Contribution on the study of allelopathic interactions between Amaranthus retroflexus L. and Secale cereale L.

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Diplôme : Master en bioingénieur : chimie et bioindustries, à finalité spécialisée
Année académique : 2022-2023
URI/URL : http://hdl.handle.net/2268.2/16825

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Host laboratory

The present his master's thesis was hosted by the Laboratory of Chemistry of Natural Molecules at Gembloux Agro-Biotech, University of Liège.

Aknowledgements

My foremost gratitude is extended to my supervisors Professor Marie-Laure Fauconnier and Laura Lheureux, that provided the best guidance and working environment that I could hope for.

I would like also to use this opportunity to thank the whole AllelopaTeam, Dr. Wasseem Mushtaq, Dr. Aurélie Gfeller, Dr Caroline De Clerck, Dr. Benjamin Delory and Pauline Canelle for their expertise and most useful feedback.

I am grateful for the collaboration with the department of plant biology (BV) for allowing us to use their facility. I'd like to mention Professor Pierre Delaplace, Maurine Antoine, Martin Quievreux and Frédéric Dresen in this regard.

A very special attention goes to the technical team of the LCMN, Frank and Thomas. Their patience, help and good mood was the key for many accomplishments. I would like to address my thankfulness to the researchers and co-TFistes that were present during my master' thesis: Manon, Laurie, Laura L (again), Laura D, Elisa, Louise and Gaelle. Having this group as colleagues was a real pleasure.

Ma gratitude la plus sincère est destiné à ma mère, mes pères, mes frères et sœurs, mes grands-parents et toute ma famille. Vous avez été mon plus grand soutient, ma bibliothèque remplie de livre de sciences qui m'ont été offert depuis ma tendre enfance en témoigne. Je vous remercie pour tous vos messages et attentions particulières au cours de mon cursus et en dehors.

Gros BigUp à ma team de Luxos, à mes Bruxellères, an meine Kölner, à mes Zers, au Kot d'Ivoire, de l'Auberge, du Dech' et du Liroux et bien sûr le saint KDR. J'ai passé un nombre incalculable de moments inoubliables que j'ai oubliés (merci Rafu pour celle-là).

Comment terminer ces remerciements sans mentionner Caro, qui a su me donner toute ma confiance en moi et ma détermination pour accomplir de grandes choses. Je suis à court de mots pour exprimer pleinement ma gratitude.

Abstract

Amaranthus retroflexus L., commonly known as redroot pigweed, is a well-known summer weed that poses significant challenges to multiple cropping ecosystems. Historically, intensive use of herbicides has been the primary method of weed control since the Green Revolution. However, this approach is becoming less effective due to the increasing adaptation and resistance of weed species to herbicides. Additionally, the negative side effects associated with herbicide use emphasize the need for the implementation of sustainable weed management practices. Considering the above considerations, the adoption of rye (*Secale cereale* L.), which is one of the most potent allelopathic crops, holds significant importance. The problem addressed in this study is the lack of knowledge concerning the causes and effects of the interactions between those two plant species.

The focal plant in this case was rye. Two populations were grown in controlled conditions and in hydroponics. One group serving as a control, and the other group being subjected to an aqueous extract of redroot pigweed. Different potential effects of this extract were monitored during 9-day long development of the rye seedlings.

Below ground interaction between the focal plant and other organisms is suspected to be strongly modulated by volatile organic compounds (VOCs). To elucidate this mystery, the volatilome inside the hydroponic system was determined using a custom-made growth apparatus that would allow the direct pumping of root volatiles using dynamic headspace sampling and followed by GC-MS analysis of the captured compounds.

The investigation of phenotypical variables in the study included gravimetric analysis of biomass allocation and root scanning to assess root architecture. These variables served as indicators of potential inhibition, stimulation, or alteration of rye's developmental pattern.

Lastly, rye is specialized in the production of allelochemicals called benzoxazinoids. Their production, accumulation and release are strongly dependent on environmental stimuli. The inducible production of the root treatment using metabolites belonging to redroot pigweed was investigated.

Results of the present experimentations show that the aqueous extract of redroot pigweed is affecting the growth pattern of rye roots. The promotion of lateral root elongation was already observed after six days of treatment. Perturbation of the auxin-ethylene growth regulation system is suspected. The overall allocation of biomass from root to shoot does not show significant variations when comparing the two populations.

Regarding the analysis of the volatilome, potential constitutive signals were identified in rye roots, both in control and treated groups. Furthermore, treatment appeared to stimulate the emission of certain volatile organic compounds (VOCs) while suppressing others. Some of the detected VOCs were attributed to emission by plant growth-promoting rhizobacteria rather than the roots themselves. Additionally, some of these compounds have been found to possess toxic activity, thereby inhibiting potential pathogenic organisms within the rhizosphere. This underscores the fact that rye employs not only benzoxazinoids or exudates, but also VOCs, to mediate subterranean interactions.

In the context of treatment with an extract, no discernible variation in the benzoxazinoid content of rye shoot was detected. Despite its susceptibility to external stimuli, the priming of the defense mechanisms of rye shoots were not observed.

Key words: Redroot pigweed, Rye, Allelopathy, Root volatile organic compounds, Phenomics

Résumé

Amaranthus retroflexus L., communément appelé "amarante réfléchie", est une mauvaise herbe estivale bien connue qui pose d'importants défis aux écosystèmes de cultures multiples. Historiquement, l'utilisation intensive d'herbicides a été la méthode principale de lutte contre les mauvaises herbes depuis la Révolution verte. Cependant, cette approche devient moins efficace en raison de l'adaptation et de la résistance croissantes des espèces de mauvaises herbes aux herbicides. De plus, les effets secondaires négatifs associés à l'utilisation d'herbicides soulignent la nécessité de mettre en œuvre des pratiques durables de gestion des adventices. Compte tenu de ces considérations, l'adoption du seigle (*Secale cereale* L.), qui est l'une des cultures allopathiques les plus prometteuse. Le problème abordé dans cette étude est le manque de connaissance concernant les causes et les effets des interactions entre ces deux espèces végétales.

La plante cible dans le cas présent est le seigle. Deux populations ont été cultivées dans des conditions contrôlées et en hydroponie. La première a servi de témoin et la seconde a été soumise à un extrait aqueux d'amarante réfléchie. Différents effets potentiels de cet extrait ont été étudiés sur le développement de semis de seigle pendant neuf jours.

Il est plus que probable que l'interaction souterraine entre la plante cible et d'autres organismes soit fortement modulée par les composés organiques volatils (COV). Pour en apprendre davantage, le volatilome à l'intérieur du système hydroponique a été déterminé en utilisant un appareil de croissance sur mesure qui permettait le pompage direct des volatiles racinaires en utilisant un échantillonnage dynamique de l'espace de tête suivi d'une analyse par GC-MS des composés capturés.

L'étude des variables phénotypiques est réalisée, comprenant l'analyse gravimétrique de l'allocation de biomasse et la numérisation des racines pour évaluer l'architecture racinaire. Ces variables ont servi d'indicateurs d'inhibition, de stimulation ou d'altération potentielles du pattern de développement du seigle.

Enfin, le seigle est spécialisé dans la production d'allélochimiques appelés benzoxazinoïdes. Leur production, accumulation et libération dépendent fortement des stimuli environnementaux. Leur production inductible par traitement des racines en utilisant des métabolites appartenant à l'amarante réfléchie a été étudiée.

Les résultats de ces expérimentations montrent que l'extrait aqueux d'amarante réfléchie affecte le schéma de croissance des racines de seigle. La promotion de l'allongement des racines latérales a été observée après seulement six jours de traitement. Une perturbation du système de régulation de la croissance par le couple auxine-éthylène est suspectée. L'allocation globale de biomasse de la racine à la partie aérienne ne présente pas de variations significatives lors de la comparaison entre les deux populations.

En ce qui concerne l'analyse du volatilome, des signaux constitutifs potentiels ont été identifiés dans les racines de seigle, à la fois dans les groupes témoins et traités. De plus, le traitement semblait stimuler l'émission de certains composés organiques volatils (COVs) tout en en supprimant d'autres. Certains des COVs détectés ont été attribués à l'émission de rhizobactéries favorisant la croissance des plantes plutôt qu'aux racines elles-mêmes. De plus, certains de ces composés sont considérés toxiques envers certains organismes pathogènes potentiels dans la rhizosphère. Cela souligne le fait que le seigle utilise non

seulement des benzoxazinoïdes ou des exsudats, mais aussi des COVs, comme moyen d'interaction avec le monde souterrain.

Dans le contexte du traitement avec un extrait, aucune variation dans le contenu de benzoxazinoïde du shoot de seigle n'a été détectée. Malgré sa sensibilité aux stimuli externes, la mise en place des mécanismes de défense des pousses de seigle n'a pas été observée.

Mots clés : Amaranthe réfléchie, Seigle, Allélopathie, Composés organiques volatiles racinaires, Phénomique

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List of abbreviations

ABC: ATP-binding cassette

MATE: Multidrug and toxic compound extrusion proteins

VOCs: Volatile organic compounds

BXs: Benzoxazinoids

DIBOA: Dihydroxybenzoxazinone

DIMBOA: Dihydroxymethoxybenzoxazinone

BOA: Benzoxazolinone

MBOA: Methoxybezoxazilinone

Glc-: Glucoside

AP: Aminophenol

APO: Aminophenoxazinone

GC: Gas chromatography

MS: Mass spectrometer

HPLC: High performance liquid chromatography

DPI: Dot per inch

LOD: Limit of detection

LOQ: Limit of quantification

d.a.g.: Days after germination

nsLTP: Non-specific lipid transfer proteins

RI: Retention index

RT: Retention time

R0: rye control group

RA: rye treated with amaranth extract

1.Introduction

1.1. Limitations of the modern weed management systems

Weeds are considered one of the major factors of food and economical loss of our agriculture system, even if their role in ecosystems has been recognized[1]. Artificial compounds targeting those weeds, herbicides, were introduced to our food production practices during the green revolution and have prevented huge loss to weed invasions, assuring food safety and demographic growth and/or stability for many[2]. Unfortunately, the extensive use of those chemicals for several decades has triggered evolutionary adaptation of the targeted organisms[3].

Indeed, conventional herbicides decline in efficiency due to the development of resistance to one or more herbicide in already 220 weed species (130 dicotyldon species and 90 monoctyledon species; data from 2013) and still increasing since 1970[4]. This phenomenon brings increases in the doses applied in fields coupled with the intensification and globalisation of modern farming practices, causing damage to non-targeted organisms, especially major plants, algae, and microorganisms[5], [6]. In addition, the active compounds, adjuvants and their metabolites tend to accumulate along the food chain and create health concerns for humans and agricultural drawbacks[7]–[11].

At present times, alternatives to conventional phytosanitary products need to be developed, as their viability as long-lasting tools for farmers is dwindling. Even if the European Union is still post-posing the ban of glyphosate-based products, the first countries, Luxemburg and Austria, have banned it from all agricultural practices[12], [13].

In this context, allelopathy offers promising results as (i) improvement of biocontrol agents in the scheme of integrated weed management, (ii) a source of bioherbicides that induce lesser environmental consequences than conventional products[1], [14]–[21]. To that end, the present master's thesis will focus on the allelopathic interaction of the weed *Amaranthus retrolflexus* L. vernacularly called "redroot pigweed" and the crop *Secale cereale* L. also known as "rye". More precisely, the potential effects of metabolites frorm redroot pigweed on the growth and the allelopathic response of rye will be investigated in a controlled environment, using anatomical and chemical features of rye as indicators.

1.2. Allelopathy

1.2.1. Definition and general concepts

Allelopathy is the ecological phenomenon of plant-plant interactions through chemical means. The chemicals causing allelopathic effect are allelochemicals (*cfr* section 1.2.3.) and are produced by an emitter (or donor) plant, effecting a receiver plant[14]. Originally, allelopathy can refer to both a positive effect or negative effect caused by the allelochemicals[14], [16] even if nowadays many studies prefer the definition of allelopathy in case of detrimental interaction and hormesis[22], [23] or mutualism[24] in case of beneficial interaction. The concept of allelopathy strictly excludes the direct competition phenomenon e.g., regarding light, nutrients, or water[25]. The combination of allelopathy and competition for resources is referred to as interference[25]. The present *consensus* is that allelopathy is observed in case these three statements below are verified:

"(1) A phytotoxic chemical is produced.

(2) The chemical is transported from the producing organisms to the target plant.

(3) The target plant is exposed to the chemical in sufficient quantity and for sufficient time to cause damage."[26]

1.2.2. The diversity of allelochemicals

The allelochemicals are classified according to their structure, very numerous compounds have been identified to have allelochemical properties. The most important classes of allelochemicals according to the review of Macìas *et* al.[27] are phenolic compounds, terpenoids, nitrogen containing compounds and parasite/host triggering compounds. How each class is subdivided is presented in Table 1 and gives an overview on the diversity of compounds and type of observed effects. The multitude of different compound classes involves the activity of different biosynthetic pathways. This causes an even higher diversity of enzymes implemented with reactions taking place in all different compartment of the plant, both on the organism level but also at subcellular level[28], [29]. This puts light on the complexity of plant physiology from an allelochemical perspective.

Table 1: Classification of allelochemicals based on the work of Macias et al,2019[27]. Every group is divided in subgroups, for which to biosynthetic pathway is given. For each subgroup, an example allelochemical is given with its emitter and receiver plant species. The observed effect of the respective allelochemicals is provided.

Group	Subgroup	Biosynthetic path	Example molecule	Emitter	Receiver	Effect	References
Phenolic comounds	Simple phenolics	shikimate pathway	R-(-)-regiolone	*Caryospora callicarpa*	*Latuca sativa;* Ra*phanus sativus; Cucumis sativus; Allium cepa; Triticum aestivum*	Plumule and Radicle shortening	[27]
	Flavonoids	phenylpropanoid pathway	Tricin	*Elymus natans*	*Echinochloa crus-galli*	Chlorophyll fluorescence pertutbation, Dyoxygenase inhibition, Growth inhibition	[28]
	Coumarins	phenylpropanoid pathway	Chalepin	*Ruta graveolens; Hortia oreadica*	*Lepidium sativum; Lycopersicon esculentum; Allium cepa*	Growth inhibition	[29]
	Quinones	acetate-malonate; shimimate pathway	Juglone	*Juglans nigra; Jregia; Carya ovata; J. sieboldiana*	*Solanum lycopersicum;*	Growth inhibition, mortality	[30]
Terpenoids	Monoterpenes	mevalonate; MEP	α-pinene and 1,8-cineole	*Eucalyptus spp.*	*Solanum elaegnifolium*	Antigerminative effect	[31]
	Sesquiterpenes	sesquiterpenoid pathway	costunolide, reynosin and santamarine	*Bidens sulphurea*	*Amaranthus viridis; Panicum maximum*	Growth inhibition	[32]
	Diterpenes	mevalonate; MEP	Mimolactone B	*Oryza sativa*	*Echinochloa crus-galli*	Inhibition of root developpement, length, depth and area	[33]
	Triterpenes and Steroids	Isopren pathway	Friedeline	*Banisteriopsis anisandra*	*Latuca sativa; Allium cepa*	Growth and germination inhibition	[34]
Allch. Containing N	Alkaloids	amino-acid derivation; polyketide pathway	Sarmentine	*Piper spp.*	*Brassica kaber; Amaranthus retroflexus Chenopodium album; Abutilon theophrasti Portulaca oleracea dandelion; Cyperus difformis; Digitaria sanguinalis; Poa annua; Echinochloa phyllopogon; Elytrigia repens*	Complete inhibition of photosystem II	[35]
	Benzoxazinoids	indole derivation pathway	MBOA	*Secale cereale*	*Amaranthus retroflexus; Portulaca oleracea*	Reduction of invasion through mulching	[36]

1.2.3. The diversity of allelopathic paths

Given the broad definition of allelopathy, the types of possible interaction modalities are numerous. The allelopathy types can be organized using three levels of classification. Firstly, the source of the emitted compounds determines if the interaction is alloallelopathic, meaning that the translocation of allelochemicals is originating from a heterospecific individual, or autoallelopathic where the source of effective compounds is coming from a conspecific individual[30]. Then, the fate of the allelochemicals of interest in the environment and the form in which they affect the receiver plant determines the second layer of classification, differentiating true allelopathy from functional allelopathy[30]. In case of true allelopathy, the emitted molecules affect the receiver plant in their original form, in opposition to functional allelopathy, where the derivatives or degradation products are responsible for the allelopathic effects. This differentiation is sometimes also descripted as direct or indirect allelopathy[27], [30]. Thirdly, the action time of the emitted compounds opposes concurrent allelopathy to residual allelopathy, meaning that the exchange of allelochemicals can take place between two living plants or, in the other case, the allelochemicals are emitted by plant residues and affecting a living plant[30], [31].

Now that the general types of allelopathy have been presented, the mechanism through which plant release their secondary metabolites in their surroundings will be addressed. Originally the synthesized allelopathic phytotoxins are present in the stems, the leaves and the roots and are for the most part stored in a bound form and thus do not present harm for the producing individual[32], [33]. Starting from there, the principal routes of release for the allelochemicals are (i)decomposition, (ii)exudation, (iii)leaching and (iv)volatilization[30], [32].

Decomposition of plant litter is driven by microbial activity and results in the release of allelochemicals in the soil[32]. Therefore, the rate at which the litter is degraded and consequently the allelochemicals are released in the environment are dependent on the quantity of cytotoxic compounds present in the decaying plant material concurrently to the availability of nutrients, water, oxygen and favourable ranges of temperatures[32], [34].

Exudation through root represents the most proficient mode of release of allelochemicals[30], but this phenomenon is not exclusive to allelopathic interaction. Indeed, plant interact with the rhizosphere through exudates for nutrient assimilation, through ion exchange with soil colloids and by releasing phytometallophores as chelating agents to capture and assimilate iron or other metals[32]. In parallel, root exudates modulate the interaction with soil microbiota like mycorrhizal fungi[35] and plant growth promoting rhizobacteria[36], but also to resist against potentially pathogenic organisms using phytoalexins[37]. Chemicals released in the rhizosphere also act as signals, and when perceived by other plant potentially inhibit or stimulate allelopathic response[24], [38], [39] (cf section 1.2.4.).

Before reaching the rhizosphere, biosynthesized compounds need to be transported first intracellularly and intercellularly and finally exudated[40]. Depending on the chemical characteristics of the allelochemicals, the transportation mechanism from the root to the rhizosphere are different[41]. Most secondary metabolites are weather charged or polar and are thus unable to cross the bilayer lipidic membranes without assistance[30], [41]. Only relatively small and lipidic compounds might cross the membranes through simple diffusion into the rhizosphere[41].

As already stated above, plants need to store the phytotoxic compounds in a way that impeaches autotoxicity, plant proceed weather a chemical alteration of the molecules into a lesser toxic form or avoid all contact of the compounds with the cytosol by encapsulating the toxin in a vesicle[33] An

overview of intracellular and transmembrane transportation is provided on Figure 1. The cellular transmembrane transportation type that leads to exudation is strongly dependant on the structure/activity of the allelochemical. The compounds are synthetized in the Golgi apparatus or the endoplasmic reticulum (ER) and then undergo vesicular transportation before sequestration in vacuoles or the exudation through exocytosis[33], [41]. Other allelochemicals are detoxified by the donor plant before exudation for example through acylation or glycosylation[33], [42]. In this case, the phytotoxins can be exudated using protein transporter located in the plasma membrane[30], [41]. This transportation is imputed to the activity of ATP binding cassette (ABC) and multidrug and toxic compound extrusion proteins (MATE). The first group uses ATP hydrolysis to actively transport bioactive molecules through membranes[41], [43]. The second uses ion exchange for transmembrane transportation, even if its direct contribution to allelopathy has not been demonstrated yet[41], [44]–[46]. Root exudation pathway not only differ through their cellular mechanisms but also by their emission tissue and location[30], [41].

Indeed, roots are organized in different specialized development zones[47], [48], the meristem zone is followed by the elongation zone and then the maturation zone where root hairs start to develop[48], [49]. In the meristem zone cells undergo division and differentiation on one pole as root cap cells and on the other pole as functional root cells[48]. The root cap is sloughing of its cells into the rhizosphere, those have a high exudation rate of polysaccharide-based mucilage to improve root penetration in the soil coupled to exudation of bioactive compounds that have shown significant interaction with soil organisms[47], [50], [51]. Sloughed of cells ultimately go through programmed cell death, releasing all their content in the soil[51]. In grasses, this rhizodeposition can represent as much as 25% of the carbon allocated to the roots[51]–[53]. Complementary to rhizodeposition, exudation at root apices is the most pronounced compared to all other compartment of the root system[41], [52]. There, not only quantitively but also from a qualitative perspective, some compounds are exudated exclusively from the root tips[52], [54]. Finally, root hairs located behind the apex of the roots in the maturation zone are significantly active in the allelochemical-releasing activity[54]. The exudation of sorgoleone for example is exclusively originating from root hairs, as transcriptomic analysis of the genes associated with the production of the molecule are only expressed there[55]. Complete metabolic profiling of different zones of root meristem, elongation and maturation zones show significant differences in barley[56], [57]. In this regard, monitoring the root architecture when studying allelopathic interactions is a relevant approach, because the exudation rate is directly dependent on the structure of the root system, this approach is backed by several sources[30], [52], [58].

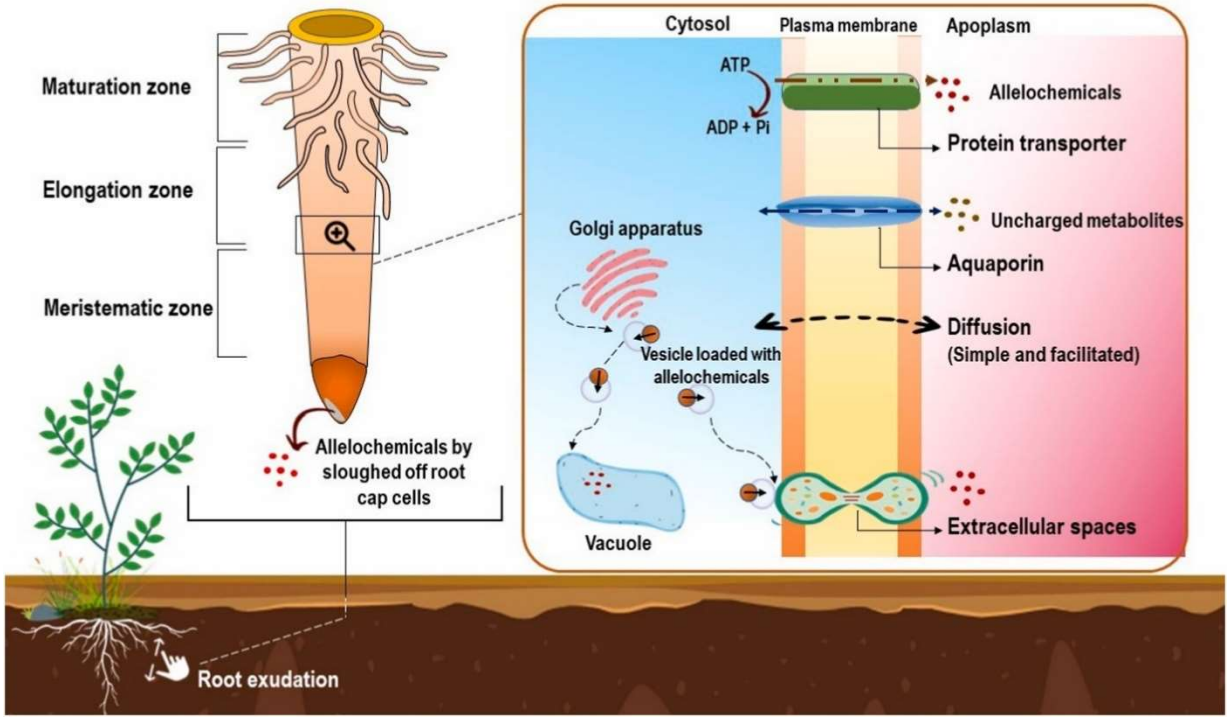

Figure 1: Schematic representation of the root tip (left) and its different development zones responsible for allelochemical activity. Zoom on the cytosol-apoplasm interface (right), allelochemicals produced in the plant root cell go through diverse transportation mechanisms. Small molecules can cross the plasma membrane through simple and facilitated diffusion. Aquaporin transportation of polar but uncharged molecules is another example of facilitated diffusion. Active transportation through ABC and MATE proteins uses ATP hydrolysis to yield the energy necessary for transmembrane transportation. Lastly, some allelochemicals are transported by vesicles originating from the Golgi apparatus and are weather sequestered in the vacuole or released in the apoplasm by exocytosis. Illustration from Choudary and al., 2023.[30]

Leaching of allelochemicals into the soil occurs on different types of plant matrixes, most illustrations were found from foliar leachates[54], [59], from decomposing plant litter on the ground[54], [60] and root and rhizome already in the soil[54], [61]. Leaching takes place when water-soluble molecules are extracted from its source and transported to the soil by rain, irrigation, mist and fog[62]. The rate at which these compounds are released in the soil is dependent on the foliar exudation rate and the quantity of precipitation in case of leaf leachate[54], [62]. In the context of plant litter laying on the ground, the initial concentration of allelochemicals coupled to the decomposition parameters stated above and finally the amount of downpour on the plant material dictate the release dynamic of the bioactive compound in the soil[63]. Leachates from leaf litter can reach up to 30% of the assimilated leaf carbon[64]. Finally, dead root leachates depend on the same parameters, with the addition of soil properties ruling the percolation speed, and consequently so the extraction of solutes from the decaying plant organs to the rhizosphere[65]. In some cases, leachates from leaves from a higher plant can directly be translocated to and absorbed by an adjacent plant, without reaching the soil[32].

Volatilisation of allelopathic compounds is mainly associated to aerial parts of the plants[66], [67]. There, epidermal tissues responsible for their biosynthesis use hydrophilic precursors and enzymatic methylation, acylation or reduction to augment the lipophilicity of those compounds and thus its volatility[68], [69]. The acquired lipophilicity allows those volatiles to diffuse trough the plant membranes and evaporate into the atmosphere[68], [70] This vision has been proven oversimplistic, as a meta-analysis shows that diffusion alone would require an unrealistic concentration of secondary metabolites in plant cells to drive the emission of volatile organic compounds (VOCs) to the observed

levels[70]. Considering that the compounds need to cross the plasma membrane, the cell wall and finally the waxy cuticle, implementation of supplementary mechanisms in the model is suitable[70]. Following this trend, most recent studies show the active contribution of ABC proteins in the cross-membrane transportation of VOCs[29], [71] and of non-specific lipid transfer proteins (nsLTP) in the cell wall[72] coupled to the potential contribution of cyclic deformation of the protoplasm as a biomechanical source of pressure to cross the cell-wall[73]. Concerning the cuticle, its waxy constituents make it a barrier to volatilisation but also acts as a concentrator of VOCs[74]. In consequence, the thickness of the cuticle dictates the emission rates and the profile of accumulation of VOCs[74]. This induces internal feedback to the emitter plant[74].

On the histological level, plants have developed other specialized structures to facilitate the propagation of volatiles in its environment, such as trichomes that are differentiated organisations of epidermal cells into hair-like structures and that have shown to directly release allelochemicals through volatilisation or leaching[28], [68], [75]. Leaves and stems also emit trough their most important leaf-atmosphere interface: the stomates leading to the spongy mesophyll (leaves only)[76]. Implications of flower VOCs in true allelopathy where not found in literature, even though those structures possess tissues specialized in the emission of VOCs, namely osmophores and crenulated epidermal cells[68], [70]. Roots also emit allelochemical VOCs[30], [67], [77], as they possess already the secretory arsenal (cf *Exudation*), and lack the resistance of the cuticle to volatilisation, except for the root cap specifically[78]. Finally, the decomposition of plant litter, be it on or under the ground, leads to the release of volatiles that act as allelochemicals[66].

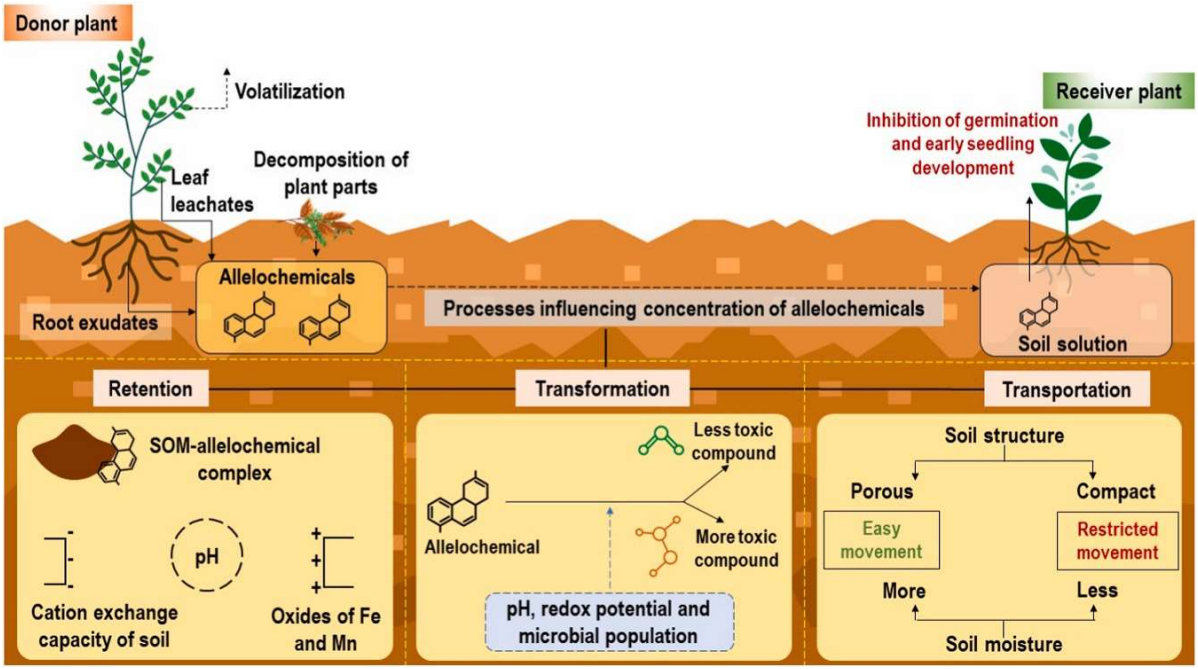

Figure 2: Schematic representation of release paths of allelochemicals by donor plant in the rhizosphere (top left); receiver plant root absorption of allelochemicals followed by their effects on germination and development (top right). Schematic overview of the main phenomena driving allelochemical translocation from donor to receiver plant: (i) retention (bottom left); (ii) transformation (bottom center); (iii) transportation (bottom right). Adapted from Choudary and al., 2023[30].

Upon arrival in the rhizosphere, allelochemicals are prone to various interactions that will modulate their efficiency, overview of this paragraph is given on Figure 2. Firstly, retention is a phenomenon of adsorption of the produced allelochemicals mainly on soil colloids. Debris of residual soil organism also

work as adsorbents, forming soil organic matter-allelochemical complexes[30]. The strength of the attraction forces, depend of course on the nature of the allelochemical, but also on the polar or non-polar components present in the soil. Typically clay or iron / aluminum / manganese oxides are considered as the main contributors to adsorption of the soil solutes on the solid colloids[79]. In addition, pH plays a large role in the cation exchange capacity of the soil which is an important factor of interaction between soil and charged allelochemicals[80]. Next, transformation of allelochemicals implements a vast diversity of factors, both abiotic and biotic. Covering this topic entirely would not fit into this study, however the specific example of benzoxazinoids of our focal plant rye is given in section 1.4.4. and shows all the different mechanisms dictating allelochemical transformation in the rhizosphere. Lastly, the concentration of allelochemicals reaching the receiver plant depends also on the transportation mechanisms. Small pores in soil have shown to heavily favor the retention phenomenon, and so reducing the mobility if allelochemical compound in soil[79]. In the contrary, meso-and macropore result in higher diffusion rates across the rhizosphere[79]. However better mobility does not always mean better allelopathic interactions, because it also means faster dilution. In sustainable weed management practices, mulching using allelopathic plant litter is common[81]. In that case, lower mobility of through the soil layers results in better allelopathic action, because the compound concentrates on the top of the rhizosphere, where future weeds seeds will potentially try to germinate[31].

1.2.4. Regulation of allelopathic response

The allelochemical production and release is determined by both genetic and environmental factors. The genotype of the individual determines which metabolites it can synthetize and pre-programs how the plant will modulate its basal immunity when confronted to environmental stress, be it abiotic or biotic[38], [39]. In case of plant-plant interaction, the communication occurs through cues, which are chemical profiles constitutively produced or induced by environmental stress (Figure 3). The perception of those cues and signals from an emitter to a receiver plant resulting in the change of transcriptomic behaviour and a switch in the pattern of the produced allelochemicals, is called inducible allelopathy. Some typical categories of plant-plant interaction can be settled to better isolate causes of inducible allelopathy: (i) stranger recognition; (ii) competition sensing; (iii) kin-recognition; (iv) danger signalisation; (v) soil feedback and legacies.

Stranger recognition is the perception of the focal plant of chemical cues that are constitutively emitted by a neighbour plant[82]. For long, attributing a single molecule as a signal eliciting plant immunity and allelopathic response for a specific biosynthetic pathway was not possible. Very recently, some ground-breaking studies are cracking the gaps of knowledge considering stranger recognition of wheat against weeds inducing DIMBOA exudation[83]. A hundred heterospecific interactions between allelopathic wheat and heterospecific individuals were studied[83]. It was demonstrated that neighbour detection and allelopathic response could be triggered by exclusively belowground signalisation, that jasmonic acid and (-)-loliolide are ubiquitously present in root exudates across a wide range of plant species, and most importantly that those compounds modulate allelochemical response of allelochemical wheat in a concentration dependent manner[83]. In the same perspective, strigolactones and allantonin have been shown to be effectively participating in underground signalisation[38], [84].

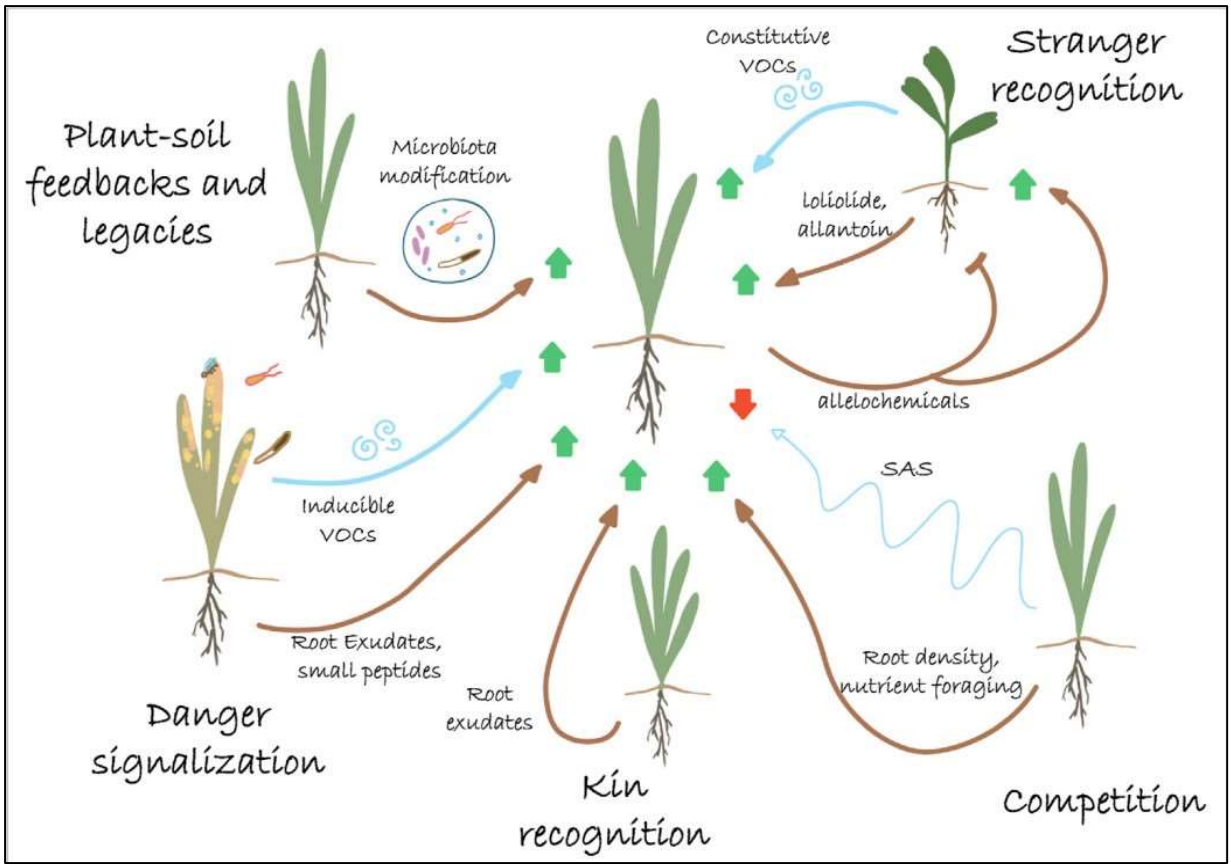

Figure 3: Representation on plant-plant interactions triggering plant immunity. The focal plant in the middle is susceptible to a diversity of signals. Brown and blue arrows represent below and above ground signals; arrowheads indicate the activation and a broken arrowhead an inhibition. Upon interaction, the plant immune system or resistance is weather increased (green arrow) or decreased (red arrow). Five different context are represented; (i) stranger recognition through constitutive volatile organic compounds (VOCs) and root exudates loliolide and allantonin; (ii) shading avoidance syndrome (SAS) and competition for nutrients in rhizosphere; (iii) kin recognition on conspecific neighbor through exudates; (iv) danger signalization through the emission of inducible VOCs when a neighbor is attacked by herbivores or pathogens; (v) alteration of soil feedback when neighbor or previous generations change the soil microbiota. Illustration from Pélissier et al, 2018.[39]

Competition sensing is a special kind of plant interaction because it can be considered an abiotic stress, but the scarcity of the abiotic resources is still caused by a plant. Low exposure of a plant to red and far-red light because of neighbour shading induces shade avoidance syndrome[85]. The phenomenon has been linked to a strong downregulation of the defence responses of the shaded plant in addition to the perturbation of many metabolic factors[86]. In some cases, nutrient or water deprivation causes a higher production of allelochemicals, even if it is hard to determine if the signal is actually caused by a neighbour plant[87].

Kin recognition is the ability of a plant to modulate its defence mechanism in accordance with the genetic relativeness of the neighbouring plant[39]. As a recent example, different rice cultivars have been cultivated together and tested in interaction with paddy weeds[88]. Results show that neighbours from the same cultivar and closely related cultivars promote the allelopathic response and weed suppression in a more efficient way in comparison to neighbours from cultivars with lower genetic similitude[88]. Genetic relatedness in allelopathic plants allows them to differentiate a potential collaborator from a competitor and prime their defence mechanisms accordingly[88].

Danger signalisation is linked to the damaging of plant organs or cells by herbivores or pathogens[89]. In this case, the plant passively releases compounds, mostly volatiles, that are sensed by the focal plant, inducing priming of their defence mechanisms against the potential aggressor[39], [89]. In case of parasite attack, *Arabidopsis thaliana* was found to actively release peptides in the rhizosphere that are perceived by neighbours as damage associated patterns and consequently induce plant resistance[90]. Upon sensing herbivore-induced VOC cis-Jasmone from conspecific neighbour attacked by aphid (*Macrosiphum euphorbiae*), potato plants defences are primed against this pest[91].

Soil feedback and legacies is the chemical-ecological phenomenon where a previous generation of plants alters the soil microbiota and leaves chemically active compounds in the soil[39]. The rice cultivars PI312777 and Liaojing-9 have shown to leave compounds in the soil they grow in that are sensible by barnyardgrass[92]. In result, the trained soils have shown to impact the development of the sensing plant barnyardgrass[92]. Moreover, DIMBOA, a benzoxazinoid from maize, has shown to be the driver of microbial feedback towards the next generation of maize growing in the soil of interest by upregulating jasmonic and salicylic acid signalisation[93]. This would result in a higher production of benzoxazinoids[93].

1.3. Plant model: *Secale cereale* L. vs *Amaranthus retroflexus* L.

This study will focus on the heterospecific interaction between typical summer weed *Amaranthus retroflexus* L., commonly called redroot pigweed, and the cover crop *Secale cereale* L. alias winter rye.

1.3.1. *Secale cereale* L., a valuable allelopathic asset

Winter rye is a species from the *Poaceae* family that accumulates the ecosystemic services it can provide to the environment it grows in. In addition to its grain production, the use of rye as a cover crop allows reduction of soil erosion and improvement of nutrient retention in soil[81], [94], [95] *Secale cereale* L. has shown to be particularly effective as a weed suppressor and its ability to resist poor soil quality and a wide range of temperature and climates make it one of the most versatile application for sustainable weed management[95]. The ability of rye to compete with and suppress heterospecific neighbors is mostly attributed to its ability to release allelochemicals in its surroundings, both actively trough root exudation while the plant is still alive and also passively, when plant liter is releasing its secondary metabolite in the soil[95]–[97]. The profile of those allelopathic compounds is made of several groups of molecules, typically polyphenols like gallic and vanillic acids in seedlings, tillering plants and crop residues and phenylpropanoids like ferulic acids in seedlings and especially in crop residues [98]. These allelochemicals are present in diverse plant families and several effects and applications have been studied [27], [98]–[100]. Most importantly, rye is biosynthesizing a range of indole derivatives that form the chemical class of the benzoxazinoids[95], [101]. These compounds are one of the focal points of this study, because their biosynthesis and storage, transcriptomic regulation, release in the environment and finally its chemical-ecological role in the environment give consequent insights on the allelopathic behavior of rye (*cfr* Section 1.4).

1.3.2. *Amaranthus retroflexus* L., a threat for our agriculture.

Substantial interference of redroot pigweed with many types of crops has been demonstrated multiple times[15], [102]–[107]. Its capacity to inhibit growth and reduce yield of plants of agronomical importance has been studied and proven in field experimentations, for instance with maize (*Zea mays*)[103], sugar beet (*Beta vulgaris*)[108] and red kidneybean (*Phaseolus vulgaris*)[109] as well as its capacity of allelopathy through aqueous extracts tested for example on cucumber (*Cucumis sativus*)

and barley (Hordeum vulgare) seedlings[107]. Complementary to its direct threat and competition to crops, redroot pigweed has also evolved in many herbicide-resistant biotypes in different zones of the world[110]–[112]. Indeed, studies report sign of resistance to ALS-inhibiting herbicides thifensulfuron-methyl; fomesafen[111], [112]; imazethapyr[110] and bentazone[113] in China. In Europe, the literature mentions early appearance resistance against triazine[114], [115] based herbicide, and more recently the development of metribuzin resistance and cross-resistance to ALS-inhibiting herbicides[116]. All those indicate clearly that the competitivity as weed, the allelopathic potential and the herbicide resistance of redroot pigweed combine into a problematic blend for our cropping system and that new solutions need to be developed to avoid further aggravation.

In the present state of knowledge, the suppression of *Amaranthus retroflexus* L. in integrated weed management practices has not been sufficiently studied, hence the appeal of this study for the topic. Nonetheless, the role of cover crops in the interference with redroot pigweed has been tested and partially explained in a recent field study in Switzerland[19], where the main effect has been demonstrated to be caused by shading of the pigweed seedling present in the field. Further investigation was fostered to determine the role of allelopathic mechanism in the observed interference[19]. More precisely, other studies focused on mulching practices using allelopathic rye and wheat, encouraging results against *Amaranthus retroflexus* L. have been observed[31], [81]. The effects were imputed to the accumulation of benzoxazinoid and their degradation products in soil complementary to the covering of the soil shading the surface[81], [117]. Extensive explanation on the production and activity of those compounds in provided in section 1.4.

1.4. The chemistry and ecology of benzoxazinoids

When studying the allelopathic potential of rye, the most important allelochemicals are the class of the benzoxazinoids (BXs) [95]. This group of compounds is predominantly synthetized in the monocotyledon *Poaceae* family, but occurrences also have been observed in dicotyledon families, namely *Acanthaceae*, *Calceolariaceae*, *Lamiaceae*, *Plantaginaceae*, and *Ranunculaceae*[101]. BXs are of the highest importance in our agricultural system because they are specialized in the defense against other plants, insects and microbiological threats for some of the globally most produced crops like wheat and maize (and in rye of course)[101], [118].

1.4.1. Biosynthesis pathway and storage

The rye biosynthetic pathway of BXs is a divergence of the tryptophan pathway, initiating in plastids from the precursor molecule indole-3-glycerolphosphate[119]. The whole biosynthetic pathway is illustrated on Figure 4. The first enzymatic reaction produces indole, which enters the cytosol in free form before further transformation on the surface of the ER[118], [120]. There, four consecutive hydroxylation through cytochrome P450 proteins result in the production of the hydroxamic acid: 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIBOA)[118]. This compound is the first metabolite of this metabolic pathway to be significantly autotoxic in the produced concentration[95]. From this step on, rye cells rely on glycosylation and to detoxify DIBOA into its glucosidated form Glc-DIBOA. The newly formed glycoside is the precursor of Glc-DIMBOA that is synthetized by a cytosolic dioxygenase and methoxygenase[121]. In rye the distribution of BXs differs strongly between shoot and root[122] and is strongly modulated by environmental stress[118]. In the shoot, DIBOA represents almost all of the total BXs concentration[122], [123]. In the roots, the distribution is shared between DIBOA, DIMBOA and their respective glucosides[122]. The very high proportion of DIBOA as a precursor in shoot, coupled to the fact that BXs are present in phloem exudates to the roots, support the hypothesis

that there is an active transportation of the synthesized compounds from the shoot to the roots. There, the BXs are sequestered in the vacuole or directly released in the environment[124].

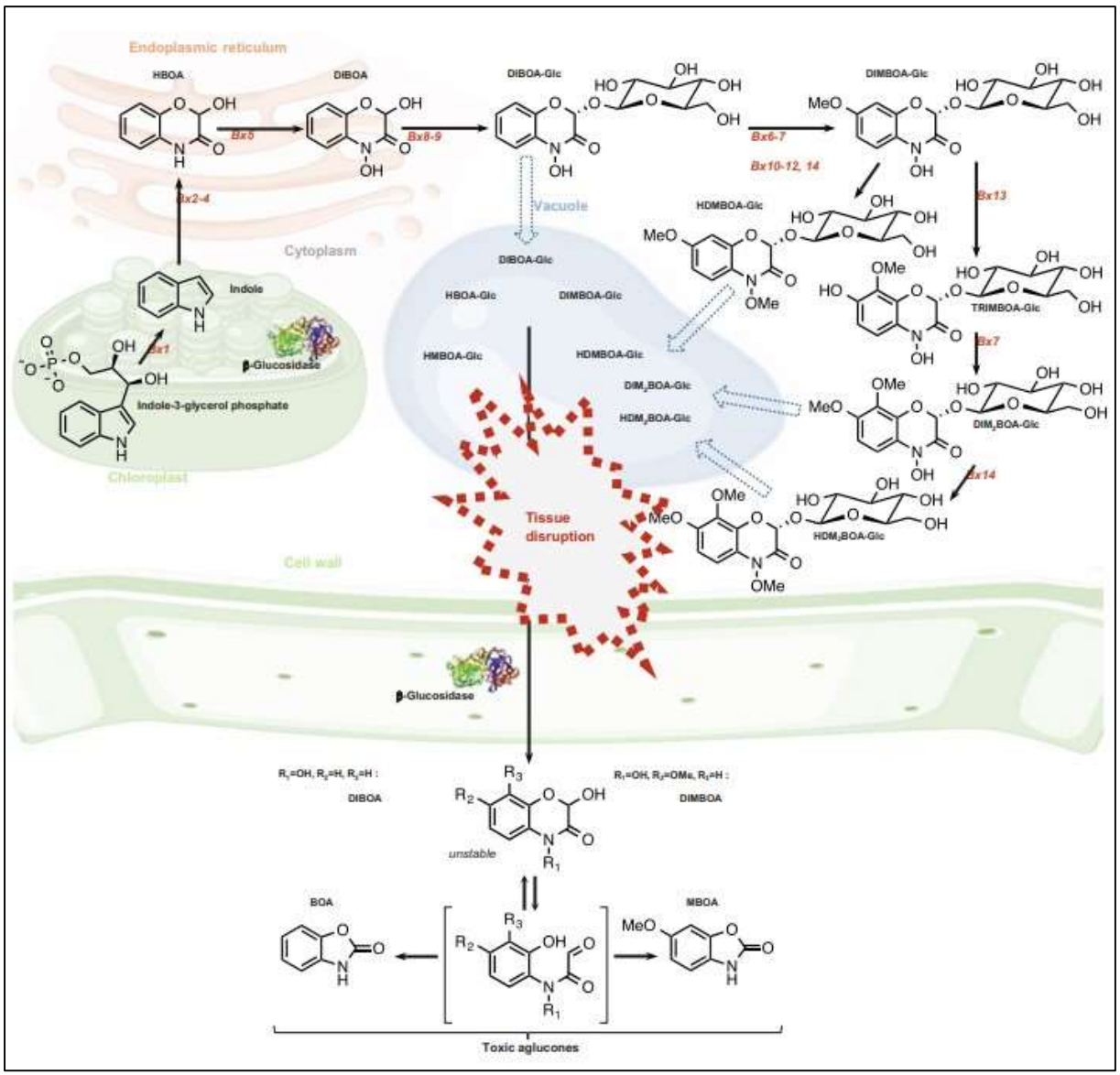

Figure 4: Schematic representation of the biosynthetic pathway of benzoxazinoids, their release through tissue disruption and the formation of the first degradation products. The pathway starts in the chloroplast with the formation of indole, continues in the endoplasmic reticulum until DIBOA is released in the cytosol and then glycosylated. The glucoside can be directly sequestered in the vacuole or undergo a multitude of enzymatic reactions before being stored in the vacuole. Upon tissue disruption the glucosidases associated to the cell wall cleave the glycosides and the toxic compounds DIBOA and DIMBOA are released in the environment. Those are unstable and are transformed into more stable BOA and MBOA respectively. Illustration from Robert et al, 2023 [125]

1.4.2. Transcriptomic regulation

Initially, the synthesis of BXs and the transcriptomic behavior linked to it was elucidated in maize and then wheat[95], [120]. Subsequent research led to the identification of the orthologue sequences in rye, which has a lesser diversity of BXs compared to wheat and maize[101]. In rye, the first step of the BX synthesis is associated with the gene ScBX1, which is constitutively expressed during the first development stages of the plant[119]. The overexpression of the gene can be induced through perception

of viral elicitors or methyljasmonate treatment and results in the increase of BXs production[119], [126]. Depending on the cultivar, its expression end after 21 to 42 days, after which it is not detected anymore[119]. Albeit, production of BXs is still observed after this period, and is still inducible, which proves the occurrence of the regulation through a supplementary gene, ScIgl[119]. This gene expression could be strongly correlated to the production of BXs, even one month after the ScBX1expression had completely stopped in rye plants[119].

In the current state of knowledge, more research has been done on the model plant wheat, which shares many genetic similarities with rye concerning BXs synthesis. The production and exudation of BXs and the expression of BX associated genes are induced by the perception of neighbors, by exudates from heterospecific plants, damaging of leaves through mechanical action, leaf consuming herbivores, or treatment with stress inducing hormones or metabolites[38], [83], [127], [128].

1.4.3. Release mechanisms of BXs

Rye releases BXs through two mechanisms, root exudation and degradation of plant material[125]. The exudation was for long suspected to take place at root tips and more recently trough the activity of ABC and/or MATE proteins[118]. A study using BXs staining method by $FeCl_3$ on maize roots showed the opposite phenomenon, BXs seem to be present on all the length of the roots, except for the root caps[118]. In addition, complete inhibition of ABC and MATE proteins with Na_3VO_4 and NH_4Cl respectively, showed no significant reduction in exudation of BXs[118]. Therefore, the latest hypothesis is that BXs cross the membrane trough passive diffusion. As mentioned (cf. *Exudation*), polarity would not allow BXs to cross the lipidic plasma membrane. Facilitation through porins is a possibility [30], [129].The transposability of this discovery from maize to rye has not been found in literature.

Once the plasma membrane is crossed, BXs reach the apoplasm, where extracellular β-glucosidase cleave Glc-DIBOA and Glc-DIMBOA into their aglycones[120], [130]. Finally, the allelochemicals reach the rhizosphere, where their ecological effect can unfold[122], [123], [131]. Latest studies concerning exudation of BXs in rye showed that only a fraction of the BXs is cleaved before entering the rhizosphere[122], [123]. Complementary to active exudation, BXs are released passively when cells are ruptured[118]. This phenomenon is observed in the context of programmed cell death in reaction to a pathogen or during cell destruction during herbivore consumption or crop termination[81], [120], [132], [133].

1.4.4. Effects of benzoxazinoids and their degradation products in the rhizosphere

When entering the rhizosphere, BXs glucosides are in contact with numerous β-glucosidases, originating for plant residues, from fungal activity or microbial activity[134]–[136]. This enzymatic activity leads to the formation of aglycons, mainly DIBOA and DIMBOA in the case of rye[95], [134]. At this step, those compounds are highly unstable and spontaneous deprotonation in aqueous solutions trigger cyclic contraction of DIBOA into BOA and DIMBOA into MBOA[95], [130]. The stability of those compounds against abiotic conditions in soil are higher compared to the aglycones, but they are still prone to detoxification and alteration by soil organisms[124], [130]. The degradation pathway depends on the organisms involved and shapes the interactions in the soil[125], [130], [137].

The first path is exclusive to plants, in the case that emitted BXs translocate and enter the receiver plant. There, the detoxification mechanism is focused on glycosylation and then alter the newly formed glycoside[137]. Most mentioned forms, malonyl glucoside carbamate and BOA-6-O-Glc, are represented on Figure 5 [130], [138], [139]. Those can then be weather metabolised, sequestered in vacuoles, deposited on the cell wall or even exudated again[137]. In plant-plant interactions, BXs are responsible for many metabolic perturbations involving reactive oxygen species (ROS) with all complications that come with it[138]–[140]. On the organism level, growth inhibition and germination inhibition have been observed BXs treated plants, leading even to high mortality [125].

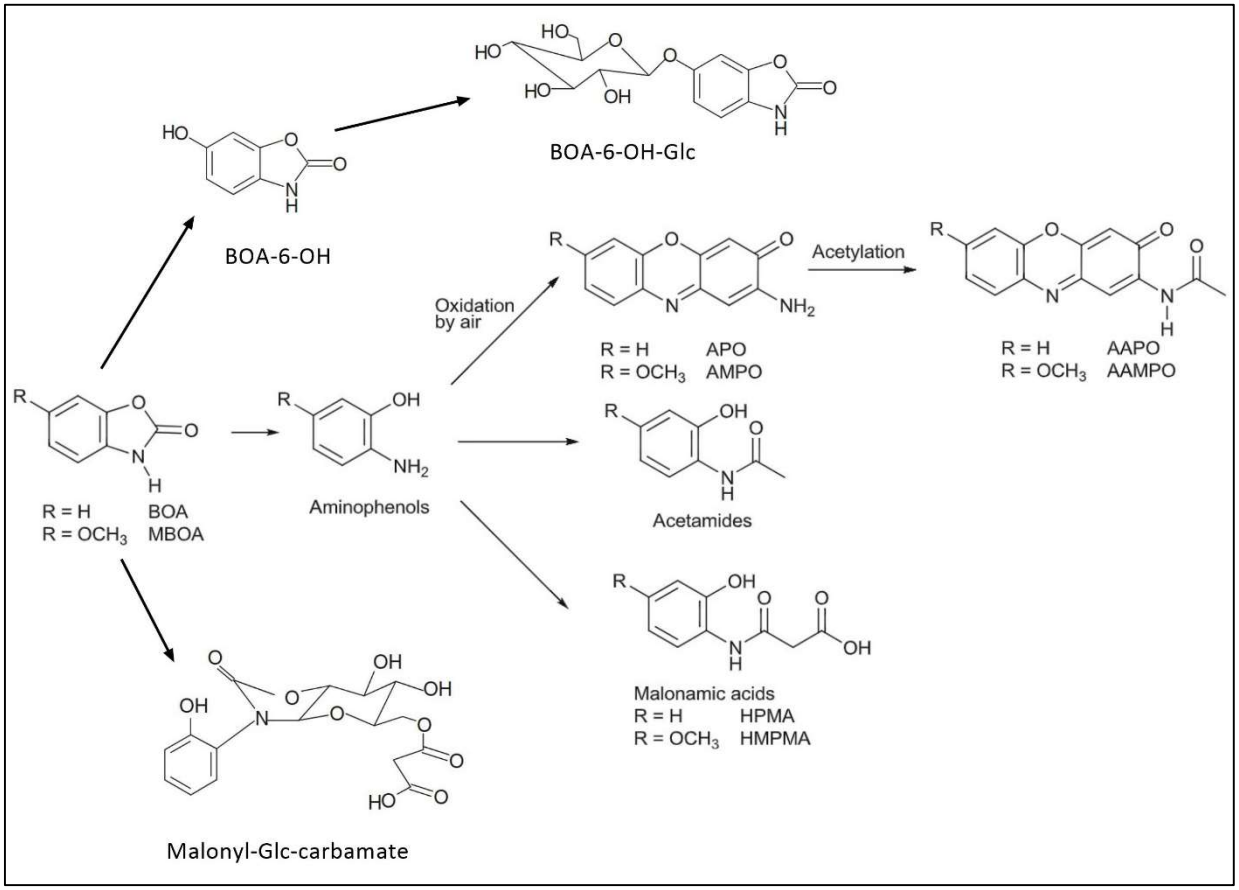

Figure 5: Degradation pathways of BOA and MBOA when reaching the environment. First branching of is exclusive to plant detoxification through glycosylation. Otherwise, bacteria and fungi degrade BOA/MBOA into aminophenols (AP) that spontaneously undergoes oxidative dimerization into APO when not metabolized by soil organisms. In that case, bacteria detoxification leads to formations of acetamides and fungal detoxification to the formation of malonamic acids. Some soil bacteria can directly metabolise AP as an energy source.

Concurrent to degradation through plant detoxification, degradation paths of BXs going through bacterial and mycotic pathways (exposed on Figure 5) are commonly present in the rhizosphere[130]. It results in the formation of aminophenol (AP) that can undergo spontaneous dimerization caused by oxidation with air into aminophenoxazinones (APO and AMPO)[141]. AP can also directly be acetylated by bacteria and fungi into acetamidephenols (AAP)[130] and even serve as energy source to some bacteria as they possess the ability to use it as energy source by entering the TCA-cycle after further enzymatic alteration[142]. Some *Fusarium* species have specialised in detoxification AP into N-(2-hydroxyphenyl)-malonamic acid by the activity of a N-malonyl transferase[143]. From all those

paths, degradation products only the aminophenoxazinones remain phytotoxic[130], [144]. Even more because those compounds are stable over time and hydrophobic, causing them to rise in concentration over time[130]. As the rye (or another BXs producing plant) is releasing BXs in the rhizosphere, APO accumulates as it is inefficiently washed away by precipitation or irrigation concurrently to its stability against abiotic factors and resistance to enzymatic degradation[95]. Most importantly bioassays show APO as the most phytotoxic compounds along the BXs metabolic and degradation chain[144].

2. Problem statement and objectives

2.1. Fundamental research goals

Rye has proven multiple times that it can effectively suppress weeds, but the precise mechanisms inducing the allelopathic response are still unknown. Concerning pigweed, almost no research concerning its secondary metabolites and their effect on other crops has been done. Even if the interference of pigweed as weed against crops has been established, the effects of its metabolites on rye have not been evaluated. No data was found on the residual allelopathy of redroot pigweed on rye. Taking those gaps of knowledge into account, a first research question can be formulated.

Does *Secale cereale L.* perceives residual metabolites of *Amaranthus retroflexus* L. in the rhizosphere? If so, how does it affect its growth pattern and systemic response?

To answer this question, different features of rye have been selected as potential indicators. First, the biomass allocation between shoot and root can give insights on how the plant is prioritizing the development of certain parts of the individual. Then, root architecture analysis is expected to give an overview on how the root system is unfolding when residual compounds of pigweed are detected by rye. The accumulation of defence molecules could be considered a valid indicator of immunity priming in plants and will be quantified in this study. The specific compounds are DIBOA and DIMBOA, as they are the most relevant molecules in rye when it comes to induced allelochemicals.

Chemical ecology of rye goes beyond the exudation of roots. The field of volatilomics is still largely underrepresented in the literature, especially concerning root volatile organic compounds. Since the emission of those volatiles is highly suspected to modulate below ground plant-plant interactions, characterizing the unknown behaviour of rye in this context is most important. Therefore, the second question of this study is:

Are rye roots emitting volatiles constitutively? Is the volatilome of rye roots altered when in contact with residual metabolites from redroot pigweed?

In order to address this question, it was decided to use a growth medium that does not emit VOCs compared to usual soil experiments. The alternative chosen was glass beads as the growth medium for hydroponics. Besides, the concentration of the VOCs in this artificial rhizosphere are suspected to be very low and their diversity very high. In addition, an apparatus specialised in root volatiles pumping was implemented in this work. Finally, it was decided that GC-MS analysis is the most suitable tool to identify and potentially quantify the components of the volatilome of rye.

2.2 Research project goals

This study was done in the context of a collaboration with the Agroscope in Switzerland. There, previous and ongoing work was done on rye-pigweed interference. The present study is settled to bring acquired knowledge and methods to Gembloux Agro-Bio Tech and to transpose these practices on the material available. From there, it was necessary to develop a strong foundation of methodology for the research project, in order to have a multitude of fully functioning and optimised tools. Practical objectives have been established to frame this study and to bring the project forward.

1) Find a method to grow rye in glass beads using a hydroponic solution in a phytotron. The growth media should maintain constant humidity levels and produce homogenous populations with similar or equal development stages throughout the experiment.

2) Find a way to induce biotic stress to rye through chemical cues from *A. retroflexus* L. without inducing competition between the two plants.

3) Develop an apparatus and analytical methods that allows to monitor the features of rye to compare a group of stressed rye plants vs a control group of unstressed rye plants. In all logic, these features are the ones formulated in our research questions above.

3. Material and methods

This section contains a detailed description of the methodology, implementing many technical aspects. As important and those can be, such a densely packed sequence of information can lead the reader to confusion in terms if general overview of how the research was done. Therefore, a narrative summary is provided here.

All plants, be it rye or pigweed were grown in glass sand, hydroponic solution and set in a phytotron. The plants were grown in a custom-made glass apparatus, based on the structure of a 500 mL Schott flask, in which an a glass inlet and an outlet was inserted welded. This would allow the setup an air pumping system directly to the growth medium.

First, an aqueous extract was prepared, using 2 weeks-old pigweed plants that were freeze dried before extraction. This extract is then added to a hydroponic solution and will be used later to induce stress in rye plants. Those are grown in a customised glass container. Two distinct populations will grow, the first is the control group or "R0" and the second is the treated group or "RA" that grows in the same conditions as R0 but using a hydroponic solution that was contaminated by the extract mentioned above.

Plants will be analysed after 3, 6 and 9 days after 24 hours germination time. The experimentation starts with the pumping of root volatiles of the plants using electric pumps and adsorbent cartridges for 4 hours. After this period, rye shoots are cut of and stored at -80°C. Roots are washed and scanned and end up stored at -80°C like shoots. All parts of the plant then go to the freeze-drying step, after which the dry mass of shoot and roots is weighted to obtain the ratios of biomass allocation. Cartridges are thermally desorbed in a GC-MS, alongside the internal standard that was directly injected in the cartridges to determine the volatilome the rye roots.

The next step is the quantification of BXs in the shoot. The dry material is crushed and its allelochemicals are extracted using a methanol/water/formic acid solution. The newly formed extract is filtered and placed in vial for HPLC analysis. Previous calibration was done using commercially available standards.

3.1. Plant culture apparatus

3.1.1. Preparation of hydroponic nutrient solution

The nutrient solution was prepared using Hoagland's No.2 basal salt mixture (Merck Life Science BV; Belgium), by dissolving 815 mg of the latter in 1 L of deionized water. This concentration is considered half of the standard concentration of Hoagland's solution. The Hoagland's solution is then autoclaved to sterilise it, avoiding the nutrient solution to be a source of microorganism interference. To reduce cost of experimentation, the formulation of Hoagland's solution was carried out in the lab, using the list of ingredients provided by the manufacturer[145].

3.1.2. Seed sterilisation

The winter rye (GR71; Sativa Seeds; Switzerland) and redroot pigweed seeds stored at 4 °C are soaked in technical ethanol for 10 seconds to reduce the microorganism count on the surface of the seeds before entering germination. After soaking, the rye seeds were placed on Whatman paper and oven dried for 20 minutes at 37 °C. To enhance germination rate of *A. retroflexus* L., the seeds are put for an hour at

50°C in a drying oven on Whatman paper. Even though this method does not provide absolute sterile environment on the surface of the seeds, it allows to maintain the viability of the seeds.

3.1.3. *In vitro* germination of *Secale cereale* L.

Glass sand is layered in the bottom of a petri dish to form a one-centimetre uniform layer and saturated with Hoagland's solution. Ten rye seeds are placed on the growth medium. Each seed was then delicately covered with a pinch of glass sand and each one received additional 1 mL of Hoagland's solution. The petri dish was covered and put 24h inside the phytotron. After this lapse of time the most vigorous seeds were selected to enter the growth step.

3.1.4. Growth apparatus

A 250 mL Schott was customized by adding a glass inlet and outlet at two ends of the container, and a perforated cover was used. In each container, approximately 600 g of glass beads humidified with 60 mL of Hoagland's solutions was added and the cover screwed back on the Schott. The cover of the container is perforated to allow placement of seedling, and application of Hoagland's solution. See Figure 6 for detailed description. Using 200 µL tips, small holes in the growth medium were perforated, and the selected seeds were placed in their respective holes, with the emerging radicle placed downwards. Then, 1 mL of Hoagland's was administered to the seedling and set to grow inside the phytotron.

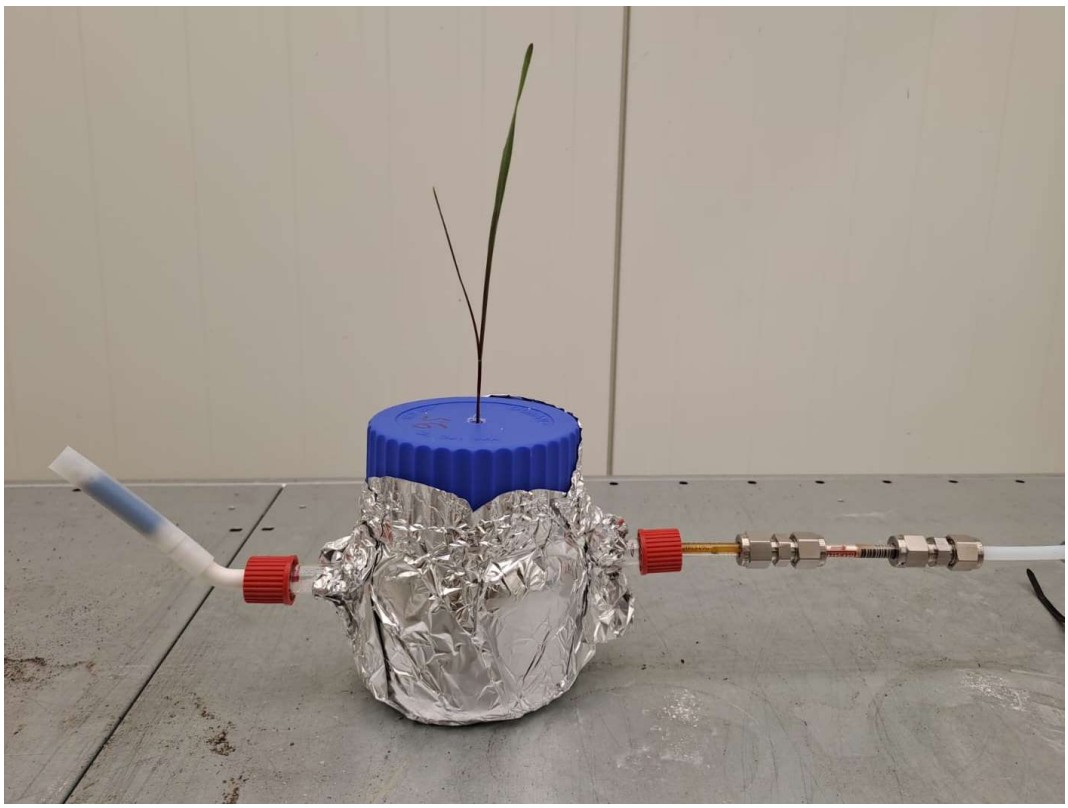

Figure 6: Image of the apparatus used to grow rye in the phytotron. Left of the image is an active charcooal filter, that is linked to a modified 500mL Schott container. The Schott is caped with a perforated cover. On the right side of the image, the air outlet is linked to two Tenax sorbent cartridges. Those cartidges are directly linked to a pump that is pulling air from the growth medium into the Tenax.

3.1.5. Growth conditions in phytotron

The growing phase takes place in a greenhouse with controlled conditions, the environment was set with a 12-hour day/12-hour night photoperiod and a thermoperiod of 21 °C-day/ 18 °C-night. The relative humidity was set at 70%. The total weight of each pot is being monitored to keep track of evaporation and every seedling is treated with a 1mL/day dose of Hoagland's. One mL of Hoagland's solution was added daily to equilibrate the loss of water in the growth medium.

3.2. Sampling and sample preparation

3.2.1. Roots volatile organic compounds (R-VOCs)

Sampling is done using dynamic headspace sampling (DHS), an activated carbon filter in PTFE tubing is screwed onto the gas inlet of the Schott. Two Tenax sorbent cartridges are connected in series to the gas outlet and connected to a Gill Air programmable pump. The pumping program runs for four hours, and the total pumping volume is 10 L (50cc/min). The cartridges are kept at -20°C until analysis. In this experimentation, the sampling was done at day 3, 6 and 9 after planting, with n=6 repetitions.

3.2.2. Rye shoots sample collection and preparation

Respectively to the timeframe explained above, rye shoots are cut using scissors just above the root collar. Plant material is immediately flash frozen using liquid nitrogen and kept at -80°C and then freeze dried during 48 hours at 200 mbar and refrigerator set at -50°C. Total dry mass is determined, and dried samples are stored at -20°C.

3.2.3. Rye roots sample collection and preparation

The roots of rye are extracted from the glass beads, followed by soaking and stirring in deionized water approximately one minute to allow the glass beads to detach from the tissues. The roots are suspended in deionized water before the root scanning (3.3.3 section).

3.2.4. Benzoxazinoid extraction

The freeze-dried plant material resulting from 3.2.2. section is crushed and 25 mg are weighted and placed in Eppendorf tubes. One mL of extraction solvent (methanol/water/formic acid; 50/50/1; v/v/v) is added, followed by 4 glassbeads (<1 mm). Extraction time was 1 h in a Heindolph Multireax Agitator set at 2000 rmp (12566 rad/min) and separation of solid phase was proceeded in an Eppendorf MiniSpin centrifuge set at 13,4 krmp (12045 g) for 8 minutes. The supernatant is syringed and filtered in 0,45 µm PTFE filter placed in a vial and finally stored at 4 °C.

During experimentation, it was necessary to try several extraction modalities to confirm that the total solubilization of the BXs was complete in our first method stated above. In this regard, a second extraction was done for 24 hours with the condition stated above. A third extraction is the same as the second, with an ultrasound bath step of 15 min between the addition of solvent and the agitation in the Multireax.

3.3. Instrumental analysis

3.3.1. Gas chromatography coupled to a mass spectrometer (GC-MS)

The cartridges were loaded on a GERSTEL Thermal desorption unit (TDU) in splitless desorption mode, set at initial temperature of 40 °C, ramping at 100 °C/min to 280 °C with 5 min hold time once reached maximum temperature.

The GERSTEL CIS was set at -10°C with a ramp of 12 °C/s to 280°C, with holding time of 5 min.

The GC oven (Agilent 7890B GC system) was set at initial temperature of 35°C for 2 min and then started ramping at 5°C/min until 300°C with 2 min holding time. Carrier gas was helium and flowrate was constant at 1 mL/min. Separation was done on an Agilent 19091S-433 HP-5ms column (dimension:30 m x 250 µm x 0.25 µm). Before analysis 100 ng of internal standard phenyloctane was injected into the cartridges, by 1 µL of hexane solution delivered by the Gerstel MultiPurpose Sampler. The mass spectrometer (Agilent 5977B MSD) was set in SCAN mode with the range of 50 to 500 a.m.u. and an ionization mode of 70 eV and gain set at 1.

3.3.2. High performance liquid chromatography coupled to UV absorption detector and diode array detector (HPLC-UVD)

The benzoxazinoids quantification was done on an Agilent 1200 HPLC System. Separation was done on an Agilent Poroshell C18 column and using a Solution A (methanol/water/ortho-phosphoric acid 85%; 10/90/0,1; v/v/v) and Solution B (methanol/ ortho-phosphoric acid 85%; 100/0,1; v/v) as eluents. Injection volume was 10 µL. Quaternary pump programming is given by Table 2. Absorption was measured at 250 nm; 280 nm and 288 nm. Before each run of samples, a set of standard solutions was injected to confirm retention times.

Table 2: Programmed sequence of the quaternary pumping system of Agilent 1200 HPLC system. Solution A (methanol/water/ortho-phosphoric acid 85%; 10/90/0,1; v/v/v) and Solution B (methanol/ ortho-phosphoric acid 85%; 100/0,1; v/v). The values represent the proportion of eluents pumped inside an Agilent Poroshell C18 separation column throughout the method.

Time (min)	Solution A (%)	Solution B (%)
0,0	77,0	23,0
2,0	77,0	23,0
5,0	70,0	30,0
8,0	20,0	80,0
20,0	10,0	90,0
21,1	0,0	100,0
28,0	0,0	100,0
29,0	77,0	23,0
35,0	77,0	23,0

3.3.3 Root Scanning

Roots were submerged in a thin layer of ethanol/water solution (70/30; v/v) inside a square petri dish of 12cm x 12 cm, and finally covered with a plastic foil. The dish is placed inside the scanner Epson Perfection V800 Photo digital ICE technologies) with a resolution set at 600DPI in acquisition software Epson Scan Professional.

3.4 Data treatment and statistical analysis

Roots were submerged in a thin layer of ethanol/water solution (70/30; v/v) inside a square petri dish of 12cm x 12 cm, and finally covered with a plastic foil. The dish is placed inside the scanner Epson Perfection V800 Photo digital ICE technologies) with a resolution set at 600DPI in acquisition software Epson Scan Professional.

3.4 Image and data treatment

3.4.1. Image treatment

Root scans were analyzed using Rhizovision explorer[146]. The settings of analysis were: enabled root pruning, contrast value kept at default 200, root classes based on diameter were set form 0,0 to 0,15 mm for the first group and following this 0,15 mm increments for the 10 first group. An 11^{th} group is set from 1,5 and above. The size of the increment size was determined in such a way that (i) lateral roots and primary roots could be discriminated from one another, (ii) the seed residue is attributed to the 11^{th} class without interfering with the measurement of the roots and lastly (iii) the root classes are attributed in a gaussian or bimodal distribution.

3.4.2. Data treatment

Data treatment from biomass measurements and HPLC-UV was done using GraphPad Prism 8 by Dotmatics[147]. Calibration curves were traced using XY linear regression without forcing through x=0, range of regression starting with the x=minimum concentration and ending with x=maximum concentration. Significance of differences was determined using multiple t-tests, with adjusted p value threshold of significance at 0,05 and $\propto$=0,05. Histograms and layout were done using the GraphPad Prism layout editor.

3.4.3. GC-MS Chromatogram

Identification and quantification of the data generated by the MS detector was processed using Agilent Unknowns analysis[148]. The analysis method was the following: TIC analysis; NIST17 list with normal search type and adjusted scoring; Use RT match with gaussian RT penalty function (6sec) and multiplicative RT mismatch penalty; max identification count 3, minimum match factor 85, minimum m/z 30, spectral search. The response factor between internal standard and analytes was estimated at 1, given the fact that it was unknown what ions might be detected.

The hit lists were then uploaded on the UGent Venn diagram webtool[149] for sorting. The webtool generated Venn diagrams and sorted listings of the match list.

4. Results and discussions

Before entering actual presentation of the results, a little overview of the data structure is necessary to avoid confusion during the interpretations. As already mentioned, a control group of rye was grown in hydroponic conditions without any treatment with the extract of pigweed. This control group is labelled R0 followed by its days after germination when sampled, namely 3, 6 or 9. The group of rye plants that went through the treatment is labelled RA followed by the number of days after germination (d.a.g.).

Table 3: Number of repetitions for each modality during experimentation; R0: rye control group; RA: rye treated with *Amaranthus retroflexus* L. extract; d.a.g: days after germination.

Modalities	3 d.a.g	6 d.a.g.	9 d.a.g
R0	5	6	3
RA	4	6	6

Data on Table 3 exposes the differences between the modalities when it comes to usable repetitions. Unfortunately, the control group supposed to come to 9 d.a.g. suffered from a very high mortality rate in the first days after gemination. In consequence, statistical comparison between R0 and RA for this modality is of lesser importance compared to the experiments using 3 and 6 d.a.g.

4.1. Biomass allocation

Starting with total biomass shown on Figure 7A, all three populations from the control group (3, 6 and 9 d.a.g.) have a higher average than their counterpart treated with the *Amaranthus retroflexus L.* extract. The same phenomenon is observed on the shoot dry mass (cf. Figure 7B) and in root dry mass (cf. Figure 7C) the averages are almost identical when comparing the two groups. Multiple t-tests comparing control vs. treated group did not show any significant differences. However, observing the mass of the individuals and their different organs is obviously not enough to identify a shift in development strategies. Using the proportional allocation of biomass is a more comprehensive approach, giving insights on how plant relocates its resources comparatively with its total biomass. In this case a slight shift is recognisable on Figures 7D, 7E and 7F, where the treated group is showing higher average allocation of biomass to its root in all three modalities. Even if a tendency is recognisable, statistical tests could not prove a significant difference in any of the three modalities when comparing the control group vs. the treated one.

However, when comparing the time modalities with one another, the allocation of biomass switches from a majority of the biomass designated for the root development (52% in R0 and 58% in RA of total dry mass) to a majority of biomass allocated to shoot development reaching 64% and 56% between the 3rd and the 6th day after germination. This change is statistically very significant (p value = 0,0006). This indicates that the seedling in both groups prioritises the establishment of a functioning root system and then rapidly shifts to the development of aerial parts. It is important to mention that the BBCH scoring of seedlings was monitored and almost no differences were observed. For the 3 d.a.g. modalities, little heterogeneity was observed upon daily observations, and seem to be linked on the different timings of coleoptile emergence after putting the germinating seeds in the glass beads. In modalities 6 and 9 d.a.g. every seedling was scoring on the same development stage, namely 11 for 6 d.a.g. and 12 for the 9 d.a.g.. These scores correspond to the unfolding of the first and the second leaf of rye plants.

If exposing rye roots to the pigweed extract is truly perceived and causes a change of the proportion of resources dedicated to the roots, it can be expected that the observation of the actual roots might give further insights. Recent studies show that the root placement in rice and wheat is strongly dependent on stranger recognition[58], [88]

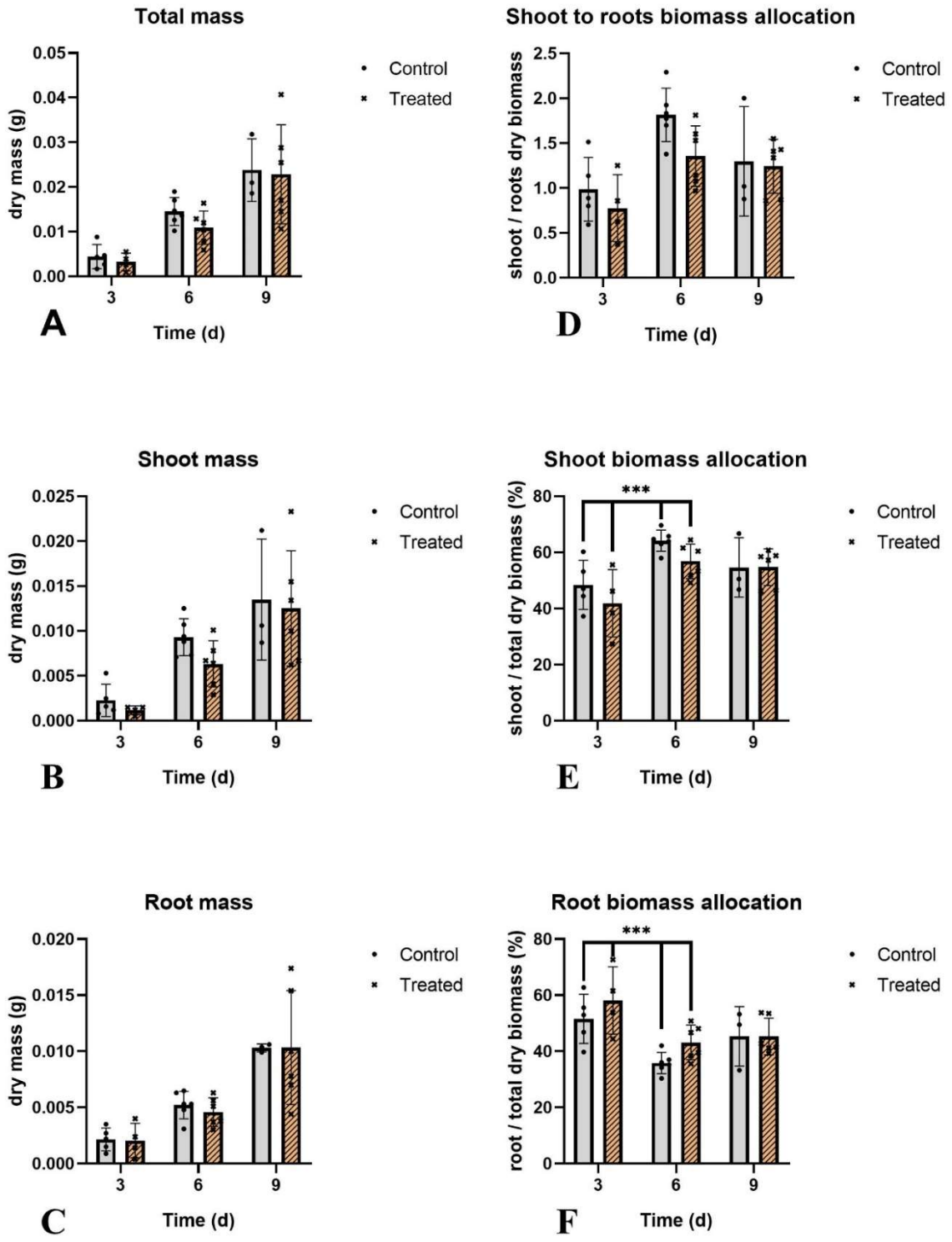

Figure 7: Six graphs describing the biomass allocation in a rye control group (grey) and rye treated with *Amaranthus retroflexus* L. extract (orange and black). Every data point is provided with control group as points and treated group as crosses. On every graph from A-F the abscissae show the time between germination and sampling and is given in days. A: total dry mass of plant material given in grams. B: dry mass of shoot given in g. C: dry mass of root given in g. D: ratio of shoot dry mass to root dry mass. E: percentage of total biomass allocated to shoot. F: percentage of total biomass allocated to root. *** indicates that modalities 3 d.a.g. compared to 6 d.a.g. are very significantly different (p=0,0006).

4.2. Root architecture

First, overall features of the rye roots system will be exposed, followed by more detailed analysis of the different repartition of the roots will be addressed. The development of the growing roots is recognizable on Figure 8A.

Without surprise the progressive increment in total root length as the plant is growing can be seen, both in the control group and the treated group. Statistical comparison however could not differentiate the two groups in any of the growth period modalities. From this perspective, it doesn't seem that the extract at the present concentration induces a reduction or enhancement of root elongation on the whole root system level.

In the context of below ground interactions and specially when it comes to exudation, root size is an important factor, but the diversity of developing tissues present and their respective proportions in the root system also dictate the secretory behavior of the organ (*cf.* sections 1.2.3 and 1.4.3). Therefore, measuring the number of root tips that emerge during exposure to *Amaranthus retroflexus L.* is important, because several specific roles when interacting with the rhizosphere are attributed to this root area. The latter is shown on Figure 8B. Interestingly, no significant differences could be identified when comparing the control group with the treated group. Still, the number of detected root tips is increasing over time, which is linked to the emergence of new primary roots from the plant and secondary root branching off the primary roots.

As root tips are the site of nutrient intake, reports of their increase in numbers can be observed in case of water deficiency stress and of high nitrogen availability[150]. Besides, plant growth promoting rhizobacteria (PGPR) have known effects on the increase of the number of root tips[151], [152]. On the contrary, allelochemicals are associated with several root growth perturbation mechanisms, including in the root tip[153], [154]. On this subject, abiotic stress was avoided and PGPR occurrence is very limited as rye was grown in glass beads and hydroponic solution. Concerning allelopathic interaction, there is a probability that (i) the concentration of the treatment was not sufficient to cause an inhibition of root tip emergence (ii) pigweed did not reach a development stage prior to the extraction of its metabolites that allowed the unfolding of its allelopathic arsenal and finally (iii) the allelochemicals in pigweed might not be water soluble and so did not end up in the extract.

Even if neither the total mass, length or number of root tips could differentiate the control group from the treated one, visual inspection of the scans directly can provide precious comprehension of potential phenomenon taking place. After 3 d.a.g. (see Figure 7), no difference is visible, the juvenile plant is developing its primary roots directly sprouting from the collar in both treated and untreated groups.

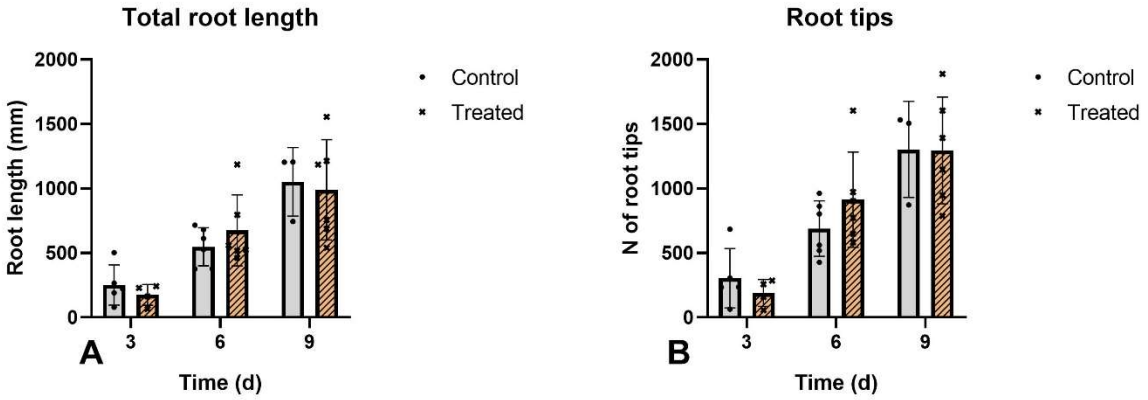

Figure 8: Two graphs comparing a rye control group (grey) and rye treated with *Amaranthus retroflexus* L. extract (orange and black). Every data point is provided with control group as points and treated group as crosses. Both abscissae represent the number of days after germination. On the graph A, the ordinate values correspond to the total root length of a whole rye root system given in mm and on graph B the ordinate values correspond to the number of root tips on a whole rye root system.

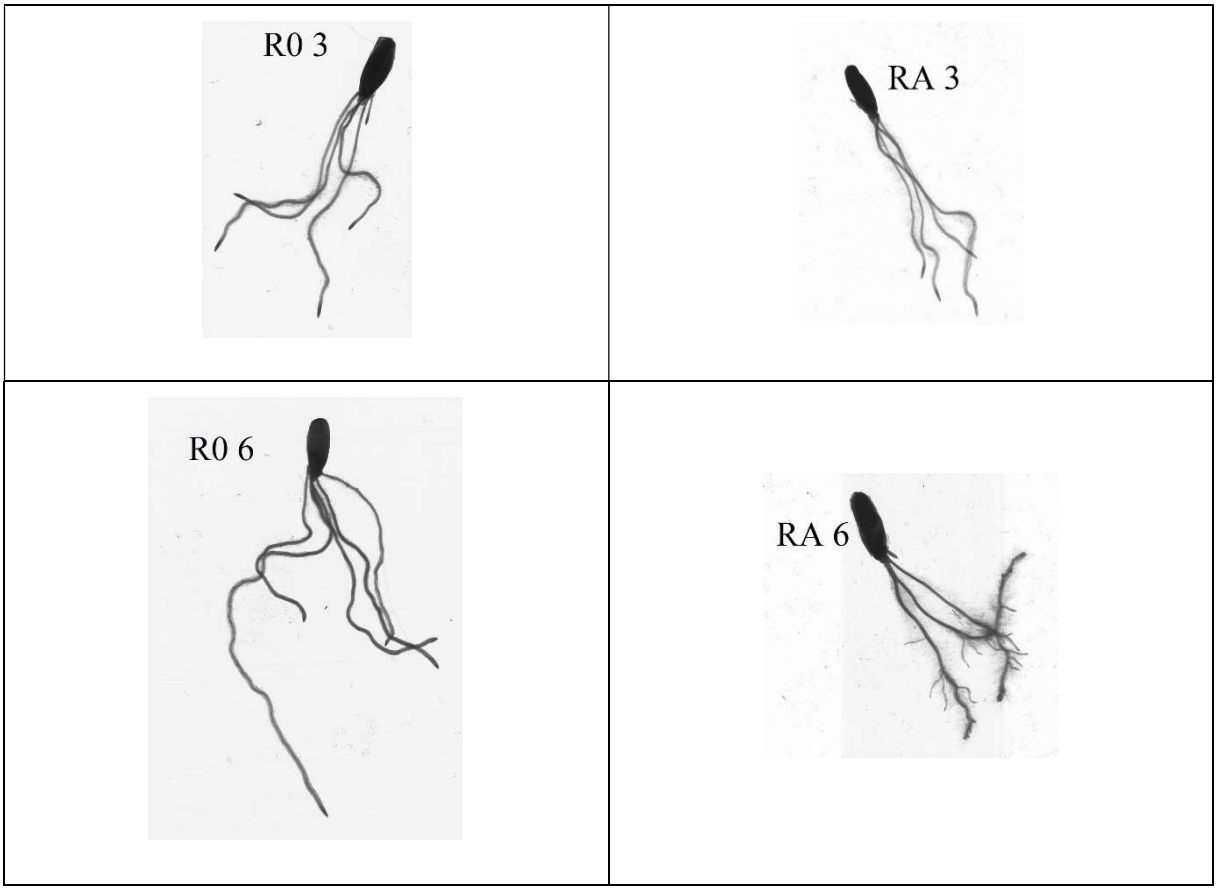

Figure 9: Images of 3 and 6 days after germination rye roots taken using scanning in ethanol/water solution (70/30 v/v) and resolution of 600DPI. R0: control group of rye grown in glass beads and hydroponic solution RA: same condition as R0 with addition of *Amaranthus retroflexus* L. aqueous extract (0,1 mg /L).

However, 6 days post germination (see Figure 9), the root systems exposed are vastly different when it comes to the organisation of the root systems. The treated rye plant seems to have developed its secondary roots way more than its counterpart from the control group. The same observation seems to be observed in the modality of 9 d.a.g. (see Figure 10). In this regard, it is unexpected that no significant differences could be identified on the number of root tips. Most probably, the scanner is detecting the sprouting of secondary root tips in R06 and R09, but as most have not elongated yet, those are not clearly visible on the images. The scans presented here are chosen as archetypes of what was observed, all scans are available in supplementary data.

Even if those scans provide useful insights on the alteration of the root architecture of rye when confronted to exogenous chemicals. Those observations need to be verified by quantitative description of the different root types of present. In this context, the representation of the distribution of root length attributed to each diameter class represented on Figure 11 gives better understand of the root architecture.

After three days (Figure 11A), most roots can be attributed to the 0,45 mm and the 0,60 mm root diameter class. To a lesser extent, the rest is mostly divided in the two smaller classes (0,15 and 0,30 mm) and the two classes just above namely 0,45 and 0,60 mm. Other classes represent a negligeable part of the root system. At least when it comes to understanding the present phenomenon. Additionally, a slightly bigger value is attributed to the ++ class, which represents any root detected with a width over 1,50 mm. Upon visual inspection this value is found because the hull of the seed is being detected and put in this ++ class in the data (visible on Figure 9 and 10). Those observations are valid for both the control and treated group.

After six days (Fig.11B), the distribution of the different roots in the control group is still slightly dominated by the 0,60 mm diameter class (26,7 %), followed by 0,30 mm then 0,75mm, 0,45mm and 0,15mm. Finally, to a lesser contribution 0,90 mm and 1,05 mm are also observed. In the treated group, a very different distribution is noticed. The highest value is observed in the 0,30 mm class (22,5 %), and then almost all equivalent 0,60 mm, 0,45 mm and 0,15 mm. Finally, 0,75 mm class dropped almost by half comparatively to the control group, representing only (8,9 %) in the treated group distribution. Multiple t-tests established that the populations of rye seedlings in the treated group had a significantly smaller proportion of root length allocated to the higher range of diameter class 0,60 and 0,75 with p-values of 0,047835 and 0,015245, respectively. Smaller diameter classes could not be differentiated using statistics, but the average structures on Figure 9B and visual observation of the root scans can allow to make some cautious assumptions.

On Figure 11C, both groups have the highest attribution of root length in the 0,30 mm class, 20,4 % and 25,5 % in control and treated group respectively. The control group overall saw its distribution of roots increase in the lower diameter classes and sink in the higher diameter classes. For the treated group, the distribution of roots cannot really be considered different than after 6 days. Even if it has obviously grown as, the pictures in Fig. 9 show. Low repetition count and very high variability of values in the control group make trends in the values difficult to detect and statistical analysis inefficient.

Globally, it can be assumed that the treatment of rye roots with *Amaranthus retroflexus L.* extract did show an effect on how the root system of treated plant develop. The incidence of the treatment can be described as an elongation of secondary roots in early development stage (1 leaf unfolded, equivalent

11 on BBCH scale). Logically this could mean that the density of the root system is increasing, because primary and long roots develop less and small roots growing on them develop more. The potential benefits of rye from this shift on the profile of roots could not be investigated in this study.

Rye is a species whose root exudation activity is considered very substantial. In consequence, augmenting the interface with the rhizosphere upon sensing exogenous compounds to potentially adapt its interaction strategy seems plausible. Even more so, because a very recent studies done on interactions between wheat and numerous weeds, show that the roots of wheat interact with *Amaranthus retroflexus* L. by stimulating intrusive root growth towards the weed[58]. It also induces a higher exudation of BXs mainly DIMBOA and consequently accumulation of MBOA in the soil [58]. However, the growth conditions in the phytotron and the glass beads are very far from the actual ecological conditions in which the rye roots might be exposed to these compounds of pigweed. Even if a controlled environment improves repeatability, it reduces the realism of the experimentation.

From a growth regulation angle, this changes in root architecture could typically be the manifestation of an auxin mediated mechanism. Most recently, exposure of maize roots to exogenous auxin was shown to induce the emission of ethylene which in return then causes elongation of lateral roots in early stages of development.

The auxin effects observed could be directly linked to the treatment using the pigweed extract, because surely enough it is supposed to contain the phytohormone. In addition, redroot pigweed was growing in the same phytotron photoperiod settings as rye of 12h day and 12h night. For a summer weed this low light treatment could be leading to higher levels of auxin in the pigweed plants.

Another possible way could be again through the action of allelochemicals, that cause perturbation of the auxin homeostasis. Instances of farnesene, coumarin and benzoic acid can be cited in this context[153].

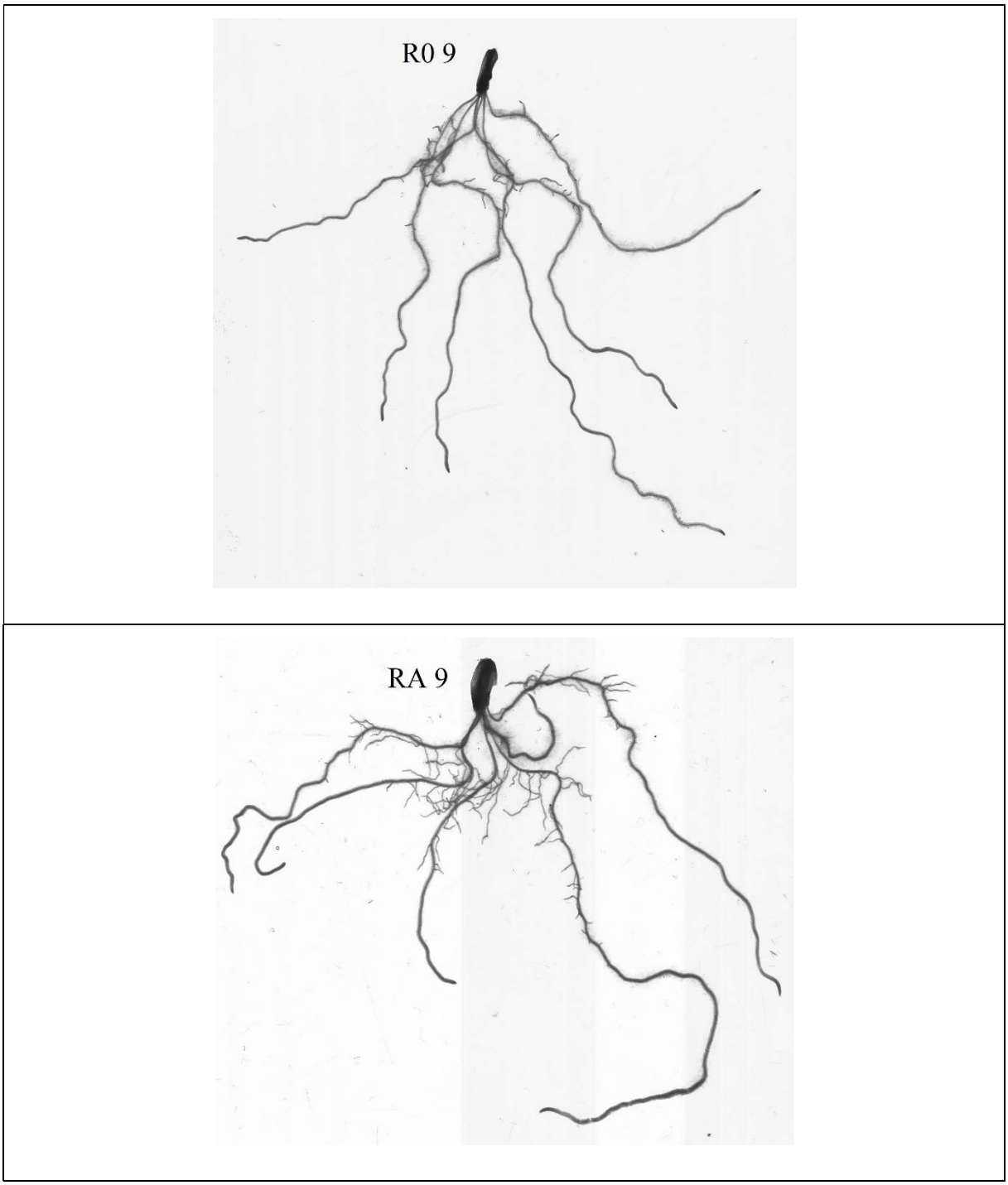

Figure 10: image of 9 days after germination rye roots taken using scanning in ethanol/water solution (70/30 v/v) and resolution of 600DPI. R0: control group of rye grown in glass beads and hydroponic solution RA: same condition as R0 with addition of *Amaranthus retroflexus* L. aqueous extract (0,1 g /L)

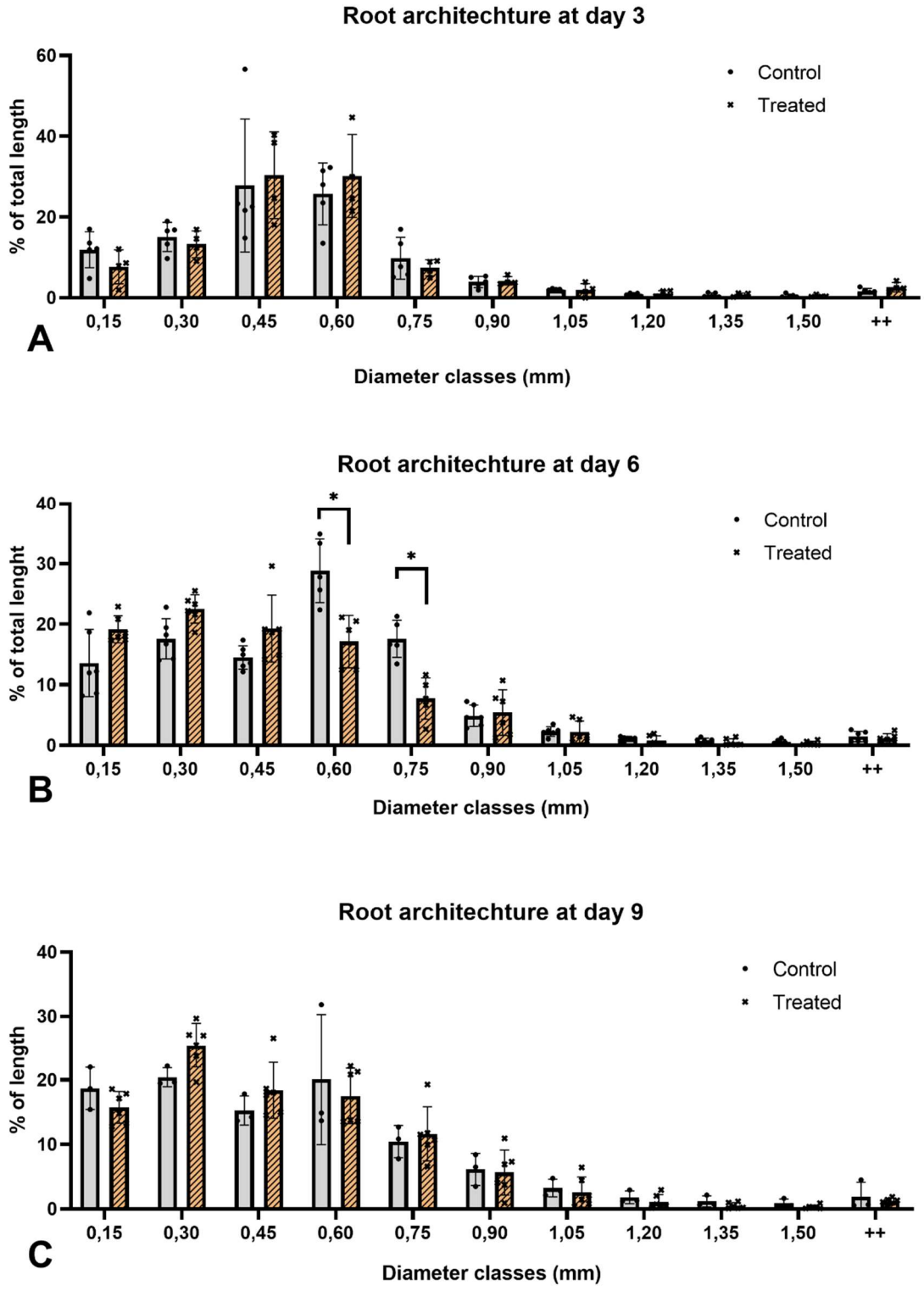

Figure 11: Distribution of rye root sizes of a rye control group (grey) and rye treated with *Amaranthus retroflexus* L. extract (orange and black). Every data point is provided with control group as points and treated group as crosses. Abscissae are the root diameters with 0,15mm increments. Ordinates are the proportion of root length attributed to each class given in percentage. Graph A is showing the distribution 3 days after germination, graph B after 6 and graph C after 9.

4.3. Shoot concentration of benzoxazinoids

The quantification results through HPLC-UV analysis are given on Figure 12. When injecting standard solutions, a signal was detected until 5,00 µg/mL for DIBOA, 10,00 µg/mL for DIMBOA and finally 6,05 µg/mL for MBOA. All signals from the detector were at least ten times higher than the baseline noise. Those concentration will be the estimation of the limit of detection, even if a full validation procedure needs to be performed to establish a valid LOD and LOQ. All samples that were analysed did have a concentration at least 6 times above the estimated LOD. In 100% of analysed rye shoots, DIBOA was detected. However, DIMBOA was never detected in any sample. Because DIBOA and DIMBOA are known to be unstable in their aglycon forms, the hypothesis that DIMBOA was degrading before analysis was formulated. To control if DIMBOA was not transforming during sampling or storage into MBOA, the latter was also quantified. In the end, no MBOA was detected in any sample, indicating that DIMBOA was not present or of so, in very small concentrations below our detection limit.

Even though Figure 12D shows a lower average after at 3 d.a.g. in the treated group, not a single significant difference could be statistically established in terms of DIBOA concentration when comparing control to the treated group. Still, a difference in peaking timings can be identified when analysing Figure 12D. In the control group, the highest concentration of DIMBOA is reached at 3 d.a.g. and then the concentration is progressively dropping. In the treated group, the highest DIBOA concentration is reached at 6 d.a.g., with the two are lower in average. Moreover, the mass of detected DIBOA was increasing during the plant development in both groups, even if the values in concentration would drop. This means the biosynthetic pathway of BXs in rye shoot are still active throughout the experiment if rye is in contact with the extract or not. Higher shoot concentrations of DIBOA are a normality, they were found to be a 1000-fold higher in concentration when compared to DIMBOA.

Even if the broad distribution of the data points does not allow to make assumptions, some possibilities could explain this different timing of peak DIBOA concentration.

Firstly, the extract could indeed be responsible for allelopathic activity on rye, given that seedlings are generally especially susceptible to chemical threats. The pigweed compounds could interfere with the production of enzymes responsible for DIBOA synthesis. Allelochemicals like phenolic compounds are known to be inducer of ROS stress, causing interference in the transcriptomic activity of plant cells and consequently the early synthesis of BXs. As the plant further develops, the allelochemical quantities of the treatment are not sufficient to induce these interferences any longer. Additionally, the right resistance mechanism could possibly not unfold in the very first stages of development, explaining why the concentrations finally reach the same levels as the control group at 6 d.a.g..

In another case, it could be possible that the extract induced an allelopathic response in rye. The main path for rye is through root exudation of BXs, which for the most part all use DIBOA as a precursor molecule. As DIBOA is present in leaf apoplasm and in the phloem to the roots, it is suspected to be transported from the shoot to the roots. Also, roots cannot synthetise the first compound of the BXs metabolic chain, because those are attributed to chloroplast activity. In our case, the active translocation of DIBOA to the roots could be further stimulated than the basal level of the control group, leading to a lower measured concentration.

On top of this, one hypothesis does not exclude the other, meaning that both could be true at the same time. Lower concentrations of DIBOA in the very early stages of development could be both caused by higher translocation to the roots for exudation and by alteration of the efficiency of biosynthesis at the

same time. These results are difficult to compare with literature, as no actual study does quantify the BXs in such a short sequence of time. Data found refer to seed and several week-old stage(s) when it comes to full BXs profiling in tissues and exudates.

In the present state of knowledge, wheat and rye have shown to modulate their root development and their exudation activity of BXs upon sensing exogenous compounds or even identifying their neighbour as non-kin. However both literature and this study seem to confirm that exposure to heterospecific metabolites is not inducing an accumulation of those BXs in the shoot, at least not in the present treatment concentration. Accumulation of BXs in shoot could be a sign of priming of the immunity of the plant. In this case however, DIBOA being a cytotoxic compound, the latter would be supposedly glycosylated and then sequestered in vacuoles in the shoot. The glycosylated compound was not quantified, as commercial standard as not available at the moment and need to be weather shared by another research team or purified in-house. Further research taking into account stress indicators and more diversity of compounds in both roots, shoot and rhizosphere could give interesting results on how rye perceives *A. retroflexus* L. metabolites and how it affects its development and allelopathic response.

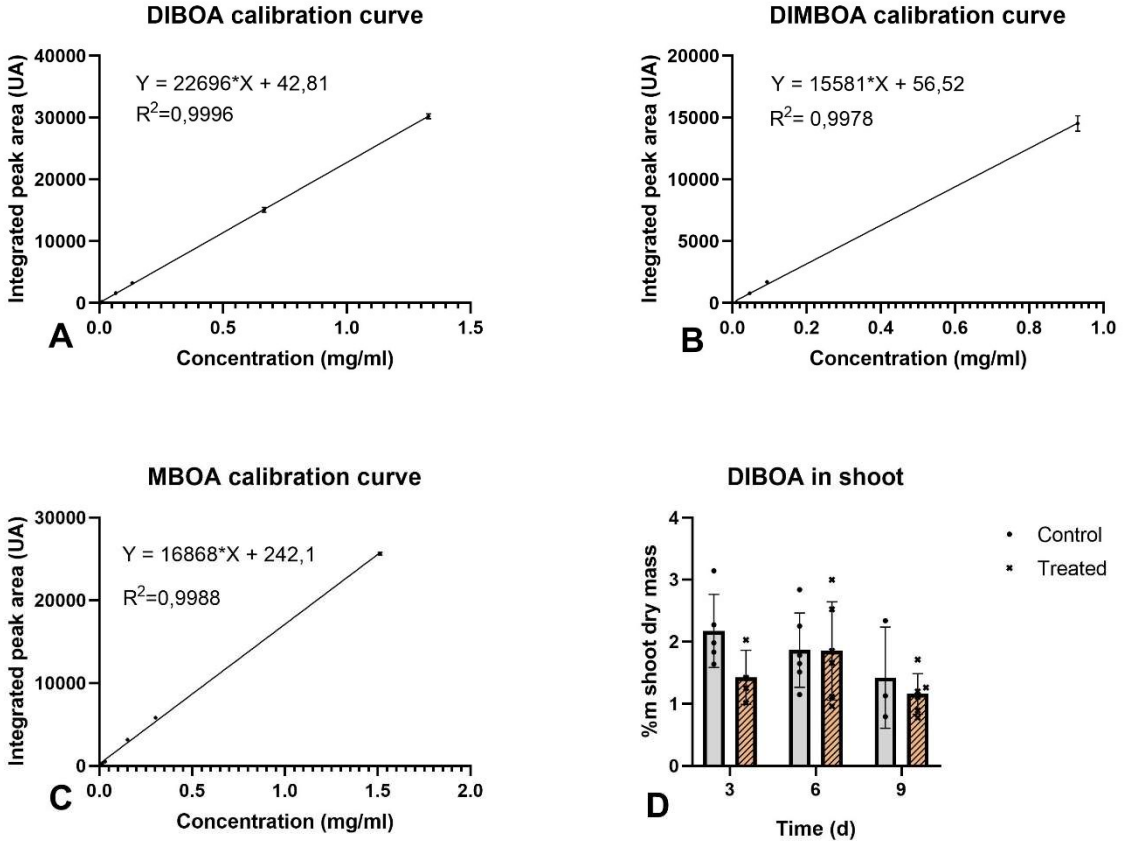

Figure 12: Graphs A-C, calibration curves of DIBOA, DIMBOA and MBOA respectively. The abscissae give the concentration of the compounds of interest in mg/mL and ordinates the integrated peak area given in numeric units of area (UA). Each of these three graphs is showing the equation of linear regression an its respective coefficient of determination. Detection was done using UV absorption asset at wavelength of 250 nm for DIBOA, 280 nm for DIMBOA and 288 nm for MBOA. Graph D represents the quantification of DIBOA in rye shoot in the control group (grey) and the group treated with Amaranthus retroflexus L. extract (orange) after 3, 6 and 9 days after germination (abscissa) in percentage of shoot dry mass (ordinate).

4.4. Volatilome analysis

4.4.1 Optimization of the volatile sampling method

In a previous work on the topic of volatiles emitted by rye roots, the VOCs were sampled using solid phase micro extraction (SPME) using a micro-perforated PTFE tube as the headspace for placement of the adsorbent fiber. The limitation of this technique is the discrimination of the analytes based on their volatility and the higher limit of detection compared to the dynamic headspace sampling method. The reason being that SPME is based on passive diffusion of the compounds from the headspace to the sorbent material. In this rhizosphere matrix, that would implicate that the compounds are first in equilibrium between the soil (in our case a hydroponic system) solution and the pores and then the compounds that did volatilize in the pores with the SPME fiber[155]. In consequence, polar compounds with a better affinity with the water solution are less volatilized and thus less adsorbed on the fiber. Higher concentrations of those analytes in the rhizosphere are necessary to be detected through SPME GC-MS analysis. By choosing dynamic headspace sampling over SPME, lower LOD can be attained because this method does not rely on passive diffusion, but on active pumping of air from the matrix to a sorbent tube[156]. This results on higher mass transfer of analytes sampled. On top of that, there is minimal analyte discrimination based on volatility, as clean air is entering the matrix, maximizing the extraction of analyte[157].

Comparatively to the last study, the volatilome in the present experiment does show an increase diversity in the detected molecules, even if the matrix is very similar. The switch from the SPME to DHS practices seems to effectively improve the analytic potential of the developed methodology to monitor the emission of root volatiles.

4.4.2. Rye root volatilome

The diversity of compounds that were identified by GC-MS is vast, regrouping many molecules from different chemical groups. The priority after the subtraction of the compounds in the blanks, is to identify further potential contaminants. The most obvious are the hits of the lists on Table 4, 5 and 6 that are made of silicon. Those are fragments that come from column bleeding and degradation of the Tenax cartridges. Then, alkanes with relative high masses, for example eicosane found in Table 4 R06 is rather a residue from the alkanes used in Kovat's index determination of a previous analysis. Those contaminants come most probably of the Tenax cartridges as these specific instances were not present in the blanks. Other that the other compounds represented in the tables are considered as biogenic, because they were not present in the cartridge blanks, nor the blanks realized by pumping the air inside a growth apparatus that did not contain any plant.

Complementary to the Table 4, 5 and 6, the Venn diagrams on Figure 13 give a good overview of the fact that many compounds were exclusively identified at one time point and exclusively in the controlled or the treated rye group. This is surprising, because most, if not all, studies that were investigated on similar subjects seem to show that the volatilome of a plant would not be altered completely every three days. The general tendency that is observed is that some compounds are less or more emitted in case there is any modulation of the volatilome through the perception of a precise stimulus. However, this is not observed here, where it seems that the treatment would almost totally suppress the release of the compounds emitted in the control group and replace them with a totally different profile. Unintentional heterogeneity in experimental and analytical conditions should not be omitted as a source of interference.

The detection of numerous new compounds when treating the roots of rye with the extract is possibly caused, firstly by the emission of VOCs by the rye roots as a reaction against the treatment, and secondly degradation products of the extract are different if it is applied in an apparatus containing a plant compared to an empty one. This different biochemical environment could induce the emission of new compounds and so generate a different volatile profile. Nonetheless, this couldn't explain the total signal suppression of the compounds emitted in the control group. On this point, it has been observed on the root scanning experiment that the root architecture is changing over time and depending on the treatment. Literature tells us that exudated and emitted compounds depend on the organ or tissue. So in our case it is visible that the treated group has a more pronounced activity of the meristems in the lateral roots than those in the primary roots. More analysis concerning a potential differential emission of volatiles in root tips or even secondary vs primary roots could elucidate this point.

From a more practical approach, it is visible that a consequent fraction of the identified peaks lacks a theoretical retention index (RI) value. This was caused by the recognition by Agilent Unknowns Analysis of molecules that seemed to be less studied when comparing the listings on the NIST database. In this case only high matching factors and homogeneous retention times in several samples can partially confirm the identity of a compound. The difficulty to establish the identity of the compound with certitude is a contributor to the high disparity of compounds detected. Mismatches could not be effectively corrected or eliminated for the lack of time to analyze more that 2000 mass spectra. On top of that, the detection of residues probably originating from previous samples or Kovat's index determination can interfere and cause this unexpected high diversity in signals. It was unexpected to find those compounds in some sample exclusively, because in total 6 cartridge blanks were analyzed.

Taking all these aspects in mind, compounds of higher interest were identified. The selection of the molecules of interest were based on several factors: (1) the match factor and retention index allowed to assume the identity of the compound with reasonable confidence (2) the molecule should be present in multiple samples (3) the molecules are well known for their bioactive proprieties.

Methyl salicylate

The emission of methyl salicylate appears only in two repetitions the first in the control group exclusively 6 d.a.g. (Table 4 R06) and the treated group exclusively 9 d.a.g. (Table 5 RA9). Both signals had the same measured retention index that was close the theoretical one (Δ=9). In R06, 16,9 ng were detected and in RA9 1,21 ng were detected. The pattern of emission does not allow to make any assumption on the reaction of rye to the extract. However, the detection of those compounds is important because methyl salicylate is known as a very powerful elicitor of plant resistance[158]. On rye specifically, the perception of methyl salicylate has been associated to the increased production of DIBOA and DIMBOA and their glucosides[159].

1-Undecene

In the control group, the table 4 gives us the compound 1-undecene that starts to be produced from 6 d.a.g. on. This compound has a very good, measured RI compared to values found in the NIST database (Δ=2) and was detected in 3 samples of the 6 d.a.g. (1,01ng;1,05 ng and 4,654 ng) and in one sample of 9 d.a.g. (0,729 ng). 1-Undecene is the most emitted volatile emitted by endophytic PGPR strains of *Pseudomas* sp.. Those bacteria show signs of growth and resistance promotion[160]. The analyte 1-Undecene when emitted by *Pseudomonas sp.* has shown effective suppression of fungal pest *Phytophtora infestants* both *in vitro* and *in vivo*[161].

Intriguingly, 1-decene was also detected in the previous study using SPME but was not detected in the control group. In that case it was exclusively detected in the group of rye plants grown with redroot pigweed. Further investigation should be done to elaborate what the source of this compound might be.

Octanyl-2-palmitate

Octan-2-yl palmitate is detected in 5 samples of RA6 and 5 samples of RA9. The detected emissions are of 5,16±1,33 ng and 3,51±0,449 ng respectively. It seems that the emission is almost omnipresent in treated roots after 6 days (Table 5). The identification seems trustworthy because both match factor are high and measured RI and theoretical RI are close (Δ=4). No allusions of the compound in this context were found in literature.

6,10-dimethyl-(E)-5,9-Undecadien-2-one

Another compound has a retention index that does fit the values found on the database, with the exact same value than the one found in the listings. The volatile 6,10-dimethyl-(*E*)-5,9-Undecadien-2-one, is produced in one repetition after 6 days in the treated roots (0,859 ng) and in one repletion after 9 days in the treated roots (0,897 ng). This compound has been described as an antifungal agent against *Phytophtora cinnamomic* by root associated PGPR strain of *Bacillus acidiceler*[162].

Bicyclo[3.1.1]heptan-3-ol, 6,6-dimethyl-2-methylene-, [1S-(1α,3α,5α)]-

A volatile was detected exclusively in treated roots in all time modalities 3, 6 and 9 days after germination: Bicyclo[3.1.1]heptan-3-ol, 6,6-dimethyl-2-methylene-, [1S-(1α,3α,5α)]- a stereoisomer of pinocarveol. The compound is detected in 2 replicates for each modality with representative average masses of emission of 1,64 ng, 0,557 ng and 0,640 ng. This molecule is probably emitted by the plants themselves because it was found in essential oils in other plant species[163]. However its chemical ecological role was, to best of the present knowledge, not investigated.

2,5-bis(1,1-dimethylethyl) 1,4-benzenediol

The phenolic compound 2,5-bis(1,1-dimethylethyl) 1,4-benzenediol is the only compounds that was detected in every modality in both the control group and the treated group of rye without being detected in blanks (Table 6). This could indicate that rye roots emit constitutive cues in both presence and absence of induced biotic stress. The presence of this metabolite was detected in 17 samples. Even if the RI is not listed in the database, every match hit did have the exact same retention index. The detected quantities were in average of 7,07 ng, 9,57 ng and 9,76 ng in the 3, 6 and 9 d.a.g. control group. In the treated group, averages of 7,84 ng, 6,23 ng and finally 9,26 ng were detected. Few data are available on this compound in the literature, but is seems that it can be synthesized in major plants and algae[164]. Interestingly it also suspected to contribute to antibacterial activity against phytopathogen *Erwinia carotovora* in the plant model *Alternanthera philoxeroides* (Mart.) Griseb[164].

o-xylene

This compound was almost present in all the samples including the apparatus treated with the extract. Proceeding to blank subtraction is most useful to show to volatiles exclusively emitted by rye. However, in case both plants do emit some volatiles in common, the signal is suppressed by the blank subtraction. O-xylene is known to be emitted and to modulate plant-herbivore interaction [165]. Indeed, when subject

to pathogenic virus it lowers the emission rate of o-xylene, this causes the plant to be more prone to aphid infestation[165]. Additionally, the emission of the repellent o-xylene has been shown to be induced by environmental factors like herbivorous activity on tomato plants or availability of silicon in a hydroponic solution in *Phaseolus vulgaris L.* The identification of o-xylene is even more interesting considering that is one of the molecules that was also detected in a previous study, in the context of rye growing alone and rye growing with redroot pigweed[166]. This strongly confirms the hypothesis that o-xylene is constitutively emitted by rye roots.

In this analysis, the measured retention index of o-xylene is slightly over the theoretical value, some samples had several hit matches on o-xylene, the hit matches attributed with closest RI were selected every time. Overall, the match factors are relatively high. The shift in retention index, could be caused by the difference of GC-MS methodology between what was done in the lab compared to the method on the data base. Most values "*" indicate that the theoretical value was taken from a methodology that was slightly different. The most common difference was the starting temperature in the GC sequence, that were higher than the present method used, taking 35°Cat starting value against 40°C or even 50°C in the database. The incidence of this difference is endured by the most volatile compound in the sample, their elution is delayed. This could potentially explain the higher values of RI of o-xylene, as it is already detected after 6,25 min (Table 6 R0AAb 369). Concerning quantification, values in the control group 13,65 ng ± 13,45 ng, 19,82 ng ± 18,22 and 5,74 ng ± 1,89 ng were detected in the 3, 6 and 9 d.a.g. respectively. In the treated group, 20,34 ng ± 8,11 ng, 30,59 ng ± 39,42ng and 10,49 ng ± 6,68 ng were detected in the 3, 6 and 9 d.a.g. respectively. Blanks with the extracts did emit 19,56 ng ± 5,33 ng. It is obvious that the distribution of data is very broad and that further statistical analysis would not make any sense because (i) The standard deviations are way too large to recognize any trend and (ii) no study on the repeatability of this apparatus has been done yet. In consequence it would be impossible to determine if the variations come from the apparatus itself or the actual variation of the biological phenomena taking place.

In summary, the volatilome analysis is giving precious insights on the below ground activity. It could be determined that even if this study is done in glass sand, PGPR most probably still contribute on the emission of volatile compounds. This is very probable because benzoxazinoids have a strong effect on the composition and diversity of the rhizobiome, and that this effect is mediated through changes in root exudation patterns. In addition, the rye plants seem to emit some compounds constitutively and upon induction of stress through treatment with exogenous metabolites. Finally, rye and its associated root microbiome emits compounds with antimicrobial and antifungal proprieties, meaning that it not does not exclusively rely on its root exudates and benzoxazinoids to suppress potential pathogens.

Table 4: List of analytes detected through GC-MS analysis of rye root volatilome. R0 stands for the control group, followed by the days after germination modality. For each molecule, the retention time (RT), the match factor (MATCH) the measured retention index (RI), the theoretical index are given. Cas identification number. n.l. stands for not listed on NIST database and the "*" is stands for the acquisition of the value on a the NIST database, referenced in a GC method that was acceptably close to the one done in this study.

	NAME	RT (min)	MATCH	measured RI	theoretical RI	CAS N°
RO3	2-Morpholinoethyl isothiocyanate	14,727	85,9	1096	n.l.	63224-35-1
	Dodecyl nonyl ether	18,2397	86,1	1215	n.l.	1000406-37-5
	(1R)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene	8,1059	89,9	926	931*	7785-70-8
	Cyclohexanol, 1-methyl-4-(1-methylethyl)-	17,062	87,5	1174	n.l.	21129-27-1
	7-Octen-2-ol, 2,6-dimethyl-	14,0521	90,5	1078	n.l.	18479-58-8
	Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl-, acetate, (1S-endo)-	20,336	88,9	1290	1285*	5655-61-8
RO 6	Undecane, 2-methyl-	20,0867	85,3	1281	1164*	7045-71-8
	Eicosane	36,4417	95,1	1999	2000	112-95-8
	Hexadecane	28,1033	90,6	1600	1600	544-76-3
	Heneicosane	38,2941	90,2	2099	2100	629-94-7
	Pentasiloxane, dodecamethyl-	19,2098	86,8	1249	n.l.	141-63-9
	9-Octadecenoic acid, methyl ester, (E)-	38,3353	89,3	2101	n.l.	1937-62-8
	Dodecane, 5,8-diethyl-	27,4339	85,8	1571	n.l.	24251-86-3
	Cyclohexanol, 5-methyl-2-(1-methylethyl)-, (1.alpha.,2.alpha.,5.alpha.)-	17,0617	88,9	1174	1194*	491-02-1
	5,9-Undecadien-2-one, 6,10-dimethyl-, (Z)-	24,6269	86,9	1455	1445*	3879-26-3
	Heptanoic acid	14,2702	89	1084		111-14-8
	Nonadecane	34,5063	94,6	1899	1900	629-92-5
	1-Octadecanol, methyl ether	38,0137	86,9	2084	n.l.	1000333-92-7
	Naphthalene, 1,3-dimethyl-	24,2791	86,4	1441	1424*	575-41-7
	Methyl salicylate	17,7051	98,5	1196	1187*	119-36-8
	Heptadecane	30,3502	89	1700	1700	629-78-7
	cis-Thujopsene	24,1702	86,2	1436	1430*	470-40-6
	1-Undecanol	15,8942	85,3	1134	n.l.	112-42-5
	Methyl formate	9,2683	94	954	n.l.	107-31-3
	Octadecane	32,4776	94,3	1800	1800	593-45-3
	2,6-Diisopropylnaphthalene	30,0179	89,9	1685	1725*	24157-81-1
	1-Nonadecene	34,3766	88,3	1893	1885*	18435-45-5
R0 9	Undecanal	20,8341	87,7	1308	1308*	112-44-7
	Cyclohexanol, 1-methyl-4-(1-methylethenyl)-, acetate	12,6825	97,3	1040	n.l.	10198-23-9
	1-Hexadecanol, acetate	34,112	85,1	1880	2010*	629-70-9
	Nonadecanenitrile	38,4233	85,7	2106	n.l.	28623-46-3
	Propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethylpentyl ester	22,619	86,6	1375	1380*	77-68-9
	Docosane	40,0681	86,2	2199	2200	629-97-0
R0 39	3-Octanol, 3,7-dimethyl-	14,8408	91,1	1099	1097*	78-69-3
R0 69	1-Undecene	14,623	96,3	1093	1091*	821-95-4

Table 5: List of analytes detected through GC-MS analysis of rye root volatilome. RA stands for the treated roup, followed by the days after germination modality. For each molecule, the retention time (RT), the match factor (MATCH) the measured retention index (RI), the theoretical index are given. Cas identification number. n.l. stands for not listed on NIST database and the "*" is stands for the acquisition of the value on a the NIST database, referenced in a GC method that was acceptably close to the one done in this study.

	NAME	RT (min)	MATCH	measured RI	theoretical RI	CAS N°
RA 3	Cyclohexanol, 1-methyl-4-(1-methylethenyl)-, acetate	12,6879	96	1041	n.l.	10198-23-9
	9-Hexadecenoic acid	35,3469	94	1943	1942*	2091-29-4
	Naphthalene, 2-methyl-	20,9323	87,9	1312	1318	91-57-6
	Hexadecanenitrile	34,5427	92,7	1901	n.l.	629-79-8
	Butanoic acid	8,8792	85,8	945	790*	107-92-6
RA 6	Undecanal	20,834	88,1	1308	1308*	112-44-7
	1-Pentadecene	25,5558	90,7	1492	n.l.	13360-61-7
	Dodecyl nonyl ether	18,2345	86,9	1214	n.l.	1000406-37-5
	Benzene, 3-cyclohexen-1-yl-	21,5138	85,7	1334	n.l.	4994-16-5
	Trimethylsilyl fluoride	7,5248	86,2	912	n.l.	420-56-4
	Octanal	11,9199	89,2	1020	1004*	124-13-0
	Isopulegol	17,5859	85,9	1192	1156*	89-79-2
	Dodecanal	23,501	91,8	1409	1412*	112-54-9
	2(3H)-Furanone, 5-dodecyldihydro-	38,4082	86,2	2105	2106*	730-46-1
	Dodecane, 1-iodo-	21,3426	85,4	1327	n.l.	4292-19-7
	n-Tridecan-1-ol	23,0496	88,4	1392	n.l.	112-70-9
RA 9	Cyclohexanol, 1-methyl-4-(1-methylethylidene)-	14,545	85,8	1091	n.l.	586-81-2
	Benzene, 1-ethyl-3,5-dimethyl-	13,6214	87,5	1066	1074*	934-74-7
	Bicyclo[3.1.1]hept-3-en-2-one, 4,6,6-trimethyl-	18,1567	94,4	1212	1206*	1196-01-6
	9-Octadecenoic acid (Z)-, methyl ester	38,3303	85,8	2101	n.l.	112-62-9
	Benzene, 1-methyl-3-(1-methylethyl)-	14,4412	91,4	1088	n.l.	535-77-3
	Fenchol	15,2924	87	1114	1121*	1632-73-1
	(-)-Isolongifolol, acetate	23,5165	89,4	1410	n.l.	1000352-28-0
	Cyclohexanol, 5-methyl-2-(1-methylethyl)-, (1.alpha.,2.alpha.,5.alpha.)-	17,0566	92,7	1174	1188*	491-02-1
	Mesitylene	12,475	88,8	1035	995*	108-67-8
	Dodecane, 4,6-dimethyl-	20,0868	85,8	1281	n.l.	61141-72-8
	Cyclohexadecane	38,0087	85,8	2083	1883*	295-65-8
	Bornyl acetate	22,7068	85,7	1379	1281*	76-49-3
	Methyl salicylate	17,6999	94,5	1196	1187*	119-36-8
	Benzene, 1,2,3,5-tetramethyl-	15,4893	93,8	1121	n.l.	527-53-7
	p-Cymene	15,3596	85,3	1116	1020*	99-87-6
	1-Undecanol	14,6127	85,6	1093	n.l.	112-42-5
RA 36	Limonene	12,6822	94,8	1040	1028*	138-86-3
RA 69	Camphor	16,2366	93,9	1146	n.l.	76-22-2
	Octan-2-yl palmitate	45,0705	94,1	2505	2501*	55194-81-5
	5,9-Undecadien-2-one, 6,10-dimethyl-, (E)-	24,6218	87,5	1454	1454*	3796-70-1
	Benzaldehyde	10,5708	91,1	985	961*	100-52-7
	Benzene, 1,3-bis(1,1-dimethylethyl)-	19,3968	91	1256	n.l.	1014-60-4
	Dodecane, 2,6,11-trimethyl-	21,3321	90,9	1327	n.l.	31295-56-4
RA 369	Bicyclo[3.1.1]heptan-3-ol, 6,6-dimethyl-2-methylene-, [1S-(1.alpha.,3.alpha.,5.alpha.)]-	16,0605	92,2	1140	1140*	547-61-5

Table 6: List of analytes detected through GC-MS analysis of rye root volatilome. R0A stands for the intersection of the volatilome of the control and the treated group. Ab is the presence of the analyte in the *Amaranthus retroflexus* L. blank, followed by the days after germination modality. For each molecule, the retention time (RT), the match factor (MATCH) the measured retention index (RI), the theoretical index is given. Cas identification number. n.l. stands for not listed on NIST database and the "*" is stands for the acquisition of the value on a the NIST database, referenced in a GC method that was acceptably close to the one done in this study.

	NAME	RT (min)	MATCH	measured RI	theoretical RI	CAS N°
ROA 3	Acetic acid	29,4108	85,8	1658	n.l.	24851-98-7
	Undecanal	20,834	96	1308	1308*	112-44-7
	1-Hexanol	7,8672	95	921	867*	111-27-3
	Dodecane, 5,8-diethyl-	27,4339	85,8	1571	n.l.	24251-86-3
	Cyclohexanol, 5-methyl-2-(1-methylethyl)-, (1.alpha.,2.alpha.,5.alpha.)-	17,0566	87	1174	1188*	491-02-1
ROA 6	Cyclohexadecane	34,1068	86,1	1880	1883*	295-65-8
	Tetracosane	43,41	90,4	2438	2400	646-31-1
	Docosane	40,0685	87,2	2199	2200	629-97-0
	Hexadecanoic acid, methyl ester	35,03	95,5	1926	1927*	112-39-0
ROA 9	Undecane, 2-methyl-	20,0816	86,6	1281	n.l.	7045-71-8
	Limonene	12,6821	97,3	1040	1028*	138-86-3
ROA 36	(-)-Isolongifolol, acetate	23,5165	90,5	1410	n.l.	1000352-28-0
ROA 69	Cyclononasiloxane, octadecamethyl-	33,157	91,4	1833	n.l.	556-71-8
	Hexadecanenitrile	34,5374	93,1	1901	n.l.	629-79-8
ROA 369	1,4-Benzenediol, 2,5-bis(1,1-dimethylethyl)-	30,9519	90,1	1728	n.l.	88-58-4
ROAAb 69	Benzene, 1-ethyl-4-methyl-	9,4967	93,8	959	n.l.	622-96-8
ROAAb 9	E-15-Heptadecenal	38,0034	92,8	2083	2084*	1000130-97-9
ROAAb 369	o-Xylene	6,2485	97,7	860	889*	95-47-6

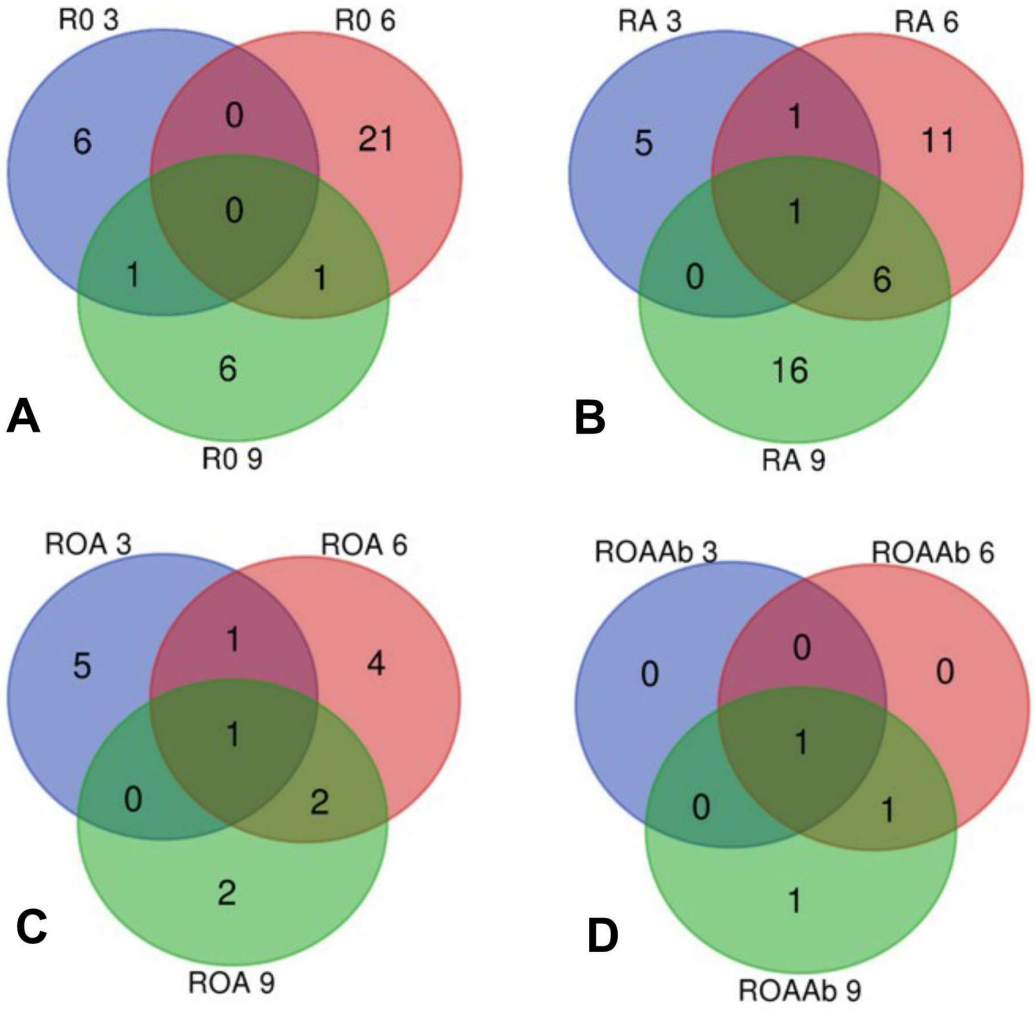

Figure 13 : Venn diagrams representing the profile of emission of rye roots. R0 stands for rye control group. RA represents the group of rye plants treated with the pigweed extract. R0A stands for volatiles detected in both the control group of rye and the group treated with the pigweed extract. The number 3, 6 and 9 refer to the days after germination modalities. R0AAb designates the group of volatiles detected in R0A and in the blank treated with pigweed extract. The numbers in the diagrams are the numbers of compounds identified through GC-MS analysis.

5. Conclusions and perspectives

5.1. Conclusions on the research questions

The best way to conclude this work, is to remind the research questions and assess how far the experiments done have contributed to the answers. Those were:

Does *Secale cereale L.* perceives residual metabolites of *Amaranthus retroflexus* L. in the rhizosphere? If so, how does it affect its growth pattern and systemic response?

This question was partially answered. Emphasis was placed on the observation of changes in the root architecture and in the volatilome. . These values are phenotypical changes and environmental measurements. It would have been even better evidence if actual metabolic stress signals or chemical defence priming could have been observed. Even more, the effects of the extract might be indirect, because this work highlights potential presence of PGPRs in the roots. The answer of the second part of the question, it can be stated that treatment of roots with the extract significantly reduces the attribution of resources to primary root elongation to favour the growth of secondary roots. Concerning systemic response more work must be done on the matter, mainly by choosing a metabolic indicator of metabolic activity linked to the perception of exogenous compounds.

Are rye roots emitting volatiles constitutively? Is the volatilome of rye roots altered when in contact with residual metabolites from redroot pigweed?

In this regard, two potential molecules are possibly constitutively produced in by rye roots, o-xylene and 2,5-bis(1,1-dimethylethyl) 1,4-Benzenediol. On top of that, instances in the literature show their role as important actors in volatile mediated plant-herbivore/pathogen interactions. Besides, the volatilome of rye seems to be strongly altered when treated with the redroot pigweed extract. Some compounds are not detected anymore after treatment, and other start to be emitted. Those newly emitted compounds are attributed both to root and potentially PGPR emission.

To conclude, the volatilome of the roots is most probably influenced by the presence of specific microbial communities in the soil. These findings provide new insights into the chemical ecology of rye plants and may have implications for the development of more sustainable agricultural practices.

5.2. Conclusions on the objectives of this study

Going through the objectives of the study allows to retrospectively assess what advancement have been reached during this work:

1) Find a method to grow rye in glass beads using a hydroponic solution in a phytotron. The growth media should maintain constant humidity levels and produce homogenous populations with similar or equal development stages throughout the experiment.

By utilizing a specifically designed glass apparatus, rye was able to be cultivated under regulated conditions. The seedlings exhibited consistent developmental stages throughout the duration of the experiment, and the growth medium remained consistently humid. This accomplishment enables reliable

replication for both volatilome and plant material sampling. Furthermore, the most suitable timeframe for observing the phenology of rye within this apparatus was determined.

2) Find a way to induce biotic stress to rye through chemical cues from *A. retroflexus* L. without inducing competition between the two plants.

By utilizing an extract rather than living plant material, it was possible to effectively isolate chemical interactions among plants from the competition phenomenon. As a result, a variation was observed in the growth pattern of the rye roots, along with a modulation of the volatilome, indicating that the plant had sensed a stimulus. To establish the occurrence of a stressor, it is recommended that measurements of stress factors be given priority.

3) Develop an apparatus and analytical methods that allows to monitor the features of rye to compare a group of stressed rye plants vs a control group of unstressed rye plants. In all logic, these features are the ones formulated in our research questions above.

In this study, a successful method was developed to investigate the phenotypical features of rye, as well as the content of benzoxazinoids in its shoot and the volatilome of its roots. By using a combination of analytical techniques, including gas chromatography and mass spectrometry, high-performance liquid chromatography it was possible to identify and quantify a range of compounds in both the shoot tissues and in the rhizosphere where rye plants were developing.

5.3 Perspectives

For the next steps of this research, it is important to prioritize the realization of a total validation method for the HPLC analysis used to quantify the benzoxazinoid compounds in rye plant tissues. Validation of the analytical method is essential to ensure that the results obtained are accurate, precise, and reliable. This involves testing the method's specificity, linearity, limit of detection, limit of quantification, accuracy, and precision. By validating the HPLC analysis method, we can have confidence in the quality of the data obtained and the conclusions drawn from it. This will also enable us to compare our results with those obtained by other researchers using similar methods, and to facilitate the reproducibility of our findings. Ultimately, the total validation of the HPLC analysis method will strengthen the scientific rigor of this study and enhance the impact of its results.

By analyzing stress indicators such as reactive oxygen species, proline, or other phytohormones, it can be determined whether rye has been exposed to biotic stress inducing conditions.

Now that this work could establish some molecules of interest in the volatilome, it is possible to proceed to the validation of the quantification of the selected analytes. First the identities of the analytes must be all confirmed, through mass spectrum comparison with databases. In addition, it would be very convenient to determine specific RI on these analytes using the present GC-MS method, thus avoiding making assumptions when comparing with similar methods on online databases. Also, the determination of the response factor of the mass spectrometer between the internal standard phenyl octane and the molecules of interest will give a more accurate quantification.

Validation of a dynamic headspace sampling method involves several steps to ensure that the method is reliable and produces accurate results. The first step is to determine the method's linearity by preparing calibration standards over a range of concentrations and testing the response of the method to each

standard. The next step is to test the method's limit of detection and limit of quantification by analyzing samples with known low concentrations of the target compounds. Accuracy and precision are then evaluated by analyzing samples with known concentrations and testing the variability between replicate measurements. Specificity is also assessed by analyzing samples with potentially interfering compounds to ensure that the method only measures the target compounds. Finally, robustness is tested by evaluating the method's performance under different experimental conditions such as changes in sample volume or analysis temperature. By following these validation steps, the dynamic headspace sampling method can be validated to ensure reliable and accurate results for its intended use.

The sorbent material used for DHS is considered not to be specific for a type of volatiles, it covers a wide range of molecules, from the small to the large one and polar to non-polar. Using specialized sorbent cartridges using for example molecular sieves technology could allow to identify small and highly volatile compounds. Precisely for this study, phenomena potentially induced by emission and sensing of ethylene were observed. Adding an additional molecular sieve cartridge in front of the present Tenax cartridge seems relevant.

Given the fact microbial colonies on the roots or in the growth medium are most probably contributing to the emission of VOCs, identify the microbes and characterize their behavior from a chemical ecological point of view is relevant. Identifying a species of root-associated bacteria can be done through a series of steps. The first step is to isolate the bacteria from the root tissue or the soil surrounding the roots[167]. Once the bacteria are isolated, they can be cultured on different media to evaluate their growth characteristics and identify their morphological features. Next, biochemical tests can be used to determine the bacteria's metabolic pathways and enzymatic activities[167]. Additionally, molecular biology techniques such as polymerase chain reaction (PCR) and DNA sequencing can be used to identify specific genes or regions of the bacterial genome that are unique to a particular species. This can allow for a more accurate identification of the species based on genetic markers[168]. Finally, physiological, and ecological characteristics of the bacteria, such as its ability to fix nitrogen or its response to different environmental conditions, can be used to confirm the identification of the species[169]. By following these steps, researchers on this project could identify and study the role of root-associated bacteria in various plant-microbe interactions. Especially since benzoxazinoids have been proven to regulate the metabolic activity of the rhizobiome, correlating the sensing of *Amaranthus retroflexus* L. residues to the exudation of rye BXs, that in return shape the rhizobiome and contribute or not to the fitness of rye in its environment could be a lead for a complete study.

6. Personal contributions

To initiate my research, I conducted a comprehensive review of the literature on allelopathy in general and in the present plant models, plant metabolism, chemical signalization, and soil ecology. This enabled me to develop the objectives and experimental plan for my master's thesis in collaboration with my supervisors. Following this, I conducted my experiments in the appropriate laboratory and made necessary adjustments when facing new challenges. Upon completing my lab work, I processed and analyzed the generated data to determine its statistical significance and generated hypothesis based in my oy observations. Subsequently, I composed this thesis, which entirely represents my own work, and adapted the content while being guided by my supervisors.

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