig. 4G**). *OXI1* plays a key role in Cd-induced ROS detection by activating the mitogen-activated protein kinases (MAPKs) MPK3/6, which induce the transcriptional activity of *ACS2* and *ACS6* [52, 55, 56]. We observed a Cd-induced increase in expression of *ACS2* (**Fig. 4H**), which is responsible for the rate-limiting step in the biosynthesis of ethylene, suggesting that ethylene production in roots is increased upon high Cd exposure. Similar results have been previously reported in leaves of 3-week-old *A. thaliana* plants exposed to 5 µM $CdSO_4$ in a hydroponic culture [52]. Our findings support the working model proposed by Schellingen *et al.* (2015) that explains the early link between ethylene biosynthesis, signal transduction and oxidative stress in *A. thaliana* exposed to Cd (**Supplementary Fig. S2**) [52]. Nevertheless, additional studies need to confirm this model in roots grown on VAPs.

Influence of biochar on RSA – To our surprise, PRLs of seedlings grown on agar amended with biochar and Cd did not differ from PRLs of seedlings grown on RM with Cd (**Fig. 1A**). This indicates that although the addition of biochar reduces the Cd concentration taken up by seedlings significantly (**Fig. 3**), inhibition of PR growth remained similar. These results are in contrast to observations of other studies that found that the application of biochar resulted in increased PRLs [41, 43]. Effects of biochar depend greatly on the pyrolysis temperature, pyrolysis duration, the type of biomass used and the biochar percentage, meaning that differences in biochar can induce different effects on the RSA. Furthermore, different cultivation techniques could result in different outcomes. Additionally, chemical differences between agar plates with and without biochar could alter root traits. Since the medium with biochar gets autoclaved at high temperatures, chemical reactions could take place that release toxic substances or change the pH. A thorough chemical examination of the media without and with biochar and Cd is needed to fully understand the differences between growth conditions (*e.g.* high-performance liquid chromatography (HPLC)).

Independent of the Cd concentration, the addition of biochar visibly reduced LRL compared to the RM conditions (**Fig. 1B**). Additionally, biochar induced dense root hair formation compared to the non-biochar conditions, which expressed no visible root hairs (**Supplementary Fig. S1**). A possible explanation could be that biochar offers many nutritive elements, providing the plant with enough nutrients and giving no reason to expand LRs further. Another explanation could be the inhibition of LR growth by biochar. Several factors can cause

inhibition of LR growth, for example, high ammonia levels (NH_4^+); high nitrate (NO_3^-); low K, Mn and Mg; drought or high salinity [26, 57, 58]. Furthermore, dense root hair formation is often linked to high NO_3^- levels [59, 60]. It could be that both biochar additions caused high NO_3^- concentrations, resulting in shorter LRs and dense root hair formation. In addition, we observed elevated expression of *CPL3* in the TB 0, 10 and 25 µM Cd conditions (**Fig. 4C**). The *CPL3* gene is involved in cell differentiation, and overproduction of root hairs was previously reported in *A. thaliana* seedlings overexpressing *CPL3* in VAPs [61]. The addition of both TB and MDF biochar showed to have a beneficial effect on DW percentages of whole seedlings compared to RM conditions (**Fig. 2C**). Increased DW after biochar application was observed by several other studies that investigated the influence of biochar on plant growth in Cd-exposed plants [44, 62, 63]. This increase in biomass could be due to the large number of nutritive elements that biochar offers. The gene expression results showed that the application of TB biochar combined with Cd lowered expression levels of *GR1*, *RBOHC*, *OXI1* and *ACS2* compared to the expression of these genes in RM conditions (**Fig. 4**). These data suggest that TB biochar alleviates Cd stress in roots by limiting the Cd concentration and consequently reducing the expression of genes involved in the roots' responses to Cd stress that was discussed earlier. How these gene expression changes specifically influence RSA is not yet clear. However, this could be studied in the future by using specific knock-out mutants for those genes.

CONCLUSION

We conclude that the application of woody biochar effectively reduces the bioavailable Cd in the medium of VAPs. Furthermore, the application of TB biochar alleviates Cd-induced stress by limiting the bioavailable Cd in the medium, resulting in lower expression of genes involved in the Cd induced stress response. Despite the reduced Cd concentrations in seedlings, it was unexpected that no increased root growth was observed. Nevertheless, biochar application resulted in reduced LRL and an increase in root hair formation, probably indicating that although the addition of biochar reduced Cd bioavailability and, thus, indirectly, the corresponding Cd-induced stress, there is still an environmental stressor affecting the RSA. In the present study, only one biochar concentration was used. However, differences in biochar concentration can induce differential responses. This should be investigated further in a follow-up study. Overall, this study reveals that the use of VAPs is an effective way of studying the influence of biochar on the RSA of plants exposed to Cd. Our findings can bring us one step closer to the eventual implementation of biochar in contaminated soils. Hence, additional research is necessary to further optimize this system and unravel what aspects of the biochar cause differences in RSA and which genes are responsible for those differences. Interesting experiments for follow-up studies could be: Determining the composition of the media with/without biochar and with/without Cd to investigate nutrient concentrations and possible toxic compounds by, for example, HPLC analysis. Additionally, a more extensive gene expression (*e.g. ACS6, MPK3/6, GSH2, AUXIN*) analysis could be performed at multiple time points combined with the use of several knock-out mutants to unravel specific processes involved in the biochar-Cd-root interactions.

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Author contributions – Wim Kuypers and Stéphanie Vandionant conceived and designed the research. Wim Kuypers performed experiments and data analysis, under guidance of Stéphanie Vandionant. Wim Kuypers wrote the paper, and Stéphanie Vandionant carefully edited the manuscript.

Supplementary data

Table S1 – **Reference genes**

Gene
ACT2
UBC21
PPR
MON1
TIP-41-like
YSL8
Fbox
RHIP1
UBQ10
EF1α

Ten reference genes that were measured and used as input for the GrayNorm algorithm. Genes that are marked in yellow were used for the calculations of the normalization factor.

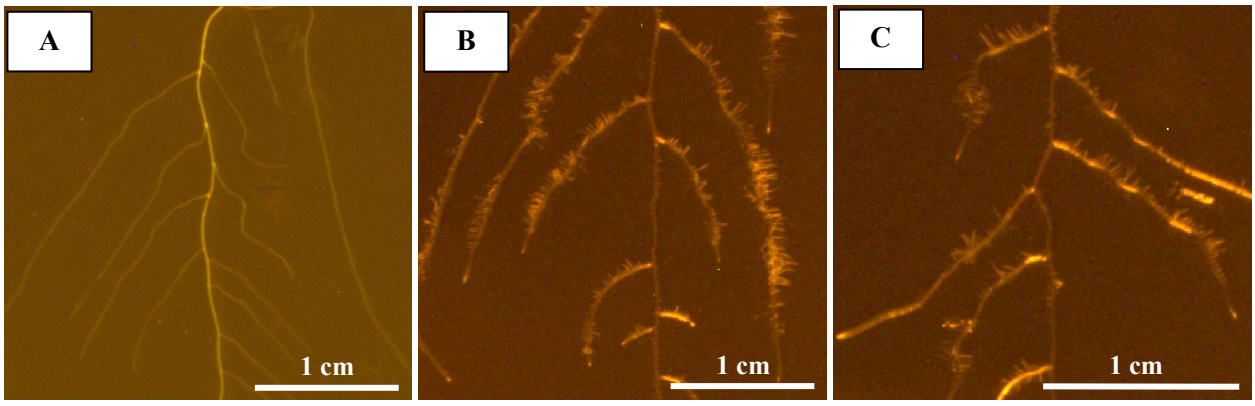

Fig. S1 – Part of the RSA of seedlings grown on **(A)** RM 10 µM Cd, **(B)** TB 10 µM Cd and **(C)** MDF 10 µM Cd.

Table S2 – Element content of *Arabidopsis thaliana* seedlings exposed to Cd and or biochar

		Element content (mg/kg DW)							
	µM $CdSO_4$	Cd	Ca	Fe	K	Mg	Na	P	Zn
RM	0	0	5948	1842	48258	2515	9300	31281	302
	10	478	5375	2199	44522	2163	7535	30424	246
	25	827	5317	1107	48761	2496	8932	35874	235
TB	0	0	10289	290	36002	1927	6619	25987	121
	10	119	9183	372	33598	1746	4948	23971	129
	25	209	7515	359	32015	1676	5509	25875	94
MDF	0	0	8782	428	38559	2214	8233	31083	122
	10	160	7643	117	39864	2092	7400	32378	133
	25	304	6025	419	41904	2213	7122	35535	138

Seven-day-old *A. thaliana* seedlings were transferred to agar plates with RM, 0,5% TB biochar or 0,5% MDF biochar in combination with 0, 10 or 25 µM $CdSO_4$ for one week. ICP-OES analysis was conducted to detect element content of Cd, Ca, Fe, K, Mg, Na, P and Zn. Element contents are shown as the mean mg/kg dry weight.

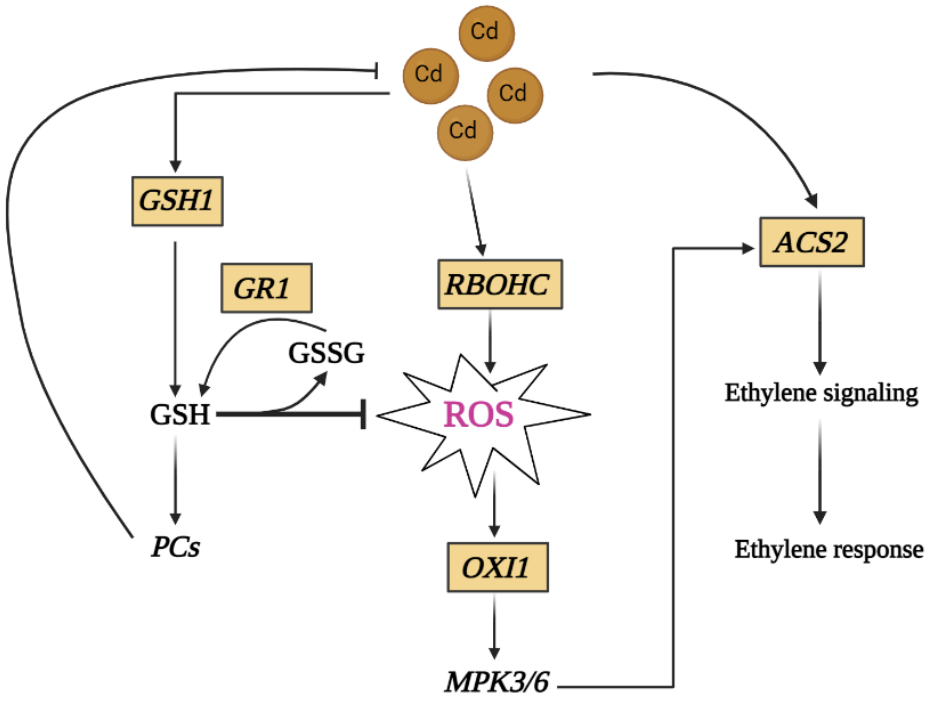

Fig. S2 – Working model proposed by Schellingen *et al.* (2015) that explains the early link between ethylene biosynthesis, signal transduction and oxidative stress in *A. thaliana* exposed to Cd, applied on the gene expression analysis of the present study. Yellow boxes indicate genes that were measured in the present study.