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Soil microbiota promotes early developmental stages of *Phelipanche ramosa* L. Pomel during plant parasitism on *Brassica napus* L.

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Abstract

Purpose

The root holoparasitic plant *Phelipanche ramosa* has become a major constraint for rapeseed cultivation in western France for the last decades and its control remains challenging. To date, few studies have considered soil microbiota as a third partner of the parasitic plant-plant interaction. Therefore, we here addressed the question of how soil microbiota interferes with host-derived signal metabolites required for host plant recognition by the parasitic plant.

Methods

Using a branched broomrape infested soil (genetic group 1) from a rapeseed field, we first provided soil physicochemical and microbiological descriptions by metabarcoding, followed by *P. ramosa* seed germination and prehaustorium formation bioassays, and by *in vitro* co-cultivation with *Brassica napus.*

Results

Co-cultivation in presence of soil microorganisms promoted parasitic plant seed germination and attachments to host's roots. Seed germination assays showed that only the combination of gluconasturtiin (main rapeseed glucosinolate) with soil extracts stimulated broomrape germination. This suggests a microbial conversion of gluconasturtiin into germination stimulants via soil microbial myrosinase enzymes. Furthermore, soil bacteria *Arthrobacter*, *Ralstonia*, *Actinobacterium, Proteobacterium* spp. and fungus *Penicillium* spp. were isolated and screened for myrosinase activity. Pre-germinated seeds treated with soil extracts or differentially filtrated soil extracts also promoted the formation of *P. ramosa* prehaustorium and led to more parasitic attachments on rapeseed roots in co-cultivation assays. This thus suggests that this enhancement of parasitic attachments could also be partly attributed to soil microbial production of haustorium inducing factors.

Conclusion

Soil microbiota influences *B. napus* - *P. ramosa* interaction by altering direct and indirect recognition signals.

Abbreviations

AMF: Arbuscular mycorrhizal fungi

ASV: Amplicon sequence variant

GNT: Gluconasturtiin

GS: Germination stimulants

GH: Glycoside hydrolase

SNG: Sinigrin

TDZ: Thidiazuron (synthetic cytokinin)

tZ: *trans*-Zeatin

INTRODUCTION

Rhizosecretion of plant-derived compounds (*i.e.* root exudates) improves plant nutrient-seeking and defense abilities, partly by allowing beneficial co-associations with neighboring organisms (Bais et al. 2006). Nevertheless, this carbon exchange can also be used as a signal to initiate host invasion (Trivedi et al. 2020; Vives-Peris et al. 2020). Such a mechanism is illustrated in *Orobanche* and *Phelipanche* spp. (broomrapes) which are obligate root holoparasites from the *Orobanchaceae* family that retrieve all of their water and nutrients from a host plant (Heide-Jørgensen 2013). Among broomrapes, *Phelipanche ramosa* L. Pomel is a major constraint, worldwide and more specifically in Mediterranean regions, to a wide range of economically important crops such as *Solanaceae* (*Nicotiana tabacum* L., *Solanum tuberosum* L., *S. melongena* L.), *Cucurbitaceae* (*Cucumis melo* L., *C. sativus* L.) and *Brassicaceae* (*Brassica napus* L., *Sinapis* spp. L.), as well as several weed species (Gibot-Leclerc et al. 2012; Parker 2013).

As an adaptive strategy for host detection, broomrape seeds only germinate under suitable abiotic conditions and in response to the fine perception of germination stimulants (GSs) found in the root exudates of many host species (Bouwmeester et al. 2021). Historically, the first class of GSs characterized were the strigolactones, plant hormones derived from the carotenoids (Cook et al. 1972; Yoneyama et al. 2010) further identified as regulating several plant developmental and signaling processes in the rhizosphere such as initiation of symbiosis with arbuscular mycorrhizal fungi (Akiyama et al. 2005; Ćavar et al. 2015). GSs also act as chemotropic signals for *Orobanchaceae* root parasitic plants to direct their radicle towards host roots (Krupp et al. 2021; Ogawa et al. 2022). Yet

another adaptive trait is the formation of a specialized intrusive organ at the apex of the parasite radicle, called haustorium. This unique development process occurs upon perception by the parasite of haustorium inducing factors (HIFs) released by the host plants (Yoshida et al. 2016). HIF perception first triggers prehaustorium formation which includes morphological changes such as radicle growth arrest, radial cellular extension, and formation of secretory papillae with adhesive functions (Joel and Losner-Goshen 1994; Goyet et al. 2017). After the prehaustorium development, the haustorial cells then penetrate host tissues to form a vascular bridge, which channels the nutrient flow towards a parasitic tubercle (storage organ). This organ then grows at the expense of the crop, develops adventitious roots and finally produces an above-ground flowering stem which will produce seeds viable for decades in the soil (Joel et al. 2007). Although HIFs triggering prehaustorium formation in hemiparasitic plants have been studied for a long time and shown to belong to the quinones and phenolic compounds family (Goyet et al., 2019), effective HIFs in the holoparasite *P. ramosa* have been recently detected in rapeseed rhizosphere and shown to belong mainly to the cytokinin class of compounds (Goyet et al. 2017).

Although described as a generalist parasitic plant, *P. ramosa* also exhibits suboptimal development on some host crops, resulting for instance in France, in its structuration in three genetic populations according to their host preference and geographical distribution (Brault et al. 2007; Stojanova et al. 2019; Huet et al. 2020). *P. ramosa* genetic group 1 is a major drawback for farmers of western France who are facing severe yield losses. To date, no mean to limit its spreading on rapeseed, nor suitable control solutions exist. The occurrence of genetic groups was shown to be due to abiotic factors such as pedo-climatic and agronomic context (Gibot-Leclerc et al. 2003; 2012) but also to differences in sensitivity to host cues during early developmental stages (Stojanova et al. 2019; Huet et al. 2020). Indeed, rapeseed, which is a non-mycorrhizal species, exudes none or only few strigolactones (Auger et al. 2012; Yoneyama et al. 2018; de Saint Germain et al. 2021). This has presumably led *P. ramosa* to adapt and perceive other GSs such as isothiocyanates (Auger et al. 2012; Miura et al. 2022). These volatile compounds are the main breakdown products of glucosinolate hydrolysis. They are nitrogen- and sulphur-containing secondary metabolites found almost exclusively in the *Brassicaceae* plant family (Fahey et al. 2001; Wittstock et al. 2016).

Because most *P. ramosa* biological cycle takes place in the rhizosphere, which is a complex and rich niche for microbial interactions, plant-associated microbiota has recently been considered as a third partner, contributing to the parasite's life history traits. Recently, microbial communities have been described as a potential tool for broomrape control (Cartry et al. 2021), but also as drivers of broomrape development on its hosts in agrosystems (Hristeva and Denev 2017; Iasur Kruh et al. 2017; Fitzpatrick and Schneider 2020; Huet et al. 2020; Durlik et al. 2021). Durlik et al. (2021) indeed

suggested that both *P. ramosa* seed endophytic bacteria *Brevibacterium frigoritolerans* and *Bacillus simplex* could promote the germination and longevity in the soil of their parasitic host. Additionally, *Sphingobacterium* spp. and *Leptosphaeria maculans* were mostly found in *P. ramosa* seeds of genetic group 1 and potentially facilitate seed germination by improving the production of isothiocyanates as GSs in the rhizosphere of rapeseed (Huet et al. 2020). Indeed, release of isothiocyanates usually occurs upon plant injury, when glucosinolates come in contact with spatially separated endogenous plant myrosinase enzymes (β-thioglucosidase glucohydrolase - EC 3.2.1.147; Wittstock et al. 2016) or alternatively, *via* exogenous myrosinases detected in several microorganisms found in glucosinolate-containing plants and soils (Al-Turki and Dick 2003; Gimsing et al. 2006). Hence, plant-associated microbes gain competitive advantages for carbon acquisition when they can tolerate isothiocyanates (Rumberger and Marschner 2003; Bressan et al. 2009; Szűcs et al. 2018). Plant microbiomes can also alter hormone levels, such as cytokinins, which can be produced in the rhizosphere by a large range of microbial species (Müller et al. 1989; García de Salamone et al. 2001; Spallek et al. 2018) resulting in possible alterations of HIF signaling in broomrape seeds. Occurrence of such microbes in the soil could contribute to the parasitic behavior of *P. ramosa* on *B. napus,* but still needs to be functionally demonstrated.

We here aimed to demonstrate that soil microorganisms are able to interfere with host-derived signaling metabolites that control the parasitic plant cycle, and that they ultimately impact the success of the parasitic plant. To do so, we used a soil from a rapeseed field of western France heavily infested with broomrape (*P. ramosa* genetic group 1). The main objectives were i/ to describe the soil-associated bacterial and fungal communities, ii/ to assess the activity of the soil microbiome on *P. ramosa* key pre-parasitic stages including germination and induction of prehaustorium formation, through its interaction with the host-parasite plant allelochemical signaling and iii/ to functionally validate microbial contribution to broomrape germination using fungal and bacterial candidates. This latest part should provide original and useful findings for the management of microbiota in the soil, in relation to the control of *P. ramosa*.

MATERIALS AND METHODS

The experimental design displaying the link between the different experiments is described in Supp. Fig.S1.

Plant materials

To preserve seed quantity throughout the study, two batches of parasite seeds were used, Pram120 and Pram123. These seed batches both display similar specific phenotypic characteristics of genetic group 1 (Auger et al. 2012; Stojanova et al. 2019; de Saint Germain et al. 2019, 2021; Huet et al. 2020).

Pram120 and Pram123 seeds were collected in rapeseed fields at Saint-Jean-d'Angély (France, 45°56'40.545" N, 0°31'10.472" O; Charente Maritime, France) in June 2015, and at Benet (46° 22'6'' N, 0° 35' 45.999'' O; Vendée, France) in July 2019 respectively, from dry mature spikes of *P. ramosa*. Seed batches were sieved through a 180-200 µm mesh, and kept in the dark at 25°C until use.

Brassica napus L. seeds from the highly susceptible to *P. ramosa* variety Veritas CL (Terres Inovia) were provided by DSV France SARL (Terminiers, France) in October 2019, and kept in a tinted glass jar at 4°C.

P. ramosa and *B. napus* seeds were similarly surface sterilized according to Lechat et al. (2012). Seeds were immersed in a 2.5% (v/v) sodium hypochlorite solution for 3-times 30 s under vigorous shaking and then rinsed 5 min in sterile distilled water. *P. ramosa* seeds were then suspended in an incubation solution (HEPES 1 *mM*; pH 7.5 adjusted with KOH; PPM 0.1% v/v) at a final seed concentration of 10 $mg.mL^{-1}$, and incubated 7 days in the dark at 21 °C as a conditioning period (Lechat et al. 2015).

Soil sampling and characterization

Description of soil sampling site, crop itinerary and physicochemical analyses are available on supplementary materials (Supp. Fig. S2).

Thirty liters of soil were collected from multiple random sampling point at a 0-20 cm depth in a rapeseed field infested by *P. ramosa* after harvest at Faye-sur-Ardin (46°25'57.72" N, 0°29'19.68" O; Deux-Sèvres, France) in July 2019. This field was under a wheat – barley – rapeseed triennial crop rotation since 2017. Prior to use, the soil was homogenized, hand sieved to remove large debris, and kept in a dark room in a closed container, under low temperature and humidity fluctuations. Physicochemical analyses were performed at the SADEF laboratory (Aspach le bas, France). Texture was loamy with 12.4% clay, 73.2% silt and 14.3% sand. Soil was slightly alkaline (pH of 7.9) with a high organic matter content (67 $g.kg^{-1}$) and a low C/N ratio (8.6 for values between 8 to 12). To measure the effect of soil microorganisms on plant parasitism, a portion of the sampled soil underwent gamma-sterilization at 35-60 kGy (IONISOS, Pouzauges, France), to eradicate most microorganisms while limiting damages to the soil's integrity (McNamara et al. 2003). Effect of sterilizing treatment was controlled by plating out soil suspensions on a solid rich medium and assessing colony forming units (CFUs). Soil suspensions were prepared as 0.5 g of soil per mL of sterile distilled water macerated for two-hour and then diluted from 1 to 10^{-3}. Two hundred µL of these suspensions were then plated on LB agar for bacterial assessment, and on potato dextrose agar (39 $g.L^{-1}$ PDA, Sigma-Aldrich, in distilled water; pH 5.6) for fungal assessment. LB and PDA plates were then incubated seven days at 28°C and 21°C respectively (Balestra and Misaghi 1997; Obire and Anyanwu 2009).

Soil DNA extraction and high-throughput sequencing

Soil DNA was extracted in triplicates from 200 mg of solid materials (bulk soil; after harvest). Sterile distilled water was added as a negative control. Samples were processed using the NucleoSpin® Soil Kit (Macherey-Nagel) according to the recommended standard instructions. Amplicon library were constructed as described by Huet et al. (2020). Two-step PCRs were required for Illumina MiSeq sequencing, with PCR1 used for amplification of the specific V4 regions of bacterial 16S rRNA genes and fungal internal transcribed spacer (ITS1) genes, and PCR2 used to add barcodes and adapters to these amplicons. For bacteria, the paired primers of taxonomic markers used for PCR1 were 16S_515f (GTGCCAGCMGCCGCGGTAA) and 16S_806r (GTGCCAGCMGCCGCGGTAA) (Caporaso et al. 2011). For fungi, paired primers used were ITS1_f (CTTGGTCATTTAGAGGAAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC) (Buée et al. 2009). PCR1 program was 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 45 s, 68 °C for 1min 30 s, final extension at 68 °C for 10 min and final cooling at 10 °C. PCR2 program consisted in 94 °C for 60 s followed by 12 cycles of 94 °C for 60s, 55 °C for 60 s and 72 °C for 60 s and a final extension step of 72 °C for 10 min. Library preparation and sequencing on Illumina MiSeq were performed in the ANAN platform (nucleic acid analysis; SFR QUASAV, Beaucouzé, France).

Bioinformatics analyses

To ensure data reproducibility, sequences were processed through an automated bioinformatics workflow microSysMics (https://bio.tools/microSysMics), as reported by Huet et al. (2020). Quality of reads was controlled with Fastqc and Multiqc (Ewels et al. 2016) on demultiplexed Fast. To preserve sample quality (Phred-score >30), 16S reads were trimmed at 225 bp and 220 bp while ITS reads were trimmed at 175 bp and 170 bp for the forward and reverse reads respectively. Prior to the denoising procedure, PCR adapters were removed using Cutadapt (Martin 2011) and reads were truncated to a uniform length. Then, Dada2 processed unique sequences into ASV (Amplicon Sequence Variant), thanks to an error rate algorithm distinguishing biological sequence variations from amplification or sequencing errors. Denoised reads were merged and chimera were omitted from the dataset. Taxonomic assignments were performed using SILVA database for bacteria (Quast et al. 2012; Yilmaz et al. 2014) and UNITE database for fungi (Abarenkov et al. 2020). For further investigations, specific unidentified ASVs were computed using the BLASTn algorithm through the NCBI database. ASVs were numbered by alphabetical order according to the SILVA or UNITE code respectively for bacteria and fungi. Their associated codes and corresponding DNA sequences are available in supplementary data tables (Supp.Table1_16S and Supp.Table1_ITS). Chloroplast and mitochondria ASVs were removed from the abundance table *via* the Phyloseq package (1.34.0). Potential external contaminants were

discarded using the package Decontam (1.10.0) with the "either" method (Davis et al. 2018), and a 0.5 decision threshold.

Soil extract procedure for biological activity analysis

Soil extracts were obtained by simultaneous extraction of soil living microorganisms and metabolites, and carried out according to an adaptation of several extraction methods using only water and mild centrifugations to avoid death of microorganisms (Morra and Kirkegaard 2002; Gimsing et al. 2005; Choi et al. 2020) The same procedure was used for both native and gamma-sterilized soils. Two replicates of five grams of 1 mm-sieved soil were suspended in 5 mL of sterile distilled water and kept in the dark at 20°C for 1 h under 400 rpm of constant shaking. After centrifugation (500 rpm for 5 min), both supernatants were pooled and pre-filtered on a 0.7 µm glass fiber filters (Merck Millipore) to remove most of soil particles. Half of the pre-filtered extracts were filtered at 0.22 µm with a syringe filter (PES Membrane Millex-GP) to remove microorganisms and extracting soil metabolites. Resulting soil extracts were kept at 4 °C for up to one day before use.

Germination and prehaustorium formation bioassays

Soil extracts were tested on conditioned and pre-germinated *P. ramosa* seeds for germination and prehaustorium formation bioassays, respectively. Sterilizing filtration treatments were carried out on native soil extracts using 0.45 µm (Nylon filter Membrane, Phenomonex) then 0.22 µm (PES Membrane syringe Filter, Millex-GP) to remove part and then all microorganisms (mostly fungi and bacteria) respectively. Additionally, a treatment with a large-spectral biocide (PPM 0.1% v/v) was used to chemically remove microorganisms from the seed suspensions. Germination and prehaustorium-formation bioassays were performed using these different soil extracts, with at least 3 technical and 3 biological independent replicates.

Germination bioassays were carried out according to Pouvreau et al. (2013) using 50 µL of conditioned seeds (10 $mg.mL^{-1}$) in 96-well plates (Cell Culture Multiwell Plate Cellstar; Greiner Bio-One). The different soil extracts were tested at 10-fold and 100-fold dilutions in triplicates in a final volume set to 100 µL in HEPES buffer (1 *mM*; pH 7.5) together with or without 10^{-6} *M* gluconasturtiin (GNT, Phenylethylglucosinolate K-salt from *Nasturtium officinale* L., Phytoplan). GNT was chosen as its main degradation products, 2-PEITC, is primarily known to induce the germination of *P. ramosa* seeds (Auger et al. 2012). Plates were sealed with gas-impermeable filters to prevent inter-well contamination due to volatile degradation products. For positive controls, seed germination was induced with 10^{-7} M *rac*-GR24 (synthetic strigolactone kindly provided by F-D Boyer (Centre National de la Recherche Scientifique, Gif-sur-Yvette, France)). Seeds were treated with (1 *mM*; pH 7.5) HEPES buffer solution as negative controls. Direct induction of germination or presence of residual

glucosinolates was assessed by using control wells without GNT, or in presence of exogenous myrosinase (thioglucosidase from *Sinapis alba*, Sigma-Aldrich; 5 $mU.mL^{-1}$ final concentration) respectively (Auger et al. 2012). Plates were incubated at 21 °C in the dark for 4 days. Seeds were stained by adding 5 µL of thiazolyl methylthiazolyldiphenyl-tetrazolium bromide (MTT; Sigma-Aldrich; 5 $g.L^{-1}$) per well, and the number of germinated seeds was then determined (purple seeds) the following day under binocular (Olympus SZX10; Olympus Europa GmbH). Germination ratio was then expressed as a ratio relative to the average ratio of germination with *rac*-GR24-treated seeds within the same plate.

Prehaustorium formation bioassays were conducted according to Goyet et al. (2017). Five mL of conditioned seeds (10 $mg.mL^{-1}$) were rinsed 3 times in sterile distilled water and suspended in 10 mL of germination medium (HEPES 1 *mM*, pH 7.5, *rac*-GR24 10^{-7} *M*) to reach 5 $mg.mL^{-1}$ seed concentration. After 5 min, 50 µL of treated seeds (around 50 seeds) were distributed in 96 well plates, sealed with parafilm, and incubated at 21 °C in the dark. After 4 days, germination was controlled and should have reached a minimum of 70% germination ratio. The incubation medium was then carefully removed and replaced with 90 µL of HEPES buffer solution (1 *mM*; pH 7.5). Ten microliters of soil extracts were then added at 10-fold and 100-fold dilutions in six replicates. In addition, seeds were treated with the synthetic cytokinin thidiazuron (TDZ; PESTANAL® analytical standard, Sigma-Aldrich;10^{-8} *M*) as positive control (Goyet et al. 2019) and with buffer solution as negative control. Plates were incubated at 21 °C in the dark. After 3 days, seeds were stained by adding 5 µL of MTT per well. Ratio of germinated seeds developing prehaustorium (occurrence of papillae and root tip swelling) was evaluated the following day under binocular (Olympus SZX10; Olympus Europa GmbH), and was reported as a ratio relative to the average ratio of prehaustorium formation on TDZ-treated germinated seeds on the same plate.

Mini-rhizotron co-cultivation for germination and aggressiveness assays

Germination of *B. napus* seeds was induced by placing surface-sterilized seeds between two sterile glass microfiber filters (150 mm diameter, Macherey-Nagel) moistened with 15 mL of sterile distilled water in a round Petri-dish (150 mm diameter, 15 mm height, Dutscher). Petri-dishes were kept 7 days in a growth chamber (21 °C, 16 h photoperiod with 110 µmol m^{-2} s^{-1} photosynthetically active radiation, PAR).

To investigate the interacting effect of soil microbiota and the host plant on broomrape seed germination and aggressiveness (*i.e.* number of broomrapes attached to host roots), two distinct co-cultivation assays were set using *P. ramosa* conditioned seeds or seeds with pre-induced prehaustorium formation for germination and aggressiveness assays respectively. These assays were conducted

according to Gauthier et al. (2012) and Billard et al. (2020) with some minor modifications: one-week old rapeseed plantlets were transferred onto glass fiber filters in mini-rhizotron systems, with either one plantlet for germination assays, or two plantlets for aggressiveness assays to ensure sufficient root surface for attachment of germinated *P. ramosa* seeds. Mini-rhizotrons consisted of a square Petri-dish (120 mm diameter, 17 mm height, Greiner) uniformly filled with sterile rock wool moistened with 50 mL of 50% (v/v) autoclaved Tadano et Tanaka medium pH 7.5 (Tadano and Tanaka, 1976) or 50% (v/v) of Coïc medium pH 6.8 (Coic and Lesaint 1975), for germination or aggressiveness assays respectively. Mini-rhizotrons were sealed on three sides with parafilm, covered with aluminum and incubated in the growth chamber under the same conditions. Plants were watered with 10 to 40 mL of medium every 2 days, depending on plant phenology requirements.

For inoculation, sterile conditioned broomrape seeds were treated with either unfiltered or 0.22 µm-filtered soil extracts (1/6-fold dilution) or buffer solution (HEPES 1 mM, pH 7.5) as a negative control for germination test, and spun before spreading on roots. Seeds with pre-induced prehaustorium formation were prepared 6 days earlier as described in Goyet et al. (2017). Sterile conditioned *P. ramosa* seeds were rinsed 3-time and were then placed in germination medium (HEPES 1mM, pH 7.5, *rac*-GR24 10^{-7} *M*) in 50 mL tissue culture flasks (Becton Dickinson, Franklin Lakes, NJ, Etats-Unis) at 21 °C in the dark. After 4 days, seeds were rinsed 3-time and suspended in a medium containing filtered or unfiltered soil extracts (1/6-fold dilution), or buffer solution (HEPES 1 *mM*, pH 7.5) as a negative control, or *trans*-zeatin (10^{-7} M, *t*Z dissolved in 50% acetonitrile, Olchemin, Czech Republic) as a positive control (Billard et al. 2020), 2 days before rapeseed root inoculation. The quality of broomrape seed sets was assessed beforehand regarding germination and prehaustorium formation. Two-week old rapeseed roots were then inoculated by spreading either 10 mg of conditioned broomrape seeds for germination assays onto the one root, or 12.5 mg of seeds pre-induced for prehaustorium formation onto the two roots after 4 weeks for aggressiveness assays.

During co-cultivation assays dedicated to germination assessment, broomrape germination ratios were determined in mini-rhizotrons on weeks 1 and 2 post inoculation by evaluating the number of germinated seeds and total seeds in 4 squares of 1 cm^2 nearby the roots, and total broomrape attachments on weeks 2 to 4 post root inoculation. Broomrape attachments were also characterized according to Gibot-Leclerc et al. (2012) as either young tubercles (no adventitious roots), spider-like tubercles (numerous adventitious roots) tubercles or older tubercles already bearing a flowering bud. Each mini-rhizotron was repeated 4 times for each treatment modality as 4 independent biological replicates. During co-cultivation assays dedicated to aggressiveness assessment, broomrape

attachments were determined daily from day 7 to day 20 post root inoculation. Each treatment was conducted as nine mini-rhizotron replicates.

Isolation of myrosinase producing microorganisms by glucosinolate enrichment in minimum media

Myrosinase producing bacteria and fungi were isolated following an enrichment isolation method using selective media with glucosinolate as the sole source of carbon (based on Albaser et al. 2016). Five hundred mg of 1 mm - sieved soil were suspended in 50 mL tissue culture flasks (Becton Dickinson) containing either 10 mL of M9-sinigrin medium (SNG; Sinigrin hydrate, Sigma-Aldrich) for bacteria and fungi, or PDB-sinigrin medium for fungi only. M9-SNG medium was prepared with 2 mL of M9 solution 5X (64 $g.L^{-1}$ Na_2HPO_4, 15 $g.L^{-1}$ KH_2PO_4, 2.5 $g.L^{-1}$ NaCl and 5 $g.L^{-1}$ NH_4Cl), 2 mL of 24.65 $g.L^{-1}$ $MgSO_4$ and 10 µL of 14.7 $g.L^{-1}$ $CaCl_2$, and 2 mL of 10 $g.L^{-1}$ SNG filtrated at 0.22 µm with syringe filter in sterile distilled water (pH 7.2). PDB-SNG medium was prepared with 8 mL of ¼ PDB solution (6 $g.L^{-1}$) with 2 mL of 10 $g.L^{-1}$ SNG filtrated at 0.22 µm with syringe filter in sterile distilled water (pH 5.6). Flasks were incubated in the dark for 2 days under 100 rpm shaking at 30 °C or 21 °C for bacteria and fungi respectively. One milliliter of culture medium was then collected and subcultured in 4 ml of fresh M9-SNG or PDB-SNG medium and incubated at 30 °C or 21 °C respectively for another two days. This step was repeated two times. One milliliter was then collected, and serial dilutions were plated onto LB agar at 30 °C or PDA at 21 °C for 4 days respectively.

In addition, to increase fungal collection, serial dilutions of soil extracts were plated onto the non-selective medium PDA and incubated at 21 °C for 7 days. Finally, distinct colonies were isolated and purified on new solid LB or PDA medium for 5 days and grown in liquid LB or PDB medium for 2 days. Pure cultures were then stored at -80 °C in 40 % glycerol (v/v) until further molecular and biological characterizations.

Validation of myrosinase producing microorganisms using germination assays

Validation of glucosinolate-degrading abilities of microbial isolates was assessed in vitro using broomrape seeds as a proxy for isothiocyanate detection. Beforehand, pure microbial isolates were suspended in buffer solution (HEPES 1 mM; pH 7.5) to maintain germination conditions for P. ramosa. Bacterial suspensions were set to OD600nm = 0.01 in plate using an Absorbance Microplate Reader (POLARstar Omega, BMG Labtech GmbH), and fungi solutions were set to 105 spores mL-1 in plate using a hemocytometer. Germination bioassays were carried out in 96-well plates as described above. Similar controls were used. Germination ratios were expressed as mentioned for germination assays, relatively to the positive control with rac-GR24.

Taxonomic identification of myrosinase producing isolated strains

Molecular analyses were conducted on either single-pure bacterial colonies or mycelia, grown on solid medium. Microbial material was suspended in 100 µL of sterile ultra-pure water and incubated for 5 min at 94 °C in order to allow cell lysis cells and release DNA. DNA amplifications were then performed using GoTaq® (G2 Flexi DNA Polymerase, Promega France) according to manufacturer's recommendations. In a final volume of 50 µL, 2 µL of heated DNA suspensions were mixed with 1 µL of dNTP mix (10 *mM*, Promega France), 1 µL of upstream and downstream primers and GoTaq® reagents (10 µL of 5X Green Buffer, 5 µL of MgCl2 Solution (25 *mM*) and 0.25 µL of GoTaq®. The paired primers 16S_515f (GTGCCAGCMGCCGCGGTAA) and 16S_806r (GTGCCAGCMGCCGCGGTAA) were used to amplify bacterial DNA (Caporaso et al. 2011). The paired primers ITS1_f (CTTGGTCATTTAGAGGAAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC) were used to amplify fungal DNA (Buée et al. 2009). PCR amplification were carried out using a MyCycler™ thermal Cycler system (Bio-Rad) according to Barret et al. (2015) with some modifications, 95 °C for 2 min followed by 35 cycles of 95 °C for 45 s, 50 °C or 52 °C for 45 s for bacteria and fungi respectively, 72 °C for 1 min, then final elongation at 72 °C for 5 min and final cooling at 10 °C. Sequencing was performed at Eurofins Genomics (Ebersberg, Germany).Taxonomic assignations were computed using the BLASTn algorithm on the NCBI database (Altschul et al. 1990). Species was assigned when it belonged to the top hit, *i.e.* single species with identity percentage above 95%.

Statistical analyses

All results were computed on RStudio v1.4.1103 (R Core v4.0.3), and analyzed with different statistical approaches according to data distribution and experimental design.

For co-culture experiments, effects of soil extracts on broomrape germination and attachment were compared across treatments (3 modalities) and weeks (from 1 to 4) with ANOVAs (analysis of variance) on Mixed-Effect Models using the Car package v3.0-10, (Fox and Weisberg 2019) and the lme4 package v1.1-26 (Bates et al. 2015). Fixed effects were compared with multi comparisons of estimated means with the emmeans package 1.5.5-1 (Lenth 2021). For germination assays, a logit transformation was required for the percentage data, and were modeled with a linear mixed model (lmer), assuming normal distribution of germinated seeds. Square position (1 to 4), biological replicate, experimenters, and mini-rhizotron identification were used as random factors. For broomrape attachments, Poisson law was selected as a large part of the dataset are small counts, and data were modeled with a generalized mixed model (glmer), with a square-root link function. For relative abundance of the different development stages, each stage was used as explanatory variable (Young, Spider, Bud and Necrosis tubercle) using a glmmPQL, a glmer based on a *quasi* law for logistic regression. GlmmPQL

were employed in this case, as it is usually required when explanatory variable is only available as a proportion but follows binomial law. For both total attachment and development stage models, biological replicate, and mini-rhizotron individuals were used as random factors.

In both germination and prehaustorium-formation bioassays, integration of positive and negative controls resulted in an unbalanced design; therefore, soil treatment was used as a unique factor to cover all modalities including soil dilution, soil sterilization and co-treatments. On the one hand, the large proportion of zeros in the germination dataset limited the identification of a known statistical law and therefore the use of parametric tests. Data were analyzed with a Kruskal rank sum test (stats package v4.0.3) and comparisons of effects were done with multiple pairwise comparison procedures (asbio package v1.6-7). On the other hand, prehaustorium-formation data were analyzed with a parametric mixed-model, using a glmmPQL based on a *quasi* law for logistic regression. Fixed effects were compared with multi comparisons of estimated, and biological and technical replicates were included as random factors.

RESULTS

Bacterial and fungal profiles of broomrape infested soil

Bacterial and fungal communities present in the rapeseed soil were described on three independent soil samples by sequencing the 16S and ITS marker genes respectively. After bioinformatics processing, the number of reads ranged from 12,519 to 14,892 for bacteria, and from 35,005 to 45,425 for fungi. Bacterial communities were composed of 877 distinct amplicon sequence variants (ASVs), clustered into 29 phyla, 163 families and 189 genera, while fungal communities were composed of 545 distinct ASVs clustered into 9 phyla, 95 families and 136 genera. For abundance description, taxa with abundance less than 0.1% were grouped into a common class named "< 0.1%". ASVs from SILVA and UNITE identifiers with corresponding DNA sequences for 16S and ITS can be found in supplementary data (Supp.TableS1; Supp.TableS2 respectively).

At the phylum level, the four most abundant bacterial phyla were *Actinobacteriota* (mean= 34.45± sd= 1.11%), *Proteobacteria* (23.31±3.04%), *Acidobacteriota* (21.91±2.71%) and *Chloroflexi* (5.29±0.90%) (Supp. Fig.S3A). Bacterial families were homogeneously distributed among the samples, and characterized by a high number of low abundant taxa, with 68.36±7% of them being less than 1% abundant (Fig.1). Nevertheless, the most abundant families were the *Pseudonocardiaceae* (11.97±3.99%), the *Vicinamibacteraceae* (10.72±0.86%) and families less than 0.1% abundant (6.71±0.45%). In addition, 4 *Streptomyces* ASVs (2.1±0.54%), one *Bacillus* ASV (0.08±0.13%), and one *Pseudomonas* ASV (0.02±0.04%) were detected. Species assignations were not available from either the SILVA or the NCBI data base.

The majority of fungal ASVs corresponded to the phyla *Ascomycota* (74.52±8.56%), followed by a smaller proportion of *Olpidiomycota* (9.86±7.58%) and *Basidiomycota* (6.15±0.42%) (Supp. Fig.S3B). A very small proportion of the phylum *Glomeromycota* (0.05±0.03%) was present, mainly composed of the genera *Funneliformis* (33.33±57.74%), *Paraglomus* (26.09±45.19%) and *Archaeospora* (28.49±38.60%), namely arbuscular mycorrhizal fungi (AMF). Fungal families were less homogeneously distributed than bacterial families and had smaller proportions of few abundant taxa (24.97±2.55%) (Fig.2). On average, *Aspergillaceae* members were dominant in the samples (29.89±14.83), followed by *Olpidiaceae* (9.86±7.58%), *Plectosphaerellaceae* (8.81±7.89%), *Nectriaceae* (6.08±1.93%), and families less than 0.1% abundant (6.42±0.72%). The *Aspergillaceae* family was mostly composed of *Aspergillus* (65.12±6.16%), *Penicillium* (34.69±6.13%) genera, and one unidentified genus (0.19±0.03%). The different *Aspergillus* species were presented in supplementary Table S3, with prevalence of *ASV 853* (76.07±1.14%). After a BLASTn analysis, *ASV 853* shared 100% identity with *A. europaeus* and *A. fumigatus* but no exact assignation could be determined.

No metabarcoding analyses were conducted on gamma on sterilized soil to search for microbial communities. Nevertheless, the average values of colony forming bacteria and fungi were respectively $4.80.10^3$±$1.11.10^3$ (mean±sd) and 11.55±4.88 CFU g^{-1}dry native soil, whereas average values were of 8.67±6.46 bacterial and 1.01±0.98 fungal CFU.g^{-1} dry sterilized soil thus confirming sterilizing treatment effectiveness.

Effect of soil microorganisms on germination signal in mini-rhizotron systems

Germination of broomrape seeds was monitored in mini-rhizotron systems at one- and two- weeks post rapeseed root inoculation. Inoculum consisted of broomrape seeds suspended either in control buffer, buffered unfiltered soil extracts or 0.22 µm-filtered soil extracts (Fig.3A). Seed viability was assessed separately *via* addition of synthetic strigolactone *rac*-GR24, and led to identical and high seed germination ratio for all treatments after 4 days (mean= 94.64±sd= 2.27%, p-value >0.05).

Control treatment with buffer and treatment with 0.22 µm-filtered soil extracts containing water-soluble soil metabolites resulted in similar germination ratios after two weeks of co-cultivations. Germination ratios were close to 0 during the first week but increased considerably during the second week to reach 47.72±27.51% (median±sd) and 41.97±34.73%, respectively (p-value >0.05). This thus suggests the release of GSs from rapeseed roots after 2 weeks of co-cultivation without any effect of water-soluble soil metabolites. Furthermore, treatment with unfiltered soil extracts significantly increased broomrape germination ratios over the two weeks compared to the control, as germination ratios reached 41.55±25.21% (p-value <0.0001) on the first week and 76.84±26.15% (p-value <0.0001) on the second week. Besides, in the presence of unfiltered soil extracts, germination ratio on week 1

was similar to the control buffer treatment on week 2 (p-value = 0.99), indicating a one-week delay of germination in the absence of microorganisms. Altogether, these results highlight the amplification of broomrape seed germination signal in the presence of microorganisms from soil extracts.

Subsequent effects of soil on broomrape attachment to rapeseed roots and subsequent tubercle development was assessed at two, three, and four weeks post inoculation (Fig.3B). Treatment with unfiltered soil extracts only impacted the number of attached parasites at three weeks post inoculation with over 6.8 times more broomrape tubercles compared to the 0.22µm filtered extract (p-value =0.004) and buffer treatment (p-value <0.0001). However, differences in number of attached parasites at four weeks post inoculation were no longer significant. There were 56.63±51.81 (mean±sd) attached tubercles for unfiltered soil extract treatment, and 42.47±46.22 for 0.22µm filtered soil extract treatment compared to 42.32±33.35 attached tubercles for buffer control treatment (p-values =0.596 and =0.376 respectively). Nevertheless, unfiltered soil extract treatments resulted in higher proportions of advanced spider tubercles at four weeks post inoculation with 59.69±19.93% compared to buffer control with 21.79±24.30% (p-values<0.0001) while treatments with buffer solution and 0.22 µm filtered soil extract displayed higher proportions of young tubercles with respectively 76.42±27.37% (p-values <0.0001) and 59.46±24.77% (p-value =0.042) compared to unfiltered extract treatment with 39.39±19.67% (Fig.3C). These results suggested that the microbial-mediated signal enabled faster kinetics of broomrape seed germination, and thus faster kinetics of attachment to host roots and subsequent post attachment development.

Effect of soil microorganisms and metabolites on broomrape seed germination in presence of glucosinolates in *in vitro* assays

Four days after induction treatment in 96-well plates, no *P. ramosa* seed germinated in the presence of either the negative control (HEPES buffer) or the soil extracts alone for any dilutions (0%, p-value >0.05; Fig.4). This suggests that both metabolites and microorganisms from soil extracts had no direct impact on the germination of broomrape seeds. Furthermore, no inhibitory effect was observed on seed germination after co-treatment with germination stimulant *rac*-GR24 (10^{-8} *M*) associated with either 0.22 µm filtered or unfiltered soil at 2 dilutions. At the 10-fold dilution, seeds germinated at relative ratios of 1.00±0.05 (median±sd) and 0.92±0.05 respectively for filtered and unfiltered soil extracts (p-value =1), similarly to the control (*rac*-GR24 only) with 1.01±0.05 (p-value >0.05; Supp. Fig.S4A).

In contrast, broomrape seed co-treatment with soil extracts and exogenous gluconasturtiin (GNT; 10^{-6} *M*) significantly enhanced seed germination ratios ranging from 0 to 0.90±0.08 (median±sd) to 0.97±0.13 for the 10-fold and 100-fold soil extract dilutions respectively (p-value <0.0001 for both dilutions), compared to seeds treated with positive control *rac*-GR24 (10^{-7} *M*). As GNT alone does not

induce any seed germination (p-value =1), this result suggests the release of GSs from GNT hydrolysis by soil microorganisms. Control co-treatments with exogenous myrosinase (5 $mU.mL^{-1}$) and 0.22 µm-filtered soil extracts confirmed the absence of residual glucosinolate among the water-soluble soil metabolites, whereas co-treatments with GNT confirmed the absence of residual extra-cellular myrosinase (null germination ratio, Supp. Fig.S4B).

All co-treatments of any of the sterilized extracts (soil extract filtrations, biocidal treatment, gamma sterilization) with GNT (10^{-6} *M*) were similarly inactive on *P. ramosa* seed germination (null germination) compared to the buffer control for all soil extract dilutions (p-values >0.05; Fig.4). In addition, both 0.45 µm and 0.22 µm filtrations of native soil extracts prevented GNT-mediated germination, suggesting that the soil activity was concentrated in unfiltered extracts, with microbes larger than 0.45 µm.

Bacterial and fungal candidates responsible for glucosinolate degradation in rapeseed soil

Isolation procedures from enrichment cultures revealed nine bacterial isolates on minimum medium M9 (8 growing on LB agar and 1 on PDA), as well as nine fungal isolates (2 on M9 and 7 on ¼ strength PDB). Bacteria were identified via sequencing of 16S rRNA amplicons, and fungi via sequencing of ITS1. Nucleotide sequences were compared in NCBI database (Table 1) and assigned to one uncultured *Actinobacterium* sp., one uncultured *Proteobacterium*, four *Arthrobacter* spp., one *Ralstonia picketti*, and one *Chryseobacterium* sp. for bacteria. As for fungi, all isolates belonged to *Penicillium* genus. Four additional strains of *Aspergillus* spp. were isolated from the soil extracts on PDA plates (non-selective medium for *Aspergillus* spp.).

All isolates were screened for their ability to degrade glucosinolates and to induce *P. ramosa* seed germination in 96-well plate germination assays using the seeds as bioindicators (Table 1). No germination activity was observed for any strains in the absence of GNT, meaning that there was no direct microbial production of effective GSs. The tested strains were reported as myrosinase-producing microorganisms when they were able to induce seed germination in presence of GNT (10^{-6} *M*). Out of the 13 tested fungi, isolates previously grown with SNG and within the *Penicillium* genus showed myrosinase activity. None of the *Aspergillus* strains showed any myrosinase activity. In total, 2 strains Pen_M9_1 and Pen_M9_2 isolated from M9 and SNG medium, as well as 1 strain Pen_PDB_5 from PDB and SNG were able to degrade GNT into broomrape GSs. For bacteria, myrosinase activity was detected for all *Arthrobacter* strains and for *Ralstonia, Actinobacterium and Proteobacterium* strains. Out of 9, all 8 isolates but Chry_M9_9 were able to induce GNT-mediated germination.

Effect of soil microorganisms and metabolites on broomrape aggressiveness

Broomrape aggressiveness was monitored using mini-rhizotron systems, by inoculating rapeseed seedlings with pre-germinated *P. ramosa* seeds (at mean= 94.78±sd= 1.48%). Prehaustorium induction on an aliquot of *P. ramosa* seeds was also assessed separately in 96-well plates the day after, with a maximum formation ratio of 79.32±9.70% for the positive control (trans-zeatin; *t*Z 10^{-7} *M*) and 2.09±3.70% for the negative control (HEPES buffer).

In mini-rhizotrons, attachments to rapeseed roots of pre-germinated and treated seeds were evaluated from day seven post inoculation. Attachments were first observed on the 10th day for each treatment (Fig.5A). Filtered and unfiltered soil extracts, and *t*Z treatments increased broomrape aggressiveness with respectively 3.89±5.62 (mean±sd), 3.22±2.05 and 5.40±5.50 attachments, compared to the control buffer with 0.63±0.74 attachments (p-values ≤ 0.01) from day 10 and throughout the assay. At the end of the experiment, the attachment number increased significantly by 21.31±4.54 % in conditions treated with unfiltered soil extracts, compared to the negative control. No significant differences (p-values >0.05) in tubercle development were nevertheless observed, all stages considered (Fig.5B). No increase of the parasitic kinetic was triggered by soil extract treatments, as all conditions had similar daily attachment ratios (number of new attachments reported to total final attachments; p-values >0.05; Supp. Fig.S5). Altogether, these results show a combined effect of soil metabolites and microorganisms on broomrape aggressiveness, through improvements of the attachment ratio of germinated seeds.

Characterization of soil activities on induction of prehaustorium formation using *in vitro* assays

After three days of treatment with unfiltered soil extracts, pre-germinated seeds exhibited shorter radicles and developed prehaustoria with ratios of 0.71±0.17 (median±sd) at the 10-fold and 0.08±0.11 at the 100-fold dilutions, relatively to seeds treated with the positive control (TDZ 10^{-8} *M*; ratio of 1.00±0.05 ≈95.15±5.53%; Fig.6). The 10-fold dilution strongly differed from the control buffer treatment which did not induced prehaustoria (0±0.004; p-value <0.0001), thus showing that soil may be involved in the release of HIFs. Prehaustorium formation ratios were lower for the 100-fold dilution for all treatment, suggesting a dilution effect of HIFs in the microbial suspension.

Prehaustorium formation ratios were also assessed using mechanic (filtrations) and chemical (PPM addition) sterilization procedures as well as soil gamma-sterilization treatment. This resulted in two divergent patterns according to the chosen method. Indeed, mechanical sterilization induced decreased activities compared to the 10-fold diluted unfiltered extracts, with relative prehaustorium formation ratios of 0.42±0.22 for soil extracts filtrated at 0.45 µm (p-value =0.0006) and 0.25±0.13 for soil extracts filtrated at 0.22 µm (p-value <0.0001). Similarly, chemical sterilization with PPM reduced

relative Prehaustorium formation ratios to 0.40±0.17 compared to 10-fold diluted unfiltered soil extracts (p-value <0.0001). These results suggest that both soil extract metabolites and microorganisms contribute to the induction of prehaustorium formation. In contrast, gamma-sterilized soil extracts considerably increased prehaustorium formation ratios up to levels similar to the positive control, without any effect of the 0.22 µm filtration for both extract dilutions (p-value =0.104). Indeed, relative prehaustorium formation ratios of the 10-fold diluted gamma-sterilized soil extracts were of 0.98±0.05 and 0.97±0.07 for the 0.22 µm filtered and unfiltered extracts respectively (p-value =0.989). Thus, this suggests an artifact effect of soil sterilization by gamma-irradiation causing the release of metabolites with HIF activity.

DISCUSSION

The confirmation of microbial contribution in the *B. napus* - *P. ramosa* interaction is of major interest in the integrated and global understanding of the parasitic plant biological cycle in its agro-ecological environment. This study shows that a rapeseed soil contains a microbiota that promotes both the parasitic plant germination and aggressiveness through the release of glucosinolate-breakdown products and HIFs.

Rapeseed soil microorganisms hydrolyze host plant glucosinolates into isothiocyanates in the rhizosphere

In the present study, using *B. napus* - *P. ramosa* co-cultivation experiments, we have assessed the effect of the soil activity on the rhizosphere signaling leading to broomrape seed germination. We first observed that, in absence of soil microorganisms, *B. napus* alone was able to induce more than 45% of broomrape seed germination after two weeks of co-cultivations, thus suggesting the occurrence of GSs in the vicinity of roots. Most likely, these compounds include 2-phenylethyl isothiocyanate (2-PEITC), as it is the main GS exuded by rapeseed (Auger et al. 2012). Indeed, rapeseed roots may release isothiocyanates, through the degradation of tissues associated with root growth, during which glucosinolates come in contact with endogenous myrosinase (Rumberger and Marschner 2003). Non-enzymatic degradation of glucosinolates in the roots have also been reported through induction by strong thermal and chemical stresses (*e.g.* pH>10; Blažević et al. 2015). Although glucosinolate stability is still under exploration, it has been shown to vary according to the side chain structure of the compound (aromatic, aliphatic, indolic) and plant species (Blažević et al. 2015; Dekker et al. 2009). In the present study, glucosinolates are thus unlikely to be easily degraded due to their structure under the used conditions (21°C in the dark in buffered medium).

Interestingly, transfer of soil microorganisms to the mini-rhizotron co-cultivation systems favored parasitic seed germination, in turn accelerating the whole parasitic cycle. This indicates an amplification of the host pre-existing germination signal by the hydrolysis of the host-exuded glucosinolates thanks to soil microbial myrosinase activities (Fahey et al. 2001; Rumberger and Marschner 2003; Bressan et al. 2009; Auger et al. 2012). This hypothesis is further supported by *in vitro* germination assays which showed that soil microorganisms stimulated broomrape seed germination, only when supplied with the main glucosinolate of rapeseed roots GNT (Kirkegaard and Sarwar 1998). This result suggests GNT hydrolysis into 2-PEITC in the incubation medium (Auger et al. 2012). Moreover, in the absence of soil microorganisms, the transformation of GNT into GSs required addition of exogenous myrosinase, which is the only enzyme able to click the S-glycosidic bond present in GNT (Naumoff 2011; Bhat and Vyas 2019). Thus, the present study provides strong evidence of microbial myrosinase-like activity in the studied rapeseed soil. The complete loss of activity observed after sterilizing treatments (0.22 µm filtrations, PPM and gamma-sterilization) further confirmed the microbial origin of the germination-promoting activity. This activity was also shown to originate from microorganisms larger than 0.45 µm, as filtration at 0.45 µm prevents the germination. Further analyses using intermediate filters or including specific fungicide or bactericide treatments are nevertheless required to establish the bacterial and/or fungal origin of the observed activity.

Interestingly, while myrosinase activity has already been reported in soils (Al-Turki and Dick 2003; Gimsing et al. 2006), this report is the first to emphasize its role as a factor contributing to the whole parasitic plant - plant interaction.

Rapeseed soil microorganisms alone do not produce germination stimulants

Direct release of GSs by soil microorganisms was not observed in the present study, as no broomrape seed germination occurred *in vitro* upon treatment with unfiltered or filtered soil extracts alone. Moreover, as no germination was observed with or without exogenous myrosinase treatment, it appears that no residual glucosinolate nor GS were detected in the soil extracts. Nevertheless, we cannot out rule that the parasitic seeds were not able to detect putative isothiocyanates, as they could be too diluted or rapidly degraded by soil microorganisms, or also because of apolar interactions may prevent them to be properly solubilized in water (Sarwar et al. 1998; Rumberger and Marschner 2003). Indeed, Auger et al. (2012) successfully extracted glucosinolate-breakdown products from rapeseed soil using dichloromethane solvent, showing that GSs can be retained by soil particles.

To date, only few studies have described the production of GSs by microorganisms. For instance, fusicoccins (carbotricyclic diterpenoids) and their derivatives isolated from the fungus *Fusicoccum amygdali*, the causal agent of peach and almond canker, as well as Ophiobolins (sesquiterpenes) from

pathogenic *Bipolaris* fungal species were shown to induce germination of *P. ramosa* and other broomrape species (Yoneyama et al. 1998; Evidente et al. 2006; Fernández-Aparicio et al. 2008; Okazawa et al. 2021). However most of these studies focused on non-crop specific or plant pathogenic microorganisms as tools for suicidal germination approaches. Yet, no study has considered the role of microorganisms naturally occurring in the soil during the parasitic plant cycle. Altogether, these results emphasize the tri-partite nature of the signaling mechanisms leading to *P. ramosa* germination in rapeseed fields.

Rapeseed microbiome is structured by the rapeseed metabolome

The prevalence of isothiocyanates in the rhizosphere of rapeseed and other *Brassicaceae* species is known to induce a strong biocidal activity against numerous soilborne bacteria and fungi (Smith and Kirkegaard 2002; Aires et al. 2009; Sotelo et al. 2015). It was also shown that, under different pH and cellular conditions (*e.g.* presence of Fe^{3+}, epithiospecifier-like proteins, glucosinolate side-chain structure), glucosinolates can rearrange into other breakdown compounds (thiocyanates , nitriles, oxazolidine-2-thiones and epithionitriles) which also bear biocidal properties (Wittstock et al. 2016). Overall, this toxicity shapes the microbiota structure in the rhizosphere of *Brassicaceae* (Bressan et al. 2009). Fungal communities are especially impacted and undergo severe reductions in diversity and richness, as they are usually more sensitive to these toxic compounds (Smith and Kirkegaard 2002; Hollister et al. 2013). This explains the absence of AMF (arbuscular mycorrhizal fungi) in rhizosphere of isothiocyanate-producing plant species (Wang et Qiu 2006; Vierheilig and Ocampo 1990; Schreiner and Koide 1993), which is also demonstrated by the very low proportion of AMF phylum *Glomeromycota* recovered in the rapeseed soil of the present study (<0.1%).

Microbial community rearrangements have also been observed after brassicaceous plant material or isothiocyanate amendment as indirect effects of isothiocyanate toxicity. They are generally associated with an increase in the proportion of *Pseudomonas* spp., *Bacillus* spp. and taxa from *Actinomycetales* including *Streptomyces* spp. (Hollister et al. 2013; Ren et al.2018; Hu et al. 2015), which are well-known to suppress bacterial and fungal soilborne pathogens of rapeseed (Cohen et al. 2005; Sun et al. 2017; Jelušić et al. 2021). Although no soil microbiota analysis was performed prior to rapeseed cropping in our study, glucosinolate-degradation products possibly induced the selection of a high proportion of the *Pseudonocardiaceae* (order *Actinomycetales*). Among this family, genus *Saccharopolyspora*, which contains numerous species producing a wide variety of biologically active secondary metabolites, is mainly represented (Sayed et al. 2020). Global effects of glucosinolates on microbial communities also result in combining toxicity effects of glucosinolate degradation products together with beneficial effects of allelopathy and nutrient release. Indeed, at the small scale, glucose

molecules resulting from the glucosinolate hydrolysis can be used as a carbon substrate, thus conferring competitive advantages for myrosinase-producing and isothiocyanate-tolerant microorganisms (Rumberger and Marschner 2003; Bressan et al. 2009; Szűcs et al. 2018). We thus hypothesize a distribution of these specific traits among several groups of microorganisms found in the rapeseed rhizosphere.

Distribution of myrosinase activity among microorganisms isolated from rapeseed soil

We demonstrated that a rapeseed cultivated soil contains microorganisms able to degrade glucosinolates thanks to their myrosinase activity and thus able to induce broomrape seed germination. Indeed, we isolated both bacterial and fungal candidates harboring myrosinase activities. Notably, this study reveals a myrosinase-like activity of *Penicillium* strains with three out of nine isolates inducing about 45% of broomrape seed germination in presence of glucosinolates. However, the myrosinase activity was not evenly distributed among the genera. Only one out of the seven PDB-isolated *Penicillium* exhibited myrosinase activities in a more complex medium (SNG and potato dextrose). This suggests the ability of *Penicillium* spp. to tolerate toxicity of glucosinolate-breakdown products and develop on different available sources of carbon, either from the medium itself or from co-metabolism with co-occurring myrosinase-containing organisms. To date, most of the identified glucosinolate-degrading fungi belong to *Aspergillus* or *Fusarium* species, such as *A. sydowii, A. clavatus, A. flavus, A. niger, A. terreus* and *F. oxysporum* (Ohtsuru et al. 1973; Smits et al. 1993; Sakorn et al. 1999; Rakariyatham and Sakorn 2002; Rakariyatham et al. 2005; Galletti et al. 2008; Wang et al. 2012; Szűcs et al. 2018). Interestingly, both *Aspergillus* and *Penicillium* genera belong to the *Aspergillaceae* family, which was the most abundant family in the studied rapeseed soil (20%). However, among all the species identified in this study, only *A. flavus*, which was present in low quantities, has previously been described to degrade glucosinolates. Nevertheless, we tested the ability of the four isolated *Aspergillus* strains to degrade glucosinolates into GSs. In our conditions, none of *Aspergillus* strains at a concentration of around 10^3–10^4 spores per mL, were able to induce broomrape germination in presence of GNT (10^{-6} *M*). However, these strains were isolated on a classic medium (PDA) without glucosinolate, which may be required to select myrosinase active strains and/or to induce their myrosinase activity. Indeed, fungal myrosinases may function similarly as bacterial myrosinases that were characterized as inducible rather than constitutive enzymes (Albaser et al. 2016). Likewise, Gimsing et al. (2006) found that degradation of glucosinolates was higher in soils that had been cultivated with glucosinolate-containing crops compared to soils cultivated with other crops. This suggests a selection and/or stimulation of myrosinase-producing microorganisms. Further experiments may be nevertheless required to properly conclude on the contribution of these isolates

to the germination-promoting signaling in rapeseed rhizosphere. No *Fusarium* strain, was isolated from the studied soil. This may be due to their low relative abundance in the studied soil (less than 3%), but also to the competition with other species on non-selective PDA medium. Nevertheless, *Fusarium* spp. have been found to both degrade glucosinolates and tolerate isothiocyanates (Smits et al. 1993; Ishimoto et al. 2000). This thus highlights the need to further investigate the contribution of *Fusarium* spp. in the germination signaling. *F. oxysporum* might be considered of great interest in this case, as this species is pathogenic to many broomrapes and was dominant in our studied soil (Andolfi et al. 2005; Aybeke 2020; Gibot-Leclerc et al. 2022).

In addition to the mycobiota, myrosinases are also found in several bacteria mostly found in human or animal gut, such as enterobacteria or lactic acid bacteria (Palop et al. 1995; Cheng et al. 2004; Mullaney et al. 2013). Bacterial myrosinase activities were also recently characterized in *Leclercia adecarboxylata* and *Citrobacter* WEY1 strains isolated from the rhizosphere of rapeseed seedlings and adult plants respectively (Albaser et al. 2016; Tie et al. 2021). Gene sequence structures differ between bacterial myrosinases and those of plants and insects suggesting different hydrolysis mechanisms and strategies for glucosinolate-breakdown. Plant myrosinase belong the glycoside hydrolase 1 (GH1) enzyme family (Francis et al. 2002; Naumoff 2011; Mahn et al. 2014), whereas bacterial ones were assigned to multiple GH families. On the one hand, Albaser et al. (2016) described a GH enzyme with myrosinase activity encoded by genes of GH3 family in a *Citrobacter* strain. On the other hand, Cordeiro et al. (2015) found GHs with possible myrosinase-like activities encoded by genes of GH1 and GH4 families in strains of SNG-degrading *Escherichia coli*. In the present work, myrosinase activity was detected in eight out of the nine soil bacterial strains isolated from M9 SNG-medium. Most of these strains were characterized as members of the genera *Arthrobacter, Ralstonia* and two uncultured *Actinobacterium and Proteobacterium*. In the same way as for fungi, incubation of bacteria with SNG did not constitutively result in myrosinase enzymatic activity, since Chry_M9_9 (*Chryseobacterium* sp.) could not degrade GNT into GSs in the tested conditions. Interestingly, *Arthrobacter* genus is known to be prevalent in soils and rhizospheres and is involved in the transformation of several organic carbon substrates, such as aromatic hydrocarbons (Stevenson, 1967). In a recent study, a variety of *Arthrobacter* strains isolated at different depth in a sediment substrate exhibited a high number of carbohydrate-active enzymes from the CAZymes database (carbohydrate-active enzyme database Drula et al. 2022), many of them being glycoside hydrolases (Gushgari-Doyle et al. 2022). They displayed high genomic capacities for utilization of carbohydrates and other carbon substrates, which is somewhat similar to their abilities to degrade glucosinolate in rapeseed soils.

Rapeseed soil microorganisms increase broomrape aggressiveness through HIF release

This study provides an original demonstration of the direct contribution (*i.e.* without requiring a host plant) of soil microorganisms in the induction of prehaustorium formation in root-parasitic plants.

Seeds treated with soil microorganisms and/or soil metabolites indeed displayed more attachments to rapeseed roots, which was also similar to treatments with cytokinins (*t*Z 10^{-7} *M*). Cytokinins are known effective HIFs that induce the formation of prehaustorial structures on *P. ramosa* germinated seeds (Goyet et al. 2017) as well as on *P. ramosa* microcalli (Billard et al. 2020). Thus, we suggest that the studied soil contains HIFs that trigger prehaustorium formation and lead to a higher attachment ratio of broomrape seeds. The hypothesis of the HIFs' microbial origin was strengthened by the *in vitro* assays. Indeed, soil microorganisms induced up to 66% of prehaustorium formation in absence of the host plant. This ratio was decreased after biological or mechanical soil sterilization. The contribution of microorganisms present in both the 0.45 µm-filtered fraction and the 0.22 µm-filtered fraction was confirmed by the progressive yet significant loss of activity after these filtrations with ratios of 0.49 and 0.22 of prehaustorium formation, respectively. The residual activity in the filtered extracts is suspected to be the outcome from HIFs released in the medium during soil extraction procedure, as it has already been observed with cytokinin-producing microorganisms (Müller et al. 1989; García de Salamone et al. 2001; Spallek et al. 2018). Chemical bio-guided analysis or pharmacological approaches using specific competitive inhibitors could be considered to further characterize these microbial HIFs (Goyet et al. 2017).

This strong HIF activity was also detected in the gamma-sterilized extracts and in both filtered and unfiltered soil extracts. This excludes a direct microbial activity. Unlike biocidal treatment, gamma-irradiation possibly results in the breakdown of microbial DNA structure. This might subsequently allow the release of adenine molecules, used as precursor for (naturally occurring) cytokinin biosynthesis which in turn leads to artefactual HIF activity (Frebort et al., 2011; Sakakibara 2010).

HIF-producers represent a large diversity of microbial species with diverse ecological roles

Host-derived quinones and cytokinins have been identified as HIFs in *Orobanchaceae* (Bandaranayake et al. 2010; Goyet et al. 2019; Aoki et al. 2022). The present study highlights that soil microorganisms can also contribute to cytokinin signaling in the rhizosphere. These phytohormones are highly conserved and widely distributed among living organisms including plants, bacteria and fungi, making them perfect candidates for allelopathic signaling across kingdoms (Tirichine et al. 2007; Spíchal 2012). A large diversity of microbial species releases cytokinins for their own development and nutrient uptake as free-living bacteria and fungi, or as chemical signaling to control the host plant growth and development during beneficial or detrimental interactions (Frebort et al. 2011; Chanclud and Morel

2016). For instance, PGP (plant-growth promoting) bacteria *Azospirillum* spp. (*A. lipoferum, A. brasilense*), *Bacillus* sp. (*B. subtilis)* and *Pseudomonas* spp. (P. *fluorescens, P. putida)* synthesize cytokinins and increase cytokinin levels *in planta* (Arkhipova et al. 2006; Esquivel-Cote et al. 2010; Pallai et al. 2012). However, in the present study, few of these organisms were detected in the studied soil (one *Pseudomonas* (0.02%) and one *Bacillus* (0.08%) ASVs). Also, phytopathogenic microorganisms produce cytokinins as effectors to dampen the host defenses upon root infection (Chanclud and Morel 2016, Spallek et al*.,* 2018). For instance, in rapeseed, Trdá et al. (2017) showed that *Leptosphaeria maculans*, the fungal agent of Blackleg disease (Howlett et al*.,* 2001), was able to release cytokinins both *in vitro*, without any precursor, and inside its host, by modifying cytokinin levels after tissue colonization. Although many potential HIF producers exist among the microorganisms in this study, no candidate can be proposed to date.

CONCLUSIONS

The present study highlights the importance of integrating the soil microbiota in the allelopathic signaling shaping of host plant- root parasitic plant interactions. We showed here that microbial contribution was characterized as beneficial for *P. ramosa*, facilitating host-recognition mechanisms during interactions for carbon acquisition in rapeseed rhizosphere. More specifically, microbe-related decomposition of glucosinolates efficiently releases germination stimulants, a.k.a. isothiocyanates, while microbe-related production of HIFs promotes broomrape attachments to host roots.

To a larger extent, these microbial driving forces, as well as rapeseed cropping intensification, may eventually contribute to host specialization of *P. ramosa* on *B. napus* in western France. These findings also disclose a dilemma for *Brassicaceae* cropping in an agroecological context. Indeed, although isothiocyanates act as biofumigants and suppress soilborne pathogens, *Brassicaceae* are likely to promote broomrape infestation and suppress mutualist symbiosis for the next crop.

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Competing Interests/Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Lisa Martinez, Lucie Poulin, Jean-Bernard Pouvreau, Christophe Jestin and Philippe Simier contributed to the study conception and design. Material preparation and data collection were performed by Ph.D student Lisa Martinez. Analyses were conducted by Lisa Martinez with the supervision of Lucie Poulin, Jean-Bernard Pouvreau and Philippe Simier. The first draft of the manuscript was written by Lisa Martinez and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The datasets generated during the current study can be found in the https://www.ncbi.nlm.nih.gov/bioproject/PRJNA854370

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Tables

Table 1 Screening for presence or absence of myrosinase-like activity in bacteria and fungi isolated from a rapeseed soil using either selective or non-selective media amended or not with sinigrin (5 mM). Germination assays were conducted in 96-well plates in presence of GNT (5 10^{-6} M) with broomrape seeds as bioindicators for perception of glucosinolate-breakdown products.

Kingdom	Taxonomic assignation [a]	Strain name	Relative germination % [b,c]	Medium
Bacteria	Uncultured *Actinobacterium*	Act_M9_1	96.68±1.86%	M9+Sinigrin
	Arthrobacter sp.	Art_M9_2	75.31±15.94%	
	Arthrobacter sp.	Art_M9_3	69.08±6.02%	
	Arthrobacter sp.	Art_M9_4	78.42±0.89%	
	Ralstonia *picketti*	Ral_M9_5	90.12±8.73%	
	Uncultured *Proteobacterium*	Prot_M9_6	74.72±5.98%	
	Arthrobacter sp.	Art_M9_7	67.94±2.18%	
	Arthrobacter sp.	Art_M9_8	94.68±11.93%	
	Chryseobacterium sp.	Chry_M9_9	0%	
Fungi	*Penicillium* sp.	Pen_M9_1	46.32±26.00%	
	Penicillium echinalutum	Pen_M9_2	44.50±25.33%	M9+Sinigrin
	Penicillium sp.	Pen_PDB_1	0%	PDB+Sinigrin
	Penicillium sp.	Pen_PDB_2	0%	
	Penicillium sp.	Pen_PDB_3	0%	
	Penicillium sp.	Pen_PDB_4	0%	
	Penicillium sp	Pen_PDB_5	46.19±21.27%	
	Penicillium sp	Pen_PDB_6	0%	
	Penicillium sp.	Pen_PDB_7	0%	
	Aspergillus ochraceus	Asp_PDA_1	0%	PDA
	Aspergillus sp.	Asp_PDA_2		
	Aspergillus versicolor	Asp_PDA_3		
	Aspergillus sp.	Asp_PDA_4		

[a] *All strains were molecularly characterized using the V4 regions of 16S rRNA (16S) and internal transcribed spacer (ITS1) phylogenetic markers for bacteria and fungi respectively.* Species was assigned when it belonged to the top hit, *i.e.* single species with identity percentage above 95%.
[b] Percentages (mean±sd) are expressed as the number of germinated seeds on total seeds, relatively to the average ratio obtained with the positive control (rac-GR24 10^{-7} *M*).
[c] Activity is validated and highlighted in red when relative germination is different than zero at the highest concentration of 0.01 OD_{600} and 10^{-5} spores mL^{-1} for bacteria and fungi respectively.

Figures

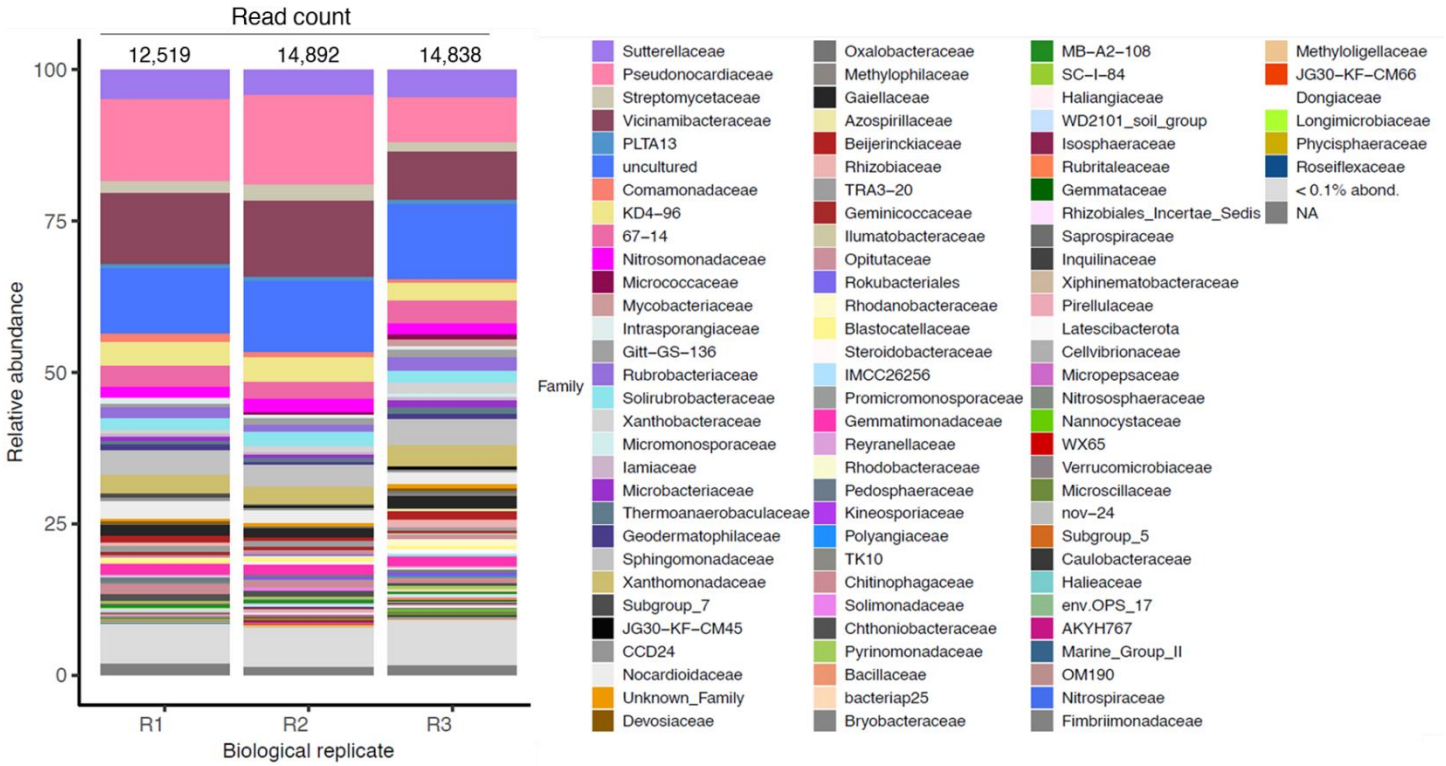


Fig.1 Relative abundance levels of bacterial families, expressed as mean percentages ± sd of all amplicon sequence variant (ASVs) detected in the rapeseed soil. DNA extraction was performed on three soil samples and amplification targeted the V4 region of the 16S rRNA genes. Taxonomic assignations were performed on the SILVA database. Taxa less than 0.1% abundant were grouped into the "< 0.1% abond." class, and taxa with no family assignation of family level were grouped into the "NA" class (not available).

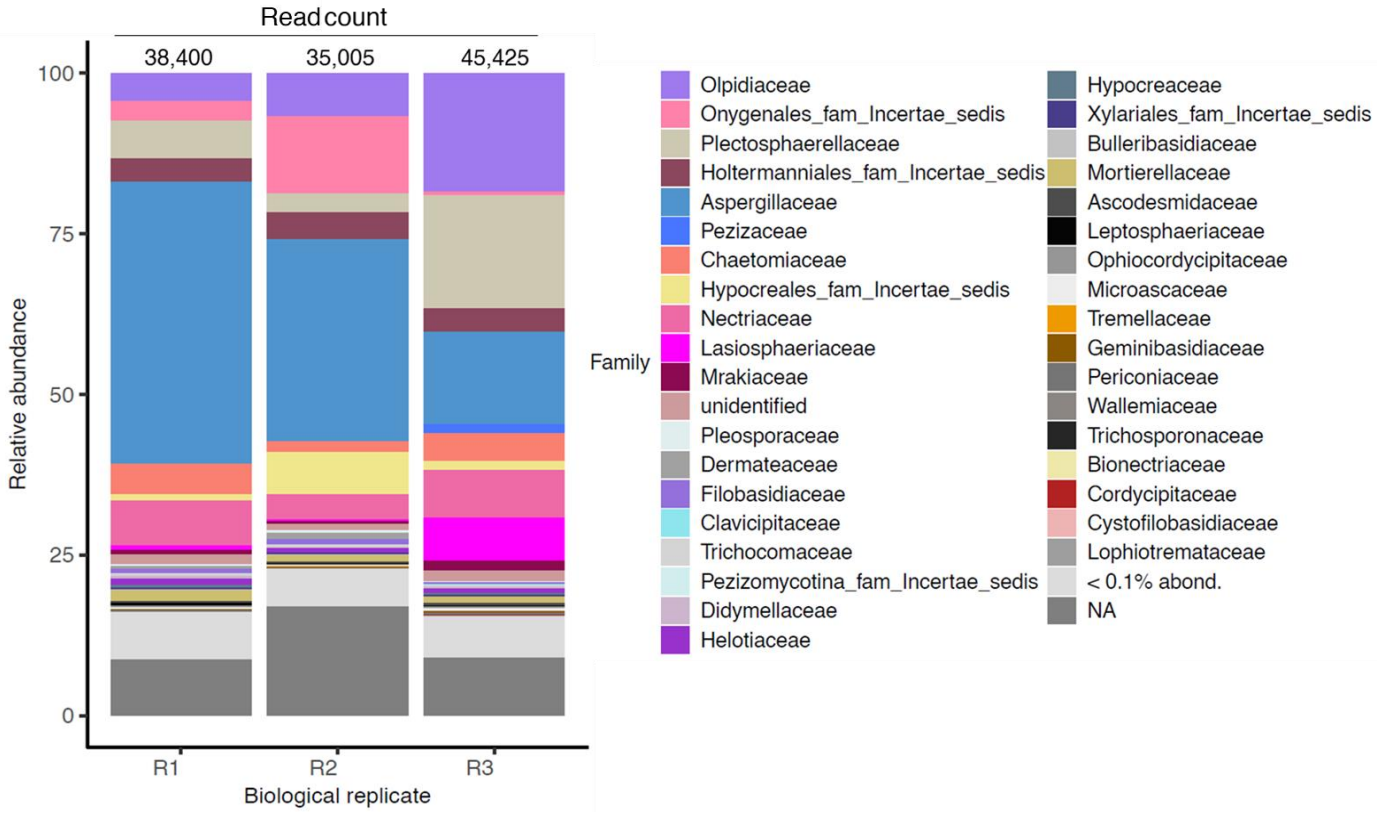
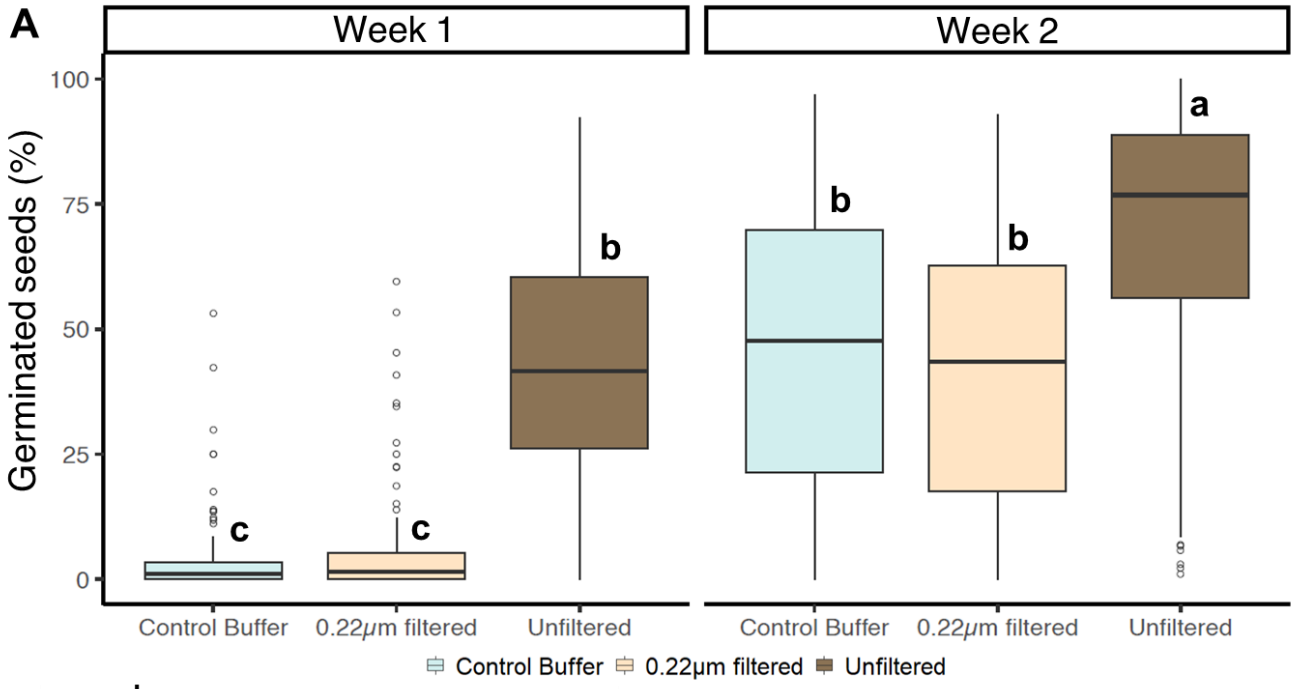
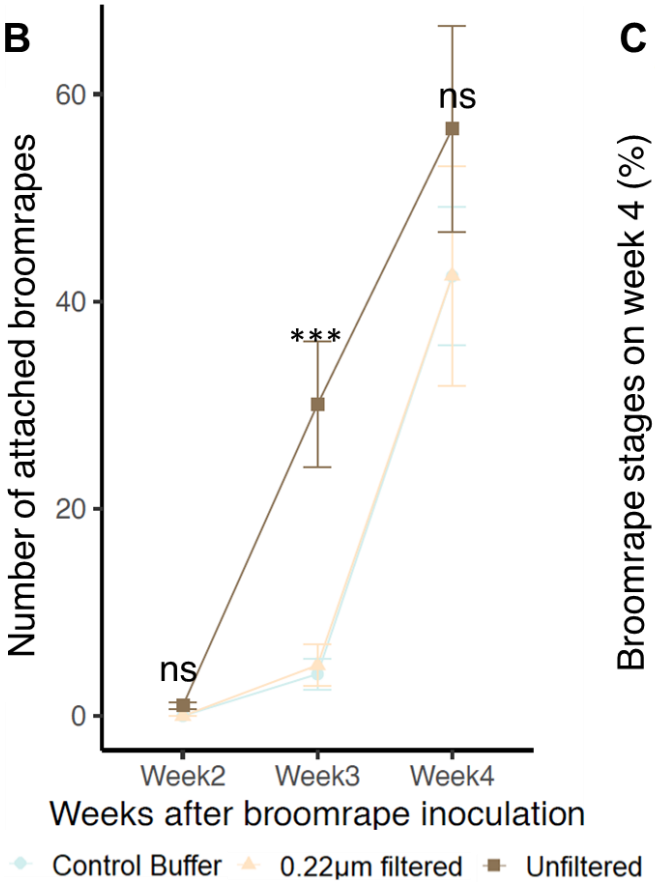
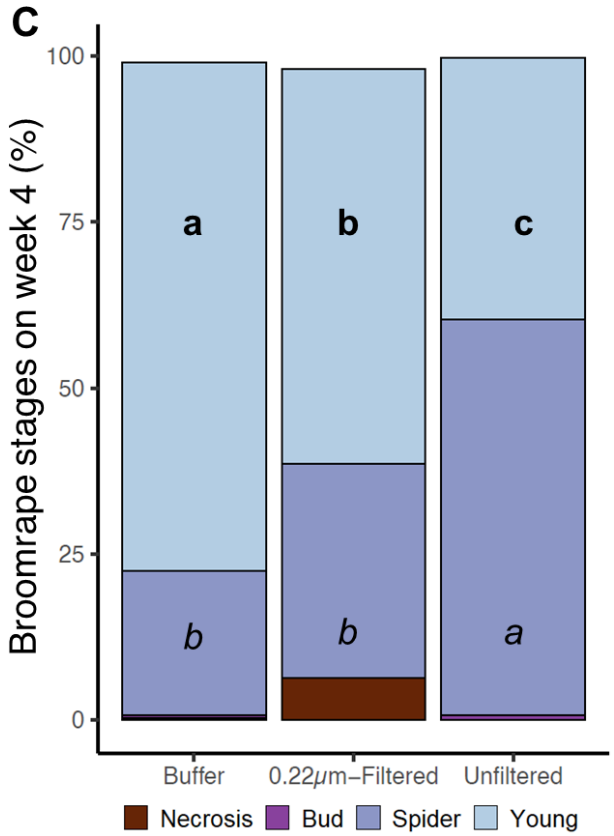


Fig.2 Relative abundance levels of fungal families, expressed as mean percentages ± sd of all amplicon sequence variant (ASVs) detected in the rapeseed soil. DNA extraction was performed on three soil samples and amplification targeted the ITS region. Taxonomic assignations were performed on the UNITE database. Taxa less than 0.1% abundant were grouped into the "< 0.1% abond." class, and taxa with no family assignation of family level were grouped into the "NA" class (not available).


A
Week 1
Week 2
Germinated seeds (%)
100
75
50
25
0
c
c
b
b
b
a
Control Buffer
0.22μm filtered
Unfiltered
Control Buffer
0.22μm filtered
Unfiltered
Control Buffer
0.22μm filtered
Unfiltered


B
Number of attached broomrapes
60
40
20
0
ns
***
ns
Week2
Week3
Week4
Weeks after broomrape inoculation
Control Buffer
0.22μm filtered
Unfiltered


C
Broomrape stages on week 4 (%)
100
75
50
25
0
a
b
c
b
b
a
Buffer
0.22μm–Filtered
Unfiltered
Necrosis
Bud
Spider
Young

1153

1154 **Fig.3A** Germination of broomrape seeds in mini-rhizotron systems, 1 and 2 weeks post broomrape
1155 inoculations on rapeseed roots. Percentages were calculated on 4 squares of 1cm^2. Different lowercase letters indicate significant differences of treatments (p-value <0.05) determined by ANOVA. **B** Successful broomrape attachment on rapeseed roots in mini-rhizotron systems, 2, 3 and 4 weeks post broomrape inoculation. Values are displayed as mean with standard deviation as error bars. Asterisks indicate significant differences 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 'not significant (ns) ', compared to the control modality and determined by ANOVA on a Poisson distributed model**. C** Average proportion of the different broomrape developmental stages, reported as a relative ratio to the average number of total attached parasites. Stages correspond to Young (young tubercle without adventitious root), Spider (old tubercle bearing adventitious roots) and bud (bud with adventitious roots but without a stem). Different lowercase letters indicate significant differences of treatments (p-value <0.05) determined by ANOVA on logistic models for each stage. Bold and italic letters indicate comparisons between young and spiders respectively. No letter was displayed when there was no significant difference between modalities (i.e. Bud and Necrosis stages).

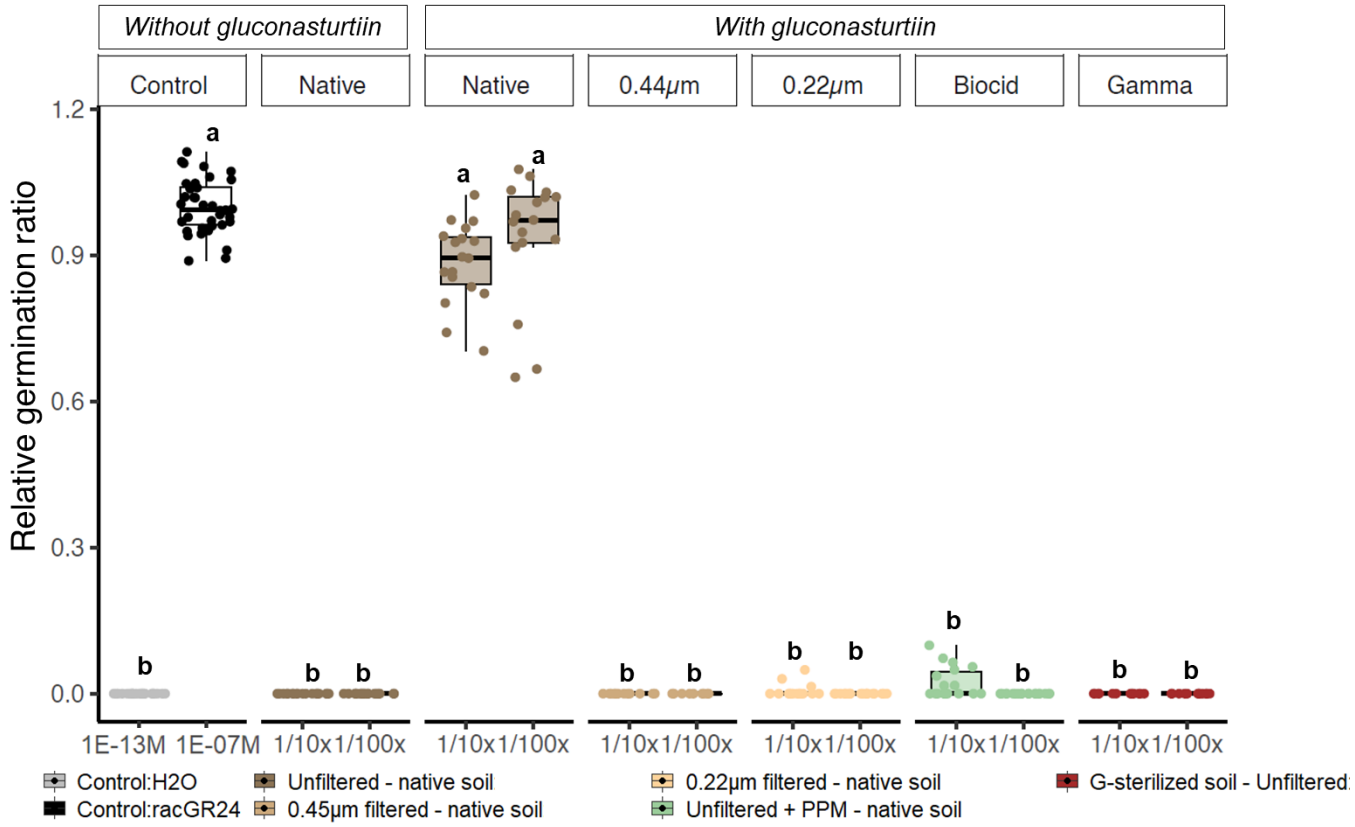


Fig.4 Effect of soil extract and exogenous gluconasturtiin on broomrape germination. Germination of broomrape seeds after a four-day treatment in 96-well plates with unfiltered soil extracts at a 10-fold and 100-fold dilution with and without gluconasturtiin (GNT 10^{-6} M), or after co-treatment with GNT and treated soil extracts: 0.45 µm and 0.22 µm syringe filtration, addition of large spectral biocide (Plant-Preservative Mixture 0.1% v/v) with the unfiltered extract, and soil gamma-sterilization. Ratios are expressed as the number of germinated seeds on total seeds, relatively to the average ratio obtained with the positive control (racGR24 10^{-7} M, a synthetic strigolactone, maximum germination ratio = 1 ≈ 70.12±4.37% of germinated seeds). Different lowercase letters indicate significant differences (p-value <0.05), and determined by a Kruskal-Wallis rank sum test (non-normal data) and multiple pairwise comparison.

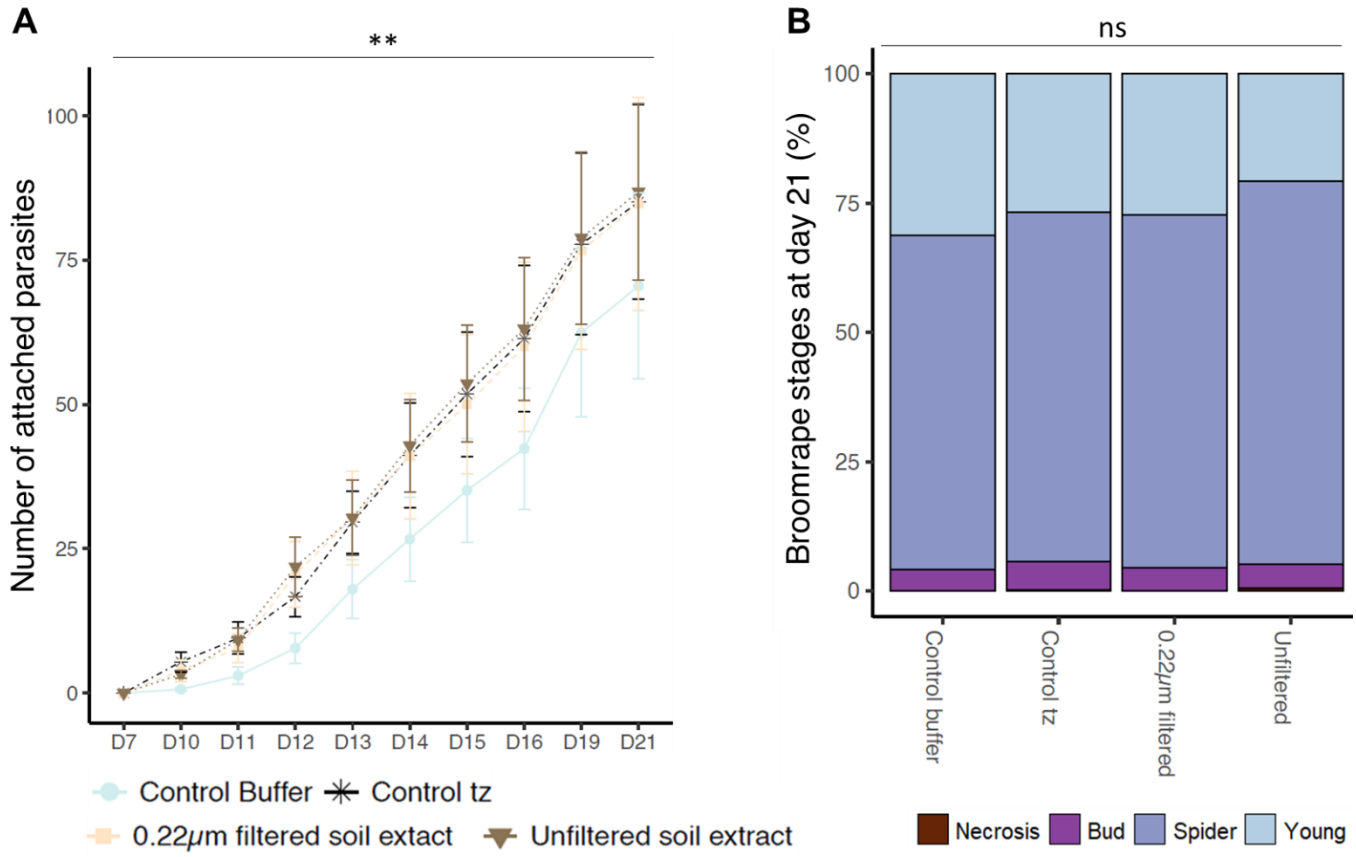


Fig.5A Broomrape attachments to rapeseed roots in mini-rhizotrons, on day 7 to 21 post seed inoculation. Values are displayed as mean with standard deviation as error bars. Asterisks indicate significative p-values compared to the control modality 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 'ns'; determined by an ANOVA on a Poisson distributed model. **B** Corresponding tubercle developmental stages of the broomrape attachments to roots in mini-rhizotrons at day 21. Stages correspond to Young (young tubercle without adventitious root), Spider (old tubercle bearing adventitious roots) and bud (bud with adventitious roots but without a stem). Aggressiveness assays were conducted on germinated and 48 h pre-treated seeds with either control buffer solution, unfiltered soil extract or 0.22µm filtered soil extract or cytokinin (tZ 10^{-7} M). Differences of treatments (p-value <0.05) are determined by ANOVA on logistic models for each stage, but are not significant for any stage between modalities (ns).

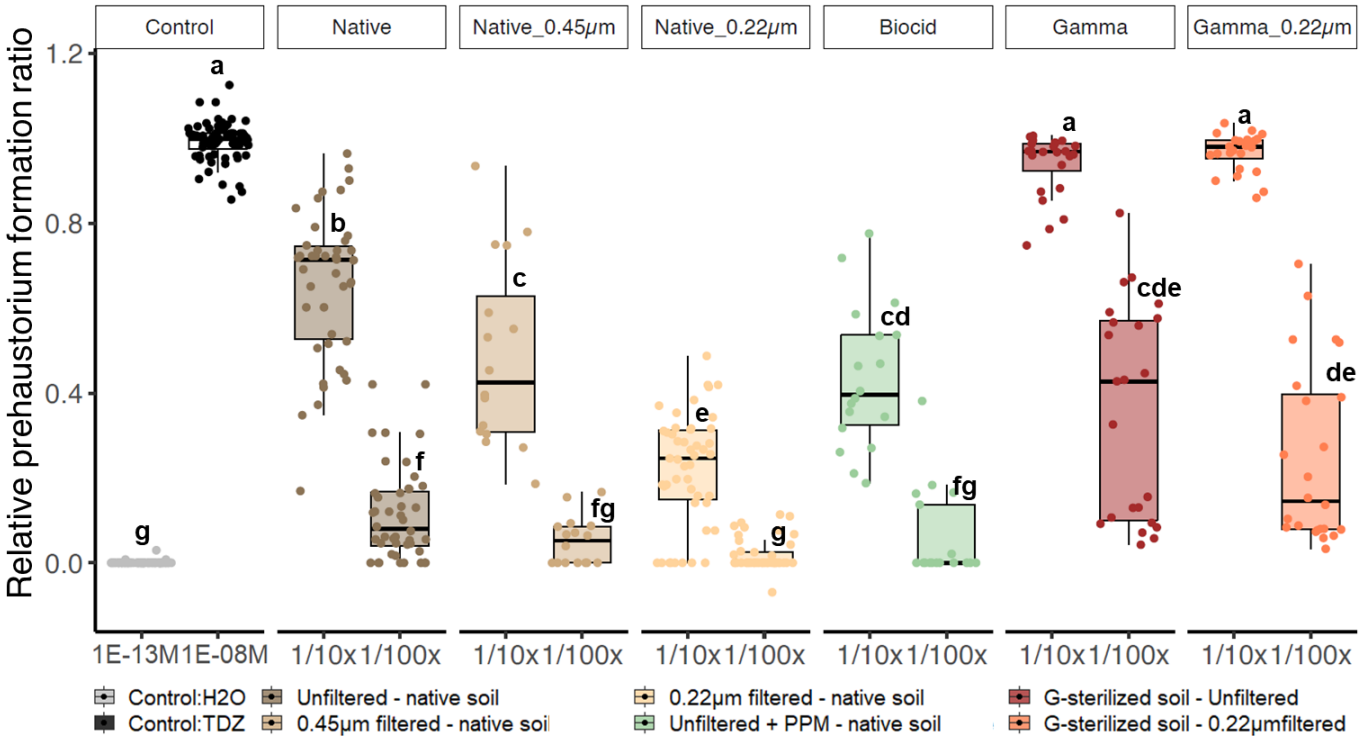


Fig.6 Effect of soil extracts on prehaustorium formation in P. ramosa. Prehaustorium formed after a three-day treatment in 96-well plates with unfiltered soil extracts or treated soil extracts: 0.45 µm and 0.22 µm syringe filtration, addition of large spectral biocide (Plant-Preservative Mixture 0.1% v/v) with the unfiltered extract and soil gamma-sterilization. Ratios are expressed as the number of germinated seeds exhibiting prehaustoria (swollen radicles with papillae) on total germinated seeds, relatively to the average ratio obtained with the positive control (TDZ 10^{-8} M, a cytokinin-like molecule). Different lowercase letters indicate significant differences of treatments (p-value <0.05), determined by an ANOVA on a logistic model.