Environmental and Experimental Botany 155 (2018) 185–194

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

How cadmium affects the fitness and the glucosinolate content of oilseed rape plantlets

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ARTICLE INFO

Keywords:
Cadmium
Oilseed rape
Glucosinolates
Sulfur allocation
Hormesis

ABSTRACT

Secondary metabolites such as glucosinolates (GSLs) are involved in plant response to biotic stress but can be significantly influenced by abiotic factors as well. Oilseed rape (*Brassica napus* L.) produces large quantities of several GSLs both in seeds and at the vegetative stage. These sulfur-containing compounds are known to play an important role in cadmium stress tolerance within the Brassicaceae family probably due to specific cross-talk between the S primary and secondary metabolism. Sulfur assimilation is in the middle of multiple metabolic pathways including Cd stress responses at physiological level. Our research focused on the assessment of GSL profiles and content in the roots and shoots of 28-day-old winter oilseed rape plantlets. The study was conducted under *in vitro* sterile conditions using concentration gradients of 0, 5, 15 and 45 µM of cadmium. A phenotypic analysis was carried out at the end of this experiment in order to evaluate the plantlets' fitness. Our results described hormetic growth curves for root elongation, root biomass and shoot biomass at Cd concentrations of 5 µM and 15 µM respectively. Our experiment shows that a concentration of 5 µM can be considered as non-toxic, while one of 45 µM represents a lethal dose. Strong relationships were found between Cd accumulated in roots or translocated to shoots and the total sulfur accumulation in the plantlets' different organs. A decrease of both indole and aliphatic GSL content associated with an increase of Cd accumulation and an increase of total sulfur accumulation was observed in the roots and shoots of the plantlets. It was also further demonstrated that Cd stress has a highly significant effect on roots' and shoots' GSL content bringing new insights into GSL's possible role in the priming of Cd stress.

1. Introduction

Cadmium is a widespread toxic trace metal with an average soil concentration of 0.3 mg kg^{-1} in Europe (Six and Smolders, 2014). The geochemical occurrence of cadmium typically reaches the 0.1–1.0 mg kg^{-1} range. In addition, Cd can be released into the environment by the metallurgic industry, waste incinerators and urban traffic, contributing to Cd accumulation in soils (Smolders and Mertens, 2013). It can become a risk for human health as a class 1 human carcinogen if consumed and for the environment if critical levels of 1 mg kg^{-1} in soils are exceeded (Tóth et al., 2016). The contamination of agricultural soils is mainly due to phosphate amendments and surveys of dynamic cadmium balances in EU arable soils are conducted to assess limits of P-fertiliser application (SCHER, 2015).

Cd uptake in the plant kingdom occurs in the rhizosphere solution and through transmembrane carriers also engaged in the assimilation of divalent cations such as Ca^{2+}, Fe^{2+}, Mg^{2+}, Cu^{2+} and Zn^{2+} (Gallego et al., 2012). Cd^{2+} accumulation can dramatically interfere with the most important physiological processes such as photosynthesis and respiration (He et al., 2017). Cd tends to accumulate in *Brassica napus* L. plants with a decreasing general trend from roots to leaves, to fruits and to seeds but its capacity to be translocated to shoots can be high depending on the genotype (Clemens, 2006; Benáková et al., 2017). This long-distance transport and distribution of metals to different cells and tissues is related to key transporters mediating Cd tolerance (Mendoza-Cózatl et al., 2011). Leaf Cd concentrations exceeding 5–10 µg g^{-1} are toxic to most plants (Lux et al., 2011). Therefore, plants have evolved mechanisms to restrict Cd delivery to the xylem such as i) the chelation process of Cd by phytochelatins in the cytoplasm with accumulation of Cd-phytochelatin complexes in the vacuole (symplasmic pathway) and ii) physical barriers to block Cd extracellular movement (apoplasmic pathway). Toxicity is partly due to the production of reactive oxygen species (ROS) such as superoxide radical, hydrogen peroxide and hydroxyl radical. Antioxidant processes through enzymatic responses such as superoxide dismutase (EC 1.15.1.1.), glutathione reductase (EC 1.8.1.10) and catalase (EC 1.11.1.6) and non-enzymatic responses such

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https://doi.org/10.1016/j.envexpbot.2018.06.008
Received 27 February 2018; Received in revised form 15 May 2018; Accepted 8 June 2018
Available online 30 June 2018

as glutathione, vitamins, flavonoids and alkaloids represent the major plant ROS-scavenging mechanisms (Azevedo et al., 2012). The detoxification of Cd is thus a complex phenomenon under polygenic control, which after long-term exposure sometimes leads to real plant tolerance of Cd (Sanità di Toppi and Gabbrielli, 1999).

During the last decade, winter oilseed rape has become a prominant oilseed crop thanks to considerable yields of 35 kg ha^{-1} $year^{-1}$ to 45 kg ha^{-1} $year^{-1}$ depending on genetic, environmental and agronomic factors (Stahl et al., 2017). The allopolyploid *Brassica napus* L., resulting from the hybridization between *B. rapa* and *B. oleracea,* is well known for its capacity to tolerate heavy metals and especially cadmium. This crop with an important biomass, a deep taproot and huge lateral root system is often mentioned as a potential candidate for cadmium phytoextraction. According to Grispen et al. (2006), a screening of 77 accessions of *B. napus* L. revealed that intraspecific natural variation in Cd accumulation can be used to phytoextract Cd from moderately contaminated soils (2.5 to 5.5 mg kg^{-1}). Carrier et al. (2003), even described a Cd translocation factor of 2.5 in comparison with soil Cd concentration. Some cultivars are able to tolerate and accumulate high Cd concentrations in shoots (Ben Ghnaya et al., 2009).

This species is thus one of the most tolerant to Cd of the Brassicaceae family. Yet the physiological and molecular mechanisms responsible for this tolerance are still poorly understood. Recent research has focused on the important role of antioxidant enzymes and the ascorbate-gluthation cycle (Wu et al., 2015b) or on the post-transcriptional level, with novel targets for microRNAs involved in plant response to Cd (Zhou et al., 2012). Another role still under discussion in Cd detoxification is the importance of sulfur-rich secondary metabolites such as β-thioglucoside-*N*-hydroxysulfates. These compounds known as glucosinolates (GSLs) and their myrosinase-catalysed hydrolysis products (isothiocyanates, thiocyanates, nitriles, goitrin and epithionitriles) have a physiological significance in plant response to different biotic and abiotic stresses (del Carmen Martínez-Ballesta et al., 2013). GSLs composition and content depend on the genotype, the climate and the cultivation conditions (Lee et al., 2014). The classification is based on the structure of different amino acid precursors, and three classes are defined: aliphatic, indole and aromatic GSLs (Fahey et al., 2001). Aliphatic are derived from alanine, leucine, isoleucine, valine and methionine; indole and aromatic are derived from tryptophan and phenylalanine or tyrosine respectively (Ishida et al., 2014).

Oilseed rape is known as a crop with a very high sulfur demand (Brunel-Muguet et al., 2015), and sulfate assimilation plays a key role in coping with Cd excess in *Brassica* spp. plants (Gill and Tuteja, 2011). After the reduction of sulfur, the primary sulfate assimilation into cysteine serves for biosynthesis of methionine as a precursor of GSLs and gluthatione (GSH) (Babula et al., 2012). On the other hand, as GSH is the principal low-molecular-weight thiol in most cells, it is regulated by sulfur supply and is the precursor of phytochelatin synthesis in response to Cd (Noctor et al., 2012). Moreover, the redox buffer GSH protects cellular compartments against ROS and operates also as a component in detoxification mechanisms which are based on glutathione S-transferase (GST; EC 2.5.1.18) (Rausch and Wachter, 2005). Cross-talk between primary and secondary sulfur metabolism certainly occurs in Cd tolerance response, and is worth investigating in more detail in species such as *Brassica napus* L. (Fig. 1). Sulfur-rich defence compounds (SDCs) including GSL may represent up to 30% of the total sulfur content of plant organs and can also serve as a potential source of sulfur for other metabolic pathways (Falk et al., 2007; Variyar et al., 2014). GSL profiles of cultivated *Brassica* spp. are subject to evolution, and the natural selection is not just due to the "mustard oil bomb" system appearing after tissue disruption (Agerbirk and Olsen, 2012). Questions clearly remain about Cd's impact on GSL composition and their possible roles in Cd stress tolerance within the Brassicaceae family. For example, cadmium has been associated with changes in GSL content in white cabbage through the stimulation of GSL biosynthesis (Kusznierewicz et al., 2012). Pongrac et al. (2010) highlighted that Cd

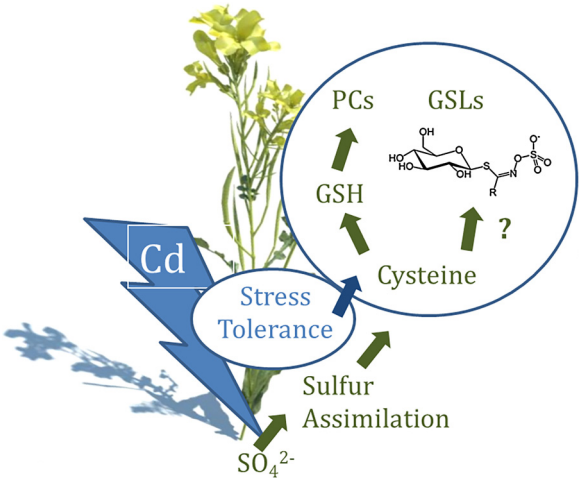

Fig. 1. Schematic view of the putative role of glucosinolates (GSLs) in Cd stress tolerance for oilseed rape crop. (GSH: glutathione, PCs: phytochelatines).

hyperaccumulation can influence GSL profile and content differently from simple Cd tolerance. A recent field study showed that cabbage and kale had a similar response to Cd exposure, possibly indicating a unique feature for the regulation of GSL content (Jakovljević et al., 2013).

Therefore we decided to investigate the GSL profile and content in the roots and shoots of 28-day-old winter oilseed rape plantlets under *in vitro* sterile conditions, and using concentration gradients of 0, 5, 15 and 45 µM of cadmium stress. At the same time, we also studied the Cd accumulated in roots and translocated to shoots in comparison with the total sulfur accumulation. The morphological development (symptoms, growth and biomass) of the *Brassica napus* L. plantlets was also analysed at the end of the experiment. The major aim of this research was to throw light on the putative role of glucosinolates in cadmium stress response under controlled and defined conditions.

2. Material and methods

2.1. Plant material and growth conditions

Shoots of winter oilseed rape, *Brassica napus* L. var. *Es Astrid* (Euralis semences, France), grown from germinated certified seeds were propagated *in vitro* using genetically very stable axillary branching proliferation. The voucher specimens (n°0312) are held at the Walloon Agricultural Research Centre (CRA-W, Belgium). Two standardised nodal segments 2.5 cm long with three primary leaves were cultivated in a home-made glass chamber system and a culture medium previously described (Durenne et al., 2018). In parallel to a control, cadmium abiotic stresses ($CdCl_2$) (Sigma-Aldrich, Diegem, Belgium) were applied to the culture media at 5 µM, at 15 µM and at 45 µM with a threefold factor. Each Cd condition including the control and the different concentrations of Cd tested were undertaken in triplicate. The set-up of culture medium homeostasis consisted of well-balanced Ca^{2+} concentration using any plant-growth regulator because i) a high amount of Ca^{2+} can alleviate Cd stress symptoms by competing with Cd uptake in the rhizosphere and can strengthen the photosynthesis organelles of *Brassica napus* L. plants during Cd exposure (Wan et al., 2011) and ii) plant-growth regulators are known to be beneficial for the metabolism of Cd-stressed plants, by regulating the antioxidative defence system, osmolytes production, Cd uptake and the activation of stress tolerance genes (Asgher et al., 2015). Twenty-eight days-old plantlets were obtained after 14 days of a hermetic *in vitro* preliminary rooting phase and 14 days of acclimation growth of shoots under sterile culture conditions. The oilseed rape plantlets were so cultivated at 23/18 °C (day/night), with a photoperiod of 16 h, 45% relative humidity and 100 µmol m^{-2} s^{-1} of photosynthetically active radiation. This experimental set-

up was duplicated five times consecutively using propagated shoots in order to obtain two plantlets per container each time: one for cadmium and sulfur determination and the other one for the assessment of GSL content.

2.2. Evaluation of growth and biomass for roots and shoots

At the end of the experiment, the roots of the two 28-day-old oilseed rape plantlets of each container were carefully immersed in tap water to remove culture medium, rinsed twice with distilled water and wiped with tissues. For phenotyping, leaf symptoms were observed and were pictured using a camera DSC-HX50™ (Sony, Belgium). The lengths of the shoot and the largest root of each plantlet were measured and recorded (mm). Each sample was kept in a glass vials, and air-dried at 70 °C for 72 h in an HL80® drying oven (Memmert, Schwabach, Germany). The dried plant materials were finally weighed (g) (DW) using a digital lab scale balance analytical AE166 Delta Range® (Mettler, Zaventem, Belgium) and stored in the dark at 2 °C until analysis.

2.3. Root and shoot glucosinolates content

Glucosinolates were extracted from dried shoots and roots and analysed according to the ISO 9167-1:1992 method. Briefly, the dried plant material was homogenised and extracted using an ethanol-water mixture (50:50) at 74 °C for 12 min in a 5 ml tube. The internal standard of pure sinigrin (10 µmol L^{-1}) (Sigma-Aldrich, Diegem, Belgium) was directly added to all samples. One millilitre of root and shoot extracts was passed through ion-exchange resin DEAE Sephadex® A-25 (Sigma-Aldrich, Diegem, Belgium) for enzymatic desulfation with purified sulfatase (EC 3.1.6.1.), type H-1, sulfatase ≥10,000 units/g solid from *Helix pomatia* (Sigma-Aldrich, Diegem, Belgium). HPLC analysis of the desulfoglucosinolates (DSGSLs) was performed using an Agilent HP1200 apparatus (Agilent Technologies Inc., Santa Clara, CA, USA) on an RP-18 Inertsil® ODS-3 column 3 µm, 100 x 3 mm (GL Sciences Inc., Eindhoven, the Netherlands). A two-solvent system consisting of water (A) and acetonitrile 20% in water (B) was used with a gradient elution mode (20:80 to 80:20). The column was eluted during 30 min at 30 °C with an eluent flow of 1 ml min^{-1} and with a UV-Diode Array Detection at 229 nm. The identification of DSGSLs was performed by comparison of their retention times with pure references and whenever possible confirmed by recording their UV spectra. Each desulfoglucosinolate was quantified (µmol g^{-1} DW) by comparison with the internal standard peak area and corrected with relative response factors according to the ISO 9167-1:1992 method.

2.4. Cadmium and sulfur determination

Samples of dried shoots and roots were pooled in order to obtain approximately 0.5 g and 0.05 g of material respectively. Sub-samples were mineralised using a mixture of 30% H_2O_2 (1 mL), 65% HNO_3 (6 mL) and H_2O in a closed high-pressure microwave system Milestone® MLS1200 Mega (Gemini BV, Apeldoorn, Netherlands). After dilution, the samples were analysed for Cd and total S content (mg g^{-1}) using an ICP-AES Ultima® spectrometer (Horiba Jobin Yvon, Edison, NJ, USA). The selected wavelengths were 228.802 nm for Cd and 180.676 nm for sulfur. The extraction and analysis methods were validated using a white cabbage powder BCR®-679 (TechLab, Metz, France) provided as a certified reference material by the European Commission (Geel, Belgium).

2.5. Statistical analysis

All statistical analyses were carried out with Minitab® package version 17 and all data sets were tested for normality and homoscedasticity. The results of phenotypic analysis (growth and biomass) were tested using one-way analysis of variance (ANOVA). This analysis was followed by a *post hoc* Tukey's range test to find significant differences among pairwise means at 0.05 level of probability. The results of Cd and sulfur accumulation were analysed using one-way ANOVA and Pearson's correlation coefficient. The glucosinolate content values (µmol g^{-1} DW) are reported as means with standard error (± SE). These data were also analysed using one-way ANOVA and ranged using Tukey's HSD test at 0.05, 0.01 and 0.001 levels of probability.

3. Results

3.1. Morphological analysis and effect of Cd on growth and biomass in oilseed rape plantlets

Symptoms were then directly observed in order to compare the morphological development of each *Brassica napus* L. plantlet after the 28 days of *in vitro* growth for the control (0 µM) and under the different Cd concentrations tested (5, 15 and 45 µM). All the plantlets from the control medium showed a perfect development and interestingly, no leaf chlorosis symptoms were observed for all plantlets stressed with Cd at 5µM. In contrast, cadmium stress at 15 µM and at 45 µM affected leave morphology of all plantlets with sporadic outbreaks of chlorosis and with visible growth retardation corroborated with obvious symptoms of significant pigment loss respectively (Fig. 2). In view of these observations, Cd at 5 µM could be considered non-toxic and Cd at 45 µM as a lethal dose for oilseed rape plantlets in our experimental conditions. The phenotyping results corresponded to the mean and the 95% confidence intervals obtained at the end of the experiment under the cadmium concentration gradient. One-way ANOVA showed that Cd had a significant effect on the 28-day-old plantlets' root growth ($F(3,111) = 18.85$, $P < 0.001$) and biomass ($F(3,111) = 10.69$, $P < 0.001$), as confirmed by the differences between the means obtained after *post hoc* Tukey's range test. A typical inverted U-shaped dose-response curve may be extrapolated for root growth (Fig. 3a) and for root biomass (Fig. 3b). Moreover, Cd at 45 µM generated a drop of 20.5% in comparison with the mean of results of unstressed root growth. One-way ANOVA also revealed that Cd had a significant effect on the 28-day-old plantlets' shoot growth ($F(3,111) = 134.77$, $P < 0.001$) and biomass ($F(3,111) = 34.06$, $P < 0.001$), as confirmed by the differences between the means. The results for shoots of the 28-day-old plantlets showed by extrapolation a typical toxic dose-response curve at Cd concentrations higher than 5 µM for the growth analysis (Fig. 3c) and simultaneously, an inverted U-shaped dose-response curve for shoot biomass (Fig. 3d). Finally, Cd at 45 µM generated a drop of 31.6% and 34.4% in comparison with the mean of results of unstressed shoot growth and shoot biomass respectively.

3.2. Cadmium and sulfur accumulation in roots and shoots

The concentrations of cadmium and total sulfur content in shoots and roots of the 28-day-old oilseed rape plantlets were assessed. As expected, an accumulation depending on the Cd concentration in the culture medium was observed for Cd accumulation in roots ($F(3,19) = 68.03$, $P < 0.001$) and for Cd translocation in shoots ($F(3,19) = 162.33$, $P < 0.001$). This can be observed in the graph of the boxplots showing the mean, median, outliers and 25^{th} and 75^{th} percentiles of the Cd accumulation in roots (Fig. 4a) and of Cd translocated in shoots (Fig. 4b). Cd accumulation for the different Cd stress conditions of 5, 15 and 45 µM averaged 1.26, 2.42 and 3.03 mg g^{-1} in roots and 0.09, 0.22 and 0.54 mg g^{-1} in shoots respectively. Cd concentration in the culture medium also had an effect on total sulfur accumulation in roots ($F(3,19) = 28.38$, $P < 0.001$) and in shoots ($F(3,19) = 6.04$, $P < 0.01$). In addition, a linear relationship between total sulfur accumulation and the different Cd concentrations tested was found in the plantlets' roots, as it can be observed with boxplots of total sulfur accumulation (Fig. 4c). For the shoots of the plantlets, the

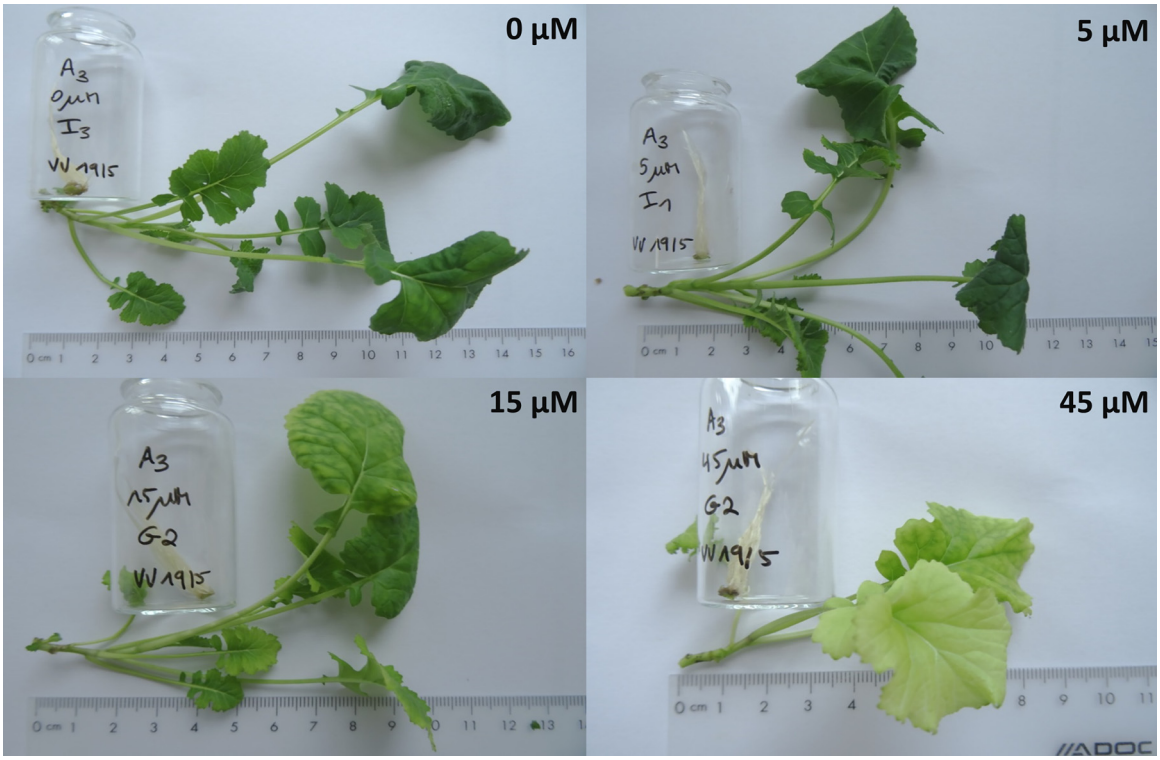

Fig. 2. Pictures of 28-day-old oilseed rape plantlets under 0, 5, 15 and 45 µM Cd stress conditions. As can be observed, no symptoms and perfect development were obtained at 0 and 5 µM of Cd. Sporadic chlorosis appeared on plantlet leaves at 15 µM and severe pigment loss with growth retardation were obtained at 45 µM of Cd stress.

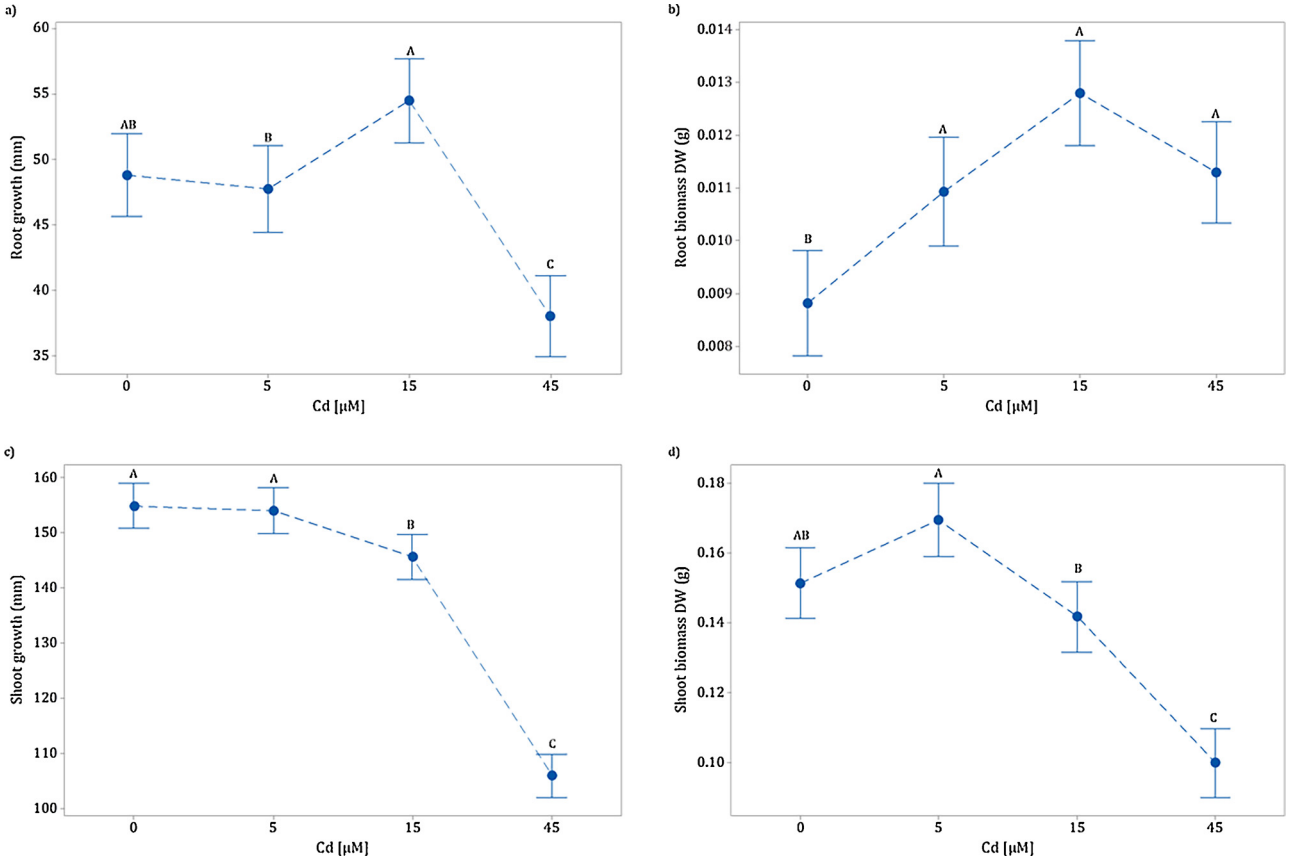

Fig. 3. Graphs of the means and 95% confidence intervals of a) root growth (mm), b) root biomass (DW) (g), c) shoot growth (mm) and d) shoot biomass (DW) (g) for 28-day-old oilseed rape plantlets under the cadmium concentration gradient of 0, 5, 15 and 45 µM (n = 28, 26, 28, 30). Data were ranged after a *post hoc* Tukey's test.

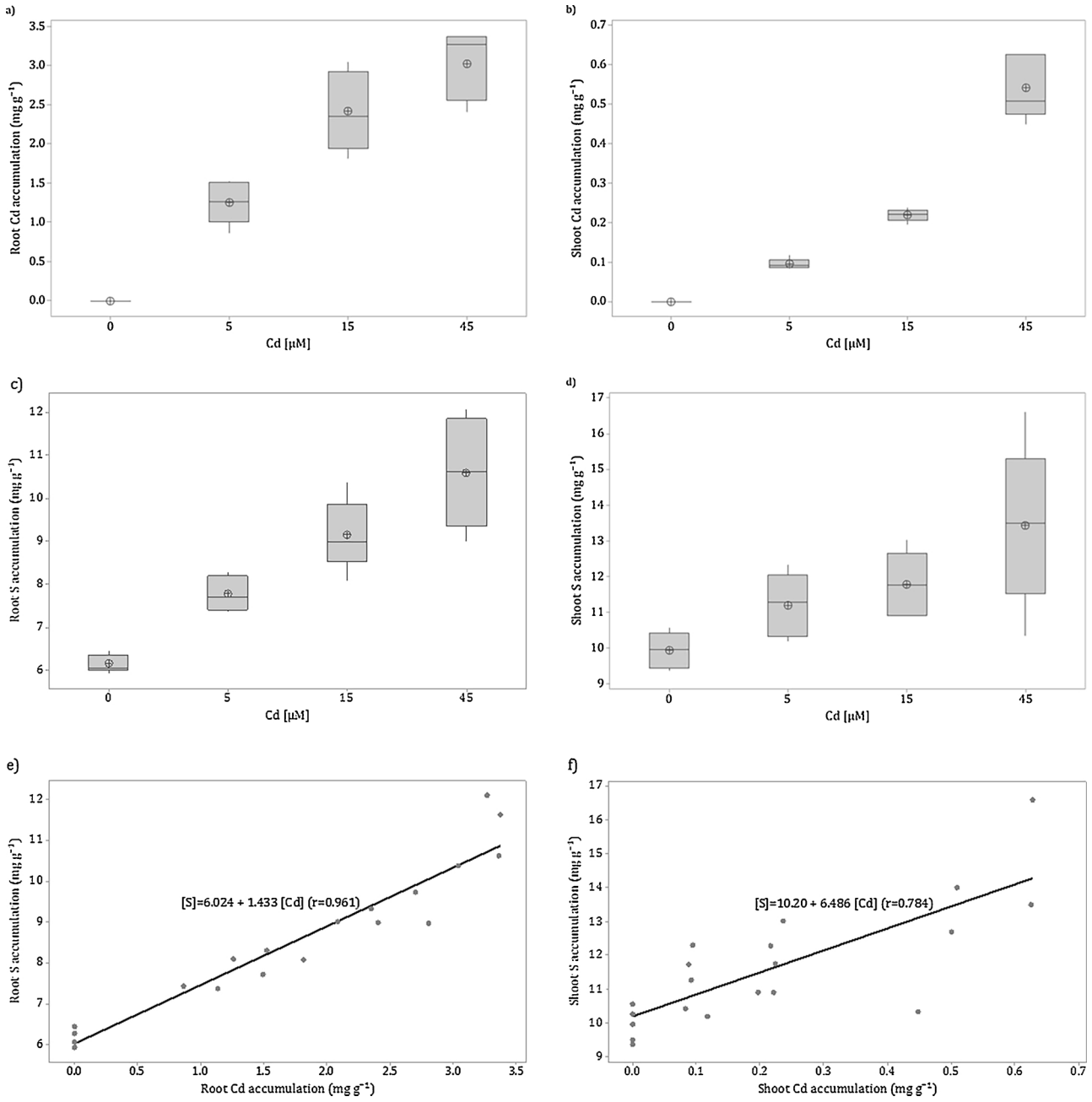

Fig. 4. Boxplots (mean (⊕), median (line), 25th and 75th percentiles and representing outliers) of a) Cd accumulation (mg g^{-1}) in roots, b) Cd translocation in shoots, c) sulfur accumulation (mg g^{-1}) in roots and d) sulfur accumulation (mg g^{-1}) in shoots (n = 5) and correlation between Cd accumulation and total sulfur accumulation (mg g^{-1}) in e) roots and f) shoots for 28-day-old oilseed rape plantlets under the cadmium concentration gradient of 0, 5, 15 and 45 µM.

boxplots of total sulfur accumulation showed more variability, but with an upward trend related to elevated Cd concentrations and particularly at 45 µM (Fig. 4d). The total sulfur accumulation for the different conditions of 0, 5, 15 and 45 µM of Cd averaged 6.16, 7.78, 9.15 and 10.60 mg g^{-1} in roots and 9.94, 11.20, 11.78 and 13.43 mg g^{-1} in shoots respectively. Strong relationships between Cd and total sulfur accumulations were observed in both roots (Fig. 4e) and shoots (Fig. 4f) with Pearson's correlation coefficients of 0.961 and 0.784 respectively. Finally, Cd accumulation was clearly restricted to plantlets' roots as it can be observed with the percentages representing the Cd proportion in roots in comparison with the total Cd accumulation in plantlets (Fig. 5).

3.3. Assessment of GSL profile and content in roots and shoots of oilseed rape plantlets

Three indole glucosinolates derived from tryptophan as glucobrassicin (3-indolylmethyl, I3M), as 4-methoxyglucobrassicin (4-methoxy-3-indolylmethyl, 4MOI3M) and as neoglucobrassicin (n-methoxy-3-indolylmethyl, 1MOI3M) were found in the roots and shoots of the 28-day-old oilseed rape plantlets from the GSL profiling analysis. In addition, four other GSLs were found only in shoots: one indole as 4-hydroxyglucobrassicin (4-hydroxy-3-indolylmethyl, 4OHI3M) and three aliphatic as progoitrin (2-hydroxy-3-butenyl, 2OH3But), as gluconapin (3-butenyl, 3But) and as glucobrassicanapin (4-pentenyl, 4Pent). A typical example of a root and a shoot chromatogram showing the GSLs separated by HPLC for unstressed plantlet is given below in Fig. 6 a) and b) respectively. The observation of the graphs of the GSL

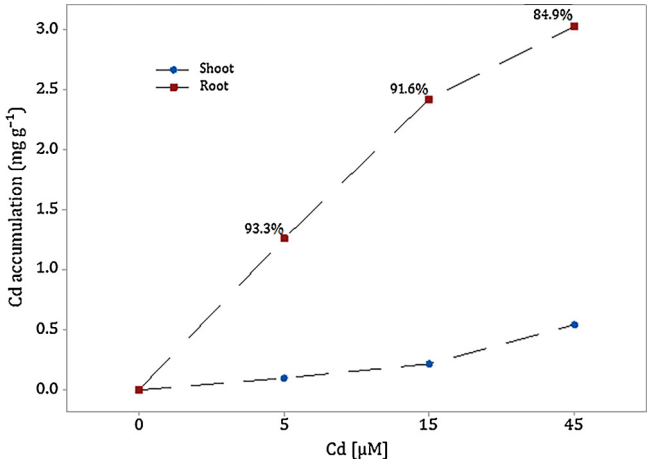

Fig. 5. Graph of the mean values of Cd accumulated in roots and translocated to shoots (mg g^{-1}) of 28-day-old oilseed rape plantlets under the cadmium concentration gradient of 0, 5, 15 and 45 µM (n = 5). The percentages represent the Cd proportion in roots in comparison with the total Cd accumulation in plantlets.

profiling for roots (Fig. 7a) and for shoots (Fig. 7b) showed that the mean value (µmol g^{-1} DW) of each GSL varied according to Cd concentration in the culture medium. One-way ANOVA confirmed that Cd concentrations had a significant effect on GSL content in roots and in shoots in all cases except for 1MOI3M in roots, as can be observed in Table 1. According to Tukey's HSD test, the mean values were ranged and a clear trend was observed corresponding to a general decrease of GSL content with a dose-dependent pattern of Cd concentrations. The most abundant GSL in roots was 4MOI3M with maxima of 4.26, 4.47, 5.01 and 3.69 µmol g^{-1} DW, while the most abundant GSL in shoots was I3M with maxima of 11.48, 11.55, 9.95 and 4.17 µmol g^{-1} DW at 0, 5, 15 and 45 µM of Cd stress respectively. Finally, as it can be observed (Fig. 8), strong relationships were found between I3M and 1MOI3M contents in roots under the cadmium concentrations of 0, 5, 15 and 45 µM.

4. Discussion

4.1. Morphological effect of Cd stress on oilseed rape plantlets

The cadmium toxicity in plants generally causes leaf roll, chlorosis and growth reduction both in roots and in shoots. A decrease of total chlorophyll content and carotenoid content and an intensive triggering of non-photochemical quenching have also been described in *Brassica napus* L. plants (Sanità di Toppi and Gabbrielli, 1999). Cd exposure in the rhizosphere can lead to root browning, as described in many plants, and to reduction in root growth (Benáková et al., 2017; He et al., 2017). The inhibition of root elongation, being one of the earliest symptoms of Cd toxicity, has been used as a morphological stress indicator (Lux et al., 2011). We described in our experiment a clear drop in root elongation at a concentration of 45 µM of Cd (Fig. 3a). The same observation was also made for shoot growth (Fig. 3c), and this decrease of elongation was clearly associated with visible symptoms of typical whole leaf chlorosis at 45 µM (Fig. 2). Cadmium at 45 µM can be considered as a lethal dose for oilseed rape in these experimental conditions because the plantlet's growth is completely inhibited and the plant is not able to complete its life cycle. In view of the results for shoot growth in comparison with those of the control (0 µM), a cadmium concentration of 5 µM can be described as a tolerant dose, as no toxic symptoms were found in morphological development at this concentration.

The observations and results at 5 µM of Cd suggest the phenomenon of plant hormesis. This can be defined as a stimulatory effect of toxic agents at low concentrations *versus* an inhibitory effect at higher doses. Hormetic effects are currently being investigated in connection with toxicology dose-response. In the plant kingdom, dose-response curves of hormetic ions such as Cd, Cr, Al and Pb are frequently observed showing typical inverted U-shaped curve at low concentrations. Many parameters such as time and concentration of exposure influence plant fitness, indicating biological plasticity through an adaptive response to stress (Poschenrieder et al., 2013). Many questions remain about the possible role of the huge structural diversity of secondary metabolites involved in the reduction of ROS as an important component in hormetic effects (Hadacek et al., 2011). For example, in *Lemna trisulca* L. (Duckweed), different adaptive mechanisms involved in response to low and high doses of Cd have been found with a drastic increase of total soluble thiols (Malec et al., 2010). The hyperaccumulator *Lonicera japonica* Thunb. showed a typical biphasic hormetic response after a Cd exposure of 10 mg L^{-1} for 28 days in terms of plant growth, leaf water and photosynthetic pigment content (Jia et al., 2013). These hormesis effects on growth and photosynthetic performance have been confirmed for the same species through a soil experiment and after 90 days of exposure (Jia et al., 2015). Another recent study demonstrated a differential response in a duality of GSH-related chelating and antioxidant capacities of *Arabidopsis* leaves and roots exposed to several Cd concentrations (Jozefczak et al., 2014).

However, we observed these typical inverted U-shaped dose-response curves for root growth (Fig. 3a), for root and shoot biomass (Fig. 3b and d) in our 28-day-old oilseed rape plantlets experiment under the concentration gradient of cadmium of 0, 5, 15 and 45 µM. A biphasic response curve of root elongation is likely to be due to ionic interactions at the root surface (Poschenrieder et al., 2013). The cations present in the culture medium decreased the external membrane surface potential in the priming of Cd stress at 5 µM, thus diminishing Cd uptake by the roots. We also observed maximum root elongation at 15 µM, and Cd accumulation grew steadily until the very toxic dose of 45 µM. Lux et al. (2011) brilliantly demonstrated that roots are able to grow in less Cd-contaminated patches. We also know that cadmium greatly influences root system architecture, such as greater root diameter with increased parenchyma cell size and enlarged cortical tissues (He et al., 2017). Moreover, endodermal suberisation and lignification of cell walls in roots can represent additional barriers by reducing the entry of Cd to the xylem and protecting shoots from excessive Cd loads. The results for root biomass at 15 µM (Fig. 3b) corroborated this finding, maybe suggesting an accelerated maturation of cells at this high Cd stress level. It is clear that in our experiment, the tolerant dose for roots may be as high as 15 µM of Cd: after this concentration, the toxicity probably can no longer be alleviated by the whole plantlet. We think that the root system played its full part in plantlet tolerance, by restricting Cd translocation to the shoots.

4.2. Cd accumulation in roots and translocation to shoots

Tolerant plants are often excluders, limiting root-to-shoot translocation and accumulating toxic ions in their roots, mainly with deposition in the cell walls (Gallego et al., 2012). Cd uptake in the roots in such cases can be up to 10 times higher than when translocated in the shoot, depending greatly on the rhizosphere Cd concentration (Lux et al., 2011). As previously described, *Brassica napus* L. can be considered as one of the most tolerant species to Cd, with intraspecific natural variation in Cd accumulation and translocation (Ben Ghnaya et al., 2009). Some studies have also mentioned oilseed rape crops' very high threshold of foliar concentration of Cd, especially after long-term growth in soil conditions and in co-cropping systems (Carrier et al., 2003; Selvam and Wong, 2009). Some cultivars seem to accumulate a high amount of Cd in shoots through mechanisms still poorly understood, although xylem transport and specific gene expression certainly play crucial roles (Wu et al., 2015a). A more recent study has shown a similar subcellular distribution in soluble fraction in roots of two

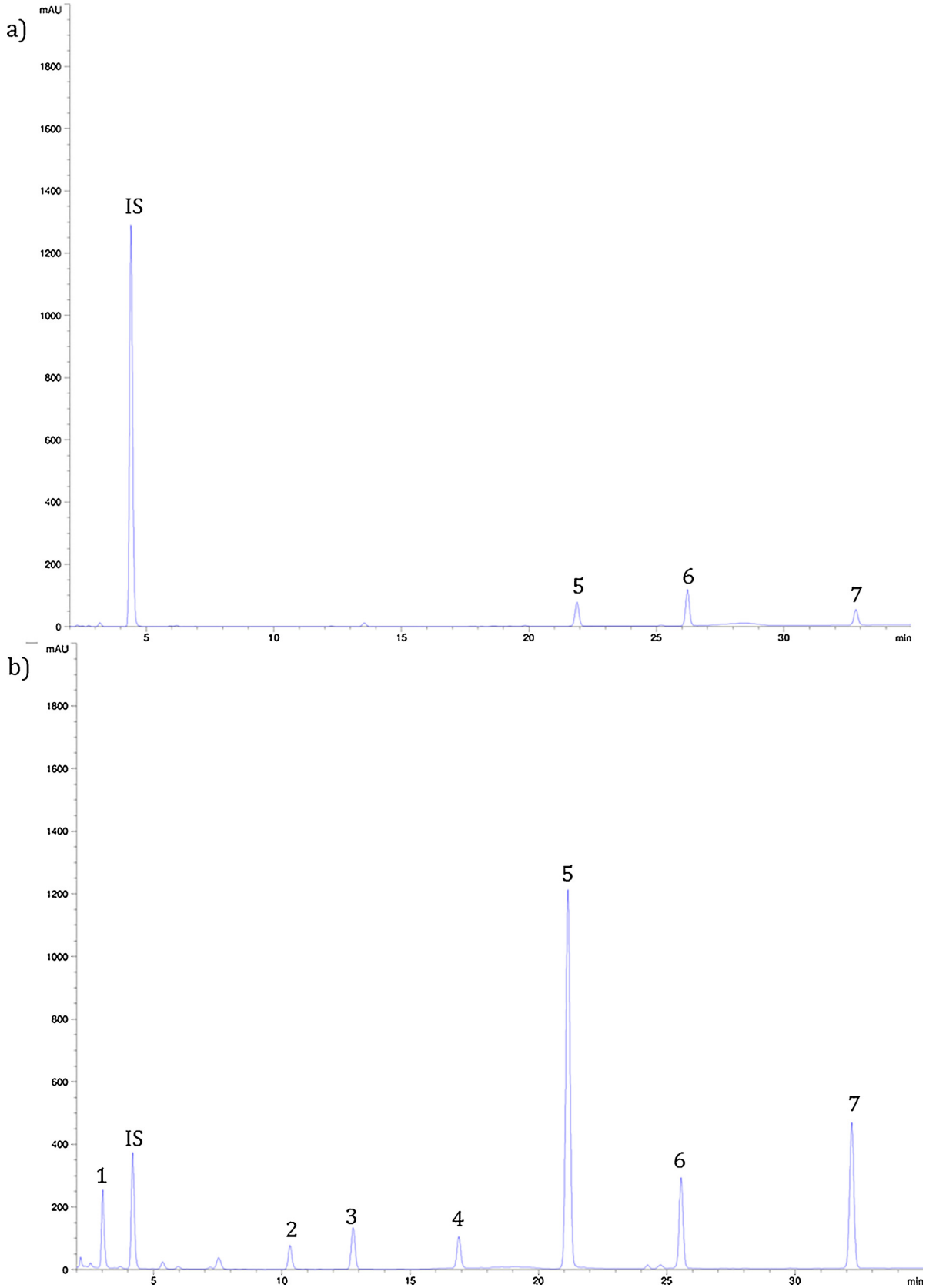

Fig. 6. Typical chromatograms of the GSL profile obtained from unstressed 28-day-old oilseed rape plantlets for a) roots and b) shoots. IS: internal standard (sinigrin), 1: progoitrin (2OH3But), 2: gluconapin (3But), 3: 4-hydroxyglucobrassicin (4OHI3M), 4: glucobrassicanapin (4Pent), 5: glucobrassicin (I3M), 6: 4-methoxyglucobrassicin (4MOI3M), 7: neoglucobrassicin (1MOI3M).

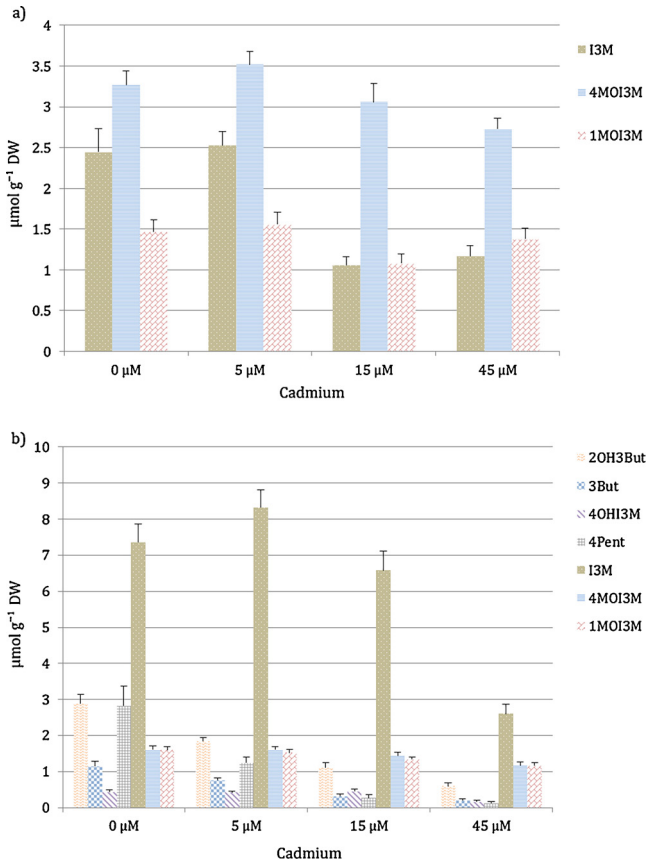

Fig. 7. Graph of the GSL profile and content (mean values ± SE) (µmol g^{-1} DW) for a) roots and b) shoots of the 28-day-old oilseed rape plantlets under the cadmium concentration gradient of 0, 5, 15 and 45 µM. (2OH3But: progoitrin, 3But: gluconapin, 4OHI3M: 4-hydroxyglucobrassicin, 4Pent: glucobrassicanapin, I3M: glucobrassicin, 4MOI3M: 4-methoxyglucobrassicin, 1MOI3M: neoglucobrassicin).

cultivars of *Brassica napus* L. plants differing in their metal tolerance, at two Cd concentrations (50 and 200 µM) (Mwamba et al., 2016). In the present study and during the time of exposure of 28 days, our oilseed rape plantlets tended to accumulate 10 times more cadmium in their roots than in their shoots (Fig. 5). This observation illustrates the high Cd stress tolerance of this variety up to a maximum of 15 µM as previously demonstrated. At 45 µM, oilseed rape plantlets showed typical Cd stress symptoms and were evidently not viable. The root system accumulated Cd as much as possible, with a dose-dependent trend described by a typical extrapolated sigmoid curve (Fig. 4a). Conversely, the translocation of Cd to shoots was hugely reduced, described by an extrapolated exponential curve (Fig. 4b). This extrapolated curve suggests that the failure of the protective root role certainly occurred between 15 µM and 45 µM of Cd. In most Cd-tolerant plants, cadmium can be accumulated to levels above 0.01% of shoot dry weight without causing toxicity symptoms (Verbruggen et al., 2009; Gallego et al., 2012). A leaf concentration of 0.06 mg g^{-1} of Cd has been reported for oilseed rape cultivars without any toxicity symptoms for the plants (Mwamba et al., 2016). Using the mean values of shoot biomass and Cd accumulation in shoots at 5 µM and 15 µM of Cd, we found a Cd accumulation average of 0.09 mg g^{-1} and 0.219 mg g^{-1} corresponding to a level of 0.009% and 0.022% respectively. These percentages confirm the very high tolerance of this variety in our experimental conditions.

4.3. Relationship between Cd and total sulfur accumulations

It was recently suggested that ethylene plays an important role in S-induced alleviation of Cd stress on photosynthesis in mustard (Masood et al., 2012). For oilseed rape, Cd at 100 mg kg^{-1} in contaminated soil has been described as responsible for a noticeable accumulation of phytochelatins in a short-term evaluation of 22 days (Carrier et al., 2003). A recent study described a significant increase of abscisic acid, a well-known stress-related phytohormone, and an increase of enzymatic antioxidant activity as physiological mechanisms involved in Cd tolerance (Yan et al., 2015). Finally, higher Cd tolerance seems to be due to enhancement of the glutathione-ascorbate cycle in three-week-old oilseed rape seedlings (Wu et al., 2015b).

It is clear that high tolerance to Cd stress is a very complex mechanism in which sulfur is involved both in plant growth recovery and in Cd detoxification. It has been demonstrated using white mustard plants (*Sinapis alba* L.) that intensive S nutrition can enhance tolerance to Cd stress (Matraszek et al., 2016). We know that sulfate uptake and assimilation are the basis of a platform for the biosynthesis of sulfur-containing defence compounds (SDCs) such as phytochelatines from glutathione (GSH) and secondary plant products such as GSLs for Brassicaceae species especially. Finally, other S-rich compounds such as metallothioneins, S-amino acids and hydrogen sulfide are also described as possibly involved in heavy metal stress response (Rausch and Wachter, 2005; Capaldi et al., 2015). Our results indicating a pronounced increase of total sulfur accumulation relative to a concentration gradient of Cd (0, 5, 15 and 45 µM) showed a net dose-dependent relationship. In the literature, the concentration of total sulfur in plant tissues has been reported as 0.5 to 1.5% of the plant dry weight (Falk et al., 2007). In our experiment, we found 1.61% and 1.89% at 0 and

Table 1
Effect of Cd concentrations (0, 5, 15 and 45 µM) on GSL content (µmol g^{-1} DW) in roots and shoots of 28-day-old oilseed rape plantlets. Mean values (± SE) ranged with Tukey's test.

	Cd test		GSL content (µmol g^{-1} DW)						
	[µM]	n =	I3M ***	4MOI3M *	1MOI3M nd	2OH3But	3But	4OHI3M	4Pent
Roots	0	14	2.45 ± 0.28 **A**	3.27 ± 0.17 **AB**	1.47 ± 0.14	/	/	/	/
	5	13	2.53 ± 0.17 **A**	3.52 ± 0.16 **A**	1.56 ± 0.15	/	/	/	/
	15	12	1.06 ± 0.10 **B**	3.06 ± 0.23 **AB**	1.08 ± 0.11	/	/	/	/
	45	15	1.17 ± 0.13 **B**	2.73 ± 0.13 **B**	1.38 ± 0.13	/	/	/	/
			***	**	**	***	***	**	***
Shoots	0	14	7.36 ± 0.50 **AB**	1.61 ± 0.10 **A**	1.60 ± 0.10 **A**	2.88 ± 0.26 **A**	1.15 ± 0.14 **A**	0.43 ± 0.06 **A**	2.82 ± 0.54 **A**
	5	14	8.33 ± 0.49 **A**	1.61 ± 0.09 **A**	1.52 ± 0.10 **A**	1.84 ± 0.11 **B**	0.76 ± 0.06 **B**	0.42 ± 0.04 **A**	1.24 ± 0.16 **B**
	15	13	6.58 ± 0.54 **B**	1.44 ± 0.10 **AB**	1.34 ± 0.07 **AB**	1.12 ± 0.12 **C**	0.32 ± 0.06 **C**	0.45 ± 0.07 **A**	0.27 ± 0.08 **B**
	45	15	2.60 ± 0.27 **C**	1.17 ± 0.09 **B**	1.16 ± 0.08 **B**	0.60 ± 0.09 **C**	0.20 ± 0.05 **C**	0.16 ± 0.05 **B**	0.12 ± 0.04 **B**

*P < 0.05, **P < 0.01, ***P < 0.001, nd (no difference), / not present.
I3M: glucobrassicin, 4MOI3M: 4-methoxyglucobrassicin, 1MOI3M: neoglucobrassicin, 2OH3But: progoitrin, 3But: gluconapin, 4OHI3M: 4-hydroxyglucobrassicin, 4Pent: glucobrassicanapin.

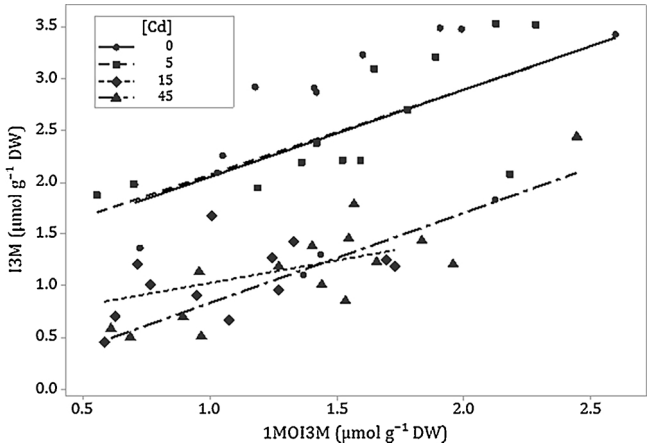

Fig. 8. Graph representing interactions between I3M, 2018 (glucobrassicin) (µmol g^{-1} DW) and 1MOI3M (neoglucobrassicin) (µmol g^{-1} DW) content in roots of 28-day-old oilseed rape plantlets under the cadmium concentration gradient of 0, 5, 15 and 45 µM.

5µM of Cd respectively, corroborating the influence of sulfur nutritional status in cadmium detoxification and tolerance. Keeping in view the possible and multiple roles of sulfur in Cd stress, the correlations that were found in roots and shoots (Fig. 4e, f) can be described as indicating the accumulation of SDCs in Brassicaceae plants in response to Cd stress. The high variability of total sulfur content in 28-day-old oilseed rape shoots at 45 µM Cd stress probably indicated huge metabolism disturbance due to the excess of Cd.

4.4. General decrease of GSL content related to the effect of Cd concentrations

GSL biosynthesis is a very complex mechanism of regulation due to its relationship with other major metabolic and signalling pathways related to plant fitness. Over 120 GSLs have been identified mainly in species belonging to the Brassicaceae. Plant organs have different GSL biosynthesis and turn-over regulation, and it is assumed that GSL content is also developmentally regulated depending on the age of the plant (Augustine and Bisht, 2016). For example, the level of cell differentiation plays a key role in the GSL profile and content in *in vitro* plant cultures (Kastell et al., 2013). Surprisingly, a recent study found no qualitative and quantitative difference for the GSL profiles and content in different embryo tissues of *Brassica napus* L. (Fang et al., 2012). The profiles of the different GSLs found in roots and shoots in our experiment were in accordance with previous published data concerning *Brassica* spp. (Fahey et al., 2001). Previous data published for a Cd hyperaccumulator, *Thlapsi praecox,* and related to severe Cd stress (50 µM), described an increase in the level of total GSL without statistically significant differences in total sulfur accumulation (Tolrà et al., 2006). Conversely, GSL content seems to exhibit a lower trend in Cd-sensitive species, given that a Cd concentration of 50 µM significantly decreased the total content of GSL in both leaves and in roots for the model plant of *Arabidopsis thaliana* (Sun et al., 2009). Metal hyperaccumulation is a specific case where plants do more than tolerate high concentrations, and possible trade-offs between glucosinolate-based organic and inorganic defences are being investigated (Pongrac et al., 2010; Kazemi-Dinan et al., 2015). A recent study clearly described how the regulation and pattern of GSL played a role in alleviating arsenic stress in two cultivars of *B. juncea* (Pandey et al., 2017). Finally, GSL content variation in young and old leaves relating to heavy metal accumulation can also influence elemental defence against chewing and sucking herbivores, suggesting a direct impact on plant fitness (Stolpe et al., 2017).

Aliphatic and indole GSLs are derived from different pathways which are regulated by specific transcription factors such as the MYB family with organ-specific expression patterns (van Dam et al., 2009). In the case of indole GSLs, CYP81 F has been identified as the gene encoding the enzymes involved in I3M oxidation to form 4OHI3M, 4MOI3M and 1MOI3M, and for aliphatic GSL, during chain elongation amino acids are elongated by introducing methylene to obtain 3But and 2OH3But with 4 carbon chain length and 4Pent with 5 carbon chain length (Sánchez-Pujante et al., 2017). We found 4MOI3M as the major compound of GSL in roots and I3M as the major compound of GSL in shoots. A strong relationship was also observed between the variations in I3M and 1MOI3M content depending on Cd concentrations tested in roots (Fig. 8).

In the roots in particular, we can suggest that sulfur uptake was increased at 15 and 45 µM of Cd stress, certainly promoting SDC biosynthesis at the cost of both I3M and 1MOI3M biosynthesis. GSL content is closely related to sulfur availability, and accounts for 2%–8% of the total sulfur accumulation in vegetative tissues (Blake-Kalff et al., 1998). Moreover, it has been suggested for some *Brassica napus* L. cultivars that aliphatic GSLs may be used as a survival strategy in case of sulfur deficiency (Yan and Chen, 2007) and that the proportion of aliphatic GSL increases more than the proportion of indole GSL when sulfur is applied to S-deficient plants (Falk et al., 2007). Finally, glucose-induced GSL accumulation in *Arabidopsis* has been found to be due to enhanced sulfur assimilation, with MYB34 and MYB51 crucial in maintaining the basal indole glucosinolate content (Miao et al., 2016). There is a direct link between indole GSL biosynthesis and the metabolism of the major auxin IAA (indol-3-acetic acid) (van Dam et al., 2009). We found that root elongation results at 15 µM of Cd were associated with a decreased level of glucobrassicin in roots. These results suggest the hypothesis that glucobrassicin is converted to auxin, a process involved in enhanced root growth under Cd stress (Jakovljević et al., 2013).

5. Conclusion

To our knowledge, this was the first time that the GSL profile and content of oilseed rape plantlets had been investigated under a concentration gradient of cadmium. We demonstrated that Cd clearly decreases GSL content in 28-day-old oilseed rape plantlets both in roots and shoots, with a dose-dependent pattern. It can be also suggested that priority is given to the use of sulfur supplies to cope with Cd stress at physiological and cellular levels, interfering with the plant defence strategy against possible biotic stresses. Further studies are needed to rule out the key role of GSL in below-ground and above-ground response to Cd tolerance in *Brassica napus* L. plants. It would be very interesting to confirm these observations using soil experimental conditions in the field.

Acknowledgements

The authors would like to thank Georges Lognay for his critical revision of the manuscript and General and Organic Chemistry of Gembloux Agro-Bio Tech for providing the equipment to carry out the liquid chromatography analysis. The research project was funded by the Walloon Agricultural Research Centre. Finally, we are also grateful to Boris Krings, Martine Leclercq, Franck Michels, Sophie Richet and Danny Trisman for their technical assistance.

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