Beloground interactions involved in plant neighbor detection and allelochemical responses: effects and quantification of potential signaling molecules on secale cereale L.

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LIÈGE université
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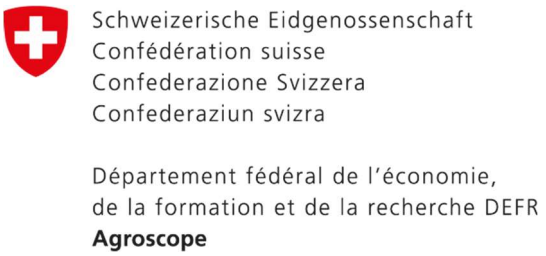

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ABSTRACT

The urge for new sustainable weed control systems in Europe is increasing as modern herbicides have proven their limit. Alternatives using allelopathic plants as cover crops to reduce weed growth, are perceived as a compelling approach. Rye is an allelopathic crop which has been shown to produce phytotoxic defensive secondary metabolites called allelochemicals such as benzoxazinoids (BXDs). In that view, the belowground interactions between, rye (*Secale cereale* L.) and pigweed (*Amaranthus retroflexus* L.), which is a widespread weed, have been studied in this master thesis. A focus has been made on the effect of two potential belowground signaling molecules (loliolide and jasmonic acid) inducing the production of BXDs in rye. Three different aspects have been developed.

Firstly, the influence of substrate characteristics on plant growth, root architecture along with BXDs composition and concentration has been analyzed. Both rye and pigweed have been cultivated, alone or in co-culture, in two different substrates: microbeads of glass and a mixture of clay and attapulgite. It has been demonstrated that both plants had greater growth in the clay and attapulgite mixture, suspected to be caused by substrate differences in particle size, water retention and/or pore space between particles. However, fewer BXDs were detected in that substrate, presumably due to the sorption capacity of clay compared to glass microbeads. These findings further support the critical importance of the physiochemical properties of soils when investigating plant morphology and plant chemistry such as allelopathy.

Secondly, the physical and chemical interactions between rye and redroot pigweed along with the effect of potential signaling molecules (loliolide and jasmonic acid) on rye root architecture and allelochemicals (e.g. BXDs) production have been studied. On the one hand, rye treated with pigweed root exudates showed greater root growth for almost all root architecture parameters and lower BXDs concentrations compared to rye grown in co-culture with pigweed. It could be assumed that the physical competition between rye and pigweed when grown in co-culture might have overcome the potential effect of chemicals from root exudates. Moreover, low concentration of pigweed root exudate might have reduced the entire chemical outcome, leading to a possible hormesis effect. On the other hand, rye treated with low dose corresponding to 0.5 nM of loliolide and jasmonic acid, which has never been pursued before, showed lower root growth for all root architecture parameters as well as higher BXDs concentrations for three compounds (DHBOA-Glc, HBOA-Glc and DIMBOA). The increase of BXDs concentration motivates the hypothesis that loliolide and jasmonic acid could act as potential belowground signaling molecules inducing the production of defensive metabolites, while the root growth reduction highlights their inhibition effect.

To close this master thesis, the detection and quantification of loliolide in rye root and shoot by HPLC-UV have been carried out. The method of loliolide extraction has been optimized by using fresh plants biomass and the extraction solution made of acetonitrile, water and formic acid. Loliolide could be detected in rye roots. Those results should nonetheless be further confirmed by repeating the experiment with more replicate.

Keywords: *Secale cereale* L., *Amaranthus retroflexus* L., plant-plant interactions, benzoxazinoïds, loliolide, jasmonic acid

RESUME

La demande de nouveaux systèmes durables de lutte contre les mauvaises herbes en Europe est de plus en plus forte, les herbicides modernes ayant montré leurs limites. Les alternatives utilisant des plantes allélopathiques comme cultures de couverture, pour réduire la croissance des mauvaises herbes, sont perçues comme une approche encourageante. Le seigle est une culture allélopathique qui produirait des métabolites secondaires défensifs phytotoxiques appelés allélochimiques, tels que les benzoxazinoïdes (BXDs). Dans cette optique, les interactions souterraines entre le seigle (*Secale cereale* L.) et l'amarante (*Amaranthus retroflexus* L.), une mauvaise herbe très répandue, ont été étudiées dans ce travail de fin d'études. Une attention particulière a été portée sur l'effet de deux potentielles molécules signales souterraines (loliolide et acide jasmonique) induisant la production de BXDs dans le seigle. Trois aspects différents ont été développés.

Tout d'abord, l'influence des caractéristiques d'un substrat sur la croissance des plantes, l'architecture des racines ainsi que la composition et la concentration des BXDs a été analysée. Le seigle et l'amarante ont été cultivés, seuls ou en co-culture, dans deux substrats différents : des microbilles de verre et un mélange d'argile et d'attapulgite. Il a été démontré que les deux plantes avaient une croissance plus importante dans le mélange d'argile et d'attapulgite, ce qui pourrait être dû aux différences de taille des particules du substrat, à la rétention d'eau et/ou à l'espace poreux entre les particules. Cependant, moins de BXD ont été détectés dans ce substrat, probablement en raison de la capacité de sorption de l'argile. Ces résultats confirment l'importance cruciale des propriétés physiochimiques des sols lors de l'étude de la morphologie et de la chimie des plantes, comme l'allélopathie.

Deuxièmement, les interactions physiques et chimiques entre le seigle et l'amarante ainsi que l'effet de potentielles molécules signales (loliolide et acide jasmonique) sur l'architecture des racines du seigle et sur la production de substances allélochimiques, comme les BXDs, ont été étudiés. D'une part, le seigle traité avec des exsudats racinaires d'amarante a montré une plus grande croissance des racines pour presque tous les paramètres d'architecture racinaire, et des concentrations plus faibles de BXDs par rapport au seigle cultivé en co-culture avec l'amarante. Il peut être supposé que la compétition physique entre le seigle et l'amarante, en co-culture, peut avoir surmonté l'effet chimique provenant des exsudats racinaires. En outre, la faible concentration d'exsudats racinaires d'amarante a pu réduire l'ensemble des effets chimiques, ce qui a pu entraîner un effet d'hormésis. D'autre part, le seigle traité avec une faible dose correspondant à 0,5 nM de loliolide et d'acide jasmonique, ce qui n'a jamais été étudié auparavant, a montré une croissance racinaire plus faible pour tous les paramètres d'architecture racinaire ainsi que des concentrations de BXDs plus élevées pour trois composés (DHBOA-Glc, HBOA-Glc et DIMBOA). L'augmentation de la concentration en BXDs motive l'hypothèse selon laquelle le loliolide et l'acide jasmonique pourraient agir comme des molécules signales souterraines potentielles induisant la production de métabolites défensifs, tandis que la réduction de la croissance des racines met en évidence leur effet d'inhibition.

Pour conclure ce travail de fin d'études, la détection et la quantification du loliolide dans les racines et les feuilles de seigle ont été réalisées par HPLC-UV. La méthode d'extraction du loliolide a été optimisée en utilisant la biomasse de plantes fraîches et la solution d'extraction composée d'acétonitrile, d'eau et d'acide formique. Le loliolide a pu être détecté dans les racines de seigle. Ces résultats devront néanmoins être confirmés en répétant l'expérience avec plus de réplicats.

Mots-clés : *Secale cereale* L., *Amaranthus retroflexus*, interactions plante-plante, benzoxazinoïdes, loliolide, acide jasmonique

TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDIXES

LIST OF ABBREVIATIONS

AOC: Allene oxide cyclase
AOS: Allene oxide synthase
BOA: Benzoxazolin-2-one
BXD: Benzoxazinoids
DIBOA : 2,4- dihydroxy-1,4-benzoxazin-3-one
DIBOA-Glc: 2-β-D-Glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one
DIMBOA: 2,4-dihydroxy7-methoxy-1,4-benzoxazin-3-one
DIMBOA-Glc: 2-β-D-Glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one
HBOA: 2-hydroxy-1,4-benzoxazin-3-one
HBOA-Glc: 2-β-D-Glucopyranosyloxy-1,4-benzoxazin-3-one
HDMBOA-Glc: 2-β-D-Glucopyranosyloxy-4,7-dimethoxy-1,4-benzoxazin-3-one
HMBOA-Glc: 2-β-D-Glucopyranosyloxy-7-methoxy-1,4-benzoxazin-3-one
JA: Jasmonic acid
JAs: Jasmonates
LOL: Loliolide
LOX: Lipoxygenase
MBOA: 6-Methoxybenzoxazolin-2-one
MeJA: Methyl-jasmonate
OPDA: 12-oxo-phytodienoic acid
OPR: 12-oxo-phytodienoic acid reductase
P: Pigweed
PRE: Pigweed root exudates
R: Rye
R+P: Rye and pigweed grown in co-culture
RBD: Root branching density
RLD: Root length density
ROS: Reactive oxygen species
RSA: Root system architecture
RSD: Root surface area density
RTD: Root tissue density
SA: Salicylic acid
SAR: Systemic acquired resistance
SEM: Standard error of the mean
SRL: Specific root length
VOC: Volatile organic compound

PART I – STATE OF THE ART

1. General context

In many respects, modern herbicides have proven their limit for weed control. Indeed, the soil and water quality are declining, and weeds are getting more resistant to herbicides every year impacting the sustainability of weed control systems worldwide. Furthermore, pesticide residues have a negative impact on both the environment and human health (Tabaglio et al., 2008; Jabran, 2017). The urge for new sustainable weed control systems in Europe is increasing as the European Union has set up the "Farm to Fork Strategy" directly correlated to the European Green Deal. This strategy aims to reduce by 50% the use of chemical pesticides as well as 25% of total farmland being under organic farming by 2030 (Wesseler, 2022).

Several alternative weed management strategies have been studied for past decades. Among them, using allelopathic plants as cover crops or using their residues are perceived as a compelling approach. In 1984, E.L. Rice defined allelopathy as "any direct or indirect harmful or beneficial effect by one plant on another through production of chemical compounds that release into the environment" (Willis, 2007). Therefore, allelopathy can be considered as one of the mechanisms involved in plant-plant interactions.

In this study, rye (*Secale cereale* L.) and pigweed (*Amaranthus retroflexus* L.) have been selected as model plants in order to further investigate the belowground interactions between them. Rye is an allelopathic crop which has been shown to produce phytotoxic defensive secondary metabolites such as allelochemicals (e.g., benzoxazinoids). Redroot pigweed is a widespread weed which has shown sensitivity to rye mulches and to benzoxazolin-2 (3H)-one (BOA) from the family of the benzoxazinoids (Tabaglio et al., 2008; Schulz et al., 2013; Jabran, 2017). Three different aspects will be developed. At first, both rye and pigweed, alone and in co-culture will be cultivated in two different substrates in order to analyze their impact on plant growth and root exudates production. Secondly, the effects of two potential signaling molecules (jasmonic acid and loliolide), which are supposed to be released through root exudates and induce the production of allelochemicals such as benzoxazinoids in rye, will be investigated. To do so, the growth and the allelopathic response of rye will be analyzed in a controlled environment, using anatomical and chemical features of rye as indicators. To close this master's thesis, the quantification of loliolide in rye roots and shoots will be attempted.

2. Mechanisms involved in plant-plant interactions

Plant survival and performance are influenced by diverse factors. Abiotic factors such as environmental conditions (e.g., agricultural methods, water or nutrient deficiency) and biotic factors (e.g., pathogens, microbes, insects and other plants) will influence plant growth and reproduction (Tukey, 1969; Dangl & Jones, 2001; Wang et al., 2021). In plant-plant interactions, plants must perceive and interact with their neighbors to survive and increase their performance (Wang et al., 2021). Plant neighbors identification and immunity responses rely on different biological processes presented in Figure 1: plant-soil feedbacks, danger signaling, intraspecific and interspecific competition, kin recognition and stranger recognition (allelopathy) (Pélissier et al., 2021).

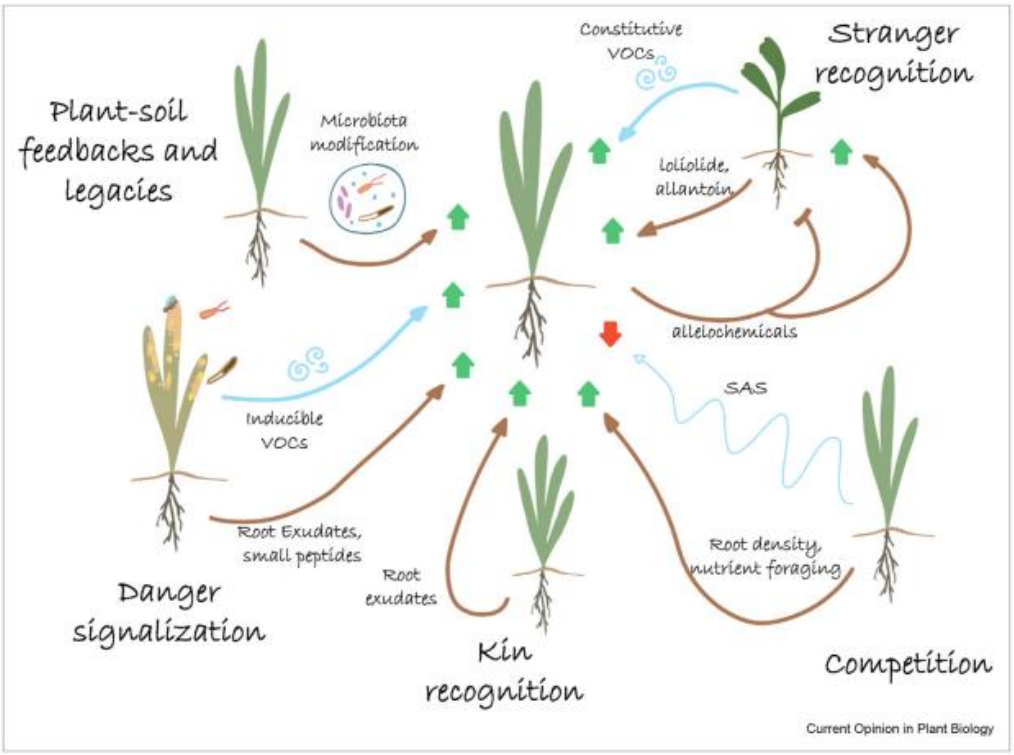


Figure 1: Conceptual framework for different mechanisms involved in plant-plant interactions

2.1. Plant Soil feedback and legacies

Soil quality, including microbial communities and nutrient availability, deeply influences plant growth. Nevertheless, already-grown plants also condition soil, through microbiome modification or soil chemical modification which impact the growth and the immunity of the next plant (Pélissier et al., 2021). Environmental changes (e.g., drought) have lasting effects on belowground communities with consequences for plant–soil feedbacks and plant–plant interaction. Thus, plant–soil feedback impacts the structure of plant communities and plant-plant competition (Kaisermann et al., 2017). Recent studies have shown that 2,4-dihydroxy7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and its derivatives released by maize roots were able to alter bacteria communities in the rhizosphere. Moreover, when other maize plants were growing in the soil already conditioned by maize plants producing DIMBOA, the growing maize plants showed significant induction of defense marker genes and defense hormones such as salicylic acid (SA) and jasmonic acid (JA) in response to the allelochemicals already present in the soil (Hu et al., 2018).

2.2. Danger signalisation

Plants can release danger signals to their neighbors in case of biotic stress such as a pathogen attack or damage caused by insects (Pélissier et al., 2021). Those released signals are above and belowground compounds such as volatile organic compounds (VOCs), root exudates or small peptides. By detecting those signals, neighboring plants can activate their own defense system (Gust et al., 2017). This phenomenon is called eavesdropping and can occur between intraspecific or heterospecific neighboring plants. Under pathogen or insect attack, the plant will induce its systemic acquired resistance (SAR) which is a defense priming mechanism between infected and uninfected tissues. VOCs such as specific monoterpene emitted by the infected plants will induce SAR in its own distant tissues but also in neighboring plants (Riedlmeier et al., 2017).

2.3. Competition: nutrient, water, light and root competition

Competition is a predominant factor that regulates the diversity and the relative dominance of a species within plant communities (Blum, 2011). Neighboring plants induce competition, not specifically by producing signals, but rather by indirectly competing for the same resources such as nutrients and water availability as well as light (Tukey, 1969, Pélissier et al., 2021). It is well known that plants need numerous nutrients acquired in several forms like nitrogen to grow properly. Nutrients availability mostly depends on their diffusivity in soil, soil properties and microbial decomposition. Thus, plant activity can increase or decrease nutrient availability leading to competition (Craine & Dybzinski, 2013). Plant resources-acquisition capacity mainly relies on root functional traits such as specific root length. Indeed, plants with longer roots have higher nutrients and water accessibility leading to better competition for soil resources (Fort et al., 2014). Root competition influences plant growth by reducing the soil and resources availability but also by root exudation through allelopathy (Schenk, 2006).

Plant competition can also be induced by the perception of light quantity and quality. Both blue light depletion and red-to-far-red light ratio indicate the presence of neighboring plants (Keuskamp et al., 2012). The reduction of the red: far-red light ratio, perceived through the phytochrome photoreceptors of the plant, can be used by the plant as an early warning signal for future competition (Pierik & de Wit, 2014). Whereas blue light depletion, detected by cryptochromes receptors, is an indicator of the current shade level (Keuskamp et al., 2012).

2.4. Kin-recognition

Kin recognition refers to a plant's ability to detect and recognize a neighboring plant, through root physical touch or chemical cues such as root exudate, at an intraspecific level meaning from the same species, or closely related plants. The ability to recognize and respond to close relatives leads plants to optimize their competitive strategies, resulting in less intra-specific competition and more cooperation among plants, maximizing the kin group performance (Xu et al., 2021). A recent study, working on herbicide resistance barnyard grass and rice, has hypothesized that the level of loliolide present in root exudates may indicate neighbor kinship by discriminating kin from non-kin and even differentiating interspecific competitors from conspecific competitors (Ding et al., 2023).

2.5. Allelopathy

The allelopathy phenomenon, inducing allelochemicals, has been defined in many ways over time. Unlike kin-recognition which reduces the competition between closely related plants, allelopathy is an interference mechanism in which plants produce and release allelochemicals due to the perception of signaling molecules from neighboring plants (Xu et al., 2021). The most common definition cited by researchers is the one provided by E.L. Rice in 1984 which has been mentioned above (Willis, 2007). However, this definition, which includes both positive and negative effects, has been controversial for many scientists for being too broad. Instead, a definition only recognizing, direct or indirect, negative effect of a plant on an inter-specific or intra-specific neighbor is preferred (Gaba et al., 2018). Such allelopathic interferences can impact the performance of neighboring plants and alter local plant coexistence (Xu et al., 2021). Unlike competition, which mainly occurs through physical mechanisms such as competition for resources or light, allelopathy is a chemical defense strategy against competing neighbors, which results from allelochemicals produced and released from plants themselves (Macías et al., 2019).

2.5.1. Allelochemicals

Allelochemicals are secondary metabolites produced by plants which have stimulatory or inhibitory effects upon the growth or neighboring plants' behavior (Zeng et al., 2008). Secondary metabolites are organic compounds which are not directly involved in the growth, development or reproduction of an organism (Wink, 2003). Most allelochemicals are defensive compounds that are energetically costly to produce, leading to the production of multifunctional compounds with high structural diversity (Wink, 2003). Allelochemicals are represented by several chemical families among which phenolic compounds, terpenoids, alkaloids and nitrogen-containing chemicals. The most extensively studied ones are simple phenolics, flavonoids and alkaloids. In general, allelochemicals are distributed in different organs of the plant such as seeds, flowers, pollen, leaves, stems, and roots (Zeng et al., 2008). Allelochemicals possess diverse modes of release. Aboveground compounds are mostly delivered through volatilization or lixiviation while belowground compounds are mostly produced via residue decay, leaching or root exudation (Zeng et al., 2008). Allelochemicals are delivered into the rhizosphere by leaching from the above aerial plant parts by precipitation, decomposition of leaf or bark litter and root exudation which activated form might depend on microbial transformation (Weir et al., 2004).

2.5.2. Signalling molecules

The chemical compounds (e.g., VOCs, root exudates) from one plant, which induce local or systemic responses, such as allelochemicals production, in neighboring plants, can be named signaling molecules even though this terminology isn't properly adopted in scientific literature.

Signaling compounds are released both aboveground and belowground leading to complex plant–plant communication. Aboveground signal compounds mediated through air-borne chemicals, such as VOC or plant volatile, have been widely studied and plant-organisms or plant-plant interaction have been well established this last decade (Kong et al., 2019). Belowground signals are mostly driven by root exudates (*cfr* section 3.1). The mechanisms and identity of soil-borne chemicals in belowground plant–plant signaling interaction have risen scientists' concerns these past few years. Recent studies have hypothesized that unstressed plants can perceive compounds emitted by drought- and osmotically stressed neighboring plants. Moreover, the unstressed plant showed similar responses than stressed plants. It can be hypothesized that plants communicate without the need for direct root contact. Indeed, those results were obtained using a split-root system in which stressed plant and unstressed plant roots could not touch (Falik et al., 2012). In 2014, Semchenko et al. showed that root exudates can carry specific information including the species identity of neighbors suggesting the presence of signaling molecules. Furthermore, those root exudates trigger different responses at the root system level through changes in root morphology (Semchenko et al., 2014). In 2018, Kong et al., demonstrated that root exudates of neighboring plants induced allelochemical production in wheat, highlighting the importance of root-secreted signaling chemicals in neighbor detection and allelochemical production. Moreover, they established that the phytohormones jasmonic acid (JA) and loliolide (*cfr* section 5.1) strongly induced allelochemical production in wheat. They suggested that those ubiquitous hormones are soil-borne signaling chemicals that can trigger plant defensive responses in belowground plant – plant interaction (Kong et al., 2018).

3. Root exudation and architecture

Roots can be classified into different systems such as primary roots, lateral roots, basal roots and shoot-borne roots (Koevoets et al., 2016, Zhang et al., 2022). The root system of dicotyledonous species typically consists of primary root and lateral roots while monocotyledonous species are characterized by the development of many adventitious roots in parallel to the primary root (Badri & Vivanco, 2009). Root hairs are tubular structures shaped by outwardly protruding epidermal cells. They are important for the root system for activities such as root exudates content, nutrient absorption and rhizosphere interactions. Diverse studies have proven that root hairs secrete secondary metabolites (Holz et al., 2018).

3.1. Root exudates

Root exudates released by root hair include different elements such as ions, free oxygen, enzymes, mucilage, inorganic acid and diverse carbon-containing primary and secondary metabolites (Bais et al., 2006). The carbon-based compounds are, most of the time, separated into two classes: low-molecular-weight compounds (e.g., amino acids, organic acids, sugars, phenolics and an array of secondary metabolites) and high-molecular-weight compounds (e.g., mucilage and proteins) (Badri & Vivanco, 2009). Root exudates have diverse functions such as modifying soil properties impacting its competitiveness against other plants, inducing beneficial symbiosis and regulating root microbiome and soil microbial communities (Wang et al., 2021). Plant root chemical signals can also influence both aboveground and belowground features such as flowering and reproduction or microbe diversity which can alter plant fitness (Badri & Vivanco, 2009; Wang et al., 2021).

Soil chemical signals can induce among other responses: root detection and recognition, chemical defense such as the production of allelochemicals and modification in the root behavior such as root architecture and placement (Kong et al., 2018, Wang et al., 2021).

3.2. Root placement

The uptake of resources affects root growth, distribution and placement patterns. However, recent studies have shown that root placement also depends on the perception of neighboring plants and thus, kin and non-kin recognition (Wang et al., 2021). In the presence of other plants, roots can distinguish interspecific roots, which constitute plants from different species, from conspecific roots which represent plants from the same species (Falik et al., 2012; Xu et al., 2021). Moreover, plant roots can also perceive intraspecific differences occurring in plants from the same species. Roots can differentiate kin and non-kin individuals (Yang et al., 2018).

Depending on diverse abiotic and biotic factors such as soil resources or neighboring plants, plants can have different root-placement shown in Figure 2: intrusive (approaching, over-proliferation), avoidance (repelling, underproliferation) or unresponsive patterns. Root exudates play a key role in root recognition, especially in intrusive and avoidance roots placement in response to the presence of neighbors (Wang et al., 2021).

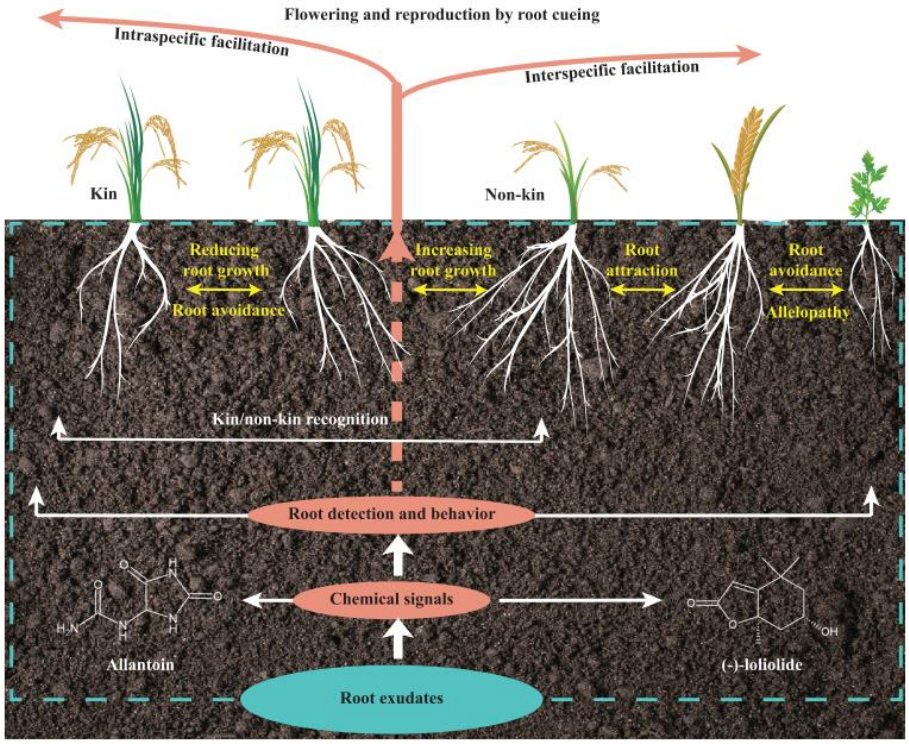


Figure 2: Rye root placement modification in response to potential chemical signals produced by redroot pigweed leading to kin/non-kin recognition (Wang et al., 2021)

3.3. Root system architecture

Like root placement, Root system architecture (RSA) can be altered by diverse factors including the presence of other neighboring plants. RSA is defined as the spatial configuration of root components and determines the soil volume that can be explored by the roots. Plants' roots can be characterized as plastic referring to the plant's ability to adapt and cope with changes in its environment (Heinz, 2012). Hence, plant shoots and roots from the same species can differ depending on their environment. RSA depends on abiotic exogenous factors, such as water, nutrient or light availability, and on endogenous factors, such as phytohormones among which auxin, cytokinin, ethylene, gibberellins and abscisic acid. Concerning biotic factors, there are increasing evidences that plant-plant interaction, especially root exudates of neighboring plants, induce a range of changes in RSA (Badri & Vivanco, 2009).

According to Zhang et al. (2022), root exudates can directly and indirectly affect RSA. The root systems from plants grown together have shown diverse behavior modifications such as an adjustment of growth and symmetry. Parameters such as root length, angle or biomass can be measured to describe root system modification. The main outcome of growth is the root biomass increase or decrease. Plants such as *Arabidopsis thaliana* can increase their lateral root number in the presence of kin or non-kin plants. It has been proven that root exudates directly mediated these differences (Palmer et al., 2016). Another study has shown that rice seedling or its root exudates applied on distantly related plants induced a larger root system compared to exposure to closely related plants (Yang et al. 2018). Other studies showed that root exudates could trigger various RSA responses, including changes in root mass, root length density, specific root length, root surface density and root branching intensity (Delory et al., 2021).

From an indirect prospect, root exudates can affect RSA by mobilizing soil nutrients such as nitrogen, phosphorus and iron that have a direct effect on the RSA modulation. Furthermore, several studies have shown evidence that the availability of nutrients affects RSA, including root biomass (van Dijk et al.,

2022), root length (Kumar et al., 2020), lateral root number (Pongrac et al., 2020) and root horizontal and vertical distribution (Zhang et al. 2020). Root exudates also indirectly affect RSA by altering soil microbial communities as it is their main source of energy (Zhang et al., 2022).

4. Plants Model: Rye (*Secale cereale L.)* and redroot pigweed (*Amaranthus retroflexus L.*)

In this study, the interaction between rye (*Secale cereale* L.) as allelopathic crop and redroot pigweed (*Amaranthus retroflexus* L.) as weed and its root exudates will be studied. This section will be divided into two sub-sections introducing the plant models. On one hand, a description of rye (*Secale cereale* L.) as a cover crop, as well as the secondary metabolite and allelochemicals it produces, will be discussed. On the other hand, redroot pigweed (*Amaranthus retroflexus* L.) and the effect of pigweed root exudates will be developed.

4.1. Rye (*Secale cereale* L.)

4.1.1. Rye as cover crop

Rye is a monocot from the family Poaceae which is closely related to wheat and barley. This winter crop is grown for its grains, fodder or even as a cover crop. For the past decades, rye has been intensively studied for its weed suppression property as a cover crop (Schulz et al., 2013; Jabran, 2017). A cover crop is a crop that is not harvested but is grown to benefit the soil and/or other crops. It can have a direct impact on the soil by physically modifying seed germination or its environment. By using allelopathic crops, cover crops can also control weeds by inducing allelopathy (Creamer et al., 1996). The most studied allelopathic field crops are wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.), maize (*Zea mays* L.), rye (*Secale cereale* L.), barley (*Hordeum vulgare* L.) and sorghum (*Sorghum bicolor* L.) (Jabran, 2017). In the case of rye, utilized as a cover crop, it suppresses weeds through shading, physical interference or allelopathy. Rye mulch can be directly applied on the field crops to control weeds. Another way is to grow the allelopathic rye cultivars as intercrops or in rotation with non-allelopathic crops (Jabran, 2017).

4.1.2. Specific allelochemicals: benzoxazinoid family

Each allelopathic crop species and varieties possess different types of allelochemicals in diverse concentrations. The allelochemicals profile and concentration produced by rye are highly dependent on internal conditions, such as the assigned organs (e.g., leaves, stem, roots) or the development stage of the plant as well as external conditions, such as the location of cultivation (green houses or field) (Wójcik-Wojtkowiak et al., 1990).

The two most abundant allelochemicals in rye shoots and roots are phenolic acids and benzoxazinoids (Carlsen et al., 2009). Phenolic acids are involved in antimicrobial activity and influence the germination and growth of different plant species. Furthermore, seedlings demonstrated higher levels of phenolics compared to crop residues or tillering plants (Wójcik-Wojtkowiak et al., 1990).

a) Biosynthesis of BXDs

Benzoxazinoid (BXD) are multi-functional allelochemical family that has a role in plant nutrition, vegetative and reproductive growth, and most importantly in the matter of allelopathy, therefore, defense. BXDs are thereupon considered to be the main family of allelochemicals involved in plant defenses against fungi, insects and weeds. Plants from the poaceae family (e.g., maize, wheat and rye) have shown a particularly significant amount of BXDs (Jabran, 2017; Robert & Mateo, 2022).

BXDs are indole-derived compounds divided into two main groups: benzoxazolinones (1,3-benzoxazol-2-one core structure) and benzoxazinones (1,4-benzoxazin-3-one skeleton). This last group can be further classified as hydroxamic acids, N-O-methylated hydroxamic acids and lactams.

Table 1: Acronym and systematic names of compounds described in the present study

Abbreviations	Systematic name
DIBOA	2,4- dihydroxy-1,4-benzoxazin-3-one
DIBOA-Glc	2-β-D-Glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one
DIMBOA	2,4-dihydroxy7-methoxy-1,4-benzoxazin-3-one
DIMBOA-Glc	2-β-D-Glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one
HDMBOA-Glc	2-β-D-Glucopyranosyloxy-4,7-dimethoxy-1,4-benzoxazin-3-one
HBOA	2-hydroxy-1,4-benzoxazin-3-one
HBOA-Glc	2-β-D-Glucopyranosyloxy-1,4-benzoxazin-3-one
HMBOA	2-hydroxy-7-methoxy-1,4-benzoxazin-3-one
HMBOA-Glc	2-β-D-Glucopyranosyloxy-7-methoxy-1,4-benzoxazin-3-one
DHBOA-Glc	2-β-D-Glucopyranosyloxy-7-hydroxy-1,4-benzoxazin-3-one
BOA	Benzoxazolin-2-one
MBOA	6-Methoxybenzoxazolin-2-one

The biosynthesis of BXDs has been mainly investigated in maize (*Zea mays*) (Frey et al., 2009). As rye is closely related to maize, the assumption can be made concerning a similar biosynthesis. As shown in Figure 3, the first step, occurring in the chloroplast, is the conversion of indole-3-glycerol phosphate (IGP) into indole by a lyase enzyme. In the endoplasmic reticulum membrane, indole will go through a series of consecutive oxidations by several other lyases leading to its conversion into lactam HBOA. The lactam HBOA will be further hydroxylated to form the hydroxamic acid DIBOA (Frey et al., 1997; Frey et al., 2009). In the cytoplasm of the plant cell, DIBOA is glucosylated and the resulting glucosylated DIBOA (DIBOA-Glc) is used as a precursor to form DIMBOA-glucoside (DIMBOA-Glc) (Von Rad et al., 2002). DIMBOA-Glc can undergo further methylation to form the methyl hydroxamate HDMBOA-Glc (Meihls et al., 2013). DIMBOA-Glc can also further be oxidized and methylated, leading to the production DIM_2BOA-Glc which can be further converted into the corresponding methyl hydroxamate (HDM_2BOA-Gl) (Handrick et al., 2016). All the stable glucosylated benzoxazinones mentioned above are stored in the vacuole of cells in young tissues of roots and leaves to prevent any degradation of the β-glucosidases located in the cytosol, plastids and cell walls (Nikus et al., 2001; Schulz et al., 2013; Robert & Mateo, 2022).

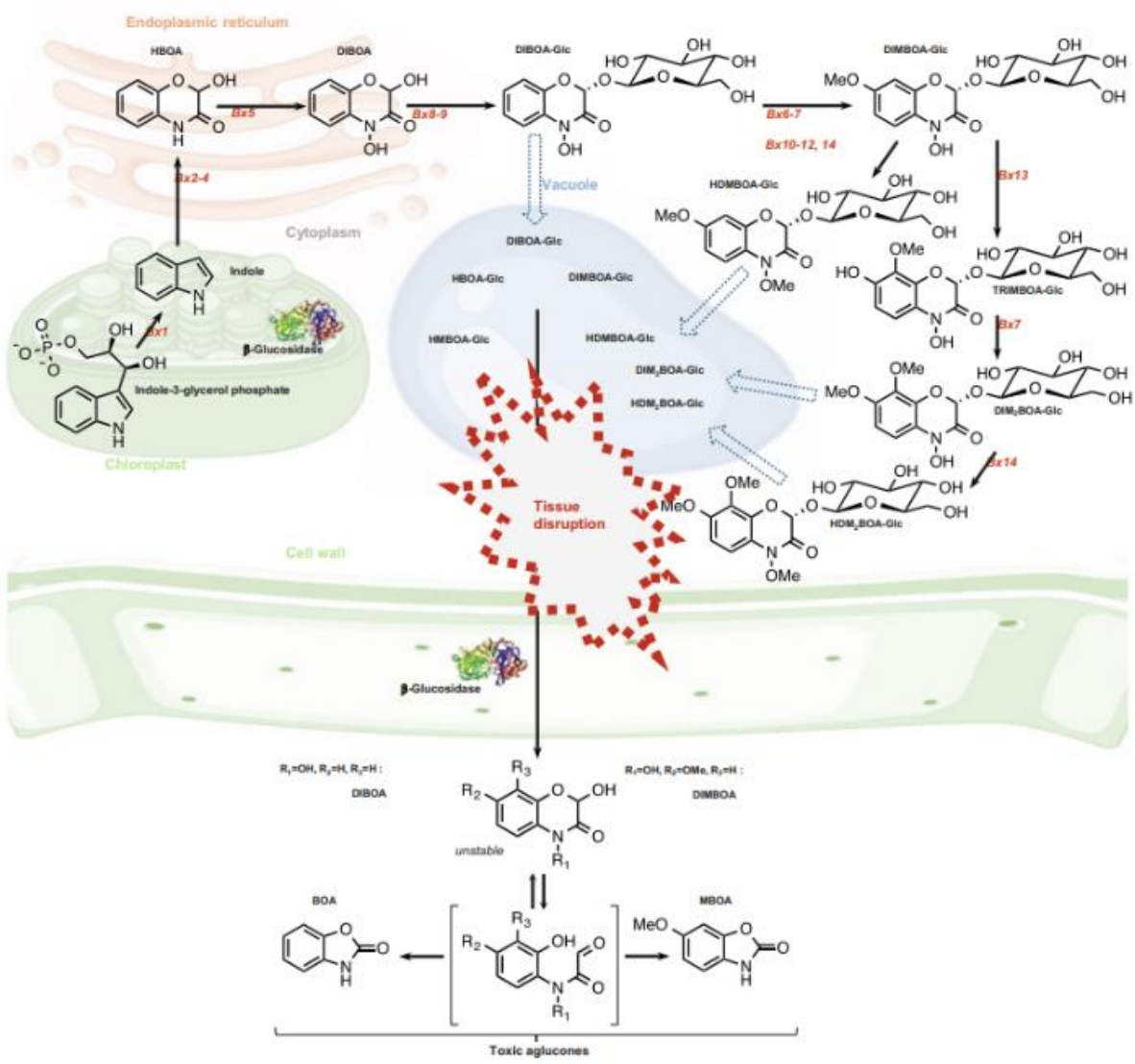


Figure 3: Known pathways involved in benzoxazinoids biosynthesis (Robert & Mateo, 2022)

b) Release mode of BXDs

Benzoxazinoids have two different modes of release: passively through plant residues, or through exudation, which is suspected to involve active mechanisms yet unknown (Robert & Mateo, 2022). As mentioned before, exudation and BXDs are deeply influenced by abiotic factors such as nutrients supply and biotic factors such as insect or plant interaction. The glucoside forms of BXDs, such as DIMBOA and DIBOA, are exuded by the roots in the rhizosphere. They will be hydrolyzed to form aglucones, which is a biologically active form. Those bioactive aglucones have been stated as the main compounds responsible for allelopathic effects of wheat and rye (Belz & Hurle, 2005). However, aglycones can be spontaneously degraded and form the more stable (M)BOA which can be detected in soil for a few days. Soil microbes are able to further degrade (M)BOA to produce the corresponding aminophenol and air can induce its transformation into aminophenoxazinones by oxidation. This final form can be detected at a stable concentration for months (Macías et al., 2004; Schütz et al., 2019).

c) BXDs Functions

Benzoxazinoids are important modulators of plant nutrition, growth, defense and reproduction. BXDs are involved in plant defense processes, such as callose deposition and ferulic acid production. They can also influence flowering time through iron nutrition or hormonal modulation in *Arabidopsis thaliana* (Chen et al., 2021). In the perspective of weed control, several experiments have been carried out with BOA, known for its stability compared to DIBOA. Many weeds such as *Amaranthus retroflexus* in the seedlings stage have been affected by BOA and rye mulch (Tabaglio et al., 2008). Regarding plant growth, studies have demonstrated that BXDs might modulate the signaling pathway of diverse hormones such as auxin, cytokinin and gibberellin. For example, in corn (*Zea mays* L.), MBOA indirectly modifies the binding affinity of auxins to specific receptor sites leading to the inhibition of shoot and root elongation in *Amaranthus* seedlings (Hussain et al., 2022). Robert & Mateo (2022) speculated interesting hypotheses on the role of BXDs concerning gibberellin and cytokinin modulations. Benzoxazinoids could modulate gibberellin pathway which is known to influence stem elongation.

4.2. Redroot pigweed (*Amaranthus retroflexus* L.)

4.2.1. Pigweed (*Amaranthus retroflexus* L.) overview

Pigweed is the third most widespread dicotyledonous weed species in the world (Konstantinović et al., 2014). *A. retroflexus* infests a wide range of crops (e.g., corn, wheat and barley) causing important economic losses. Its negative impact includes reduction of crop yield and quality as well as toxicity to livestock. Those weeds can act as an alternative host for crop pathogens and insect pests. Pigweed herbicide-resistance is a growing concern for farmers worldwide. In China, herbicides such as thifensulfuron-methyl, imazethapyr, fomesafen, and others have failed to control pigweed growth (Cao et al., 2021; Du et al., 2021). In recent years, several mutations targeting a critical enzyme (acetolactate synthase ALS) gene in *A. retroflexus* have led to ALS-inhibiting herbicide-resistance (Cao et al., 2021). New alternatives involving cover crops or allelopathy might be a solution to tackle weed resistance.

4.2.2. Pigweed root exudates

Many studies demonstrated the allelopathic effects of *A. retroflexus* on different crops such as maize, barley, wheat and cucumber (Costea et al., 2004, Shahrokhi et al., 2012; Hamideh Bakhshayeshan-Agdam et al., 2015). Costea et al. (2004) showed that pigweed could produce secondary metabolites inducing allelopathic effects on crops. Indeed, extracts made of *A. retroflexus* L. root showed inhibitory effect on maize seeds' hypocotyl length. Shahrokhi et al. (2012) demonstrated that the germination and initial growth of two wheat cultivars were affected by aqueous extracts of pigweed organs (leaf, root and stem) in all concentrations tested (2,5, 5 and 10%). Higher allelopathic effects of pigweed extracts were observed for an increasing extract concentration on measured traits. Pigweed leaf extract showed higher levels of toxicity for wheat germination than extract of other organs (Shahrokhi et al., 2012). Several potential allelochemicals were detected in pigweed extracts such as aldehydes, alkaloids, apocarotenoids, flavonoids, steroids, xyloid and saponins (Shahrokhi et al., 2012).

5. Belowground interactions focusing on potential signalling molecules and their effects on allelochemical responses

In this section, the two potential signaling molecules (jasmonic acid and loliolide) involved in rye (*Secale cereale* L.) and pigweed (*Amaranthus retroflexus* L.) interaction will be described.

5.1. Potential signalling molecules

5.1.1. The particular case of jasmonic acid

Jasmonic acid and its derivatives referred as jasmonates (JAs) influence various biochemical and physiological functions (e.g., seed germination, root growth, trichome formation, embryo development, seedling development, fruit ripening, and leaf senescence). Recent studies have been focusing on the involvement of JA in plant resistance mechanisms from defence genes activation (e.g., pathogenesis-related genes or proteinase inhibitors) to the accumulation of defensive compounds (e.g., phenolic compounds) (Zhu & Tian, 2012; Wasternack, 2014).

JAs are fatty acids belonging to the family of oxygenated fatty acid derivatives also cited as oxylipins. Linolenic acid is a precursor in JA synthesis process. As shown in Figure 4, linolenic acid, located in the chloroplast membranes, is converted to 12-oxo-phytodienoic acid (OPDA) by the action of three enzymes: lipoxygenase (LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC). In the peroxisome, OPDA is further converted into JAs through the action of 12-oxo-phytodienoic acid reductase (OPR) and three steps of β-oxidation. Several conjugates can be formed, among which methyl jasmonate and jasmonoyl-isoleucine (JA-Ile) (Yu et al., 2019).

At the time of writing, it has been demonstrated that almost all higher plants possess JA. However, JA concentrations vary according to tissue types, developmental stage, and environmental stimuli. Flowers and reproductive tissues have the highest JA levels, whereas roots and mature leaves have the lowest concentrations (Hewedy et al., 2023).

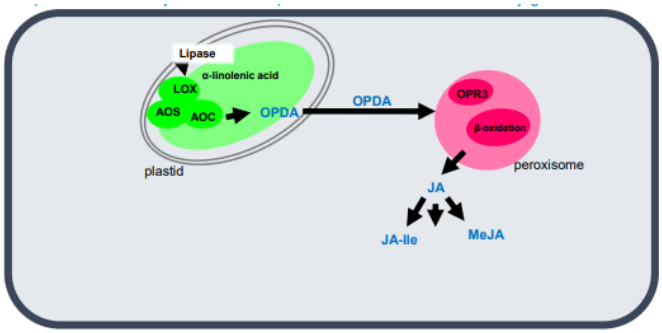


Figure 4: Biosynthesis of jasmonates from linolenic acid in the chloroplast membranes by means of the octadecanoid pathway

Diverse phytohormones, especially JA, are essential in the development of plant root systems. As a reminder, phytohormones are endogenous substances derived from plant biosynthetic pathways that can act either locally (at the site of their synthesis) or transported to other plant organs to mediate growth and development responses under stressful conditions (Peleg & Blumwald, 2011). In the particular case of JA, it affects root growth more precisely: primary root inhibition, root regeneration and adventitious root reduction. It also stimulates lateral root growth. Those physiological modifications are regulated by several crosstalk between JA and other plant hormones such as auxin (Lakehal et al., 2019; Hewedy et al., 2023). Furthermore, exogenous application of JA, has shown plant root elongation decreases due to the reduction of cells in *Arabidopsis thaliana* roots meristem (Chen et al., 2011). Those results have been further confirmed by Corti Monzon et al. (2012) who have proven that the external application of JA at different concentrations represses root architecture through inhibition of the primary root growth and reducing the number of lateral roots in sunflower seedlings.

5.1.2. The particular case of loliolide

One of the first mentions of loliolide was made in 1964 by R. Hooces where it has been isolated from *Lolium perenne* (perennial ryegrass) (Jayawardena et al., 2021). Loliolide has also been described as an active compound having germination inhibitory property and is naturally present in higher plants (Hiraga et al., 1997). In the past decade, loliolide has mostly been studied for its function in plant-plant interaction as a general soil-borne signal that induced an allelochemical response as explained above.

Loliolide is an apocarotenoid metabolite such as abscisic acid and strigolactones, resulting from several oxidations of carotenoids into β-carotene. Those oxidative processes can be either spontaneous through reactive oxygen species or catalyzed by carotenoid cleavage dioxygenase enzymes. The biosynthesis of loliolide is still being studied. However, its production is closely related to other apocarotenoids synthesis. Biotic or abiotic stress (e.g., high-light stress) induces the production of apocarotenoids by triggering the O_2 signaling cascade (hypersensitive response) leading to the oxidation of β-carotene in photosystem II (PSII) of the chloroplast. Those oxidized compounds can be transported to the cytosol by unknown transporters leading to xenobiotic detoxification (Moreno et al., 2021). The following steps remain unclear.

5.1.3. Effect of jasmonic acid and loliolide on allelochemicals production

Interestingly, phytohormones such as jasmonic acid, methyl-jasmonate (MeJA) and others influence the accumulation of benzoxazinoids in wheat seedlings. According to Sue et al. (2021) studies, hormone treatments trigger tissue- and gene-specific responses resulting in a variation in benzoxazinoid concentrations in different leaf tissues of wheat. In the context of plant-plant interactions through root exudates, recent studies have demonstrated the presence of JA in the root exudates of both rice and barnyard grass placed in a coexistence system. Moreover, endogenous JA exuded from barnyard grass roots induced the production of rice allelochemicals such as momilactone B and tricin (You et al., 2011; Li et al., 2019).

Kong et al. (2018) identified four main components among which JA and loliolide, present in the root exudates of wheat and other neighboring plants including *Amaranthus retroflexus*. Li et al. (2023) demonstrated that both loliolide and JA induced allelochemical DIMBOA response in wheat. Moreover, the exogenous application of a mixture of both loliolide and JA at a concentration of 50 µM induced allelochemical (e.g., DIMBOA) in wheat roots, underlining a potential synergic effect (Kong et al., 2018). It has also been shown that loliolide directly regulates wheat genes related to herbivore and pathogen resistance similarly to JA but it also significantly induces JA production at early incubation periods in wheat roots and shoots. Those results, confirmed via comprehensive transcriptome results, indicate that loliolide might mediate defense-related pathways connected to JA via Ca^{2+}, JA, and ROS (Reactive Oxygen Species) signaling (Li et al., 2023).

All in one, those studies suggest that loliolide might be a systemic and universal signal that regulates plant defense chemicals in plants, rather than a local and species-specific signal (Kong et al., 2018). Besides gene regulation, exogenous loliolide would also induce defensive metabolite production in both exposed roots and untreated shoots. Thus, loliolide, similarly to JA, may have both external elicitor and internal hormonal functions (Li et al., 2023).

5.2. Global scheme of the molecules involved in the Rye (*Secale cereale* L.) and Pigweed (*Amaranthus retroflexus* L.) interactions

In this study, rye is the donor plant which produces allelochemicals (e.g., DIMBOA) while redroot pigweed is the receiver plant which is affected by rye root exudates. Redroot pigweed also produces allelochemicals nonetheless, it is not the interaction studied in this master thesis. Signaling chemicals (e.g., jasmonic acid and loliolide) are potentially produced by the receiver plant inducing the synthesis of allelochemicals in rye (Kong et al., 2018). As shown in Figure 5, the donor plant produces and releases allelochemicals while the receiver plant is affected by them (Gaba et al., 2018).

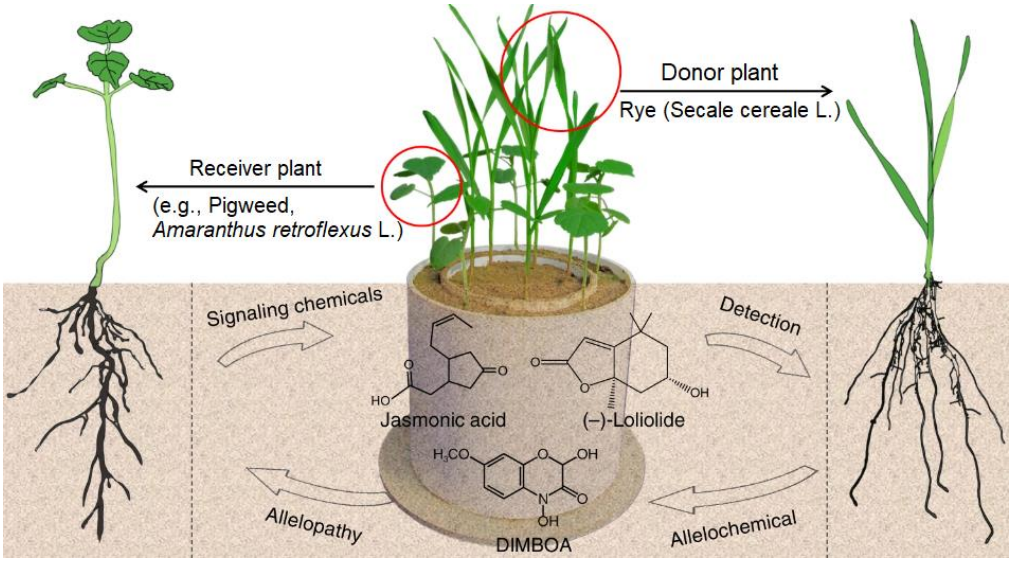


Figure 5: Global scheme of the molecules and mechanisms involved in the rye (Secale cereale L.) and pigweed (Amaranthus retroflexus L.) interaction adapted from C.H.K., S.Z.Z., and Y.H.L

PART II – OBJECTIVES

This master thesis is a part of a collaborative project between the Laboratory of Chemistry of Natural Molecules of Gembloux Agro-Bio Tech and the Weed Science Department of the Agroscope in Switzerland. This project is nested within a larger project aiming to study rye and pigweed interaction. Indeed, precedents master theses' have been focusing, among other, on VOCs emitted by rye roots, BXDs composition present in root exudates, roots and shoots of rye, the effect of stress on rye plants and, most recently, the priority effect of pigweed on rye. The overall scheme of the project has been included in Appendix 1.

The main purpose of this work is to quantify potential signal molecules, loliolide and jasmonic acid in *Secale cereale* L. leaves and roots as well as study their effects on rye root architecture and on the benzoxazinoid composition and/or concentration in rye roots. Therefore, three main objectives have been settled for this study.

Objective A – "Comparison of substrates": Do *Secale cereale* L. and *Amaranthus retroflexus* L. have a better growth in a different culture substrate than glass microbeads? Does it affect the secondary metabolites composition and/or concentration from root exudates in the rhizosphere and their chemical analysis?

Soil features affect plant morphology, growth, root microbiome and rhizosphere chemistry. Even in a controlled environment, substrate particle size and chemistry influence root morphology and exudation. For the past year, glass microbeads have been utilized as growth substrate both in Gembloux and in the Agroscope. Its main asset is being an inert system with defined sphere diameter, facilitating the root exudate analysis. However, *Amaranthus retroflexus* L. called redroot pigweed seems to have difficulties growing in this medium as shown by few seed germinations, stretched stems and folded leaves. In this context, a new growth substrate, consisting of a mixture of clay beads and attapulgite, has been tested. This medium has been promoted by the UMR Agroecology of Dijon for its capacity to grow plants easily.

Two hypotheses are settled for this objective:

- H_A1: The substrate consisting of a mixture of clay beads and attapulgite offers better growth conditions for both *Secale cereale* L. and *Amaranthus retroflexus* L. based on root architecture and shoot parameters.
- H_A2: This new substrate is adapted for the analysis of secondary metabolites such as benzoxazinoid present in root exudates of rye.

Objective B – "Treatment incubation": Do the *Amaranthus retroflexus* L. root exudates, jasmonic acid and loliolide influence rye root architecture and does it induce the synthesis of defensive metabolites from the family of benzoxazinoid in rye roots?

Recent studies have hypothesized the role of loliolide and jasmonic acid as potential belowground signals mediating chemical defense in plants. They have demonstrated that exogenous application of jasmonic acid and loliolide in high concentration modifies the concentration of DIMBOA from the family of benzoxazinoid in wheat (Li et al, 2023). As those potential signaling molecules would be global chemicals nonspecific to any plant species, the idea is to test this hypothesis on rye at low dosage. Pigweed root exudates have also been tested to analyze the induction of BXDs in rye roots without the physical presence of weeds, suppressing any competitive effect between the crop and the weed.

Five hypotheses are settled:

- H_B1: Pigweed root exudates induce root architecture changes and the production of benzoxazinoids in rye.
- H_B2: Pigweed root exudates induce changes of benzoxazinoid composition and/or concentration in rye root similar to those of pigweed and rye growing in co-culture.
- H_B3: Exogenous application of loliolide induces root architecture changes and the production of benzoxazinoids in rye.
- H_B4: Exogenous application of jasmonic acid induces root architecture changes and the production of benzoxazinoids in rye.
- H_B5: Loliolide and Jasmonic acid induce similar changes in root architecture and benzoxazinoid composition and/or concentration in rye root.

Objective C – "Loliolide detection and quantification": Does *Secale cereale* L. produce loliolide in its roots and shoots? If it does, is it possible to detect and quantify loliolide by HPLC-UV analysis?

According to recent studies, loliolide would be produced by both the allelopathic crop and weed (Kong et al., 2018). The goal is to optimize a method to extract loliolide from rye roots and shoots by modifying the quantity of fresh material and the solution of extraction as well as developing a detection method by HPLC-UV.

Two hypotheses are settled for this objective:

- H_C1: Improving loliolide extraction from roots and shoots is possible by modifying different parameters (e.g., quantity of fresh materials and solution of extraction).
- H_C2: Loliolide from rye roots and shoots can be detected and quantified by HPLC-UV.

PART III – MATERIAL AND METHODS

This section consists of two main subdivisions: a summary of the experimental set up per objective and a detailed description of the experimental protocols, containing many technical aspects.

1. Experimental setup

Every experiment has been carried out with three to five repetitions (n) for each modality. A scheme summarizing the different experiments and their modalities can be found in Figure 6.

1.1. Objective A: "Comparison of substrates"

This experiment aims to analyze different root parameters of rye and pigweed to determine whether or not plants grow better in a substrate of glass microbeads or in a mixture of clay beads and attapulgite. Thereby, rye (R) and pigweed (P) were grown, alone (R or P) and in co-culture (R+P) in the two substrates and the divers root and shoot parameters were analyzed. Moreover, the root exudates of rye (R) and rye in co-culture (R+P) were analyzed by UPLC-TOF-MS to measure the BXDs composition and concentration in each substrate to determine whether or not the substrate influences the release of allelochemicals in the rhizosphere or their extractability.

1.2. Objective B: "Incubation of treatments"

The idea behind this objective is to find whether or not different treatments (pigweed root exudates, jasmonic acid and loliolide) induce the production of BXs in rye roots and if it influences root architecture.

Firstly, a pre-test was carried out to find out which stage of rye development was the most sensitive to the different treatments by analyzing the root architecture. Two types of applications (unique and continuous) have been tested for the pigweed root exudates treatment. Thereby, pigweed root exudates and highly concentrated jasmonic acid (50 µM) were applied once at different rye development stages: day 0 (seed stage), day 3 (seedling stage 1) and day 6 (seedling stage 2). For the continuous treatment, only pigweed root exudates have been applied daily on rye. Loliolide has not been applied for the pre-test considering its price and the non-optimal treatment conditions.

Once the sensitive rye development stage and the optimal type of treatment application were selected according to the pre-test results, the final test, aiming to analyze the BXDs composition in rye roots, could be carried out. To do so, pigweed root exudates, low concentrated jasmonic acid and loliolide were applied continuously on the rye starting at the 3rd day of rye development stage. All the analyses, root architecture and BXDs composition, for the pretest and test, respectively, were executed 10 days after sowing rye.

1.3. Objective C: "Loliolide quantification"

The main purpose of this experiment is to develop a method to extract loliolide from rye roots and shoots. To do so, several parameters were modified to either optimize the extraction: quantity of fresh materials and solution of extraction or to optimize the detection: resuspension volume and injection volume. The resulting extracts were analyzed by HPLC-UV.

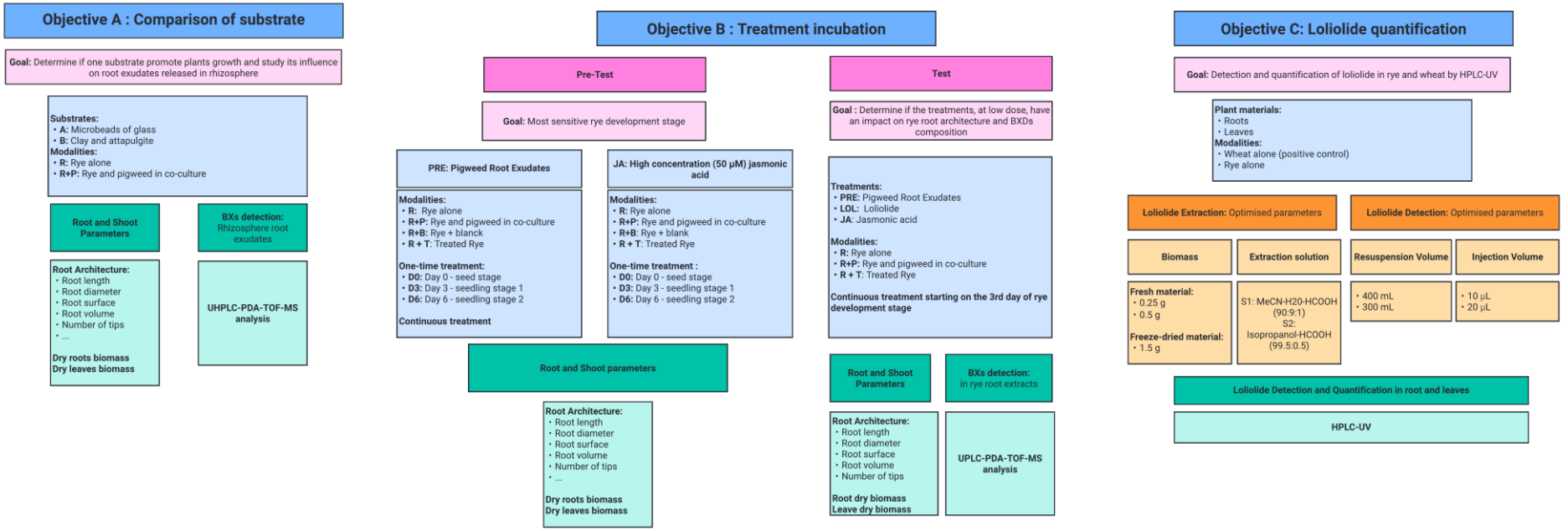


Figure 6 : Experimental set up summary

2. Experimental protocols

2.1. Plant cultivation

Three different seeds have been employed: the winter rye (*Secale cereale* L., Sativa, 2021/2022), the autumn wheat (*Triticum* L., Baretta Bio) and redroot pigweed seeds (*Amaranthus retroflexus* L.) harvested in Changins (Nyon, Switserland, 2018). Before sowing, the pigweed seeds were heated in a drying oven at 50°C to boost their germination rate.

Plants were cultivated in Solid Phase Extraction tube (SPE) of 60 mL (Agilent, BondElut Straight Barrel, catalog no: 12131018) covered with black plastic film on the outside to avoid direct light exposure. Frits of 20 µm (Agilent, catalog no.1:131012) were set at the bottom of the tube to avoid the roots to grow outside the tube and to retain the growth substrate. Approximately 105 g of glass microbeads (Guyson, Honite 09; 250 µm-425 µm) were added in the SPE tubes while 85 g of the mixture made of clay beads and attapulgite, kindly offered by UMR Agroecology of Dijon, were used (Jeudy et al., 2016). A picture of the two substrates is described in Appendix 2 to highlight their differences especially concerning their particle size. As a reminder, this clay substrate has only been used for objective A "Substrate comparison". Plants seeds were sown and watered as explained in Appendix 3. All analyses concerning plant root architecture, biomass and BXDs extraction from root exudates or roots were carried out 10 days after sowing the seeds.

2.2. Growth chamber conditions

Plants were placed in a growth chamber with controlled conditions. The environment parameters were set with a photoperiod of 16/8 hours at a temperature of 28/24°C (day/night). The relative humidity was set to 70% and light intensity at 200 µmol.$(m^2.s)^{-1}$(Aralab, Clitec Phytotron).

2.3. Nutrient solution

The daily watering was performed with a nutrient solution corresponding to Hoagland ½ solution No.2 basal salt mixture (Sigma-Aldrich, catalog number: H2395), previously autoclaved. The nutrient solution preparation protocol and the nutrient's list and their concentrations are presented in Appendix 4. In the specific case of objective B "Incubation of treatments", the different treatments were prepared with Hoagland 1 Solution by doubling the amount of Hoagland solution No.2 basal salt mixture. As plants were incubated continuously with pigweed root exudate (PRE), loliolide (LOL) or jasmonic acid (JA), nutritive solution was mixed with the treatment solutions (1:1, v/v).

2.4. Treatments preparation and application

This section only refers to objective B "Treatments incubation". As mentioned previously, a pretest has been set up in order to find the optimal day of application (seedling stage at day 0, 3 or 6) and type of treatment (one-time treatment or continuous) by analyzing root architecture (*cfr* section 2.6.1**).** The treatments conditions and preparation of the pretest are described in Appendix 5.

Concerning the final test, the solutions containing pigweed root exudates (PRE), low concentrations jasmonic acid (JA) and loliolide(LOL) have been applied continuously starting at the 3rd day of rye development stage. As a reminder, on the 10th day of the plant's development, BXDs will be extracted from rye roots (*cfr* section 2.5.2) and analyzed by UHPLC-MS (*cfr* section 2.6.2). Every day, rye (R+T) was watered with 2.5 mL of a solution mixing the treatment (PRE, JA or LOL) and Hoagland 1 (100%) solution (1:1, v/v) by applying the mix on the surface of the substrate with a serological pipette. The treatment solutions preparation is reported below.

2.4.1. Pigweed root exudates

Pigweed root exudates were collected from two weeks old pigweeds by following the same protocol as BXDs extraction from rye and/or pigweed root exudates except for the extraction solvent which was only made of nanopure water (*cfr* section 2.5.3). Additionally, a prior step was needed to wash any excess of nutrient from Hoagland solution. The day before, 30 mL of nanopure sterile water has been injected through the microbeads of glass. Then, the pigweeds were watered with 15 mL of nanopure water and put back in the growth chamber for a night. To collect the pigweed root exudates, 15 mL of nanopure sterile water has been injected through the microbeads of glass and the solution was stored in a 50 mL Falcon tube in the freezer at -80°C. For daily watering, aliquots were made in 15 mL Falcon tube by mixing 7 mL of pigweed root exudate solution from the 50 mL Falcon tube and 7 mL of Hoagland 1 solution (1:1, v/v).

2.4.2. Jasmonic acid and loliolide

For the continuous treatment of jasmonic acid and loliolide, the optimal concentration has been selected according to the pretest and Li et al., (2023) latest results. A detailed explanation concerning the parameters choices will be provided in the "Results and discussions" section (*cfr* section IV). The optimal concentration for both treatments is the loliolide concentration found in wheat root at 3-leaf stage. This concentration, closer to real soil condition, is around 0.5 $\mu g.g^{-1}$ of dry weight which has been converted into a molar concentration corresponding to 0.51 nM.

To obtain the low JA concentration solution, 10 µL of the stock solution of JA at 10 $mg.mL^{-1}$ had to be diluted in 50 mL of nanopure sterile water. Similarly, 10 µL of the stock solution of loliolide at 0.5 $mg.mL^{-1}$ had to be diluted in 50 mL of nanopure sterile water, to obtain the low loliolide concentration solution. Then, an intermediate solution with twice the concentration of the final solution (1.02 nM) had to be made by mixing 27 µL of JA or 500 µL of loliolide previous solution with 250 mL of nanopure sterile water. The daily treatments could be produced in 15 mL Falcon tubes by mixing the intermediate solution at 1.02 nM and Hoagland 1 solution (1:1, v/v). The JA and loliolide treatments were stored at -80°C and thawed before application.

2.5. Sample preparation

This section contains diverse protocols related to sample preparation such as plant roots and shoots collection, extraction of BXDs from rye roots or root exudates and extraction of loliolide from roots and shoots. The protocol for loliolide extraction from root and shoots will be further developed as the extraction method and parameters had to be optimized during this study. On the other hand, a detailed description of the extraction protocols of BXs from root, shoot and root exudates will be added in the appendix as the methods have been optimized in previous studies.

2.5.1. Plant roots and shoots collection

With the aim of analyzing the root architecture, rye and pigweeds roots and shoots have been collected for objective A "Substrate comparison" while only the rye roots and shoots were collected for objective B "Treatment incubation".

The root and shoot collection occurred on the 10^{th} day of plant development stage. The day of collection has been chosen to optimize the concentration of BXDs, which increase during plant's early growth stage, while avoiding any competition effect between roots from the same tubes. The plants roots were separated from the aerial part by cutting above the root collar. Rye leaves were isolated from the stem

by cutting just under the auricle of the first leaves while pigweed leaves were detached with a pair of tweezers from the stem. The roots and shoots were washed with distilled water and gently rubbed to remove any glass microbeads.

2.5.2. Extraction of BXDs from rye roots

The extraction of BXDs from rye roots has only been carried on for objective B "Treatment incubation". Once rye roots were collected, rinsed and blotted as mentioned above (*cfr* section 2.5.1), the plant material was directly frozen in liquid nitrogen. Samples of rye roots were extracted according to the protocol of Dr. Gaétan Glauser, Institute of Chemistry, University of Neuchâtel, Neuchâtel, Switzerland which is described in Appendix 6.

2.5.3. Extraction of BXDs from rye and/or pigweed root exudates

The collection of root exudates and extraction of BXDs from the rhizosphere has only been performed regarding objective A "Substrate Comparison". The extraction protocol is detailed in Appendix 7.

2.5.4. Extraction of loliolide from roots and shoots of rye and wheat

This section only refers to objective C "Loliolide quantification". As a reminder, a few parameters have been modified in order to optimize the loliolide extraction (quantity of fresh biomass and extraction solution) and detection (resuspension volume and injection volume). Both rye and wheat have been grown in microbeads of glass or soil, in the controlled environment offered by the growing chamber. Wheat has been selected as a positive control based on the results obtained by Li et al., (2023). Indeed, they could detect loliolide in wheat roots and leaves with higher concentration, between 0.45 and 0.6 $\mu g.g^{-1}$dry weight at early growth stage (1- or 3-leaf stage). To ensure sufficient fresh biomass, it has been decided to collect the plants at the 3-leaf stage. Also, to induce higher loliolide concentration, both rye and wheat roots were mechanically wounded using a pair of tweezers by gently stirring inside the substrate. Three hours later, the roots and shoots were collected. The extraction protocol of loliolide is detailed in Appendix 8.

2.6. Instrumental analysis

2.6.1. Root Architecture analysis: Root Scanning

Plant materials were preserved in a plastic rack containing distilled water till the end of the root and shoot analysis. The root architecture of rye and/or pigweed was analyzed using WinRHIZO^{TM} Image analysis for Plant Science 2021 (Regent Instruments Inc., Canada). The following root parameters were studied: length, volume, surface, diameter and number of tips. Then, roots and leaves were dried at 50°C for 48 hours. Finally, the total dry roots and leaves biomasses were determined with an analytical balance. Five additional measures have been analyzed: the specific root length (SRL), the root length density (RLD), the root surface area density (RSD), the root branching density (RBD) and the root tissue density (RTD) (Table 2).

Table 2: Additional calculated root architecture parameters

Parameter	Definition
Specific root length (SRL)	The total root length divided by root dry weight (Delory et al., 2021)
Root length density (RLD)	The total length of roots contained in unit soil volume (cm/cm^3) (Gong et al., 2020)
Root surface area density (RSD)	The total root surface area divided by root volume (cm^2/cm^3) (Gong et al., 2020)
Root branching density (RBD)	The number of root tips per root length of first and second order roots (tips cm^{-1}) (Liese et al., 2017)
Root tissue density (RTD)	The dry root biomass divided by the root volume

2.6.2. UHPLC analysis: BXDs quantification

The BXDs were analyzed using the method of Dr Gaétan Glauser, Institut de Chimie, University of Neuchâtel, Neuchâtel, Switzerland. BXDs in root exudates and leaf and root extracts were identified using an ultra-high performance liquid chromatography system (Acquity UPLC Waters) coupled with Synapt G2 time-of-flight mass spectrometry (Waters). The conditions are detailed in Appendix 9. Data processing was performed using TargetLynx (Waters). Calibration curves from the standards DIMBOA-Glc, DMBOA, HDMBOA-Glc, MBOA and HMBOA-Glc standards were prepared in order to calculate the BXDs concentrations. The concentrations of the calibration points were 0.08, 0.04, 2, 10 and 50 $\mu g.mL^{-1}$ for the five BXDs. BOA was quantified as MBOA equivalents, HBOA-Glc_2-β-D-glucopyranosyloxy-7-hydroxy-1,4-benzoxazin-3-one (DHBOA-Glc), DIBOA-Glc and DIM2BOA-Glc were quantified as DIMBOA-Glc equivalents and HDM_2BOA-Glc was quantified as HDMBOA-Glc equivalents. The predicted retention times and quantification ion of the quantified BXD are described in Table 3. The limit of detection for each BXD has been set, by Dr. Gaétan Glauser, at the same level as the limit of quantification (10*SD/S), which is based on the standard deviation of the response (SD) of the curve and the slope of the calibration curve (S).

Table 3: Predicted retention times (min) and quantification ions for identified benzoxazinoids.

Compound	Predicted retention time (min)	Ions of quantification m/z
DIMBOA-Glc	2.43	372.930
DIMBOA	2.76	149.014
HDMBOA-Glc	2.97	432.116
MBOA	3.36	164.039
HMBOA-Glc	2.34	356.100
DIM_2BOA-Glc	2.43	402.107
HDM_2BOA-Glc	2.98	462.125
DIBOA-Glc	1.49	342.085
DHBOA-Glc	2.13	342.085
HBOA-Glc	2.06	326.090
BOA	3.06	134.025

2.6.3. HPLC-UV analysis: Loliolide detection and quantification

The loliolide detection and quantification were performed on an high-performance liquid chromatography (HPLC) (Agilent 1260; Agilent Technologies Inc., Waldbronn, Germany) equipped with a C18 reverse-phase column (Agilent Eclipse XDB, 150 mm, 4.6 mm, 5 µm) and a UV detector at 220 nm. The injection volume was 10 µL. The elution gradient was carried out with a binary solvent system consisting of 0.1% H_3PO_4 in H_2O (solvent A) and 0.1% H_3PO_4 in MeCN (solvent B) at a constant flow rate of 0.7 $mL.min^{-1}$ at 15°C. Simultaneous separations were completed using a gradient elution described in Table 4.

Table 4: Programmed sequence of the quaternary pumping system of HPLC Agilent 1260 system.

Time (min)	Solution A (%)	Solution B (%)
0	75	25
15	50	50
18	0	100
22	0	100
23	75	25
26	75	25

The peak of loliolide was identified by its retention time. Working standard solutions ranging from 0.1 to 50 $\mu g.mL^{-1}$ were prepared to establish a calibration curve. Loliolide was quantified by regression analysis of the peak areas against standard concentrations. Additionally, the limit of detection (LOD) and the limit of quantification (LOQ) were determined for loliolide, according to European commission recommendation 2002/657/CE.

2.7. Statistical analysis

When relevant, statistical tests were performed on data with the RStudio software. The Shapiro-Wilk test and Bartlett's test were used to test the normality of the data and the equality of the variance respectively. As the normality and the equality of variance were not consistently verified, the Kruskal Wallis test has been carried out to evaluate the statistical significance between the different conditions means, with differences considered non-significant (p-value > 0.05), significant (p-value ≤ 0.05), very significant (p-value ≤ 0.01) or highly significant (p-value ≤ 0.001). In some tables, the value of the mean ± the standard error of the mean (SEM) are given. Graphics were created with the GraphPad Prism 8 software.

PART IV – RESULTS AND DISCUSSIONS

To facilitate data interpretation, results and discussions will be combined per objective.

1. Objective A: Comparison of substrates

As a reminder, this experiment aims at analyzing the effect of two variables: substrate and co-culture, with a focus on substrate comparison. To compare the substrate effect, two substrates have been selected: microbeads of glass (A) and a mixture of clay beads and attapulgite (B). Moreover, to study the co-culture effect, two plant growth modalities have been tested: pigweed and/or rye alone (P or R) and in co-culture (R+P). To pursue that, both root architecture and BXDs, from root exudates, were analyzed. Thus, the effect of two independent variables (substrate and growth modalities) on two dependent variables (root architecture parameters and BXDs concentration) has been studied. The two variables have been analyzed individually (Kruskal Wallis test) and combined (Wilcoxon Test). A summary of the experiment scheme can be found hereby (Figure 7).

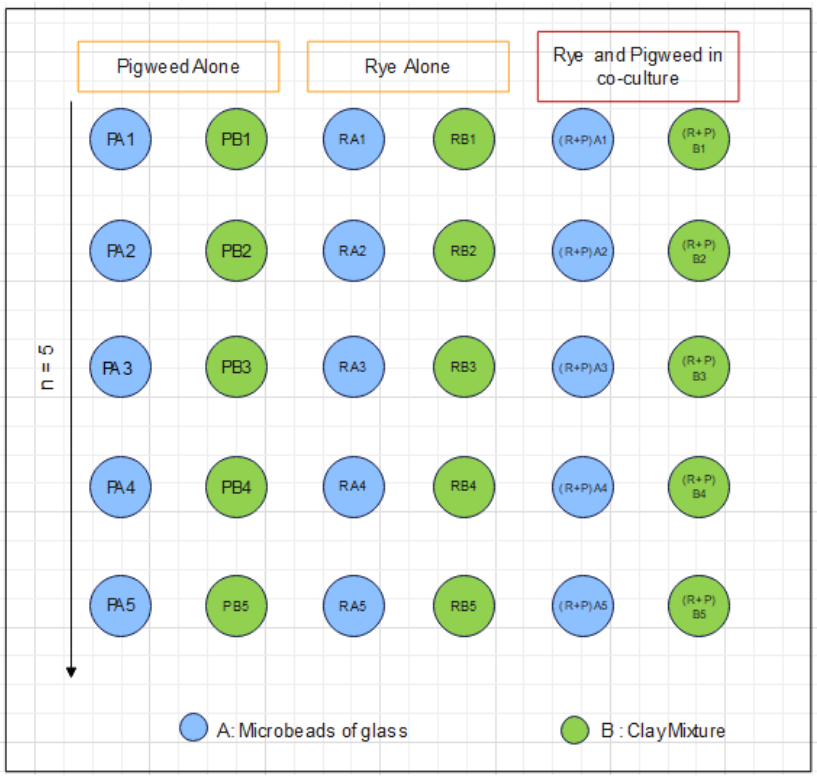


Figure 7 : Experimental set-up for objective A - For the co-culture effect, two biological modalities were tested: pigweed (P) or rye (R) alone and in co-culture (R+P). For the substrate effect, two substrates were tested: microbeads of glass (A) and clay mixture (B). In total, five replicates were carried out.

1.1. Root architecture analysis

Firstly, it is important to mention that pigweed and rye have been collected 10 days after sowing in both substrates. At that time, the cultivated plants in both substrates had the same development stage: 2 to 3 leaves-stage for both rye and pigweed. Visual differences were observed for both shoots and roots (Figures 8 and 9). As shown in Figure 8, the leaves of rye and pigweed are more developed when grown in the substrate of clay mixture regardless of the co-culture modalities (P/R or R+P).

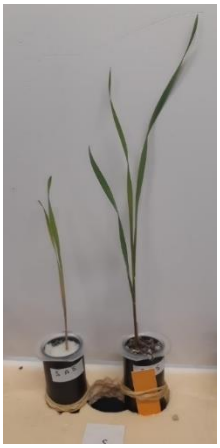
Pigweed alone (P)		Rye alone (R)		Pigweed and Rye in co-culture (R+P)	
A: Microbeads of glass	B: Clay mixture	A: Microbeads of glass	B: Clay mixture	A: Microbeads of glass	B: Clay mixture
					

Figure 8: Pictures of pigweed (P) and rye (R) shoots, alone and in co-culture (R+P), in two different substrates: microbeads of glass (A) and clay and attapulgite mixture (B) at day 10 after sowing.

Figure 9 gives a visual representation of the roots, illustrating the graphs in Figure 10. Pigweed (P) is more developed and it has more secondary roots in the clay and attapulgite mixture (B) than in the microbeads of glass substrate (A). The same observation was made for rye. Additionally, rye grown in clay mixture and in co-culture seems to have more root hairs, which cannot be measured by WinRHIZO™ Image. A few reports indicate that root cap and root hair cells are involved in the secretion of compounds such as allelochemicals (Badri & Vivanco, 2009).

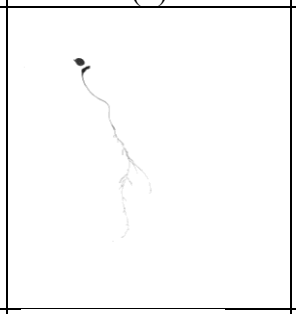
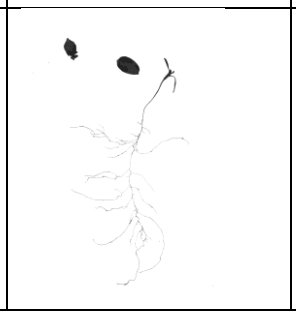
	Pigweed alone (P)	Pigweed in co-culture (P*(R+P))	Rye alone (R)	Rye in co-culture (R*(R+P))
Micro beads of glass (A)				
Clay mixture (B)				

Figure 9: Images of scans of rye and pigweed roots obtained using WinRHIZO software.

1.1.1. Comparison of two substrates

Different root parameters were measured in order to compare the two substrates as shown in Figure 10. For both ryes grown alone (R) and in co-culture (R*(R+P)), the parameters presented in Table 5 are significantly higher for rye cultivated in a mix of clay and attapulgite (substrate B). This significant difference between substrates is nonetheless slightly higher for rye cultivated alone (R). However, significantly higher root diameter (Figure 10.C), branching density (Figure 10.H) and root tissue density (Figure 10.K) are observed when rye is cultivated in microbeads of glass (substrate A).

Table 5: Comparison of substrates, microbeads of glass (A) and mix of clay and attapulgite (B), for rye cultivated alone (R) and rye in co-culture (R(R+P)) by measuring different root parameters. Values are means ± SEM for each condition (n=5) and bold values show which condition has higher mean for a particular parameter. Asterisk indicates significant difference (p-value) between two groups: * P < 0.05, ** P < 0.01, *** P < 0.001 and NS = no significant difference.*

Figure 10	Root parameters	Rye cultivated alone (R)			Rye in co-culture (R*(R+P))		
		Microbeads of glass (A)	Mix of clay and attapulgite (B)	Significant difference	Microbeads of glass (A)	Mix of clay and attapulgite (B)	Significant difference
A	Root length (cm)	106.2 ± 8.091	**272.9 ± 7.533**	**	117.8 ± 16.93	**285.9 ± 36.41**	*
B	Root surface Area (cm^2)	14.01 ± 1.294	**28.74 ± 0.419**	**	15.27 ± 1.714	**27.67 ± 3.578**	*
D	Root Volume (cm^3)	0.148 ± 0.017	**0.241 ± 0.006**	**	0.159 ± 0.013	**0.213 ± 0.030**	NS
E	Number of root tips	457.6 ± 83.37	**755.8 ± 23.50**	**	404.6 ± 55.81	**688.2 ± 104.3**	*
F	Root length density ($cm.cm^{-3}$)	1.771 ± 0.135	**4.549 ± 0.126**	**	1.964 ± 0.282	**4.766 ± 0.607**	*
G	Root surface area density ($cm^2.cm^{-3}$)	95.88 ± 3.134	**119.3 ± 2.650**	**	95.45 ± 3.874	**130.7 ± 4.80**	*
I	Dry root biomass (g)	0.013 ± 0.001	**0.018 ± 0.001**	*	0.015 ± 0.001	**0.016 ± 0.002**	NS
J	Dry shoot biomass (g)	0.017 ± 0.002	**0.041 ± 0.002**	**	0.019 ± 0.002	**0.036 ± 0.003**	*
L	Specific Root length ($cm.g^{-1}$)	8507 ± 527.1	**15444 ± 836.4**	**	7788 ± 1301	**17913 ± 1190**	*

Similar observations can be made for pigweed as shown in Table 6. Indeed, those parameters showed a significant difference between substrates (A vs B) when pigweed is cultivated alone (P). Similarly to rye, all parameters are higher when pigweed is cultivated in the mixture of clay beads (substrate B), except for the root branching density (H) (Figure 10). No particular differences between substrates can be observed when pigweed is grown in co-culture (P*(R+P)).

*Table 6: Comparison of substrates, microbeads of glass (A) and mix of clay and attapulgite (B), for pigweed cultivated alone (P) by measuring different root parameters. Values are means ± SEM for each condition (n=5) and bold values show which condition has higher mean for a particular parameter. Asterisk indicates significant difference (p-value) between two groups: * P < 0.05, ** P < 0.01, *** P < 0.001.*

Figure 10	Root parameters	Pigweed cultivated alone (P)		
		Microbeads of glass (A)	Mix of clay and attapulgite (B)	Significant difference
A	Root length (cm)	22.216 ± 2.289	**67.081 ± 6.363**	*
B	Root surface area (cm^2)	1.797 ± 0.349	**5.397 ± 0.680**	***
D	Root volume (cm^3)	0.013 ± 0.004	**0.036 ± 0.006**	***
E	Number of root tips	88.429 ± 13.945	**167.400 ± 16.297**	***
F	Root length density ($cm.cm^{-3}$)	0.370 ± 0.038	**1.118 ± 0.106**	***
H	Root branching density (nbr of root tips.cm^{-1})	**3.897 ± 0.377**	2.545 ± 0.149	***

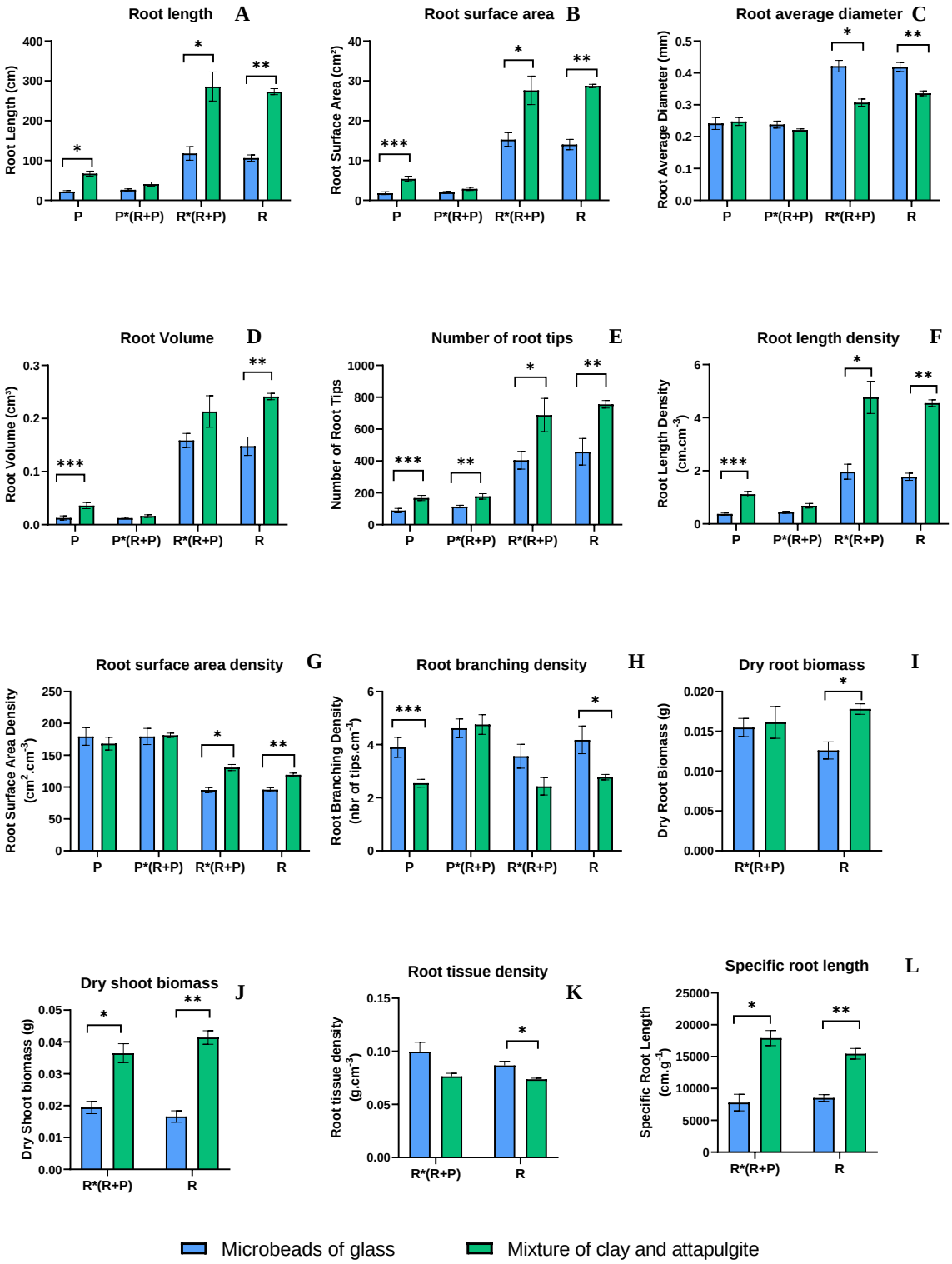


*Figure 10: Comparison of two substrates, microbeads of glass and mixture of clay and attapulgite, for rye and pigweed cultivated alone (R or P) and in co-culture (R+P) by measuring different root parameters : root length (A), root surface area (B), root average diameter (C), root volume (D), number of tips (E), root length density (F), root surface area density (G), root branching density (H). Graphs comparing two substrates by measuring dry root biomass (I), dry shoot biomass (J), root tissue density (K) and specific root length (L) for rye alone (R) and in co-culture(R+P). Asterisk indicates significant difference between two groups: * P < 0.05, ** P < 0.01, *** P < 0.001.*

The first trend that emerges from the analysis of root architecture is the greater development of plants in the mixture of clay and attapulgite compared to the microbeads of glass substrate. The modification of root morphology is tightly linked to the substrate's physical and chemical properties such as its particle size, water retention and soil chemistry. Smaller particles size (<1 mm) have been demonstrated to reduce root weight, length and the number of root tips (Sasse et al, 2020). In this experiment, the particles size of the glass microbeads substrate varies from 250 to 425 µm which, according to Sasse et al. (2020), can be considered small. In contrast, the particle size of the clay and attapulgite, being heterogeneous, is bigger and around 1 to 5 mm. As shown in Figure 10.A, the root length is significantly lower in the glass beads substrate compared to clay beads, all plant and modalities combined. Similar observations can be made for root surface area (Figure 10.B), the number of root tips (Figure 10.E) and the dry root biomass (Figure 10.I). Those results align with the data obtained by Sasse et al, (2020). Thus, it can be hypothesized that root architecture is influenced by the particle size of the substrate. This leads to the conclusion that smaller particles size such as microbeads of glass (<1 mm) reduces the general growth of the plant, whereas larger particles size such as the clay and attapulgite beads enhances the root growth.

Particles size also determines the pore space between the particle and consequently, the water-holding capacity of the substrate (Rellán-Álvarez et al., 2016). For instance, soils with smaller particles have less pore space and hold water tightly due to capillary forces. Although a substrate such as glass microbeads shows higher water availability, it tends to dry more quickly compared with clay soils, which have higher water retention. Rellán-Álvarez et al. (2016) concluded that even though the root system is able to extract water more easily from glass or sandy soils, the plant might suffer more from a water deficit as the soil dries. Therefore, it can be hypothesized that the clay beads substrate has a higher water-holding capacity, leading to less water availability for the plant but, it retains water for a longer time due to its retention ability. This hypothesis aligns with the results obtained, as more nutritive solution had to be poured initially to saturate the substrate. Indeed, 10 mL of Hoagland ½ solution had to be added for microbeads of glass compared to 25 mL for the clay and attapulgite substrate. Thus, plants in the clay substrate might have regular access to water leading to greater plant growth, which can be interpreted as higher dry shoot biomass (Figure 10.J) and higher specific root length (Figure 10.L). Indeed, plants with higher specific root length grow more root length for a given dry-mass investment and are thus, generally considered to have improved nutrient and water uptake (Fort et al., 2014).

Another important soil property is the presence of void due to heterogeneous particle size in the clay and attapulgite substrate. Air pockets might facilitate water and air flow as well as root growth especially new lateral roots (Rellán-Álvarez et al., 2016). On the contrary, glass microbeads are evenly distributed and well compact, which does not favor root growth or higher root length. Instead, the root diameter is promoted as shown in Figure 10.C.

1.1.2. Comparison of two growing modalities

Even though comparing the growth modalities (alone or co-culture) wasn't the main purpose of this experiment, it is still interesting to investigate how the substrate influences the differences between the growth modalities (alone or in co-culture) and whether or not one substrate highlights those differences. The same parameters were measured in order to compare the two growth modalities (alone and in co-culture) for both substrates as shown in Figure 11. Less significant differences between growth modalities can be observed. Indeed, for both pigweed (PA) and rye (RA) cultivated in the microbead of glass, there is no significant difference between modalities (alone vs co-culture) for any of the parameters, except for the number of tips of pigweed (Figure 11.E). Pigweeds cultivated in the microbeads of glass (PA) seem to have a higher number of root tips when grown in co-culture. Indeed,

cultivated in the microbeads of glass, pigweeds have on average a number of tips of 88.43 and 113.7 for growth alone and in co-culture respectively.

However, pigweed grown in the clay and attapulgite substrate (PB), showed significant differences between modalities for all parameters except the number of root tips (Figure 11.E) and root surface area density (Figure 11.G). It can be noted that parameters from A to F, presented in Table 7, are higher when pigweed is cultivated alone in the clay substrate. However, pigweed root branching density is higher when pigweed is cultivated in co-culture in the clay and attapulgite mixture.

*Table 7: Comparison of pigweed grown alone and in co-culture in mix of clay and attapulgite (PB) by measuring different root parameters. Values are means ± SEM for each condition (n=5) and bold values show which condition has higher mean for a particular parameters. Asterisk indicates significant difference (p-value) between two groups: * P < 0.05, ** P < 0.01, *** P < 0.001 and NS = no significant difference.*

Figure 11	Root parameters	Pigweed in clay and attapulgite substrate (PB)		
		Alone	Co-culture	Significant difference
A	Root length (cm)	**67.08 ± 6.363**	40.96 ± 5.199	**
B	Root surface area (cm^2)	**5.397 ± 0.680**	2.881 ± 0.387	**
C	Root diameter (mm)	**0.248 ± 0.012**	0.221 ± 0.004	*
D	Root volume (cm^3)	**0.036 ± 0.006**	0.016 ± 0.002	*
F	Root length density ($cm.cm^{-3}$)	**1.118 ± 0.106**	0.683 ± 0.087	**
H	Root branching density (nbr of root $tips.cm^{-1}$)	2.545 ± 0.149	**4.758 ± 0.372**	***

As no significant differences between modalities can be observed for root architecture except for pigweed grown in the clay mixture, it can be hypothesized that the difference between growth modalities (alone or co-culture) was enhanced for root parameters such as root length, root surface area, root diameter, root volume in those conditions (Figure 11).

In this paragraph, the variables will be combined and analyzed by performing a pairwise comparison using the Wilcoxon test. A summary of the results can be found in Appendix 10. The main tendencies observed are similar for rye and pigweed. The first tendency with the most significant difference is between plants cultivated alone in the two different substrates (Alone*B >< Alone*A). This result shows that the substrate, where the plants are cultivated, has the biggest influence on plant root architecture all parameters combined if the plants are cultivated alone. The second trend with the highest significant difference concerns plant cultivated alone in a mixture of clay and plants cultivated in co-culture in microbeads of glass (Alone*B >< Co-culture*A). A hypothesis concerning the influence of this interaction would be that plants have a lower development when they are co-cultivated in microbeads of glass, whereas they thrive when they are cultivated alone in a mixture of clay and attapulgite. It enhances their differences and thus, it shows a significant difference between the two interactions. Another trend which mostly influences pigweed growth is observed between growth modalities (alone or in co-culture) when pigweed is cultivated in a mixture of clay and attapulgite (Alone*B >< Co-culture*B). This interaction shows that, for pigweed, the difference between modalities is accentuated in the clay substrate.

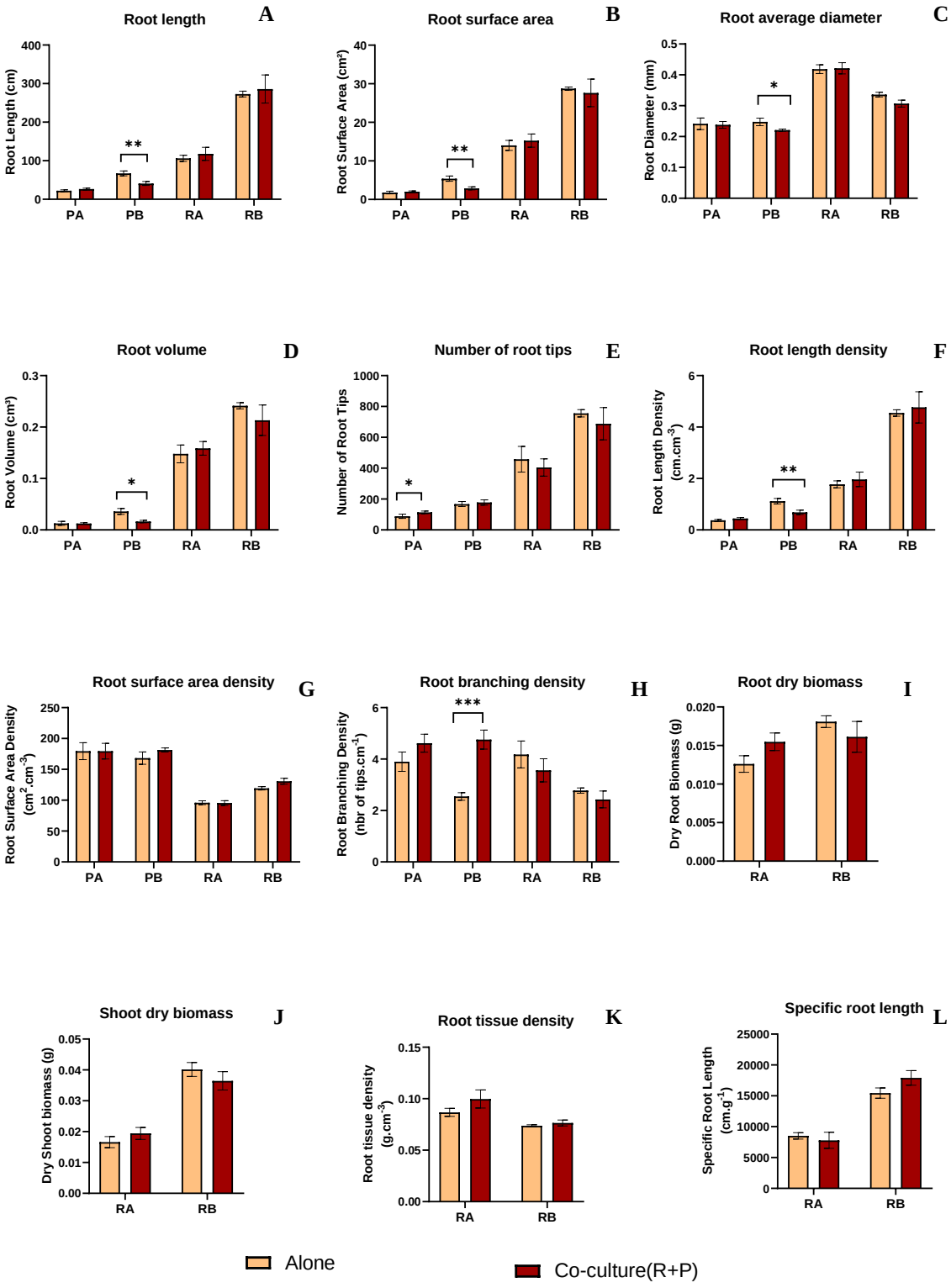


*Figure 11: Comparison of two growth modalities, alone and in co-culture, for pigweed and rye cultivated in microbeads of glass (PA or RA) and mix of clay and attapulgite (PB or RB) by measuring different root parameters: root length (A), root surface area (B), root average diameter (C), root volume (D), number of tips (E), root length density (F), root surface area density (G), root branching density (H). Graphs comparing two modalities, alone and co-culture, by measuring dry root biomass (I), dry shoot biomass (J), root tissue density (K) and specific root length (L) for rye in microbeads of glass (RA) or in clay and attapulgite substrate (RB). Asterisk indicates significant difference between two groups: * P < 0.05, ** P < 0.01, *** P < 0.001.*

1.2. BXDs analysis

To compare the two substrates (microbeads of glass and clay mixture) and the two growth modalities (alone and co-culture), the BXDs extracted from the rhizosphere have also been analyzed in Figures 12 and 13 respectively. Fewer statistical tests could be carried out, especially in the substrate of clay and attapulgite, as some BXDs could not be detected in all samples leading to less than two replicates per modality. Besides, a number of samples presented BXDs levels that were below the limit of detection and their BXDs content was thus considered null. Those low concentrations in BXDs are expected when BXDs are extracted from the rhizosphere (Li et al., 2023).

1.2.1. Comparison of two substrates

In Figure 12.A, corresponding to rye cultivated alone, nine BXDs could be detected in the substrate of microbeads of glass whereas only five could be detected in the substrate of clay and attapulgite (n=5, with more than 3 replicates with detectable levels of BXDs). Moreover, the concentration of the BXDs detected for both substrates is always higher in the microbeads of glass (Table 8). The BXDs concentration presented in Table 8 are in $ng.mL^{-1}$ unlike the one in Figure 12, which are in $\mu g.mL^{-1}$. The BXDs concentration of DIMBOA-Glc and HDMBOA-Glc is significantly different between the two substrates.

In Figure 12.B, corresponding to rye and pigweed cultivated in co-culture, nine BXDs could be detected in the substrate of microbeads of glass whereas five could be detected in the substrate of clay and attapulgite (n=5, with more than 3 replicates with detectable levels of BXDs). As a reminder, the limit of detection of each BXD has been set, by Dr. Gaétan Glauser, at the same level as the limit of quantification. Although BXDs concentration is almost always higher in the microbeads of glass, there is no significant difference between the two substrates for the BXDs detected for both substrates (Table 8).

Table 8: Comparison of two substrates, microbeads of glass (A) and mix of clay and attapulgite (B), for rye cultivated alone (R) and rye in co-culture (R(R+P)) by measuring different BXDs concentration ($ng.mL^{-1}$). Values are means ± SEM for each condition (n=5) and bold values show which condition has higher mean for a particular BXD. Asterisk indicates significant difference (p-value) between two groups: * P < 0.05, ** P < 0.01, *** P < 0.001 and NS = no significant difference. ND stands for BXDs which have not been detected in any of the replicate, while r < 3 indicates that less than 3 replicates had detectable levels of BXDs.*

BXD ($ng.mL^{-1}$)	Rye cultivated alone (R) Figure 12.A			Rye in co-culture (R*(R+P)) Figure 12.B		
	Microbeads of glass (A)	Mix of clay and attapulgite (B)	Significant difference	Microbeads of glass (A)	Mix of clay and attapulgite (B)	Significant difference
DIMBOA-Glc	**8.6 ± 1.61**	1.83 ± 0.64	**	**5.414 ± 0.9818**	5.086 ± 2.26118	*NS*
DIMBOA	0.8 ± 0.361	*ND*	*NS*	**0.6286 ± 0.24474**	0.4429 ± 0.3755	*NS*
HDMBOA-Glc	**2.586 ± 0.694**	1.914 ± 0.042	*	1.829 ± 0.0175	1.971 ± 0.6942	*NS*
MBOA	32.26 ± 11.06	r < 3	*NS*	35.04 ± 6.2143	r < 3	*NS*
HMBOA-Glc	**3.543 ± 0.889**	0.071 ± 0.04	*NS*	**1.186 ± 0.140335**	0.7143 ± 0.6965	*NS*
HDM_2BOA-Glc	**1.457 ± 0.370**	1.343 ± 0.336	*NS*	1.014 ± 0.415	r < 3	*NS*
DIBOA-Glc	*ND*	*ND*	*NS*	r < 3	*ND*	*NS*
DHBOA-Glc	**4.886 ± 2.424**	4.357 ± 2.602	*NS*	**4.314 ± 2.4283**	2.671 ± 0.8644	*NS*
HBOA-Glc	1 ± 0.484	*ND*	*NS*	1.243 ± 0.790763	*ND*	*NS*
BOA	2.914 ± 0.3915	*ND*	*NS*	5.7 ± 0.83586	*ND*	*NS*

As more BXDs have been detected and quantified in the glass beads substrate (Figure 12.A and 12.B), it can be hypothesized that some of the BXDs are sorbed on clay particles. Indeed, it has been demonstrated that clay particles might sorb around 20% of the compounds released in the clay substrate (Sasse et al., 2020). Clay structure (e.g., accessible surface areas) and surface charge might interfere with dissolved organic compounds and thus, altering the exudation composition in soil. The lower

amount of BXDs detected in the clay beads substrate might also come from the lack of compound extraction efficiency. The extraction solution has been optimized for BXDs extraction from plants grown in the glass beads substrate where compounds are more available, which is not the case for the clay and attapulgite substrate. Improving the extraction solution, by using a solvent with a higher affinity for metabolite than soil-compound affinity, might help to better desorb compounds from clay. An optimal extraction solvent must preserve the integrity of plant roots. The choice of solvent is, therefore, limited. Water (polar solvent) is the less destructive solvent that could be used to extract root exudates from the rhizosphere, explaining the use of acidified water with 0.5% of formic acid in this experiment (Abubakar & Haque, 2020). Non-polar solvents are more adequate to extract BXDs from any substrate. However, hexane (non-polar solvent) might disrupt cell membranes releasing compounds from inside the root, leading to the extraction of root compounds. Whereas the aim is to study root exudates from the rhizosphere or compounds from the root surface. Ethanol or methanol are less polar than water, nonetheless, they could also disrupt cell membranes at a lower level than hexane. An increasing extraction duration, more than one minute which has been performed in this experiment, may also help to better desorb compounds from clay.

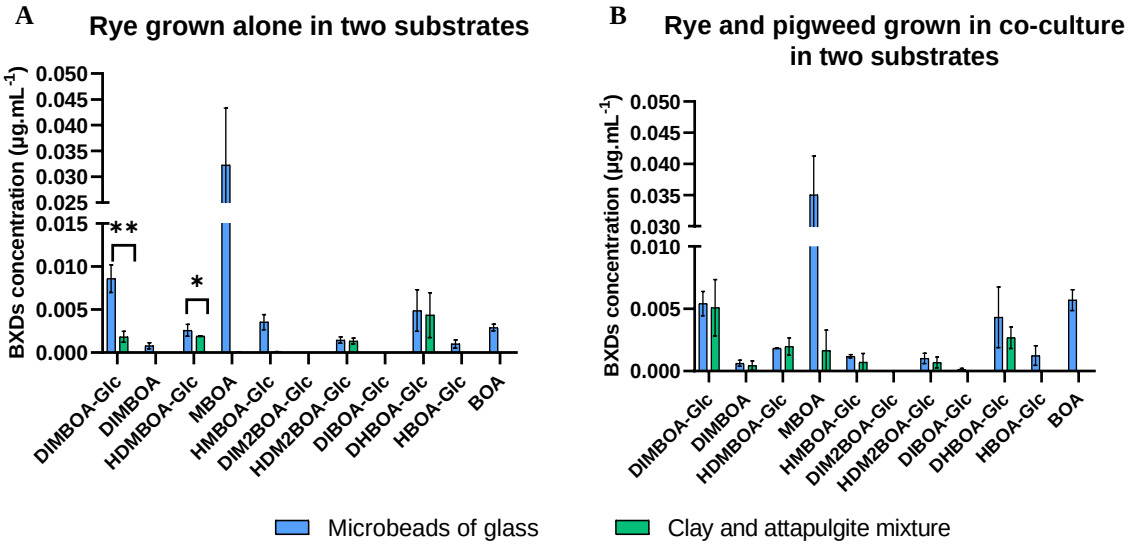


*Figure 12: Comparison of two substrates, microbeads of glass and a mixture of clay and attapulgite, for rye cultivated alone (12.A) and in co-culture (12.B) by measuring different BXDs extracted from the rhizosphere of rye and/or pigweed. Asterisk indicates significant difference between two groups: * P < 0.05, ** P < 0.01, *** P < 0.001.*

1.2.2. Comparison of two growing modalities

Figure 13.A compares the two growth modalities (alone and in co-culture) in the substrate of glass microbeads. Even though there is no significant difference between rye grown alone and in co-culture expected for HMBOA-Glc, two trends appear when rye is cultivated in the microbeads of glass (A). Out of the nine BXDs detected, when rye is cultivated alone, six of them have a higher concentration among which DIMBOA-Glc, DIMBOA, HDMBOA-Glc, HMBOA-Glc(*), HDM_2BOA-Glc and DHBOA-Glc (Table 9). Meanwhile, three BXDs (MBOA, BOA, HBOA-Glc) have a higher concentration when rye is co-cultivated with pigweed (Table 9). Thus, the emerging trends are : (1) the total glucosylated BXDs are more abundant when the plant is cultivated alone and (2) the total non-glucosylated BXDs appeared in higher concentrations in co-culture (e.g., MBOA or BOA). (1) The glucosylated form is the non-active form of BXDs stored in plant vacuoles (Robert & Mateo, 2022), which is expected when rye is cultivated alone as the crop does not need to inhibit neighboring plant

growth. Nonetheless, it is surprising to find glucosylated BXDs in the rhizosphere. Cellular lysis, which acts as a tissue disruption, occurs naturally during plant growth leading to a release of glucosylated BXDs in the rhizosphere. Once released in the cytoplasm, the glucose is removed by the β-glucosidase enzyme causing the transformation into non-glucosylated BXDs (Robert & Mateo, 2022). However, this transformation does not occur in the present experiment. This may be explained by the presence of formic acid 0.5% in the extraction solution made of acidified water. Formic acid has indeed the property to stop the enzymatic activity. (2) The non-glucosylated form is the active form of BXDs which is expected in the rhizosphere of rye grown in co-culture with pigweed as they are known to inhibit germination and root growth of neighboring plants (Macías et al., 2004). Indeed, the most abundant BXD in the rhizosphere, when rye and pigweed are grown in co-culture in the microbeads of glass, is MBOA with a concentration of 0.035 $\mu g.mL^{-1}$ (Table 9). MBOA and BOA come from the spontaneous degradation of DIMBOA and DIBOA respectively in aqueous solution, which explains their higher concentrations in the rhizosphere (Macías et al., 2004). Those hypotheses must be interpreted carefully as the BXDs concentration in the rhizosphere are close to the limit of quantification which varies between 0.01 and 0.05 $\mu g.mL^{-1}$ depending on the BXDs.

*Table 9: Comparison of growth modalities, alone or in co-culture, for rye and pigweed cultivated in microbeads of glass (A) and in a mix of clay and attapulgite (B), by measuring different BXDs concentration ($ng.mL^{-1}$), from the rhizosphere. Values are means ± SEM for each condition (n=5) and bold values show which condition has higher mean for a particular BXD. Asterisk indicates significant difference (p-value) between two groups: * P < 0.05, ** P < 0.01, *** P < 0.001 and NS = no significant difference. ND stands for BXDs which have not been detected in any of the replicate, while r < 3 indicates less than 3 replicates had detectable levels of BXDs.*

BXD ($ng.mL^{-1}$)	Rye cultivated in microbeads of glass (RA) Figure 13.A			Rye cultivated in clay and attapulgite (RB) Figure 13.B		
	Alone	Co-culture	Significant difference	Alone	Co-culture	Significant difference
DIMBOA-Glc	**8.6 ± 1.61**	5.41 ± 0.98	*NS*	1.83 ± 0.64	**6.36 ± 2.41**	*NS*
DIMBOA	**0.8 ± 0.36**	0.63 ± 0.24	*NS*	*ND*	0.55 ± 0.46	*NS*
HDMBOA-Glc	**2.58 ± 0.69**	1.83 ± 0.017	*NS*	1.91 ± 0.042	**2.46 ± 0.631**	*NS*
MBOA	32.26 ± 11.06	**35.04 ± 6.21**	*NS*	r < 3	2.054 ± 2.054	*NS*
HMBOA-Glc	**3.54 ± 0.89**	1.19 ± 0.14	*	0.071 ± 0.040	**0.89 ± 0.87**	*NS*
DIM2BOA-Glc	*ND*	*ND*	*NS*	*ND*	r < 3	*NS*
HDM2BOA-Glc	**1.46 ± 0.37**	1.01 ± 0.41	*NS*	1.343 ± 0.34	r < 3	*NS*
DIBOA-Glc	*ND*	r < 3	*NS*	*ND*	*ND*	*NS*
DHBOA-Glc	**4.89 ± 2.42**	4.31 ± 2.43	*NS*	**4.35 ± 2.60**	3.34 ± 0.708	*NS*
HBOA-Glc	1 ± 0.48	**1.24 ± 0.80**	*NS*	*ND*	*ND*	*NS*
BOA	2.91 ± 0.39	**5.7 ± 0.83**	*NS*	*ND*	*ND*	*NS*
Total non-glycosylated	35.9700	**41.3700**		0.0000	**2.6076**	
Total glycosylated	**22.0700**	15.0000		9.5144	**13.0529**	

The same trends between growing modalities (alone and in co-culture) could not be observed in the clay and attapulgite substrate. In Figure 13.B and Table 9, which compares the two modalities in the clay mixture, six BXDs could be detected, when rye is co-cultivated (n=5, with more than 3 replicates with detectable levels of BXDs), whereas five BXDs could be detected when rye is cultivated alone. Out of the four BXDs detected in both modalities, three of them have a higher concentration when rye is cultivated in co-culture among which DIMBOA-Glc, HDMBOA-Glc and HMBOA-Gl (Table 9). Nonetheless, non-glucosylated BXDs (e.g., DIMBOA and MBOA) have also been detected at an average concentration of 0.55 and 2.04 $ng.mL^{-1}$ respectively. Meanwhile, only DHBOA-Glc has a higher concentration when rye is cultivated alone (Table 9). More BXDs are detected in the rhizosphere of rye and pigweed in co-culture, as physical or chemical interactions is expected between an allelopathic crop and weed through the production of allelochemicals, which further lead rye to inhibit the growth of neighboring plants (Kong et al., 2018). The total BXDs shows that both glucosylated and non-glucosylated BXDs are present in high amounts in the rhizosphere when the plant is cultivated in co-

culture. Furthermore, total glucosylated has a higher concentration in the rhizosphere than the total non-glucosylated BXDs, 13.05 and 2.6 ng.mL^{-1} (Table 9) respectively, which contrast with the trends (1) and (2) addressed in the previous paragraph. It may be hypothesized that the non-glucosylated BXDs exuded by rye throughout plant growth, were sorbed by clay leading to a lower concentration in the rhizosphere. Whereas glucosylated BXDs arise from the naturally occurring cell lysis as mentioned in the paragraph above. It must be mentioned that those explanations are speculation and thus, must be interpreted carefully.

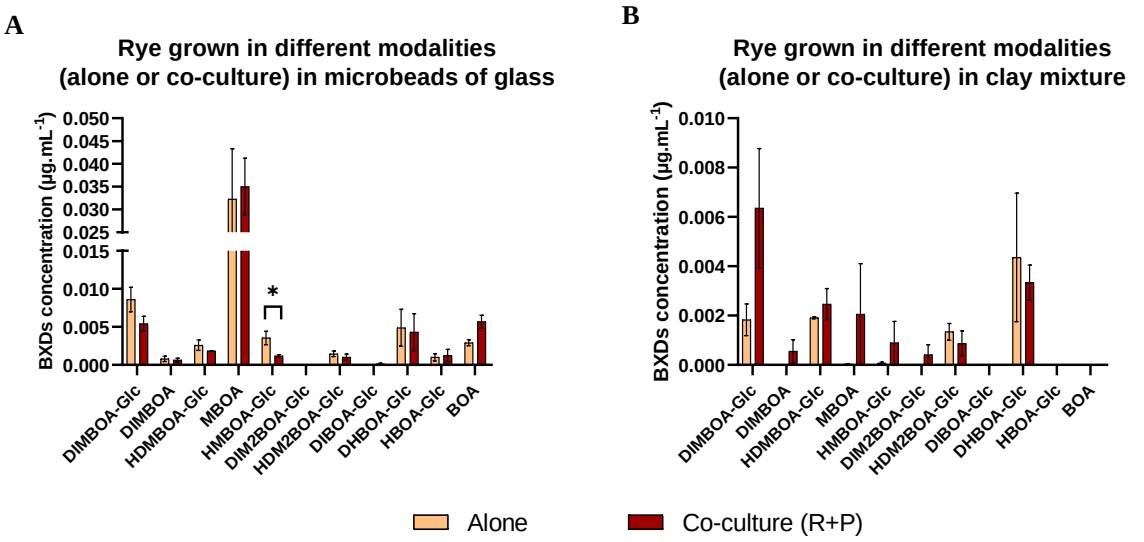


*Figure 13: Comparison of two growth modalities, alone and in co-culture, for pigweed and rye cultivated in microbeads of glass(13.A) and a mix of clay and attapulgite (13.B) by measuring different BXDs concentration from the rhizosphere. Asterisk indicates significant difference between two groups: * P < 0.05, ** P < 0.01, *** P < 0.001.*

In summary, for objective A, the effect of substrate on plant growth has been confirmed by a pairwise comparison (Wilcoxon test). As expected, the main influence on both root architecture and BXDs composition is the substrate, especially when plants are cultivated alone (Alone*B >< Alone*A). Indeed, both plants (rye and pigweed) tend to grow better in the clay and attapulgite mixture (B), as indicated notably by differences in root architecture. These differences in plant growth could be explained by substrate differences in particles size, water retention and/or pores space between particles. But while clay and attapulgite mixture (B) substrate seems to enhance plant growth, fewer BXDs were detected from plants cultivated. This may be due to the sorption capacity of clay compared to glass microbeads. In this view, using glass microbeads with higher particle size (>1mm) might help to enhance plant growth while maintaining an inert sorption-free system for BXDs chemical analysis.

2. Objective B: Treatment effects

This experiment aimed at better understanding how root architecture and BXDs production can be influenced by the root exudates of another plant, possibly containing unknown signaling molecules. Thus, root architecture, along with BDXs content in the rhizosphere, were investigated once the different treatments were applied on rye grown alone. On the one hand, rye grown in co-culture with pigweeds (R+P) will be compared to rye treated daily with pigweed root exudates (PRE), the control being the rye cultivated alone (R). This first comparison thus evaluates the potential kin and non-kin recognition between rye and pigweed through physical contact or competition and chemical cues (R+P vs PRE). On the other hand, rye treated with loliolide (LOL) and jasmonic acid (JA) at 0.5 nM will be compared to one another and to a control which is rye cultivated alone (R). The low concentration was selected based

on the loliolide concentration found in wheat roots at the 3-leaf stage (Li et al, 2023). The concentration is around 0.5 $\mu g.g^{-1}$ of dry weight which has been converted into mass concentration and finally a molar concentration corresponding to 0.51 nM. Thus, this comparison explores whether or not LOL and/or JA at low concentrations could act as signaling molecules and therefore, influence root architecture and BXDs production in rye.

2.1. Root architecture analysis

2.1.1. Effect of physical or chemical presence of pigweed or pigweed root exudates

Comparison studying the differences between rye co-cultivated (R+P) and rye treated with pigweed root exudate (PRE) will be studied first. Figure 14 represents the different root parameters measured for each modality. The comparison is made between rye cultivated alone (R) and rye treated with pigweed root exudates (PRE) as presented in Figure 14 and Table 10. The rye treated with pigweed root exudates (PRE) shows similar root development as rye cultivated alone (R), for all the measured parameters presented in Table 10. It must be mentioned that none of the parameters were significantly different from the rye cultivated in co-culture with pigweed (R+P).

Table 10: Comparison of rye cultivated alone (R) and rye treated with pigweed root exudates (PRE) by measuring different root parameters. Values are means ± SEM for each condition (n=5). NS = no significative difference.

Figure 14	Root parameters	Rye cultivated alone (R)	Rye treated with pigweed root exudate (PRE)	Significant difference
A	Root length (cm)	102.8 ± 7.665	99.24 ± 13.68	NS
B	Root surface Area (cm^2)	14.01 ± 0.877	13.76 ± 1.489	NS
C	Root Diameter (mm)	0.436 ± 0.0112	0.4536 ± 0.0345	NS
D	Specific Root length ($cm.g^{-1}$)	6597 ± 543.9	6835 ± 1019	NS
E	Root Volume (cm^3)	0.152 ± 0.009	0.156 ± 0.020	NS
F	Number of root tips	187.2 ± 24.76	189.5 ± 46.94	NS
G	Root length density ($cm.cm^{-3}$)	1.713 ± 0.128	1.654 ± 0.228	NS
H	Root surface area density ($cm^2.cm^{-3}$)	92.04 ± 2.320	90.48 ± 6.303	NS
I	Root branching density (nbr of $tips.cm^{-1}$)	1.831 ± 0.222	1.897 ± 0.298	NS
J	Dry leaves biomass (g)	0.0134 ± 0.0014	0.0155 ± 0.002	NS
K	Dry roots biomass (g)	0.0157 ± 0.0007	0.015 ± 0.0014	NS
L	Root tissues density ($g.cm^{-3}$)	0.105 ± 0.007	0.0989 ± 0.0054	NS

Although no significant differences between rye in co-culture and rye treated with pigweed root exudates (R+P vs PRE) have been determined for any root architecture parameters, slight differences can be underlined. Table 11 shows the parameters that are higher for rye treated with pigweed root exudates (PRE) compared to rye co-cultivated with pigweed (R+P).

Table 11: Comparison of rye in co-culture (R+P) and rye treated with pigweed root exudates (PRE) by measuring different root parameters. Values are means ± SEM for each condition (n=5) and bold values show which condition has higher mean for a particular parameter. NS = no significative difference.

Figure 14	Root parameters	Rye co-cultivated (R+P)	Rye treated with pigweed root exudate (PRE)	Significant difference
A	Root length (cm)	96.23 ± 7.63	**99.24 ± 13.68**	NS
B	Root surface Area (cm^2)	12.38 ± 0.73	**13.76 ± 1.49**	NS
C	Root Diameter (mm)	0.415 ± 0.013	**0.453 ± 0.034**	NS
E	Root Volume (cm^3)	0.128 ± 0.006	**0.156 ± 0.020**	NS
F	Number of root tips	172.7 ± 33.22	**189.5 ± 46.94**	NS
G	Root length density ($cm.cm^{-3}$)	1.60 ± 0.127	**1.654 ± 0.228**	NS
J	Dry leaves biomass (g)	0.012 ± 0.0004	**0.0155 ± 0.002**	NS

The root architecture parameters being higher for rye cultivated alone (R) or treated with pigweed root exudate (PRE) compared to rye co-cultivated with pigweed (R+P) might be explained by the competition induced by the physical presence of pigweed. Indeed, plants grown together in one soil volume will compete for the same resources in favor of their growth and development leading to root system rearranging (Kumari et al., 2023). Those root architecture changes may be assigned to competition and/or to non-kin recognition where plants recognize their neighbors, through physical or chemical cues, leading to an adjustment in their growth patterns (Wang et al., 2021). Nonetheless, hypothesizing the involvement of non-kin recognition in the plant responses might be an extrapolation as the differences between competition and non-kin recognition are not yet clearly defined. To improve competition and resource uptake, the roots respond by either stimulating their own growth or inhibiting the neighbor's growth of non-self-roots which is not the case in this experiment as rye in co-culture (R+P) has lower root growth (Wang et al., 2021). It may be hypothesized that rye and pigweed grown in co-culture (R+P) are less developed due to a competition effect for resources and physical space.

Regarding rye treated with pigweed root exudates (PRE), the roots and shoots seem to have a greater development than rye cultivated in co-culture (R+P). This may seem surprising as some papers have demonstrated that pigweed root exudates have an inhibiting effect on maize epicotyl's growth (Konstantinović et al., 2014.). It has also been demonstrated that plants treated with exudates obtained from individuals from a different population (non-kin) produced significantly lower root lengths than plants receiving exudates from the same population (kin recognition) (Semchenko et al., 2014). However, in this experiment, the application of root exudates from the non-kin population, being the pigweed, on rye does not induce lower root development. Three hypotheses explaining those results arise: (1) the lack of root inhibition in the case of rye treated with pigweed root exudates (PRE) might originate from the remaining presence of nutrients from the Hoagland solution, despite a preliminary cleaning step (*cfr* section 2.4.1). (2) root growth differences between rye in co-culture (R+P) and rye treated with pigweed root exudates (PRE) might be caused by a combination of the absence of physical competition between plants and a low concentration of root exudates. (3) the low concentration of pigweed root exudates stimulating the rye root growth might be explained by the hormesis effect (An, 2005). The hormesis effect can be defined as a phenomenon in which a substance gives stimulating (beneficial) effects on living organisms (e.g., animals or plants) when the quantity is small (Pickrell, 2009). However, analyzing the BXDs may help to further investigate those hypotheses.

2.1.2. Effect of loliolide and jasmonic acid on rye roots

Comparison studying, among others, the differences between rye cultivated alone (R) and rye treated with loliolide (LOL) and jasmonic acid (JA) will be studied and presented in Figure 14 for different root parameters.

Compared to rye cultivated alone (R), rye continuously treated with loliolide (LOL) has significantly lower root growth, for the parameters B, E, K and lower root growth but non-significantly for the other parameters, all shown in Table 12. Those results are matching previous conclusions from the literature. According to Kato-Noguchi et al. (2014), loliolide inhibits the growth of cress and ryegrass at concentrations greater than 3 and 10 µM, even though those concentrations are higher than the one naturally found in wheat root, around 0.5 $\mu g.g^{-1}$ of dry weight (Li et al., 2023). However, in this present work, significant differences in root architecture have been observed with a lower concentration of 0.5 nM of loliolide. As a low concentration of loliolide and jasmonic acid has been used in the present work, it may be interesting to repeat the experiment with a higher concentration of loliolide as for Kato-Noguchi et al. (2014) or to expand the treatment period, more than ten days of treatment, in order to confirm trends observed.

*Table 12: Comparison of rye cultivated alone (R) and rye treated with loliolide (LOL) at 0.5 nM by measuring different root parameters. Values are means ± SEM for each condition (n=5) and bold values show which condition has higher mean for a particular parameter. Asterisk indicates significant difference (p-value) between two groups: * P < 0.05, ** P < 0.01, *** P < 0.001 and NS = no significative difference.*

Figure 14	Root parameters	Rye cultivated alone (R)	Rye treated with loliolide (LOL)	Significant difference
A	Root length (cm)	**102.8 ± 7.665**	82.7 ± 6.460	NS
B	Root surface area (cm^2)	**14.01 ± 0.877**	11.08 ± 0.828	*
E	Root volume (cm^3)	**0.152 ± 0.0094**	0.1187 ± 0.009	*
F	Number of root tips	**187.2 ± 24.76**	127 ± 20.67	NS
G	Root length density ($cm.cm^{-3}$)	**1.713 ± 0.128**	1.378 ± 0.107	NS
I	Root branching density (nbr of $tips.cm^{-1}$)	**1.831 ± 0.222**	1.557 ± 0.234	NS
J	Dry leaves biomass (g)	**0.0134 ± 0.0014**	0.0106 ± 0.0007	NS
K	Dry roots biomass (g)	**0.0157 ± 0.0007**	0.011 ± 0.0004	**
L	Root tissues density ($g.cm^{-3}$)	**0.105 ± 0.0074**	0.095 ± 0.005	NS

Similar trends to loliolide are observed for the jasmonic acid treatment (JA), which shows lower root growth than the control (R). Even though no significant differences are observed, some root architecture parameters, presented in Table 13, displayed lower values for plants treated with JA compared to control. Those results also correlate with previous work: exogenous application of jasmonic acid on sunflowers has demonstrated primary and lateral root length reduction as well as a decrease in primary and lateral roots number (Corti Monzón et al., 2012). Interestingly, auxins also reduce primary and lateral root length and some reports suggest a cross-talk between the auxin and the JA pathways (Staswick, 2009). However, rye treated with JA also shows significantly greater root diameter (Figure 14.C) and higher dry leaves biomass (Figure 14.J) than rye in co-culture (R+P). Those parameters are nonetheless not significantly different from the control of rye cultivated alone (R). The dry root biomass (Figure 14.K) and the root tissue density (Figure 14.L) are also higher for rye treated with JA.

*Table 13: Comparison of rye cultivated alone (R) and rye treated with jasmonic acid (JA) at 0.5 nM by measuring different root parameters. Values are means ± SEM for each condition (n=5) and bold values show which condition has higher mean for a particular parameter. Asterisk indicates significant difference (p-value) between two groups: * P < 0.05, ** P < 0.01, *** P < 0.001 and NS = no significative difference.*

Figure 14	Root parameters	Rye cultivated alone (R)	Rye treated with jasmonic acid (JA)	Significant difference
A	Root length (cm)	**102.8 ± 7.665**	88.22 ± 4.150	NS
D	Specific Root length ($cm.g^{-1}$)	**6597 ± 543.9**	5473 ± 375.2	NS
F	Number of root tips	**187.2 ± 24.76**	143.6 ± 11.45	NS
G	Root length density ($cm.cm^{-3}$)	**1.713 ± 0.128**	1.47 ± 0.069	NS
H	Root surface area density ($cm^2.cm^{-3}$)	**92.04 ± 2.320**	87.62 ± 1.765	NS
I	Root branching density (nbr of $tips.cm^{-1}$)	**1.831 ± 0.222**	1.658 ± 0.162	NS

A pairwise comparison using the Wilcoxon test was performed to analyze root architecture differences between loliolide (LOL) and jasmonic acid (JA) treatments. Out of the 12 parameters studied, 10 parameters, shown in Table 14, were lower for rye treated with loliolide (LOL) compared to rye treated with jasmonic acid (JA). Thus, it can be hypothesized that loliolide has a higher reduction effect on rye root growth compared to jasmonic acid for a similar treatment concentration at 0.5 nM.

*Table 14: Comparison of rye treated with loliolide (LOL) and rye treated with jasmonic acid (JA) both at 0.5 nM by measuring different root parameters. Values are means ± SEM for each condition (n=5) and bold values show which condition has higher mean for a particular parameter. Asterisk indicates significant difference (p-value) between two groups: * P < 0.05, ** P < 0.01, *** P < 0.001 and NS = no significative difference.*

Figure 14	Root parameters	Rye treated with loliolide (LOL)	Rye treated with jasmonic acid (JA)	Significant difference
A	Root length (cm)	82.7 ± 6.46	**88.22 ± 4.15**	NS
B	Root surface area (cm^2)	11.08 ± 0.8283	**12.63 ± 0.4786**	NS
C	Root diameter (mm)	0.4284 ± 0.0104	**0.4573 ± 0.009**	NS
E	Root volume (cm^3)	0.1187 ± 0.0093	**0.1443 ± 0.0053**	NS
F	Number of root tips	127 ± 20.67	**143.6 ± 11.45**	NS
G	Root length density ($cm.cm^{-3}$)	1.378 ± 0.1077	**1.47 ± 0.07**	NS
I	Root branching density (nbr of $tips.cm^{-1}$)	1.557 ± 0.2343	**1.658 ± 0.162**	NS
J	Dry leaves biomass (g)	0.0106 ± 0.0007	**0.014 ± 0.0007**	**
K	Dry roots biomass (g)	0.0110 ± 0.0004	**0.0165 ± 0.001**	***
L	Root tissues density ($g.cm^{-3}$)	0.0952 ± 0.0050	**0.1141 ± 0.0063**	*

All in one, the most significant differences between the control and one of the treatments are found between rye cultivated alone (R) and loliolide (LOL), regardless of specific parameters. This leads to the conclusion that globally, exogenous application of loliolide significantly reduces rye root growth and biomass.

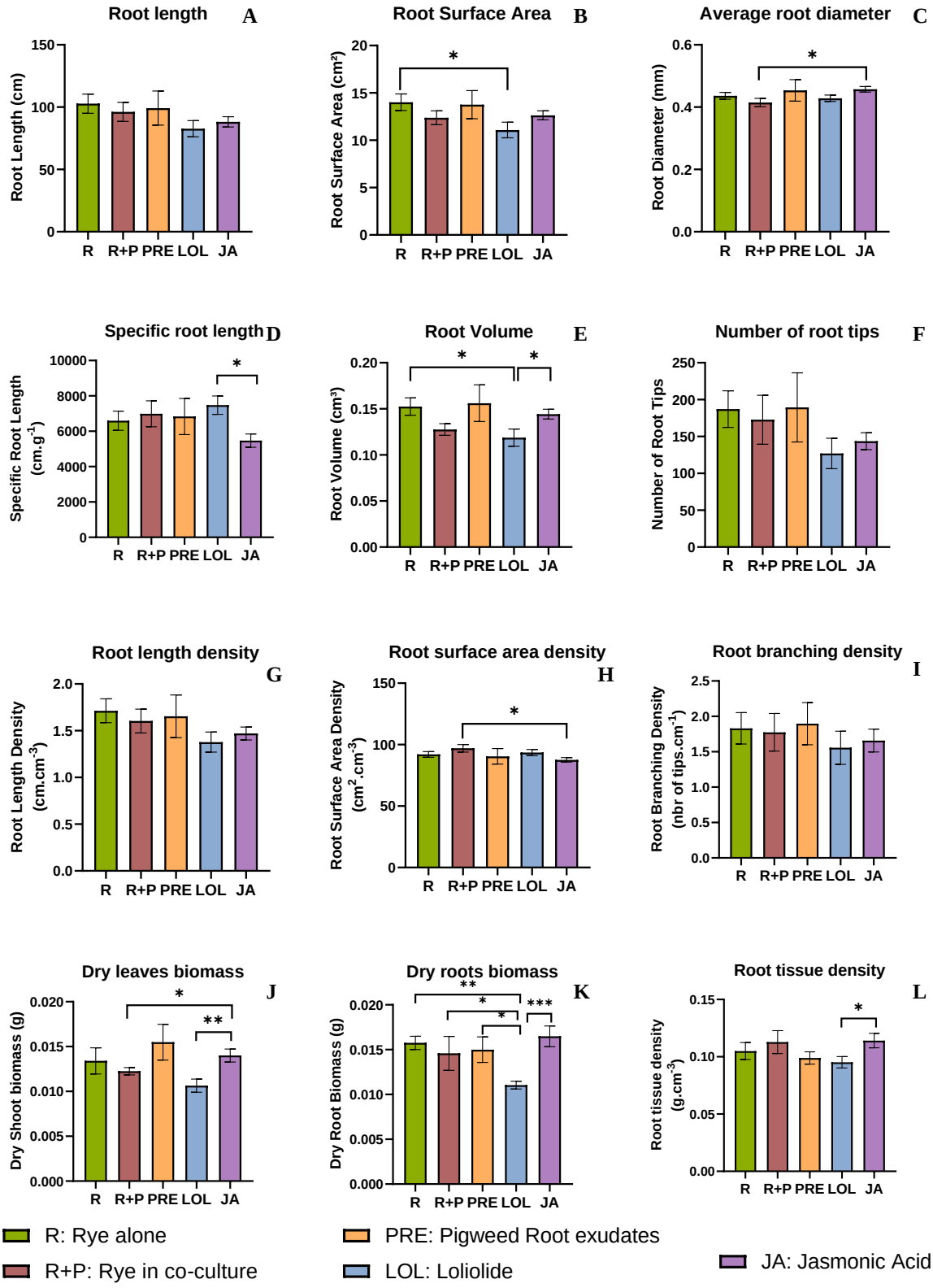


*Figure 14: Comparison of three treatments, pigweed root exudates (PRE), loliolide (LOL) at 0.5 nM and jasmonic acid (JA) at 0.5 nM, applied exogenously on rye, by measuring different root architecture parameters: the root length (A), root surface area (B), average root diameter (C), specific root length (D), root volume (E), number of tips (F), root length density (G), root surface area density (H), root branching density (I), dry leaves biomass (J), dry roots biomass (K) and root tissue density (L). Asterisk indicates significant difference between two groups: * P < 0.05, ** P < 0.01, *** P < 0.001.*

2.2. BXDs analysis

The present work also investigated if and how the presence of another plant – physically or chemically with root exudates – influenced BXDs production in rye. The potential of loliolide and jasmonic acid as signaling molecules inducing BXDs production in rye was also explored through an exogenous application on rye. More precisely, the effect of three treatments (pigweed root exudates, loliolide and jasmnonic acid), mimicking the presence of a neighboring plant without inducing competition or root physical contact, was studied.

Figure 15 indicates the concentration of different BXDs in rye roots according to the treatments applied to rye. The two most abundant BXDs with higher concentrations are DHBOA-Glc and DIMBOA-Gl, which match previous results obtained by Pauline Canelle in her master thesis (Canelle, 2023). The concentration varies from 200 to 700 $\mu g.g^{-1}$ of fresh root biomass. HMBOA-Glc and HBOA-Glc also appear with a lower concentration varying from 25 to 120 $\mu g.g^{-1}$ of fresh root biomass. DIBOA-Glc and DIMBOA stand at the limit of quantification which is 0.025 $\mu g.mL^{-1}$.

Therefore, the most abundant BXDs described in this experiment are glucosylated which is the typically stored form of non-toxic glucose conjugates (Rice et al., 2022). As BXDs were extracted from rye roots and not from the rhizosphere, it is thus expected to have a non-toxic glucosylated form. However, one may be surprised by the presence of DHBOA-Glc at such a high level as this is not commonly described in the literature, except for Canelle (2023). It must be stated that the composition and abundance of BXDs highly depend on the geographical location, growing conditions or cultivars (Carlsen et al., 2009). According to Tanwir et al. (2017), the amounts of different BXDs compounds in the developing seedling might also be due to a combination of new compound biosynthesis based on transcriptional regulation of ScBx genes and a biochemical turnover which together lead to different BXDs transformations. At a later stage, high accumulations of HMBOA-Glc and DIMBOA-Glc in maize and rye seedlings and root tissues, respectively, were observed (Sue et al., 2000, Tanwir et al., 2017). HBOA-Glc would be a starting point for the biosynthesis of lactams leading to the production of DHBOA-Glc, which might be an intermediate in HMBOA- Glc synthesis (de Bruijn et al., 2018). It can be furthermore hypothesized that DHBOA-Glc is the predominant storage form in rye which, in order to be released, should undergo further transformation i.e. HMBOA-Glc, explaining a higher concentration of DHBOA-Glc than HMBOA-Glc in rye roots.

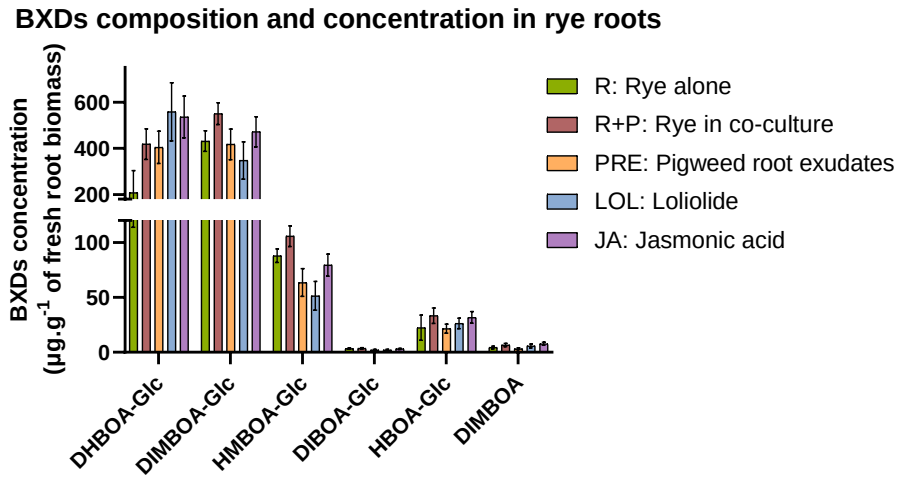


Figure 15: Composition and concentrations of diverse BXDs (DHBOA-Glc, DIMBOA-Glc, HMBOA-Glc, DIBOA-Glc, HBOA-Glc and DIMBOA) extracted from rye roots after the exogenous application of three treatments: pigweed root exudates (PRE), loliolide (LOL) at 0.5 nM and jasmonic acid (JA) at 0.5 nM.

2.2.1. Effect of physical or chemical presence of pigweed or pigweed root exudates

At first, a focus is made on the comparison between rye cultivated in co-culture with pigweed (R+P) and rye treated with pigweed root exudates (PRE) with the control being rye cultivated alone (R). It could be expected that the presence of pigweed triggers the production of BXDs in rye roots. The exogenous application of pigweed root exudates (PRE) could also be expected to induce BXDs production, although at a lower level than co-culture due to the absence of physical contact.

Figure 16 presents BXDs concentration in rye roots for each BXD analyzed separately depending on the treatment applied. For each BXD presented in Table 15, the concentration is higher when rye is co-cultivated compared to rye cultivated alone, although the difference is only significant for DHBOA-Glc (*). It could therefore be concluded that BXDs production in rye is induced upon the presence of pigweed through physical and/or chemical cues. Similar results have been obtained in different plant contexts. For instance, in wheat, allelochemicals concentration such as DIMBOA also significantly increased with the density of heterospecific neighbors such as redroot pigweed (Zhang et al., 2016; Kong et al., 2018). Altogether, the data of the present work is consistent with the literature and points out that interactions with other plant species, especially weeds, lead to the production of BXDs in rye.

*Table 15: Comparison of rye cultivated alone (R) and rye in co-culture (R+P) by measuring different BXDs concentration. Values are means ± SEM for each condition (n=5) and bold values show which condition has higher mean for a particular BXD. Asterisk indicates significant difference between two groups: * P < 0.05, ** P < 0.01, *** P < 0.001 and NS = no significative difference.*

Figure 16	BXD concentration (µg.g^{-1} of fresh biomass)	Rye cultivated alone (R)	Rye co-cultivated (R+P)	Significant difference
A	DHBOA-Glc	208.7 ± 94.83	**418.6 ± 66.34**	*
B	DIMBOA-Glc	431.6 ± 44.88	**549.9 ± 47.39**	NS
C	HMBOA-Glc	87.96 ± 6.107	**105.8 ± 9.432**	NS
D	DIBOA-Glc	3.327 ± 0.631	**3.543 ± 0.7434**	NS
E	HBOA-Glc	22.37 ± 11.53	**33.32 ± 7.079**	NS
F	DIMBOA	4.366 ± 1.189	**6.564 ± 1.439**	NS

To further explore this phenomenon, root exudates of pigweed (PRE) have been applied to rye roots to determine if they play a role in BXDs production. While the concentration of DHBOA-Glc (A) was higher, the concentrations of HMBOA-Glc (C), DIBOA-Glc (D) and DIMBOA (F) were lower in treated plants (PRE) compared to rye cultivated alone (R) (Figure 16 and Table 16). The levels of DIMBOA-Glc (B) and HBOA-Glc (E) seemed unaffected by root exudates. Although differences are not significant, these trends are pronounced and it would be constructive to repeat the experiment with more replicates to lower the variability. These trends are all the more interesting considering that while Zhang et al. (2016) observed an increased DIMBOA concentration in wheat when grown in co-culture at a 5:8 wheat/pigweed density ratio, the root exudates of pigweed did not show significant induction of allelochemicals. Besides, other plants root exudates, such as *Abutilon theophrasti* and *Alopecurus japonicus*, significantly increased DIMBOA concentration in wheat (Zhang et al., 2016). It, therefore, seems that BXDs induction is largely dependent on the crop species and the weed species considered and that in the case of rye-pigweed interactions, root exudates of pigweed seem to affect BXDs production, especially DHBOA-Glc, in rye roots.

Table 16: Comparison of rye cultivated alone (R) and rye treated with pigweed root exudates (PRE) by measuring different BXDs concentration. Values are means ± SEM for each condition (n=5) and bold values show which condition has higher mean for a particular BXD. NS = no significative difference.

Figure 16	BXD concentration ($\mu g.g^{-1}$ of fresh biomass)	Rye cultivated alone (R)	Rye co-cultivated (PRE)	Significant difference
A	DHBOA-Glc	208.7 ± 94.83	**404.4 ± 70.17**	NS
B	DIMBOA-Glc	431.6 ± 44.88	417.7 ± 66.20	NS
C	HMBOA-Glc	**87.96 ± 6.107**	63.54 ± 12.59	NS
D	DIBOA-Glc	**3.327 ± 0.6311**	2.011 ± 0.670	NS
E	HBOA-Glc	22.37 ± 11.53	21.59 ± 4.099	NS
F	DIMBOA	**4.366 ± 1.189**	3.22 ± 0.86	NS

2.2.2. Effect of loliolide and jasmonic acid on rye roots

Because loliolide (LOL) and jasmonic acid (JA) are candidates as signaling molecules in allelopathy, these molecules were applied exogenously and the BXDs content in rye roots was analyzed. In this view, JA and LOL treatments could be expected to also induce BXDs production, as it previously occurred for DIMBOA in wheat (Kong et al., 2018).

As presented in Figure 16, this was the case for DHBOA-Glc (A), HBOA-Glc (E) and DIMBOA (F), for LOL as well as JA. Exogenous application of jasmonic acid (JA) and loliolide (LOL) significantly increased DHBOA-Glc at an average concentration of 535.9 and 558.1 $\mu g.g^{-1}$ of fresh biomass, respectively, in rye roots compared to rye cultivated alone (R) which shows an average concentration of 208.7 $\mu g.g^{-1}$ of fresh biomass (Table 17). Jasmonic acid (JA) treatment also significantly increased the concentration of HBOA-Glc (31.7 $\mu g.g^{-1}$ of fresh biomass) and DIMBOA (7.7 $\mu g.g^{-1}$ of fresh biomass) compared to rye cultivated alone (R) (22.4 and 4.4 $\mu g.g^{-1}$ of fresh biomass, respectively). Although it is not significant, the same trend can be observed for the loliolide (LOL) treatment, which enhanced HBOA-Glc (26.3 $\mu g.g^{-1}$ of fresh biomass) and DIMBOA (5.7 $\mu g.g^{-1}$ of fresh biomass) concentrations in rye roots compared to rye cultivated alone (R) (Figure 16 and Table 17).

Kong et al. (2018) demonstrated that loliolide at a low concentration (5 $nmol.g^{-1}$ dry soil) and jasmonic acid at a medium concentration (50 $nmol.g^{-1}$ dry soil) could increase DIMBOA concentration in wheat. Those treatment concentrations more or less match the one used in this experiment, which was around 0.5 nM for both JA and loliolide. In this experiment, low concentrations of JA and loliolide could not only increase DIMBOA concentration but also those of HBOA-Glc and DHBOA-Glc in rye roots. Li et al (2023) also demonstrated that increasing concentration, from 0 to 50 µM, of loliolide and jasmonic acid, could rise DIMBOA concentration from 400 to 800 $\mu g.g^{-1}$ dry weight in wheat. These differences in LOL and JA concentrations applied on rye, between the present work and other articles likely explain the lower DIMBOA content found in this experiment (Kong et al., 2018; Li et al., 2023).

*Table 17: Comparison of rye cultivated alone (R) and rye treated with jasmonic acid (JA) and loliolide (LOL) by measuring different BXDs concentration. Values are means ± SEM for each condition (n=5) and bold values show which condition has higher mean for a particular BXD. Asterisk indicates significant difference between two groups: * P < 0.05, ** P < 0.01, *** P < 0.001 and NS = no significative difference.*

Figure 16	BXD concentration (µg.g^{-1} of fresh biomass)	R vs JA			R vs LOL		
		Rye cultivated alone (R)	Rye treated with jasmonic acid (JA)	Significant difference	Rye cultivated alone (R)	Rye treated with loliolide (LOL)	Significant difference
A	DHBOA-Glc	208.7 ± 94.83	**535.9 ± 90.81**	*	208.7 ± 94.83	**558.1 ± 126.0**	*
B	DIMBOA-Glc	431.6 ± 44.88	**471.9 ± 65.45**	NS	**431.6 ± 44.88**	347.5 ± 80.93	*
C	HMBOA-Glc	**87.96 ± 6.107**	79.38 ± 10.04	NS	**87.96 ± 6.107**	51.45 ± 12.99	NS
D	DIBOA-Glc	**3.327 ± 0.6311**	3.045 ± 0.5695	NS	**3.327 ± 0.6311**	2.076 ± 0.733	NS
E	HBOA-Glc	22.37 ± 11.53	**31.76 ± 5.239**	*	22.37 ± 11.53	**26.26 ± 4.754**	NS
F	DIMBOA	4.366 ± 1.189	**7.730 ± 1.366**	*	4.366 ± 1.189	**5.664 ± 1.765**	NS

Besides, it seems that the JA treatment induces more BXDs production than the LOL treatment, although differences are not significant. Indeed, out of the six BXDs detected, five of them (DIMBOA-Glc, HMBOA-Glc, DIBOA-Glc, HBOA-Glc and DIMBOA) are found at a higher concentration when rye is treated with jasmonic acid (JA) than loliolide (LOL) (Table 18). Those results might seem surprising as previous works have demonstrated that DIMBOA levels are similar when LOL and JA are applied at a low concentration and at a medium concentration respectively (Kong et al., 2018). Also, loliolide has shown higher soil mobility which facilitates its movement in the rhizosphere and thus, its potential effect on allelochemicals production (Kong et al., 2018). Nevertheless, Kong et al. (2018) worked with wheat while rye was used in the present work. It may therefore be possible that wheat and rye do not react the same way when LOL or JA is applied to their roots. The ability of loliolide to induce the production of various allelochemicals (including DIMBOA) in wheat is hypothesized to depend partially on jasmonic acid biosynthesis pathway (Li et al., 2023). LOL and JA pathways thus seem interconnected *in plantae*. Gaining more insight on these interconnections might help to understand why JA induces more BXDs production in rye than LOL, while the opposite is observed in wheat (Kong et al., 2018).

Table 18: Comparison of rye treated with loliolide (LOL) and rye treated with jasmonic acid (JA) by measuring different BXDs concentration. Values are means ± SEM for each condition (n=5) and bold values show which condition has higher mean for a particular BXDs. NS = no significative difference.

Figure 16	BXD concentration (µg.g^{-1} of fresh biomass)	Rye treated with loliolide (LOL)	Rye treated with jasmonic acid (JA)	Significant difference
A	DHBOA-Glc	**558.1 ± 126.0**	535.9 ± 90.81	NS
B	DIMBOA-Glc	347.5 ± 80.93	**471.9 ± 65.45**	NS
C	HMBOA-Glc	51.45 ± 12.99	**79.38 ± 10.04**	NS
D	DIBOA-Glc	2.076 ± 0.733	**3.045 ± 0.5695**	NS
E	HBOA-Glc	26.26 ± 4.754	**31.76 ± 5.239**	NS
F	DIMBOA	5.664 ± 1.765	**7.730 ± 1.366**	NS

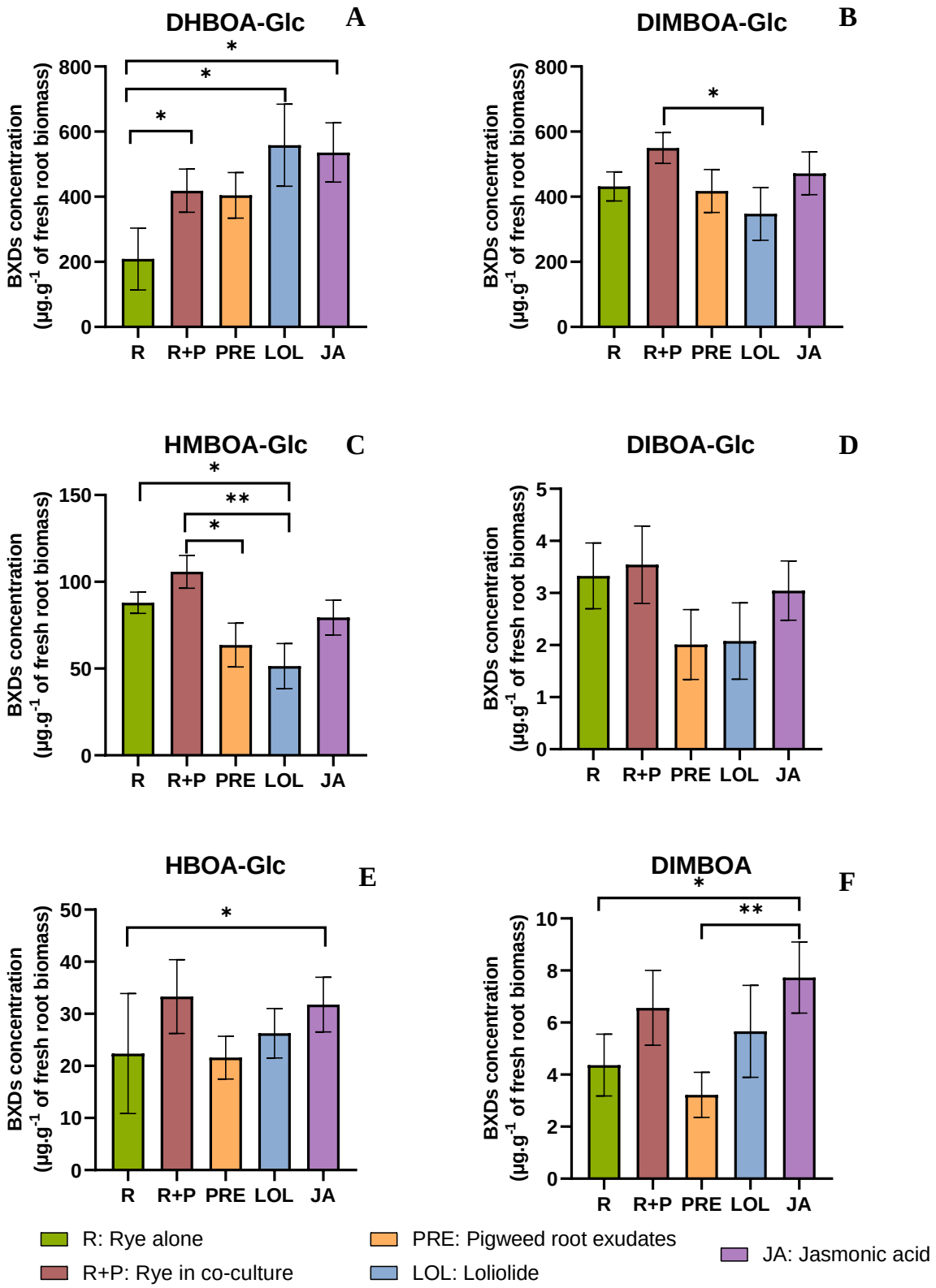


*Figure 16: Concentrations of diverse BXDs ((DHBOA-Glc (A), DIMBOA-Glc (B), HMBOA-Glc (C), DIBOA-Glc (D), HBOA-Glc (E) and DIMBOA (F)) extracted from rye roots (µg.g^{-1} of fresh root biomass). The control is rye grown alone (R) while the different treatment conditions were: rye in co-culture with pigweed (R+P), exogenous application of pigweed root exudates (PRE), loliolide (LOL) at 0.5 nM and jasmonic acid (JA) at 0.5 nM.Asterisk indicates significant difference between two groups: * P < 0.05, ** P < 0.01, *** P < 0.001*

In summary, this part of the work, objective B, explored how pigweed could chemically impact rye root architecture and BXDs production. This was achieved by supplying roots rye with either pigweed exudates or potential signal molecules, that are loliolide and jasmonic acid. Rye root architecture and BXDs levels in rye roots were then evaluated.

Rye in co-culture with pigweed (R+P) showed lower root growth for all root architecture parameters and higher BXDs concentrations for all BXDs compared to rye treated with pigweed root exudates (PRE) (Figure 16). Three main hypotheses arise from these results: (1) the competition effect is higher when the redroot pigweed is physically present in the same soil volume as rye, reducing root growth and enhancing BXDs production, (2) the concentration of the PRE treatment might not have been sufficient to reduce rye root growth and increase allelochemicals concentration in rye root and (3) the low concentration of pigweed root exudates stimulating the rye root growth might be explained by the hormesis effect (An, 2005). In conclusion, the chemical effect on both rye root architecture and BXDs production might have been overcome by the physical competition between rye and pigweed in the R+P situation while in the PRE situation, limited quantities of root exudates, might have reduced it entirely. On the one hand, the competition effect could be tested by cultivating rye and pigweed in the same pot while separating them with a mesh to study effects due to diffusion. Similar techniques have been performed to study whether other weeds are also suppressed by buckwheat and if the presence of weeds is necessary to induce growth repression (Gfeller et al., 2018). By performing this technique, rye and pigweed would not be able to compete for space while root exudates could still be transferred from one plant to another through the mesh. On the other hand, it may be possible to concentrate pigweed root exudates to better observe its effects on rye. Indeed, pigweed root exudates from three pigweeds of 2 weeks old were extracted with 15 mL of water and further mixed with the same volume of Hoagland solution, leading to a diluted treatment. To avoid this dilution effect, it may be possible in the future to dry pigweed exudates using a nitrogen flow/rotary evaporator/lyophilization, and to resuspend these exudates directly in the Hoagland solution. Increasing the number of pigweed in the tube or delaying the root exudate extraction to a 5-leaf stage pigweed may also help to increase the number of compounds and/or the concentration of these compounds extracted from the pigweed rhizosphere. Finally, it would be interesting to also collect rye root exudates (kin) or other weeds exudates (non-kin) such as for pigweed exudates. This would enable further investigation on the kin and non-kin recognition between rye and different weeds along with its effects on rye root architecture and BXDs induction.

Loliolide (LOL) and jasmonic acid (JA) were also investigated as potential signaling molecules from pigweed roots inducing allelochemical responses in rye roots. The impact of their exogenous application on rye roots was evaluated in terms of rye root architecture and rye root content in BXDs. Rye treated with LOL and JA showed lower root growth for all root architecture parameters, particularly for the LOL treatment, as well as higher BXDs concentrations, especially for the JA treatment (Figure 16). This effect of rye root growth inhibition by these compounds seems consistent with the literature as previous studies also demonstrated the root growth inhibition effect of jasmonic acid on sunflower and of loliolide on water hyacinth (Corti Monzón et al., 2012, Kato-Noguchi et al., 2014). Those treatments (LOL and JA) also increase, at a low level, the concentration of three BXDs: DHBOA-Glc (A), HBOA-Glc (E) and DIMBOA (F) in rye roots (Figure 16), which motivates the hypothesis that they could act as potential belowground signaling molecules inducing the production of defensive metabolites (Kong et al., 2018). Synergic effects between JA and LOL should also be investigated in future studies as they were already observed in wheat (Li et al., 2023). It would also be interesting to compare rye with wheat for BXDs production, as the results of the present work globally contrast with those published for wheat (Kong et al., 2018; Li et al., 2023). Finally, it would be worth to analyze the flowering mechanism as few studies showed that loliolide modulates plant belowground defense and aboveground flowering (Li et al., 2023).

3. Objective C: Loliolide detection and quantification

The third objective focus on the quantification of loliolide in rye using an HPLC-UV. As a reminder, a few parameters have been modified in order to optimize loliolide extraction and detection. Loliolide was extracted from wheat and rye. In this experiment, wheat act as a positive control as Li et al. (2023) were able to find between 0.45 and 0.6 $\mu g.g^{-1}$ dry weight of loliolide at an early growth stage (1- or 3-leaf stage).

Nonetheless, method sensitivity was expected to be an issue. In this view, plants were grown with glass microbeads but also in soil, as soil culture allows the production of more leaves and thus, the obtention of more plant material. Furthermore, all the roots/leaves from plants belonging to the same treatment, approximately ten plants grown alone in a SPE tube, were combined to obtain enough plant material (250 to 500 mg). Extraction was performed on either 250 mg fresh plant material (FW1), 500 mg fresh plant material (FW2) or 1500 mg fresh plant material that was freeze-dried before extraction (LYO). The latter allows for sample concentration before extraction. These samples were then extracted either with acetonitrile: water: formic acid (90: 9: 1, v/v/v) (S1) or cold isopropanol: formic acid (99.5: 0.5, v/v) (S2) (Glauser et al., 2013 and Wang et al, 2023).

Sample content in loliolide was then analyzed in HPLC-UV, with 220 nm as wavelength (Wang et al., 2023). Peak identification was mainly based on the retention time of an external standard of loliolide at various concentrations. However, some samples presented a peak with a retention time slightly different than the loliolide standard. Therefore, standard addition method was performed on two different sets of samples and chromatograms from the same sample before (blue curve) and after standard addition (red curve) were overlapped for comparison. From this comparison (Figure 17), the peak observed before standard addition does not seem to correspond to loliolide. In this view, some samples likely contain a compound which also absorbs at 220 nm and has a retention time close to loliolide. This standard addition experiment also allows to confirm that the slight difference in retention time is not caused by improper column equilibration.

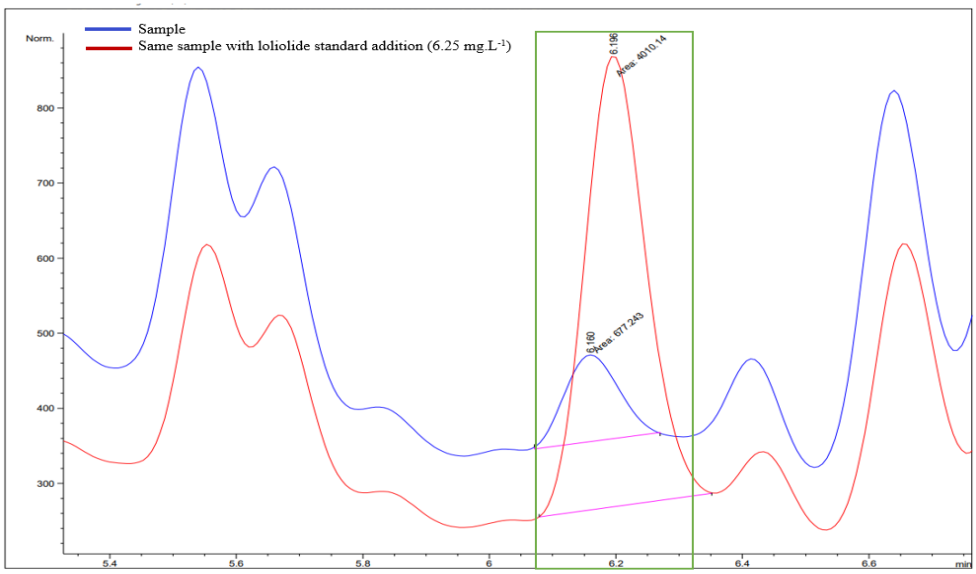


Figure 17: Overlapping of two chromatograms of the same sample before (blue curve) and after standard addition (red curve). The loliolide peak is observed at 6.19 min.

Moreover, this "loliolide" peak is low, usually close to the noise limit and closely surrounded by other higher peaks. Because of that, the peak does not appear clearly on the chromatogram, which further

hinders loliolide detection. As a mean to solve this issue, 1 500 mg of fresh material was harvested, instead of 250-500 mg, and then freeze-dried to concentrate samples before extraction. For the same purpose, the resuspension volume was changed from 400 to 300 µL and a higher injection volume was used. However, these modifications did not improve the appearance of chromatograms.

Finally, it must be noted that a peak with a very similar retention time to loliolide can be observed in blanks (Figure 18.A). This may even more enhance the above-mentioned issues as this implies that even if loliolide is detected in some samples, this may be due to the resuspension solvent itself and not the sample. Indeed, the blanks only contained the resuspension solvent, which consists of methanol: water (50/50, v/v). Most likely, this peak results from contamination during sample preparation. As standards also have been diluted with the same solvent mix, the more diluted standard with lower concentrations of loliolide such as 0.8 µg.mL^{-1} presented an absorption at 220 nm being loliolide and another one between 240 and 280 being the hypothetical contamination (Figure 18.B and 18.C). In contrast to this resuspension solvent, the injection of pure methanol or pure water did not issue such a peak. It is therefore hypothesized that the contamination comes from the glassware used to prepare the resuspension solvent. As a consequence of the above-mentioned issues and as no statistical test was performed, the results regarding loliolide quantification from samples must be discussed cautiously.

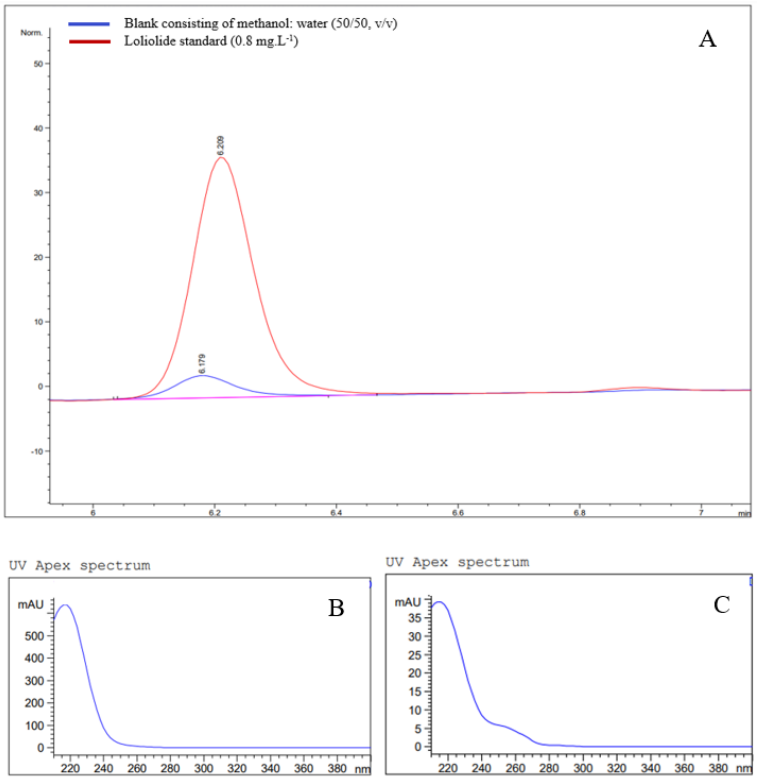


Figure 18: (A) Chromatograms of a blank consisting of methanol: water (50/50, v/v) (blue curve) presenting a peak at 6.179 min and a loliolide standard at a concentration of 0.8 µg.mL^{-1} (red curve) presenting a peak of loliolide at 6.2 min. (B) UV-spectrum of a loliolide standard at a concentration of 6.25 µg.mL^{-1} representing the absorption of loliolide at 220 nm. (C) UV-spectrum of a loliolide standard at a concentration of 0.8 µg.mL^{-1} representing the absorption of loliolide at 220 nm and an unknown compound between 240 and 280 nm.

According to Table 19, loliolide might have been detected in rye leaves (six out of 12 samples), wheat leaves (one out of 12 samples) and wheat roots (two out of two samples). Loliolide could not be detected in rye roots in this experiment. All the samples have a concentration higher than the limit of detection which is 0.15 µg.mL^{-1}, while all samples except for b and e, have higher concentrations than the limit of quantification which is around 0.42 µg.mL^{-1} (Table 19). The concentration of loliolide in wheat roots

extracted with the mix of acetonitrile, water and formic acid (a), which is around 0.73 µg.g^{-1} of dry weight, matches the results obtained by Li et al. (2023).

In rye leaves, five out of the six samples were treated with the extraction solution based on acetonitrile, water and formic acid (S1). Thus, it can be hypothesized that this solution extracts more loliolide at a higher concentration. Nevertheless, it should be mentioned that the extraction solution is not yet fully optimized as a lot of different compounds are extracted at the same time as loliolide as shown in Figure 18. However, an additional purification step in the sample preparation, using a C18 Sep-Pak cartridges for example, would reduce the noise from the other compounds extracted (Wang et al., 2023). Dry rye leaves (LYO) which have undergone lyophilisation seem to have higher concentrations of loliolide compared to fresh rye leaves (i vs h). Some samples show similar loliolide concentrations (e, g and h) to Li et al. (2023) results, with concentrations between 0.15 to 0.67 µg.g^{-1} of dry weight, while others (d, f and i) have greater concentrations at around 1.5 to 8.8 µg.g^{-1} of dry weight. The higher concentrations might come from contaminations which are released at the same time as loliolide and thus, are overestimating the real amount of loliolide present in rye leaves. As side note, loliolide has also been found in pigweed leaves at a concentration of 3.02 µg. g^{-1} of dry weight which might also have been overestimated because of contamination.

Table 19: Loliolide concentration (µg.g^{-1} of dry weight) measured for wheat and rye in different conditions: organs (roots or leaves), substrate (microbeads of glass or soil), fresh or freeze-dried, quantity of fresh biomass (0.25, 0.5 or 1.5 g) and extraction solution (S1 = acetonitrile:water:formic acid or S2 = isopropanol:formic acid). Only the samples with assumed loliolide are shown in the Table.

Plants	Organs	Substrate	Fresh or Freeze Dried	Biomass name	Fresh biomass (g)	Dry biomass (g)	Extraction solution	Sample name	Loliolide concentration (µg.g^{-1} of dry weight)	ID
Wheat	Roots	Microbeads of glass	Fresh	FW2	0.4	/	S1: Acetonitrile: water: formic acid (90:9:1, v/v/v)	BR_S1	0.7277	**a**
					0.4	/	S2: isopropanol: formic acid (99.5:0.5, v/v)	BR_S2	0.2959	**b**
	Leaves	Soil	Freeze-dried	LYO	2.5804	0.3819	S2: isopropanol: formic acid (99.5:0.5, v/v)	BT_LYO_S2	0.5737	**c**
Rye	Leaves	Soil	Fresh	FW1	0.2713	/	S1: Acetonitrile: water: formic acid (90:9:1, v/v/v)	ST_FW1_S1	1.3616	**d**
				FW2	0.514	/	S1: Acetonitrile: water: formic acid (90:9:1, v/v/v)	ST_FW2_S1	0.1605	**e**
			Freeze-dried	LYO	2.501	0.3375	S1: Acetonitrile: water: formic acid (90:9:1, v/v/v)	ST_LYO_S1	7.8311	**f**
					2.5063	0.3444	S2: isopropanol: formic acid (99.5:0.5, v/v)	ST_LYO_S2	0.6248	**g**
		Microbeads of glass	Fresh	FW1	0.2547	/	S1: Acetonitrile: water: formic acid (90:9:1, v/v/v)	SA_FW1_S1	0.6676	**h**
			Freeze-dried	LYO	1.0026	0.196	S1: Acetonitrile: water: formic acid (90:9:1, v/v/v)	SA_LYO_S1	8.8649	**i**

In summary, the method of loliolide extraction and quantification has been optimized to some extent. The quantity of fresh materials between 250 (FW1) and 500 (FW2) mg does not have a huge influence on the quantity of loliolide extracted as similar concentrations were obtained. Even though freeze-dried samples (LYO) showed higher concentration, it cannot be determined whether or not it is because of higher loliolide quantity or the presence of other compounds which are released at the same time and absorb at the same wavelength as loliolide. Likewise, the samples treated with the extraction solution made of acetonitrile, water and formic acid (S1) showed higher concentrations than samples treated with a solution of isopropanol and formic acid (S2), whether it is caused by an optimal loliolide extraction or other compounds extraction. As mentioned above, running the sample with an uncontaminated resuspension solution and adding a purification step would reduce the noise signals and the presence of other compounds. Even with those additional steps, HPLC-UV might not be the correct technique to quantify loliolide. Indeed, the loliolide concentration in rye root and leaves is too low and too close to the noise signal. It is not possible to confirm the presence of loliolide with an HPLC-UV as a lot of compounds also absorb at 220 nm, using an HPLC coupled with a photodiode-array might help to

validate the loliolide spectrum. The best option would be to quantify loliolide by HPLC coupled with a mass spectrometer to confirm the presence of loliolide based on its mass, which would give more accurate results. An entire master thesis dedicated to the optimization of loliolide extraction and quantification would help to validate the hypotheses suggested in the present work.

PART V – CONCLUSION AND PERSPECTIVES

The present master's thesis investigated the physical and chemical interactions between rye (*Secale cereale* L.) and redroot pigweed (*Amaranthus retroflexus* L.) along with the presence and the role of potential signaling molecules (loliolide and jasmonic acid) on rye root architecture and allelochemicals (BXDs) production. Especially, the continuous and exogenous application of loliolide and jasmonic acid at low dosage, similar to real soil conditions, has never been performed on any allelopathic crop before with such detailed BXDs profile characterization associated with complete root architecture analysis. This thesis also focused on studying a new growing substrate whose properties are closer to agricultural soil features by analyzing its effects on root architecture and allelochemicals (BXDs) production.

Feedback will be carried out for each objective to assess the progress reached and new discoveries made during this work.

1. Objective A: "Comparison of substrates"

Do *Secale cereale* L. and *Amaranthus retroflexus* L. have better growth in a different culture substrate than glass microbeads? Does it affect the secondary metabolites composition and/or concentration from root exudates in the rhizosphere and their chemical analysis?

Both rye (*Secale cereale* L.) and pigweed (*Amaranthus retroflexus* L.) had greater growth in the clay and attapulgite mixture, as indicated notably by higher root length, root surface area, root volume, number of root tips, root length density, root surface area density, shoot biomass and specific root length in root architecture. These differences in plant growth could be explained by substrate differences in particle size, water retention and/or pore space between particles. But while clay and attapulgite mixture enhanced plant growth, fewer BXDs were detected from plants cultivated in this substrate. Furthermore, as an inert substrate, microbeads of glass amplify the differences between growing modalities as more non-glucosylated/active BXDs were detected in co-culture while glucosylated/non-active BXDs were identified when rye was cultivated alone. Those observations may be partially due to the sorption capacity of clay compared to glass microbeads. This experiment demonstrates the effect of different characteristics of particles on root architecture and plants. These findings further support the critical importance of the physiochemical properties of soils when investigating plant morphology and plant chemistry such as allelopathy.

Analyzing root exudates from the rhizosphere seems, according to the above conclusions, optimal with microbeads of glass substrate. Thus, using glass microbeads with higher particle size (>1mm) might help to enhance plant growth, especially redroot pigweed which appears to suffer from abiotic stress, while maintaining an inert sorption-free system for BXDs chemical analysis. Nonetheless, the study of rye roots and leaves extract could be easily performed in the clay and attapulgite mixture with additional cleaning steps to remove any dust. Accordingly, a comparison of the root architecture and BXDs production and release, between rye grown in the clay and attapulgite substrate and rye directly cultivated in the field, could investigate to what extent this substrate is close to the soil profile in reality. In opposition to lab experiments, rye is not only influenced by neighboring plants but also by soil micro-organisms, insect-plant interactions and abiotic factors. Studying the impact of all these interactions on the root architecture and BXDs regulation and biosynthesis would provide a basis for future field applications.

2. Objective B: “Treatment incubation”

Do the *Amaranthus retroflexus* L. root exudates, jasmonic acid and loliolide influence rye root architecture and does it induce the synthesis of defensive metabolites from the family of benzoxazinoid in rye roots?

2.1.Effect of physical or chemical presence of pigweed or pigweed root exudates on rye

Rye treated with pigweed root exudates showed greater root growth for almost all root architecture parameters and lower BXDs concentrations for all BXDs compared to rye grown in co-culture with pigweed, which lead to the rejection of the HB2 hypothesis. Nevertheless, pigweed root exudates induced similar changes in root architecture and, in a subtler way, in BXDs concentrations than rye cultivated alone. Three main hypotheses arise from these results: (1) the competition effect is higher when the redroot pigweed is physically present in the same soil volume as rye, reducing root growth and enhancing BXDs production. To confirm this hypothesis, the competition effect could be studied by cultivating rye and pigweed in the same pot while separating them with a mesh to analyze the effects due to diffusion and not physical presence (Gfeller et al., 2018); (2) the concentration of the PRE treatment might not have been sufficient to reduce rye root growth and increase allelochemicals concentration in rye root. To concentrate the root exudates, pigweed exudates could be dried using a nitrogen flow, a rotary evaporator or through lyophilization, and then being resuspend directly in the Hoagland solution. Increasing the number of pigweed in the tube or delaying the root exudate extraction to a 5-leaf stage pigweed may also help to increase the number of compounds and/or the concentration of these compounds extracted from the pigweed rhizosphere; (3) the low concentration of pigweed root exudates stimulating the rye root growth might be explained by the hormesis effect (An, 2005).

In conclusion, the physical competition between rye and pigweed when grown in co-culture might have overcome the potential effect of chemicals while in the case of pigweed root exudates, limited quantities of root exudate might have reduced the entire chemical effect on both rye root architecture and BXDs production. To further investigate those hypotheses and their link with competition and/or non-kin recognition, it would be interesting to also collect rye root exudates (kin) or other weeds exudates (non-kin) such as for pigweed exudates. Characterizing the chemical profile of pigweed root exudates, such as signaling molecule (e.g., loliolide or jasmonic acid) by LC-MS would give further explanation on how root exudates influence rye root architecture and allelochemicals production.

2.2.Effect of loliolide and jasmonic acid on rye

Rye treated with a low dose corresponding to 0.5 nM of loliolide and jasmonic acid showed lower root growth for all root architecture parameters as well as higher BXDs concentrations for DHBOA-Glc, HBOA-Glc and DIMBOA, which confirm hypotheses HB3 and HB4. In opposition to other papers such as Kong et al. (2018) and Li et al. (2023), the most abundant BXD was DHBOA-Glc instead of DIMBOA. Further research into the biosynthesis pathway of certain BXDs in rye, which is still poorly understood, would help to completely interpret those results. However, the increase of BXDs concentration motivates the hypothesis that loliolide and jasmonic acid could act as potential belowground signaling molecules inducing the production of defensive metabolites. It is still important to mention that loliolide and jasmonic acid showed different effects on rye roots in this experiment. While loliolide had more effect on root architecture, jasmonic acid had greater effect on BXDs concentration, which rejects the hypothesis HB5. Synergic effects between JA and LOL should also be investigated in future studies as they were already observed in wheat (Kong et al., 2018). Finally, it would be worth to analyze not only root architecture but also the flowering mechanism as few studies showed that loliolide modulates plant belowground defense and aboveground flowering (Li et al., 2023).

3. Objective C: “Loliolide detection and quantification”:

Does *Secale cereale* L. produce loliolide in its roots and shoots? If it does, is it possible to detect and quantify loliolide by HPLC-UV analysis?

The method of loliolide extraction and quantification has been optimized to some extent. The quantity of fresh materials does not seem to have a huge influence on the quantity of loliolide extracted as similar concentrations were obtained. However, the ratio quantity of fresh biomass and quantity of extraction solution could be improved. The best extraction solution was the one made of acetonitrile, water and formic acid (90:9:1, v/v/v). An additional purification step would reduce the noise signals and the presence of other compounds, which happened in the present work. If those hypotheses are confirmed and loliolide could be detected, the next step would be to validate the analytical method by evaluating the method's specificity, linearity, limit of detection, limit of quantification, accuracy, and precision to ensure that the results obtained are accurate, precise and reliable. If the additional steps do not help to improve loliolide quantification by HPLC-UV, it could be explained by HPLC-UV sensitivity which might not be strong enough for low amount of loliolide present in plants or because the wavelength use in this experiment is not specific enough. In that case, it can be considered to use a (HP)LC coupled with a photodiode-array detector, to confirm the loliolide spectrum or a (HP)LC coupled with a mass spectrometer to confirm the presence of loliolide based on its mass, which would give more accurate results. An entire master thesis dedicated to the optimization of loliolide extraction and quantification would help to validate the hypotheses suggested in the present work.

To complete this study, it would be interesting to pursue similar experiments for both rye and wheat, as the results of the present work, especially for BXDs, globally contrast with those published for wheat (Kong et al., 2018; Li et al., 2022). On the one hand, repeating the application of loliolide and jasmonic acid at low dose on both plants would determine which BXDs are specifically produced by rye. On the other hand, quantifying loliolide in rye and wheat but also in pigweed or other weeds would help to determine whether or not loliolide is a common belowground signal in both allelopathic crops and weeds (Kong et al.,2018). Indeed, Kong et al. (2018) speculated that all species could produce loliolide and the plant response would depend on the variation in level of soil signaling chemicals produced. Complete analysis from a molecular, genetic and enzymatic point of view would provide a better understanding of the overall chemical interactions between rye and pigweed or wheat and pigweed. Finally, whether it is for fundamental research or for a field application, a better understanding of the mechanisms involved in potential signaling molecules production and their effect on BXDs production in rye would help limit the use of herbicides by inhibiting weed growth and thus, avoiding yield losses.

In conclusion, the results obtained, for the allelochemical and root architecture responses to loliolide and jasmonic acid exogenous application, as well as their quantification in rye, are promising. Nonetheless, it is extremely difficult to isolate chemical-mediated belowground signaling interactions or to determine their actual effects due to the complexity of plant-soil and plant-plant interactions. It is, therefore, necessary to further characterize those soil-borne chemical signals and their effects in order to develop, in the near future, a practical application in the field to reduce weed growth.

PART VI – PERSONAL CONTRIBUTIONS

To start with, I investigated the literature on plant-plant interactions, allelopathy and more precisely the belowground interactions inducing signaling molecules between plants. In that manner, I could elaborate on precise objectives and experimental protocols to pursue the present master's thesis, in agreement with my supervisors. Afterwards, I carried out my experiments in the appropriate laboratories and adjusted my experimental plan if necessary. The BXDs analysis by UHPLC-MS has been, partially, carried out by Dr. Gaétan Glauser from the University of Neuchâtel. At the end of my lab work, I processed the data generated and analyzed its statistical significance when applicable. Finally, I assembled the state of the art, the objectives and hypotheses, the protocols along with the results and their discussion altogether in the present work. This master thesis is therefore entirely written by myself, however, revised comments made by my supervisors have been considered.

PART VII – BIBLIOGRAPHY

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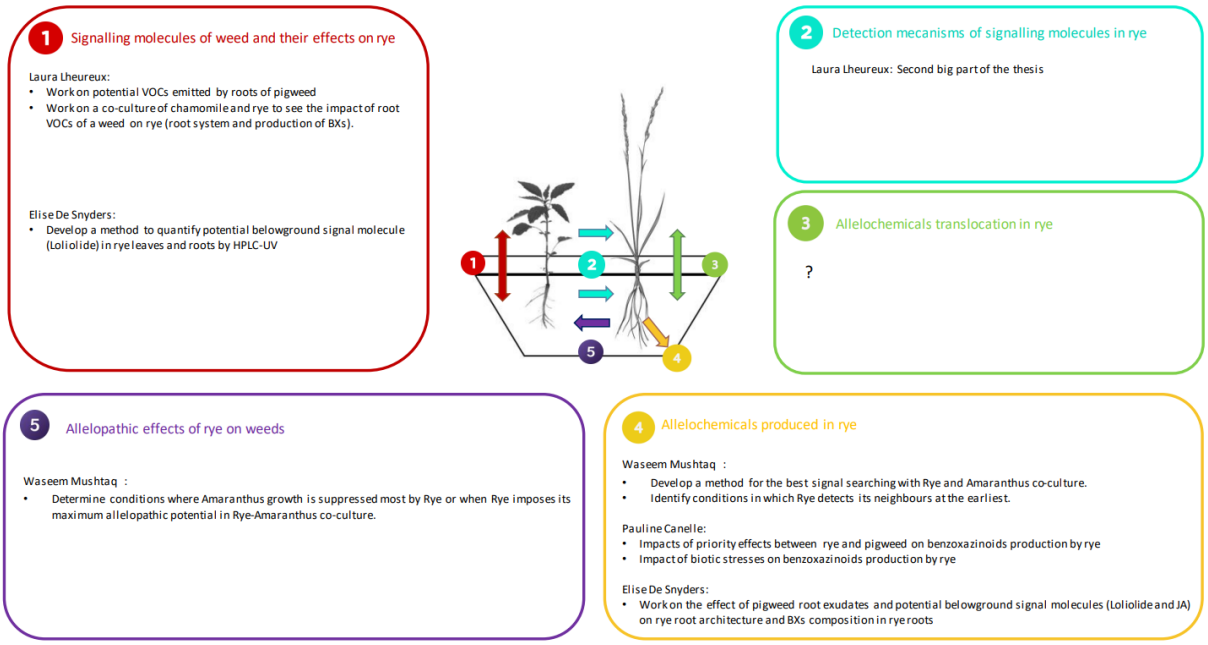
PART VIII – APPENDIXES

Appendix 1: Global scheme of the allelopathy project



Appendix 2: Picture of the two substrates : mix of clay and attapulgite (left) and microbeads of glass (right)

Appendix 3: Seeds sowing and watering per modality and objective

Objective	Modality	Nbr of seeds before germination	Nbr of seeds after germination
A, B, C	Rye alone	Rye: 2 seeds	Rye: 1 seed
A, B	Rye and pigweed in co-culture	Rye: 2 seeds	Rye: 1 seed
		Pigweed: 10 seeds	Pigweed: 5 seeds
B	Pigweed alone	Pigweed: 10 seeds	Pigweed: 5 seeds
C	Wheat alone	Wheat: 2 seeds	Wheat: 1 seed

The seeds were covered with three cm of substrate and watered, drop by drop, with Hoagland ½ solution, with 10 mL for the tube filled with microbeads and 25 mL for the one filled with the mixture of clay beads, with a serological pipette (Greiner, catalog nbr: 7760180). A black plastic bag was wrapped around the tubes to imitate the belowground darkness until the seeds germinated. The tubes, covered with the bag, were placed in a growth chamber. After 48h, the black cover was removed and only five seeds of pigweed and one seed of either rye or wheat were kept in the tube. Every day, the tubes were weighed before and after watering in order to quantify the daily water evaporation. The amount of Hoagland ½ Solution added per day and per tube was based on the water evaporation which was generally around 2.5 mL for the glass microbeads while it was around 5 mL for the clay and attapulgite substrate. In that respect, water and nutrient supplies were optimal and the plants were not suffering from any deficiency.

Appendix 4: Nutrient's list and their concentrations

The solution was prepared as follows: 0.815 g of the Hoagland powder was dissolved in 900 mL nanopure sterile water. The optimal pH at 5.8 was reached by adding a few drops of NaOH solution at 1 M. Finally, 100 mL of nanopure water was added to reach a total volume of 1 L.

Hoagland's No. 2 Basal Salt Mixture 100%	**mg.L^{-1}**
Ammonium phosphate Monobasic	115.03
Boric acid	2.86
Calcium citrate	656.4
Cupric sulfate.5 H_2O	0.08
Ferric tartrate.2 H_2O	5.32
Magnesium sulfate	240.76
Manganese chloride.4 H_2O	1.81
Molybdemun trioxyde	0.016
Potassium nitrate	606.6
Zinc sulfate.7 H_2O	0.22

Appendix 5: Treatments for pre-test: conditions and preparation

As a reminder, a pre-test was carried out to find out which rye development stage was the most sensitive to the different treatments by analyzing the root architecture. For the pre-test concerning the treatment application, pigweed root exudate and jasmonic acid have been tested. For a matter of cost and non-optimal conditions, loliolide hasn't been used in the pretest. The pigweed root exudates were applied one-time at different rye development stages: day 0 (seed stage), day 3 (seedling stage 1) and day 6 (seedling stage 2) but also continuously starting at day 0 (seed stage). Whereas, highly concentrated jasmonic acid was applied only one-time at different rye development stages: day 0 (seed stage), day 3 (seedling stage 1) and day 6 (seedling stage 2) but not continuously. After 10 days, the rye root architecture has been analyzed using WinRHIZO™ Image.

a) Pigweed root exudates

For a one-time treatment, 5mL of the thawed pigweed root exudates solution (*cfr* section 2.4.1) was directly added to the surface of the substrate with a serological pipette. Whereas for the continuous daily treatment, the pigweed root exudates solution was mixed with Hoagland 1 Solution (1:1, v/v) and the volume of treatment solution depended on the daily water evaporation which was around 2.5 mL per day and tube in glass microbeads substrate.

b) Jasmonic acid at 50 µM

The concentration of JA has been selected according to Li et al. (2023) latest results. At first, JA was dissolved in a small amount of pure methanol for a final concentration of 10 $mg.mL^{-1}$. Then, 157 µL of the JA solution was diluted with nanopure water to obtain a final concentration of 150 µM in 50 mL. To obtain a final JA concentration of 50 µM in the SPE tube, a solution of 150 µM has been prepared to compensate the dilution induced by the 15 mL Hoagland solution already present in the tube. The JA treatments were stored at -80°C and thawed before application. For the pre-test, 5 mL of the 150 µM JA solution was applied on-time at different rye development stages (0, 3 and 6 day-old) on the surface of the glass microbeads with a serological pipette.

Appendix 6: Detailed protocol for BXDs extraction from rye roots

Frozen rye roots were ground to a fine powder in liquid nitrogen using a mortar and pestle. The freeze-dried powder is weighed and 25 mg of each sample is placed in Eppendorf tubes with safety closure. Then, the powder is mixed with 1 mL of extraction solvent (methanol/water/formic acid; 1/1/0.5%; v/v/v) (Methanol for spectroscopy,Merck Uvasol® catalog number: 1.06002 ; Formic acid ≥99%, VWR, HiPerSolv Chromanorm® for LC-MS, catalog number: 84865.260). The final solution is vigorously mixed. Five to six glass beads (HONITE 09, 250-425 nm) (<1 mm) are added to each tube and, samples were extracted for 3 minutes at 30 Hz in a TissueLyser II (Qiagen, Hilden, Germany). Then, samples were centrifuged at 12,000 g for 3 minutes (SIGMA 1-14KLaborzentrifugen GmbH, Osterode am Harz, Germany). The supernatant is collected and stored in vials at -80°C until analysis.

Appendix 7: Detailed protocol for BXDs extraction from rye and/or pigweed root exudates

BXDs extraction from the rhizosphere was performed by using an SPE vacuum manifold (Macherey-Nagel™ CHROMABOND™, catalog no:730151) which was connected, on one hand, to the SPE tubes holding the plants and, on the other hand, to a vacuum pump (Buchi Syncore - Model V-300). In order to maintain a constant pressure of 5 mmHg in the glass chamber, the vacuum pump was set at 780 mbar during the extraction process. Plastic valves and stainless-steel needles (Macherey-Nagel™, catalog no: 730152) were placed respectively above and under the SPE vacuum manifold lid. Once the vacuum pressure had been applied, 30 mL of extraction solution, made of acidified nanopure water with 0.5%

formic acid, was injected on the substrate's surface with a serological pipette for 30 seconds. The rhizosphere was rinsed under vacuum pressure for a further 30 seconds. Thus, root exudates were extracted for a total of 1 minute. The root exudates aqueous solutions were collected in 50 mL Falcon tubes placed under the stainless-steel needles. The samples were stored at -80°C.

To prepare the sample for chromatographic analysis, the root exudates in the 50 mL Falcon tube were centrifuged using the Avanti® J-HC centrifuge (Beckman Coulter™, reference nbr 08647). This prior step isn't mandatory if the root exudates are collected from glass microbeads. Nevertheless, the root exudates collected from the substrate of clay beads and attapulgite mixture showed additional dust particles that should be removed. The root exudates were centrifuged for 3 minutes at 12,000g and the supernatant was transferred in a 15 mL falcon tube. The root exudates were, then freeze-dried using the ALPHA 1-4 LSC freeze-dryer (CHRIST, reference nbr 102041) for 96 hours to obtain freeze-dried powder. The dried extracts were resuspended in 1 mL acidified H_2O/Methanol (50:50 v/v; 0.5% formic acid). The extracts were sonicated for 1 minute, vortexed and centrifuged (SIGMA 1-14K Laborzentrifugen GmbH, Osterode am Harz, Germany) for 3 minutes at 12,000g. Finally, the supernatants were transferred in vials and stored at -80°C, ready for analysis.

Appendix 8: Detailed protocol for loliolide extraction from rye and wheat roots and shoots

The roots and shoots of wheat and rye were collected as described above (*cfr* section 2.5.1) at the 3-leaf plant development stage, three hours after the mechanical wounding. The method of loliolide extraction is a mix between two different existing protocols from Glauser et al., 2013 and Wang et al, 2023. Roots and shoots from the same plant were collected together in order to obtain enough fresh biomass. The plant materials were frozen in liquid nitrogen. They were grounded to a fine powder in liquid nitrogen using a mortar and pestle. The freeze fresh material powder was weighed and 250 or 500 mg was placed in Eppendorf tubes with safety closure. Then, the powder was mixed with 1.5 mL of extraction solvents. Additionally, 2.5 g of fresh freeze powder was placed in a 15 mL Falcon tube and freeze-dried for 72h using the ALPHA 1-4 LSC freeze-dryer (CHRIST, reference nbr 102041). The lyophilized powder was mixed with 15 mL of extraction solvents. The first solution was a mix of acetonitrile: water: formic acid (90: 9: 1, v/v/v) and the second solution was a mix of cold isopropanol: formic acid (99.5: 0.5, v/v). The final solutions were vigorously mixed. Five to six glass beads (<1 mm) were added to each tube and, samples were extracted for 3 minutes at 30 Hz in a TissueLyser II (Qiagen, Hilden, Germany). To induce a phase separation, 0.25 g of sodium chloride (NaCl) was added to the samples extracted with the mix of acetonitrile: water: formic acid for both 250 and 500 mg of fresh materials whereas 1.25 g of sodium chloride was added in the 1.5 g freeze dried powder samples. Then, samples were centrifuged at 12 000 g for 3 minutes (SIGMA 1-14KLaborzentrifugen GmbH, Osterode am Harz, Germany). The supernatant was collected and stored in a new Eppendorff at -80°C. Then, the samples were transported under dry ice at the Chemistry of Natural Molecules at Gembloux Agro-Bio Tech.

There, the supernatant was dried under nitrogen flow. The residue was resuspended in 300 µL or 400 µL aqueous methanol (1:1, v/v) and sonicated for one minute. The samples were filtered with a 0.45 µm PTFE syringe filter. Then, 200 µL of the filtrate was transferred in vials and stored at -80°C until the HPLC-UV analysis.

Appendix 9: Conditions for the analysis of benzoxazinoids by UHPLC- PDA- TOF-MS

The detection and quantification of BXDs in root exudates, leaf and root extracts were performed using a high-performance liquid chromatography system (Acquity UPLC Waters) coupled with a Synapt G2 time-of-flight mass spectrometer (Waters) and equipped with an Acquity UPLC® BEH C18 1.7µm column (50 mm x 2.1 mm, 1.7 µm). Gradient elution was performed using two solvents: water (H_2O) + 0.05% formic acid as phase A and acetonitrile (MeCN) + 0.05% formic acid as phase B. The gradient

program is shown in the table below. The column temperature was maintained at 25°C. Injection volume was 2.5 µL. UV spectra were acquired over the range from 190 to 400 nm at a resolution of 1.2 nm. The Q-TOF-MS operated in negative electrospray mode. The source parameters were as follows: capillary and cone voltages 2 kV and 40 V, respectively, source temperature 120°C, desolvation flow rate and temperature 900 $L.h^{-1}$ and 400°C, respectively, cone gas flow 50 $L.h^{-1}$. The system was controlled by Masslynx 4.2 (Waters).

Time (min)	Flow rate ($mL.min^{-1}$)	Solution A (%)	Solution B (%)
0	0.4	98.0	2
3.50	0.4	72.8	27.2
4.50	0.4	0	100
5.50	0.4	0	100
5.55	0.4	98	2

Appendix 10: Summary of the pigweed and rye score obtained for each variables combination for all root architecture parameters combined

The score is assigned depending on the p-value and the significance different acquired from a pairwise comparison using Wilcoxon rank sum exact test for each variable combination. “A” stands for the microbeads of glass substrate and “B” stands for the clay and attapulgite substrate.

Modalities*Substrate	Pigweed score	Rye score
Alone*A >< Co-culture*A	1*	0*
Co-culture*B >< Co-culture*A	2*	7*
Co-culture*B >< Alone*A	10*	8*
Alone*B >< Co-culture*A	19*	19*
Alone*B >< Alone*A	21*	20*
Alone*B >< Co-culture*B	12*	0*