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Targeted metabolomic study in *Brassica napus* L. under cadmium and epoxiconazole stress

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Résumé

L'objectif poursuivi par cette thèse de doctorat était la mise en évidence de marqueurs métaboliques volatils et non-volatils chez une plante modèle d'intérêt agronomique, le colza d'hiver (*Brassica napus* L.). Celle-ci a été soumise à deux stress abiotiques spécifiques représentant une menace potentielle pour les sols agricoles : le cadmium (Cd), un élément trace métallique cancérogène, et l'époxiconazole, un fongicide systémique rémanent. Une approche métabolomique ciblée a été utilisée en sélectionnant la famille des terpènes, composés organiques volatils (COVs), impliqués notamment dans les stress abiotiques, ainsi que la classe des glucosinolates, métabolites secondaires non-volatils présentant un intérêt majeur au sein des Brassicacées.

Un nouveau dispositif combinant la croissance des plantules *in vitro* et la capture de manière non-invasive des COVs a été développé avec succès. Les manipulations et analyses des plantules de colza ont été effectuées en conditions stériles et contrôlées, sur milieu gélosé dans le cas des expériences concernant le cadmium et avec un substrat composé de perlite, mimant les conditions du sol, dans le cas du stress époxiconazole. En outre, un phénotypage se basant sur des observations et des mesures physiologiques : i) symptômes caractéristiques (chloroses), ii) croissance racinaire et caulinaire et iii) biomasse a été réalisé en relation avec les différents niveaux de stress engendrés au niveau du colza au stade végétatif et en complément de l'approche métabolomique. Les teneurs en Cd et en soufre (S) ont également été déterminées dans les parties aériennes et racinaires des plantules en lien avec l'analyse des teneurs en glucosinolates afin de pouvoir comprendre l'importance de ces derniers dans l'étude de la tolérance au Cd. Concernant le stress époxiconazole, une caractérisation de la présence de la molécule au sein des plantules a précédé l'expérience visant l'analyse des terpènes et des produits de dégradation des glucosinolates tels que les isothiocyanates.

Des marqueurs métaboliques ont été mis en évidence pour les deux stress abiotiques étudiés et ce en lien avec l'intensité du stress appliqué. Parmi les COVs, les sesquiterpènes sont clairement ressortis de notre étude en tant que possibles marqueurs de stress. Dans le cas du Cd, il a également été démontré que les glucosinolates jouent un rôle dans les mécanismes de tolérance et dans le maintien du métabolisme primaire soufré.

L'analyse des profils COVs de manière non destructrice et précoce, ainsi que la quantification des terpènes au niveau du colza pourraient indéniablement servir à étudier les relations entre les COVs émis et d'autres stress abiotiques et/ou biotiques. Enfin, cette technique pourrait être facilement adaptée à d'autres plantes (pommes de terre, betteraves, cultures maraîchères) et d'autres substrats de culture, comme le sol, afin de rechercher différents marqueurs de stress potentiels.

Abstract

Investigations carried out during this thesis consisted in the research of volatile and non-volatile abiotic stress markers using winter oilseed rape (*Brassica napus* L.), a major crop worldwide. Two specific abiotic stresses involved in current agricultural soil threats were studied such as cadmium (Cd), a carcinogen trace heavy metal, and epoxiconazole being a persistent systemic fungicide. A targeted metabolomic approach was therefore used through the analysis of volatile organic compounds (VOCs) profiles, targeting terpenoid compounds largely described in plant abiotic stress response, and through the profiling of glucosinolates (GSLs) as non-volatile sulfur-containing metabolites within *Brassicaceae*.

A new glass device allowing the plantlets growth and the non-invasive dynamic sampling of emitted VOCs was successfully developed. It can be described as an innovative laboratory and high-throughput plant chambers system. Oilseed rape plantlets analysis was performed under sterile and controlled conditions, using *in vitro* medium in the case of experiments related to cadmium exposure and using perlite as a soil-similar substrate for testing epoxiconazole. A phenotyping based on plantlets observations and physiological measurements under cadmium and epoxiconazole stress: i) characteristic symptoms (chloroses), ii) root and shoot growth and iii) biomass was performed at vegetative stage, in complementarity with the targeted metabolomic approach. Different levels of stress were tested on oilseed rape plantlets through dose-response experiments in order to obtain a concentration gradient representing defined stress conditions (i.e. low, middle and severe). With respect to cadmium stress, the concentration of Cd and sulfur (S) has been also measured in the different plant organs such as roots and shoots. GSL profile and content in plantlet organs were also investigated in order to highlight their putative involvement in Cd stress tolerance. About epoxiconazole stress, a characterisation of molecule concentration in the plantlets was carried out before studying its impact on terpenes profiles and on the breakdown products of GSLs (i.e. isothiocyanates) as putative metabolic markers.

Overall results showed that metabolic markers could be identified for both kinds of stress such as cadmium and epoxiconazole. These molecules were also involved in adaptive response of plantlets to stress. The sesquiterpenes clearly emerged from the experiments as VOC stress markers. In addition, the role of GSLs in the mechanisms of Cd-tolerance was highlighted with an emphasis on importance of oilseed rape primary S metabolism.

The non-invasive method of rapid analysis of VOCs emitted by oilseed rape plantlets and terpenes quantitation could certainly be used for studying the relationships between plant-emitted VOCs and other abiotic or biotic stresses. Finally, this customised glass chambers system could be used in regards to other plants such as potatoes, sugar beets and vegetable crops using soil substrate in order to discover new putative metabolic markers.

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Je voudrais remercier tout d'abord le Centre wallon de Recherches Agronomiques (CRA-W) qui, via la mise en place d'un projet financé par la loi Moerman, m'a permis de réaliser ma thèse de doctorat au sein du département des sciences du vivant et de l'unité du génie biologique. Je tiens à remercier également les deux coordinateurs d'unité successifs qui m'ont accueilli, le Dr. Pascal Geerts et tout spécialement le Dr. Philippe Druart en tant que co-promoteur de cette thèse.

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Chapter 0

Context and objectives

Context and objectives

In 2015, the Walloon Agricultural Research Centre (CRA-W) started a research project entitled "Solindic". The main goal was to study the biological activity of the plant-soil interactions, in order to preserve agricultural soil functions, focusing on cadmium (Cd) and epoxiconazole exposures as potential threats. It was prospected to monitor the biological activity with an emphasis on specialised metabolites such as volatile organic compounds (VOCs) for plants and using metagenomic and microbiological tools for soil experiments especially.

Brassica napus L. was investigated as the model plant during this thesis. Oilseed rape is effectively a crop of global economic significance, with 72 10^6 tons recently produced worldwide in a year and from an area of 36.5 10^6 ha (Chikkaputtaiah et al., 2017). Furthermore, oilseed rape is mainly cultivated for human consumption due to its high-quality vegetable oil and as a renewable source for fuels (Brunel-Muguet et al., 2015). The residual meal can be used as protein feed for animals and there have been also recent discussions about its potential interest for human nutrition (Stahl et al., 2017). The genome of this crop plant has been widely sequenced. Extensive breeding efforts led to the development of "00" cultivars with: i) less than 2% of erucic acid (i.e. potentially cytotoxic to mammals), and ii) less than 30 µmol g^{-1} of glucosinolates concentration in seeds which can negatively affect the meal palatability (Derbyshire and Denton-Giles, 2016). Lately, metabolites profiling was essentially focused on lipidomic profiling studies for oil biosynthesis study and extensive omics datasets from seed filling stages have become available (Misra, 2016; Gupta et al., 2017).

Cd is a widespread toxic trace heavy 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 carcinogen substance and when critical levels exceed 1 mg kg^{-1} in soils (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 have been achieved to establish limits of P-fertiliser applications (SCHER, 2015). Toxicity symptoms observed in plants due to excessive amounts of Cd may result from a wide range of tolerance mechanisms at cellular level that promote physiological adaptive responses. Recently, Gallego et al. (2012) described schematic representations about the different Cd targets involved in plant metabolism to cope with Cd stress: i) antioxidant responses, ii) reactive oxygen species (ROS) detoxification or signaling to activate/repress gene expression, and iii) relationship with very important nitrogen and sulfur (S) metabolism (Fig. 0.1).

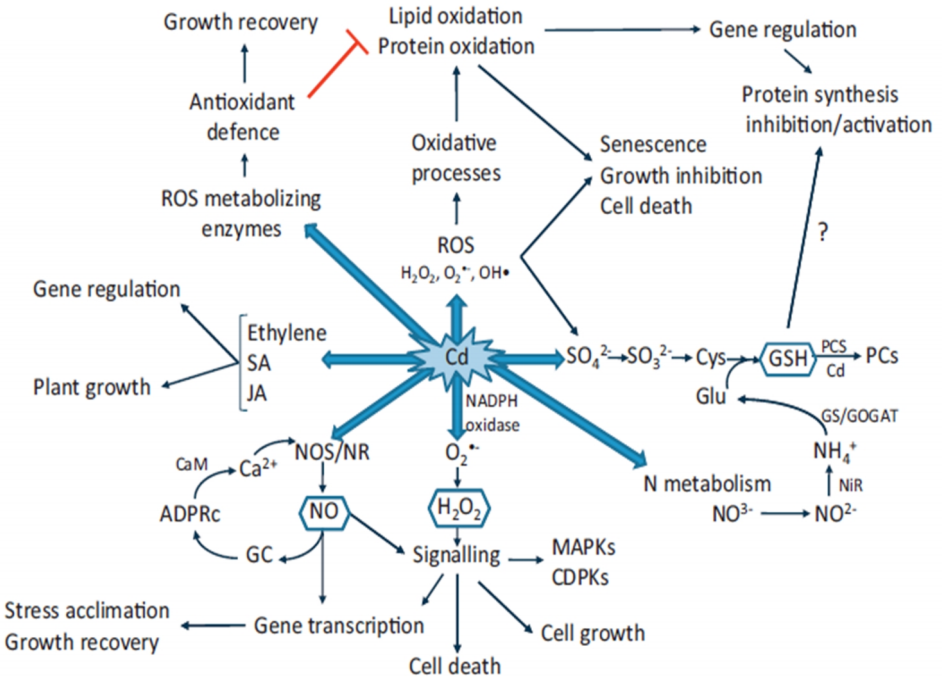

Figure 0.1: Schematic representations about the different Cd targets involved in plant metabolism to cope with Cd stress. ADPRc, adenosine diphosphate ribosyl cyclase; CaM, calmodulin; Cd, cadmium; CDPKs, calcium dependent protein kinases; Cys, cysteine; GC, guanylate cyclase; Glu, glutamate; GOGAT, glutamine oxoglutarate aminotransferase; GS, glutamine synthase; GSH, glutathione; JA, jasmonic acid; MAPKs, mitogen-activated protein kinases; NiR, nitrite reductase; NO, nitric oxide; NOS, nitric oxide synthase; NR, nitrate reductase; PCs, phytochelatin synthase; PCs, phytochelatins; ROS, reactive oxygen species; SA, salicylic acid. (from Gallego et al., 2012).

Pesticides are substances widely used for agricultural purposes which reach the soil through rain and wind when they are applied to crops (Marican and Durán-Lara 2018). Some pesticides such as broad-spectrum triazole fungicides persist in soils and their sediments. This is especially true for epoxiconazole which has a half-life time of more than two years (at 10°C and 80% of field capacity) (Bromilow et al., 1999). Epoxiconazole is extensively used to protect cereal crops against fungal pathogens. Recently, Silva et al. (2019) described through their analysis of pesticide residues in European agricultural soils that this systemic fungicide was among the most frequently detected compounds at important concentrations. Crop plants seem to be able to detoxify adsorbed pesticide residues through a system including enzymes, gluthatione (GSH) and sequestration in the vacuole but research is still needed about the role of S-containing metabolites (Shahzad et al., 2018).

In the literature, terpenoids are largely described to be involved in abiotic stress VOC response, but showing a high phenomic plasticity. Some questions effectively remain about constitutive versus induced emissions and about their putative release

during plant stress response. On the other hand, GSLs represent secondary metabolites in the *Brassicaceae* family being deeply studied under biotic and/or abiotic stress conditions for understanding their importance in plant fitness. It is known that the GSL content in plant's organs can be affected by the magnitude and duration of biotic stress impact (herbivore feeding or pathogen attack) (van Dam et al., 2009; Gols et al., 2018). Interestingly, abiotic factor such as heavy metal stress can also modulate the GSL profiles through the involvement of hormones or signalling molecules (Pongrac et al., 2010; Variyar et al., 2014). Sulfur assimilation is effectively in the middle of multiple metabolic pathways, including Cd-stress responses at cellular level via detoxification through GSH and phytochelatines (PCs) synthesis (Gill and Tuteja, 2011; Hasanuzzaman et al., 2018). Metal-complexing peptides such as PCs are widely accepted as a major product for plant Cd detoxification and tolerance in most non-hyperaccumulators species (Cai et al., 2011). However, questions remain about how metal ions can affect the GSL composition and possible roles of S-containing compounds in stress tolerance (Fig. 0.2). About Cd-stress, GSLs seem to play an important role in tolerance within the *Brassicaceae* family probably due to specific cross-talk between the S primary and secondary metabolism.

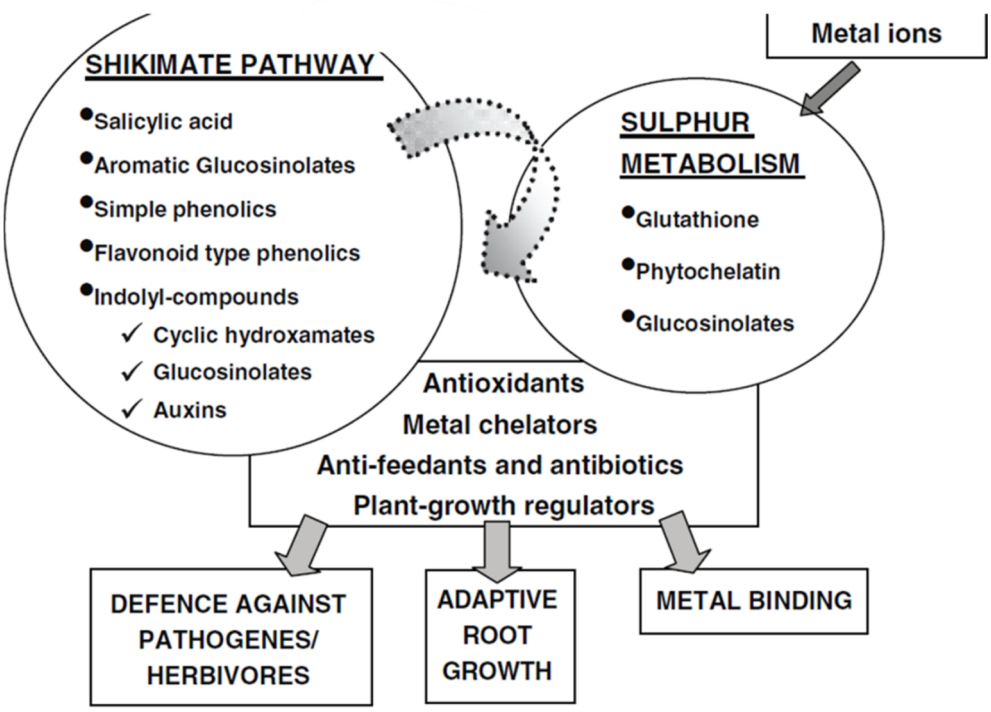

Figure 0.2: Metabolic pathways representing antioxidants, metal binding substances and plant growth regulators involved in adaptive responses under metal ions stress (adapted from Pongrac et al., 2010).

The purpose of this PhD thesis was to understand the adaptive response of oilseed rape (*Brassica napus* L.) plantlets by targeting potential plant metabolic markers under cadmium and epoxiconazole stress. A profiling of specialised metabolites involved in abiotic stress plant response was performed using mass spectrometry methods coupled with gas or liquid chromatography (GC/LC). Furthermore, an innovative glass device allowing the plantlets growth and the non-invasive dynamic sampling of emitted VOCs under controlled conditions was successfully developed. It was decided to phenotype i) characteristic symptoms (chloroses), ii) root and shoot growth and iii) biomass) winter oilseed rape plantlets using Cd and epoxiconazole dose-response experiments in complementarity to a targeted metabolomic approach. Experiments were performed at the laboratory scale in sterile conditions using culture medium for Cd-stress and using perlite substrate for epoxiconazole exposure. The concentrations that were tested through both experiments were established in order to obtain a concentration gradient representing several relevant stress conditions (i.e. low, middle and severe). In addition, phenotyping and further analyses have been achieved in comparison with a control without Cd or epoxiconazole exposure. Investigations consisted in the research of volatile and non-volatile metabolic markers. The major concern was focused on terpenes after a first round of a non-targeted screening and on glucosinolates (GSLs) as non-volatile S-containing compounds. In conclusion, key objectives of this current research in *Brassica napus* L. under Cd and epoxiconazole stress were to analyse:

i)	Induced emission of terpenoids,
ii)	Variations in GSL profile and content,
iii)	Changes of sulfur-containing volatiles in plantlet tissues.

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After the current introduction of context and objectives, the manuscript is structured around five chapters:

- **Chapter 1**: Literature review of recent trends in oilseed rape metabolomic studies under abiotic stress.
- **Chapter 2**: A laboratory high-throughput glass chamber using dynamic headspace TD-GC/MS method for the analysis of whole *Brassica napus* L. plantlet volatiles under cadmium-related abiotic stress.
- **Chapter 3**: How cadmium affects the fitness and the glucosinolate content of oilseed rape plantlets.
- **Chapter 4**: Phenotyping of *Brassica napus* L. plantlets affected during *in vitro* growth in the presence of epoxiconazole.
- **Chapter 5**: Smelling the stress of *Brassica napus* L. plantlets exposed to epoxiconazole residues using TD-GC-MS through a targeted approach.

Briefly, the first chapter draws a literature review of metabolomic studies in oilseed rape under abiotic stress, describing existing analytical platforms, and discussing the potentialities of VOC analysis for crop phenotyping. The following four chapters of the thesis develop experiments that were performed using *Brassica napus* L. plantlets. Chapters 2 & 3 concern cadmium investigations and chapters 4 & 5 report epoxiconazole exposure respectively.

The first research step was to set-up an innovative experimental device for dynamically trapping VOCs emitted by oilseed rape plantlets under cadmium stress. Chapter 2 fully explains this laboratory high-throughput glass chambers system and the related GC-MS method using thermal desorption and adsorbent cartridges. Chapter 3 is dedicated to the study of oilseed rape plantlets physiology submitted to cadmium-related abiotic stress and to the analysis of glucosinolate profiles and contents in association with Cd-stress tolerance. A first characterisation of epoxiconazole stress was achieved in chapter 4 with the analysis of plantlets growing *in vitro* and through an assessment of the fungicide exposure. Finally, chapter 5 investigates putative terpenes metabolic markers and glucosinolate breakdown products involved in epoxiconazole stress plant-response, using perlite substrate and our customised device. On the basis of these results, the end of this manuscript focuses on a discussion where prospects are mentioned as potential opportunities for future research on oilseed rape. Ultimately, concluding remarks are also drawn.

Chapter 1

Literature review of recent trends in oilseed rape metabolomic studies under abiotic stress

Plant metabolomics may represent a powerful tool for crop phenotyping in the future, making possible to analyse plant-responses and metabolism cross-talks at any given time. *Brassica napus* L. is known as a fast-growing crop cultivated around the world. Despite some remaining challenges related to huge amount of data involved, the targeted or non-targeted metabolite profiling could enhance the understanding of oilseed rape response to biotic or abiotic stresses.

Major developments in metabolomic platforms have been achieved through analytical progresses and the improvement of data processing. For example, modern hyphenated MS methods are increasingly used for detecting changes in plant metabolomes under varying environmental conditions. In addition, the discovery of metabolic markers due to their high specificity and some emitted metabolites such as volatile organic compounds (VOCs) could certainly serve as indicators of plant performance in the future.

After an introduction to existing analytical tools and related plant metabolome investigations, the present chapter draws the literature review of recent trends in oilseed rape metabolomic studies under abiotic stress. The discussion also focuses on the interest of VOC profiling and on glucosinolates, a very interesting group of sulfur-containing compounds within the *Brassicaceae* family which are described to be involved in stress response as non-volatile secondary metabolites.

1. Literature review of recent trends in oilseed rape metabolomic studies under abiotic stress

1.1 Phenotyping of oilseed rape based on metabolomics

Molecular studies have provided evidence that *Brassica napus* L. (2n = 38, AACC) has multiple polyploid origins, suggesting that its hybridisation comes from southern Europe or the Mediterranean region, where the ranges of the two parental taxa, *B. rapa* and *B. oleracea,* may have overlapped (Warwick, 2011). The plant phenotype can be defined as the set of structural, physiological and performance-related traits of a genotype in a given environment (Großkinsky et al., 2015). Despite some limitations, future developments in the use of data from plant metabolomes may offer new possibilities to predict plant performance of available genetic resources (Nakabayashi and Saito, 2015). The use in modern plant breeding of conventional genetic markers issued from amplified fragment length polymorphisms (AFLPs) or simple sequence repeats (SSRs) can be still problematic for species with a complex polyploid genome and if they are not directly linked with highly polygenic traits (Steinfath et al., 2010). Some key metabolic markers could certainly be used in functional genomics studies of plants and to predict the phenotypical properties of crop core collections (Frank and Engel, 2013). In particular, metabolite-based genome-wide association studies (mGWAS) could be used to link the chemical diversity of metabolomic profiles with specific locations in the genome in order to identify quantitative trait loci (QTLs) (Luo, 2015).

Among ‘omics’ approaches, the study of the metabolome is the most complex and has received little attention in crop science so far (Kumar et al., 2017). Despite the fact that this kind of research is the final and most representative state of a biological system in response to environmental and genetic perturbations. In complementarity with traditional molecular approach, the improvement of plant breeding strategies could be possible using metabolomics tools, by highlighting some interesting metabolic markers or metabolites profiling and through a more sophisticated phenotyping of oilseed rape crops under abiotic or biotic stress (Fig. 1.1). Furthermore, using innovative techniques such as modern hyphenated mass spectrometry methods coupled with gas or liquid chromatography (GC/LC), the profiling of metabolites, including volatile organic compounds (VOCs), could lead to the discovery of interesting metabolic markers and for a better understanding of the oilseed rape response to stress.

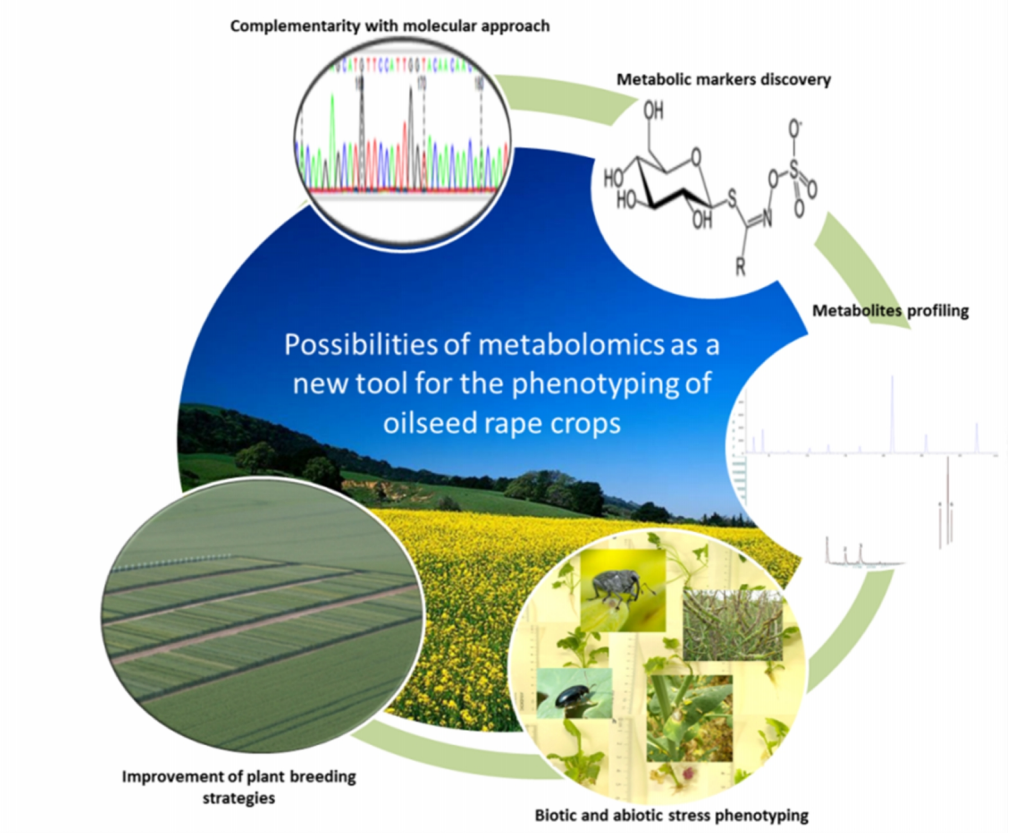

Figure 1.1: Schematic view of some possibilities of metabolomics as a new tool for the phenotyping of oilseed rape crops. In complementarity with traditional molecular approach, the improvement of plant breeding strategies could be possible using metabolomic tools.

1.2 Analytical platforms using targeted versus non-targeted approaches

Nowadays, the most common platforms involved in metabolomics are liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance spectroscopy (NMR). The use of one or the other technique depends on whether a targeted or non-targeted strategy is planned, as the number of quantified metabolites differs hugely between these two approaches. Mass spectrometry (MS) methods are often used in targeted studies thanks to their selectivity where the analysis focuses only on selected substances. While NMR, a non-destructive and non-selective method, is widely used in non-targeted analysis of almost all metabolites present in a sample (Lankadurai et al., 2013). No single analytical technology can cover the analysis of the whole metabolome (i.e. all metabolites), because each plant contains a huge number of

compounds corresponding to various physico-chemical properties. LC-MS is clearly emerging as the most widely used approach for the acquisition of global plant metabolomes. The analytical approach should therefore be chosen according to the scientific purpose of the study and the characteristics of the compounds to be analysed For example, primary or secondary metabolites vary according to their structure, their molecular weight and their polarity. The use of fragmentation spectra (MS/MS and MS^n) is a growing area with significant applications in agricultural sciences (Tian et al., 2016) and can help with structural identification, but series of databases are needed for the interpretation of results (Ernst et al., 2014). Multidimensional separation systems such as two-dimensional gas chromatography (GC x GC) and two-dimensional liquid chromatography (LC x LC) are used to enhance the chromatographic separation of complex mixtures and detect a low abundance of metabolites (Ghatak et al., 2016).

Profiling refers to a detailed chromatographic analysis, generally using hyphenated mass spectrometry techniques, and is especially suitable in cases of targeted analysis, covering a tiny fraction of the metabolome. Targeted methods of screening are already used to identify metabolic markers as predictive of phenotypical features independently of environmental variations, and to overcome the limitations of genetic markers (Steinfath et al., 2010; Fernandez et al., 2016). Alternatively, metabolic fingerprinting refers to more rapid and non-targeted screening methods such as direct mass spectroscopy (MS) (e.g. quadrupole-time of flight (Q-TOF) and matrix-assisted laser desorption/ionisation (MALDI)) or NMR and Raman spectroscopy (Halket et al., 2005). NMR-based approaches can detect and quantify the most abundant metabolites from the total pool, but due to its poor sensitivity and its poor dynamic range relative to mass spectroscopy, is regarded as more useful for giving a rapid high-throughput screening of all detectable analytes (Jorge et al., 2015). In conclusion, such a technique is largely used to identify metabolic signatures or patterns associated with a particular stress response, but without identification or quantification of the different metabolites in the sample (Shulaev et al., 2008).

Although it is still necessary to reduce costs by methodological adaptations, phenotyping platforms also involved in metabotyping are the way forward. Metabolites effectively give a more realistic picture of plant performance than molecular markers. Recently, a study using *Sorghum bicolor* L. breeding lines showed that many secondary metabolites such as glycosylated flavonoids and chlorogenic acids may be associated with morpho-physiological traits such as plant biomass and photosynthesis (Turner et al., 2016). Another study shown that a greater accumulation of proline and antioxidant enzymes such as superoxide dismutase (SOD; EC 1.15.1.1) is associated with leaf biomass reduction for *Brassica oleracea* L. cv. Bronco under alkaline stress (de la Torre-González et al., 2018). Finally, Kang et al. (2018) found that some metabolites such as zeatin-9-glucoside could be useful metabolic markers for the selection of a high-yield clone of *Quercus acutissima,* with the obvious advantage of predictive phenotyping in clonal seed orchards.

1.3 Using VOCs as a new tool for phenotyping

Metabolic markers are a sub-category of biomarkers that can be defined as "an objective characteristic measured as a predictor of plant performance". There are three types of plant metabolic marker: i) traits of agricultural importance, whether desirable (e.g. ascorbic acid and aromas) or undesirable (e.g. erucic acid in oilseed rape and toxins), ii) diagnostic markers to evaluate stress damage or resistance, such as glutathione (GSH), malondialdehyde, lipids or phenols and iii) markers of genotype performance (e.g. lignin precursors such as p-coumaric acid and caffeic acid) (Fernandez et al., 2016). Metabolite extraction from plant tissues can be very labour-intensive, expensive and time-consuming and represents a crucial step for insuring reproducibility. Stopping the metabolic processes in a cell through the use of low temperatures such as flash-freezing with liquid nitrogen is usually required if the objective is to gain an insight into the plant's molecular biochemistry at a given time (Gemperline et al., 2016). Another challenge is that a huge amount of data is obtained from metabolomic platforms, so that mathematical and statistical tools such as principal component analysis (PCA), partial least squares (PLS), boxplot and multivariate analysis or the use of other data interpretation sets are an essential prerequisite in order to extract as much valuable information as possible (Großkinsky et al., 2015).

It is estimated that between 200.000 and 1.000.000 plant metabolites are produced in the plant kingdom. These compounds share some basic functional groups (i.e. hydroxyls, alcohols, steroids, alkyls, benzyl rings, etc.), and a single plant is able to biosynthetise 5.000 to 25.000 compounds at any given time (Beckles and Roessner, 2012). Primary metabolites such as amino acids, polyamines, carbohydrates and organic osmolyte such as glycine betaine are principally responsible for plant growth, whereas secondary metabolites such as phenolic compounds, terpenoids, alkaloids and glucosinolates (GSLs) are considered to be more related to plant responses to both biotic and abiotic stress (Luo, 2015). In fact, many metabolites of lower molecular-weight protect plants from oxidative damage: i) compounds such as antioxidants or osmoprotectants, responsible for the priming of acclimation and for the scavenging of reactive oxygen species (GSH, anthocyanins, carotenoids); ii) stress by-products appearing in cells after disruption of normal homeostasis such as phytoalexins and iii) signal transduction molecules coming from adaptive responses: salicylic acid, methyl salicylate, jasmonic acid, methyl jasmonate (Shulaev et al., 2008).

Interestingly, some of the secondary metabolites produced in various tissues can be emitted by plants into the atmosphere. Such a mechanism, known as "plant language", can be used to communicate with other plants and organisms. About 1.700 substances can so be emitted in the plant kingdom throughout the life cycle, usually at specific developmental stages such as the maturation of leaf and needle, senescence, flowering or fruit ripening (Loreto and Schnitzler, 2010). Plant VOCs are lipophilic low-molecular-weight compounds (from 100 to 200 Da) representing up to 10% of photosynthetically fixed carbon but questions remain about how

volatiles are released from plant cells (active versus passive diffusion) (Widhalm et al., 2015). Volatile isoprenoids emitted by plants are largely described in the literature to be involved in abiotic stress response (Vickers et al., 2009) and the most important of these compounds are thought to be volatile terpenes biosynthesised in epidermal cells or in specialised structures such as trichomes and glandular cells (Loreto and Schnitzler, 2010). Numerous studies have demonstrated an increase in the emission of these protective volatiles from vegetative parts of plants under high temperature and high light (Dong et al., 2016).

VOCs can be emitted constitutively with differentiation inter- and intra-species, or after *de novo* synthesis induced by different stresses. These stresses may be biotic (e.g. wounding or herbivore feeding) or abiotic (light, temperature, CO_2 and nutrient deficiency being the most studied) (Blande et al., 2014). These volatile emissions can also provide useful information on the plant phenology, thus offering a picture of the plant's specific physiological state at a given point in time (Rosenkranz and Schnitzler, 2016). Volatile-based methods are among the advanced tools available for plant disease detection. Because VOCs are generally a first sign of early infections before primary symptoms are visible (Martinelli et al., 2015). From an analytical point of view, GC-MS remains the most widely used method for VOC analysis, thanks to its reproducibility, accuracy and selectivity. Moreover, due to the development of new dynamic or static headspace sampling methods and on-line measurement techniques such as proton transfer reaction-mass spectrometry (PTR-MS), VOC phenotyping can be considered as a fast and non-invasive measure of abiotic stress plant response (Niederbacher et al., 2015).

The dynamic collection of VOCs emitted from plants must take into account the regulation of the rhythmic emission by the circadian clock (Zeng et al., 2017). VOCs could possibly serve as a metabolite marker. However, there is an overlap between abiotically and biotically induced volatiles under field conditions. Moreover, a large number of volatile compounds can be also emitted by plant roots into the rhizosphere, playing a role in between- and within-plant signalling in below-ground interactions (Delory et al., 2016). Finally, after VOC emission by plants, oxidation reactions can occur directly into the atmosphere, leading to the production of secondary organic aerosols (SOA) with an impact on cloud formation and climate (Blande et al., 2014). For these reasons, a lot of additional data is still needed to achieve the ambitious goal of volatile sensors development for direct applications in agricultural research purposes (Martinelli et al., 2015).

1.4 Oilseed rape metabolomic investigations under abiotic stress

The metabolic networks in higher plants are known to be highly complex, and are usually investigated using the model plant *Arabidopsis*. However, data from metabolomic platforms using relevant stress scenarios of agricultural and vegetable crops could be useful to illuminate plants' response to abiotic stress (Hong et al., 2016). Agriculture has to cope with many stresses, but abiotic factors (temperature,

drought, salinity) may be responsible for over 50% yield reduction in major crop plants, depending on the plant's development stage, the intensity, the duration and the beginning of the stress (Rodziewicz et al., 2014). It is known that drought, salinity and heat influence plant development negatively by disturbing cell homeostasis, but the function of secondary metabolites in abiotic stress tolerance is still relatively little understood (Tian et al., 2016). The photosynthetic metabolism can be affected through stomata closure by a decrease of the internal CO_2 concentration, and by the perturbation of the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) due to the presence of tight-bound inhibitors (Rodziewicz et al., 2014). Moreover, oxidative stress can lead to the production of singlet oxygen, superoxide anion radicals, hydrogen peroxide and hydroxyl radicals. However, the ability to counteract the effect of reactive oxygen species (ROS) by non-enzymatic processes such as the accumulation of specialised antioxidant metabolites (e.g. ascorbic acid, GSH, α-tocopherols, proline, sugars, carotenoids, chlorogenic acid, saponins, GSLs, phenolamides, phenylpropanoids and flavonoids) really needs further research (Nakabayashi and Saito, 2015).

The main European oil crop, oilseed rape (*Brassica napus* L.), is the largest source of high-quality vegetable oil, and is mainly cultivated for human nutrition purposes (Stahl et al., 2017). Less is known about the impact on the oilseed rape metabolome of unfavourable abiotic stress conditions occurring with variable timing and intensity. Nokhrina et al. (2014) have used the lines of *Brassica napus* L. with an overexpressed gene of phospholipase C2 (*BnPCL2*), known to enhance drought tolerance, to highlight the triggering of different metabolite patterns related to low temperature stress tolerance. Non-targeted metabotyping could also be used to investigate the important leaf senescence process. Interestingly, Heat maps of metabolite changes have been created in laminae and midvein tissues of oilseed rape leaves during senescence (Clément et al., 2018).

It is well established that future climatic changes will include an increase of average temperature and more important atmospheric CO_2 levels. Several publications have focused on oilseed rape metabolomics and how it may be affected by climate change. For example, high-temperature stress can occur in oilseed rape above 25°C. Koscielny et al. (2018) identified 25 metabolic markers from heat-tolerant and heat-sensitive genotypes exposed to a heat treatment of 31°C/14°C (day/night) during 14 days of flowering. Recently, 268 metabolites were also quantified in a profile change analysis of two different cell types (guard and mesophyll cells) in response to increased CO_2 and through supplementation with bicarbonate, in order to investigate CO_2 sensing and the response mechanisms of stomata (Misra et al., 2015). More recently, a non-targeted metabolomics study using LC-MS/MS and GC-MS/MS was performed to identify the metabolic signatures response of guard cell protoplasts to abscisic acid (ABA) also involved in stomatal closure (Zhu and Assmann, 2017). The function of secondary metabolites of *Brassica napus* L. in abiotic stress tolerance is still poorly understood. Nevertheless, identification of metabolite such as polyphenols, flavonoids,

terpenoids and GSLs, underscoring interesting oilseed rape genotypes, could represent valuable markers in the response to environmental stresses (Beckles and Roessner, 2012). Finally, it would be also interesting to focus oilseed rape metabolomic studies on abiotic factor such as metal stress, given that *Brassica* species are well known as tolerant-metal accumulators (Zhuang et al., 2014; Durenne et al., 2018). For example, Hédiji et al. (2010) demonstrated that proline and total ascorbate amounts were reduced in Cd-treated leaves of tomato plants, whereas α-tocopherol, asparagine and tyrosine accumulation increased principally at 100 μM of Cd. Another recent study has also shown the importance of the response of the plant volatilome to excessive Zn and described a new tool for smelling the metal (Bibbiani et al., 2018).

1.5 Prospects of lipidome and glucosinolates profiling

Plant metabolomics can range from phytochemistry up to single-cell type studies, and is essential to understanding the metabolic pathways (Chikkaputtaiah et al., 2017). An understanding of *Brassica napus* L. seeds' lipidome profiling and of the mechanisms involved in oil biosynthesis could be useful in plant phenotyping for breeding strategies. Misra (2016) recently catalogued the seeds metabolome with enrichment of fatty acids, glucosinolates, phenylpropanoids, flavonoids, and phytohormones, among other compounds and described metabolic pathway showing the map of 110 metabolites. On the other hand, a NMR-based analysis has been used to compare the composition of oilseed rape and turnip rape, using multivariate models followed by a PCA, leading to the finding that oilseed rape has a higher overall oil content and sinapine level (Kortesniemi et al., 2015). Another study using NMR-based method showed an effective distinction between low-erucic acid from high-erucic acid rapeseed oil by hierarchical cluster analysis (Gupta et al., 2017). More recently, Lu et al. (2018) using the MALDI-MSI technique, evaluated lipid metabolism in a spatial context in the seeds of two low-erucic acid genotypes with high and low oil content respectively. Finally, other organs such as root, stem, leaf and inflorescence also contain phytochemical compounds of relevance to health promotion, and polyphenol composition in organs has been mapped using an analysis via UPLC-QTOF-MS, with the vast majority of identified metabolites being found to be flavonol glycosides (Farag et al., 2012).

Glucosinolates (GSLs) are sulfur-containing compounds from *Brassica* crops. A wide variety of them exist, but all share a β-thioglucoside *N*-hydroxysulfate common structure with a β-D glucopyranosyl moiety and a variable side-chain (Velasco et al., 2011). A recent area of research consists of studying dietary glucosinolates and related metabolites isothiocyanates as precursor of cancer-preventive metabolites using mainly LC-MS/MS with positive electrospray (Song et al., 2005). Lately, Feng et al. (2012) brilliantly reported the characterisation of metabolite quantitative trait loci (mQTL) and metabolic networks controlling GSL concentration in the seeds and leaves of *Brassica napus* L. GSLs could therefore serve as metabolite markers of stress situations. Some studies give the results of change in GSL

composition under abiotic stress such as drought or water deficit (Rodziewicz et al., 2014). The recent literature suggests that the place of GSLs in future investigations will become more and more important (Misra, 2016). Because, these secondary metabolites are not only involved in plants' growth or development but their production can enhance plant fitness under both biotic and abiotic stresses (van Dam and Bouwmeester, 2016).

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Chapter 2

A laboratory high-throughput glass chamber using dynamic headspace TD-GC/MS method for the analysis of whole *Brassica napus* L. plantlet volatiles under cadmium-related abiotic stress

Durenne, B., Blondel, A., Druart, P., Fauconnier, M-L. (2018). A laboratory high-throughput glass chamber using dynamic headspace TD-GC/MS method for the analysis of whole *Brassica napus* L. plantlet volatiles under cadmium-related abiotic stress. *Phytochem Anal* 29, 463–471.

The literature review revealed that the profiling of volatile organic compounds (VOCs) and specialised metabolites such as glucosinolates could serve as indicators of oilseed rape response to abiotic stresses. Experiments performed in sterile and controlled conditions can be effectively useful for VOC metabolism investigations. This chapter concerns the set-up of a laboratory high-throughput glass chambers system for the analysis of whole *Brassica napus* L. plantlets VOCs. After a first round of non-targeted volatiles screening, the system was then successfully used for investigating the relationship between low emission of induced terpene and cadmium-related abiotic stress.

The dynamic headspace sampling technique and gas chromatography coupled with mass spectrometry (TD-GC/MS) is a powerful method for analysing plant VOC emissions. 28-day-old *Brassica napus* L. plantlets were cultivated *in vitro* and, VOCs were trapped with our device using adsorbent cartridges that were thermally desorbed before cryofocusing with a cooled injection system and programmable temperature vaporising inlet into an HP-5ms GC column.

Briefly, terpene detection and quantitation from chromatogram profiles were acquired through a targeted metabolomics approach using selected-ion monitoring (SIM) mode during full scan analysis. Mass spectra were obtained with a quadrupole-type mass spectrometer. This new trapping method produced reliable qualitative profiles of oilseed rape VOCs. Typical emissions of monoterpenes (myrcene, limonene) and sesquiterpenes (β-elemene, (E,E)-α-farnesene) were found for the different concentrations of cadmium tested.

2. A laboratory high-throughput glass chamber using dynamic headspace TD-GC/MS method for the analysis of whole *Brassica napus* L. plantlet volatiles under cadmium-related abiotic stress

2.1 Introduction

Volatile organic compounds (VOCs), representing about 1% of plant secondary metabolites with around 1700 substances, mediate plant semiochemistry and are involved in abiotic stress responses (Dudareva et al., 2006; Zhao et al., 2017). Strategies for dynamically collecting plant-emitted VOCs depend hugely on recent advances in sampling methods and commonly target plant organs such as limb, leaf or flowers. The technique of volatile analysis poses analytical challenges and must be adapted either to laboratory or field experiments. Portable GC-MS represents the best compromise for studies investigating both biotic and abiotic stresses in field research (Beck et al., 2015). The isoprene molecule (C_5H_8) has been extensively studied with real-time volatile collection methods using proton transfer reaction/mass spectrometry (PTR-MS) technology (Jardine et al., 2016). Conversely, *in vitro* headspace analysis in connection with abiotic stress studies is usually performed under laboratory conditions. A number of studies described in recent literature have used methods involving plant biological sample destruction such as volatile extraction under air-dried conditions at 40°C for 72 hours, or hydrodistillation performed from cut plant materials (Alvarenga et al., 2015; Bassolino et al., 2015). We therefore decided to develop a glass collection chamber that would not harm the plant during its *in vitro* physiological development or during the VOC sampling procedure. Another goal for this innovative *in vitro* system was to use the plant's clonal regeneration ability, leading to genetically stable propagation. Another advantage of this method is that it enables VOCs' putative phenotype biomarkers to be detected as soon as possible during plant growth and under abiotic stress without hurting the plant. Our sampling method is used over a 24-hour period in order to take account of plant's circadian emission rhythms (Loreto et al., 2010).

There are many techniques for studying leaf reservoirs of stored VOCs, and practical approaches to investigating plant volatiles have been reviewed by Tholl et al., 2006. More recently, microwaves or supercritical fluid extraction (SFE), often combined with solid-phase microextraction, have been suggested for plant VOC analysis under laboratory conditions (Ormeño et al., 2011). However, these techniques are highly time-consuming and destructive, and frequently depend on the equilibrium of the sample and the headspace. The system described in our study is of particular interest for the analysis of volatiles emitted in sterile conditions and without any stress disturbance. Moreover, volatiles freely emitted from plants are

dynamically trapped without any other laboratory manipulation (in comparison with solvent extraction methods) and after only a few days of plant growth under controlled and defined conditions.

VOC sampling can either be static, using a solid phase microextraction (SPME) device providing semi-quantitative information, or dynamic, using continuous airstream flows within glass cuvette chambers coupled with adsorbent material. As the major purpose of the present study was to create an innovative design for the analysis of whole plantlet volatiles, a dynamic headspace TD-GC/MS method using Tenax® TA adsorbent cartridges was developed. Adsorbent material selection is always one of the critical steps of the procedure. Large numbers of molecules of different chemical natures, molecular weights and polarities have to be adsorbed jointly, quantitatively (i.e. without any breakthrough) and reversibly, if possible in a single "sampling run". Gas chromatography-mass spectrometry can then be used to separate and detect volatile compounds trapped dynamically on adsorbent material, which makes it a suitable technique to study whole plant terpene emissions under controlled conditions (Pinto et al., 2007; Lin et al., 2016). Moreover, the use of a thermal desorption unit (TDU) avoids any use of solvents to extract volatiles from the sorbent, while the cooled injection system (CIS) concentrates samples by cryofocusing before their injection into the GC column (van Drooge et al., 2009; Jochmann et al., 2014). Finally, selected ion monitoring allows analyses with higher sensitivity and improved signal to noise ratios as it removes much of the matrix noise (Jia et al., 2006).

Volatile terpenes are the most important compounds in plant VOC emissions in terms of functional diversity and plant protection. They can be constitutive and highly controlled by genetic and environmental factors. Emission rates vary with genotype (variety for example), season and physiological development of the plant (Niederbacher et al., 2015). Moreover emissions can be induced by abiotic stresses (temperature, light, drought, salt, ozone and UV-B radiation). Plant's constitutive VOCs are well known to be stored within internal structures such as resin ducts (pines) and glandular cells (*Lamiaceae*) or within external structures such as glandular trichomes (*Lamiaceae*, *Solanaceae*) (Loreto et al., 2010). These compounds are synthesised via two interconnected isoprenoid pathways within the plant cell: the formation of homoterpenes, sesquiterpenes and triterpenes from cytosolic mevalonic acid (MVA) and the formation of hemiterpenes, monoterpenes, diterpenes and tetraterpenes from chloroplastic 2-C-methyl-D-erythritol 4-phosphate (MEP) (Vickers et al., 2009; Lange and Akhami, 2013). Flowers produce large numbers of terpene volatiles involved in the attraction of pollinators (Zu et al., 2016) and of seed-disperser (Adebesin et al., 2017).

However, many plant species also emit constitutive VOCs from their foliage without any storage pool structure, depending on factors such as the plant's phenological stage, ambient temperature and light intensity. Our system allows VOCs to be trapped at a wide range of plant development stages, e.g. flowering. VOCs such as monoterpenes and sesquiterpenes are lipophilic compounds with high

vapour pressures which cross membranes freely. They can therefore be directly emitted in the plant's biosphere depending on abiotic or biotic factors. Their release is greatly restrained in normal conditions due to the plant's carbon balance, with the apparent exception of sesquiterpenes (Oikawa et al., 2013). The optimisation of the sample chamber, the design of the sampling lines and the adsorbent properties of the cartridges used can improve the experimental recovery of these sesquiterpenes (Ormeño et al., 2011). However, leaf stomatal closure resulting from abiotic stresses such as temperature may prevent volatile transmission. Emitted constitutive monoterpene and sesquiterpene VOCs have been shown to serve as non-destructive markers of phenotypic abiotic stress using measurements under sterile and controlled conditions (Niederbacher et al., 2015). The present study was therefore designed to develop a sensitive sampling-analysis technique that would make two things possible: firstly, the investigation of large numbers of *in vitro* replicates with the guarantee of genetic stability, thus overcoming any response plasticity, and secondly, the measurement of minute amounts of secondary volatile emitted products such as putative abiotic stress biomarkers. The system developed was tested and applied to clonal oilseed rape *Brassica napus* L. regenerated plants at the vegetative stage using *in vitro* axillary shoot branching method.

Brassica napus L. is a worldwide crop of high interest as one of the main sources of oil and protein for food and feed or biofuel production (Evans et al., 2010). Monoterpenes and sesquiterpenes emitted from oilseed rape in the field were first investigated as potential causal irritation agents leading to human allergic reaction (McEwan et al., 1998; Murphy et al., 1999). Higher emissions of monoterpenes (α-thujene, sabinene, myrcene and limonene) have also been observed during the flowering stage of oilseed rape (Müller et al., 2002) and the effect of N fertilisation on bud and flower volatile bouquets has been demonstrated under laboratory conditions with higher emission of several monoterpenes at increased N dosages (Veromann et al., 2013). A multivariate study demonstrated that high concentrations of CO_2 related stress (720 µl/L) lead to enhanced emissions of α-thujene, sabinene, limonene, 1,8 cineole and γ-terpinene for *Brassica napus* spp. *oleifera* at the vegetative stage (Himanen et al., 2009). Most previous investigations of *Brassica napus* L. VOCs have involved the collection of the floral scent. Floral volatile plasticity may be environmentally and genetically based and any evolution may be mediated by pollinators (Zu et al., 2016). Push-pull or multi-chamber cuvette systems described in the literature in connection with investigations of oilseed rape at the plant-vegetative stage only take parts of the plant into account for VOC capture. For example, several abiotic stress studies have been performed under field conditions using the panicle of the main raceme (Veromann et al., 2013), using foliage in order to compare VOC emission in relation to different soil types (Ibrahim et al., 2008), or using foliage of CO_2-stressed oilseed rape plants (Himanen et al., 2009). The initial step of our research was therefore to set up an innovative glass VOC trapping system in which whole oilseed rape plantlets could grow under sterile, controlled and strictly defined conditions. When experiments are performed effectively using whole plantlets or plants, all constitutive or induced VOCs emitted

can be trapped. The TD-GC/MS method was optimised so that volatiles could be actively trapped on adsorbent material at any moment.

The impact of abiotic stresses such as temperature, drought, ozone and UV-B radiation on terpene emission in oilseed rape is poorly understood. Winter et al., (2012) reported that heavy metal stress has a strong influence on terpene emissions. Moreover, diffuse cadmium sources, notably P-fertilisers and atmospheric deposits, can contribute to the concentration of Cd in agricultural soils (Six et al., 2014), and Cd as a hazardous pollutant can lead to inhibition of plant growth processes and to chlorosis (decreasing of photosynthetic activity) (Gallego et al., 2012). Cadmium stress-induced VOC profiles were therefore investigated in order to study the relationship between this specific stress and emissions from winter oilseed rape plantlets. Finally, this sterile laboratory high-throughput system revealed an easy-to-use method for *in vitro* plants regeneration ensuring genetic stability with advantages for studying the impact of one stress at a time without other disturbances to the plant's secondary VOC metabolism.

2.2 Materials and methods

2.2.1 Plants

Winter oilseed rape (*Brassica napus* L. var. *Es Astrid*) shoots grown from germinated seeds (Euralis semences, France) were propagated *in vitro* using genetically very stable axillary branching proliferation and voucher specimens (n°0312) held at the Walloon Agricultural Research Centre (Belgium). Twenty-eight-day-old plantlets were obtained after 14 days of a hermetic *in vitro* preliminary rooting phase and 14 days acclimation growth of shoots under sterile culture conditions. After the plantlets' growth and leaf development had been tested, two standardised shoots were cultivated in each glass chamber. The culture medium consisted of 400 mg/L NH_4NO_3; 800 mg/L KNO_3; 300 mg/L $Ca(NO_3)_2$; 180 mg/L $MgSO_4$; 150 mg/L KH_2PO_4; 1.5 mg/L $MnSO_4$; 0.5 mg/L $ZnSO_4$; 3 mg/L H_3BO_3; 0.5 mg/L KI; 0.25 mg/L Na_2MoO_4; supplemented with 20 mg/L Na_2EDTA and 15 mg/L $FeSO_4$. Sucrose (3%) and 0.5% dehydrated powdered agar Pastagar B (Biorad, Temse, Belgium) as a strong gelling agent were added to the culture medium. In parallel to three controls (0 µM of Cd), cadmium abiotic stress ($CdCl_2$) was added (Sigma-Aldrich, Diegem, Belgium) to three culture media at 5 µM (low stress 0.56 ppm), three at 15 µM (middle stress 1.68 ppm) and three at 45 µM (severe stress 5.04 ppm). The oilseed rape was cultivated under controlled environmental conditions at 23/18°C (day/night), a long-day photoperiod of 16h, 45% relative humidity and 100 µmol of photons /m²/s PAR (photosynthetically active radiation). The entire experiment was repeated 5 times. Phenotyping consisted of leaf symptom observation, and phenotypic results consisted of the two plantlets' growth (mm) and fresh weight biomass (g) data recorded after VOC collection.

2.2.2 Volatiles trapping system

An open enclosure glass system was developed based on existing gas wash Drechsel® bottles without filter disc of 500 mL capacity (borosilicate 3.3 glass bottle and head, overall height of 275 mm, standard ground joint neck size of 29/32, hose nozzles with outer diameter of 11 mm (Brand, Wertheim, Germany). These bottles were manually modified by cutting away the glass tube in order to allow the growth of two oilseed rape plantlets under cadmium-related abiotic stress and the collection of volatile terpenes (Fig. 2.1).

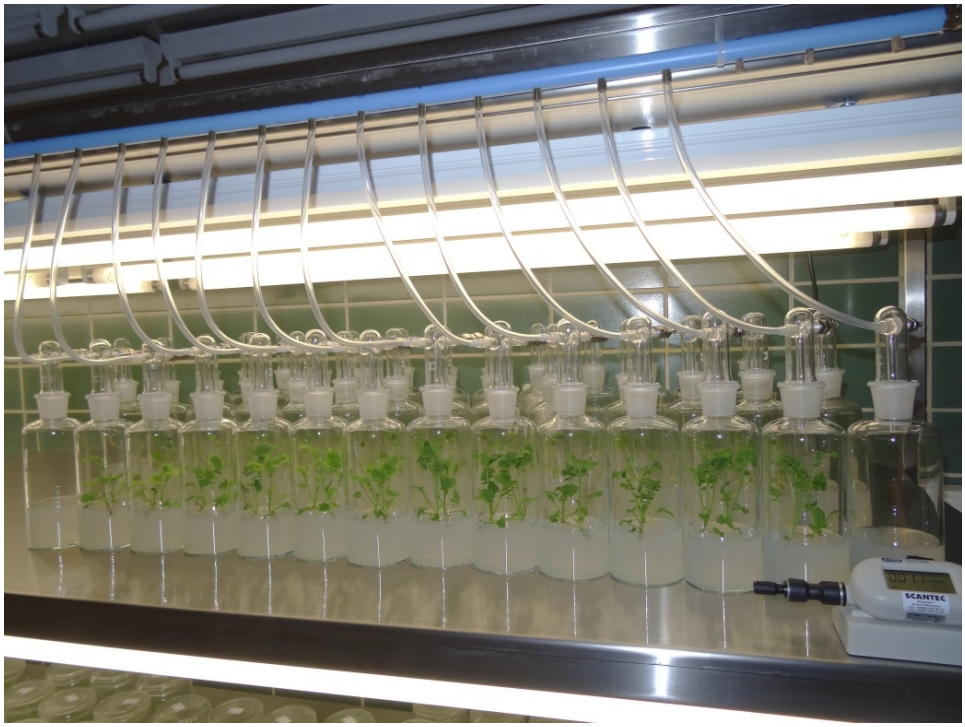

Figure 2.1: Picture of the laboratory glass chamber sterile system allowing the *in vitro* growth of *Brassica napus* L. plantlets and the VOC trapping at any time under cadmium-related abiotic stress.

Clean sterile air (filtered via activated charcoal and Acro™ 37 TF 0.2 µm PTFE) was supplied continuously to the homemade system by a portable diaphragm pump with 2.4 bar operating pressure and 6 L/min delivery (N86 KN.18, KNF, Neuberger, Germany) connected with Teflon® and connection tubes. Blank tests (bottles with culture medium only) and the trapping of VOCs from 28-day-old plantlets were performed using Tenax® TA (Supelco, Bellefonte, PA, USA) adsorbent cartridges (Gerstel, Mülheim an der Ruhr, Germany) in order to minimise water vapour collection (Fig. 2.2).

Figure 2.2: Picture of the high-throughput dynamic headspace sampling technique using Tenax® TA (2,6-diphenylene oxide polymer) cartridges connected to the cuvette system with swageloks® stainless steel links for a sampling time of 24 h.

Tenax TA is a porous polymer (2,6-diphenylene oxide) commonly used for trapping volatile and semi-volatile compounds from C7 to C26. The cartridges were connected to the cuvette system with swageloks® stainless steel links for a sampling time of 24 hours at a flow of approximately 0.200 L/min (calibrated using air flow calibrator TSI™ model 4199). No volatile compound breakthrough was observed using sampling conditions after analysis of a second cartridge placed after the first one. All cartridges were preliminarily conditioned at an elevated temperature of 280°C for 10 hours under a flow of nitrogen (ultra-high purity grade, Air Liquide, Belgium) independently of the analytical system using a tube conditioner TC 2® (Gerstel, Mülheim and der Ruhr, Germany). The cartridge cleaning protocol was

checked with blank analyses on these conditioned cartridges. Before use and after all conditioning procedures, the cartridges were stored directly in a GERSTEL Twister® rack suitable for manual operations and capable of storing up to 15 cartridges. Five repetitions of volatile collection were performed successively using micropropagated oilseed rape clones and in triplicate for each cadmium concentration (0 µM, 5 µM, 15 µM and 45 µM).

2.2.3 TDU/CIS coupled to GC-MS profile analysis

Each sample was loaded and injected using a MultiPurpose Sampler (MPS). The VOCs were firstly thermally desorbed from Tenax TA cartridges with a thermal desorption unit (TDU; Gerstel, Mülheim an der Ruhr) running in splitless mode from 40°C to 120°C (110°C/min) in order to prevent thermal degradation and for 2 min, and then at 280°C (200°C/min) for 5 min. Cryofocusing with a programmable temperature vaporising inlet (CIS4/PTV inlet) was performed at -10°C before injection into the GC column by heating the CIS/PTV inlet to 260°C for 5 min at a rate of 12°C/s. VOC separation was performed using gas chromatography (7890A; Agilent Technologies, Palo Alto, CA, USA), with an HP-5ms capillary column (30 m length x 0.25 mm internal diameter x 0.25 µm film thickness; Agilent Technologies, Palo Alto, CA, USA). High-purity helium (Air Liquide, Liège, Belgium) was used as the carrier gas at a constant flow of 1.6 ml/min. The oven temperature programme started at 40°C with an heating rate of 4°C/min to 90°C and followed by a heating rate programme at 20°C/min to 300°C with a final hold for 5 minutes at this temperature.

2.2.4 Identification of volatile organic compounds

VOC detection was performed using a quadrupole-type mass spectrometer (MS 5975C; Agilent Technologies, Palo Alto, CA, USA). Mass spectra were obtained using electron impact mode (70 eV) and operated in SCAN mode with a range of 35 to 350 amu for m/z ratios. Both SIM (with only ion 93 recorded) and full-scan modes were used in the same run of 28 minutes. Mass spectrometry was performed with a source temperature of 230°C and a quadrupole temperature of 150°C. GC-MS data were analysed using the Agilent MSD Chemstation E 02.00.493 (Agilent Technologies, Palo Alto, CA, USA). Identification of emitted terpenes was performed by comparing the data with a Wiley 275 mass spectral database and further confirmed by comparison to retention times and fragmentation patterns of commercially available analytical standards for myrcene, β-elemene and (E,E)-α-farnesene (Sigma-Aldrich, Diegem, Belgium). Kovats indices were also calculated using a saturated n-alkanes (C7 – C30) standard solution (Sigma-Aldrich, Diegem, Belgium) on the HP-5ms column. SIM mode was used for quantification, based on terpene's most representative m/z 93 ion response with a dwell time set at 100 ms. In this way representative single-ion peaks with respective relative abundance of myrcene (23.03%), β-elemene (7.29%) and (E,E)-α-farnesene (9.45%) were integrated and compared with the equivalent single-ion response of 1 µl of hexane solution containing an internal standard of octylbenzene (0.5 mg/mL) (2.69%) (Sigma-Aldrich, Diegem, Belgium). The internal standard was injected directly on

Tenax TA cartridges with a 10 µl Hamilton gastight syringe, and adjusted chromatograms with a 3-minute solvent delay were used to remove hexane. The terpenoid emission rate was calculated as pg/g/L of fresh weight plantlet and air extracted.

2.2.5 Statistical analysis

Statistical analysis was carried out with Minitab® package version 17 and all data sets were tested for normality and equality of variances. Phenotypic results for growth and fresh weight biomass for 28-day-old oilseed rape plantlets were analysed using one-way analysis of variance (ANOVA) followed by a post hoc Dunnett's 95% confidence intervals test comparing the mean of the control group (0 µM of Cd) with the mean of every other group (5 µM, 15 µM and 45 µM of Cd). The values are reported as means with standard error for all results. One-way ANOVA was also used to test the impact of the Cd concentration factor on (E,E)-α-farnesene emission rates followed by a post hoc Tukey range test to find significant difference among pairwise means.

2.3 Results and discussion

2.3.1 Volatile collection system set-up

The developed open enclosure glass system successfully enabled two whole winter oilseed rape plantlets to be grown *in vitro* in sterile and controlled conditions. The plantlet growth was achieved inside the glass chamber without biotic stresses such as moisture, as was confirmed by clean culture media at the end of all experiments. The main advantages of the system are that it allows whole plant VOCs to be trapped and is a simple yet high-throughput system. The dynamic headspace sampling method using thermal desorption and gas chromatography combined with mass spectrometry produced reliable qualitative data for each triplicate of the control and of each cadmium concentration tested (5 µM, 15 µM and 45 µM). This very inexpensive volatile sampling cuvette is a useful tool for collecting complete whole plant VOCs at defined and repeated intervals under sterile and controlled conditions. The plant choice can be adapted to the purpose of the study, suggesting that this new system represents, for example, a non-invasive opportunity for quality checking of aromatic plants on the basis of the terpenes typically emitted.

This innovative method can also be used in order to screen putative abiotic or biotic phenotypic plant indicators. The capacity of the bottle (using several volumes) can also be adapted to a large numbers of plants or to further *in vitro* culture research. It can also be used to follow VOC emission at different developmental stages in numerous plant species, or, for example, to study the considerable quantities of volatiles emitted by flowering plants. Finally, other substrates such as soil (inside the bottles) could be used as an alternative model of plant growth, leading to new possibilities for investigation. The most recent literature describes *in vitro* studies of VOCs using SPME techniques leading to difficult quantitative interpretations (Bassolino et al., 2015) or using destructive methods such as

incubation of the harvested tissue at a non-biological temperature followed by headspace analysis (Alvarenga et al., 2015). The glass chamber set-up was based on preliminary tests focusing on the size of the cuvette system, the development-age pattern of the plantlets (i.e. their ability to emit VOCs), the optimal number of plantlets to use (showing a perfect foliage development without disturbance) and the flow rate applied into the developed system.

All these points can be crucial for accurate recording of plant VOC emissions. Unappropriated gas flow inside the cuvette due to the use of an incorrect volume can lead to air stagnation, CO_2 depletion and condensation that disrupt the trapping of VOCs on adsorbent material (Tholl et al., 2006; Niederbacher et al., 2015). It was also decided to push and not pump the filtered air across our system between experiment repetitions to avoid any memory effects of Teflon lines and silicone tubing. Our expertise in trapping plant VOCs was experimentally based, and we tested numerous combinations in terms of the number and the physiological age of the oilseed rape plantlets as this affects the emission of volatile terpene. The most relevant profiles were obtained when analysis was performed for 24 hours using two 28-day-old plantlets with well-developed foliage. The extent of plant community growth and associated specific microclimate, the foliage atmosphere and circadian rhythms could have a significant impact on plant-discrete VOC emission detection (Loreto et al., 2010; Blande et al., 2014). We therefore used the CIS injection system, as part of our study had the objective of detecting discrete amounts of plant VOCs. Ultimately, our system thus provides a new tool for the rapid detection of VOC emissions from very small plantlets.

2.3.2 VOCs qualitative results using TD-GC/MS and Tenax TA

We firstly investigated the whole profile of emitted VOCs using the TD-GC/MS method, which is a new high-throughput approach to analysing plant volatiles within undisturbed and sterile environment. To our knowledge, no work has been reported on the potential effect of Cd in VOC metabolism cross talk. Because the results of chromatograms for the whole profile using full-scan mode exhibited too much noise, we were unable to find any VOC qualitative differences in our investigated test conditions. This can be observed in Figure 2.3 for a) total ion chromatogram (TIC) of whole profile of blank test of growing medium and b) TIC of whole profile of plant volatiles (28-day-old plantlets of *Brassica napus* L. var. *Es Astrid*). We therefore decided to perform volatile analysis targeting terpenes emitted by oilseed rape using SIM mode (monitoring one single of m/z 93 being the most relevant ion) and Tenax TA cartridges, as this polymer is regarded as the best compromise for terpene and volatile trapping (Tholl. et al., 2006).

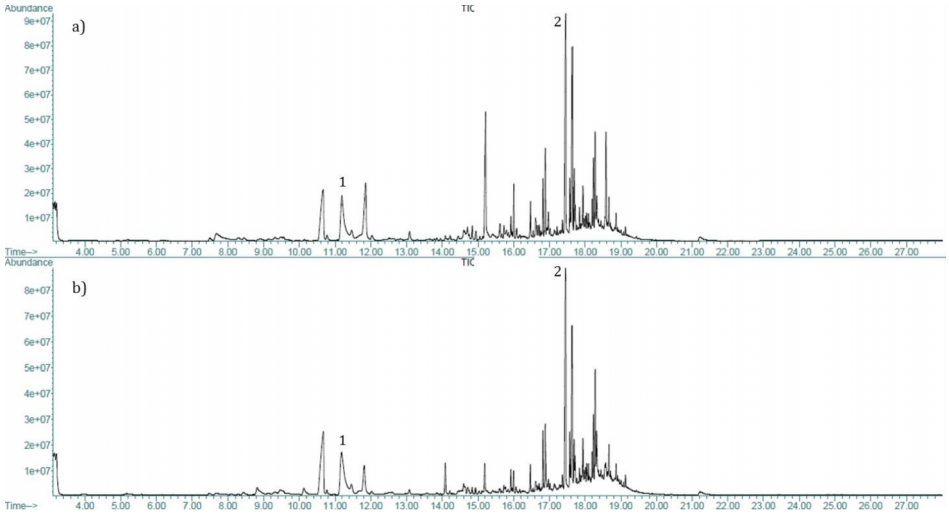

Figure 2.3: Total Ion Chromatogram (TIC) for a) whole profile of blank test performed on growing medium and b) whole profile of 28-day-old plantlets of *Brassica napus* L. var. *Es Astrid* volatiles. Peak identification: 1: n-butyl benzene internal standard (IS) not used, 2: octylbenzene (IS).

Qualitative terpene profiles were specifically achieved for the control group of plantlets (0 µM of Cd) and at different cadmium concentrations (5 µM, 15 µM and 45 µM). As confirmed by phenotypic results showing plantlets' physiological symptoms and analysis of plantlets' growth reduction, these concentrations can be considered as low (0.56 ppm), middle (1.68 ppm) and severe (5.04 ppm) cadmium stress respectively. In our study no significant effect of Cd was found on qualitative results of emitted terpenes from the 28-day-old plantlets of *Brassica napus* L. var. *Es Astrid*, which means that no specific compound related to cadmium stress could be identified. Figure 2.4 shows the typical chromatogram achieved using data acquisition in SIM mode (m/z 93) for blank tests using growing medium, while Figure 2.5 shows the profiling of monoterpenes (myrcene, limonene) and sesquiterpenes (β-elemene, (E,E)-α-farnesene) emitted by the two 28-day-old oilseed rape plantlets. Kovats indices were 983.55 for myrcene, 1019.76 for limonene, 1408 for β-elemene and 1500.18 for (E,E)-α-farnesene. Terpene identifications were in line with mass spectral data and published retention indices (Adams, 2007). Finally, they were consistent with previously published data on oilseed rape terpenoid emissions at the vegetative stage (Himanen et al., 2009; Veromann et al., 2013). As described before, a plant-free glass cuvette containing only the same quantity of *in vitro* culture medium was used as a blank to determine the chemical background. Small amounts of limonene coming from these growing media were detected, as confirmed by also testing an empty glass cuvette without culture medium, and limonene was therefore disregarded for the further analysis of the results. Selected ion monitoring mode was very useful for focusing on m/z 93, which most accurately

represents monoterpene and sesquiterpene emissions, excluding impurities and contaminants volatiles from the homemade system (such as plasticisers for example clearly coming from silicone connection tubes).

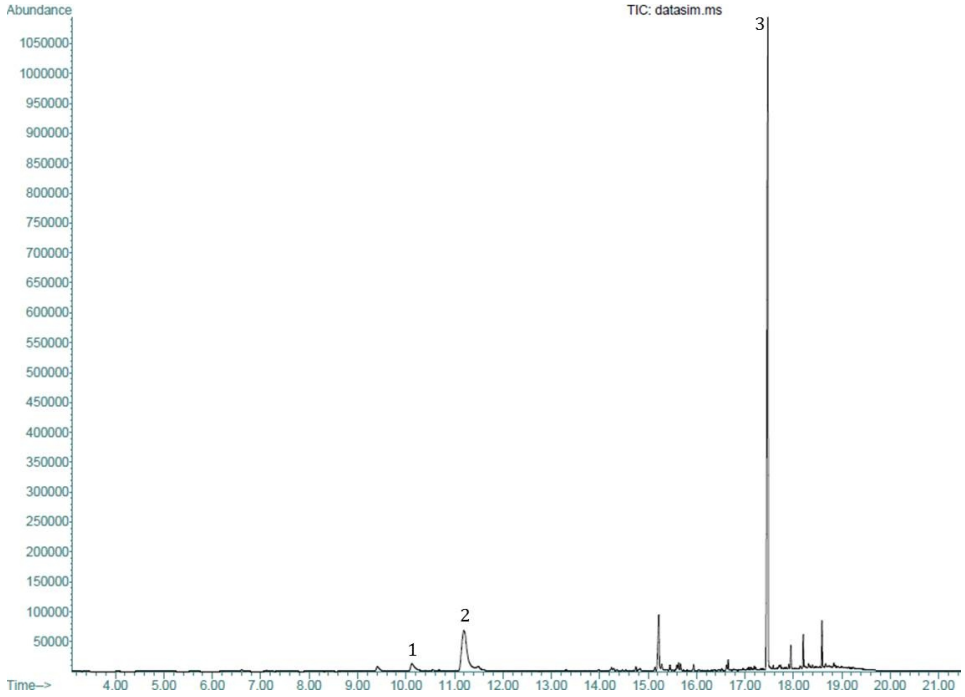

Figure 2.4: Typical chromatogram of growing medium using selected-ion monitoring (SIM Mode). Peak identification: 1: limonene, 2: n-butyl benzene internal standard (IS) not used, 3: octylbenzene (IS).

Tenax TA adsorbent material was selected in view of the characteristics of this polymer, which are ideal for plant VOC analysis. Preliminary experiments using Carbotrap® and Carbosieve® polymers were performed, but the results were not usable because these polymers have too strong affinity for water. Most studies have focused on developing new types of sorbents to extract a wide range of plant volatiles, but Tenax TA adsorbent can reversibly bind high-molecular-weight compounds and is particularly recommended for trapping target analytes such as volatile terpenes. Finally, the polymer material has a low affinity to water, thus avoiding water vapour troubleshooting during GC injection, especially with the cooled injection system.

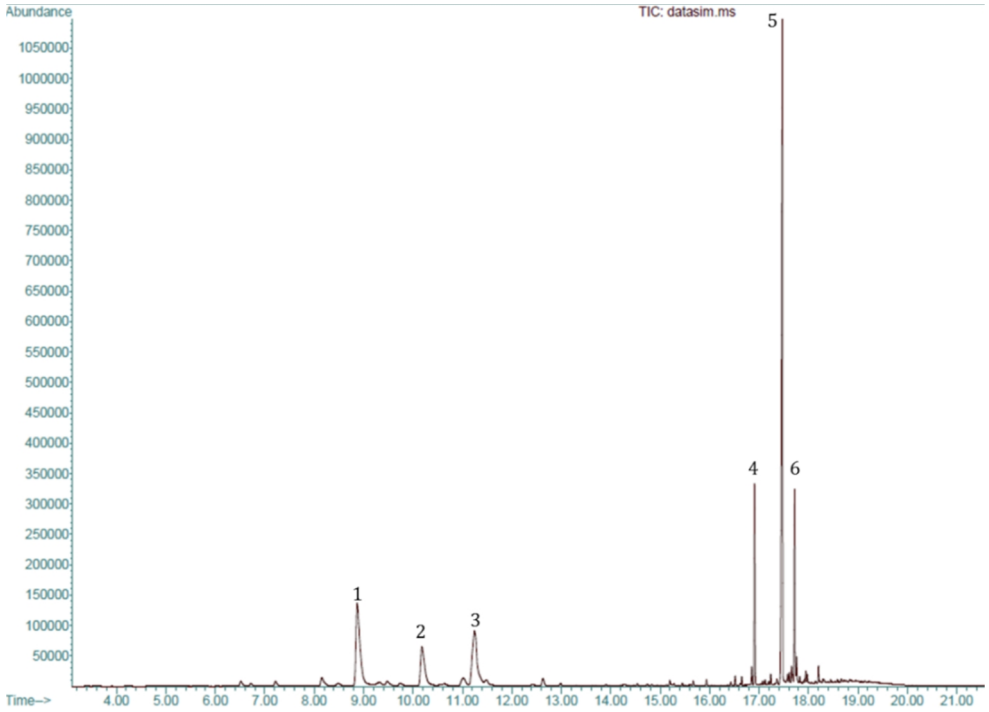

Figure 2.5: Typical chromatogram of 28-day-old plantlets of *Brassica napus* L. var. *Es Astrid* using selected-ion monitoring (SIM Mode). Peak identification: 1: myrcene, 2: limonene, 3: n-butyl benzene internal standard (IS) not used, 4: β-elemene, 5: octylbenzene (IS), 6: (E,E)-α-farnesene.

2.3.3 Cadmium-related stress and induced terpenoids

Heavy metal stress related to terpenoid emission is poorly documented. We therefore decided to quantitatively investigate the concentration of myrcene, β-elemene and (E,E)-α-farnesene induced by the different levels of cadmium stress (5 µM, 15 µM and 45 µM) in comparison with a control group (0 µM of Cd). It is commonly known that cadmium induces reactive oxygen species (ROS) playing a multitude of signalling roles within abiotic stress response, but leading in excess to the destabilisation of thylakoid membranes (Gallego et al., 2012). Leaf chlorosis was observed in 28-day-old plantlets cultivated under 15 µM and 45 µM cadmium concentrations, confirming that chlorosis represents a typical phenotypic symptom of Cd contamination. One-way ANOVA showed that Cd significantly affects the plantlets' growth ($F_{(3,111)}$ = 134.77, P<0.001) and fresh weight biomass ($F_{(3,111)}$ = 39.39, P<0.001). Cd was responsible for a significant decrease of growth and fresh weight biomass at a concentration of 15 µM and for very severe stress at the 45 µM concentration. No leaf chlorosis and no phenotypic effect were observed for plantlets cultivated under 5 µM (corresponding to 0.56 ppm), confirming that *Brassica napus* L. is one of the most tolerant species to Cd (Mwamba et al., 2016).

Dunnett's 95% confidence intervals tests comparing the mean from the control group (0 µM of Cd) with the mean of every other group (5 µM, 15 µM and 45 µM of Cd) for the growth and fresh weight biomass of 28-day-old plantlets are shown in Fig. 2.6 a) and b), respectively. As can be observed from the graphs, the confidence intervals of both growth and fresh weight biomass means show an overlap for the control condition (0 µM) and the first cadmium concentration. These results confirm the tolerance of *Brassica napus* L. var. *Es Astrid* plantlets at 5 µM of cadmium.

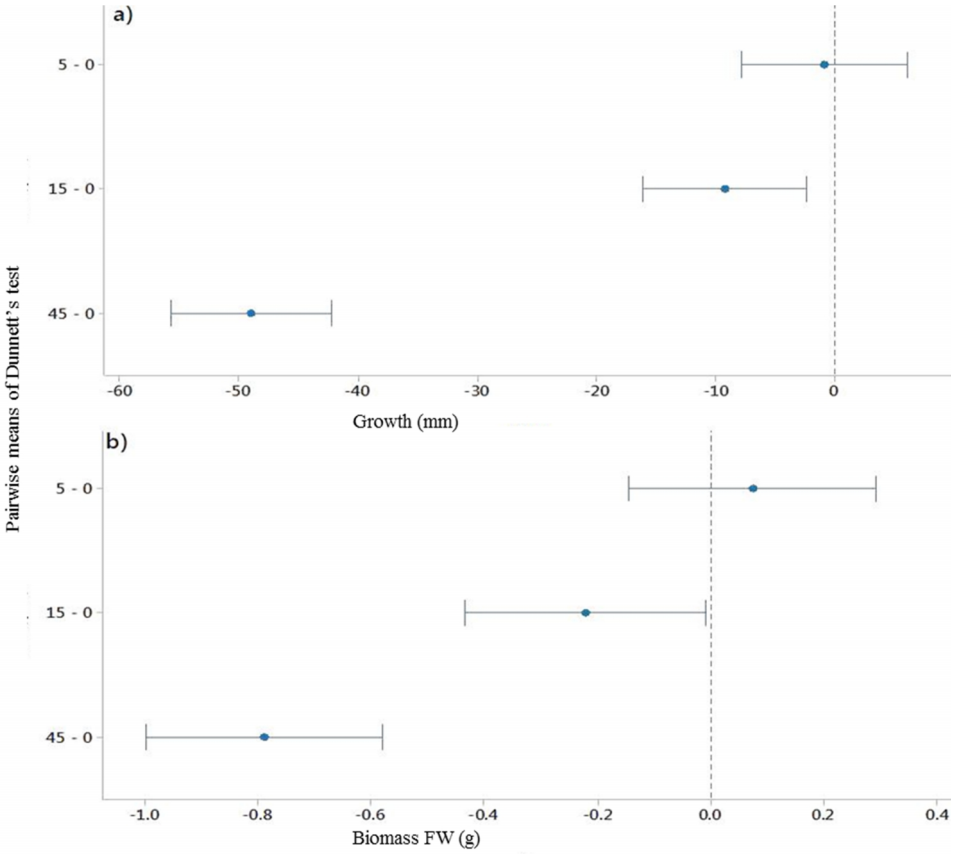

Figure 2.6: Graphs of Dunnett's 95% confidence intervals tests comparing the mean from the control group (0 µM of Cd; n=28) with the mean of every other group (5 µM, 15 µM and 45 µM of Cd; n= 26, 28, 30 respectively) for the growth a) and fresh weight biomass b) of 28-day-old plantlets of *Brassica napus* L. var. *Es Astrid*.

Volatile terpene emission from plants represents more than half of the total emission of plants VOCs (Loreto et al., 2010). In addition to constitutive terpenes, abiotic stress can induce a dynamic and multifaceted response reflecting variations in volatile terpene metabolism and biological response (Niederbacher et al., 2015). Induced VOC emission is a *de-novo* emission and is closely associated with the

photosynthetic activity of the plant. Table 2.1 summarises the data obtained from quantitative results showing the mean (± SE) of emission rates (pg/g/L) for myrcene, β-elemene and (E,E)-α-farnesene emitted by oilseed rape under the different cadmium stress conditions (0 µM, 5 µM, 15 µM and 45 µM). Emission rates for myrcene and β-elemene showed very high plasticity and no influence of cadmium abiotic stress could be found.

Table 2.1: Mean (±SE) of emission rates (pg/g/L) of terpene emitted (myrcene, β-elemene and (E,E)-α-farnesene) by 28-day-old plantlets of *Brassica napus* L. var. *Es Astrid.* under the different cadmium stress conditions (0 µM, 5 µM, 15 µM and 45 µM).

	Emission rates (pg/g/L) Mean (±SE)		
Cadmium concentrations (µM)	Myrcene	β-elemene	(E,E)-α-farnesene
0[a]	27.72 ±5.6	37.89 ±7.5	19.94 ±3.8
5[a]	22.86 ±4.9	38.33 ±6.8	37.99 ±5.1
15[a]	27.73 ±3.8	52.26 ±6.4	29.23 ±4.3
45[b]	31.98 ±5.0	58.04 ±13.6	17.25 ±3.3
[a]:n=10; [b]:n=11			

However, one-way analysis of variance test performed on (E,E)-α-farnesene emission rates revealed a significant effect for Cd stress ($F_{(3,40)} = 5.17$, $P = 0.004$). A significant difference was found using Tukey's *post hoc* test between means of emission rates for (E,E)-α-farnesene at 0 µM and 5 µM of Cd, as can be observed in Figure 2.7.

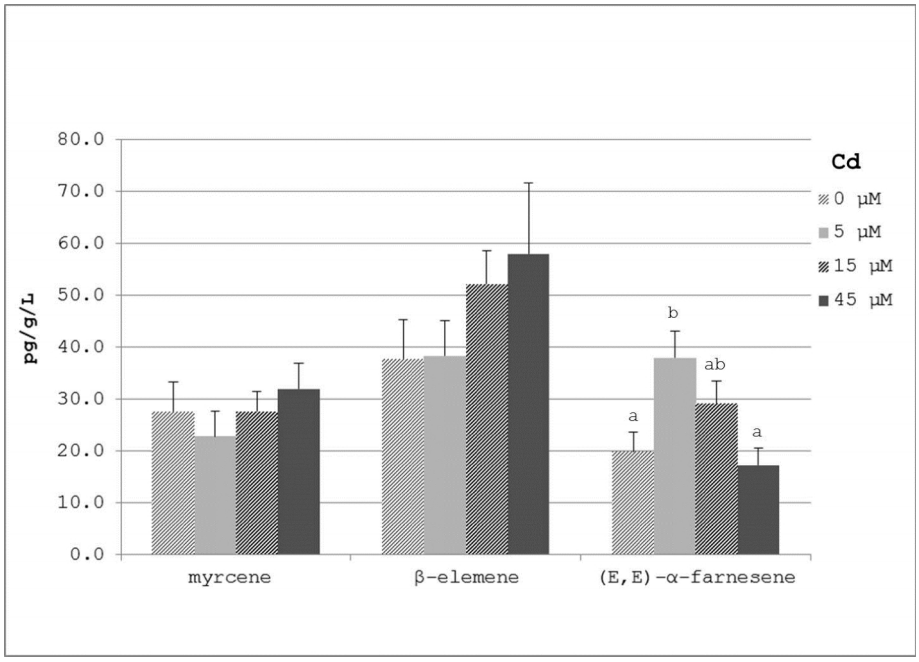

Figure 2.7: Graph of terpene emission rates (pg/g/L) for 28-day-old plantlets of *Brassica napus* L. var. *Es Astrid*. under different cadmium stress conditions (0 µM, 5 µM, 15 µM and 45 µM) and Tukey's *post hoc* test between means of emission rates (pg/g/L) for (E,E)-α-farnesene.

This may suggest that the sesquiterpene (E,E)-α-farnesene could be implicated in an elastic and reversible response allowing tolerance to low Cd stress conditions, such as in our experiment at 5 µM of Cd, where the plantlets showed no stress symptoms. This increase of (E,E)-α-farnesene (47.5%) at 5 µM could also represents a potential biomarker of Cd presence within the culture medium without biotic stress interaction. According to the literature, the sesquiterpene (E,E)-α-farnesene is also involved with stress related to biotic damage (Lin et al., 2016). A trend of elevation of myrcene and β-elemene emissions correlated to cadmium stress concentration can also be observed in Figure 2.7.

Some studies have reported similar results concerning a putative variation of emitted terpene under abiotic stress conditions (Himanen et al., 2009; Winter et al., 2012; Zhao et al., 2017). It seems to be clear that VOCs, especially terpenoids, mediate plants' resilience to abiotic stress related to heavy metals, but more research is needed. It would also be very interesting to perform other experiments using the developed system in order to recover plant volatiles using other substrate of plant growth such as soil.

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Chapter 3

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

Durenne, B., Druart, P., Blondel, A., Fauconnier, M-L. (2018). How cadmium affects the fitness and the glucosinolate content of oilseed rape plantlets. *Environ Exp Bot* 155, 185–194.

We found previously that (E,E)-α-farnesene emission rates showed clearly a Cd concentration effect. These results were achieved after targeting terpene emissions under cadmium stress using our inexpensive glass chambers system. We decided to investigate, using the same experimental conditions of Cd-stress, the glucosinolates (GSLs) profiles and contents of oilseed rape plantlets. It is known that secondary metabolites such as GSLs are involved in plant response to biotic stress, but can be significantly influenced by abiotic factors as well. *Brassica napus* L. produces large quantities of several GSLs both in seeds and at the vegetative stage. These sulfur-containing compounds seem to play an important role in cadmium stress tolerance within the *Brassicaceae* family probably due to a specific cross-talk between sulfur (S) primary and secondary metabolism.

Our research focused on the assessment of GSL profile and content in the roots and shoots of 28-day-old winter oilseed rape plantlets cultivated in sterile conditions using concentration gradients of 0, 5, 15 and 45 µM of cadmium. Briefly, a phenotypic analysis was carried out at the end of this experiment in order to evaluate the plantlets' fitness. Strong relationships were found between concentrations of Cd in roots or in shoots and the sulfur concentration in the different organs of the plantlets. A decrease of both indole and aliphatic GSL content associated with an increase of Cd and an increase of total sulfur concentration 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.

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

3.1 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 like 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 S., 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 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 due to considerable yields between 35 to 45kg ha^{-1} $year^{-1}$ depending on genetic, environmental and agronomic factors (Sthal 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., 2015) 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. 3.1).

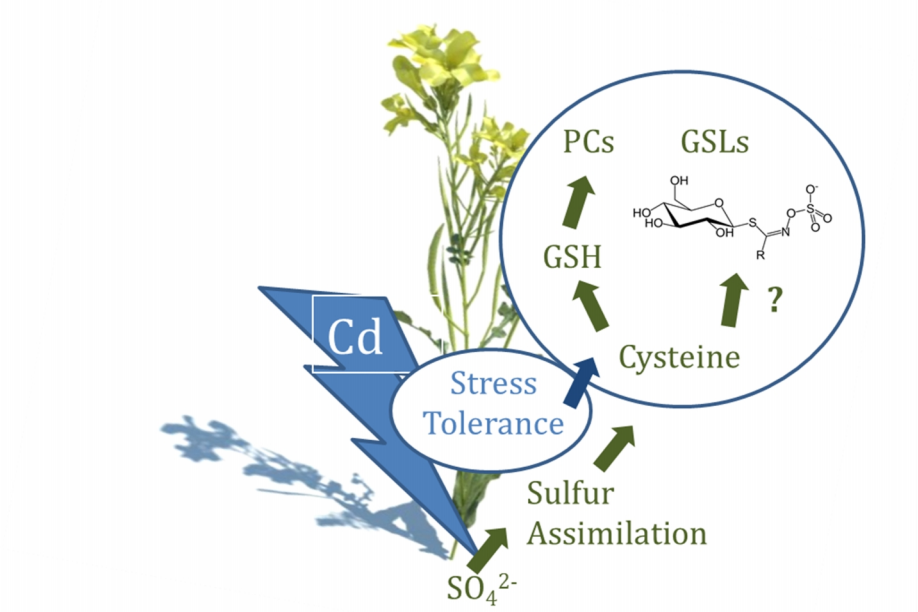

Figure 3.1: Schematic view of the putative role of glucosinolates (GSLs) in Cd stress tolerance for oilseed rape crop. (GSH: glutathione, PCs: phytochelatines).

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 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.

3.2 Materials and methods

3.2.1 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 16h, 45% relative humidity and 100 µmol of photons 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.

3.2.2 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 72h 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.

3.2.3 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^{-1} 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 3µm, 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.

3.2.4 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 contents (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).

3.2.5 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.

3.3 Results

3.3.1 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 symptom was observed for all plantlets stressed with 5µM of Cd. In contrast, Cd doses of 15 µM and 45 µM affected leaf morphology of all plantlets with sporadic chloroses and with visible growth retardation corroborated with obvious symptoms of pigment loss respectively (Fig. 3.2).

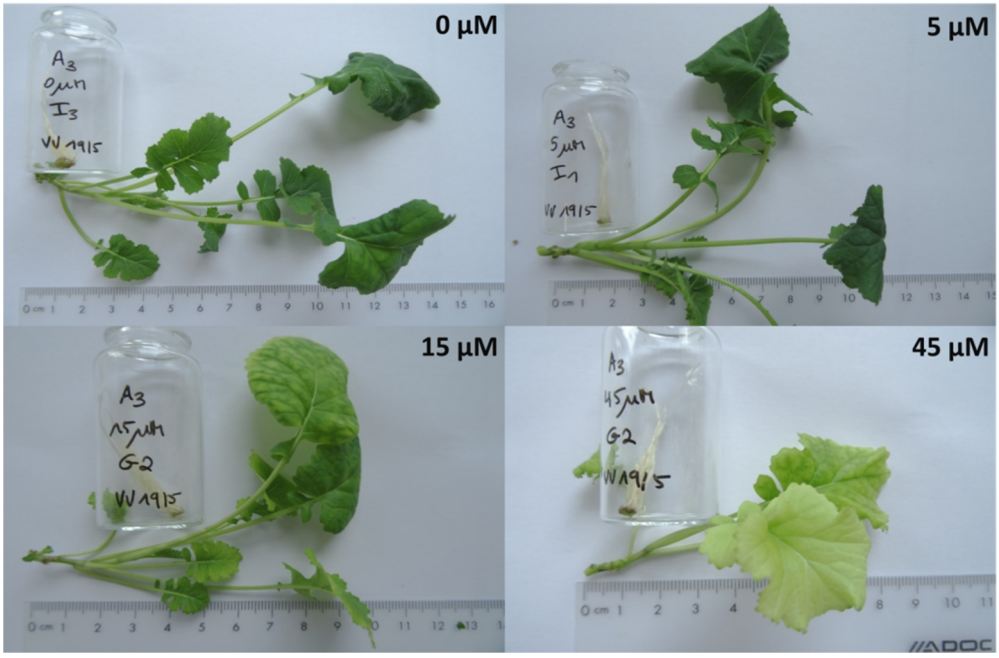

Figure 3.2: Pictures of 28-day-old oilseed rape plantlets under 0, 5, 15 and 45 µM Cd stress conditions. No symptom and perfect development were observed at 0 and 5 µM Cd. Sporadic chlorosis appeared on plantlet leaves at 15 µM and severe pigment loss with growth retardation were observed at 45 µM Cd.

In view of these observations, treatment with 5 µM of Cd could be considered non-toxic and with 45 µM of Cd 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. 3.3 a) and for root biomass (Fig. 3.3 b). 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 above than 5 µM for the growth analysis (Fig. 3.3 c) and simultaneously, an inverted U-shaped dose-response curve for shoot biomass (Fig. 3.3 d). 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.

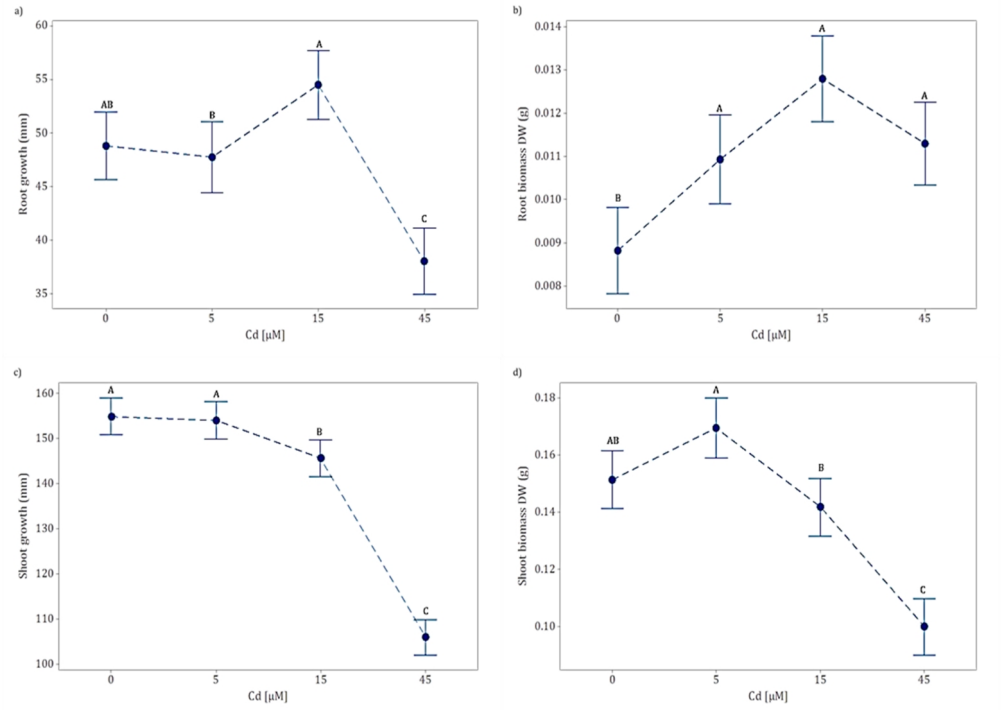

Figure 3.3: Graphs of the means and 95% confidence intervals of a) root growth (mm), b) root biomass (DW) (g), c) shoot growth (mm) and d) shoot biomass (DW) (g) for 28-day-old oilseed rape plantlets under the cadmium concentration gradient of 0, 5, 15 and 45 µM (n= 28, 26, 28, 30). Data were ranged after a *post hoc* Tukey's test.

3.3.2 Cadmium and sulfur accumulation in roots and shoots

The concentrations of Cd and total S 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 25th and 75th percentiles of the Cd accumulation in roots (Fig. 3.4 a) and of Cd translocated in shoots (Fig. 3.4 b). 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 S 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 S accumulation (Fig. 3.4 c). For the shoots of the plantlets, the boxplots of total sulfur accumulation showed more variability, but with an upward trend related to elevated Cd concentrations and particularly at 45 µM (Fig. 3.4 d). 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. 3.4 e) and shoots (Fig. 3.4 f) 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. 3.5).

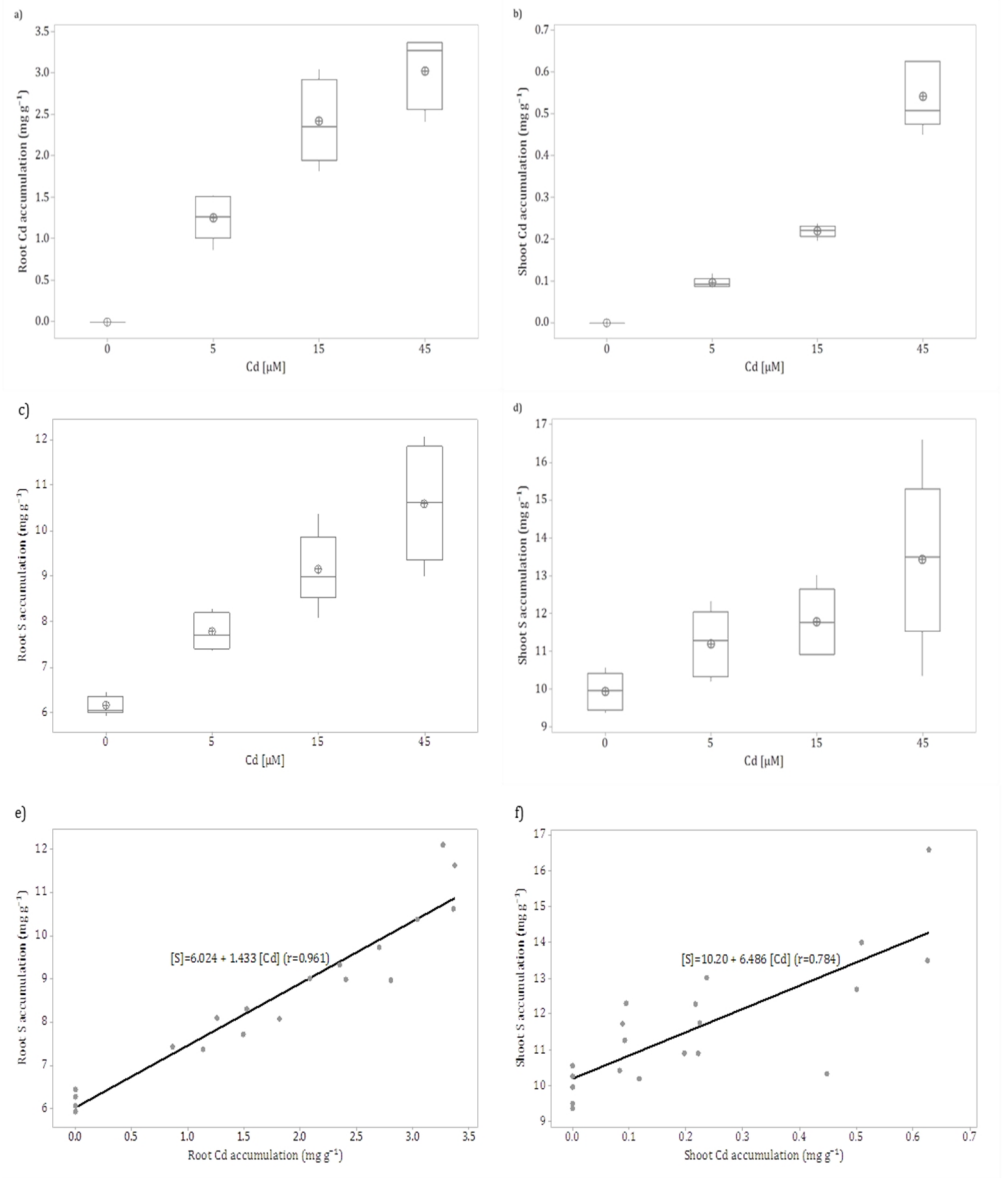

Figure 3.4: Boxplots (mean (⊕), median (line), 25th and 75th percentiles and representing outliers) of a) Cd accumulation (mg g^{-1}) in roots, b) Cd translocation in shoots, c) sulfur accumulation (mg g^{-1}) in roots and d) sulfur accumulation (mg g^{-1}) in shoots (n=5) and correlation between Cd 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.

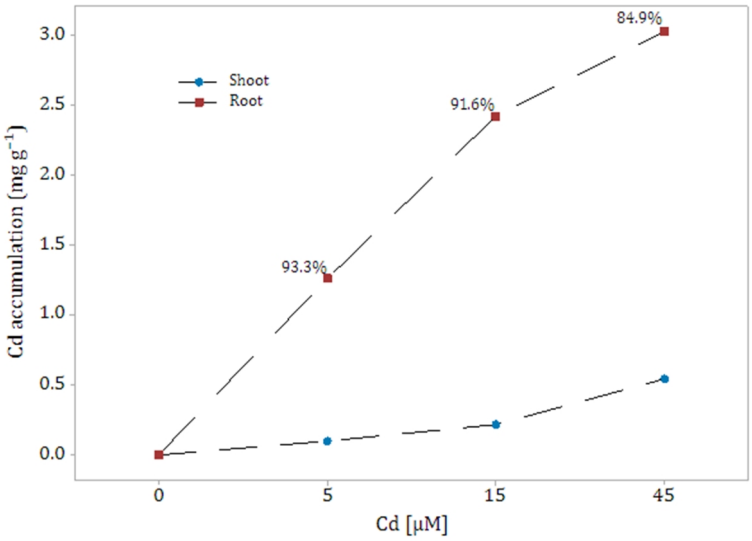

Figure 3.5: Graph of the mean values of Cd accumulated in roots and translocated to shoots (mg g^{-1}) of 28-day-old oilseed rape plantlets under the cadmium concentration gradient of 0, 5, 15 and 45 µM (n=5). The percentages represent the Cd proportion in roots in comparison with the total Cd accumulation in plantlets.

3.3.3 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 Figure 3.6 a) and b) respectively.

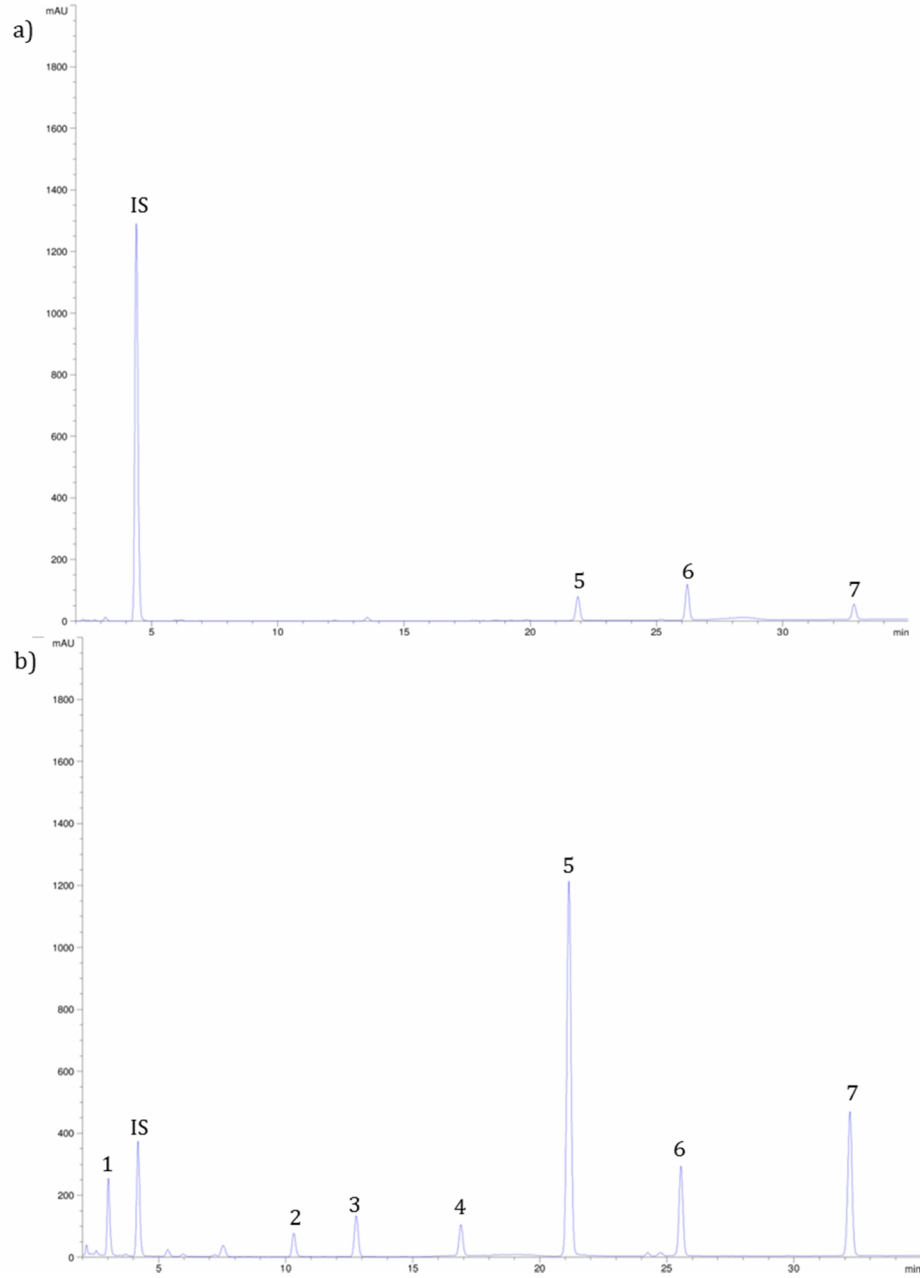

Figure 3.6: Typical chromatograms of the GSL profile obtained from unstressed 28-day-old oilseed rape plantlets for a) roots and b) shoots. IS: internal standard (sinigrin), 1: progoitrin (2OH3But), 2: gluconapin (3But), 3: 4-hydroxyglucobrassicin (4OHI3M), 4: glucobrassicanapin (4Pent), 5: glucobrassicin (I3M), 6: 4-methoxyglucobrassicin (4MOI3M), 7: neoglucobrassicin (1MOI3M). (mAU: micro absorbance unit).

The observation of the graphs of the GSL profiling for roots (Fig. 3.7 a) and for shoots (Fig. 3.7 b) showed that the mean value (µmol g^{-1} DW) of each GSL varied according to Cd concentration in the culture medium.

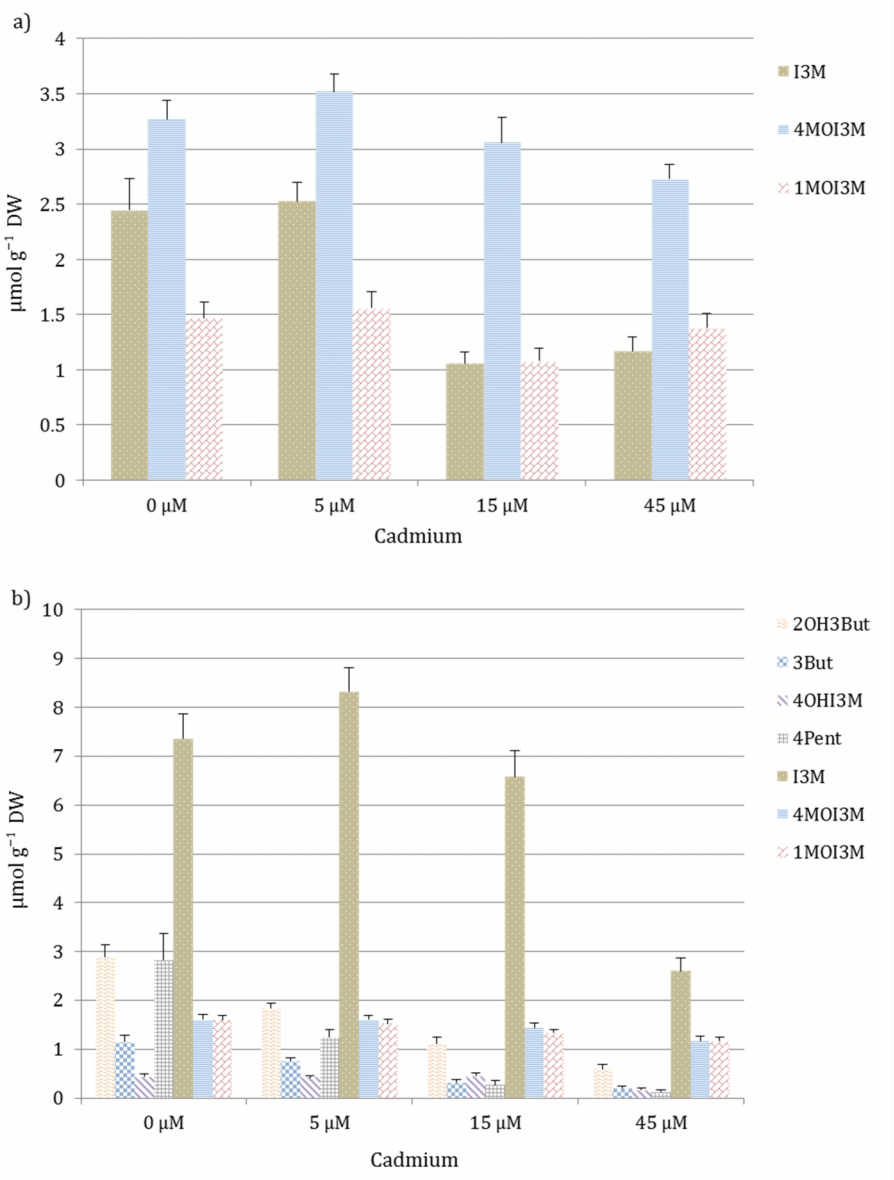

Figure 3.7: GSL profile and content (mean values ±SE) (µmol g^{-1} DW) for a) roots and b) shoots of the 28-day-old oilseed rape plantlets under the cadmium concentration gradient of 0, 5, 15 and 45 µM. (2OH3But: progoitrin, 3But: gluconapin, 4OHI3M: 4-hydroxyglucobrassicin, 4Pent: glucobrassicanapin, I3M: glucobrassicin, 4MOI3M: 4-methoxyglucobrassicin, 1MOI3M: neoglucobrassicin).

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 3.1. 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. 3.8), strong relationships were found between I3M and 1MOI3M contents in roots under the cadmium concentrations of 0, 5, 15 and 45 µM.

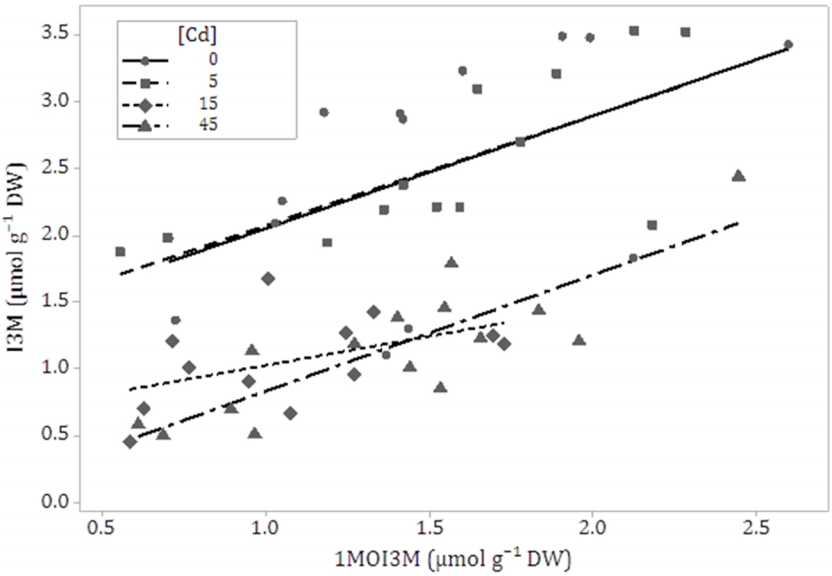

Figure 3.8: Interactions between I3M (glucobrassicin) (µmol g^{-1} DW) and 1MOI3M (neoglucobrassicin) (µmol g^{-1} DW) content in roots of 28-day-old oilseed rape plantlets under the cadmium concentration gradient of 0, 5, 15 and 45 µM.

Table 3.1: 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	2OH3But	3But	4OHI3M	4Pent
			***	*	nd				
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

3.4 Discussion

3.4.1 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. 3.3 a). The same observation was also made for shoot growth (Fig. 3.3 c), and this decrease of elongation was clearly associated with visible symptoms of typical whole leaf chlorosis at 45 µM (Fig. 3.2). 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. 3.3 a), for root and shoot biomass (Fig. 3.3 b and Fig. 3.3 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. 3.3 b) 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.

3.4.2 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., 2015). A more recent study has shown a similar subcellular distribution in soluble fraction in roots of two 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. 3.5). 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

3.4 a). Conversely, the translocation of Cd to shoots was hugely reduced, described by an extrapolated curve (Fig 3.4 b). 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.

3.4.3 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., 2015).

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 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. 3.4 e; Fig. 3.4 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.

3.4.4 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 (Kastel 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, CYP81F 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. 3.8).

In the roots in particular, we can suggest that sulfur uptake was increased at 15 and 45 µM of Cd, 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% to 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).

3.5 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 under field conditions.

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Chapter 4

Phenotyping of *Brassica napus* L. plantlets affected during *in vitro* growth in the presence of epoxiconazole

Durenne, B., Blondel, A., Ducat, N., Pigeon, O., Fauconnier, M-L., Druart, P. (2018). Phenotyping of *Brassica napus* L. plantlets affected during *in vitro* growth in the presence of epoxiconazole. *Acta Hortic* 1202: 101–106.

Epoxiconazole like other triazole fungicides are known to be persistent in soils. Furthermore, several studies using foliar application demonstrated the effect of its triazole metabolite as plant growth regulator through an anti-gibberellin activity. This chapter describes an *in vitro* experiment studying the relationship between epoxiconazole presence in culture medium (0 mg L^{-1}, 0.120 mg L^{-1} and 0.200 mg L^{-1}) and the phenotyping (root and shoot growth) of three varieties of winter oilseed rape (*Brassica napus* L. var. *Catalina*, var. *ES Astrid* and var. *Toccata*). Three different varieties of winter oilseed rape were used in order to test such effect of growth inhibition.

Plantlets fungicide content was quantified using the Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) extraction method following by an automated UHPLC-MS/MS analysis. Briefly, results showed that the shoot and root growth of *Brassica napus* L. plantlets was significantly inhibited by epoxiconazole at 0.120 mg L^{-1} independently of the variety. The concentration of 0.200 mg L^{-1} leaded to necroses and anthocyanoses and can be considered as lethal for *in vitro* growing explants. The huge epoxiconazole absorption by oilseed rape plantlets showed a dose-dependent relationship and was closely similar for the three varieties.

4. Phenotyping of *Brassica napus* L. plantlets affected during *in vitro* growth in the presence of epoxiconazole

4.1 Introduction

The epoxiconazole chemical substance, (2RS,3SR)-1-[3-(2-chlorophenyl)-2,3-epoxy-2(4-fluorophenyl) propyl]-1H-1,2,4-triazole, is a synthetic broad-spectrum triazole fungicide interfering with the biosynthesis of ergosterol, a fungal essential membrane component, by competitively inhibiting the enzyme lanosterol 14α-demethylase (Chambers et al., 2014). Foliar application to the field corresponding to the agronomic homologated dose of 125 g ha^{-1} is mainly used for preventive and curative actions regarding cereals, sugar beets, apple trees, oilseed rape and ornamentals (Liang et al., 2012; Lichiheb et al., 2015). The triazole fungicides exhibit long persistence in soil and especially, epoxiconazole with a half-life time greater than two years at 10°C and 80% of field capacity (Bromilow et al., 1999). Field accumulation studies showed a plateau concentration of 0.167 mg Kg^{-1} into soils (EFSA, 2008). Triazole-type compounds such as metconazole and tebuconazole are also frequently used on oilseed rape crop for both their plant growth regulatory effect and fungicidal properties (Berry and Spink, 2009). Experiments with foliar application of epoxiconazole at laboratory-scale clearly indicated that phytosterol biosynthesis is affected (Benton and Cobb, 1997) and that epoxiconazole reduces electron transport capability of thylakoids (Petit et al., 2012). A study in hydroponic conditions described a decrease of oilseed rape plant height and shoot biomass related to the use of a triazole type plant growth regulator (Bruns et al., 1990). In fact, triazole compounds show an anti-gibberellin activity by inhibition of the early steps of their biosynthesis at the stage of conversion of ent-kaurene to ent-kaurenoic acid (Rademacher, 2000; Yamaguchi, 2008). We decided therefore to set up experiments in order to test for a first time the *in vitro* phenotyping effect of epoxiconazole presence on plantlet growth of three varieties of winter oilseed rape (*Brassica napus* L. var. *Catalina*, var. *ES Astrid* and var. *Toccata*) and to evaluate the epoxiconazole absorption by the oilseed rape plantlets.

4.2 Materials and methods

4.2.1 Plantlets growth

The three winter oilseed rape varieties of *Brassica napus* L. var. *Catalina* (Dekalb, France), var. *ES Astrid* (Euralis semences, France) and var. *Toccata* (Maïsadour semences, France) germinated *in vitro* from certified seeds and micropropagated by axillary branching. Three explants were cultivated using *in vitro* home-developed container system (weck®). Plantlets (rooting and development of the standardized shoots) were obtained after 36-days of culture without plant growth regulator (medium composition: 400 mg L^{-1} NH_4NO_3; 800 mg L^{-1} KNO_3; 300 mg L^{-1} $Ca(NO_3)_2$; 180 mg L^{-1} $MgSO_4$; 150 mg L^{-1} KH_2PO_4; 1.5 mg L^{-1} $MnSO_4$; 0.5 mg L^{-1}

$ZnSO_4$; 3 mg L^{-1} H_3BO_3; 0.5 mg L^{-1} KI; 0.25 mg L^{-1} Na_2MoO_4; 20 mg L^{-1} EDTA*Na_2; 15 mg L^{-1} $FeSO_4$ supplemented with 3% sucrose and 0.5% agar). Plantlets were cultivated under controlled environmental conditions within a climate room at 23/18°C (day/night), using a 16h photoperiod, 40% relative humidity and 100 µmol of photons m^{-2} s^{-1}. In parallel to a control medium, epoxiconazole (Sigma-Aldrich, Diegem, Belgium) was added by filtration using 0.2 µm supor membrane syringe filter (nonpyrogenic AcrodiscR®) after autoclaving at a concentration of 0.120 mg L^{-1} and 0.200 mg L^{-1}. Experiment was performed in triplicate for each variety. Phenotyping corresponded to leaf symptoms observation and to plantlets height and roots length measurement.

4.2.2 Epoxiconazole extraction and liquid chromatography analysis

Extraction of epoxiconazole from plantlets was performed using the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method. The three whole plantlets were crushed together with a blender before making 2-g of minced sample weighed into a 50-mL centrifugation tube, homogenized with 5 mL of deionized water and macerated 30 minutes before adding of 10 mL acetonitrile. QuEChERS salts were added to perform phase-separation following by agitation and centrifugation (4500 r min^{-1}). The supernatant was filtered through a 0.20 µm filter (PTFE) into a sample vial for UHPLC-MS/MS analysis. The liquid chromatograph used was a Waters and separation was performed using a C18 column (50 mm x 2.1 mm x 1.7 µm) with mobile phase composed of a mixture of H_2O/methanol/formic acid (90:10:0.1, v/v/v) and methanol/formic acid (100:0.1, v/v) with an elution gradient (80-20%) at 35°C and at flow rate of 0.3 mL min^{-1}. An Acquity® (Waters) triple-quadrupole detector mass spectrometer equipped with an electrospray ionization source was used for MS/MS analysis. The transitions of precursor ion (m/z 330) to production (m/z 121 and 101) of epoxiconazole were detected with multiple reaction monitoring (MRM) in positive ion (ESI+) mode. The collision energies for m/z 121 and 101 were respectively 22 and 50 eV and the ion m/z 121 was used for quantification based on matrix-matched calibration curve obtained from linear regression of plotted area for respective epoxiconazole concentrations gradient (0.001-0.5 µg mL^{-1}).

4.3 Results and discussion

4.3.1 Phenotyping results

Epoxiconazole presence in the culture medium was clearly responsible of a visible effect on *Brassica napus* L. plantlets growth physiology and after 36 days of *in vitro* culture (Fig. 4.1). Root development was tremendously inhibited and it can be observed for 0.120 mg L^{-1} and 0.200 mg L^{-1} epoxiconazole concentrations both. The three winter oilseed rape varieties (var. *Catalina*, var. *ES Astrid* and var. *Toccata*) were also similarly affected by a reduction of plantlets internode certainly due to the triazole metabolite anti-gibberellin activity (Bruns et al., 1990; Berry and Spink, 2009). Phytotoxic symptoms such as leaf chlorosis and decreasing growth were found at 0.120 mg L^{-1} confirming the photosynthetic apparatus perturbation by

epoxiconazole (Petit et al., 2012). Finally 0.200 mg L^{-1} epoxiconazole concentration led to severe chloroses, anthocyanoses, mainly for var. *ES Astrid*. It could be considered as lethal in regards to harvested plantlets morphology and specially, for the inhibition of root system development.

Figure 4.1: Pictures of *Brassica napus* L. plantlets after 36 days of culture on control medium (a: var. *Catalina*, b: var. *ES Astrid*, c: var. *Toccata*); on medium with 0.120 mg L^{-1} of epoxiconazole (d: var. *Catalina*, e: var. *ES Astrid*, f: var. *Toccata*) and on medium with 0.200 mg L^{-1} of epoxiconazole (g: var. *Catalina*, h: var. *ES Astrid*, i: var. *Toccata*).

Visual interpretation of plantlets observation was corroborated with data obtained from phenotyping results (plantlets height and roots length) concerning the three winter oilseed rape varieties (var. *Catalina*, var. *ES Astrid* and var. *Toccata*) (Table 4.1).

Table 4.1: Phenotyping results (plantlet height and root length mean ±SE) of *Brassica napus L.* plantlets cultivated under 0, 0.120 mg L^{-1} and 0.200 mg L^{-1} of epoxiconazole (var. *Catalina*, var. *ES Astrid* and var. *Toccata*).

Epoxiconazole Concentration (mg L^{-1})	var. *Catalina*	var. *ES Astrid*	var. *Toccata*
Plantlet height mean[a] (mm)			
0	117±2	123±4	128±7
0.120	75±6	65±6	63±6
0.200	52±3	46±3	51±4
Root length mean[a] (mm)			
0	116±15	159±11	71±8
0.120	30±2	21±4	28±2
0.200	17±2	6±1	15±3

[a] : *n=9*

A significant decrease can be observed directly from 0.120 mg L^{-1} of epoxiconazole concentration regarding plantlets height and roots length means both, independently of the variety (Figure 4.2 a) and b) respectively).

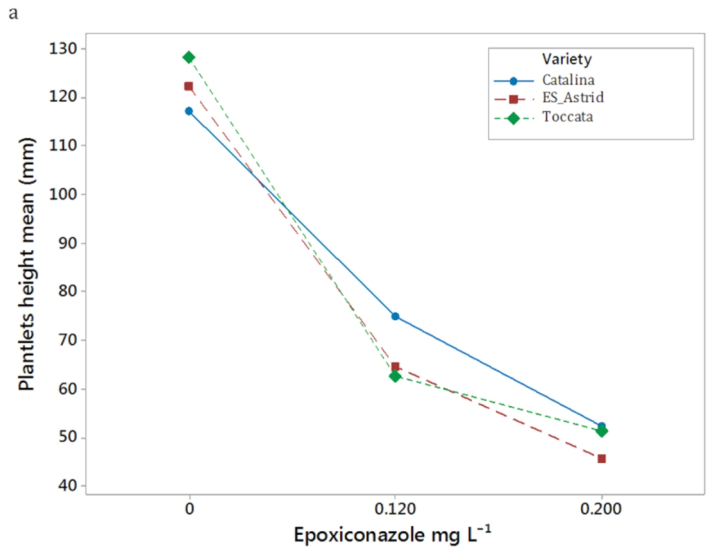

Figure 4.2 a): Phenotyping results (mean of plantlet heights) for *Brassica napus* L. (var. *Catalina*, var. *ES Astrid* and var. *Toccata*) plantlets cultivated with 0, 0.120 mg L^{-1} and 0.200 mg L^{-1} of epoxiconazole (n=9) (36 days of culture).

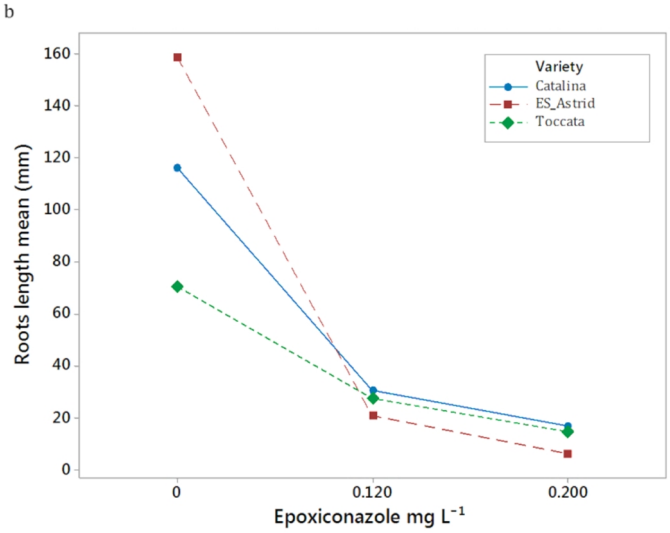

Figure 4.2 b): Phenotyping results (mean of root lengths) for *Brassica napus* L. (var. *Catalina*, var. *ES Astrid* and var. *Toccata*) plantlets cultivated with 0, 0.120 mg L^{-1} and 0.200 mg L^{-1} of epoxiconazole (n=9) (36 days of culture).

4.3.2 Epoxiconazole concentration

UHPLC-MS/MS analysis of epoxiconazole content of the *Brassica napus* L. plantlets showed an obvious dose-dependent relation between its level in the culture medium and its absorption (Figure 4.3). Means (±SE) of epoxiconazole absorption (µg g^{-1}) were closely similar for the three varieties respectively (var. *Catalina*, var. *ES Astrid* and var. *Toccata*): 0.59 (±0.08), 0.57 (±0.03), 0.61 (±0.01) for 0.120 mg L^{-1}; 1.30 (±0.05), 1.6 (±0.07), 1.6 (±0.17) for 0.200 mg L^{-1}. It could be concluded in comparison with phenotyping results that epoxiconazole plantlets absorption lead to severe stress physiology from 0.6 µg g^{-1}. Liquid chromatography research and development could help to evaluate in the future the epoxiconazole metabolisation rate into triazole compounds for a better understanding of how the morphology can be affected resulting in such symptoms.

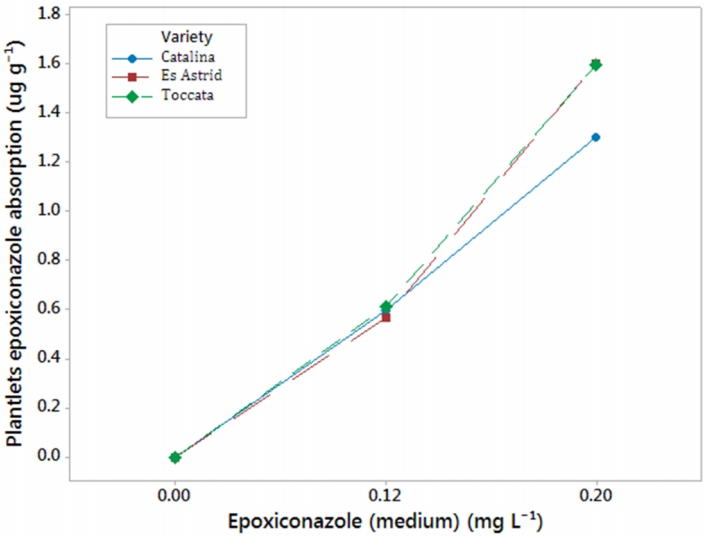

Figure 4.3: Graph of epoxiconazole absorption mean (n=3) in the plantlets of *Brassica napus* L. (var. *Catalina*, var. *ES Astrid* and var. *Toccata*) cultivated with 0, 0.120 mg L^{-1} and 0.200 mg L^{-1} of epoxiconazole during 36 days.

4.4 Conclusions

The experiments performed with *in vitro* grown *Brassica napus* L. explants (var. *Catalina*, var. *ES Astrid* and var. *Toccata*) led to the following conclusions: i) confirmation that the presence of epoxiconazole affected clearly the plantlets growing physiology within sterile and controlled conditions, ii) decreasing growth (plantlet height), inhibition of the roots development and severe chloroses were observed at 0.120 mg L^{-1} of epoxiconazole and iii) epoxiconazole at 0.200 mg L^{-1} can be considered to be a lethal concentration. It will be interesting to confirm these observations with future experiments using oilseed rape cultivated on artificially treated substrate to evaluate putative phytotoxicity related to triazole fungicides persistence (Bromilow et al., 1999; Liang et al., 2012) and in the frame of multiple abiotic stress conditions.

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Chapter 5

Smelling the stress of *Brassica napus* L. plantlets exposed to epoxiconazole residues using TD-GC-MS through a targeted approach

Durenne, B., Blondel, A., Druart, P., Fauconnier, M-L. Smelling the stress of *Brassica napus* L. plantlets exposed to epoxiconazole residues using TD-GC-MS through a targeted approach. *Environ Sci Pollut Res* (submitted on 5th October 2018).

We demonstrated previously that epoxiconazole, a broad-spectrum fungicide described as highly persistent in soil, can be considered as an abiotic agent disturbing the physiology of *Brassica napus* L. plantlets. Shoot and root growth of the plantlets was significantly inhibited by epoxiconazole presence in the culture medium. Furthermore, the non-invasive monitoring of plant-emitted VOCs may be useful for identification of putative abiotic stress markers. While we demonstrated that terpenes emitted could be linked to Cd-stress response, we decided to profile oilseed rape VOCs through a dose-response experiment under several epoxiconazole concentrations (0, 0.01, 0.1 and 1 mg L^{-1}).

VOC collections of 35-day-old plantlets were performed under defined and controlled conditions using our high-throughput glass chambers system and dynamic sampling technique. The perlite was used as growing substrate in order to mimic plant-soil interactions. Briefly, chromatograms of emitted terpenes were achieved accurately for the 35-day-old oilseed rape plantlets. We also demonstrated through this experiment that sesquiterpenes such as β-elemene and (E,E)-α-farnesene are involved in epoxiconazole plant dose-response. Finally, we analysed the presence of sulfur-containing volatiles in samples of shoot and root tissues, but no difference was found between qualitative profiles.

5. Smelling the stress of *Brassica napus* L. plantlets exposed to epoxiconazole residues using TD-GC-MS through a targeted approach.

5.1 Introduction

Pesticides are widely used compounds in farming and can reach the soil through rain, irrigation water and wind when they are applied to crops (Marican and Durán-Lara 2018). Some pesticides such as triazole fungicides persist in soil and sediments due to low bioavailability. This is especially true for epoxiconazole which has a half-life time of more than two years (at 10°C and 80% of field capacity) (Bromilow et al. 1999). Epoxiconazole is a synthetic broad-spectrum fungicide interfering with the biosynthesis of the steroid ergosterol, an essential membrane component of yeast and fungi, by competitively inhibiting the enzyme lanosterol 14 α-demethylase (Chambers et al. 2014). Low application rates of 25-125 g ha^{-1} of epoxiconazole's active substance are highly effective for the control of diseases caused by Ascomycetes, Basidiomycetes and Deuteromycetes through foliar application in cereals, rice, grapes and other crops worldwide such as oilseed rape (Liang et al. 2012). This curative and preventive fungicide is therefore extensively used, but strict requirements in line with good agricultural practices must be adhered to respect maximum residue levels (MRLs) in plants and soil (Yan et al. 2015). One of the most important metabolites of this fungicide in soil is 1,2,4 triazole, which is rapidly degraded by soil micro-organisms with low persistence (EFSA 2008; Blondel et al. 2018).

Oilseed rape (*Brassica napus* L.), belonging to the *Brassicaceae* family, is an allotetraploid crop species resulting from a natural hybridisation of the diploid species *B. oleracea* and *B. rapa* (Chalhoub et al. 2014). Global oilseed rape production has tremendously increased in the last decade and these oil-rich seeds are processed into edible oil, biodiesel and high-quality animal feed (Derbyshire and Denton-Giles 2016). In addition to its use against *Sclerotinia sclerotorium* to prevent the annual oilseed rape yield losses caused by this fungus, epoxiconazole can also regulate plant growth (Li et al. 2015). It is well known that triazole compounds are involved in the inhibition of gibberellin biosynthesis at the stage of conversion of *ent*-kaurene to *ent*-kaurenoic acid (Rademacher 2000; Yamaguchi 2008). Several publications have also described this plant growth regulatory effect on oilseed rape crops after foliar application or uptake by roots (Bruns et al. 1990; Berry and Spink 2009; Durenne et al. 2018). The metabolism of epoxiconazole in plants using foliar application is limited but a significant uptake of some triazole derivative metabolites (triazole alanine and triazole acetic acid) has been demonstrated for cereals (EFSA 2008). Although detoxification mechanisms of pesticide residues have been widely studied in mammalian cells, the regulation network in plants remains elusive (Zhou et al. 2015). Recent research described that crop plants seem to be able to detoxify

adsorbed pesticide residues through a system including enzymes, gluthatione (GSH) and sequestration in the vacuole (Shahzad et al. 2018).

Agrochemical products commonly used in agriculture could be investigated as such an abiotic factor with metabolomic study purposes (Kráľová et al. 2012). Metabolites profiling related to plants' pesticide response is also becoming increasingly common in ecotoxicological risk assessment, as a means of investigating the modes-of-action of bioactive substances and discovering new compounds (Aliferis and Chrysayi-Tokousbalides 2011). Petersen et al. (2011) have also discussed the putative use of environmental metabolomics to detect *Brassica napus* L. exposure to glyphosate. From an analytical point of view, gas and liquid chromatography coupled with mass spectrometry (GC/LC-MS) through a non-targeted full-scan approach or a targeted approach using selected-ion monitoring (SIM) are both cutting-edge technologies, not to mention nuclear magnetic resonance (NRM) spectrometry. NMR-based methods have been used to describe some changes in plant metabolite content and composition for *Agrostis capillaris* and *Arabidopsis thaliana* after epoxiconazole exposure (Standberg et al. 2013) and have recently be used to monitor plant metabolic changes in association with pesticide exposure in major crops such as maize (Blondel et al. 2016). However, NMR-screening is expensive and time-consuming. Principal component analysis (PCA) is frequently needed to obtain response patterns as putative indicators. Methods based on GC-MS and derivatisation were recently used to study the metabolomic profile of rice under pesticide stress through a pseudotargeted approach (Zhao et al. 2015) and to show the integration of *Lolium perenne* metabolic responses after exposure to glyphosate and tebuconazole (Serra et al. 2015). Derivatisation protocols remain also time-consuming involving additional sample handling and chemical steps (Jorge et al. 2016). In order to identify specialised metabolites as markers of abiotic stress response as accurately as possible, technologies such as GC or LC-MS must be used with a strong emphasis on secondary metabolism (Nakabayashi and Saito 2015). Finally, the SIM mode available with mass spectrometry can be performed at the same time as a full-scan and can greatly help to target metabolites of interest (Delory et al. 2016).

Secondary metabolism, especially terpenoids and glucosinolates within *Brassica* spp., is clearly identified to play a crucial role in tolerance to environmental stresses resulting from agriculture challenges (Rodziewicz et al. 2014). Some secondary metabolites such as volatile organic compounds (VOCs) are emitted from plants and, represent a specific and non-invasive way to phenotype plants' response to abiotic stress (Niederbacher et al. 2015). This is particularly true if the data can be obtained from a high-throughput system with high repeatability in order to describe the most representative state of the plant's metabolism in response to environmental stressors. Volatile isoprenoids are well known to be involved in abiotic stress (Vickers et al. 2009), and terpenes in particular can represent an attractive target as a marker of adaptive response to abiotic stress (Loreto and Schnitzler 2010; Durenne et al. 2018). Specialised terpenoids have fundamental functions in plants' growth

and development, coupled with roles in their environmental interaction (Tholl 2015). In addition, sulfur-containing volatiles such as nitriles, epithionitriles and isothiocyanates (ITCs), representing well-known breakdown products of glucosinolates (GSLs), are frequently mentioned in investigations of oilseed rape's volatile response to biotic stress (van Dam et al. 2012). They are not normally emitted by oilseed rape in response to abiotic factors, but can be analysed using the GC-MS method and dynamic headspace (DHS) sampling after flash-freezing of the plant tissue with liquid nitrogen. Such a technique can be used to profile the metabolites in plant tissues by stopping metabolic processes in cells through the use of very low temperatures (Jorge et al. 2015; Delory et al. 2016; Gemperline et al. 2016). We therefore decided to investigate the volatile response of *Brassica napus* L. plantlets under several concentrations of epoxiconazole using a thermal desorption and gas chromatography-mass spectrometry (TD-GC-MS) method with a targeted approach, based on SIM mode acquisition data and focusing on terpenes and sulfur-containing volatiles. The dose-response experiment was performed under controlled and defined conditions using perlite substrate in order to highlight putative metabolic markers for oilseed rape as indicators of fungicide exposure.

5.2 Materials and methods

5.2.1 Plant material and growth conditions

The winter oilseed rape plantlets were grown from germinated certified seeds of *Brassica napus* L. var. *Es Astrid* (Euralis semences, France). Seeds were surface-sterilised in 70% ethanol for 1 min, followed by immersion in calcium hypochlorite (7% W/V) for 45 min, rinsed 2 times with sterile water for 15 min and sown in Petri dishes (100 x 15 mm) with distilled water in order to germinate. Two standardised seedlings were transferred to a home-made glass cuvette system previously described (Durenne et al. 2018) containing sterile perlite substrate with the addition of 40 mL of a modified Hoagland's nutrient solution (590 mg L^{-1} $Ca(NO_3)_2$; 70 mg L^{-1} KH_2PO_4; 250 mg L^{-1} KNO_3; 750 mg L^{-1} $MgSO_4$; 0.1 mg L^{-1} $ZnSO_4$; 0.8 mg L^{-1} $MnSO_4$; 1.5 mg L^{-1} H_3BO_3; 0.1 mg L^{-1} $CuSO_4$ and 65 mg L^{-1} Fe-EDTA). The plantlets were cultivated for 35 days in a climate room equipped with LED lighting (Valoya L28 Spectrum NS12 Clear), at 23/18 °C (day/night), with a photoperiod of 16 h, 45% relative humidity and 130 µmol of photons m^{-2} s^{-1} of PAR. The plantlets were watered every three days with 5 mL of the nutrient solution under sterile conditions.

5.2.2 Epoxiconazole dose-response experiment

Analytical standard epoxiconazole, LC-MS grade 99% (Sigma-Aldrich, Darmstadt, Germany), was diluted in purified water Milli-Q (Millipore, Bedford, USA) to obtain a stock solution of 4 mg L^{-1} that was stored in the dark at 6°C. For the dose-response experiment, the fungicide was added to the initial nutrient solution volume (40 mL) in order to achieve final epoxiconazole concentrations of 0, 0.01, 0.1 and 1 mg L^{-1}. Each concentration of epoxiconazole was tested in triplicate on

two oilseed rape plantlets per cuvette system (Fig. 5.1). A blank consisting of a plant-free glass cuvette (containing only perlite substrate and nutrient solution) and an empty cuvette was also included in the dose-response experiment.

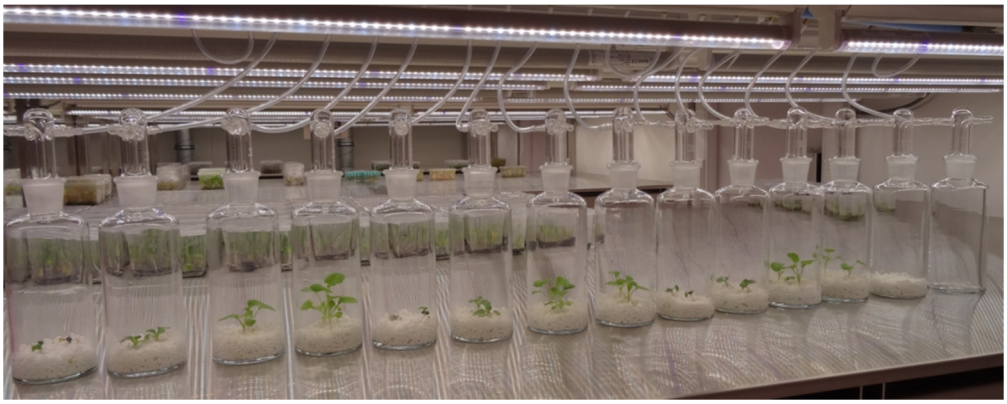

Figure 5.1: Experimental set-up to study epoxiconazole dose-response with two oilseed rape plantlets (at the 21-day-old stage) with customised cuvette system using perlite substrate.

5.2.3 Phenotyping of plantlets

At the end of the experiment, oilseed rape plantlets were gently harvested from the glass chamber system for physiological and biochemical analysis. The roots were carefully immersed in tap water to remove perlite substrate, rinsed with distilled water and wiped with tissues. Phenotyping consisted of plant observation at each concentration of epoxiconazole, and a picture of each plantlet was taken using the DSC-HX50™ (Sony, Belgium). The fresh weight biomass (g), the length of the shoot (cm) and the length of greatest root (cm) of each 35-day-old *Brassica napus* L. plantlet were measured and recorded. Shoot and root samples were obtained by cutting plantlets with a scalpel and were carefully stored at -25°C before further analysis.

5.2.4 Collection and quantitation of terpenes emission

Volatile terpenes from the 35-day-old oilseed rape plantlets were analysed and quantitated according to the non-destructive TD-GC-MS method, fully described in Durenne et al. 2018. Briefly, terpenes were trapped for 24 hours on Tenax® TA adsorbent cartridges that were desorbed with a thermal desorption unit before cryofocusing with a CIS/PTV into an HP-5ms GC column. The terpene detection and quantitation from chromatogram profiles were acquired with SIM mode using the most representative m/z 93 ion during full-scan analysis. The mass spectra were obtained with a quadrupole-type mass spectrometer. Identification of emitted terpenes was performed by comparing the data with a Wiley 275 mass spectral database and further confirmed by comparison to retention times and fragmentation patterns of commercially available analytical standards for sabinene, myrcene, β-elemene and (E,E)-α-farnesene (Sigma-Aldrich, Diegem, Belgium). Retention

indices were also calculated using a saturated n-alkanes (C7 – C30) standard solution (Sigma-Aldrich, Diegem, Belgium). Single-ion peaks of m/z 93 with relative abundance of sabinene (25.44%), myrcene (23.03%), β-elemene (7.29%) and (E,E)-α-farnesene (9.45%) respectively were integrated and compared with the equivalent single-ion response of 1 µL of hexane solution containing an internal standard of octylbenzene (0.58 mg mL^{-1}) (2.69%) (Sigma-Aldrich, Diegem, Belgium). The terpenoid emission rate was calculated as pg g^{-1} L^{-1} of fresh weight plantlet and air extracted.

5.2.5 Analysis of sulfur-containing volatiles in plantlet tissues

Sulfur-containing volatiles contained in plant organs (not emitted) were analysed at the end of the dose-response experiment in the shoot and root tissues respectively. Shoot and root samples of 35-day-old oilseed rape plantlets were frozen in liquid nitrogen before being pulverised in a mortar. The root and shoot powders were placed in a 20 mL glass vial supplied with a silicone/PTFE septum (FilterService, Eupen, Belgium), and stored at -80°C before automated DHS-TD-GC-MS analysis. The sulfur-containing volatiles were collected using a DHS system (Gerstel, Mülheim an der Ruhr, Germany) during an incubation time of 2 min at 23°C under constant agitation (500 rpm). They were trapped on Tenax TA cartridges with a 500 mL volume of trapping phase and using a helium flow rate of 20 mL min^{-1}. Finally, VOCs were thermally desorbed with a TDU (Gerstel, Mülheim an der Ruhr, Germany) running in splitless mode from 40°C to 120°C (110°C min^{-1}) for 2 min in order to prevent thermal degradation, and then at 280°C (200°C min^{-1}) for 5 min. Cryofocusing with a programmable temperature vaporising inlet was performed at -30°C before injection into the GC column by heating the CIS/PTV inlet to 260°C for 5 min at a rate of 12°C s^{-1}. VOC separation was performed using gas chromatography (7890A; Agilent Technologies, Palo Alto, CA, USA), with an HP-5ms capillary column (30 m length x 0.25 mm internal diameter x 0.25 µm film thickness; Agilent Technologies, Palo Alto, CA, USA). High-purity helium (Air Liquide, Liège, Belgium) was used as the carrier gas at a constant flow of 1.6 ml/min. The oven temperature programme started at 40°C with increasing at a rate of 10°C min^{-1} to 65°C, then of 5°C min^{-1} to 90°C and then 20°C min^{-1} to 300°C with finally, 5 minutes at this temperature. VOC detection was performed using a quadrupole-type mass spectrometer (MS 5975C; Agilent Technologies, Palo Alto, CA, USA). Mass spectra were obtained using electron impact mode (70 eV) and operated in SCAN mode with a range of 35 to 450 amu for m/z ratios. Accurate profiles of sulfur-containing volatiles were obtained using SIM mode targeting the most representative 72 m/z ion in the same full-scan run of 23 min. GC-MS data were analysed using the Agilent MSD Chemstation E 02.00.493 (Agilent Technologies, Palo Alto, CA, USA). Because no commercial analytical standard was available, a tentative compound identification was performed by comparing the data with a Wiley 275 mass spectral database, with the database of the National Institute Standard and Technology (NIST08) which consists of more than 62000 patterns, and with previously published mass spectral data (m/z and relative

abundance) (Al-Gendy and Lockwood 2003; Taveira et al. 2009; Hong and Kim 2013).

5.2.6 Statistical analysis

All statistical analyses were carried out with Minitab® package version 17 and all data sets were tested for normality and equality of variances. Phenotypic results of shoot and root growth (cm) for 35-day-old oilseed rape plantlets were analysed using one-way analysis of variance (ANOVA). One-way ANOVA was also used to test the impact of the epoxiconazole concentration factor on sabinene, myrcene, β-elemene and (E,E)-α-farnesene emission rates. This analysis was followed by a *post hoc* Tukey's range test to find significant differences among pairwise means at a 0.05 level of probability. The values are reported as means with standard error for all results.

5.3 Results and discussion

5.3.1 Phenotypic results of 35-day-old oilseed rape plantlets

It was apparent that a progressive plantlet growth inhibition was found at the end of the dose-response experiment for each concentration of epoxiconazole (0, 0.01, 0.1 and 1 mg L^{-1}) which was tested in triplicate using perlite substrate (Fig. 5.2).

Figure 5.2: 35-day-old oilseed rape plantlets at the end of the epoxiconazole dose-response experiment with each concentration tested in triplicate: 0, 0.01, 0.1 and 1 mg L^{-1}.

Boxplots showing the mean, median, outliers and 25th and 75th percentiles of shoot and root growth have confirmed the morphological responses of growth inhibition with a dose-dependent pattern (Fig. 5.3 a) and b). In addition, one-way ANOVA showed that epoxiconazole significantly affects shoot growth (cm) ($F_{(3.23)} = 249.18$, $P < 0.001$) and significantly affects root growth (cm) ($F_{(3.23)} = 17.43$, $P < 0.001$)

measured from the 35-day-old oilseed rape plantlets. In our experimental conditions, the concentration of 0.1 mg L^{-1} corresponds to a subtoxic condition test and, the concentration of 1 mg L^{-1} corresponds to the dose where no plantlet can normally grow. The concentration of 1 mg L^{-1} was therefore disregarded for the further analysis of volatiles induced by epoxiconazole. Berry and Spink (2009) have previously described anti-gibberellin activity of triazole compounds affecting the growth of oilseed rape and these compounds can be also used for their fungicidal and regulatory properties. Recently, a field experiment showed also that nine triazole and strobilurin fungicides significantly influenced the plant height and green area index of winter oilseed rape (*Brassica napus* L.) (Ijaz and Honermeier 2012). The presence of epoxiconazole, a well-known soil-persistent systemic fungicide, in the rhizosphere of *Brassica napus* L. was also demonstrated to act as a plant growth regulator and in excess in agar medium, severe stress symptoms such as chlorosis and anthocyanosis can also occur (Durenne et al. 2018).

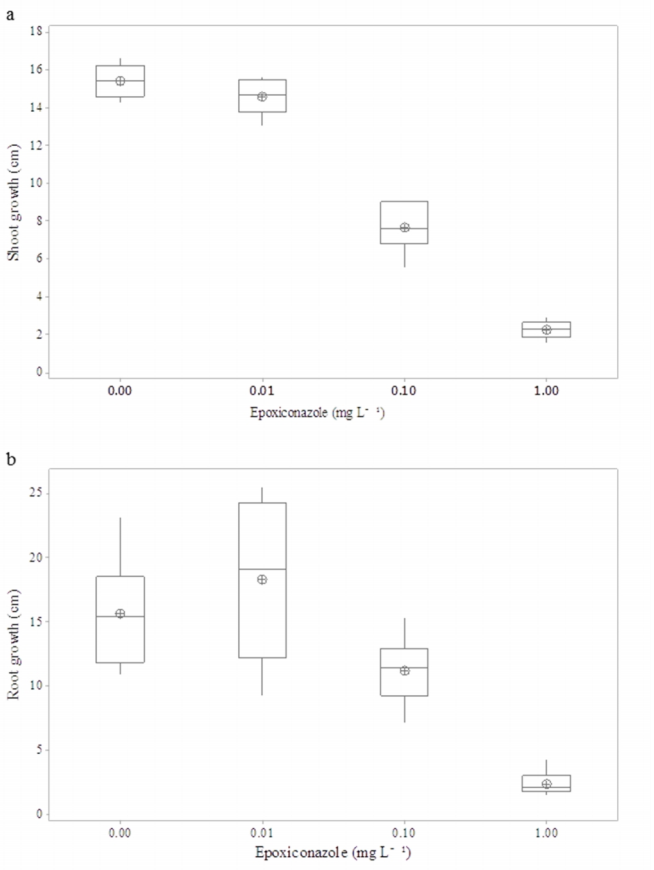

Figure 5.3: Boxplots (showing mean (⊕), median (line), 25th and 75th percentiles and outliers) of a) shoot growth and b) root growth for 35-day-old oilseed rape plantlets under different concentrations of epoxiconazole (0, 0.01, 0.1 and 1 mg L^{-1}) (n=6).

5.3.2 Volatile terpenes and epoxiconazole exposure

At laboratory-scale, foliar application of epoxiconazole on *Galium aparine* L. can affect phytosterol profiles and modify photosynthetic electron transport (Benton and Cobb 1997; Petit et al. 2012). To our knowledge, there is no scientific information about terpene emission related to fungicide exposure, and the influence of pesticide residues on oilseed rape plant metabolome is as yet poorly documented. We therefore used the GC-MS technique and our sampling method to compare differences in VOCs emitted by the 35-day-old oilseed rape plantlets for each concentration of epoxiconazole tested (0, 0.01 and 0.1 mg L^{-1}). We first investigated the data of the full-scan chromatogram, but this yielded no reliable evidence. Typical chromatograms of a blank (a plant-free glass cuvette containing only perlite substrate with the nutrient solution) and terpenes emitted by the 35-day-old oilseed rape plantlets were therefore achieved using selected-ion monitoring (SIM) mode (m/z 93) (Fig. 5.4 a) and b). Except obviously at 1 mg L^{-1}, the two plantlets significantly emitted three monoterpenes (sabinene, myrcene and limonene) and two sesquiterpenes (β-elemene and (E,E)-α-farnesene). These results were consistent with previously published data relating to oilseed rape terpene emission at vegetative stage and under abiotic stress (Veromann et al. 2013; Durenne et al. 2018). No difference was found between qualitative profiles of terpenes at the different concentrations of epoxiconazole tested.

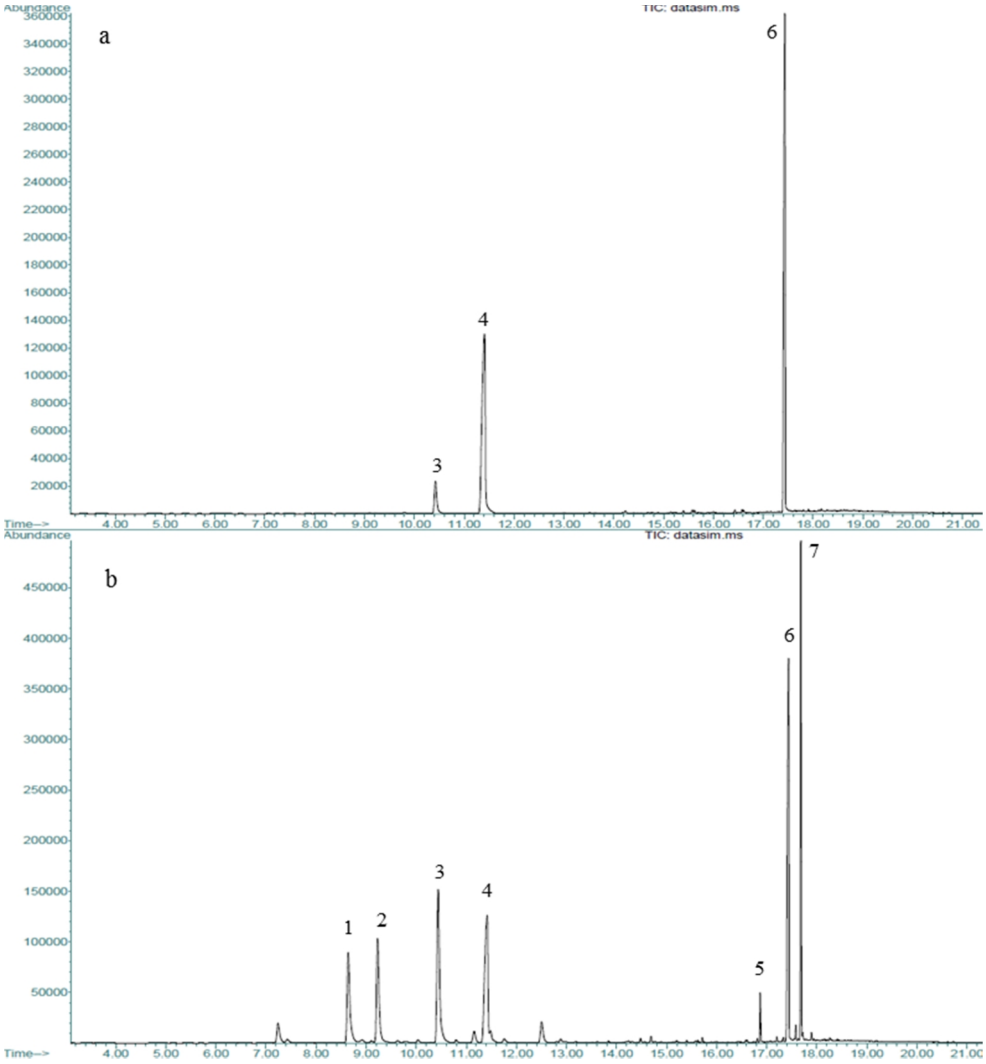

Figure 5.4: Typical chromatograms achieved using SIM mode (m/z 93) of a) blank and b) terpenes emitted by the two 35-day-old plantlets of oilseed rape. Peak identification: 1: sabinene 2: myrcene, 3: limonene, 4: n-butyl benzene (IS) not used, 5: β-elemene, 6: octylbenzene (IS), 7: (E,E)-α-farnesene.

We decided to quantitatively investigate the terpene response under epoxiconazole exposure in order to identify any induced emission. Limonene results were disregarded because very small amounts were found in blank tests. One-way ANOVA followed by a *post hoc* Tukey's range test showed no difference between means of emission rates (pg g^{-1} L^{-1}) for the two monoterpenes sabinene and myrcene but showed differences between means of emission rates for two

sesquiterpenes β-elemene and (E,E)-α-farnesene ($F_{(2,8)} = 32.69$, $P < 0.001$ and $F_{(2,8)} = 8.64$, $P < 0.05$, respectively) (Fig. 5.5).

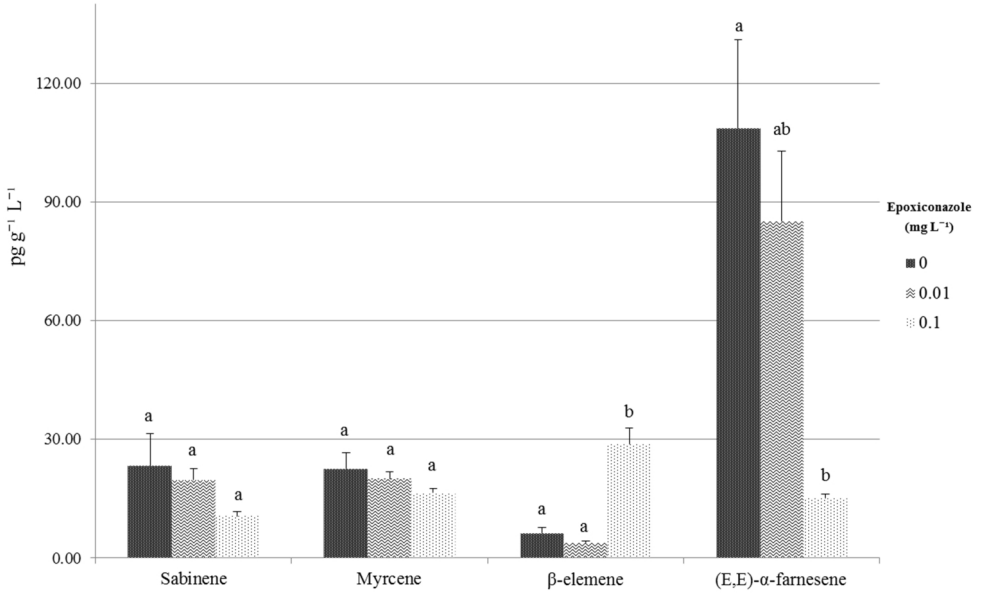

Figure 5.5: Graph of means (±SE) of terpene emission rates (pg g^{-1} L^{-1}) for 35-day-old plantlets of oilseed rape and Tukey's *post hoc* test between means for sabinene, myrcene, β-elemene and (E,E)-α-farnesene at 0, 0.01 and 0.1 mg L^{-1} of epoxiconazole (n=3).

As can be observed on the graph, the ranges of β-elemene and (E,E)-α-farnesene depended on the concentration of epoxiconazole in the perlite substrate, with a tremendous increase for β-elemene at 0.1 mg L^{-1} and a dose-dependent decrease for (E,E)-α-farnesene. Monoterpenes and sesquiterpenes are synthesised via two distinct and interconnected isoprenoid pathways (Vickers et al. 2009, Tholl 2015). It seems clear from this dose-response experiment that sesquiterpenes are more influenced by epoxiconazole exposure and that (E,E)-α-farnesene emission is particularly affected. Further investigations are needed, possibly involving testing several concentrations of epoxiconazole in subtoxic conditions in order to identify any crosstalk between related pathways of (E,E)-α-farnesene synthesis and the control of elongation growth by gibberellins (Davidson et al. 2006; Yamagushi 2008). It is known that volatile terpenes are involved in abiotic stress response (Loreto and Schnitzler 2010), and we can conclude that pesticide residues also affect the volatilome of oilseed rape plantlets through an adaptive emission. We also demonstrated that they could possibly serve as metabolic markers of fungicide exposure, but this should be confirmed in association with biotic stress.

5.3.3 Profiling of sulfur-containing volatiles in shoot and root samples

We tried to highlight glucosinolate breakdown products in association with epoxiconazole exposure after the flash-freezing of the 35-day-old oilseed rape plantlet tissues (roots and shoots) and analysis using an innovative DHS-TD-GC-MS method. First as expected, we found well-known green leaf volatiles (GLVs) in our full-scan chromatograms, resulting from damage to oilseed rape plantlet tissues (crushing in liquid nitrogen) and the peroxidation of polyunsaturated fatty acids. The same GLV compound profiles were found in roots and shoots of oilseed rape plantlets at the different epoxiconazole concentrations tested (0, 0.01 and 0.1 mg L^{-1}). The *Brassicaceae* family is known to contain very interesting secondary metabolites such as GSLs that are involved in abiotic stress response (Rodziewicz et al., 2014). These consist of a β-thioglucose, a sulfonated oxime and a variable aglycone side chain derived from an α-amino acid. In the cell after disruption of the vacuole, they are hydrolysed with myrosinase, resulting in the production of isothiocyanates (ITCs), thiocyanates, nitriles, goitrin and epithionitriles depending on the pH conditions (Ishida et al. 2014). Three isothiocyanates (3-butenyl ITC, 4-pentenyl ITC, 4-methylpentyl ITC), resulting from hydrolysis of GSL secondary metabolites were found by profiling ITCs using SIM mode and the most representative ion (m/z 72), and we observed that 4-methylpentyl ITC was only present in the sample of root tissues (Fig. 5.6).

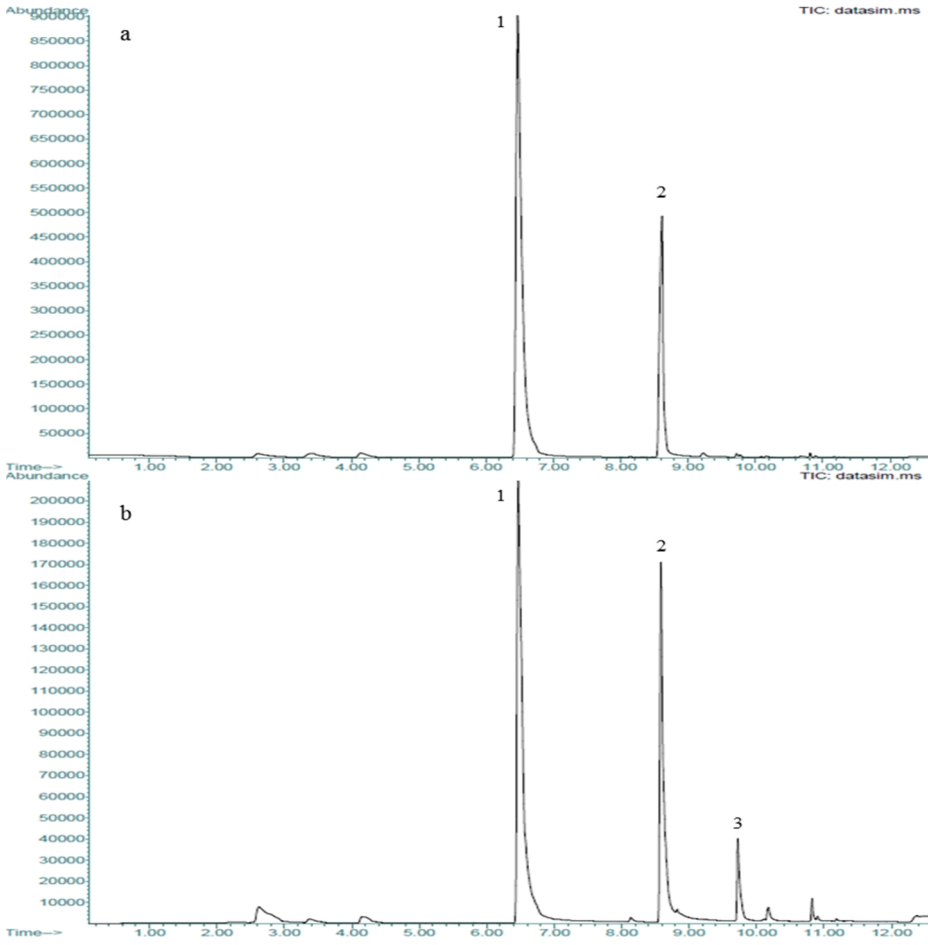

Figure 5.6: Typical chromatograms achieved using SIM mode (m/z 72) of a) sample of shoot tissue and b) sample of root tissue of 35-day-old oilseed rape plantlets. Peak of tentatively identified compound: 1: 3-butenyl isothiocyanate, 2: 4-pentenyl isothiocyanate, 3: 4-methylpentyl isothiocyanate.

No qualitative difference in our ITC profiles was found for root and shoot samples in relation to fungicide exposure of 0.01 mg L^{-1} and in comparison to the control without epoxiconazole. The results of plantlets' physiological stress under 0.1 mg L^{-1} of epoxiconazole have been previously described and can simply be determined by visual observations. We suggest that ITC cannot be used as a metabolic marker of epoxiconazole exposure for oilseed rape plantlets, but we have demonstrated with this DHS-TD-GC-MS method targeting a single ion (m/z 72) the possibility of studying ITCs as metabolic markers for others stresses (e.g. biotic). Numerous *Brassica* species investigations relating to biotic stress have concerned GSL and their relative breakdown products such as ITCs (van Dam et al., 2012).

5.4 Concluding remarks

Plant metabolic profiling, under various subtoxic conditions of chemical stress, such as that caused by pesticide residue, can reveal complex metabolic shifts and physiological perturbations (Serra et al. 2015). VOC profiling and GC-MS studies seem to be a convenient and non-invasive approach for identifying some metabolic markers for pesticide exposure. It will be also interesting to confirm the results and observations obtained from these experimental conditions using other substrates such as soil and with other pesticide residues, for example. Finally, further research is needed to gain a more accurate understanding of crop plant pesticide detoxification, and brassinosteroids also seem to play an important role in the alleviation of pesticide physiological stress (Zhou et al. 2015; Sharma et al. 2016; Shahzad et al. 2018).

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Discussion

Agriculture has to cope with many abiotic stresses among which temperature, drought and salinity may be responsible for over 50% yield reduction in major crop plants (Rodziewicz et al., 2014). The rapid industrialisation and urbanisation can be also a source of toxic substances accumulation such as trace heavy metals in soils via airborne pollutant contamination (Kumar et al., 2018). In addition, an intensive agriculture tends to increase the pesticides accumulation in soils, and especially those which are known to be persistent (Silva et al., 2019). The quality of edible crops could be therefore degraded including putative health risks via their consumption (Dappe et al., 2018). In this thesis, we used a targeted metabolomic approach to find in oilseed rape (*Brassica napus* L.) volatile and non-volatile metabolic markers in connection with two soil-related abiotic stresses: cadmium (Cd) and epoxiconazole exposure. We decided mainly to focus on volatile organic compounds (VOCs) such as terpenes after a first round of a non-targeted screening and on glucosinolates (GSLs), including their breakdown products as non-volatile compounds.

Literature reported that VOCs emitted from leaf surfaces are terminal metabolites indicating the physiological plant health status (Martinelli et al., 2015). At the same time, volatile response involved in abiotic stress could represent also a metabolic picture at a given time. The study of the plant volatilome seems to be therefore one of the most realistic view of a biological state, but is very complex and led for now to a poor attention in crop science. On the basis of our results, we suggest that terpenes could serve as non-invasive metabolic markers to follow oilseed rape physiological activity. One biggest challenge remains the high temporal and spatial variability related to environmental influence and then the need of a real-time VOC recording (Niederbacher et al., 2015). We give in the current section some elements of discussion about future research possibilities related to VOCs and GSLs as metabolic markers. Ultimately, concluding remarks are also drawn.

Discussion

Emphasis on terpenes

The profiling and the magnitude of emissions can vary with genotype (intra- and inter species), phenology, diurnal rhythm and plant part such as green foliage, buds, and flowers. VOCs come from amino acids, fatty acids and five-carbon precursors such as isoprenoids, but terpenes remain the most abundant volatiles emitted by plants (Rosenkranz and Schnitzler, 2016). We discussed previously (cfr. 2.3 & 5.3) that terpenoids could also be used to phenotype some important agricultural core crops such as oilseed rape in controlled conditions. Literature describes that most typical terpenoids emitted by *Brassica napus* L. foliage include monoterpenes and sesquiterpenes such as sabinene, myrcene, δ-3-carene, α-terpinene, limonene, (E,E)-α-farnesene and others depending on the variety (Veromann et al., 2013; Shannon et al., 2016; Durenne et al., 2018).

Plants emit many volatiles into their immediate surroundings that serve essential functions in their growth, communication and defense (Martinelli et al., 2015). Most studies profiling terpenes in *Brassica* focus on biotic stress investigations (Himanen et al., 2017). However, a multivariate study demonstrated that high concentrations (720 µl L^{-1}) of CO_2 related stress lead to enhanced foliage emissions of α-thujene, sabinene, limonene, 1,8 cineole and γ-terpinene for *Brassica napus* spp. *Oleifera* (Himanen et al., 2009). Winter et al. (2012) reported that heavy metal stress can also have a strong influence on terpene emissions in *Zea mays,* while we also demonstrated the influence of Cd in *Brassica napus* L emissions. Recently, Bibbiani et al. (2018) described the identification of twenty-three different VOCs in the *Tetradenia riparia* volatilome Zn stressed-plants and hypothesised an active role of such compounds in the adaptive plant response.

Influence of Cd or epoxiconazole stress on terpenes synthesis is poorly investigated. Terpenoids constitute the largest and most diverse class of secondary metabolites. This wide diversity comes from the terpene synthase (TPS) gene family (Dudareva et al., 2013). Both isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), by acting as connecting metabolites, facilitate metabolic cross-talks between compartmentally separated cytosolic mevalonic acid (MVA) and chloroplastic 2-C-methyl-D-erythritol 4-phosphate (MEP) pathways, but how precisely remains unclear (Dudareva et al., 2013). Kanagendran et al. (2018) studied how ozone and wounding stresses temporally can influenced the genes regulation and expression of isoprene, 1,8 cineole and isoledene in *Eucalyptus globulus*. Using a same approach would be very interesting to study the terpene synthase genes regulation involved in oilseed rape plantlets response under Cd and epoxiconazole stress.

Terpenes release and abiotic stress tolerance

It remains unclear how exactly VOCs can cross barriers from intact cells for a direct release to the environment (Widhalm et al., 2015). It is largely presumed that they can passively diffuse across cellular barriers. However, Adebesin et al. (2017) demonstrated that volatiles diffusion across the plasma membrane relies on active transport in petunia flowers. We know that the constitutive VOCs biosynthesis depends on the primary metabolism and the availability of carbon, nitrogen and sulfur. Recent research has also revealed the importance of VOC catabolism in plant kingdom with implications for plant carbon (C) balance (Oikawa and Lerdau, 2013). Interestingly, in response to abiotic stresses, plants seem often to invest an increasing portion of assimilated carbon into constitutive VOC synthesis whereas, the blend of VOC profile emitted by plants after herbivory damage largely consist of induced VOCs (Catola et al., 2018). Furthermore, questions remain because volatiles emitted in response to abiotic factors may constitute informative cues to other community members but under such stress, plants may also become a better quality host for herbivores than under regular conditions (Blande et al., 2014).

We know that isoprene emission is considered to play a role in mechanisms involved in severe stress damages due to reactive oxygen species production (ROS). Literature described the thermoprotective function on photosynthetic processes under abiotic stress conditions such as light or temperature (Loreto et al., 2006; Loreto and Schnitzler 2010). Nevertheless, the response of monoterpenes and sesquiterpenes needs to be yet clarified (Rosenkranz et al., 2016). In this thesis, we observed contrasting sesquiterpenes results related to plantlets abiotic stress-adaptive response. Most described biological functions of sesquiterpenes are ecological as being nonspecific toxins active against a wide range of organisms (i.e. bacteria, fungi, plants and animals) (Rosenkranz et al., 2016). Oilseed rape sesquiterpenes field emission certainly plays a role in tritrophic communication with indirect or direct defense against possible biotic attacks (i.e. herbivores or pathogens). Further researches are still needed for understanding the terpenes release role into oilseed rape stress tolerance mechanisms and in the frame of multiple abiotic and biotic stress conditions. For example, Catola et al. (2018) described results where tomato responses to water deficit and aphid attack showed an additive impact on VOC emission.

Using VOC analysis for biotic stress purpose

It is known that plants release VOCs for acting as chemical signals between neighbouring plants. They can mediate also bitrophic and/or tritrophic interactions (Delory et al., 2016), and are involved at the phytobiome level by recruiting, repelling and coordinating interactions among different organisms and environment (Leach et al., 2017). Microbes and insects perception by plants leads mainly to activation of phytohormone signals corresponding to salicylic acid, jasmonic acid and ethylene (Winter et al., 2012). This plant hormone signaling cascade conveys

messages among the different plant parts and can serve to communicate with neighbouring plants (Leach et al., 2017).

A lot of common major pests of oilseed rape, including *Phyllotreta* sp., *Meligethes aeneus* (Fabricius), several Lepidopteran species are economically important. It is known that characteristic *Brassica*-emitted VOCs induce olfactory and behavioural responses ranging from repellence to attraction and depending on species and growth stage in many of these herbivores. Literature is replete with examples of studies where *Brassica* pests are responding to VOCs such as terpenoids, isothiocyanates and green leaf volatiles (GLVs) at some points in their life cycle, but VOC-based management strategies are really hard to set-up at field level (Himanen et al., 2017). Recently, Shannon et al. (2016) reported that a mixture of monoterpenes emitted by oilseed rape seedlings from differing cultivars was identified as a putative attractant for mollusc attack while glucosinolate profiles were unrelated to snail feeding behaviour.

It is known that the VOC emission in plant is species specific, reflecting not only environmental conditions but giving also the quality or healthy status of the plant (Lubes and Goodarzi, 2017). While serological and PCR-based methods, now being the most available, are effective to confirm diseases diagnosis, volatile sensors could provide instantaneous results for identifying infections at asymptomatic stages (Martinelli et al., 2015). Recently, Kasal-Slavik et al. (2017) described changes in VOC emissions in tomato (*Solanum lycopersicum*) during *Myzus persicae* attack and before occurrence of visual symptoms. It would be very interesting to compare oilseed rape emissions using biotic stress such as *Sclerotinia sclerotorium,* a necrotrophic fungal pathogen being able to infect more than 400 species of plants and causing tremendous oilseed rape yield losses worldwide (Li et al., 2015). Phoma stem canker is another important disease of oilseed rape caused by closely related species of *Leptosphaeria maculans* and *L. biglobosa* (Sewell et al., 2016) and could constitute also an interesting research issue.

Using a non-targeted approach could be relevant in order to increase the possibility of finding metabolite markers. VOC analysis should be so integrated into physiological phenotyping during genotypes assessment because research is needed to prospect if some VOCs, coming from oilseed rape breeding population, could confer potential resistance against economically important pathogens. On the other hand, manipulating plant VOC emission by transforming plant volatiles blend via genetic engineering or via exogenous elicitor treatments may effectively represent an alternative to the pesticide use in order to improve pest control (Himanen et al., 2017), but such an effect of modified VOC profiles that could enhance plant defense needs to be evaluated in an agricultural setting (Dudareva et al., 2013). Furthermore, the complexity of the regulatory system does not necessarily bring expected results and for example, the overexpression of foreign *S*-linalool synthase in transgenic *petunia* led to accumulation of *S*-linalyl-β-D-glucoside instead of accumulation of free linalool (Lubes and Goodarzi, 2017). Finally, the study of plant-associated microbiota volatilomes could constitute an untapped source of biocontrol agents,

new valuable molecules and farming strategies (Bailly and Weisskopf, 2017). Research on bacterial volatile compounds (BVCs) is effectively at the forefront for the discovery of missing pieces of the plant-bacteria interaction puzzle (Sharifi and Ryu, 2018). In the case of oilseed rape, it would be useful to investigate plant endophytic bacteria potential for reducing sclerotial production and mycelial growth through emission of specific VOCs (Massawe et al., 2018).

Field challenges of VOC phenotyping

A lot of cuvette systems such as our customised glass chambers are used at the laboratory scale to measure gas exchange and VOC emissions by controlling temperature and light conditions. VOCs are in these conditions trapped with polymers before being thermally desorbed or analysed after very time-consuming solvent extraction. Most common platform involved in offline volatile analyses is gas chromatography coupled with mass spectrometry. We demonstrated that GC-MS remains a powerful technique to separate terpenoids emitted in minute amounts. Moreover, terpenoids quantitation can be largely improved using selected-ion monitoring (SIM) mode in complementarity with total ion chromatograms (TIC) (cfr. fig. 2.5).

We know that there are fluctuating factors in agricultural *versus* in controlled environment. Novel approaches and techniques are required to characterise the plasticity of the plant volatile phenome (Araus and Cairns, 2014; Großkinsky et al., 2015). Our multiple cuvette system was designed in order to follow young plantlets VOC emissions at the whole-plant level and under reproducible conditions. The possibility of using Teflon™ bags could resolve our sampling's dilemma in field conditions by focusing at leaf or branch scale and with zero air to perform the headspace analysis. Morrison et al. (2016) described convenient possibilities for dynamic vegetation enclosure using polycarbonate or polyethylene terephthalate (PET) materials. Solid-phase microextraction (SPME) technique using adsorbent-coated fibres remains a possibility but static headspace sampling leads to a very difficult interpretation of quantitative results. Moreover, agricultural crops (i.e. maize, wheat and rice) typically have low volatile emissions (Rosenkranz et al., 2016). VOCs must be initially pre-concentrated through the set-up of dynamic sampling strategies. Key limitations of our VOC trapping system are that they can be time-consuming and they don't achieve simultaneous and time-resolved monitoring of different VOC compounds (e.g. isoprenoid hydrocarbons, C_1-C_2 alcohols). Some alternative solutions for real-time detection of VOC emissions exist: i) the novel proton-transfer-reaction time of flight mass spectrometry technology (PTR-TOF-MS) (Jordan et al., 2009), ii) portable GC-MS such as Torion® T-9 or zNose® using ultra-fast gas chromatography and surface acoustic wave technologies and, iii) electronic nose (e-nose) using specialised metal oxide sensors and generating impedance response. Nevertheless, some limitations remain such as a lack of MS information for zNose® and a lack of chromatographic separation and

ion fragmentation for PTR-MS (Kallenbach et al., 2014). These technologies are still maturing in terms of reproducibility, data processing and robustness.

Innovative technologies for phenotyping needs

A solution could be therefore to use real-time analysis possibilities of PTR-TOF-MS technology. A lot of studies describe effectively its interest, by targeting 10-15 ions, for the plant volatilome real-time analysis (Niederbacher et al., 2015; Bibbiani et al., 2018; Mu et al., 2018). Jardine et al. (2016) described results about emitted isoprene oxidation products from mango branches under abiotic stress. Mozzafar et al. (2018) reported, in their study, the tracking of VOC emissions from senescent maize leaves. Monoterpenes and sesquiterpenes, presenting a same molecular weight, are poorly separated using this technology, in comparison to classical TD-GC-MS. A fast-GC must be coupled to PTR-MS for allowing terpene compounds analysis (Materic et al., 2015; Palozzi et al., 2016). Evaluation of isoprene emission by our plantlets as a model molecule by targeting the metabolism response under abiotic stress would be already a promising tool.

In addition, a solution for high-throughput phenotyping purpose could be to link VOC emitted by oilseed crops in field or semi-field conditions with other remote sensing methods. For example, the integration of volatile sensors or trapping polymers in flexible and affordable plant phenotyping solution such as the 'phenobox' could be relevant (Czedik-Eysenberg et al., 2018). Such phenobox technology is actually investigated to reach the gap between phenotypic traits and stress-related effects under different biotic and abiotic conditions. Remote sensing innovative technologies are based mostly on information provided by visible/near-infrared (VIS-NIR) radiation reflected (or transmitted) and far-infrared (thermal) radiation. Fully automated systems based on non-destructive and non-invasive sensors in greenhouses, growth chambers and semi-field conditions are currently operating within European research centre in order to integrate sensor-based phenotyping into physiological breeding programmes (Großkinsky et al., 2015). Recent improvements in imaging sensor technologies (i.e. red-green-blue, multispectral, hyperspectral, thermal) and data analyses are now making high-throughput root, shoot, whole-plant and canopy phenomic studies possible. However, big-data challenge must be faced from data collection to meta-analyses levels (Tardieu et al., 2017). In the future, electronic noses could also have its place among potentialities of new sensor development. Despite the fact that highly complex data from it require processing via multivariate statistical methods to be accurately interpreted (Martinelli et al., 2015). This technology is at the moment more suggested in complementarity with traditionally adopted diagnostic techniques (Cellini et al., 2017). Furthermore, using gas sensors based on the creation of molecularly imprinted polymers (MIPs) could be a way forward by targeting molecules of interest in the field (Zhang et al., 2017).

Glucosinolates and their breakdown products as non-volatile markers

Identification of specialised metabolite functions in plants becomes more and more affordable due to the development of innovative methodologies and high-throughput analytical platforms. Interestingly, glucosinolates (GSLs) and their myrosinase-catalysed hydrolysis products (i.e. isothiocyanates, thiocyanates, nitriles, goitrin and epithionitriles) are known to have physiological significances in plant response to different biotic and abiotic stresses (del Carmen Martínez-Ballesta et al., 2013). In order to give more insights, it would be interesting to confirm our results in semi-field conditions by testing several accessions of *Brassica napus* L. using soil experiments. An issue could be to prospect GSLs and phytochelatins analysis in relation with sulfur supplies. The biotic stress such as clubroot disease could be also challenged. Recent literature described effectively that aliphatic GSLs are involved in this disease which is caused by the obligate biotrophic protist *Plamodiophora brassicae* (Xu et al., 2018).

We analysed also sulfur-containing volatiles such as nitriles, epithionitriles and isothiocyanates because they are frequently mentioned in investigations of oilseed rape's volatile response to biotic stress (van Dam et al. 2012). These compounds are not normally emitted by oilseed rape at vegetative stage, but can be analysed using dynamic headspace (DHS) sampling after flash-freezing of the plant tissue with liquid nitrogen. Using this cost-effective technique, they can also give a picture of the status of GSLs pool in different organs of oilseed rape plantlets, bypassing the time-consuming HPLC analysis of desulfoglucosinolates. The analytical method using SIM mode and focusing on 72 m/z ion that we developed in our last experiments could really serve through other research protocols. Very reliable and clean chromatograms were achieved with distinction between profiling of root and shoot plant organs. Organ-specific GSL distribution from roots to leaves is yet poorly described in oilseed rape and could be explored for agricultural research purpose. For example, it could be investigated for highlighting the mediator role of myrosinase-system on the activity of belowground organisms (Kumar and Verma, 2018). Rightly, GSL breakdown products such as isothiocyanates are actually under consideration for their ability to: i) control weeds, ii) restrict the growth of neighbouring plants (allelopathy), and iii) possibly alleviate soil pathogens proliferation in agricultural co-cultivation systems (van Dam et al., 2009; Rosenkranz and Schnitzler, 2016). We could therefore easily imagine using our customised device as a model-system for studying interactions between plants, soil and microorganisms. It would be also interesting to test allelopathy possibilities with *Brassica* plants such as mustard or *Eruca* spp. (biofumigation process).

The possibility to assess some metabolic markers using targeted approaches and modern hyphenated mass spectrometry methods could bring responses to a more efficient primary metabolism use and to secondary metabolism needs for scavenging adverse environmental conditions. GC-MS was recently used to investigate heat map

of metabolite changes for understanding how oilseed rape grows under various applications of nitrogen fertilisers (Clément et al., 2018). Large research projects investigating and generating genetic diversity for nitrogen use efficiency (NUE) in oilseed rape are currently in progress in Europe (Bouchet et al., 2016; Stahl et al., 2017). Oilseed rape is also known to assimilate large amount of inorganic sulfur (S) and for its metabolisation to essential organic sulfur compounds such as cysteine and methionine (Kopriva et al., 2016; Zheng and Leustek, 2017). It will be then relevant to investigate metabolite changes related to S allocation in order to better predict the growth of this very high S-demanding crop (Brunel-Muguet et al., 2015; Poisson et al., 2018).

Concluding remarks

Analytical methods based on gas and liquid chromatography coupled with mass spectrometry were principally used for this oilseed rape plantlets analysis. Higher accuracy, greater selectivity and better sensitivity can be mentioned among numerous advantages of a targeted approach in order to discover metabolic marker or for pathway mapping (Beckles and Roessner, 2012). The set-up of our innovative glass chambers system under sterile and controlled conditions was a very important step. Furthermore, a major analytical development was achieved through the use of selected-ion monitoring (SIM) mode for data acquisition. Undoubtedly, this led to considerable progresses for chromatograms analysis and interpretation, because the signal-to-noise ratio is a strong determinant for whether the compounds quantitation will be successful. Our results were therefore easier to interpret using SIM mode and this method could be relevant in future research about VOCs.

Volatile and non-volatile metabolic markers have been identified through this thesis. Moreover, these dose-response experiments provided useful insights for linking the physiological status of oilseed rape plantlets to putative metabolic pathway adaptations. We found effectively that terpene emissions were affected under Cd and epoxiconazole stress. After a first round of a non-targeted volatile screening, we found very reliable results of mono- and sesquiterpenes emitted by our plantlets using our customised device (cfr. 2.3). Nevertheless, no specific signal molecule related to Cd and epoxiconazole stress was found. We demonstrated through these dose-response experiments that VOCs were emitted in relation to oilseed rape plantlets stress-response, at more important concentrations tested for both stresses in particular.

Sesquiterpenes such as β-elemene and (E,E)-α-farnesene emission rates were clearly affected under epoxiconazole stress (cfr. 5.3) and interestingly, (E,E)-α-farnesene emission rate was also influenced under Cd-stress (cfr. 2.3). We suggest that the different emission rates could be the result of induced variations at physiological level among terpenoids synthesised by oilseed rape plantlets. In fact, it is known that under non-stressed conditions, plants emit volatiles constitutively and that abiotic stress episodes can induce or inhibit plant VOC emission responses

(Kanagendran et al., 2018). Based on these observations, we suggest also that sesquiterpenes such as β-elemene and (E,E)-α-farnesene could serve as potential metabolic markers of physiological perturbations in *Brassica napus* L.

Ultimately, our results related to Cd-stress provide strong evidence that glucosinolates are involved in Cd-stress oilseed rape response. Cd clearly decreased GSL content in our 28-day-old oilseed rape plantlets both in roots and shoots, with a dose-dependent pattern (cfr. 3.3). About root phenotyping, it would be interesting for future investigations to estimate the important root/shoot ratio. This parameter could help to understand the growth physiology and allocation of primary metabolism. We have also suggested that priority is given to the use of sulfur supplies to cope with Cd stress at physiological and cellular levels corroborating also implications for plant defense against biotic factors.

Finally, the plant-stress response under abiotic factor such as Cd is accomplished through sophisticated multilevel regulatory processes (e.g. transcriptional, post-transcriptional, post-translational and epigenetic) (D'Alessandro et al., 2013; Haak et al., 2017). It is obvious that metabolomics must be integrated into omics for a comprehensive understanding of the responses and tolerance to abiotic stresses such as Cd and epoxiconazole in oilseed rape.

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Posters

- Using TDU-GC-MS to investigate the VOC emission of *Brassica napus* L. plantlets cultivated *in vitro* and exposed to cadmium abiotic stress. Durenne B., Blondel A., Druart P., Fauconnier M-L. 40th ISCC and 13th GCxGC Symposium. Riva del guarda, Italy. 2016.
- Could the profiling of root indolic glucosinolates be correlated with *Brassica napus* L. cadmium stress tolerance? Durenne B., Blondel A., Druart P., Fauconnier M-L. Glucosinolates2017. Berlin, Germany.
- Using phenotyping and complementary VOC profiling approach to investigate physiological response of *Brassica napus* L. plantlets under cadmium and epoxiconazole abiotic stresses. Durenne B., Geerts P., Druart P., Blondel A., Fauconnier M-L. "Plant Phenotyping Forum: integrating European plant phenotyping community" Tartu, Estonia, 2017.

Oral communication

- *Brassica napus* L. plantlets affected during *in vitro* growth in the presence of epoxiconazole. Durenne B. *Brassica*2017, International Society for Horticultural Science (ISHS) Pontevedra, Spain.

Publications

- Durenne, B., Blondel, A., Druart, P., Fauconnier, M-L. (2018). A laboratory high-throughput glass chamber using dynamic headspace TD-GC/MS method for the analysis of whole *Brassica napus* L. plantlet volatiles under cadmium-related abiotic stress. *Phytochem Anal* 29, 463–471.
- Durenne, B., Druart, P., Blondel, A., Fauconnier, M-L. (2018). How cadmium affects the fitness and the glucosinolate content of oilseed rape plantlets. *Environ Exp Bot* 155, 185–194.
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