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Impact of Alternative Substrates on Plant Growth and Root Exudates in Plant Interactions: A Study on *Secale cereale* L. and *Amaranthus retroflexus* L.

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Abstract: This study investigates the effects of substrate composition on root architecture, plant growth, and allelopathic secondary metabolites, specifically benzoxazinoids (BXs), in the rhizospheres of rye (*Secale cereale* L.) and redroot pigweed (*Amaranthus retroflexus* L.). Given the complexities of root exudate analysis, including the influence of substrate on root morphology and exudation, the experiment compared plant growth and BX release in two substrates: glass microbeads and a mixture of clay beads and attapulgite. Rye, pigweed, and co-cultures of the two were grown under controlled conditions, with root and shoot parameters measured to assess substrate suitability. Additionally, UPLC-QTOF-MS was used to analyze BXs in rye and rye–pigweed co-cultures. The results demonstrated that the clay bead and attapulgite mixture provided better growth conditions and was effective for BX extraction, making it a suitable substrate for studying allelopathy in controlled environments. The findings highlight the critical role of substrate composition in both plant development and the study of root exudates, with implications for better understanding of crop–weed interactions and allelopathy.

Keywords: *Secale cereale*; rye; *Amarathus retroflexus*; benzoxazinoids; substrate composition; allelopathy; root exudates

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1. Introduction

Plants release a wide variety of metabolites via their roots into the soil. These root exudates (REs) include a range of compounds, such as sugars, amino acids, organic acids, phenolics, enzymes, and several other secondary metabolites [1]. REs play crucial roles in plant nutrition, defense against pathogens, interactions with other organisms (such as microbes, fungi, insects, and plants), and soil structure and affect the plant rhizosphere, which is defined as the soil zone influenced by roots and root exudates [2].

Expanding knowledge of the exudome of root systems is subject to several limitations. First, REs exhibit significant chemical complexity, necessitating the choice of extraction methods based on specific research questions. RE concentrations are often very low due to dilution effects, soil sequestration, volatilization, or rapid transformation by soil microorganisms and chemical reactions, making their detection and analysis difficult [3,4]. Local and temporal variability in REs is influenced by a plant's developmental stage, root architecture, and environment and interactions in the soil [5]. Isolation of REs is also a major issue due to the matrices in which roots grow and the variability of soil matrices themselves [6]. For these reasons, several substrates and techniques have been used and developed, such as hydroponics, which allows clean collection of root exudates but does

not represent real conditions of root development and interactions in the soil; growing plants in inert substrates, such as sand, vermiculite, and glass beads, which reduces contamination from soil particles and microorganisms while allowing root development in a matrix that somewhat mimics soil resistance constraints; and growing plants in real or controlled soils [6]. In both inert substrates and soil, experimental setups still rely on the use of dedicated techniques to extract root exudates, either by gently extracting the roots from the soil, washing them, and collecting the root exudates in a liquid or by using systems designed to collect root exudates with minimal disturbance to the root system [7,8]. Finally, identification and quantification are challenging, as databases are less comprehensive for root exudate metabolites than for metabolites originating from other plant parts [9].

Crop–weed interactions are complex and influence crop development and yield due to competition for resources such as light, water, and nutrients, leading to an average productivity loss of 34% [10]. Crop–weed interactions also involve allelopathy, a mechanism in which released compounds affect the germination and growth of neighboring plants [11–13]. Allelopathy triggered via root exudates is difficult to characterize due to technical challenges and the fact that the effects of allelopathy are confounded with or difficult to separate from those related to other environmental factors, such as competition and defense [11,13]. Soil features affect plant morphology, growth, the root microbiome, and rhizosphere chemistry, leading to high variability in target plant responses to allelopathy [14–16]. We aimed to reduce the number of variables by growing plants under controlled conditions in two distinct substrates: a crop known for its allelopathy, rye, and a weed, redroot pigweed. 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. Moreover, pigweed responds well to the presence of another crop by changing the root exudation of the neighboring plant [8] Thus, we evaluated the suitability of RE extraction for one well-known family of allelochemicals, the benzoxizanoids (BXs) [17,18]. BXs are a family of plant secondary metabolites produced by several gramineous species like maize, wheat, and rye and present a strong allelopathy effect against several weed species. The two main BXs are 2,4-dihydroxy-7-methoxy-(2H)−1,4-benzoxazin-3(4H)-one (DIMBOA) and 2,4-dihydroxy-(2H)−1,4-benzoxazin-3(4H)-one (DIBOA), which are present in plants in a glycosylated form (Glc) and released into soil as aglycones are, in turn, transformed in the soil chemically or by microorganisms into a complex array of metabolites [18]. Even in a controlled environment, substrate particle size and chemistry influence root morphology and exudation [19]. These aspects were explored in this article based on the following hypotheses.

H1. *The substrate consisting of a mixture of clay beads and attapulgite offers better growth conditions for both Secale cereale L. and Amaranthus retroflexus L. (redroot pigweed) based on root architecture and shoot parameters than glass beads.*

In root exudate analysis, glass microbeads are often employed as a growth substrate due to their inert nature and uniform sphere diameters. However, in previous internal studies in Agroscope, *Amaranthus retroflexus* L. (redroot pigweed) appeared to face growth challenges in this medium, as proven by low seed germination rates, elongated stems, and distorted leaves. Consequently, a new growth substrate, consisting of a blend of clay beads and attapulgite, was tested. This substrate is noted for its ability to support plant growth more effectively [20]. The experiment aimed to analyze different root parameters of rye and pigweed to determine whether the plants grow better in a substrate of glass microbeads or in a mixture of clay beads and attapulgite. Therefore, rye (R), pigweed (P), and rye in co-culture with pigweed (R + P) were grown in the two substrates, and various root and shoot parameters were analyzed.

H2. *The substrate of clay beads and attapulgite is suitable for the analysis of secondary metabolites such as BXs present in the root exudates of rye.*

Moreover, the root exudates of rye (R) and rye in co-culture (R + P) were analyzed by UPLC-QTOF-MS to measure the BX composition and concentration in each substrate to determine whether the substrate influences the release of allelochemicals in the rhizosphere or their extractability.

2. Materials and Methods

2.1. Plant Growth

Plants were cultivated in solid-phase extraction tubes (SPEs) of 60 mL (BondElut Straight Barrel, catalog no. 12131018, Agilent Technologies, Palo Alto, CA, USA) covered with black plastic film on the outside to avoid direct light exposure. Frits of 20 µm (Catalog no. 1:131012, Agilent Technologies, Palo Alto, CA, USA) were set at the bottom of the tube to prevent the roots from growing outside the tube and to retain the growth substrate. Approximately 105 g of glass microbeads (Guyson, Honite 09; 250–425 µm, DM CONSEIL La Chaux-de-Fonds, Switzerland; referred to as substrate (A)) was added to the SPE tubes, while 85 g of a mixture of equal volumes of clay beads (Sorbix US-Special G, Damolin, Etrechy, France) and attapulgite (ARGEX NV, Burcht, Belgium) (referred to as substrate (B)) [20] was moistened with half-strength Hoagland solution (Sigma Aldrich, St. Louis, MO, USA,) adjusted to a pH of 5.8 and autoclaved. One rye seed was sown in every SPE tube for the conditions including rye, whereas ten pigweed seeds were sown in each tube for the conditions including pigweed, and after 3 days of germination three pigweed seeds were left in each tube. The experiment included three plant growth modalities: rye grown alone (R), pigweed grown alone (P), and rye grown in co-culture with pigweed (R + P). Each modality was evaluated in the two distinct substrates, A and B, to assess the effects of substrate type on root architecture and exudation (Figure 1; Table A1).

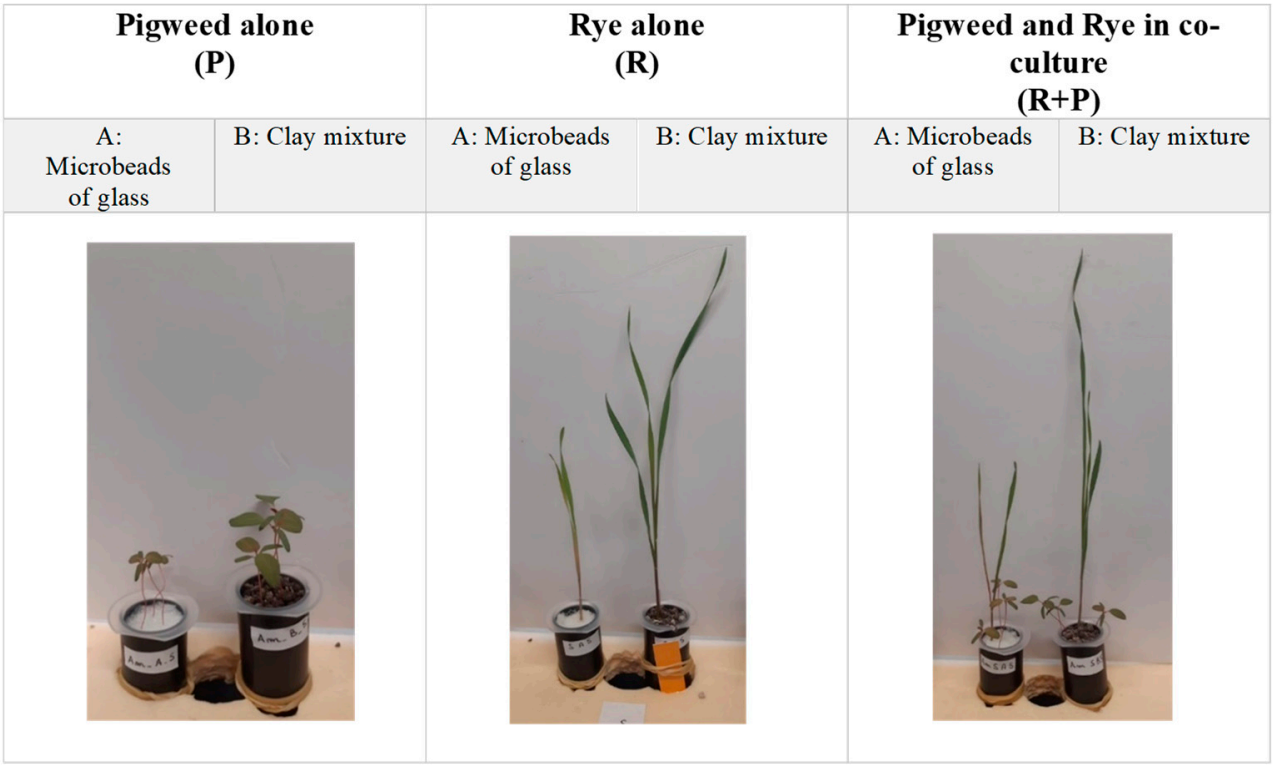

Figure 1. Pictures of pigweed (P) and rye (R) shoots, alone and in co-culture (R + P), in two different substrates: microbeads of glass on the left (A) and clay beads and attapulgite mixture on the right (B) at day 10 after sowing.

Daily watering was performed with half-strength Hoagland solution. The plants were placed in a growth chamber under controlled conditions. The environmental parameters

were set with a photoperiod of 16/8 h at a temperature of 28/24 °C (day/night). The relative humidity was set to 70%, and the light intensity was set to 200 µmol·$(m^2 \cdot s)^{-1}$ (Clitec Phytotron, Aralab, Rio de Mouro, Portugal). All analyses concerning plant root architecture, biomass, and BX extraction from root exudates or roots were carried out 10 days after sowing with N = 5 repetitions, each SPE tube system representing one replicate.

2.2. Root Architecture Analysis

After ten days, the root system was gently separated from the growth substrate to study the root architecture. The root architecture of rye and/or pigweed was analyzed using WinRHIZO™ Image Analysis for Plant Science 2021 (Regent Instruments Inc., Québec, QC, Canada). The following root parameters were studied: length, volume, surface area, diameter, and number of tips. Then, roots and leaves were dried at 50 °C for 48 h. Finally, the total dry root and leaf biomass was determined with an analytical balance. Five additional measures were analyzed: specific root length (SRL), root length density (RLD), root surface area density (RSD), root branching intensity (RBI), and root tissue density (RTD).

2.3. Root Exudate Extraction

Root exudate extraction from the rhizosphere was performed using an SPE vacuum manifold (Macherey-Nagel™ CHROMABOND™, catalog no. 730151, Düren, Germany) connected to the SPE tubes holding the plants and to a vacuum pump (Büchi Labortechnik AG, Flawil, Switzerland). To maintain a constant pressure of 5 mmHg in the glass chamber, the vacuum pump was set to 780 mbar during the extraction process. The pressure was standardized between every sample and through the whole extraction procedure. Plastic valves and stainless-steel needles (Macherey-Nagel™, catalog no. 730152, Düren, Germany) were placed above and under the SPE vacuum manifold lid, respectively. Once the vacuum pressure was applied, 30 mL of extraction solution, made of acidified nanopure water with 0.5% formic acid, was injected onto the substrate's surface with a serological pipette for 30 s, avoiding any contact with the stem and leaves and thus preventing any lixivation from aboveground parts of the plant. The rhizosphere was rinsed under vacuum pressure for a further 30 s. Thus, root exudates were extracted for a total of 1 min. The samples were stored at −80 °C. To prepare the samples for chromatographic analysis, the root exudates were centrifuged. This step was not mandatory for the root exudates collected from substrate (A). Nevertheless, the root exudates collected from substrate (B) showed additional dust particles that had be removed. The root exudates were centrifuged for 3 min at 12,000× *g*, and the supernatant was transferred. The root exudates were then freeze-dried (ALPHA 1-4 LSC freeze-dryer, Martin CHRIST, Osterode am Harz, Germany) for 96 h to obtain a 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 min, vortexed, and centrifuged for 3 min at 12,000× *g*. Finally, the supernatants were transferred into vials and stored at −80 °C, ready for analysis.

2.4. Benzoxizanoid Analysis and Quantification

The detection and quantification of BXs in root exudates were performed using a high-performance liquid chromatography system (Acquity UPLC Waters Corporation, Milford, MA, USA) coupled with a Synapt G2 time-of-flight mass spectrometer (Waters Corporation, Milford, MA, USA) and equipped with an Acquity UPLC BEH C18 1.7 µm column (Waters Corporation, Milford, MA, USA). Mobile phases were composed of 0.05% formic acid in nanopure water (Solution A) and 0.05% formic acid in acetonitrile (Solution B). Gradient elution started with 2% B, increased to 100% B over 5 min, and returned to 2% B over 2 min. The column temperature was maintained at 25 °C. The sample 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 Corporation, Milford, MA, USA).

Data processing was performed using TargetLynx (Waters Corporation, Milford, MA, USA). Calibration curves from the standards DIMBOA-Glc (2-β-D-glucopyranosyloxy-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one), DIMBOA (6,7-dimethoxy-2-benzoxazolinone), HDMBOA-Glc (2-β-D-glucopyranosyloxy-4,7-dimethoxy-1,4-benzoxazin-3-one), MBOA 6-methoxybenzoxazolin-2-one, and HMBOA-Glc (2-β-D-Glucopyranosyloxy-7-methoxy-1,4-benzoxazin-3-one) were prepared in order to calculate the BX concentrations. The concentrations of the calibration points were 0.08, 0.04, 2, 10, and 50 µg/mL for the five BXs. BOA was quantified as MBOA equivalents; DHBOA-Glc (2-(beta-D-Glucopyranosyloxy)-7-hydroxy-2H-1,4-benzoxazin-3(4H)-one), DIBOA-Glc (2-β-D-glucopyranosyloxy-4-hydroxy-1,4-benzoxazin-3-one), and DIM_2BOA-Glc (2-β-D-glucopyranosyloxy-4-hydroxy-7,8-dimethoxy-1,4-benzoxazin-3-one) were quantified as DIMBOA-Glc equivalents; and HDM_2BOA-Glc (2-(2-hydroxy-4,7,8-trimethoxy-1,4-benzoxazin-3-one)-β-d-glucopyranose) was quantified as HDMBOA-Glc equivalents. The predicted retention times and quantification ions of the quantified BXs are described in Table A2. The limit of detection of each BX was set at the same level as the limit of quantification (10*SD/S), which was based on the standard deviation of the response (SD) of the curve and the slope of the calibration curve (S).

2.5. Statistical Analysis

When relevant, statistical tests were performed on data using R within the RStudio environment (Version 2022.12.0+353, RStudio Team, Boston, MA, USA). 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 was carried out to evaluate the statistical significance of the differences between the conditions' means, with differences considered non-significant (p-value > 0.05), significant (p-value $\leq$ 0.05), very significant (p-value $\leq$ 0.01), or highly significant (p-value $\leq$ 0.001). Thus, the effect of two independent variables on two dependent variables was studied. The independent variables were the type of substrate (A or B) and the growth modality (alone or in co-culture). The two dependent variables were the root architecture parameters (e.g., root length) and the BX concentrations (Table A1). The independent variables were analyzed individually (Kruskal–Wallis test) and in combination (Wilcoxon test). Graphics were created with the GraphPad Prism 8 software.

3. Results

This experiment aimed to analyze the effects of two variables: substrate and co-culture, with a focus on substrate comparison. To compare the substrate effect on plant growth and BX characterization from root exudates, two substrates were 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 were tested: pigweed and rye alone (P and R) and in co-culture (R + P). To pursue this aim, both root architecture and BXs were analyzed.

3.1. Root Architecture Analysis

The plants cultivated in both substrates exhibited the same development stage: the two-to-three leaf stage was observed in both rye and pigweed. Visual differences were observed for both shoots and roots (Figures 1 and 2). The leaves of rye and pigweed were more developed when grown in substrate (B) regardless of the co-culture modalities (P/R or R + P). Based on visual observation, pigweed (P) was more developed and had more secondary roots in (B) than in (A). The same observation was made for rye (Figure 2). Additionally, rye grown in substrate (B) and in co-culture seemed to have more root hairs, which could not be measured by WinRHIZO™ Image. A few recent reports indicate that root cap and root hair cells are involved in the secretion of compounds such as allelochemicals [21].

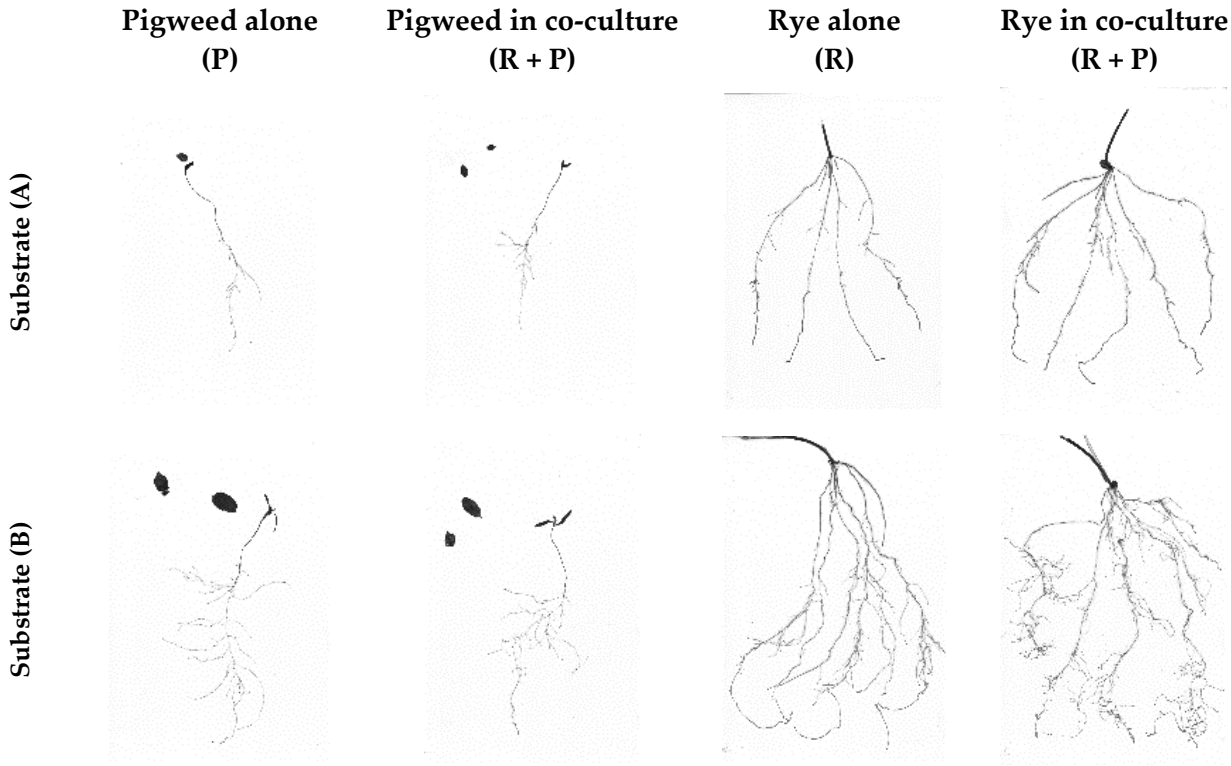

Figure 2. Images of scans of rye and pigweed roots in two different substrates obtained using WinRHIZO™ Basic 2021 software: microbeads of glass (**A**) and clay beads and attapulgite mixture (**B**) at day 10 after sowing.

3.1.1. Rye and Pigweed Root Architecture in Substrates (A) and (B)

Different root parameters were measured in order to compare the two substrates (Figure 3; Tables A4 and A5). For both rye grown alone (R) and in co-culture (R*(R+P)), the parameters presented in Figure 3 are significantly higher than for rye cultivated in (B). This significant difference between the substrates was nonetheless slightly higher for rye cultivated alone (R). However, significantly higher root diameter and branching density were observed when rye was cultivated in (A).

Similar observations were made for pigweed, as shown in Figure 3 and Table A5. Indeed, these parameters showed a significant difference between substrates ((A) vs. (B)) when pigweed was cultivated alone (P). Similarly to rye, all parameters were higher when pigweed was cultivated in (B), except for the root branching density.

3.1.2. Rye and Pigweed Root Architecture in Two Growing Modalities

We investigated how the substrates influenced the differences between the growth modalities (alone or in co-culture) and whether or not one substrate emphasized these 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 4, where less significant differences between modalities can be observed. Indeed, for both pigweed (PA) and rye (RA) cultivated in (A), there was no significant difference between modalities (alone vs. co-culture) for any of the parameters, except for the number of tips of pigweed (Figure 4E). Pigweed cultivated in (A) seemed to have a higher number of root tips when grown in co-culture. Indeed, pigweed cultivated in the glass microbead substrate had, on average, 88.4 and 113.7 tips when grown alone and in co-culture, respectively.

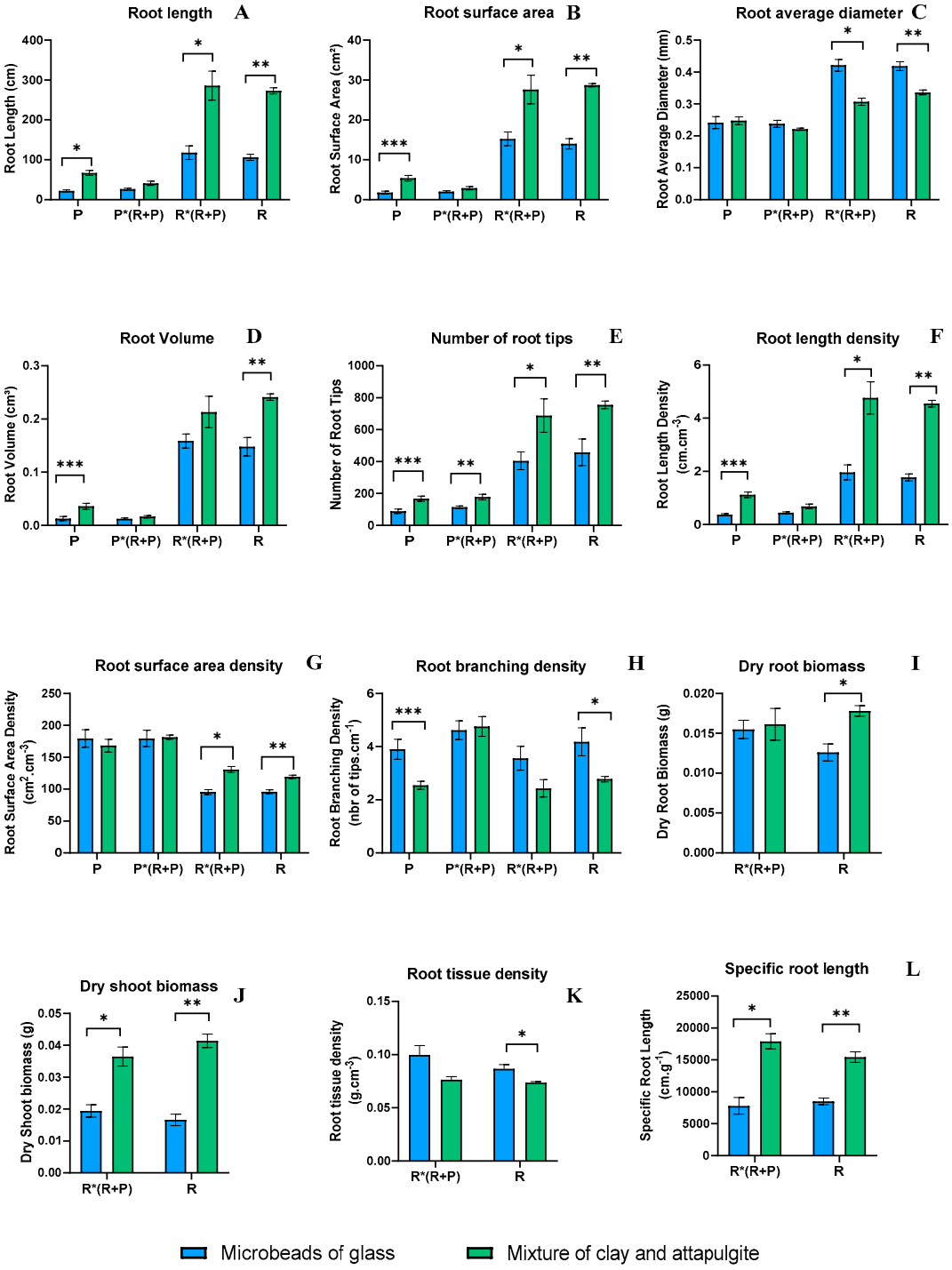

Figure 3. Comparison of two substrates, microbeads of glass (substrate A) and a mixture of clay and attapulgite (substrate B), for rye and pigweed cultivated alone (R or P) and in co-culture (R + P) by measuring different root parameters: root length (**A**), root surface area (**B**), root average diameter (**C**), root volume (**D**), number of tips (**E**), root length density (**F**), root surface area density (**G**), and 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). Asterisks indicate significant differences between two groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

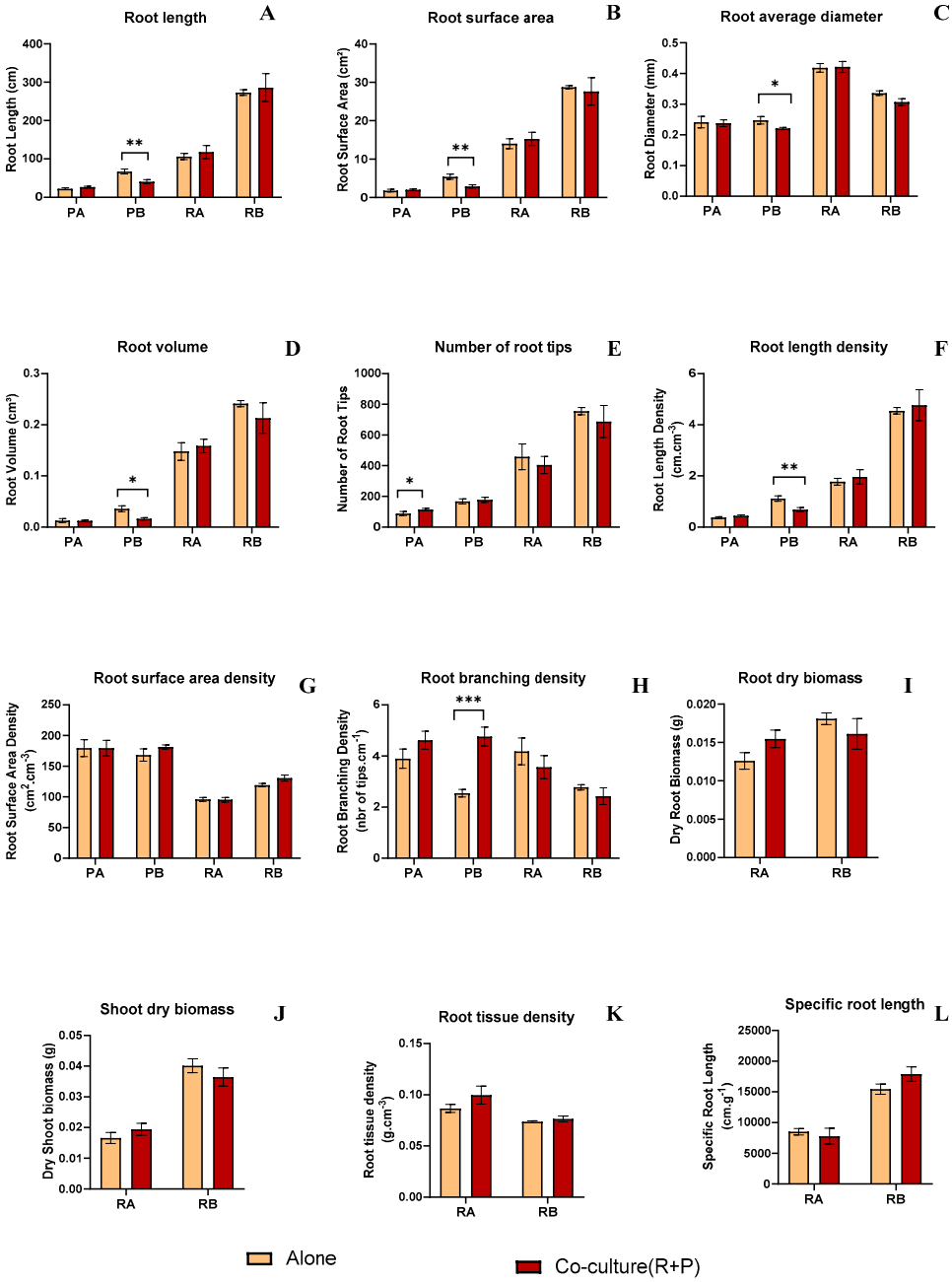

Figure 4. Comparison of two growth modalities, alone and in co-culture, for pigweed and rye cultivated in microbeads of glass (PA or RA) and a mix of clay beads and attapulgite (PB or RB) by measuring different root parameters: root length (**A**), root surface area (**B**), root average diameter (**C**), root volume (**D**), number of tips (**E**), root length density (**F**), root surface area density (**G**), and 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 the clay bead and attapulgite substrate (RB). Asterisks indicate significant differences between two groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

However, pigweed grown in (B) showed significant differences between modalities for all parameters except the number of root tips (Figure 4E) and root surface area density (Figure 4G). It can be noted that various parameters (root length, root surface area, root diameter, root volume, and root length density) were higher when pigweed was cultivated alone in substrate (B) (Figure 4; Table A6). However, pigweed's root branching density was higher when it was cultivated in co-culture (Figure 4H).

When variables were combined and analyzed by performing a pairwise comparison using the Wilcoxon test (Table A3), the main tendencies observed were similar for rye and pigweed. The first tendency with the most significant difference was observed in the plants cultivated alone in the two different substrates (Alone*B >< Alone*A). The substrate in which the plants were cultivated had the biggest influence on plant root architecture when the plants were cultivated alone regardless of the parameter. Another trend which mostly influenced pigweed growth was observed between growth modalities (alone or in co-culture) when pigweed was cultivated in substrate (B) (Alone*B >< Co-culture*B). This interaction showed that the difference between modalities was accentuated in substrate (B).

3.2. Benzoxizanoid Analysis

3.2.1. Benzoxizanoid Analysis in Two Different Substrates

To compare the two substrates ((A) and (B)) and the two growth modalities (alone and co-culture), the BXs extracted from the rhizosphere are shown in Table 1 and Figure A1. Fewer statistical tests could be carried out, especially for (B). When the BX concentration did not reach the limit of detection in at least three replicates, statistical analysis was not pursued and thus not discussed.

Table 1. 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 beads and attapulgite (B) by measuring different BX concentrations. Values are means for each condition, and bold values show which condition had the higher mean for a particular BX. Asterisks indicate significant differences (*p*-values) between two groups: * $p < 0.05$. NS: no significant difference. ND stands for BXs which were not detected in any of the replicates, while r < 3 indicates less than 3 replicates with detectable levels of BXs.

BX	Rye Cultivated in Substrate (A)			Rye Cultivated in Substrate (B)		
(ng·mL^{-1})	Alone	Co-Culture	Significant Difference	Alone	Co-Culture	Significant Difference
DIMBOA-Glc	**8.60**	5.41	NS	1.83	**6.36**	NS
DIMBOA	**0.80**	0.63	NS	ND	0.55	NS
HDMBOA-Glc	**2.59**	1.83	NS	1.91	**2.46**	NS
MBOA	**32.26**	**35.04**	NS	r < 3	2.05	
HMBOA-Glc	**3.54**	1.19	*	0.07	**0.89**	NS
DIM_2BOA-Glc	ND	ND		ND	r < 3	
HDM_2BOA-Glc	**1.46**	1.01	NS	1.34	r < 3	
DIBOA-Glc	ND	r < 3		ND	ND	
DHBOA-Glc	**4.89**	4.31	NS	**4.36**	3.34	NS
HBOA-Glc	**1.00**	**1.24**	NS	ND	ND	
BOA	**2.91**	**5.70**	NS	ND	ND	
Total non-glycosylated	**36.00**	41.40		0.00	**2.60**	
Total glycosylated	**22.10**	15.00		9.50	**13.10**	

When rye was cultivated alone, nine BXs could be detected in (A), whereas only five could be detected in (B). Moreover, the concentrations of BXs detected for both substrates were always higher in (A) (Figure A1). The BX concentrations of DIMBOA-Glc and HDMBOA-Glc were significantly different between the two substrates (Figure A1).

In the rye and pigweed co-culture, nine BXs could be detected in (A), whereas five could be detected in (B). Although BX concentrations were almost always higher in (A), there was no significant difference between the two substrates in the BXs detected for both substrates (Figure A1). The effect of substrate on plant growth was confirmed by a pairwise comparison (Wilcoxon test). As expected, the main influence on both root architecture and BX composition was the substrate, especially when plants were cultivated alone (Alone*B >< Alone*A; Table A3).

3.2.2. Benzoxizanoid Analysis in Two Different Growing Modalities

Even though there was no significant difference in the BXs measured between rye grown alone and in co-culture, except for HMBOA-Glc (Table 1), two trends appeared when rye was cultivated in (A). Out of the nine BXs detected, six of them were found at a higher concentration when rye was cultivated alone, namely, DIMBOA-Glc, DIMBOA, HDMBOA-Glc, HMBOA-Glc(*), HDM_2BOA-Glc, and DHBOA-Glc (Table 1). Meanwhile, three BXs (MBOA, BOA, and HBOA-Glc) had a higher concentration when rye was co-cultivated with pigweed (Table 1).

4. Discussion

4.1. Plant Growth Conditions in SPE Tubes

Allelochemical concentrations, particularly benzoxazinoids (BXs), exhibit irregular dynamic changes during different growth stages. In general, the greatest concentrations are correlated with the highest allelopathic effects, which are typically observed when crops reach the two–three leaf stage [22,23]. This was the rationale for growing rye for 10 days, as this period was sufficient under our experimental conditions for the plants to reach the two–three leaf growth stage, and it did not require larger containers than the 60 mL SPE tubes. It is important to note that the tube size may have influenced root architecture, potentially limiting certain growth parameters. However, the size of the tubes did not obscure the differences in root development between the two substrates, which remained statistically significant and could potentially be even more pronounced in larger growing tubes. These findings align with those reported by Sasse et al. [19].

Rye plants were also grown in 60 mL SPE tubes to facilitate the extraction of root exudates from the growth substrates without disturbing the root systems [8]. This protocol, which we previously developed to study belowground crop–weed interactions, also simplified the separation of the root system from the growth substrate for root system architecture (RSA) analysis. By gently draining water from the bottom of the SPE tubes, we could remove the root system intact, preventing damage.

4.2. Comparison of Substrates (A) and (B)

The first trend that emerged from the analysis of root architecture was the greater development of plants in (B) compared to (A) (Figure 3). 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 and length and the number of root tips [19]. In this experiment, the particle size of the glass microbead substrate varied from 250 to 425 µm, which, according to [19], can be considered small. In contrast, the particle size of the clay beads and attapulgite, being heterogeneous, was bigger at around 1 to 5 mm. The root length was significantly lower in (A) compared to (B) for all plant growth modalities. Similar observations were made for root surface area, the number of root tips, and the dry root biomass (Figure 3). Thus, it can be hypothesized that root architecture is influenced by the particle size of the substrate. This leads to a first hypothesis that smaller particles, such as microbeads of glass (<1 mm), reduce the general growth of a plant, whereas larger particles, such as clay beads and attapulgite particles, enhance root growth. However, this hypothesis must be confirmed with further physical and chemical analysis of the two substrates.

The particle size also determines the pore space between particles and consequently the water-holding capacity of the substrate [24]. 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. Ref. [24] concluded that even though the root system is able to extract water more easily from glass or sandy soils, a plant might suffer more from a water deficit as the soil dries. Therefore, it can be hypothesized that substrate (B) has a higher water-holding capacity, leading to less water availability but retaining water for a longer time due to its retention ability. Plants grown in (B) might

have regular access to water, leading to greater plant growth, which can be interpreted as higher dry shoot biomass and higher specific root lengths (Figure 3). Indeed, plants with higher specific root lengths grow longer roots for a given dry mass investment and are thus generally considered to have improved nutrient and water uptake [25].

Another important soil property is the presence of voids due to the heterogeneous particle size in (B). Air pockets might facilitate water and airflow, as well as root growth, especially of new lateral roots [24]. On the contrary, glass microbeads are evenly distributed and well compacted, which does not favor root growth or higher root length. Instead, root diameter is promoted (Figure 3).

Concerning the response to neighboring plants, our data reveal that they strongly repress root morphogenesis, including inhibiting primary root growth, lateral root formation, and root hair elongation (Figure 4). Auxin is a key hormone in root and shoot development and root–shoot communication well-known for being influenced by plant density [26–28]. Specifically, the number of neighboring plants affects auxin levels in the primary root and the expression of auxin transporters. While low plant density promotes robust root systems, benefiting growth and resource uptake in *Arabidopsis*, high plant density inhibits root development by interfering with auxin biosynthesis, transport, and sensing. Neighbor detection also influences shoot development patterns, likely via auxin feedback loops and phase transitions in the plant life cycle [28,29].

As more BXs were detected and quantified in (A) (Figure A1; Table 1), it can be hypothesized that some of the BXs were sorbed on the clay. Indeed, it has been demonstrated that clay might sorb around 20% of the compounds released in substrate (B) [19]. A hypothesis can be made that clay structure (e.g., accessible surface areas) and surface charge might interfere with dissolved organic compounds and thus alter the exudate composition in soil. The lower amounts of BXs detected in the clay bead substrate might also have been due to the lack of compound extraction efficiency. The extraction solution was optimized for the extraction of BXs from plants grown in (A), where compounds were more available than in (B). Improving the extraction solution by using a solvent with a higher affinity for metabolites than soil compounds might help to better desorb compounds from clay but could have deleterious effect on root surfaces. An optimal extraction solvent must preserve the integrity of plant roots, which was the case in our study [30]. The choice of solvent is therefore limited. Water (a polar solvent) is a 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 [31]. Non-polar solvents are more suitable for extracting BXs from any substrate. However, hexane (a non-polar solvent) might disrupt cell membranes, releasing compounds from inside the root, leading to the extraction of root compounds. The aim here was to study root exudates from the rhizosphere or compounds from the root surface. Ethanol and methanol are less polar than water; nonetheless, they could also disrupt cell membranes at a lower level than hexane. A longer extraction duration of more than one minute, as in this experiment, may also help to better desorb compounds from clay. Additionally, the use of an internal standard could help assess recovery from different substrates and increase the accuracy of the quantification.

4.3. Comparison of Two Growing Modalities

Thus, the trends that emerged were as follows: (1) total glycosylated BXs were more abundant when the plants were cultivated alone and (2) total non-glycosylated BXs appeared in higher concentrations in co-culture (e.g., MBOA and BOA; Table 1). (1) The glycosylated forms are the non-active forms of BXs stored in plant vacuoles [18], which are expected to appear when rye is cultivated alone, as the crop does not need to inhibit neighboring plant growth. Nonetheless, it was surprising to find glycosylated BXs in the rhizosphere. Cellular lysis, which disrupts tissues, occurs naturally during plant growth, leading to a release of glycosylated BXs in the rhizosphere. Once released into the cytoplasm, glycose is removed by the β-glucosidase enzyme, causing the transformation into non-glycosylated BXs [18]. However, this transformation did 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 indeed has the property of being able to stop enzymatic activity. (2) The non-glycosylated forms are the active forms of BXs, which are 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 [32]. Indeed, the most abundant BX in the rhizosphere when rye and pigweed were grown in co-culture in (A) was MBOA, with a concentration of 35 ng·mL^{-1}. MBOA and BOA derive from the spontaneous degradation of DIMBOA and DIBOA, respectively, in aqueous solutions, which explains their higher concentrations in the rhizosphere [32]. These hypotheses must be interpreted carefully, as the BX concentrations in the rhizosphere were close to the limit of quantification.

The same trends could not be observed between growing modalities (alone and in co-culture) in (B). Table 1, which compares the two modalities in substrate (B), shows that six BXs could be detected for rye in co-cultivation (n = 5, with more than three replicates with detectable levels of BXs), whereas five BXs could be detected when rye was cultivated alone. Out of the four BXs detected in both modalities, three of them had a higher concentration when rye was cultivated in co-culture, namely, DIMBOA-Glc, HDMBOA-Glc, and HMBOA-Glc (Table 1). Nonetheless, non-glycosylated BXs (e.g., DIMBOA and MBOA) were also detected at an average concentration of 0.55 and 2 ng·mL^{-1}, respectively. Meanwhile, only DHBOA-Glc had a higher concentration when rye was cultivated alone (Table 1). More BXs were detected in the rhizosphere of rye and pigweed in co-culture, as physical or chemical interactions can be expected between an allelopathic crop and a weed through the production of allelochemicals, which led to rye inhibiting neighboring plants' growth [33]. The total BX results show that both glycosylated and non-glycosylated BXs were present in high amounts in the rhizosphere when the plants were cultivated in co-culture. Furthermore, the total glycosylated BXs were found at a higher concentration in the rhizosphere than the total non-glycosylated BXs—their concentrations being 13.1 and 2.6 ng·mL^{-1}, respectively—which contrasts with trends (1) and (2) addressed in the previous paragraph. It may be hypothesized that the non-glycosylated BXs exuded by rye throughout plant growth were absorbed by clay beads, leading to a lower concentration in the rhizosphere, whereas glycosylated BXs arose from naturally occurring cell lysis, as mentioned in the paragraph above. It must be mentioned that these explanations are speculative and thus must be interpreted carefully.

5. Conclusions

In summary, the main influence on both root architecture and BX composition is the substrate, especially when plants are cultivated alone. Indeed, both plants (rye and pigweed) tended to grow better in (B), as indicated notably by differences 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. However, while substrate (B) seems to better allow for enhanced plant growth, fewer BXs were detected in plants cultivated in this substrate. This may be due to the sorption capacity of clay compared to glass microbeads. In this view, using glass microbeads with a higher particle size (>1 mm) might help enhance plant growth while maintaining an inert sorption-free system for BX chemical analysis.

This article highlights the importance of cultivation conditions when studying chemical interactions between plants, particularly emphasizing the crucial role of the growth substrate. The importance of the growth substrate in studies on belowground plant interactions is crucial for all research conducted under controlled conditions, as in this study, but also for field studies where conditions are not controlled and where the soil's composition, structure, and water content could drastically influence the observations made.

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draft preparation, E.D.S., A.G. and M.-L.F.; writing—review and editing, A.G. and M.-L.F.; visualization, A.G. and M.-L.F.; supervision, A.G., M.-L.F. and J.W.; project administration, J.W. and A.G.; funding acquisition, A.G. and J.W. All authors have read and agreed to the published version of the manuscript.

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Appendix A

Table A1. List of variables.

Independent variables	Growing modalities	Rye alone (R) Pigweed alone (P) Rye and pigweed in co-culture (P + R)
	Type of substrate	Substrate (A) Substrate (B)

Table A2. Predicted retention times (min) and quantification ions for identified benzoxazinoids.

Compound	Predicted Retention Time (min)	Quantification Ions (*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

Table A3. Summary of the pigweed and rye scores obtained for each variable's combination for all root architecture parameters combined. The scores were assigned depending on the *p*-values and the significance of the differences acquired from pairwise comparisons using the Wilcoxon rank-sum exact test for each variable combination. "A" stands for the glass microbead substrate and "B" stands for the clay bead and attapulgite substrate.

Modality*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

Appendix B

Table A4. Comparison of substrates, microbeads of glass (A) vs. mix of clay beads and attapulgite (B), for rye cultivated alone (R) and rye in co-culture (R*(R + P)) by measuring different root parameters. Values are means ± SEMs for each condition, and bold values show which condition has a higher mean for a particular parameter. Asterisks indicate significant differences (*p*-values) between two groups: * $p < 0.05$, ** $p < 0.01$. NS = no significant difference.

Root Parameters	Rye Cultivated Alone (R)			Rye in Co-Culture (R*(R+P))		
	Substrate (A)	Substrate (B)	Significant Difference	Substrate (A)	Substrate (B)	Significant Difference
Root length (cm)	106.2 ± 8.091	**272.9 ± 7.533**	**	117.8 ± 16.93	**285.9 ± 36.41**	*
Root surface area (cm^2)	14.01 ± 1.294	**28.74 ± 0.419**	**	15.27 ± 1.714	**27.67 ± 3.578**	*
Root volume (cm^3)	0.148 ± 0.017	**0.241 ± 0.006**	**	0.159 ± 0.013	**0.213 ± 0.030**	NS
Number of root tips	457.6 ± 83.37	**755.8 ± 23.50**	**	404.6 ± 55.81	**688.2 ± 104.3**	*
Root length density ($cm \cdot cm^{-3}$)	1.771 ± 0.135	**4.549 ± 0.126**	**	1.964 ± 0.282	**4.766 ± 0.607**	*
Root surface area density ($cm^2 \cdot cm^{-3}$)	95.88 ± 3.134	**119.3 ± 2.650**	**	95.45 ± 3.874	**130.7 ± 4.80**	*
Dry root biomass (g)	0.013 ± 0.001	**0.018 ± 0.001**	*	0.015 ± 0.001	**0.016 ± 0.002**	NS
Dry shoot biomass (g)	0.017 ± 0.002	**0.041 ± 0.002**	**	0.019 ± 0.002	**0.036 ± 0.003**	*
Specific root length ($cm \cdot g^{-1}$)	8507 ± 527.1	**15444 ± 836.4**	**	7788 ± 1301	**17913 ± 1190**	*

Table A5. Comparison of substrates, microbeads of glass (A) vs. mix of clay beads and attapulgite (B), for pigweed cultivated alone (P) by measuring different root parameters. Values are means ± SEMs for each condition, and bold values show which condition has a higher mean for a particular parameter. Asterisks indicates significant differences (*p*-values) between two groups: * $p < 0.05$, *** $p < 0.001$.

Root Parameters	Pigweed Cultivated Alone (P)		
	Substrate (A)	Substrate (B)	Significant Difference
Root length (cm)	22.216 ± 2.289	**67.081 ± 6.363**	*
Root surface area (cm^2)	1.797 ± 0.349	**5.397 ± 0.680**	***
Root volume (cm^3)	0.013 ± 0.004	**0.036 ± 0.006**	***
Number of root tips	88.429 ± 13.945	**167.400 ± 16.297**	***
Root length density ($cm.cm^{-3}$)	0.370 ± 0.038	**1.118 ± 0.106**	***
Root branching density (nbr of root $tips.cm^{-1}$)	**3.897 ± 0.377**	2.545 ± 0.149	***

Table A6. Comparison of pigweed grown alone and in co-culture in the mix of clay and attapulgite (PB) by measuring different root parameters. Values are means for each condition, and bold values show which condition has a higher mean for a particular parameter. Asterisks indicates significant differences (*p*-values) between two groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Root Parameters	Pigweed in Clay Beads and Attapulgite Substrate (PB)		
	Alone	Co-Culture	Significant Difference
Root length (cm)	**67.08 ± 6.363**	40.96 ± 5.199	**
Root surface area (cm^2)	**5.397 ± 0.680**	2.881 ± 0.387	**
Root diameter (mm)	**0.248 ± 0.012**	0.221 ± 0.004	*
Root volume (cm^3)	**0.036 ± 0.006**	0.016 ± 0.002	*
Root length density ($cm.cm^{-3}$)	**1.118 ± 0.106**	0.683 ± 0.087	**
Root branching density (nbr of root $tips.cm^{-1}$)	2.545 ± 0.149	**4.758 ± 0.372**	***

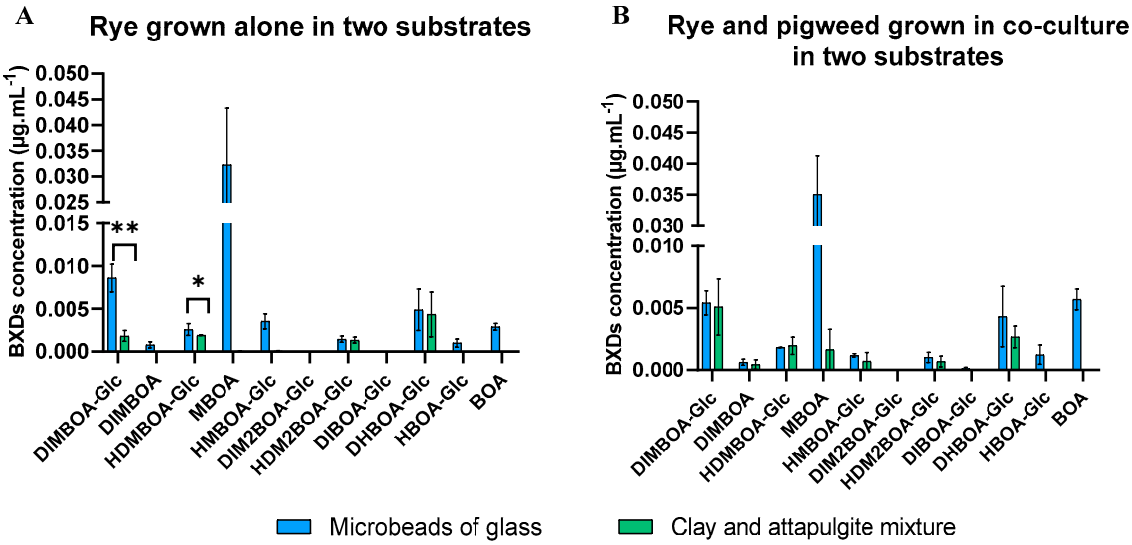

Figure A1. Comparison of two substrates, microbeads of glass (**A**) and mix of clay beads and attapulgite (**B**), for rye cultivated alone and rye in co-culture by measuring different BX concentrations ($\mu g \cdot mL^{-1}$). Values are means ± SEMs for each condition. Asterisks indicate significant differences (p-values) between two groups: * $p < 0.05$, ** $p < 0.01$.

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