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Faba bean root exudates alter pea root colonization by the oomycete *Aphanomyces euteiches* at early stages of infection

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ABSTRACT

Aphanomyces euteiches is an oomycete pathogen that causes the pea root rot. We investigated the potential role of early belowground defense in pea (susceptible plant) and faba bean (tolerant plant) at three days after inoculation. Pea and faba bean were inoculated with *A. euteiches* zoospores. Root colonization was examined. Root exudates from pea and faba bean were harvested and their impact on *A. euteiches* development were assessed by using *in vitro* assays. *A. euteiches* root colonization and the influence of the oomycete inoculation on specialized metabolites patterns and arabinogalactan protein (AGP) concentration of root exudates were also determined. In faba bean root, *A. euteiches* colonization was very low as compared with that of pea. Whereas infected pea root exudates have a positive chemotaxis index (CI) on zoospores, faba bean exudate CI was negative suggesting a repellent effect. While furanoacetylenic compounds were only detected in faba bean exudates, AGP concentration was specifically increased in pea.This work showed that early in the course of infection, host susceptibility to *A. euteiches* is involved *via* a plant-species specific root exudation opening new perspectives in pea root rot disease management.

1. Introduction

Aphanomyces euteiches Drechsler is a soil-borne oomycete pathogen that causes root rot disease in several legume species including pea and faba bean [1–4]. The disease is a major limitation to pea crop production worldwide [5]. Except prophylactic procedures that are mainly based on crop rotation and biodetection of potential inoculum sources in the soil, no efficient methods are currently available to control the pea root rot disease [6–9].

A. euteiches is a diploid, homothallic oomycete, which belongs to Telonemids, Stramenopiles, Alveolates, and Rhizaria (TSAR) clade [10]. Its life cycle includes both sexual and asexual reproduction stages by the succession of oospore and zoospore formation. Oospores can survive in the soil for more than ten years without host crops [11–13]. Under favorable conditions and at close vicinity of a host plant, oospores produce a mycelium that can directly infect root tissues or form a zoosporangium [14]. The zoosporangium releases a large number of zoospores [15] that are chemo-attracted by root exudates [16–18]. At the root surface, the zoospores pass from a mobile state to an immobile state. The zoospores encyst preferentially at the elongation zone within 30 min after reaching the root [19–21]. The cysts then germinate and form coenocytic hyphae that penetrate the intercellular spaces of root cortex within hours [22]. Within a few days of infection, *A. euteiches* mycelium produces oogonia, which are fertilized by antheridia resulting in the formation of new oospores [6,23]. Infected roots become soft, water-soaked and turn blackish-brown. In severe cases, seed production is reduced and plants die prematurely [5].

Although several resistance genetic studies have been conducted, no resistant pea cultivars have been identified so far [24–26]. According to Moussart et al., (2008) [27], partial levels of resistance have been

Abbreviations: AGP, arabinogalactan protein; *A. euteiches*, *Aphanomyces euteiches*; CI, chemotaxis index; dpi, days post-inoculation; RET, root extracellular trap.

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reported for common vetch, faba bean, clover and other species with a very high level of resistance observed in chickpea. Knowledge on the defense mechanisms established by legumes in response to *A. euteiches* infection are essentially based on transcriptional data obtained from the model plant *Medicago truncatula* [28–31]. Using RNA-sequence analysis, Gaulin et al., (2008) [32] have shown that *A. euteiches* pathogenicity is correlated with the production of a specialized secretome including carbohydrate-active enzymes.

Comprehension of *A. euteiches* life cycle during the first stage of infection could have an important role in limiting root infection and enhancing plant resistance. At early stages of infection, pea root tip remains free of colonization [21]. This has been attributed to the presence of highly secretory cells at the root apex called border cells [33–36] or root-associated, cap-derived cells (AC-DCs) [37]. These cells are the most mature cells of the root cap, which detach when the root apex is placed in water [34]. At the soil-root interface, AC-DCs play a key role in plant-microorganisms interactions in the rhizosphere and ensure root apical meristem protection in response to biotic and abiotic stresses. [21] Cannesan et al. (2011) demonstrated that *A. euteiches* infection induced an increase of pea root AC-DCs production and altered their morphology. Root AC-DCs secrete large amounts of mucilage containing polysaccharides and proteins, combined with extracellular DNA and other metabolites [21,34,38–42]. The mucilage, together with root AC-DCs, form a protective web-like structure named the "Root Extracellular Trap" (RET) [37,43]. It is commonly accepted that over half of the root exudates originate from the RET namely by the secretion of AC-DCs [44,45]. Root exudates are defined as the suite of chemical compounds released by diffusion or secretion from the whole plant roots into the rhizosphere [46]. They contain both low- and high molecular weight compounds. The low-molecular weight categories include amino or organic acids, sugars and specialized metabolites, while the high-molecular weight compounds consist mostly of polysaccharides and proteins [47]. The production of root exudates is constitutive [48]; however under the influence of biotic and abiotic factors [49] their composition varies qualitatively and quantitatively [50] driving interactions with soil communities [51].

A few studies relating to both the impact of legume root exudates on *A. euteiches* behavior and the effect of *A. euteiches* infection on root exudate composition have been carried out [21,52–54]. The flavonoid, prunetin (5,4'-dihydroxy-7-methoxy-isoflavone) from pea root exudate is involved in *A. euteiches* zoospore attraction [52]. Pea root exudates contain specific antimicrobial compounds such as the phytoalexin pisatin whose level increases in response to *A. euteiches* [21,53]. The presence of the arabinogalactan proteins (AGPs) in the RET of pea attracts zoospores, induce their encystment and prevent the cyst germination [42].

In this study, early stages of root infection by *A. euteiches* were explored in pea (*Pisum sativum* L.) and faba bean (*Vicia faba* L.). Pea is the most susceptible host [55] whereas french faba bean was described as less sensitive and even tolerant [27]. Given that first injuries of root rot appear in the root between 3 and 4 days after inoculation [11,55], root colonization was examined at 3 days post inoculation. Pea and faba bean root exudates were harvested to investigate the early belowground responses. The effect of root exudates on *A. euteiches* development was assessed. Influence of the oomycete inoculation on specialized metabolite and glyco-polymer contents of root exudates were also determined. Our data show that faba bean root exudates may have a repellent effect on *A. euteiches* zoospores. This study highlights promising future applications in pea crop management.

2. Materials and methods

2.1. Plant material

Pea (*Pisum sativum*, AVENGER variety) and faba bean (*Vicia faba,* FANFARE variety) seeds were surface sterilized with 70% ethanol (v/v) and 0.9% sodium hypochlorite solution (v/v) for 10 min. Seeds were immersed in sterile water for 6 hours at 24 °C and placed in a sterile culture box (Eco box 2, Dutscher) with 1.2% agar at 24 °C and 16 hours photoperiod. Pea and faba bean seedlings with 3 cm root length were used for optical microscopy analyses and infection assays.

2.2. A.euteiches strain culture

Aphanomyces euteiches isolate RB84 (the French reference strain for pea) was supplied by Anne Moussart (INRAE). Isolate was grown and maintained on Potato Dextrose Agar (PDA) plates in the dark at 24 °C. Zoospores were produced according to [42] Cannesan et al. (2012) and adjusted at a concentration of 10^5 zoospores ml^{-1} water.

2.3. Histochemical staining and light microscopy

Pea and faba bean roots were inoculated with 10^5 zoospores ml^{-1} during two and half hours in dark at 24 °C. Seedlings were grown with sterile source water at 24 °C and 16 hours photoperiod. Seedlings were harvested 1, 2 and 3 days post-inoculation (dpi), fixed in 100 % methanol and conserved at 4 °C. *A. euteiches* was labeled with a fluorescein isothiocyanate–wheat germ agglutinin (FITC–WGA) conjugate, according to [21] Cannesan et al. (2011). Infected roots were observed using a Leica DMI 6000B inverted microscope using epifluorescence mode (excitation filter: 480 nm; emission filter: 527 nm).

2.4. A.euteiches DNA quantification in root infection bioassay

Inoculated pea and faba bean roots were harvested at 1, 2 and 3 dpi for total DNA extraction. Non-inoculated seedlings were used as controls. Three independent biological replicates were used. Total genomic DNA was extracted from roots using the PowerPlant ® Pro DNA isolation kit (MoBio Laboratories, USA) according to the manufacturer's instructions, and eluted in 50 µl and stored at -20 °C. DNA was quantified by fluorimetry using the Fluorescent DNA quantitation Kit (Hoechst 33258, Bio-Rad, CA). Total genomic DNA of *A. euteiches* was extracted from mycelium. *A. euteiches* isolate was grown on 60 ml of Sabouraud Dextrose broth 30 % (w/v) in a cell culture flask during 7 days at 24 °C in the dark. Total genomic DNA was extracted from 50 mg crushed lyophilized mycelium using the E.N.Z.A ® HP Fungal DNA Kit (Omega bio-tek, USA). Total DNA was eluted in 50 µl and was quantified using the Eppendorf BioPhotometer. DNA extracts were stored at -20 °C. Real-time quantitative PCR was performed using the LightCycler® 480 Instrument II Real Time PCR system (Basel, Switzerland, Roche) in a total volume of 25 µl. The qPCR mix was prepared as follows: 4 ng of plant DNA, 12.5 µl of Thermo Scientific ABsoluteqPCR Mix no rox (Thermo Fischer Scientific), 0.25 mM bovin serum albumin (GeneOn, DE) and 250 nM of each primer Ae_ITS1_39 F and Ae_ITS1_167R targeting the ITS region, described by [8] Gangneux et al. (2014). Influence of plant DNA and component co-extracted from roots was previously assessed. A first range with serial dilution from 4 ng to 4.10^{-5} ng of *A. euteiches* DNA was performed in triplicate as control. A second range was achieved in triplicate with serial dilution from 4 ng to 4.10^{-5} ng of *A. euteiches* DNA, add to 10 ng of plant DNA extracted from pea or faba bean roots. Amplification results were compared between the two ranges. After an initial enzyme activation step of 15 min at 95 °C, 50 cycles of PCR were performed as follows: 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. In the last step, melting curves were added with the following conditions: 10 s at 95 °C, 30 s at 60 °C and slowly heating to 97 °C, with 1.1 °C increase every 10 seconds with continuous measurement of fluorescence at 520 nm.

2.5. Root exudate collection from seedling roots

Seedlings with a root length of 3 cm were placed in an ecobox within 200 ml of source water. One hundred seedlings were used per condition.

Infection assays were carried out by inoculating roots with a solution of 10^5 zoospores ml^{-1} during two and half hours in the dark at 24 °C. Control conditions were done by maintaining seedlings two and half hours in water. Roots were then rinsed and placed in 200 ml of water. Both control and inoculated seedlings were incubated at 24 °C and 16 hours photoperiod during 3 days. Root exudates from pea and faba bean were performed by adapting the method previously described by [56] Carreras et al. (2019). Root incubation medium was centrifugated at 9 000 rpm for 15 min. Root exudates (supernatant) were freeze dried and weighed for subsequent biological assay and biochemical analyses.

2.6. Chemotaxis bioassay on A.euteiches zoospores

Chemotaxis tests were performed by adapting the method previously described by [42] Cannesan et al. (2012). Zoospore attraction and germination were explored on a microscopy wells-slide (Fig. 3A). A droplet of zoospores containing 500 zoospores (20 µl) was added in the middle well, source water (20 µl) was added into the right well and test compounds into the left well (20 µl). Water was used as control and Gum Arabic (GA) (0.02 mg) as standards, whereas pea or faba bean root exudates from inoculated or non-inoculated seedlings are tested (4 mg). A bridge was made between the wells. The three wells were connected to each other by water. Only the zoospores, which moved out of the middle well were counted after 4 hrs. A chemotaxis index (CI) was calculated using equation based on previous studies [57–60] as: [(Number of zoospores within the Z1 – Number of zoospores within the Z2) / (Number of zoospores which made a choice Z1 + Z2)]. Slides were incubated 4 hours in the dark at room temperature. The percentage of germinated encysted zoospores was calculated as: [(Number of germinated cysts per zone / Total number of attracted cysts per zone) X100]. Observations were made with a Leica DM IL with a 10x objective.

2.7. A.euteiches mycelial growth bioassay

Pea and faba bean root exudates were evaluated for their effect on *A. euteiches* mycelium growth. A mycelium plug was cut from the margin of 7 days-old on PDA, deposited on the Petri plate center containing PDA medium and 50 µg ml^{-1} of ampicillin and 12.5 µg ml^{-1} of streptomycin. A droplet of 50 µl containing 4 mg of pea or faba bean exudates was deposited at 1.5 cm from the mycelium plug. Water was used as control. Petri plate was placed at 24 °C in the dark. Mycelium growth was measured at 3, 4, 5, 6 and 7 days post-incubation.

2.8. UPLC-MS analyses

Freeze dried root exudates were resuspended in methanol before UPLC-MS analyses. The system consisted in an ACQUITY™ Ultra Performance Liquid Chromatography system coupled to a photo diode array detector (PDA) and a Xevo TQD mass spectrometer (Waters, Milford, MA) equipped with an electrospray ionization (ESI) source controlled by Masslynx 4.1 software (Waters, Milford, MA). Analyte separation was achieved by using a Waters Acquity HSS T3 C18 column (150 × 2.1 mm, 1.8 µm) with a flow rate of 0.4 ml min^{-1} at 55 °C. The injection volume was 5 µl. The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). Chromatographic separation was achieved using an 18-min linear gradient from 5 to 70% (v/v) solvent B. MS detection was performed in both positive and negative modes. The capillary voltage was 3,000 V and sample cone voltages were 30 and 50 V. The cone and desolvation gas flow rates were 60 and 800 l h^{-1}. A list of mass-to-charge (m/z) ratios corresponding to molecular ions was established for both plant species, based on total ion current chromatograms from switching positive (ES^+) and negative modes (ES-). Identification of analytes was based on retention times, m/z values, and UV spectra and by comparison with commercial standards or data from literature when no authentic standards were available (Supplementary Table S1 and S2). UPLC-MS analyses were achieved using selected ion monitoring (SIM) mode of the targeted molecular ions. SIM chromatograms were integrated using the subroutine QuanLynx 4.1 for data mining. Peak integration was performed using the ApexTrack algorithm with a mass window of 0.1 Da and relative retention time window of 1 min followed by Savitzky–Golay smoothing (iteration = 1 and width = 1). The resulting pairs of m/z values and retention times were also manually controlled. Tryptophan, *p*-coumaric acid, *t*-ferulic acid and formononetin were purchased from Sigma Chemical Company (St. Louis, MO) and pisatin from Apin Chemicals Ltd (Abingdon,UK).

2.9. Preparation of alcohol insoluble residues (AIR) and monosaccharide analysis

Root exudates was incubated with 96% ethanol (4:1) 24 hours at 4 °C. Samples were centrifuged at 4 000 g during 15 min at 4 °C and precipitated molecules (AIR of root exudates) was dried and stored at -20 °C. Two mg of AIR of root exudates from pea and faba bean were treated in 2 M trifluoroacetic acid (TFA) for 2 hours at 110 °C to release monosaccharides. TFA from samples was removed twice with 50% isopropanol: water solution washings. After freeze-drying, monosaccharide hydrolysates were converted in methyl-glycosides in 1 M HCl/methanol (Supelco) for 16 hours at 80 °C. Samples were washed twice with methanol and were treated with hexamethyldisilazane (HMDS): trimethylchlorosilane (TMCS): pyridine solution (3: 1: 9) (Supelco), for 20 min at 80 °C. The resulting trimethylsilyl methyl glycosides were dried, resuspended in 1 ml of cyclohexane and injected in the 3800 Varian GC-FID system equipped with a CP-Sil5-CB capillary column (Agilent Technologies) and Helium was the gas vector. Elution was performed with the following method: 40 °C for 3 min, 15 °C min^{-1} from 40 °C to 160 °C, 1.5 °C min^{-1} from 160 °C to 220 °C and finally 20 °C min^{-1} from 220 °C to 280 °C. Relative quantification of each monosaccharide was carried out using external monosaccharide standards for their respective retention times and response factors and using inositol as internal standard [61].

2.10. Arabinogalactan protein (AGP) quantification by rocket gel electrophoresis

Yariv reagent (β-d-Glc Y) [62–64] was used to detect free or attached type II arabinogalactan (AGII) to AGPs in root exudates using rocket electrophoresis. AGPs were quantified in root exudates precipitated (AIR of exudates) or not by ethanol. For the two plant species, the analyses were applied on extracts from seedlings inoculated or not by *A. euteiches*. Gels were composed of 1% agarose containing 90 mM Tris (pH 8.4), 90 mM boric acid, 2 mM EDTA, and 20 mg.ml^{-1} of β-d-Glc Y reagent according to van Holst and Clarke (1986). Root exudate fractions (1 mg) were loaded into each well and run for 16 h at 200 V/5 mA/10 W. Gum Arabic from acacia (Fisher Scientific) was used as a standard. 1 mg of *A. euteiches* mycelium and 1 mg of mycelium exudates were also loaded as control. After migration, gels were rinsed with 2% NaCl (w/v). Quantification of AGP was estimated using peak areas of Gum Arabic as standard. Peak areas of AGII were from the sample fractions and Gum Arabic control were calculated using ImageJ software.

2.11. Statistical analysis

GraphPad Prims 7.0 software was used for statistical analysis. Nonparametric tests (Kruskal-Wallis and Mann-Withney) were performed with 5% level of significance.

3. Results

3.1. Root colonization by A. euteiches during the first three days of infection

Pea root surface colonization was assessed by *A. euteiches* staining at 1, 2 and 3 dpi (Fig. 1). The colonization of pea roots was much stronger than that of faba bean roots. At 1 dpi, encysted zoospores and germinated cysts were localized over both the elongation and maturation zones (Fig1B). Mycelial hyphae were mostly observed longitudinally at the maturation zone. At 2 dpi, mycelium was extended and grown on the root elongation and maturation zones. At 3 dpi, it fully surrounded the root tip and elongation zones (Fig.1B). On faba bean root surface, at 1 dpi, a lower number of cysts was observed. Cysts were distributed throughout the root and a few cysts germinated preferentially above the meristematic zone. At 2 dpi, cyst germination was weak. At 3 dpi, a few cysts had produced a germ tube on both elongation and maturation zone root parts.

3.2. Effect of pea and faba bean co-cultures on the colonization of the roots by the pathogen

We monitored the colonization of roots of both species by the pathogen in a co-culture by quantifying *A. euteiches* DNA from roots of both species at 1, 2, and 3 dpi (Fig. 2 and Fig. S2). We also assessed the effect of faba bean root exudates on the colonization of pea root by the pathogen. Three experimental conditions were used. To test the impact of co-culture on zoospore encystment, both species were co-inoculated with zoospores and co-cultivated during 3 days (condition 1). In condition 2, the plants were inoculated separately and then co-cultivated in order to evaluate the effect of co-culture on mycelium growth. Pea plants were inoculated and then cultured in the presence of faba bean root exudates in condition 3. Controls corresponding to peas and faba beans inoculated and cultured separately have been made (Supplementary Fig. S1). Major data are first that there is more pathogen DNA in pea than in faba bean roots (Fig. 2A, 2B and Fig. S1) and secondly that *A. euteiches* DNA content decreased in both plants when they are co-cultivated (Fig. 2A, 2B). While DNA content of *A. euteiches* is strictly reduced in faba bean at 1 dpi, it is significantly altered in pea at 3 dpi. Third, the addition of faba bean root exudates in the pea culture medium

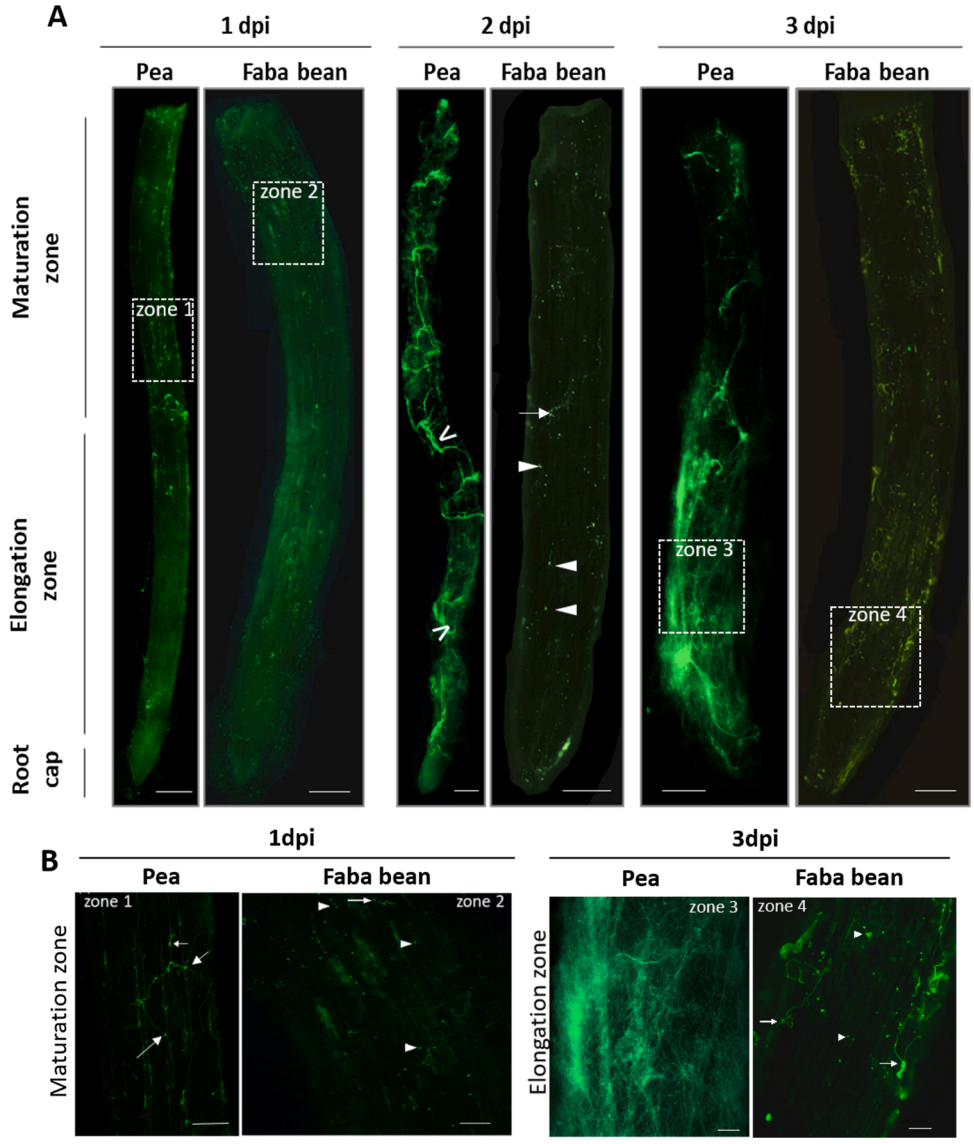

Fig. 1. *A. euteiches* colonization in pea and faba bean roots at early stage of infectionby epifluorescence microscopy. dpi, days post-inoculation; non-germinated cysts (white triangles), germinated cyst (white arrows) and mycelium (arrows heads). Scale bars: 500 μm (A); 100 μm (B).

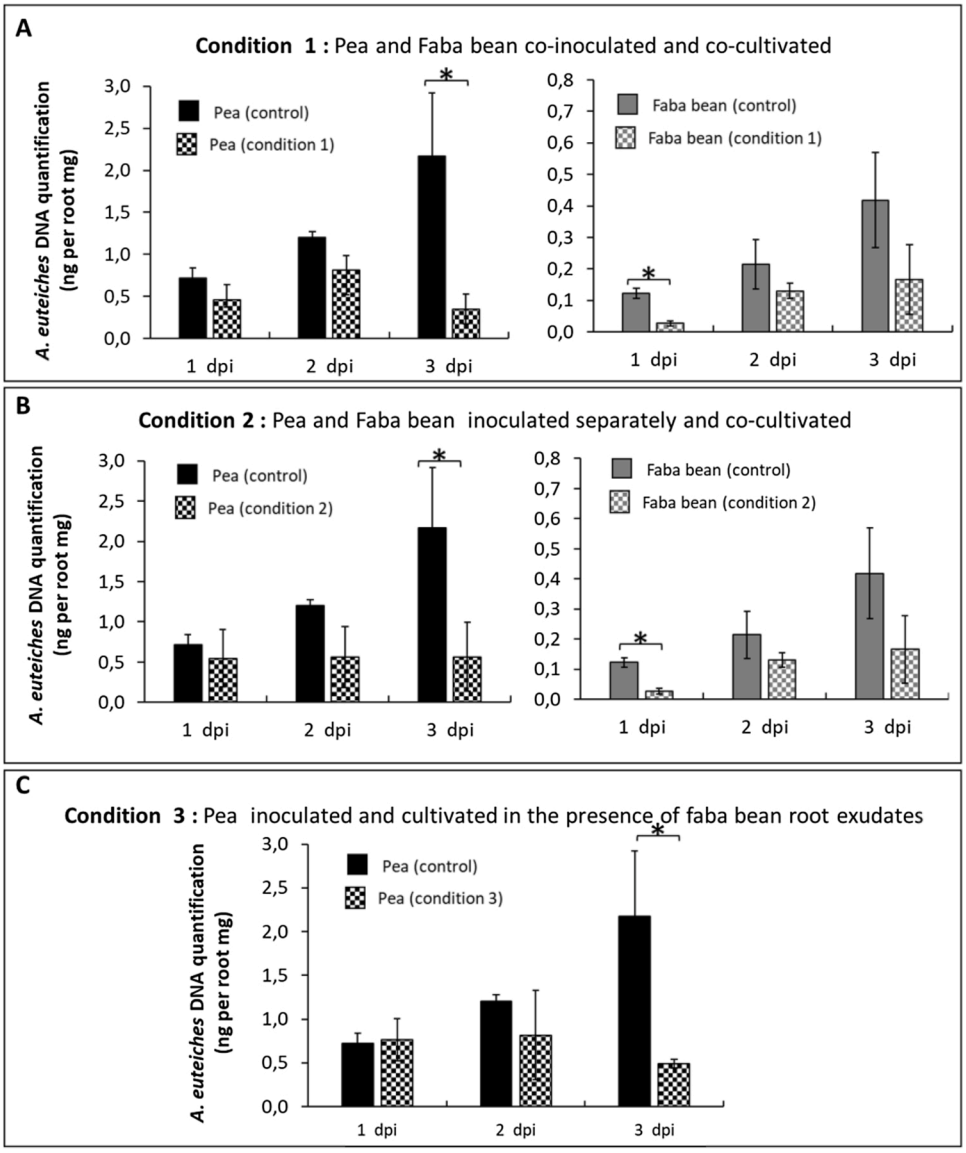

Fig. 2. Quantification of *A. euteiches* DNA in pea and faba bean roots.
A, DNA quantification of *A. euteiches* by qPCR in pea and faba bean roots co-inoculated and co-cultivated (condition 1). **B**, DNA quantification of *A. euteiches* in pea and faba bean roots inoculated separately and co-cultivated (condition 2). **C**, DNA quantification of *A. euteiches* in pea roots inoculated and cultivated in the presence of faba bean root exudates from non-inoculated seedlings (condition 3). For each condition, control is pea (black colored histograms) or faba bean (grey colored histograms) inoculated and cultivated separately. Each histogram represents the mean of three biological replicates ($n = 3$) and error bars indicate the standard error. Statistical analysis was performed using Mann-Whitney test (*$P < 0.05$) (5% level of significance). dpi, days post-inoculation.

induced a significant reduction of infection (measured by the content of the pathogen DNA) (Fig. 2C). For pea, there are no significant difference between the three conditions (Fig. S2). The comparison of the number of ITS copies between *A. euteiches* DNA control and *A. euteiches* DNA supplemented by plant DNA did not reveal any significant differences (Supplementary Table S1).

3.3. Effect of pea and faba bean root exudates on A. euteiches

To study the effect of root exudates on zoospore behavior, we have performed chemotaxis and germination *in vitro* assays using the experimental device shown in Fig. 3A. We have assessed the effect of bean and pea root exudates as well as Gum Arabic (Fig.3B, 3C). Our data show that root exudates from non-inoculated peas had no chemoattractive effect on zoospores whereas those from inoculated plants presented a significant positive CI of 0.31 ± 0.03. Interestingly, we found that root exudates from both non-inoculated and inoculated faba bean plants had a significant negative CI equal to -0.52 ± 0.12 and -0.49 ± 0.06 (Fig. 3B). Remarkably, among the root exudates tested, only those from non-inoculated peas promoted the germination of cysts (13.64 ± 2.84%) as compared with the controls 1.67 ± 1.11% (Fig. 3C). Gum Arabic, had a strong attractive effect (CI = 0.82 ± 0.14) but did not promote cyst germination. Chemotaxis index corresponding to the neutral condition (water *vs* water) was 0.17 ± 0.19 and the percentage of germinated cysts in Z1 and Z2 areas were 7.25 ± 4.57 % and 5.02 ± 3.15 % (Fig. 3C).

The impact of root exudates from non-inoculated plants on *A. euteiches* mycelium growth was determined (Fig. 3D). Except a slight positive effect of pea root exudates on mycelium growth at days 4 and 5, the growth of *A. euteiches* mycelium was neither inhibited nor stimulated by the exudates of both plants. Also, no alteration of mycelium growth was observed in the presence of antibiotics in the PDA medium (Supplementary Table S2).

3.4. UPLC-MS based analyses of root exudates

UPLC-DAD-MS-based metabolite profiling was carried out on pea and faba bean root exudates. Major peaks were annotated and assigned according to their retention time, UV and mass spectra by comparison with standards or data from the literature. MS experiments were performed in both positive and negative ionization modes. For the two species, while the metabolic profiles were not qualitatively altered by infection, the concentration of metabolites often increased (tables 1 and 2; Fig. S3).

In pea root exudate extracts, five peaks were assigned to tryptophan,

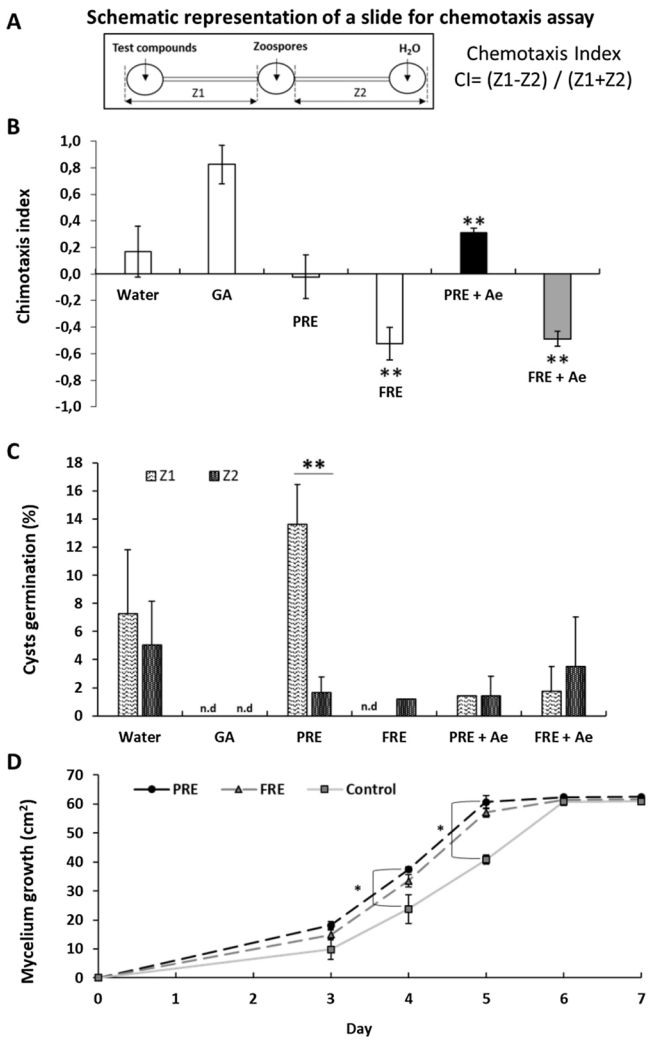

Fig. 3. Effect of pea and faba bean root exudates on *A euteiches* behavior.
A, Experimental device for chemotaxis and cyst germination assays. Zoospores were put in the middle well. Water, used as control, was put in the right. Water, Gum Arabic or root exudates were been put in the left. Chemotaxis index was calculated as: [(number of zoospores within the Z1 – number of zoospores within the Z2) / (number of zoospores which made a choice Z1 + Z2)]. The percentage of cysts that germinate is determined for each zones as : [(Number of germinated cysts per zone / Total number of cysts per zone) X100].
B, *A. euteiches* zoospores chemotaxis response. **C**, Germination of cysts in zones 1 and 2. **B**, **C**, Statistical analysis was performed using Mann-Whitney test comparing the test compounds with water control (* $P < 0.05$; ** $P<0.01$) (5% level of significance). Each histogram represents the mean of five biological replicates ($n = 5$) excepted for GA ($n = 3$). Error bars indicate the standard error.
D, Effect of root exudates from non-inoculated seedlings on mycelium growth. Surfaces of mycelium were compared with water control condition. Error bars indicate the standard error of five biological replicates ($n = 5$) excepted for water control conditions ($n = 3$). Statistical analysis was performed using Mann-Whitney test comparing the test compounds with water control (* $P <$ 0.05) (5% level of significance). Ae, *A. euteiches* ; GA, Gum Arabic ; FRE, faba bean root exudates from non-inoculated seedlings ; PRE, pea root exudates from non inoculated seedlings ; FRE + Ae, faba bean root exudates from inoculated seedlings ; PRE + Ae, pea root exudates from inoculated seedlings ; Z1, zone 1; Z2, zone 2.

p-coumaric acid, trans-ferulic acid, formononetin and pisatin by comparison with authentic standards (Table 1 and Fig. S3A). All these metabolites have been previously described in pea [65,66]. At a retention time of 5.38 min, a peak showed a molecular ion [M-H]- at m/z 609 and [M+H]+ at m/z 611. In negative mode this analysis produced a characteristic fragment at m/z 301 [M-H-glucose-rhamnose]- corresponding to a quercetin moiety. Therefore, this compound was identified as quercetin-3-rutinoside as previously described in pea seed coat extracts [67]. A peak at 7.10 min produced a molecular ion [M-H]- at m/z 302. The corresponding UV spectrum and MS fragmentation features allowed to annotate this peak as DMDI (7,2'-dihydroxy-4', 5'-methylene-dioxyisoflavanol). In the same way, the peak at 10.22 min showing molecular ions [M-H]- at m/z 297 and [M+H]+ at m/z 299 as well as specific UV spectrum was assigned as DMD (7,2'-dihydroxy-4', 5'-methylenedioxyisoflavone). These two last compounds are known to be biosynthetic intermediates in the pathway of pisatin a phytoalexin of pterocarpan type with formononetin as precursor [66]. In faba bean exudates, tryptophan and formononetin were undoubtedly identified by comparison with pure standards (Table 2 and Fig. S3B). The peak at retention time 6.32 min, showing a molecular ion [M-H]- at m/z 385 and a fragment ion 223 [M-H-162]- corresponding to the loss of a glucose moiety was assigned to sinapoyl-glucoside, a hydroxycinnamic acid derivative previously described in faba bean leaf extracts (Neugart et al., 2015). Peak at 8.11 min produced a [M-H]- molecular ion at m/z 273 and fragments ions at m/z 224 and 183. It was assigned to wyerone epoxide as reported in faba bean (Hargreaves et al. 1976). Peak at 10.41 min gave a molecular ion [M-H]- at m/z 261 producing two fragments at m/z 217 and 191, and was assigned to dihydrowyerol. This metabolite was previously reported in *Vicia faba* [68]. Peak at 10.57 min showed a [M-H]- ion at m/z 243 and a fragment ion at m/z 225 and was attributed to wyerone acid as previously described [68]. These three last compounds belong to furanoacetylene family, a non-flavonoid class of phytoalexins. The peak at 12.09 min showed a molecular ion [M-H]- at m/z 269 and was assigned to the pterocarpan medicarpin as previously described in broad bean [69].

3.5. AGPs content in root exudates

Given that AGPs impacted *A. euteiches* development [42] and that the relative levels of Ara and Gal monosaccharides were modulated following inoculation (Fig. S4; Table S3), we focused on AGPs analysis. A semi-quantitative analysis of type II arabinogalactans (AG-II), a typical side chain of AGPs, was performed (Table 3). Rocket gel electrophoresis of root exudates (AIR or raw fractions) detected AGP in pea and faba bean extracts (Supplementary Fig. S5). The amount of AGP strongly increased in extracts from infected pea roots as compared with non-infected roots (Table 3). In contrast, no significant variations were observed in extracts from faba bean roots. It is important to note that AGPs, were detected neither in *A. euteiches* mycelium nor in the mycelium culture medium (Supplementary Fig. S5).

4. Discussion

Although the life cycle of *A. euteiches* is characterized by rapid colonization of the root system within hosts, better understanding of the early stages of the disease development is necessary. We studied the *A. euteiches* colonization, during the first three days of infection, within pea and faba bean roots respectively sensitive and tolerant to this root rot agent.

4.1. Host specificity of A. euteiches infection at early stages

Compared with faba bean, pea roots are more intensely colonized by *A. euteiches* during the first three days of infection. Microscopical and qPCR data showed that colonization of pea root is high at 3 dpi. These results are in line with those reported by Billard et al. (2019) [54] showing that root necrosis is observed much earlier in pea than in faba bean roots. We show that host sensitivity is involved in the oomycete infectious process at early stages and attest that *A. euteiches* strain RB84, is preferentially aggressive on the susceptible plant. As previously observed [19,21], we show that pea root tips remained free of infection.

Table 1
List of polyphenols identified in pea root exudates.

RT (min)	Compound assignement	Compound class	MW	[M-H]-	[M+H]+	Fragments (ES-)	Fragments (ES+)	References
3.39	tryptophane*	amino acid	204	203	205	188; 146		standard
5.38	quercetin-3-rutinoside	flavonol	610	609	611	131	301 [M+H-gluc-rha]+ 287; 141	Mullen et al., 2003
5.76	*p*-coumaric acid*	phenolic	164	163	165	119	147; 119	standard
6.32	trans-ferulic acid*	phenolic	194	193	195	178; 134	177	standard
7.1	DMDI[a]	isoflavanol	302	301		273; 163		Celoy and VanEtten., 2013
10.22	DMD[b]	isoflavone	298	297	299		321 [M + Na]+	Celoy and VanEtten., 2013
11.19	formononetin*	isoflavone	268	267	269	252		standard
11.76	pisatin*	pterocarpan	314	313		299; 181		standard

[a] 7,2'-dihydroxy-4',5'-methylene-dioxyisoflavanol.
[b] 7,2'-dihydroxy-4',5'-methylenedioxyisoflavone.

Table 2
List of polyphenols identified in faba bean root exudates.

RT (min)	Compound assignement	Compound class	MW	[M-H]-	[M+H]+	Fragments (ES-)	Fragments (ES+)	References
3.39	tryptophane*	amino acid	204	203	205		188.1; 146.0	standard
6.32	sinapoyl-glucoside	phenolic	386	385		269; 223 [M-H-Gluc]-; 171		Neugart et al. 2015
8.11	wyerone epoxide	furanoacetylene	274	273		224; 183		Hargreaves et al. 1976
10.41	dihydrowyerol	furanoacetylene	262	261	263	217; 191	207	Mansfield et al. 1979
10.57	wyerone acid	furanoacetylene	244	243	245	225	227	Mansfield and Widdowson. 1973
11.15	formononetin*	isoflavone	268	267	269	252		standard
12.09	medicarpin	pterocarpan	270	269	271	254; 163	165; 137	Abu-Reidah et al. 2014

Table 3
AGP content in root exudates at three days after inoculation.

	AGP content (mg ml $^{-1}$)	
	Control	Plant + *Ae*
Pea	**0.15 ± 0.09**	**0.42 ± 013** *
Faba bean	**0.14 ± 0.15**	**0.05 ± 0.09 ns**

To quantify AGP, the quantification of β-glucosyl Yariv precipitate in AIR of root exudates for peas and faba beans was assessed. Controls consist in peas or faba beans non-inoculated. Each histogram represents the mean of four technical replicates ($n = 4$) ± the standard error. For statistical analysis Mann-Whitney nonparametric test (5% level of significance) was performed (*$P < 0.05$). Ae, *A. euteiches*; AGP, arabinogalactan protein.

These results highlight the protective function of root cap-derived border cells and their secretions toward the root apical meristem at early stages of infection. The primary function of motile zoospores is to connect oomycetes to the host [70]. Several host-specific molecular attractants have been reported such as isovaleraldehyde, which attracts *Phytophthora palmivora* [71], prunetine a pea compound that attracts *A. euteiches* zoospores [52] or indole-3-aldehyde isolated from cabbage that promotes *Aphanomyces raphani* zoospores attraction [72]. Root exudates from pea induce a higher rate of oospore germination than those from vetch and faba bean [54]. Although, pea root exudates from non-inoculated plants affect zoospore germination it has little effect on zoospore attraction. The chemotaxis index (CI) of exudates originated from non-inoculated peas is nearly null. In contrast, the CI is positive for exudates originated from inoculated peas. Kong and Hong (2010) [73] reported that *Phytophthora nicotianae* zoospores secrete a fluid able to promote successful infection in *Arabidopsis thaliana*. Exudates from zoospores of *Phytophthora* and *Pythium* species act as a *quorum-sensing* signals impacting zoospore homing and triggering infection [73–75]. Herein, exudates from *A. euteiches* zoospores and/or mycelium may be present in harvested pea exudates and would promote zoospore attraction. The CI for root exudates from non-inoculated or inoculated faba beans was negative which suggests that faba bean exudates had a repellent effect towards *A. euteiches* zoospores. Host repellent signals have been mostly demonstrated in plant-nematode interactions [76–79]. As for oomycete-host interactions, a phenolic extract from the root of the non-host plant *Portulaca oleracea*, was shown to exhibit a repellent activity on *Aphanomyces cochlioides* zoospores [80]. Bazghaleh et al., (2018) [81] showed that, in response to *A. euteiches* infection, the root polyphenolic composition of lentil is dependent on the plant genotypes. The metabolic profiling of pea and faba bean root exudates based on UPLC-MS analyses revealed the presence of two main classes of plant specialized metabolites, phenolic compounds and furanoacetylenic derivatives. Among phenolic compounds, flavonoids are ubiquitously found in the plant kingdom, whereas isoflavones are restricted to the subfamily of Papilionoideae [82]. Within Fabaceae, almost all species of the subfamily Papilionoideae accumulate isoflavones and derivatives, including phytoalexins of the pterocarpan type [82]. Here, the presence of pterocarpan biosynthetic pathway was observed in both legumes confirming previous chemotaxonomy observations in whole plants [66, 69]. Focusing on the root exudate composition, the presence of the phytoalexin pisatin in pea root exudates was already reported [21], however the presence of pterocarpan-type phytoalexin (medicarpin) in faba bean root exudates has never been reported in earlier studies. Although different susceptibility levels toward *A. euteiches* were observed, pterocarpans were identified in both legumes suggesting that they might not be strongly involved in the susceptibility/tolerance during early stages of infection by *A. euteiches*. A search for the distribution of furanoacetylene in the metabolite-plant species database Knapsack indicates a presence restricted to the two genus *Vicia* and *Lens* [83]. Furanoacetylenic derivatives were described as markers of resistance of faba bean to chocolate spot disease [84]. The ability of *Botrytis fabae* and not *B. cinerea* to metabolize the phytoalexin wyerone acid was of primary significance in the pathogenicity towards faba bean [68]. Therefore, we suggest that the presence of furanoacetylenic in root exudates of faba bean might contribute to tolerance mechanisms of faba bean to the pathogen *A. euteiches*.

Coupled to small molecules, root exudates contain macromolecules like cell wall polymers [85,86] (Chaboud, 1983; Knee et al., 2001). Hinch and Clarke (1980) [87] demonstrated that zoospore adhesion of *Phytophthora cinnamomi* depends on carbohydrate components present at the *Zea mays* L. root surface. Pea root exudate monosaccharide profiles are quite similar to those previously described [86,88,89]. For the two legumes, Ara and Gal are among the major sugars detected suggesting the presence of arabinogalactan motifs. The precipitation by the β-glucosyl Yariv reagent assessed that AG-II are present in the root exudates. AG-II are the major side chain of arabinogalactan proteins

(AGP). AGP is a family of non-enzymatic cell surface hydroxyproline-rich glycoproteins found in plant cell walls, in the plasma membrane or in plant secretions [63,90] especially the root cap-associated mucilage and root exudates [42,56,91]. These proteoglycans control cell morphology and growth, and play a major role in plant-micro-organism interactions [38,91–93]. Here, a high CI was recorded with the Gum Arabic (an AGP-like molecule) whereas the cyst germination was not detectable. These observations are in line with the results described by Cannesan et al. (2012) [42] using purified pea root AGPs that show that AGPs stimulate zoospore attraction and encystement but also reduce cyst germination. The present results revealed an increase of AGP content in root exudates concomitantly to infection in pea. Exudates from non-inoculated peas were favorable to cyst germination while those from inoculated seedlings had no impact. This could be linked to the increase of AGP in pea root exudates after infection.

4.2. *Pea susceptibility towards A. euteiches is reduced by faba bean*

Moussart et al. (2013) [99] indicated that faba bean resistant cultivars contribute to reduce the inoculum potential of soils naturally infested by *A. euteiches*. We revealed that *A. euteiches* colonization in pea root at 3 dpi is significantly reduced when pea is co-cultured with faba bean or when pea is inoculated and cultured in the presence of faba bean root exudates. As a significant decrease of *A. euteiches* DNA content is strictly recorded at 1dpi in faba bean and at 3dpi in pea, we hypothesize that co-inoculation and co-culture experiments have distinct effect in the two plants. Co-culture have a minor impact on zoospore attraction but affect the mycelium colonization in pea. Conversely, we could postulate that co-culture prevents zoospore attraction in faba bean. As a whole, these results suggest that faba bean might have a protective effect on pea. This protective effect of faba bean could be related to the repulsive or biocide effects of its exudates, and /or to another effect such as an activation of pea root defense responses. Our data reveal that faba bean exudates have no biocide effect on mycelium and their repulsive effect is suggested by chemotaxis *in vitro* assay. In response to oligogalacturonide elicitor in pea, Sameh et al. in 2017, [100] showed an upregulation of genes involved either in the basal defense (genes encoding proteins involved in salicylic acid and ROS signaling pathways) and either in the antifungal defensins, lignans and the phytoalexin pisatin pathways. Expression of pea genes involved in cell wall modifications such as those encoding callose synthase, pectin esterase and pectin esterase inhibitor are also reported to be modulated during *A. euteiches* infection [31]. Supplementary experiments such as monitoring of plant defense genes or marker enzymatic activities could be performed to investigate pea defense responses to *A. euteiches* infection at early stages in presence of faba bean exudates. To assess the protective effect of faba bean towards pea it also would be interesting to explore it throughout the plant's life.

5. Conclusions

The present study provides new findings related to *A. euteiches* colonization at early stages of infection in pea and faba bean, respectively susceptible and tolerant hosts. We revealed that faba bean root exudates presented a negative CI towards zoospores. We showed that at three days after zoospore inoculation, *A. euteiches* was more abundant in pea compared with faba bean root. We also reveal that pea root colonization by *A. euteiches* can be significantly reduced by faba bean root exudates. These provide promising perspectives for new pest management toward Aphanomyces root rot in the field pea crops.

Author Contributions

Y.L, M-L.F-G and C.G planned and designed the research. Y.L performed experiment and wrote the first version of the manuscript with the assistance of M-L.F-G, A. G. and A.D. B.G contributed in GC-FID experiment, A.B contributed in biological assays in Petri plate, A.L and T.M performed the phenolic composition of root exudates by UPLC-MS analyses. All authors read, provided critical feedback and approved the final version of the manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.plantsci.2021.111032.

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