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

Yohana Laloum, Christophe Gangneux, Bruno Gügi, Arnaud Lanoue, Thibaut Munsch, Adrien Blum, Adrien Gauthier, Isabelle Trinsoutrot-Gattin, Isabelle Boulogne, Maité Vicré, et al.

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1 **ORIGINAL RESEARCH ARTICLE**

2 **Title**

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

5

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18 **Running title: Control of *A. euteiches* pea root rot agent**

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

3

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

19 **Key words:** *Aphanomyces euteiches*, *Pisum sativum*, specialized metabolites; root  
20 colonization, root exudates, *Vicia faba*

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

23

## 1        **1. Introduction**

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

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

21            Although several resistance genetic studies have been conducted, no resistant pea  
22 cultivars have been identified so far [24–26]. According to Moussart *et al.*, (2008) [27] ,  
23 partial levels of resistance have been reported for common vetch, faba bean, clover and other

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

7         Comprehension of *A. euteiches* life cycle during the first stage of infection could have  
8 an important role in limiting root infection and enhancing plant resistance. At early stages of  
9 infection, pea root tip remains free of colonization [21]. This has been attributed to the  
10 presence of highly secretory cells at the root apex called border cells [33–36] or root-  
11 associated, cap-derived cells (AC-DCs) [37]. These cells are the most mature cells of the root  
12 cap, which detach when the root apex is placed in water [34]. At the soil-root interface, AC-  
13 DCs play a key role in plant-microorganisms interactions in the rhizosphere and ensure root  
14 apical meristem protection in response to biotic and abiotic stresses. [21] Cannesan *et al.*  
15 (2011) demonstrated that *A. euteiches* infection induced an increase of pea root AC-DCs  
16 production and altered their morphology. Root AC-DCs secrete large amounts of mucilage  
17 containing polysaccharides and proteins, combined with extracellular DNA and other  
18 metabolites [21,34,38–42]. The mucilage, together with root AC-DCs, form a protective web-  
19 like structure named the “Root Extracellular Trap” (RET) [37,43]. It is commonly accepted  
20 that over half of the root exudates originate from the RET namely by the secretion of AC-DCs  
21 [44,45]. Root exudates are defined as the suite of chemical compounds released by diffusion  
22 or secretion from the whole plant roots into the rhizosphere [46]. They contain both low- and  
23 high molecular weight compounds. The low-molecular weight categories include amino or  
24 organic acids, sugars and specialized metabolites, while the high-molecular weight  
25 compounds consist mostly of polysaccharides and proteins [47] . The production of root

1 exudates is constitutive [48]; however under the influence of biotic and abiotic factors [49]  
2 their composition varies qualitatively and quantitatively [50] driving interactions with soil  
3 communities [51].

4 A few studies relating to both the impact of legume root exudates on *A. euteiches* behavior  
5 and the effect of *A. euteiches* infection on root exudate composition have been carried out  
6 [21,52–54]. The flavonoid, prunetin (5,4'-dihydroxy-7-methoxy-isoflavone) from pea root  
7 exudate is involved in *A. euteiches* zoospore attraction [52]. Pea root exudates contain specific  
8 antimicrobial compounds such as the phytoalexin pisatin whose level increases in response to  
9 *A. euteiches* [21,53]. The presence of the arabinogalactan proteins (AGPs) in the RET of pea  
10 attracts zoospores, induce their encystment and prevent the cyst germination [42].

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

## 23 **2. Materials and methods**

### 24 *2.1. Plant material*

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

## 7 2.2. *Aphanomyces euteiches* strain culture

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

## 12 2.3. *Histochemical staining and light microscopy*

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

## 20 2.4. *Aphanomyces euteiches* DNA quantification in root infection bioassay

21 Inoculated pea and faba bean roots were harvested at 1, 2 and 3 dpi for total DNA extraction.  
22 Non-inoculated seedlings were used as controls. Three independent biological replicates were

1 used. Total genomic DNA was extracted from roots using the PowerPlant<sup>®</sup> Pro DNA  
2 isolation kit (MoBio Laboratories, USA) according to the manufacturer's instructions, and  
3 eluted in 50 µl and stored at -20°C. DNA was quantified by fluorimetry using the Fluorescent  
4 DNA quantitation Kit (Hoechst 33258, Bio-Rad, CA). Total genomic DNA of *A. euteiches*  
5 was extracted from mycelium. *A. euteiches* isolate was grown on 60 ml of Sabouraud  
6 Dextrose broth 30 % (w/v) in a cell culture flask during 7 days at 24°C in the dark. Total  
7 genomic DNA was extracted from 50 mg crushed lyophilized mycelium using the E.N.Z.A<sup>®</sup>  
8 HP Fungal DNA Kit (Omega bio-tek, USA). Total DNA was eluted in 50 µl and was  
9 quantified using the Eppendorf BioPhotometer. DNA extracts were stored at -20°C. Real-time  
10 quantitative PCR was performed using the LightCycler<sup>®</sup> 480 Instrument II Real Time PCR  
11 system (Basel, Switzerland, Roche) in a total volume of 25 µl. The qPCR mix was prepared as  
12 follows: 4 ng of plant DNA, 12.5 µl of Thermo Scientific ABsoluteqPCR Mix no rox  
13 (Thermo Fischer Scientific), 0.25 mM bovin serum albumin (GeneOn, DE) and 250 nM of  
14 each primer Ae\_ITS1\_39F and Ae\_ITS1\_167R targeting the ITS region, described by [8]  
15 Gangneux *et al.* (2014). Influence of plant DNA and component co-extracted from roots was  
16 previously assessed. A first range with serial dilution from 4 ng to 4.10<sup>-5</sup> ng of *A. euteiches*  
17 DNA was performed in triplicate as control. A second range was achieved in triplicate with  
18 serial dilution from 4 ng to 4.10<sup>-5</sup> ng of *A. euteiches* DNA, add to 10 ng of plant DNA  
19 extracted from pea or faba bean roots. Amplification results were compared between the two  
20 ranges. After an initial enzyme activation step of 15 min at 95°C, 50 cycles of PCR were  
21 performed as follows: 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. In the last step, melting  
22 curves were added with the following conditions: 10 s at 95°C, 30 s at 60°C and slowly  
23 heating to 97°C, with 1.1°C increase every 10 seconds with continuous measurement of  
24 fluorescence at 520 nm.



## 1        2.5. *Root exudate collection from seedling roots*

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

## 12       2.6. *Chemotaxis bioassay on Aphanomyces euteiches zoospores*

13       Chemotaxis tests were performed by adapting the method previously described by [42]  
14       Cannesan *et al.* (2012). Zoospore attraction and germination were explored on a microscopy  
15       wells-slide (Fig. 3A). A droplet of zoospores containing 500 zoospores (20  $\mu\text{l}$ ) was added in  
16       the middle well, source water (20  $\mu\text{l}$ ) was added into the right well and test compounds into  
17       the left well (20  $\mu\text{l}$ ). Water was used as control and Gum Arabic (GA) (0.02 mg) as standards,  
18       whereas pea or faba bean root exudates from inoculated or non-inoculated seedlings are tested  
19       (4 mg). A bridge was made between the wells. The three wells were connected to each other  
20       by water. Only the zoospores, which moved out of the middle well were counted after 4 hrs.  
21       A chemotaxis index (CI) was calculated using equation based on previous studies [57–60] as:  
22        $[(\text{Number of zoospores within the Z1} - \text{Number of zoospores within the Z2}) / (\text{Number of}$   
23        $\text{zoospores which made a choice Z1+Z2})]$ . Slides were incubated 4 hours in the dark at room

1 temperature. The percentage of germinated encysted zoospores was calculated as: [(Number  
2 of germinated cysts per zone / Total number of attracted cysts per zone) X100]. Observations  
3 were made with a Leica DM IL with a 10x objective.

#### 4 2.7. *Aphanomyces euteiches* mycelial growth bioassay

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

#### 11 2.8. UPLC-MS analyses

12 Freeze dried root exudates were resuspended in methanol before UPLC-MS analyses. The  
13 system consisted in an ACQUITY™ Ultra Performance Liquid Chromatography system  
14 coupled to a photo diode array detector (PDA) and a Xevo TQD mass spectrometer (Waters,  
15 Milford, MA) equipped with an electrospray ionization (ESI) source controlled by Masslynx  
16 4.1 software (Waters, Milford, MA). Analyte separation was achieved by using a Waters  
17 Acquity HSS T3 C18 column (150 × 2.1 mm, 1.8  $\mu\text{m}$ ) with a flow rate of 0.4  $\text{ml min}^{-1}$  at  
18 55°C. The injection volume was 5  $\mu\text{l}$ . The mobile phase consisted of solvent A (0.1% formic  
19 acid in water) and solvent B (0.1% formic acid in acetonitrile). Chromatographic separation  
20 was achieved using an 18-min linear gradient from 5 to 70% (v/v) solvent B. MS detection  
21 was performed in both positive and negative modes. The capillary voltage was 3,000 V and  
22 sample cone voltages were 30 and 50 V. The cone and desolvation gas flow rates were 60 and  
23 800  $\text{l h}^{-1}$ . A list of mass-to-charge (m/z) ratios corresponding to molecular ions was

1 established for both plant species, based on total ion current chromatograms from switching  
2 positive (ES<sup>+</sup>) and negative modes (ES<sup>-</sup>). Identification of analytes was based on retention  
3 times, m/z values, and UV spectra and by comparison with commercial standards or data from  
4 literature when no authentic standards were available (Supplementary Table S1 and S2).  
5 UPLC-MS analyses were achieved using selected ion monitoring (SIM) mode of the targeted  
6 molecular ions. SIM chromatograms were integrated using the subroutine QuanLynx 4.1 for  
7 data mining. Peak integration was performed using the ApexTrack algorithm with a mass  
8 window of 0.1 Da and relative retention time window of 1min followed by Savitzky–Golay  
9 smoothing (iteration = 1 and width = 1). The resulting pairs of m/z values and retention times  
10 were also manually controlled. Tryptophan, *p*-coumaric acid, *t*-ferulic acid and formononetin  
11 were purchased from Sigma Chemical Company (St. Louis, MO) and pisatin from Apin  
12 Chemicals Ltd (Abingdon,UK).

### 13 *2.9.Preparation of alcohol insoluble residues (AIR) and monosaccharide analysis*

14 Root exudates was incubated with 96% ethanol (4:1) 24 hours at 4°C. Samples were  
15 centrifuged at 4 000 g during 15 min at 4°C and precipitated molecules (AIR of root exudates)  
16 was dried and stored at -20°C. Two mg of AIR of root exudates from pea and faba bean were  
17 treated in 2 M trifluoroacetic acid (TFA) for 2 hours at 110°C to release monosaccharides.  
18 TFA from samples was removed twice with 50% isopropanol: water solution washings. After  
19 freeze-drying, monosaccharide hydrolysates were converted in methyl-glycosides in 1 M  
20 HCl/methanol (Supelco) for 16 hours at 80°C. Samples were washed twice with methanol and  
21 were treated with hexamethyldisilazane (HMDS): trimethylchlorosilane (TMCS): pyridine  
22 solution (3: 1: 9) (Supelco), for 20 min at 80°C. The resulting trimethylsilyl methyl glycosides  
23 were dried, resuspended in 1 ml of cyclohexane and injected in the 3800 Varian GC-FID  
24 system equipped with a CP-Sil5-CB capillary column (Agilent Technologies) and Helium was

1 the gas vector. Elution was performed with the following method: 40°C for 3 min, 15°C min<sup>-1</sup>  
2 from 40°C to 160°C, 1.5°C min<sup>-1</sup> from 160°C to 220°C and finally 20°C min<sup>-1</sup> from 220°C to  
3 280°C. Relative quantification of each monosaccharide was carried out using external  
4 monosaccharide standards for their respective retention times and response factors and using  
5 inositol as internal standard [61].

#### 6 2.10. *Arabinogalactan protein (AGP) quantification by rocket gel electrophoresis*

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

#### 19 2.11. *Statistical analysis*

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

### 1 **3. Results**

#### 2 *3.1. Root colonization by A. euteiches during the first three days of infection*

3 Pea root surface colonization was assessed by *A. euteiches* staining at 1, 2 and 3 dpi (Fig. 1).  
4 The colonization of pea roots was much stronger than that of faba bean roots. At 1 dpi,  
5 encysted zoospores and germinated cysts were localized over both the elongation and  
6 maturation zones (Fig1B). Mycelial hyphae were mostly observed longitudinally at the  
7 maturation zone. At 2 dpi, mycelium was extended and grown on the root elongation and  
8 maturation zones. At 3 dpi, it fully surrounded the root tip and elongation zones (Fig.1B). On  
9 faba bean root surface, at 1 dpi, a lower number of cysts was observed. Cysts were distributed  
10 throughout the root and a few cysts germinated preferentially above the meristematic zone. At  
11 2 dpi, cyst germination was weak. At 3 dpi, a few cysts had produced a germ tube on both  
12 elongation and maturation zone root parts.

#### 13 *3.2. Effect of pea and faba bean co-cultures on the colonization of the roots by the pathogen*

14 We monitored the colonization of roots of both species by the pathogen in a co-culture  
15 by quantifying *A. euteiches* DNA from roots of both species at 1, 2, and 3 dpi (Fig. 2 and Fig.  
16 S2). We also assessed the effect of faba bean root exudates on the colonization of pea root by  
17 the pathogen. Three experimental conditions were used. To test the impact of co-culture on  
18 zoospore encystment, both species were co-inoculated with zoospores and co-cultivated  
19 during 3 days (condition 1). In condition 2, the plants were inoculated separately and then co-  
20 cultivated in order to evaluate the effect of co-culture on mycelium growth. Pea plants were  
21 inoculated and then cultured in the presence of faba bean root exudates in condition 3.  
22 Controls corresponding to peas and faba beans inoculated and cultured separately have been  
23 made (Supplementary Fig. S1). Major data are first that there is more pathogen DNA in pea

1 than in faba bean roots (Fig. 2A, 2B and Fig. S1) and secondly that *A. euteiches* DNA content  
2 decreased in both plants when they are co-cultivated (Fig. 2A, 2B). While DNA content of *A.*  
3 *euteiches* is strictly reduced in faba bean at 1 dpi, it is significantly altered in pea at 3 dpi.  
4 Third, the addition of faba bean root exudates in the pea culture medium induced a significant  
5 reduction of infection (measured by the content of the pathogen DNA) (Fig. 2C). For pea,  
6 there are no significant difference between the three conditions (Fig. S2). The comparison of  
7 the number of ITS copies between *A. euteiches* DNA control and *A. euteiches* DNA  
8 supplemented by plant DNA did not reveal any significant differences (Supplementary Table  
9 S1).

### 10 3.3. Effect of pea and faba bean root exudates on *A. euteiches*

11 To study the effect of root exudates on zoospore behavior, we have performed  
12 chemotaxis and germination *in vitro* assays using the experimental device shown in Fig 3A.  
13 We have assessed the effect of bean and pea root exudates as well as Gum Arabic (Fig.3B,  
14 3C). Our data show that root exudates from non-inoculated peas had no chemoattractive effect  
15 on zoospores whereas those from inoculated plants presented a significant positive CI of  $0.31$   
16  $\pm 0.03$ . Interestingly, we found that root exudates from both non-inoculated and inoculated  
17 faba bean plants had a significant negative CI equal to  $-0.52 \pm 0.12$  and  $-0.49 \pm 0.06$  (Fig.  
18 3B). Remarkably, among the root exudates tested, only those from non-inoculated peas  
19 promoted the germination of cysts ( $13.64 \pm 2.84\%$ ) as compared with the controls  $1.67 \pm$   
20  $1.11\%$  (Fig. 3C). Gum Arabic, had a strong attractive effect (CI =  $0.82 \pm 0.14$ ) but did not  
21 promote cyst germination. Chemotaxis index corresponding to the neutral condition (water vs  
22 water) was  $0.17 \pm 0.19$  and the percentage of germinated cysts in Z1 and Z2 areas were  $7.25 \pm$   
23  $4.57\%$  and  $5.02 \pm 3.15\%$  (Fig. 3C).

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

#### 6 3.4. UPLC-MS based analyses of root exudates

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

13 In pea root exudate extracts, five peaks were assigned to tryptophan, *p*-coumaric acid, trans-  
14 ferulic acid, formononetin and pisatin by comparison with authentic standards (Table 1 and  
15 Fig. S3A). All these metabolites have been previously described in pea [65,66]. At a retention  
16 time of 5.38 min, a peak showed a molecular ion [M-H]<sup>-</sup> at *m/z* 609 and [M+H]<sup>+</sup> at *m/z* 611.  
17 In negative mode this analysis produced a characteristic fragment at *m/z* 301 [M-H-glucose-  
18 rhamnose]<sup>-</sup> corresponding to a quercetin moiety. Therefore, this compound was identified as  
19 quercetin-3-rutinoside as previously described in pea seed coat extracts [67]. A peak at 7.10  
20 min produced a molecular ion [M-H]<sup>-</sup> at *m/z* 302. The corresponding UV spectrum and MS  
21 fragmentation features allowed to annotate this peak as DMDI (7,2'-dihydroxy-4',5'-  
22 methylene-dioxyisoflavanol). In the same way, the peak at 10.22 min showing molecular ions  
23 [M-H]<sup>-</sup> at *m/z* 297 and [M+H]<sup>+</sup> at *m/z* 299 as well as specific UV spectrum was assigned as

1 DMD (7,2'-dihydroxy-4',5'-methylenedioxyisoflavone). These two last compounds are known  
2 to be biosynthetic intermediates in the pathway of pisatin a phytoalexin of pterocarpan type  
3 with formononetin as precursor [66]. In faba bean exudates, tryptophan and formononetin  
4 were undoubtedly identified by comparison with pure standards (Table 2 and Fig. S3B). The  
5 peak at retention time 6.32 min, showing a molecular ion [M-H]<sup>-</sup> at m/z 385 and a fragment  
6 ion 223 [M-H-162]<sup>-</sup> corresponding to the loss of a glucose moiety was assigned to sinapoyl-  
7 glucoside, a hydroxycinnamic acid derivative previously described in faba bean leaf extracts  
8 (Neugart *et al.*, 2015). Peak at 8.11 min produced a [M-H]<sup>-</sup> molecular ion at m/z 273 and  
9 fragments ions at m/z 224 and 183. It was assigned to wyerone epoxide as reported in faba  
10 bean (Hargreaves *et al.* 1976). Peak at 10.41 min gave a molecular ion [M-H]<sup>-</sup> at m/z 261  
11 producing two fragments at m/z 217 and 191, and was assigned to dihydrowyerol. This  
12 metabolite was previously reported in *Vicia faba* [68]. Peak at 10.57 min showed a [M-H]<sup>-</sup>  
13 ion at m/z 243 and a fragment ion at m/z 225 and was attributed to wyerone acid as previously  
14 described [68]. These three last compounds belong to furanoacetylene family, a non-flavonoid  
15 class of phytoalexins. The peak at 12.09 min showed a molecular ion [M-H]<sup>-</sup> at m/z 269 and  
16 was assigned to the pterocarpan medicarpin as previously described in broad bean [69].

### 17 3.5. AGPs content in root exudates

18 Given that AGPs impacted *A. euteiches* development [42] and that the relative levels of Ara  
19 and Gal monosaccharides were modulated following inoculation (Fig. S4; Table S3), we  
20 focused on AGPs analysis. A semi-quantitative analysis of type II arabinogalactans (AG-II), a  
21 typical side chain of AGPs, was performed (Table 3). Rocket gel electrophoresis of root  
22 exudates (AIR or raw fractions) detected AGP in pea and faba bean extracts (Supplementary  
23 Fig. S5). The amount of AGP strongly increased in extracts from infected pea roots as  
24 compared with non-infected roots (Table 3). In contrast, no significant variations were



1 observed in extracts from faba bean roots. It is important to note that AGPs, were detected  
2 neither in *A. euteiches* mycelium nor in the mycelium culture medium (Supplementary Fig.  
3 S5).

#### 4 **4. Discussion**

5 Although the life cycle of *A. euteiches* is characterized by rapid colonization of the root  
6 system within hosts, better understanding of the early stages of the disease development is  
7 necessary. We studied the *A. euteiches* colonization, during the first three days of infection,  
8 within pea and faba bean roots respectively sensitive and tolerant to this root rot agent. ~~To the~~  
9 ~~best of our knowledge, this work reveals for the first time that pea root colonization by~~  
10 ~~*A. euteiches* can be significantly reduced by faba bean root exudates.~~

##### 11 *4.1. Host specificity of A. euteiches infection at early stages*

12 Compared with faba bean, pea roots are more intensely colonized by *A. euteiches* during the  
13 first three days of infection. Microscopical and qPCR data showed that colonization of pea  
14 root is high at 3 dpi. These results are in line with those reported by Billard et al. (2019) [54]  
15 showing that root necrosis is observed much earlier in pea than in faba bean roots. We show  
16 that host sensitivity is involved in the oomycete infectious process at early stages and attest  
17 that *A. euteiches* strain RB84, is preferentially aggressive on the susceptible plant. As  
18 previously observed [19,21], we show that pea root tips remained free of infection. These  
19 results highlight the protective function of root cap-derived border cells and their secretions  
20 toward the root apical meristem at early stages of infection. The primary function of motile  
21 zoospores is to connect oomycetes to the host [70]. Several host-specific molecular attractants  
22 have been reported such as isovaleraldehyde, which attracts *Phytophthora palmivora* [71],  
23 prunetine a pea compound that attracts *A. euteiches* zoospores [52] or indole-3-aldehyde

1 isolated from cabbage that promotes *Aphanomyces raphani* zoospores attraction [72]. Root  
2 exudates from pea induce a higher rate of oospore germination than those from vetch and faba  
3 bean [54]. Although, pea root exudates from non-inoculated plants affect zoospore  
4 germination it has little effect on zoospore attraction. The chemotaxis index (CI) of exudates  
5 originated from non-inoculated peas is nearly null. In contrast, the CI is positive for exudates  
6 originated from inoculated peas. Kong and Hong (2010) [73] reported that *Phytophthora*  
7 *nicotianae* zoospores secrete a fluid able to promote successful infection in *Arabidopsis*  
8 *thaliana*. Exudates from zoospores of *Phytophthora* and *Pythium* species act as a *quorum-*  
9 *sensing* signals impacting zoospore homing and triggering infection [73–75]. Herein, exudates  
10 from *A. euteiches* zoospores and/or mycelium may be present in harvested pea exudates and  
11 would promote zoospore attraction. The CI for root exudates from non-inoculated or  
12 inoculated faba beans was negative which suggests that faba bean exudates had a repellent  
13 effect towards *A. euteiches* zoospores. Host repellent signals have been mostly demonstrated  
14 in plant-nematode interactions [76–79]. As for oomycete-host interactions, a phenolic extract  
15 from the root of the non-host plant *Portulaca oleracea*, was shown to exhibit a repellent  
16 activity on *Aphanomyces cochlioides* zoospores [80]. Bazghaleh *et al.*, (2018) [81] showed  
17 that, in response to *A. euteiches* infection, the root polyphenolic composition of lentil is  
18 dependent on the plant genotypes. The metabolic profiling of pea and faba bean root exudates  
19 based on UPLC-MS analyses revealed the presence of two main classes of plant specialized  
20 metabolites, phenolic compounds and furanoacetylenic derivatives. Among phenolic  
21 compounds, flavonoids are ubiquitously found in the plant kingdom, whereas isoflavones are  
22 restricted to the subfamily of Papilionoideae [82]. Within Fabaceae, almost all species of the  
23 subfamily Papilionoideae accumulate isoflavones and derivatives, including phytoalexins of  
24 the pterocarpan type [82]. Here, the presence of pterocarpan biosynthetic pathway was  
25 observed in both legumes confirming previous chemotaxonomy observations in whole plants

1 [66,69]. Focusing on the root exudate composition, the presence of the phytoalexin pisatin in  
2 pea root exudates was already reported [21], however the presence of pterocarpan-type  
3 phytoalexin (medicarpin) in faba bean root exudates has never been reported in earlier studies.  
4 Although different susceptibility levels toward *A. euteiches* were observed, pterocarpan-type  
5 phytoalexins were identified in both legumes suggesting that they might not be strongly involved in the  
6 susceptibility/tolerance during early stages of infection by *A. euteiches*. A search for the  
7 distribution of furanoacetylene in the metabolite-plant species database Knapsack indicates a  
8 presence restricted to the two genus *Vicia* and *Lens* [83]. Furanoacetylenic derivatives were  
9 described as markers of resistance of faba bean to chocolate spot disease [84]. The ability of  
10 *Botrytis fabae* and not *B. cinerea* to metabolize the phytoalexin wyerone acid was of primary  
11 significance in the pathogenicity towards faba bean [68]. Therefore, we suggest that the  
12 presence of furanoacetylenic in root exudates of faba bean might contribute to tolerance  
13 mechanisms of faba bean to the pathogen *A. euteiches*.

14 Coupled to small molecules, root exudates contain macromolecules like cell wall polymers  
15 [85,86] (Chaboud, 1983; Knee *et al.*, 2001). Hinch and Clarke (1980) [87] demonstrated that  
16 zoospore adhesion of *Phytophthora cinnamomi* depends on carbohydrate components present  
17 at the *Zea mays* L. root surface. Pea root exudate monosaccharide profiles are quite similar to  
18 those previously described [86,88,89]. For the two legumes, Ara and Gal are among the major  
19 sugars detected suggesting the presence of arabinogalactan motifs. The precipitation by the  $\beta$ -  
20 glucosyl Yariv reagent assessed that AG-II are present in the root exudates. AG-II are the  
21 major side chain of arabinogalactan proteins (AGP). AGP is a family of non-enzymatic cell  
22 surface hydroxyproline-rich glycoproteins found in plant cell walls, in the plasma membrane  
23 or in plant secretions [63,90] especially the root cap-associated mucilage and root exudates  
24 [42,56,91]. These proteoglycans control cell morphology and growth, and play a major role in

1 plant-micro-organism interactions [38,91–93]. Here, a high CI was recorded with the Gum  
2 Arabic (an AGP-like molecule) whereas the cyst germination was not detectable. These  
3 observations are in line with the results described by Cannesan *et al.* (2012) [42] using  
4 purified pea root AGPs that show that AGPs stimulate zoospore attraction and encystment  
5 but also reduce cyst germination. The present results revealed an increase of AGP content in  
6 root exudates concomitantly to infection in pea. Exudates from non-inoculated peas were  
7 favorable to cyst germination while those from inoculated seedlings had no impact. This  
8 could be linked to the increase of AGP in pea root exudates after infection.

9 ~~Attraction of zoospores and their encystment do not necessarily guarantee successful~~  
10 ~~infection and mycelium colonization mostly depends on their capacity to penetrate host~~  
11 ~~tissues [94]. Van West *et al.* (2003) [70] suggest that host specificity is involved during the~~  
12 ~~penetration phase. The estimation of *A. euteiches* root content, indicate that mycelium~~  
13 ~~developed more importantly in pea root as compared with faba bean showing a better ability~~  
14 ~~to pea root penetration. Expression of pea genes involved in cell wall modifications such as~~  
15 ~~those encoding callose synthase, pectin esterase and pectin esterase inhibitor are modulated~~  
16 ~~during *A. euteiches* infection [31]. Gaulin *et al.* (2018) [95] have also shown that *A. euteiches*~~  
17 ~~pathogenicity towards *Medicago truncatula* is correlated with the production of specialized~~  
18 ~~secretome including carbohydrate active enzymes such as cellulase, cellobiohydrolase,~~  
19 ~~xylanase, polygalacturonase and pectin lyase. During penetration and colonization stages,~~  
20 ~~*A. euteiches* is likely to secrete specific cell wall degrading enzymes that facilitate mycelium~~  
21 ~~penetration and development within the host [96–98].~~

22 4.2. Pea susceptibility towards *A. euteiches* is reduced by faba bean

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

1        ~~Root exudates directly influence root-root interaction in the rhizosphere [51,53,100–102].~~  
2        ~~Regarding plant species communication, Wentzell and Kliebenstein (2008) [103] showed that~~  
3        ~~glucosinolate activation in *Arabidopsis* was regulated by the density of neighboring plants.~~  
4        ~~More recently, Chen *et al.* (2019) [104] showed that tobacco plants respond to the presence of~~  
5        ~~belowground neighbors by activating different defense pathways.~~

## 6        **Conclusions**

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

## 14       **6. Author Contributions**

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

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### 3 **Declaration of Competing Interest**

4 The authors declare no conflict of interest.

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1 **Figures**

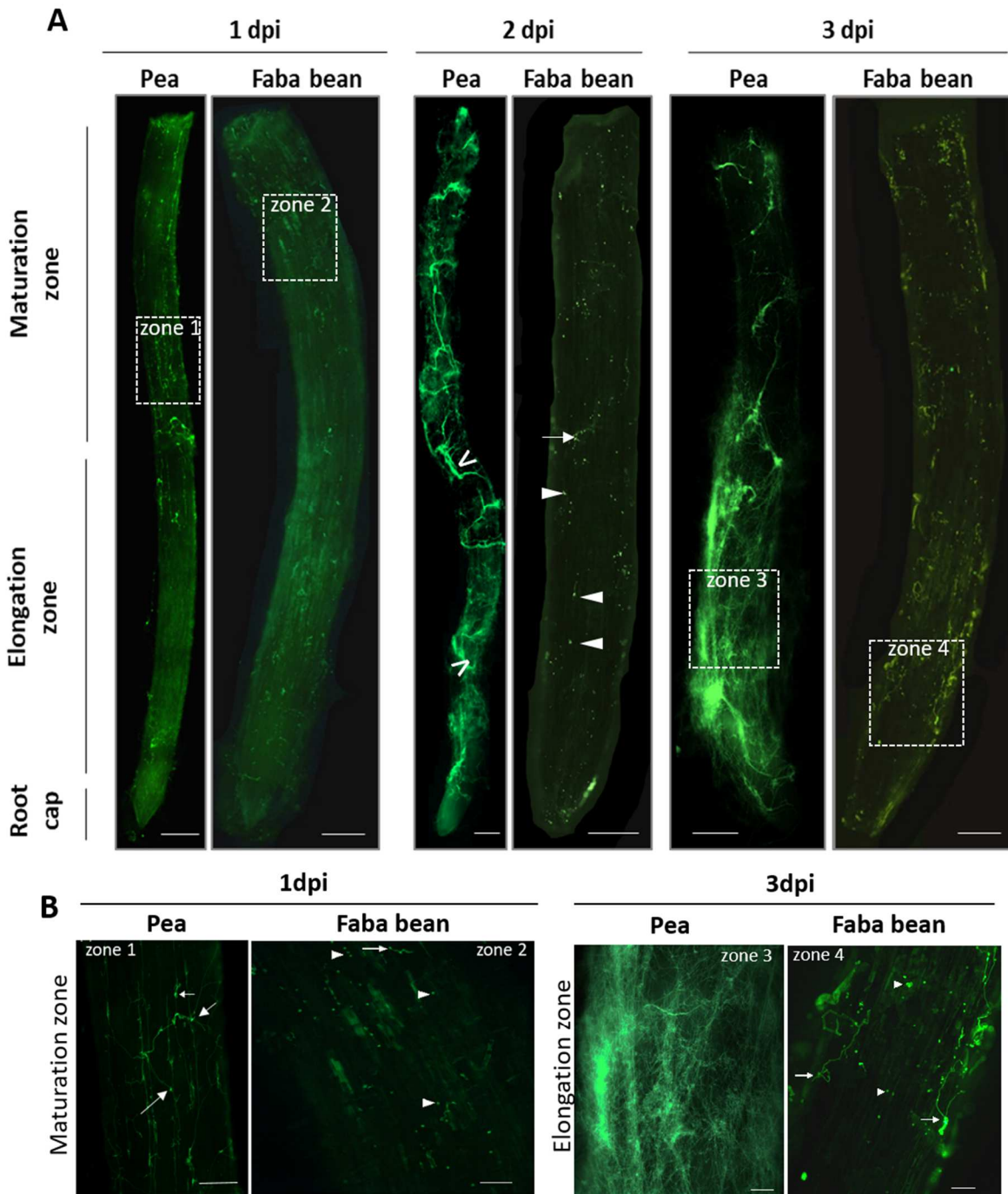


Figure 1

2

3 **Fig.1. A. euteiches** colonization in pea and faba bean roots at early stage of infection. at  
 4 1, 2 and 3 dpi by epifluorescence microscopy. dpi, days post-inoculation; non-germinated

- 1 cysts (white triangles), germinated cyst (white arrows) and mycelium (arrows heads). Scale
- 2 bars: 500  $\mu\text{m}$  (A); 100 $\mu\text{m}$  (B).

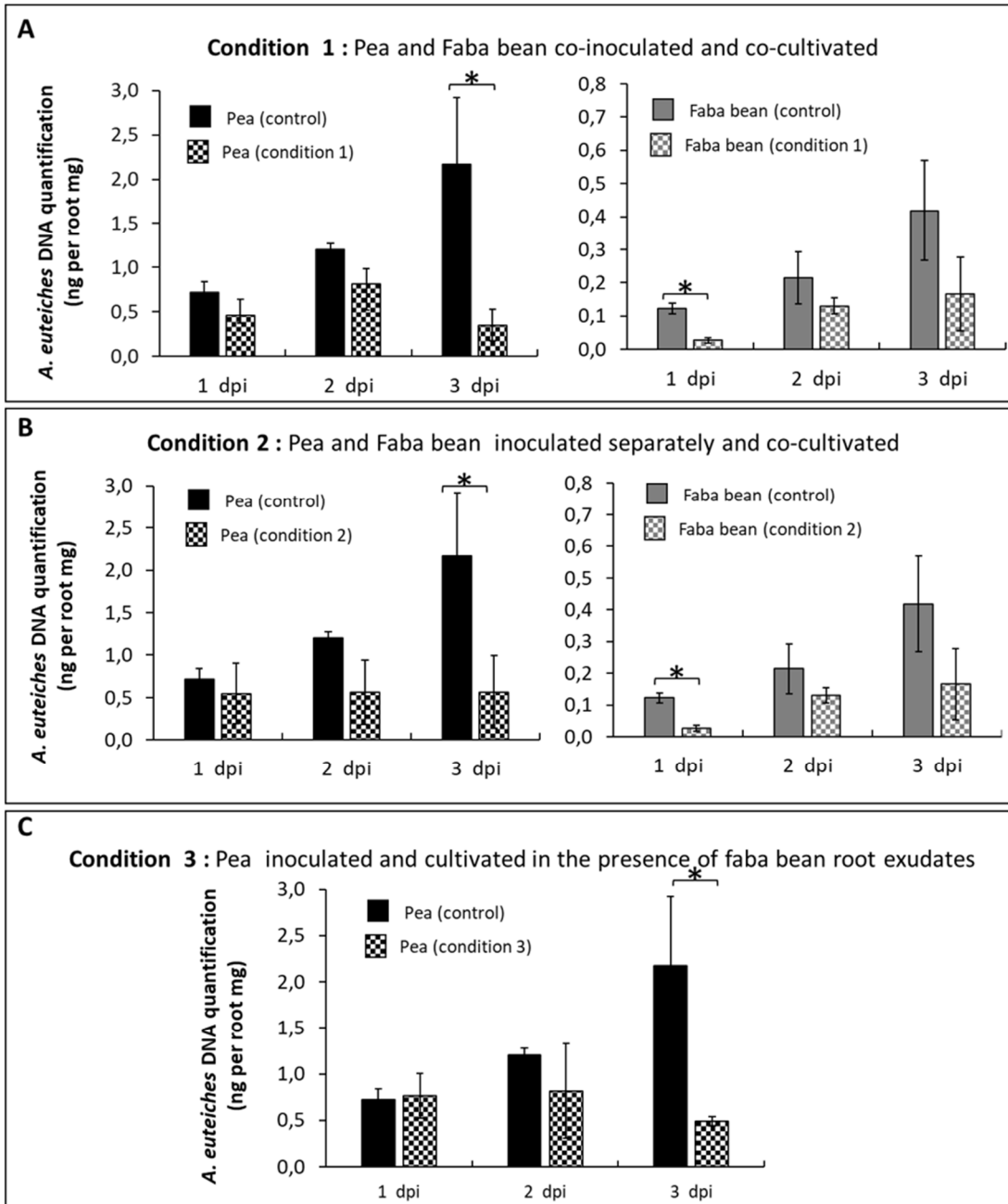


Figure 2

3

4 Fig. 2 Quantification of *A. euteiches* DNA in pea and faba bean roots

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

10

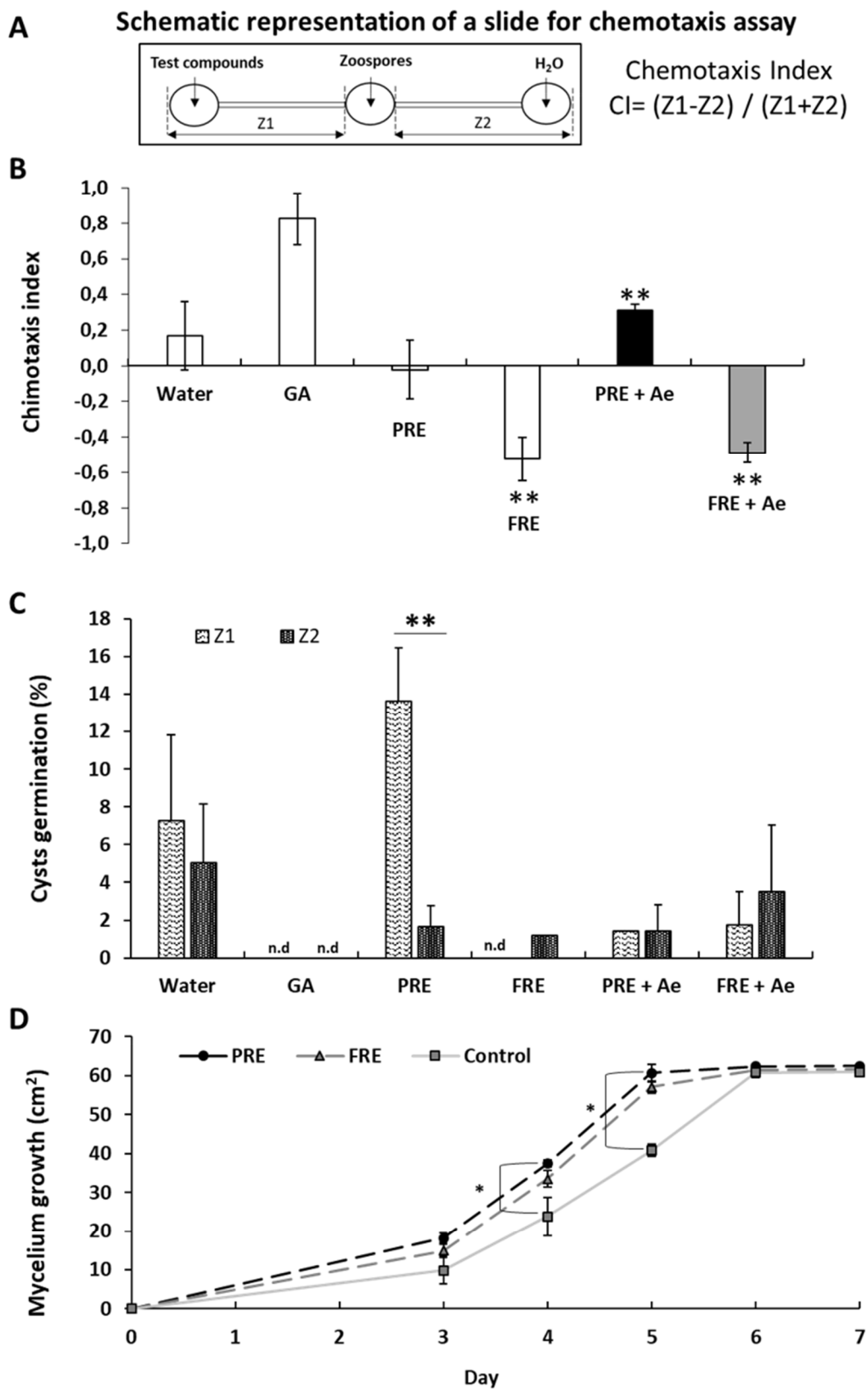


Figure 3

1 **Fig. 3. Effect of pea and faba bean root exudates on *A euteiches* behavior**

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

21

22 **Tables**

23 **Table 1:** List of polyphenols identified in pea root exudates.

RT (min)	Compound assignment	Compound class	MW	[M-H]-	[M+H]+	Fragments (ES-)	Fragments (ES+)	References
3.39	tryptophane <sup>a</sup>	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	<i>p</i> -coumaric acid <sup>a</sup>	phenolic	164	163	165	119	147; 119	standard
6.32	trans-ferulic acid <sup>a</sup>	phenolic	194	193	195	178; 134	177	standard
7.1	DMDI <sup>a</sup>	isoflavanol	302	301		273; 163		Celoy and VanEtten. 2013
10.22	DMD <sup>b</sup>	isoflavone	298	297	299		321 [M+Na]+	Celoy and VanEtten. 2013
11.19	formononetin <sup>a</sup>	isoflavone	268	267	269	252		standard
11.76	pisatin <sup>a</sup>	pterocarpan	314	313		299; 181		standard

1 <sup>a</sup>7,2'-dihydroxy-4',5'-methylene-dioxyisoflavanol

2 <sup>b</sup>7,2'-dihydroxy-4',5'-methylenedioxyisoflavone

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

RT (min)	Compound assignment	Compound class	MW	[M-H]-	[M+H]+	Fragments (ES-)	Fragments (ES+)	References
3.39	tryptophane <sup>a</sup>	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	dihydroxyerol	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 <sup>a</sup>	isoflavone	268	267	269	252		standard
12.09	medicarpin	pterocarpan	270	269	271	254; 163	165; 137	Abu-Reidah et al. 2014

4

5

6 **Table 3. AGP quantification in root exudates at three days after inoculation**

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

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<b>AGPs quantification (mg ml<sup>-1</sup>)</b>		
	<b>Control</b>	<b>Plant + <i>Ae</i></b>
<b>Pea</b>	<b>0.15 ± 0.09</b>	<b>0.42 ± 0.13 *</b>
<b>Faba bean</b>	<b>0.14 ± 0.15</b>	<b>0.05 ± 0.09 ns</b>

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