

Comparative analysis of two neighboring conducive and suppressive soils towards plant parasitism caused by *Phelipanche ramosa* L. Pomel on *Brassica napus* L.

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Abstract

Background

In western France, rapeseed farmers face significant yield losses due to root parasitism caused by *Phelipanche ramosa*, a holoparasite plant of the *Orobanchaceae* family. Recently, a reduction of parasitic plant development has been observed in fields with history of severe infestation. In a same given pedoclimatic environment, this so-called soil-suppression of parasitism might presumably result from microbial mechanisms targeting *P. ramosa*. We tested this hypothesis on two soils from neighboring rapeseed fields, with similar physicochemical properties but contrasted parasitism, characterized as suppressive and conducive. We assessed these soils in a hydroponic co-cultivation system of *P. ramosa* and *B. napus*, and simultaneously sampled rhizosphere exudates weekly, as well as rhizosphere and rapeseed roots at three time points along parasite development. Comparisons were thus drawn between conducive and suppressive soils, both in untreated or gamma-sterilized conditions, regarding the effects of soil derived signaling metabolites on broomrape key early parasitic stages (*i.e.* germination and pre-haustorium development), late parasite development as well as soil bacterial and fungal structures.

Results

We demonstrate that the suppressive soil mitigates broomrape parasitism by reducing both parasite attachments and development, while causing tubercle necrosis. Activity assays on initial soils as well as co-cultivation rhizosphere exudates reveal that pre-attachment stages of broomrape are not affected by suppression. Root exudates, either they were collected from plants cultivated on suppressive or conducive soils, exerted similar contribution on the induction of broomrape seed germination and on haustoria formation. Thus, attachment and post-attachment stages are more likely to be suppressed by soil microbiota. Given the observed dissimilarities in microbial composition between the soils, we suggest that rather than bacteria, fungi play a greater part in parasitism suppression. Because suppression is not correlated with fungal diversity during co-cultivation, we suspect that in addition to general microbial interactions, specific groups of fungi participate in parasitism reduction. Further correlation network analyses (WGCNA) propose that three clusters of ASVs are indeed correlated with suppressive parasitic traits by their abundance. In these clusters, 7 ASVs are inversely correlated with parasitic attachment and one is positively correlated with the occurrence of necrotic tubercles, while being more abundant in the suppressive soil (DEseq2). These 7 ASVs are from *Nectriaceae*, *Niessliaceae*, *Bartaliniaceae*, *Holtermanniaceae* families as well as from *Helotiales* and *Pleosporales* orders, while the last ASV is identified as a *Berkeleyomyces* sp., a necrotrophic fungal genus known as a causal agent of black root rot.

Conclusion

This study explains the observed contrasted parasite development on two physiochemically similar soils by linking parasitic infestation to fungal dynamics in the rhizosphere. This finding extends our knowledge of disease suppressive soils to plant parasitism and proposes biological leads for potential biocontrol of broomrape.

Background

Broomrapes are root holoparasitic *Orobanchaceae* that belong to the *Orobanche* and *Phelipanche* genera and entirely rely on their host to fulfil their nutrient and water supplies [1]. Broomrapes are indeed physically connected to host plant roots through the haustorium, a specific invasive parasitic organ that allows the connection between their respective vascular systems and the spoliation of host nutrients and water [2, 3]. Broomrape infestations on several major crops (including sunflower, tomato, tobacco, legumes and rapeseed) remain difficult to control, making them some of the most agronomically noxious parasitic plants worldwide [4, 5]. The parasitic plant seeds undergo a two-step recognition process as they first perceive germination stimulants (GSs) and haustorium inducing factors (HIFs), which trigger their germination and then the formation of the prehaustorium respectively [6–8]. These fine-tuned key processes allow parasite seeds to synchronize the protagonists' life cycles and remain dormant in the soil for a long time [9].

Branched broomrape, *Phelipanche ramosa*, is widely spread across the world on various host families ranging from crops such as *Solanaceae*, *Brassicaceae*, *Cannabaceae*, *Cucurbitaceae* crops to weedy species [4, 10]. Western-France farmers are facing a major threat due to an aggressive population of *P. ramosa* attacking rapeseed that causes heavy damages, yield penalties and identified as genetic group 1 [11, 12]. This host preference results from both the interactions between abiotic - pedoclimatic and agronomic circumstances - but also biotic factors [13]. Indeed, soil and seed microorganisms were shown to promote parasite seed germination by enhancing the activity of selective brassicaceous GS, mainly through the increase of isothiocyanate availability [12, 14–16]. 2-Phenylethyl isothiocyanate (2-PEITC) was shown to be the main GS found in rapeseed exudates [15]. Isothiocyanates are bioactive compounds essentially found in the *Brassicaceae* plant family that result from the hydrolysis by myrosinase enzymes of the sulfur heteroside glucosinolates [17, 18]. These secondary metabolites are well-known for their roles in the plant's constitutive defenses and for shaping the host microbial communities, due to their strong antimicrobial properties [19–21].

In recent years, the use of microbes to control broomrape has been extensively considered for a lot of different crops. Through approaches relying on direct or indirect interactions with either broomrape-specific pathogens or host plant symbionts [22, 23]. Until now, promising fungal agents have been studied for their various antagonistic activities towards broomrapes. For instance, several *Fusarium* species can directly suppress broomrape at both pre- and post-attachment stages. Notably, fungi produce secondary metabolites with mycoherbicidal properties which inhibit the parasite seed germination [24, 25]. Some fungi can also be parasites and colonize broomrape tissues, including seeds, radicles and later underground tissues. This eventually results in fatal damages, as observed with *F. oxysporum* or

Trichoderma spp. [26, 27]. In addition, *F. oxysporum* and *Aspergillus alliaceus* have also been shown to induce lethal impacts through changes in broomrape hormone and phenolic substance levels [28, 29]. Other fungal strategies rely on disrupting the flow of signaling molecules required for host recognition, such as the universal GSs of broomrape species, strigolactones [30, 31]. Indeed, strigolactones are known to act as signaling hormones in plants [32, 33], but also to be key signaling molecules during the symbiotic arbuscular mycorrhizal fungi (AMF) colonization of plant roots [34, 35]. Although very promising, the introduction of single-isolated microbes within stable and competitive niches is challenging and usually results under poor colonization and survival efficiency in the soil, especially in field conditions [36, 37]. A different and more suitable approach would rather be to focus on microbial consortia and harness the crop protection traits conferred by soil microbiome, especially the ones from disease suppressive soils [38].

Since the early 2010's, Terres Inovia (the French technical institute for oilseed and protein crops, formerly CETIOM) has reported several contrasted broomrape infestations in a number of fields during rapeseed variety trials in Western France (Terres Inovia, personal communication). Some fields exhibited few parasitic emergences with underground necrotic symptoms while they had a history of severe broomrape infestation. Some other fields located in the same area exhibited high infestation rates and severe crop damages. Interestingly, these observations match with the concept of disease suppression by soils. Indeed, suppressive soils are defined as soils harboring advantageous physicochemical (pH, clay content, *etc*) and/or biological (soil and root-associated microbes) properties which reduces disease incidence or severity whereas conducive soils display high disease occurrence [39, 40]. Therefore, such soils appeared to be very promising for microbiome-based crop protection against branched broomrape.

In this study, we compared neighboring soils which had very contrasting broomrape infestation levels and were characterized accordingly as conducive or suppressive soils. The primary objective of the study was to characterize these dissimilarities in laboratory and put an emphasize on the broomrape lifecycle stages that eventually differed depending on the soil used. As a second objective, we sought to shed light on the importance of the soil microbiota in the overall interaction and validate its involvement in the success or failure of broomrape development and parasitic behavior. For those purposes, the two chosen soils were characterized for their physicochemical properties and biological activities on parasite seeds. Rapeseed and broomrape were then co-cultivated in the soils using single-pot system which allows us to sample weekly rhizosphere exudates but also and rhizosphere soils in single pots at three time points during parasitism. This allowed us to carry out:

- (i) bio-guided analyses with rhizosphere exudates in order to compare soil metabolomic profiles and effect on early parasitic stages, *i.e.* germination and prehaustorium formation;
- (ii) post-attachment parasitic plant phenotyping in order to characterize soil effects on late parasitic behavior;
- (iii) metabarcoding analyses on sampled rhizosphere soils in order to describe the microbial community dynamics during the co-cultivation and to eventually identify possible groups of microbes involved in

suppressive mechanisms.

Methods

A summary of the experimental design is reported in Fig. 1 and displays the system we used for simultaneous sampling.

Plant material

P. ramosa seeds were collected from dry mature spikes of *Phelipanche ramosa* growing on rapeseed crops at Saint Jean d'Angély (France, 45°56'40.545" N, 0°31'10.472" W) in June 2015. Harvested seeds were sieved with a 180–200 µm mesh, and characterized as laboratory reference batch Pram120 (89% maximum germination ratio; $2.25 \cdot 10^{-11}$ M as 50% effective concentration of *rac*-GR24, genetic group 1 by genetic and phenotyping assays [41, 42]). Seeds were kept in the dark at 25°C until use.

Rapeseed seeds (variety Veritas CL highly susceptible to *P. ramosa*) were kindly provided by DSV France SARL (Terminiers, France) in October 2019, and kept in a dark glass jar at 4°C until use.

For *in vitro* assays, broomrape seeds were surface sterilized according to Lechat *et al.* [43]. Seeds were suspended in a 2.5% (v/v) sodium hypochlorite solution under agitation, then thoroughly rinsed 3 times 30 s and 3 times 5 min in sterile distilled water under agitation. After sterilization, seeds were resuspended in an incubation solution (HEPES 1 mM; pH 7.5; Plant Preservative Mixture 0.1% v/v) at 10 mg seeds mL⁻¹, and placed 7 days in the dark at 21°C for conditioning [44].

Soil sampling and physicochemical characterization

The conducive soil (46°22'50.82" N, 0°27'19.02" W; high infestation and crop damage levels) and the suppressive soil (46°22'47.07" N, 0°27'423" W; low emergences and necrotic symptom levels) were sampled in adjacent fields, in the Mursay site (Echiré, France; Fig.S1, A). Since 2007, the conducive native soil (CN) underwent four cycles of wheat cropping, and was cultivated following a wheat - barley - sunflower rotation since 2015 and not with rapeseed (Fig.S1, B). The suppressive native soil (SN) was however cultivated according shortened rapeseed rotations for experimental field trials, whether triennial or biennial rotations with wheat or barley. Prior to sampling, both soils were cultivated with rapeseed. Rapeseeds showed strong variations of broomrape infestations (*i.e.* near zero for the SN soil versus high for the CN soil). Following rapeseed harvests (early July 2019), thirty liters of both soils were sampled on ten random patches nearby rapeseed roots - infested by *P. ramosa* or not, at a 0–20 cm depth.

Physicochemical analyses of these native soils were performed at the SADEF laboratory (Aspach le bas, France;). Parameters analyzed are presented in Additional table S1. Prior to use, soils were homogenized, hand sieved to remove large debris and kept in a room with few temperature and humidity fluctuations. To study the biological effect of soil on plant parasitism, a portion of each soil was subjected to gamma-

sterilization (35 kGy) at Ionisos (Pouzauges, France) in order to eradicate most microorganisms while limiting damages to the soil integrity [45].

Experimental system set-up for rapeseed - broomrape co-cultivations in soil

To compare levels of parasitism in the different soils, rapeseed host plants were co-cultivated along with seeds for seven weeks. Plants were grown in four different soil modalities: the conducive or suppressive soils either native or sterilized which were labelled throughout the study as CN (conductive native), CS (conductive sterile), SN (suppressive native) and SS (suppressive sterile).

Each soil modality consisted of twenty pots, filled with 100 mg of soil and mixed with an inoculum of 20 mg of Pram120 seeds (the excess quantity permits to neglect the soil's initial parasite seed stock). Single pots (or gift pots) were placed onto a coconut fiber cloth on a growth tray on top of a hydroponic container (nutrient film technique growth tanks; Fig.S2). Pots were watered by capillarity using a water pump, recirculating autoclaved Tadano and Tanaka nutrient medium diluted by two pH 7.5 (TT/2, 50% v/v) [46] onto the coconut fiber cloth for one hour every 3 hours (Fig. 1). After a one-week acclimation period in the growth chamber during which weeds or native rapeseed plantlets were removed, three seeds of *Brassica napus* var. Veritas were sowed in each pot. After a one-week acclimation period during which weeds or native rapeseed plantlets were removed, three seeds of *Brassica napus* var. Veritas were sowed in each pot. These co-cultivations were conducted three times independently in 2020, in a growth chamber with a 16/8 h photoperiod with $240 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation (PAR), 21°C constant, and relative humidity of 70%.

Assessment of soil effects on broomrape development: pre- and post-attachment stages

Initial activity of CN and SN soils was assessed on pre-attachment parasitic stages, *i.e.* germination and prehaustorium formation induction. Extracts of soils were prepared according to Martinez et al. [14] by macerating 5 g of 1 mm-sieved soil and 5 mL of sterile distilled water twice for 1 h. To test for microbial and metabolic activities, effect of soil extracts was tested without treatment or after sterilizing filtration (0.22 μm ; PES Membrane syringe Filter, Millex-GP).

Effect of soil was then tested on parasitic infestation at two, five and seven weeks after rapeseed sowing on the whole root system (three plants per pot) and on five or six randomly selected pots per modality. Soil was gently detached in sterile distilled water and a 2 mL aliquot of rhizosphere soil was systematically stored at -80°C until further analysis. The number of parasitic plant attachments per pot were counted under a magnifying binoculars (Euromex StereoBlue) and ranked according to their developmental stages following S1: attachment to host roots, S2: young tubercle with no adventitious roots, S3: spider-like tubercle bearing numerous adventitious roots, S4: tubercle bearing a bud and S5: tubercle bearing an under-ground stem [47]. In addition, we added an abnormal stage N, corresponding to

tubercle presenting necrotic symptoms, *i.e.* brownish or rotted tissues. To account for the soil effect on the host growth, root dry weight (RDW) and shoot dry weight (stems and leaves; SDW) were separately weighted for each pot after 72 h at 80°C.

Exudate sampling and treatment

Rhizosphere exudates were obtained by weekly collecting the recirculated growth medium from co-cultivation tanks from week 0 to 7 post rapeseed sowing. The collected medium was simultaneously clarified and filtered through glass fiber filters (retention of 1.6 µm particles; Merck Millipore) combined with nitrocellulose membrane filter (0.22 µm, MF-Millipore™) to remove soil particles and microorganisms. All rhizosphere exudates were first adjusted to 2.2 L (maximum volume sampled) with TT/2 and were then purified and concentrated according to Goyet et al. [48] on 200 mg Solid Phase Extraction columns (SPE; strata-X 33µm Phenomenex). Briefly, after conditioning columns with acetonitrile and washing them twice with distilled water, samples were loaded. The polar compounds passed into the resulting aqueous flow-through fraction whereas more apolar compounds were trapped on the column and subsequently eluted with 4 mL acetonitrile, evaporated using a R II Rotavapor (Buchi) under reduced pressure (93 mbar) at 40°C and dissolved in 50% v/v acetonitrile/water (final volume between 200–800 µL; approx. 20-fold volume reduction) for solubility and conservation purposes. The eluted fractions were kept at – 20°C before use.

Glucosinolate and isothiocyanate quantification

Both glucosinolates and glucosinolate-breakdown products - *a.k.a* isothiocyanates - were quantified in the eluted fractions. Glucosinolate contents were analysed by UPLC-TQD (Ultra Performance Liquid Chromatography – Triple Quadripole Detector; Waters), using a protocol based on Glauser et al. [49]. Briefly, 2 µL of samples were injected on a C₁₈ column (CSH 2.1 x 100 mm, 1.7 µm, Waters), with water-acetonitrile gradient (see details below), a flow of 0.4 mL.min⁻¹, and a column temperature of 25°C. Details of the elution gradient were as follows: Solvent A = water + 0.05% formic acid; Solvent B = acetonitrile + 0.05% formic acid; %B: 2–46 for 6 min, then 45–100 for 0.5 min, then 100% for 2 min, then back to 2% for 2 min. For mass detection, the capillary voltage was of 2.9 kV, the cone voltage was of 37 V, the source and desolvation temperature were of 150°C and 400°C, and the desolvation flow was of 800 L/h. 4-methylsulfinylbutyl-, 5-methylsulfinylpentyl-, 3-indolylmethyl-, and phenylethyl- glucosinolates were quantified based on standard curves made with commercially available authentic standards. 3-methylthiopropyl-, methylpropyl-, 5-methylthiopentyl-, and the two methoxy-3-indolylmethyl- glucosinolates isomers were quantified based on standard curves made with most structurally related commercially available authentic standards: 3-(methylsulfinyl)propyl-, 2-hydroxy-3-butenyl-, 4-methylthiobutyl-, and 3-indolylmethyl-glucosinolates, respectively.

For the GC-MS, isothiocyanates were first extracted by adding 200 µL of hexane with internal standard Eucalyptol (10 µM) to each sample (200 µL). Tubes were vortexed (10 s) and then centrifugated for 5 min at 5,000 RCF and 4°C to produce a biphasic separation. The upper hexane phase, containing isothiocyanates, was then transferred to clean vials for subsequent injection. GC-MS measurements were

carried out on Shimadzu GC-2010Plus GC coupled to a Shimadzu GCMS-TQ8040 Quadrupole MS, equipped with a Gerstel Multipurpose Sampler Robotic Pro. One microliter of each sample was injected to a ThermoFisher Scientific TG-5-MS (26098 – 1430) column (30 m x 0.25 mm internal diameter, 0.25 μ m film thickness) in split mode with a ratio of 1:10. The inlet temperature was of 280°C. During the analyses, the carrier gas (helium) flow rate was set at 1 mL.min⁻¹. The GC oven temperature was first set to 80°C and increased by 10°C.min⁻¹ up to 300°C. The temperature of the transfer line was set to 300°C, and the MS source to 200°C. The electron ionization (EI) source was set to 70 eV. For identification, we used full scan mode in the m/z 50 to 650 range. Sample composition was determined with eight standards and quantified using an external calibration curve with the most abundant fragment: iberiverin (3-Methylthiopropyl isothiocyanate, m/z 101), erucin (4-Methylthiobutyl isothiocyanate, m/z 115), 2-PEITC (2-Phenylethyl isothiocyanate, m/z 91), goitrin (5-ethenyl-1,3-oxazolidine-2-thione, m/z 129), iberin (3-Methylsulfinylpropyl isothiocyanate, m/z 101), Sulforaphane (1-isothiocyanato-4-(methylsulfinyl)butane, m/z 61), 3-Butenyl ITC (m/z 72) and berteroin (5-Methylthiopentyl isothiocyanate, m/z 61). For each sample, the eucalyptol fragment (m/z 81) was used as internal standard to determine the response factor for quantification. For each molecule, the kinetics of exudation during the seven weeks of co-cultivation was determined using the area under the curve (AUC in μ M.week⁻¹) of each trapeze during seven weeks using the following formula:

$$AUC = \frac{\sum(\text{concentration}_{\text{week}n} + \text{concentration}_{\text{week}n+1})}{(\text{Week}n+1 - \text{Week}n)} \times \frac{1}{2}$$

Germination bioassays

Activities of soil extracts as well as rhizosphere exudates, flow through and eluted fractions were tested on parasite seed germination. Soil extracts were applied at 10¹ and 10²-fold dilutions either unfiltered or filtered (0.22 μ m). Rhizosphere exudates and flow through fractions were both tested at 2-fold and 10-fold dilutions, whereas eluted fractions were tested at 10², 10³, and 10⁴-fold dilutions to limit solvent toxicity (acetonitrile < 1% v/v).

Germination bioassays were conducted according to Pouvreau et al. [50] on 50 μ L of sterilized and conditioned *P. ramosa* seeds (10 mg seed mL⁻¹) distributed on 96-well plates (Cell Culture Multiwell Plate Cellstar; Greiner Bio-One), by adding 10 μ L of the tested solution in triplicates. Each solution of rhizosphere exudates and resulting fractions were tested with and without myrosinase enzymes (thioglucosidase from *Sinapis alba*, Sigma-Aldrich; 5 mU.mL⁻¹ final concentration) which cleave inactive glucosinolates into active products, namely isothiocyanates. Each well was adjusted up to 100 μ L with buffer (HEPES 1 mM, Plant Preservative Mixture 0.1% v/v; pH 7.5). Each soil extract solutions were tested with and without glucosinolate, using gluconasturtiin (Phenylethylglucosinolate K-salt from Nasturtium officinal, Phytoplan; 10⁻⁶ M) to test for presence of myrosinase-like activity. Gluconasturtiin was chosen as 2-PEITC (2-phenethyl isothiocyanate), its corresponding isothiocyanates, is known to induce germination of *P. ramosa* seeds [15]. As previously, each well was adjusted up to 100 μ L with buffer

(HEPES 1 mM, no Plant Preservative Mixture; pH 7.5). For both tests, synthetic strigolactone *rac*-GR24 (10^{-7} M) kindly provided by F-D Boyer (Centre National de la Recherche Scientifique, [CNRS]-INRAE, Gif-sur-Yvette, France) was used as a positive control, whereas seeds treated with buffer solution were used as a negative control. Positive control (*rac*-GR24; 10^{-7} M) was accepted when germination ratio was at least 60% of germinated seeds. To control enzyme activity, additional wells with myrosinase co-treated with gluconasturtiin (10^{-6} M) were prepared. Inter-well contaminations due to volatile degradation products were avoided by sealing plates with gas-impermeable films. After 4 days of incubation at 21°C in the dark, seeds were stained by adding 5 µL of thiazolyl methylthiazolyldiphenyl-tetrazolium bromide (MTT; Sigma-Aldrich; 5 g.L⁻¹). For soil extracts, the ratio of germinated seeds was assessed the following day under a binocular magnifier (Olympus SZX10; Olympus Europa GmbH) and was reported as a ratio relative to the average ratio of *rac*-GR24-treated germinated seeds, within the same plate. For rhizosphere exudates, the day after MTT salts were solubilized in the well by incubating the seeds at 21°C in the dark overnight with 100 µL lysis buffer (Triton X-100 10% v/v, HCL 0.04 M in isopropanol). Germination ratios were approximated by spectrophotometric reading based on the method developed by Pouvreau et al. [50]. Absorbance was read in each well at 570 nm from which a reference wavelength of 690 nm was subtracted ($A_{570-690nm}$). Relative ratios were then calculated by subtracting average minimum absorbance (negative controls within the same plate), reported to the average maximum absorbance (positive controls with *rac*-GR24 within the same plate) subtracted by average minimum absorbance using the following formula:
$$\text{relative absorbance}_{\text{well}} = \frac{\text{Absorbance}(\text{well}) - \text{Average absorbance}(\text{min})}{\text{Average absorbance}(\text{max}) - \text{Average absorbance}(\text{min})}$$

Early Haustorium Structure induction bioassays

Soil extracts and eluted fractions only were tested on pre-haustorium formation in *P. ramosa* germinated seeds. For this, 10^1 and 10^2 -fold dilutions as well as unfiltered or filtered (0.22 µm) for soil extracts and 10^2 or 10^3 -fold dilutions for eluted fractions to prevent solvent toxicity (acetonitrile < 1%; v/v) were tested.

Prehaustorium formation bioassays were conducted as described by Goyet et al. [48]. Briefly, 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. Germination was induced by resuspending three-time rinsed and conditioned seeds in a germination medium (HEPES 1 mM; pH 7.5; *rac*-GR24 10^{-7} M). After five minutes, 50 µL of treated seeds were distributed in 96 well plates, sealed with parafilm. Incubation was conducted at 21°C in the dark for 4 days. Germination was then controlled and should have reached a minimum of 70% germination ratio. The incubation medium was carefully removed and replaced with 90 µL of buffer solution (HEPES 1 mM; pH 7.5). Ten microliters of the different test solutions were then added in five replicates. In addition, seeds were treated with the synthetic analog of cytokinetic thidiazuron (TDZ; PESTANAL® analytical standard; Sigma-Aldrich; 10^{-8} M) as positive control [6] and for negative control with buffer solution. After 3 days of incubation at 21°C in the dark, seeds were stained by adding 5 µL MTT (Sigma-Aldrich; 5 g.L⁻¹). The following day, the ratio of germinated seeds developing prehaustorium (occurrence of papillae and root

tip swelling) was evaluated 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.

Rhizosphere DNA extraction and amplicon illumina sequencing

Microbiome analyses were conducted by extracting DNA on different types of samples all along the co-cultivation experiments. First, initial bulk soils were sampled from the storage container before each co-cultivation (week – 1). After one week in the growth chamber with TT/2 supply, bulk soil was collected from the pots preceding rapeseed seed sowing (week 0). Then, rapeseed roots were sampled on week 2, 5 and 7 after sowing both for parasitic phenotyping and rhizosphere sampling (Fig. 1; root experimental set-up). According to the “pull and shake” methods designed by Barillot et al. [51], roots were pulled out of the pots and hand-shaken to remove non-rhizosphere soil, *i.e.* non-adhering to roots, while rhizosphere soil, *i.e.* tightly associated with roots, was retrieved by soaking the roots into 100 mL of distilled sterile water. Several aliquots of collection water were stored for metabarcoding controls. DNA extractions were performed on 200 µL or 200 mg for liquid (rhizosphere soils and water controls) or solid materials (bulk soil) respectively. Samples were processed via the NucleoSpin® Soil Kit (Macherey-Nagel), according to the recommended standard instructions.

Amplicon library was constructed as in Huet et al. [12] and Martinez et al. [14] following a two-step PCRs process required for Illumina MiSeq sequencing. The first PCR amplified V4 regions of bacterial 16S rRNA using 16S_515f/16S_806r primers [52] or fungal internal transcribed spacer (ITS1) genes using ITS1_f/ITS2 (Buée et al. 2009). The second PCR was required to add barcodes and adapters. Briefly, PCR1 program started with denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, primer annealing at 50°C for 45 s, extension at 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).

Bioinformatic analysis

Illumina Miseq amplicons were processed via an automated bioinformatics workflow microSysMics (<https://bio.tools/microSysMics>). Quality filtration of reads, trimming, taxonomic assignation, as well as ASV processing and decontaminations were conducted as in Huet et al. [12] and Martinez et al. [14]. ASVs were assigned to taxa according to UNITE database (classifier v.8–99 2020.02.04) for fungi [54] and SILVA database (classifier 138-99-nb) for bacteria [55, 56]. ASVs of interests that were not assigned were further computed in the NCBI database with a BLAST of the nucleotide sequence. ASVs were numbered by alphabetical order according to the SILVA or UNITE code, respectively for bacteria and fungi. Their associated codes and corresponding DNA are available in supplementary data (Tables S2 and S3).

Microbial community analyses

All analyses were performed via R (R Core v4.0.3), on RStudio 2022.07.1 + 576.

Secondary analyses were computed via the Phyloseq package v.1.34.0 [57]. To simplify analyses, low-abundance taxa (< 0.1%) were grouped into a common class “<0.1% abund.”.

For α - and β -diversity analyses, abundance tables were rarefied to the minimum library size (across samples and weeks) to account for sampling intensity variations [58]. Analyses of α -diversity were performed using richness (*i.e.* number of ASVs detected) and Shannon (*i.e.* number of ASVs detected and their evenness) indices, and compared with two-sided Wilcoxon Rank Sum tests. Analyses of β -diversity were performed via permutational analysis of variance (PERMANOVA) via the Adonis function in the Vegan package v.2.6.2 (Oksanen et al. 2022), using a Bray-Curtis dissimilarity matrix. Dissimilarities were interpreted via multivariate analysis on principal coordinate (PCoA).

Differential abundant analysis was performed using the DEseq2 R package v.1.30.1 [59], to assess the differences between the abundance of ASVs from the conducive and suppressive soil at the initial time.

Screening of microbial ASVs functionally correlated to parasitic traits

To describe correlation patterns between fungal dynamics and parasitism suppression, we employed a weighted gene co-expression network analysis (WGCNA v.1.70.3 [60]). This allowed to detect clusters of highly correlated taxa in relation with external sample traits (total attachments, relative proportion of developmental stages, germination and prehaustorium formation ratios). Composition of clusters was described by measuring the relative abundance of their ASVs, and plotting the results onto a bubble plot (<https://github.com/alex-bagnoud/OTU-table-to-bubble-plot>).

Finally, a cross-sectional analysis of DEseq2 and WGCNA results was conducted to detect ASVs most likely associated with parasitism soil suppression. First, using a Pearson's correlation test, abundances of ASVs over abundant in SN soil (DEseq2) were tested against previously highlighted suppressive parasitic traits (total parasitic attachment and necrosis ratio). Second, the presence of correlated ASVs were investigated in clusters (WGCNA). In summary, this strategy arbitrarily considered ASVs as suppressive candidates when they were 1) initially overabundant in the SN soil compared to the CN soil, 2) present in any of the clusters and 3) significantly correlated with suppressive parasitic traits.

Statistical methods

Analyses were performed on RStudio 2022.07.1 + 576 with different statistical approaches according to data distribution and experimental design.

Differences of parasitic attachments between the soils were modeled with a Poisson distribution, adapted to fit discrete count data with low value, *e.g.* comprised between 0 and 113 attachments, and compared with analysis of variance using ANOVAs on a generalized linear mixed effects models (glmer) with the Car package v3.0-10, (Fox and Weisberg 2019) and the lme4 package v1.1-29 (Bates et al. 2015).

Parasitic stages were analyzed by calculating their relative abundance among the total attachment observed. Each stage was used as explanatory variable with a glmmPQL, a glmer based on a *quasi* distribution for logistic regression with a probit link function. GlmmPQL were employed to model proportion data following a binomial law as our data were continuous and comprised between 1 and 0. For both total number of attachments and relative stages, the fixed effects tested were: sampling week, soil effect (conducive or suppressive) and nature (native or sterile). Their effects on the explanatory variables were compared with multi comparisons of estimated means with the emmeans package 1.7.5 [63]. Biological replicates of co-cultivations (1, 2 or 3), SDW and RDW, position of pots in the tray (1–20), number of rapeseed plantlets (1, 2 or 3) and the experimenters (2 persons) were used as random effects to account for biological variability in the model.

RDW and SDW were modeled according to a Normal distribution after logarithmic transformation, and compared with analysis of variance using ANOVAs on a linear mixed effects models (lmer). Two models with respectively sampling week, soil effect (conducive or suppressive) and nature (native or sterile) were used as fixed effects in interactions, and were compared with multi comparisons of estimated means with the emmeans package 1.7.5 [63]. Biological replicates of co-cultivations (1, 2 or 3), were used as random effects to account for biological variability in the model, whereas position of pots in the tray (1–20), number of rapeseed plantlets (1, 2 or 3) and the experimenters (2 persons) introduced singular fits and were removed from the models. To check for potential correlations between host growth (SDW, RDW) and parasitic attachments, a Pearson correlation tests was performed via the Hmisc package v.4.7-0.

Germination and prehaustorium formation kinetics were first analyzed using a glmmPQL model glmer based on a *quasi* law for logistic regression. The soil (CN, SN, CS or SS), week (from 1 to 7) and myrosinase (with or without) factors were used to explain rhizosphere exudate activity, and co-cultivation replicate was used a random factor to account for biological variability. Global effects were compared with multi comparisons of estimated means with the emmeans package. To facilitate global effect of rhizosphere exudate on broomrape seeds, both the global germination and prehaustorium formation data were further processed. Basically, the area under the curve (AUC) of the 7-week kinetics was calculated as a proxy for stimulation power of each soil modality using the following formula: $AUC = \sum (\text{Relative ratio}_{\text{week } n} + \text{relative ratio}_{\text{week } n+1}) / (\text{Week}_{n+1} - \text{Week}_n) * 2$. Values of AUC ranged from 0 to 7 relative ratio.week⁻¹ given relative ratios are repositioned to 0 and 1 values for 7 weeks. AUC variable did not fit Normal distribution and was analyzed using a non-parametric Kruskal-Wallis test (p value ≤ 0.05) by rank from the stats package v.4.0.3 using soil modalities (CN, SN, CS, SS) as the response variable. Soil effects were determined with Dunn's Kruskal-Wallis Multiple Comparisons between group levels, from the FSA package v.0.9.3. Correlations between broomrape seed germination (relative absorbance of the eluted fraction) and isothiocyanate quantifications were checked with a Pearson's correlation test from the Hmisc package v.4.7-0.

Germination and prehaustorium response in presence of soil extracts were also analyzed using a non-parametric Kruskal-Wallis test by rank. As 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

controls, soil dilution, soil sterilization and co-treatments. Effect was determined with Dunn's Kruskal-Wallis Multiple Comparisons (p value ≤ 0.05).

Results

Characterization of soil initial conditions and activities

Similar physicochemical properties of CN and SN soils

Overall, CN and SN soils had similar physicochemical properties, *i.e.* texture, calcic state, clay-humus complex, as well as content in major exchangeable elements and assimilable oligo-elements (Fig. 2). Indeed, both SN and CN soils were sandy loam soils either composed of 8.5 % clay, 60.7 % silt and 30.8 % sand, or 9.2 % clay, 59.4 % silt and 35.24 % sand respectively. Both soils had a pH of 7.9, C/N ratios of 8.3 for SN soil and 8.2 for CN soil (usual values ranging between 8-12), slightly low total carbonate levels of 26.8 % for SN soil and 28.7 % for CN soil and organic matter contents of 82.1 g/kg for SN soil and 76.5 g/kg for CN soil. Only minor differences were observed in Mn content with 20.3 mg/kg for CN soil and 34.5 mg/kg for SN soil and in K_2O/MgO ratios, with values of 4.2 for SN soil and 1.5 for CN soil (optimal values comprised between 1 and 2).

CN and SN soils enable broomrape seed germination, triggered by microbial myrosinases

The initial activities of CN and SN soil metabolites and/or microorganisms on broomrape seed germination were compared through *in vitro* assays (Fig.3). For CN and SN soils, applications of filtered or unfiltered soil extracts alone resulted in zero relative germination (*i.e.* no germinated seeds) for the two dilutions, suggesting no direct activities from metabolites or microorganisms. In contrast, the combination of unfiltered soil extracts at the 10^1 -fold dilution with the gluconasturtiin aromatic glucosinolate (10^{-6} M) significantly increased relative germination ratios, and was similar for CN (0.58 ± 0.19) and SN (0.86 ± 0.12) soils (p value = 0.35). After a 10^2 -fold dilution, this effect was reduced to 0.25 ± 0.39 in SN soil (p value = 0.03 with buffer) or was absent from CN soil, with germination ratio of 0 ± 0.33 (no difference with negative control; p value = 0.19). Finally, this effect was removed after sterilization by filtration, since relative germination for all soils and dilutions were null. The following results highlight that the combined presence of either CN or SN soil microorganisms and glucosinolates induce broomrape germination, suggesting that the hydrolysis products generated from exogenous gluconasturtiin by the microbial enzymatic activity may act as GSs. Indeed, gluconasturtiin alone does not induce seed germination [15]. whereas combined application of gluconasturtiin and myrosinase enzymes (5 U.L^{-1}) induced relative germination ratio of 1.01 ± 0.11 . All in all, no suppressive activity was detected during broomrape seed germination initially in the SN soil.

CN and SN soils enable broomrape seed prehaustorium formation, triggered by microbial activities

The initial activity of CN and SN soil metabolites and microorganisms on broomrape seed prehaustorium formation was compared. This time, 0.22 µm filtered and unfiltered soil extracts were tested on germinated seeds (Fig.4). Three days after radicle protrusion, 95.33±5.33% (median±sd) of radicles had developed prehaustoria with positive control (TDZ; 10⁻⁸ M), corresponding to a relative prehaustorium formation ratio of 1.00±0.03. In contrast, zero prehaustorium was observed with the negative control buffer. For CN and SN, unfiltered soil extracts were active at the 10¹-fold dilution compared to the negative control. They induced between 0.26±0.09 (*p* value = 5.35E⁻⁴) and 0.87±0.17 (*p* value = 2.32E⁻⁸) prehaustoria, respectively for CN and SN soils (*p* value = 0.045). At the 10²-fold dilution, SN soil still induced 0.26±0.16 prehaustoria compared to the negative control (*p* value = 0.002) whereas CN soil no longer did (*p* value = 0.34). In addition, sterilizing filtration had a strongly negative effect on the formation of prehaustoria. Compared to the negative control, SN filtered soil extracts were active and relatively induced 0.51±0.10 prehaustoria at the 10¹-fold dilution (*p* value = 0.02). However, they were no longer active at the 10²-fold dilution (0.07±0.05; *p* value = 0.16). No prehaustorium was induced by CN filtered soil extracts for both the 10¹ (*p* value = 0.59) or the 10²-fold dilution (*p* value = 1.00) compared to the negative control. This suggests that both soils contain microorganisms capable of releasing HIF, and a residual activity was found in the SN soil filtered extracts. They also point out that, no suppressive activity was genuinely found in the initial SN soil during the formation of the prehaustoria.

Changes in soil conditions and activities throughout co-cultivation

Similar effects of soils on glucosinolate and isothiocyanates content in rhizosphere exudates

Levels of the main metabolites signaling broomrape germination (*i.e.* glucosinolates and then isothiocyanates) were compared between conducive and suppressive soils by mass spectrometry. UPLC-MS analyses on eluted fractions permitted to detect 9 glucosinolates in rhizosphere exudates from native soils in at least one sample, identified as 4-methylsulfinylbutyl-, 5-methylsulfinylpentyl-, 3-methylthiopropyl-, methylpropyl-, 3-indolylmethyl-, 5-methylthiopentyl-, 2-phenylethyl-, 4-methoxy-3-indolylmethyl- and 1-methoxy-3-indolylmethyl- glucosinolates. However, aside from 4-methoxy-3-indolylmethyl-glucosinolate which was present in higher but very variable concentrations (AUC of median= 75.13 ± sd= 77.05 - 17.20±12.81 for CN and SN respectively; Fig.S3), all contents were below the detection threshold (<1µM) and were not receivable for comparisons. For sterilized soils, no glucosinolate could be detected above the threshold limit (<1µM), aside from 4-methoxy-3-indolylmethyl-glucosinolate, still present in higher and very variable concentrations (AUC of 198.45±214.10 – 21.89±8.64 for CS and SS respectively; Fig.S3).

Over the eight isothiocyanates searched with GC-MS from eluted fractions rhizosphere exudates, only 2-PEITC and erucin (4-methylthiobutyl isothiocyanate) were at least once above their detection threshold: 0.1µM for 2-PEITC and 1µM for Eurcin. However, their respective contents remained low in all eluted fractions and throughout co-cultivation, which never exceeded 2.5 µM during the 7 weeks of co-

cultivation (Fig.5). Besides, global content - calculated as AUC - of both 2-PEITC and erucin was similar in the CN and SN soils (p value = 0.97 for 2-PEITC and p value = 0.11 for erucin). Additionally, isothiocyanate contents in eluted fractions was not affected by sterilization, compared to their respective native soils for both CS (p value = 0.97 for 2-PEITC and p value = 0.23 for erucin) and SS soils (p value = 0.54 for 2-PEITC and p value = 1.00 for erucin; Fig.S4).

Rhizosphere exudates from plants cultivated on CN and SN soils similarly stimulate broomrape seed germination

Rhizosphere exudates from co-cultivation media were weekly collected and the resulting SPE fractions (*i.e.* exudates, flow-through and eluted fractions) were tested for their ability to differentially induce broomrape seed germination. For instance, the example shown in supplementary figure Fig.S5, rhizosphere exudates and eluted fractions collected after 2 weeks post sowing induced germination compared to the negative control (both p values < 0.0001).

Germination ratios were not affected by the addition of myrosinase enzymes, suggesting the absence of glucosinolates from the fractions (p value = 0.19 for the myrosinase effect). The activity from rhizosphere exudates was successfully concentrated in the eluted fractions, since both rhizosphere exudates and eluted fractions both stimulated broomrape seed germination (p value = 0.09) while the flow through fractions never induced broomrape germination (p value = 0.94 compared to negative control). Besides, flow through fractions were inactive with and without myrosinase (p value = 1.00) confirming the absence of glucosinolate and active degradation products of glucosinolates respectively, in this fraction (Fig.S5). In addition, *in vitro* application of eluted fractions on broomrape seeds at 10^3 -fold and 10^4 -fold dilutions seemed to similarly affect germination, whereas 10^2 -fold dilution seemed to have slightly reduced germination, due to the toxicity of acetonitrile solvent (Fig.S6; [50]). Hence, comparative analyses were further conducted on the 10^3 -fold diluted eluted fractions (Fig.6).

Eluted fraction of exudates from plants cultivated in CN and SN soils both had a stimulant effect on broomrape seed germination (Fig.6). Germination was first induced at two weeks post sowing, with relative germination ratios of 0.56 ± 0.07 (mean $\pm$ se) and 0.45 ± 0.08 respectively for CN and SN soil (p value = 0.42), which were both different from the negative control (two p values < 0.0001). At seven weeks post sowing, relative germination ratio strongly decreased to values of 0.29 ± 0.08 for the CN soil and 0.11 ± 0.01 for the SN soil (p values = 0.011 – 1.33×10^{-12} respectively for CN and SN soils compared to the negative control). For comparisons of global germination induction during the complete 7-week-kinetics, the areas under the curves (AUC) were calculated (Fig. 6; boxplots). At the 10^3 -fold dilution, global germination stimulation was similar between the soils (p value= 0.32) with values of 2.15 ± 0.30 relative germination ratio.week⁻¹ (median $\pm$ sd) for CN soil and 2.38 ± 0.50 relative germination ratio.week⁻¹ for SN soil. This suggested that there was no effect of either soil on germination induction, especially no suppressive effect. Nevertheless, global germination stimulation was similarly reduced after soil sterilization for both CS and SS soils (p value = 0.51) compared to their respective native soils, with AUC value of 1.03 ± 0.40 for CS (p value = 3.25×10^{-5}) and for the suppressive soil with AUC value of 1.63 ± 0.92 for

SS (p value = $1.03E^{-3}$) (Fig.S7). This suggested a promoting effect from both soil native microbiota on broomrape seed germination.

Rhizosphere exudates from plants cultivated on CN and SN soils similarly stimulate broomrape seed prehaustorium formation

Eluted fractions from rhizosphere exudates were tested this time on germinated broomrape seeds *in vitro*, at 10^2 -fold (Fig.S8) and 10^3 -fold dilutions (Fig.7).

As with germination assays, soil type similarly affected prehaustorium formation, at both dilutions. Ratios of prehaustoria induced by eluted fractions from CN or SN soils were similar for all tested dilutions. As a result, analyses were only displayed at the 10^3 -fold dilution for consistency with germination assays. Contrary to germination induction, soil lixivate (growth medium collected before rapeseed installation) initially contained HIFs. Prehaustorium formation ratios at week 0 were low but significantly different from the negative control with relative ratios of 0.21 ± 0.06 (mean $\pm$ se; p value = 0.003) for CN soil and 0.16 ± 0.03 (p value = 0.02) for SN soil. Comparisons of the AUC of relative prehaustorium formation of the whole 7-week-kinetics showed no differences between the two soils (Fig.7; boxplots) with 4.73 ± 0.21 (median $\pm$ sd) and 4.87 ± 0.17 relative prehaustorium formation ratio.week⁻¹ respectively for CN and SN (adjusted p value = 1.00).

Eluted fractions from sterilized CS and SS soils were also tested on broomrape prehaustorium formation at the 10^3 -fold dilution (Fig.S9). Global comparisons showed that soil sterilization had no effect on prehaustorium formation. In comparisons with the respective native soils, AUC values were of 5.17 ± 0.59 for CS (p value = 0.23) and of 5.08 ± 0.28 for SS (p value = 0.15). Besides, soil type had no effect on prehaustorium formation as CS and SS soils had similar AUC values (p value = 0.45). As with germination, this suggested a similar effect from both soil native microbiota on broomrape seed prehaustorium formation.

Differential effect of CN and SN soils on post-attachment parasitic development

Rapeseed roots infestation levels by broomrape were contrasted between the two CN and SN soils, pointing towards a suppressive effect in SN soil (Fig.8, A). From week 2 to week 7 after sowing, there were fewer broomrape attachments on SN soil roots than on CN soil roots (p values: < 0.0001 week 2; 0.0024 week 5; < 0.0001 week 7). At the end of the experiment, attachments were 3.0 ± 1.1 times less in SN soil (p value = $9.93E^{-13}$), which only had 7.0 ± 8.4 (median $\pm$ sd) attachments, compared to 21.0 ± 9.5 attachments on CN soil roots. In addition, parasitic stages were less advanced on SN soil roots (Fig.8, B). Except for the second week, when only young attachments were observed (p value = 1.00), there were higher proportions of young tubercles compared to older stages (spider or bud tubercles) on SN soil roots than on CN soil roots (p value = $8.68E^{-6}$). In week 5 and 7 post sowing, young stages accounted for $62.3 \pm 5.1\%$ (mean $\pm$ se) and then $22.2 \pm 6.7\%$ of total tubercles in SN soil, whereas in CN soil, young stages accounted for only $36.5 \pm 11.8\%$ and then $4.1 \pm 8.8\%$ of total tubercles. At seven weeks post sowing,

broomrape attachments decreased to the same levels observed in week 2 (2.0 ± 6.7 – 7.0 ± 8.4 total attachments respectively, p value = 0.86) whereas the number of attachments decreased slightly but not significantly on CN soil roots, between week 5 and week 7 post sowing (33.0 ± 2.9 – 21.0 ± 9.5 total attachments respectively; p value = 0.25). In addition, after 7 weeks post sowing, higher proportions of necrotic tubercles were observed in SN soil roots compared to CN soil roots (p value = 1.19×10^{-10}). Necrotic symptoms were observed on $60.8 \pm 6.1\%$ of total attached tubercles, whereas in CN soil roots, only $29.2 \pm 6.2\%$ of total attached tubercles exhibited necrosis (p value < 0.001).

In Addition to the native soils, co-cultivations were conducted in the respective gamma-sterilized CS and SS soil (Fig.10). Notably, broomrape attachments were strongly delayed from the second week, on both CS and SS soil roots, compared to their respective native (*i.e.* not sterilized) soils (p values < 0.0001). Almost no parasite attachments were found neither on CS soil roots (median= $0.0 \pm \text{sd}= 0.3$) or SS soil roots (0.0 ± 0.7) 2 weeks post sowing. Thereafter, highly variable broomrape attachments were observed in each sterilized soil compared to their native soils in week 5 (p values = 0.87 CN/CS and SN/SS) and 7 (p values = 0.96 for CN/CS and for SN/SS). At the end of the experiment (*i.e.* 7 weeks), parasitic plants from sterilized soils were more developed, with less necrotic symptoms. More specifically, proportions of spider and bud tubercles were higher on SS than SN soil roots (p values < 0.0001 for spider and bud). Proportion of bud tubercles was slightly higher on CS soil roots compared to CN soil roots. In addition, tubercles developed less necrotic symptoms on CS (mean= $10.9 \pm \text{se}= 3.7\%$) and SS soil roots ($28.2 \pm 9.5\%$) compared to the proportions of necrosis found on the roots of CN soil ($29.2 \pm 6.2\%$; p value < 0.0001) and SN soil ($60.8 \pm 6.1\%$; p value < 0.0001).

To account for a potential host growth effect induced by the soil, RDW (dry root matter) and SDW (dry shoot matter) were evaluated during co-cultivation (Fig.S11). Neither CN nor SN soils differentially impacted the host RDW or SDW during the whole co-cultivation. Hence, this result excludes any effect of the soil type on the host plant growth and indirectly on the suppressive effect. However, sterilization had a promoting effect on the host growth, with similar increases of SDW in CS and SS soils, all along co-cultivations compared to respective native soils (Fig.S11 C,D), whereas RDW was slightly increased only in the first two weeks compared to native soils from 0.05 ± 0.05 g (median $\pm$ sd) to 0.07 ± 0.21 g respectively for CN and CS soils (p values < 0.0001), as well as from 0.03 ± 0.06 g to 0.06 ± 0.06 g, respectively for SN and SS soils (p values < 0.0001).

Characterization of soil initial microbial communities

Similar compositions of initial bacterial communities between CN and SN soils

Initial composition of bacterial communities was very similar between CN and SN soils. Briefly, 258,957 16S sequencing reads were retained in initial CN (112,615) and SN (146,342) soils, which were associated to 2,757 ASVs. Of those ASVs, 895 were unique to CN soil, 1442 were unique to SN soil and 420 were shared between the two. In terms of relative abundance, initial soil bacterial communities were mainly composed of families of *Vicinamibacteraceae* ($9.97 \pm 2.61\%$ in CN soil and $11.11 \pm 1.71\%$ in SN

soil), 67-14 from the *Solirubrobacterales* order ($8.62 \pm 1.12\%$ in CN soil and $7.33 \pm 1.65\%$ in SN soil), KD4-96 from the *Chloroflexi* phylum ($4.04 \pm 0.68\%$ in CN soil and $4.41 \pm 0.45\%$ in SN soil) as well as uncultured bacteria (mean = $15.23 \pm \text{sd} = 0.80\%$ in CN soil and $16.57 \pm 1.67\%$ in SN soil) and less than 0.1% abundant bacteria (6.00 ± 0.84 in CN soil and $10.02 \pm 4.03\%$ in SN soil) (Fig. 9, A). Minor differences were found in SN soil, as for the proportions of *Pseudonocardiaceae* ($2.11 \pm 1.26\%$ in CN soil and $8.47 \pm 4.50\%$ in SN soil) and *Streptomycetaceae* ($5.93 \pm 1.84\%$ in CN soil and $1.05 \pm 0.21\%$ in SN soil).

Statistically, the DESeq2 analysis revealed that only 12 ASVs were significantly more abundant in SN soil compared to CN soil, and 12 ASVs were also significantly more abundant in CN soil relatively to SN soil (Fig. 9, B). In SN soil, the 5 most differentially abundant detected ASVs belonged to the *Luteitalea* sp. (*Vicinamibacteraceae* family), *Lechevalieria* sp. (*Pseudonocardiaceae* family), the *Vicinamibacteraceae* family (ASV_12501), uncultured species (ASV_25744; order *Vicinamibacterales*) and *Streptomyces* sp. (*Streptomycetaceae* family). Blastn alignment of ASV_12501 and ASV_25744 DNA sequences on NCBI database was not conclusive to determine further taxonomic assignation. Nevertheless, these ASVs were quite rare in SN soil (33 to 177 reads), which confirms a lack of structural differences between CN and SN soils.

Measures of α -diversity showed that bacterial richness was slightly higher but variable in the SN soil with 447 ± 116.49 ASVs (median $\pm$ sd) compared to 352 ± 35.40 ASVs in the CN soil (p value = 0.01; Table 1). However, this tendency was not significant (*i.e.* p value > 0.05) with either Shannon (5.26 ± 0.08 for CN soil and 5.39 ± 0.28 for SN soil; p value = 0.13) or Evenness indices (0.89 ± 0.01 for CN soil and 0.88 ± 0.02 for SN soil; p value = 0.17).

After soil sterilization, 125,949 reads were retained in the initial sterilized soils, with 68,093 reads in CS soil and 57,856 reads in SS soil. These reads (probably from environmental DNA or dead microbes) were associated to 713 ASVs in CS soil and into 767 ASVs in SS soil (Fig.S12). After rarefaction (threshold of 16,487 reads), bacterial richness was similar between conducive (median = $353.0 \pm \text{sd} = 38.21$ ASVs in CN soil and 374.0 ± 95.7 ASVs in CS soil; p value = 0.90) and suppressive soils (446.5 ± 115.2 ASVs for SN soil and 355.0 ± 50.51 ASVs in SS soil; p value = 0.13). Thus, composition seemed globally similar after sterilization (Fig.S12).

Contrasted compositions of initial fungal communities between CN and SN soils

As opposed to bacteria, initial soil fungal communities in CN and SN soils showed major structural differences. In total, 440,919 reads were retained after sequencing on ITS amplicons in initial CN (206,255 reads) and SN (234,664 reads) soils. These reads were associated to 877 ASVs with 269 ASVs unique to CN soil, 442 unique to SN soil and 166 ASVs shared between the two. Initial soil fungal communities were relatively stable and consistent throughout the three biological repetitions, as shown by the low standard deviations of relative abundance in CN and SN soils (Fig.10, A). In terms of relative abundance, the most abundant genera in CN soil were the *Chrysosporium* (mean = $35.05\% \pm \text{sd} = 6.86\%$), *Microascus* ($19.83 \pm 4.21\%$), *Aspergillus* ($10.51 \pm 2.37\%$), *NA* (not attributed; $6.62 \pm 1.89\%$) and *Botryotrichum* ($5.72 \pm 1.46\%$). In SN soil, the genus *Aspergillus* ($69.53 \pm 6.75\%$) was the most abundant,

followed by genera less than 0.1% abundant ($5.25 \pm 0.74\%$), *Penicillium* ($4.76 \pm 1.76\%$), *Fusarium* ($4.33 \pm 1.53\%$) and *NA* ($3.03 \pm 1.16\%$). Among the *Aspergillus* genus, 37 distinct ASVs were detected: 9 ASVs in CN soil and 28 ASVs in SN soil. The top three species of total *Aspergillus* ASVs in SN soil were ASV_853 ($88.48 \pm 84.89\%$), *A. protuberus* ($2.83 \pm 1.43\%$) and *A. penicillioides* ($2.59 \pm 1.16\%$). In CN soil, the three species were *A. alliaceus* ($66.84 \pm 8.14\%$), ASV_853 (25.77 ± 6.23), and *A. pseudodeflectus* ($3.36 \pm 4.36\%$). ASV_853 was not attributed to a unique *Aspergillus* species after blastn of DNA sequence into NCBI database. Among the *Fusarium* genus, 9 distinct ASVs were detected: 8 ASVs in CN soil and 7 ASVs in SN soil. The top three species of *Fusarium* in SN soil were ASV_2553, ($63.05 \pm 9.51\%$), *F. domesticum* ($27.87 \pm 8.82\%$) and ASV_1542 ($5.16 \pm 1.57\%$). No species assignment was found neither in UNITE or NCBI database. In CN soil, the top three species of *Fusarium* were ASV_2553 ($90.68 \pm 2.23\%$) and two *F. solani* (ASV_2338: $3.50 \pm 2.14\%$; ASV_1744: $3.45 \pm 1.33\%$).

Statistically, the DEseq2 analysis revealed that 69 ASVs were differentially abundant in SN soil compared to 63 in CN soil (Fig.10, B). Of these 69 ASVs abundant in SN soil, the top five were identified as three *Aspergillus* spp. (one *A. caesiellus* and two *A. penicillioides*), *Fusarium domesticum*, and *Wallemia tropicalis*. Other notable species such as *Alternaria* sp., *Talaromyces purpureogenus*, *Mortierella elongate*, *Trichoderma* spp. (species *T. lixii* and unknown), *Fusarium* sp. (ASV_1542) and ASV_2609 from *Ceratocystidaceae* family were also significantly differentially abundant in SN soil, however it was less significant ($\log_2\text{FoldChange} < 10$). Of the 63 ASVs more abundant in CN soil, the top five ASVs were identified as *Chaetomium* sp., *Chrysosporium pilosum*, and three *Microascus atrogriseus*. There was also one *Talaromyces rugulosus*, two *Aspergillus alliaceus*, *A. affinis* and *Mortierella elongate* and *Thielavia basicola* differentially, but differences were also less pronounced ($\log_2\text{FoldChange} > -10$).

Measures of α -diversity showed that fungal richness was slightly lower in the CN soil and ranged from 178.50 ± 15.13 (median $\pm$ sd) ASVs to 222.50 ± 23.84 ASVs respectively in the CN and SN soils (p value = 0.005) (Table 1). However, this tendency was opposite when measured with Shannon and Evenness indices. Both indices were lower in SN soil and very close to CN soil, due to uneven repartition of taxa in SN soil, especially from the genus *Aspergillus*.

Regarding sterilized soils, 253,774 reads were detected in the initial CS (136,047 reads) and SS (117,727 reads) soils. These reads were assigned to 365 ASVs in CS soil and to 64 ASVs in SN soil (Fig.S13). After rarefaction (threshold of 22,865 reads), alpha diversity metrics were similar but highly variable regarding fungal richness in both the conducive (median = $30.00 \pm \text{sd} = 186.65$ ASVs in CS soil and 180.00 ± 15.27 ASVs in CN soil) and the suppressive soils (268.00 ± 129.48 ASVs in SS soil and 221.00 ± 20.31 ASVs in SN soil). Nonetheless, high variable alpha diversity indices between replicates as well as highly variable relative abundance of genera suggests that sterilization still affected fungal structures (Fig.S13).

Microbiota dynamics throughout co-cultivation

Bacterial rhizosphere dynamics throughout the co-cultivation

In total, 2,313,980 bacterial reads were detected during co-cultivation and assigned to 13,628 unique bacterial ASVs. Comparisons of α - and β -diversity during week -1 to week 7 in the soils confirmed that bacterial communities were close in richness as well as in composition, and had similar dynamics during plant parasitism. After one week of acclimation in the growth chamber and soil watering, bacterial richness became even more similar between CN and SN soils. This effect lasted throughout the entire co-cultivation experiment, *i.e.* during week 0 (465.00±90.35 ASVs in CN soil and 454.00±112.17 ASVs in SN soil; p value = 0.81), week 2 (602.00±75.82 ASVs in CN soil and 614.00±92.40 ASV in SN soil; p value = 0.90), week 5 (572.00±93.65 ASVs in CN soil and 576.00±90.06 ASVs in SN soil; p value = 1.00) and week 7 post rapeseed sowing (573.00±84.47 ASVs in CN soil and 629.00±99.28 ASVs in SN soil; p value = 0.05) (Fig. 11, A). Within the watering period, bacterial richness increased in CN soil, from 352.00±35.40 ASVs (median±sd) in week -1, to 465.00±90.35 ASVs in week 0 before sowing (p value = 0.03); While it stayed similar in SN soil with 447.00±116.49 ASVs in week -1 to 454.00±112.17 ASVs in week 0 before sowing (p value = 0.94). In addition, bacterial composition never significantly differed between the two soils and throughout the co-cultivation, since confidence ellipses of diversity measures overlapped (95% confidence; Fig.11, B1). Instead, bacterial composition was rather affected by the duration of co-cultivation ($R^2 = 0.32$; Fig.11, B2), especially between week 0 and week 2 following rapeseed sowing. This resulted in an increase of bacterial richness of 29.46% in CN soil (p value = 0.01) and of 35.24% in SN soil (p value = 0.04), along with significant shift in bacterial composition. Relative abundances of bacterial families during co-cultivation are presented in supplementary materials (Fig.S14).

In sterilized soils, rarefaction of data was done at the minimum library size of native samples (16,487 reads) due to very few reads, leading to removal of 21 samples in week 0, 2, 5 and. Nevertheless, soil sterilization appeared to have affected bacterial diversity from week 0 to week 7 (Fig.S15). Bacterial richness was initially similar between the conducive soils (p value = 0.90) and the suppressive soils (p value = 0.13), but was thereafter strongly reduced in sterilized soils (Fig.S15, A). This effect was confirmed by a shift of bacterial composition in both CS and SS soils after only one week of co-cultivation (Fig.S15, B2). The effect of soil sterilization ($R^2 = 81.60$) was the more discriminating between the soils, followed by the co-cultivation weeks ($R^2 = 12.42$; Fig.S15, C).

Fungal rhizosphere dynamics throughout the co-cultivation

In total, 7,493,992 fungal reads were detected throughout co-cultivations and were assigned to 2,758 unique fungal ASVs. Comparisons of α - and β -diversity during week -1 to week 7 in the soils confirmed initial differences between fungal communities of CN and SN soils, while revealing important shifts in fungal community i) before sowing, during the initial regular watering of the soil (week-1 to week 0) and ii) after rapeseed seed sowing, during host plant growth (week 2 post sowing) (Fig. 12).

Initial watering homogenized the fungal richness and composition between the two soils. First, the resulting richness in week 0 was no longer significantly different between the two soils which harbored in week 0 209.00±59.68 ASVs in CN soil and 208.5±54.52 ASVs in SN soil (p value = 0.58; Fig. 12, A). Second, confidence ellipses of diversity measures were significantly different at week -1 but thereafter

overlapped at week 0 (95% confidence; Fig.12, B1). Contrary to bacteria, the introduction of host plant *Brassica napus* at week 0 induced a sharp decrease in fungal richness in both soils, plus a significant shift in their composition. Fungal richness decreased of 71.77% and 53.64% respectively in CN and SN soils between week 0 and week 2, and remained similar throughout the co-cultivations. For instance, in week 7, 68.00 ± 29.49 ASVs were detected in CN soil, and 48.00 ± 21.46 ASVs in SN soil (p value = 0.23) post sowing (Fig. 12, A). Fungal compositions in CN and SN soils were mostly affected by rapeseed sowing (week 2; Fig.12, B2). These compositional changes were linked with the increased and high prevalence of one ASV, *Olpidium brassicae* (ASV 3,371; Fig.S16, A) which represented more than 90% of total ASVs from week 2 to 7 of co-cultivations in both soils. At the end of the experiment, ASV 3,371 represented $87.62 \pm 11.1\%$ (mean $\pm$ sd) and $97.17 \pm 0.78\%$ of total taxa respectively in CN and SN soils. As for other taxa, families *Chaetomiaceae*, *Sordariales*, *Ceratocystidaceae*, *Nectriaceae* and *Microasaceae* were the 5 most abundant in CN soil (all weeks considered) and families *Nectriaceae*, *Aspergillaceae*, *Mortierellaceae*, unidentified, *Chaetomiaceae* and *Ceratocystidaceae* were the 5 most abundant in SN soil (Fig.S16, B).

Contrary to bacteria, soil sterilization only affected fungal richness (but not composition), which was reduced in sterilized soils compared to native soils, from week 2 and until week 7 (Fig.S17, A). This time, soil watering did not change fungal richness in either CS (30.00 ± 187.23 ASVs in week -1 and 137.00 ± 96.93 ASVs in week 0; p value = 0.52) nor SS (271.00 ± 129.88 ASVs in week -1 and p value = 0.37) soils. However, rapeseed sowing again induced a loss of 81.16% (median) or 83.48% of fungal richness, respectively for CS and SS soils in week 2. In addition, fungal composition in sterilized soils remained similar to that of native soils (95% confidence ellipses overlapped; Fig.S17, B2). Composition was mostly affected by co-cultivation weeks ($R^2 = 43.75$) rather than soil sterilization ($R^2 = 18.91$).

Research for groups of soil microorganisms statistically associated with plant parasitic suppression

For fungi, correlation network analysis (WGCNA) enabled us to find ten clusters of co-abundant ASVs, highly correlated to one another (close distance metrics; Fig 13, A). Of those 10 clusters, the 3 cluster Turquoise, Pink, and Blue were correlated to suppressive parasitic traits (p value <0.05; Fig 13, B). More precisely, the Turquoise and Pink clusters were positively correlated to ratio of necrotic symptoms (Turquoise: $R^2 = 0.29$; p value = 0.04 and Pink: $R^2 = 0.29$; p value = 0.04), while the Blue cluster was negatively correlated with parasite attachments ($R^2 = -0.3$; p value = 0.03). In addition, the Turquoise cluster was negatively correlated to stimulation of prehaustorium formation ($R^2 = -0.45$; p value = 0.0006) and positively correlated to the proportion of stem tubercle ($R^2 = 0.42$; p value = 0.0009). The Blue cluster contained 71 total ASVs including 57 ASVs unique to SN soil. The pink cluster contained 37 total ASVs including 31 ASVs unique to SN soil. There was no ASV unique to CN soil in the Blue and Pink clusters. In contrary, in the Turquoise cluster, there were 83 total ASVs including 57 ASVs unique to CN (Fig. 13, C). Cluster composition was described in supplementary materials (Fig.S18). Several clusters appeared positively correlated to ratio of broomrape attachment stages; however, correlations were not significant (p value > 0.05).

To propose individual microbes, or groups of microbes, associated with plant parasitism suppression, a decisive analysis was performed within the clustered ASVs. ASVs were considered of interest when they were i) initially overabundant in SN soil (DESeq2), ii) individually correlated to suppressive parasitic traits (*i.e.* total attachment and necrotic symptom ratio) and iii) present in one of the three clusters (WGCNA; Table 2). Following this strategy, eight fungal candidates were detected. The six ASVs *Holtermanniella takashimae* (ASV_756), ASV from *Nectriaceae* family (ASV_951), *Monocillium mucidum* (ASV_2460), *Fusarium domesticum* (ASV_2920), *Tetracladium* sp. (ASV_2949) and ASV from *Bartaliniaceae* family (ASV_3836), were differentially abundant in SN soil, found in the Blue cluster and negatively correlated to broomrape attachments. Additionally, one ASV_3513 (*Pyrenochaeta terrestris* or *P. lycopersici* according to NCBI database; percent identity = 100.00%) was differentially abundant in SN soil, found in Pink cluster and negatively correlated to broomrape attachments (instead of necrotic ratio as initially expected). Finally, ASV 2609 from *Ceratocystidaceae* family (*Berkeleyomyces basicola* or *B. rouxiae*, *syn. Thielaviopsis basicola* according to NCBI data base; percent identity = 100%) from the Turquoise cluster, was the only ASVs positively correlated (Person's correlation coefficient = 0.32; *p* value = 0.02) to the ratio of necrotic symptoms and was also differentially abundant in SN soil.

With respect to bacteria, given the similarities in diversity and composition between CN and SN soils and the scarcity of differentially abundant ASV, no correlative approach was conducted. Nevertheless, among the 24 ASVs differentially abundant in CN or SN soil, only 4 ASVs were positively correlated to parasitic attachment or negatively to necrotic tubercles (Table.S4). Thus, correlations were reversed relatively to the suppressive effect and were not further investigated for this study.

Discussion

Disease-suppressive soils have been well-characterized against many soil-borne bacteria, fungi, oomycetes, nematodes [64–67]. It was not until very recently that suppressive soils have been considered for parasitic plants, on *Striga hermonthica* [68]. Our objectives were to extend this knowledge to the case of rapeseed parasitism by *Phelipanche ramosa* and to disentangle the underlying suppressive mechanisms.

Soil microbiota reduces overall branched broomrape installation

The differential infestation success that was originally observed in the two neighboring rapeseed fields was verified in the lab with three independent co-cultivations of broomrape and rapeseed in the soils of interest. Throughout co-cultivation, rapeseed plants were less parasitized when grown in the suppressive soil rather than the conducive soil. Ultimately, parasitic development was delayed in the suppressive soil, and the established tubercles were subject to higher necrotic symptoms, suggesting lethal physiological damages to the parasitic plant.

A large number of studies have demonstrated that disease-suppressive soils owe their activity to their microbial properties [39, 69, 70]. To show that suppression was of microbial origin, our first hypothesis

was that within fields subjected to similar pedoclimatic conditions and agronomic conditions (Terres Inovia personal communication), variation in microbiota could explain a substantial part of the different behaviors toward parasitism. Therefore, and similarly to studies on disease-suppressive soils [67, 71], our strategy was to compare the structure of both bacterial and fungal communities in the conducive and suppressive soils. In the two initial soils, bacterial communities were reported to be substantially similar in terms of diversity and composition. Fungal communities on the other hand, were more contrasted. Notable differences between the soils were the very high proportion of *Aspergillus* spp. (70% in SN soil vs. 10% in CN soil) and the 69 ASVs that were more abundant in the suppressive soil.

With a second strategy, we aimed to compare parasitic infestation between native and sterilized soils. However, in both suppressive and conducive soils, sterilization lead to very variable and thus inconclusive patterns of parasitism. Microbial composition and diversity analyses also revealed that microbial recolonization of sterilized soils proceeded rapidly, and varied hugely from sample to sample, especially for fungal communities. Besides, soil sterilization resulted in greater shoot growth in rapeseed plants from both suppressive and conducive soils. This is consistent with many studies, showing that soil sterilization often promotes plant growth, by eliminating plant pathogens, reducing competition for nutrients, or promoting plant-beneficial microorganisms [72, 73]. We thus suspect that the modification of microbial communities caused by sterilization could have altered the plant - parasitic plant interaction. However, such interactions are still poorly investigated on plant parasitism.

Soil microbiota induces broomrape pre-attachment stages

Independent of subsequent suppression of parasitism, germination-inducing activities were detected in both soil extracts and were associated with the presence of microorganisms capable of degrading exogenous glucosinolates into GSs. Furthermore, during co-cultivation, the overall germination activity of rhizosphere exudates from both soils was reduced after soil sterilization. Thus, altogether those results demonstrate the contribution of native soil microorganisms in signaling broomrape seed germination in association with the host plant metabolites, which is consistent with our previous work [14]. Using a different rapeseed soil, we previously demonstrated that soil microbiota and isolated strains promoted germination of broomrape seeds either supplied with glucosinolates or with rapeseed roots thanks to microbial myrosinase-like enzymes [14]. Although Auger et al. [15] found that 2-PEITC was the main GS for *P. ramosa*, in the present study similar content of 2-PEITC and erucin were found in the native and sterilized soils, both underneath the detection threshold of measuring instruments.

In addition, activities of prehaustorium formation-induction were detected in both the conducive and suppressive soil extracts, suggesting initial release of HIFs, possibly cytokinins (only known HIFs of *P. ramosa* [48]). Their microbial origin is supported by the decrease or loss of activity after sterilization by filtration of soil extracts, as demonstrated in our previous work [14]. This initial signal partly explains the residual HIF activity found in the growth medium collected after one-week recirculation in native and

sterilized soils. Nonetheless, this microbial contribution is likely to be minor compared to the host plant's contribution, as seen after 2 weeks of co-cultivation.

Finally, broomrapes attached more rapidly in the native conducive and suppressive than in the two sterilized soils, providing further support for the microbial contribution. Although, it remains to be tested, we suggest that the germination delay also resulted in a delay in parasitic attachment in the onset of parasitism, since prehaustorium stage was not impacted by sterilization.

Soil microbiota only suppresses broomrape post-attachment stages

Because both CN and SN soils contributed positively to parasitic pre-attachment stages and also did not inhibit broomrape germination nor haustorium formation, we suggest that the overall alleviation of parasitism reflects post-attachment resistance mechanisms mediated by the soil microbiota.

Most post-attachment resistance resembles typical plant defense mechanisms against pathogenic microorganisms. They mainly involve i) production of toxic chemical (*e.g.* phytoalexins), ii) formation of physical barriers by thickening the cell-wall to prevent penetration (*e.g.* accumulation of phenolic compounds, lignification, suberisation, callose depositions), iii) activation of enzymatic activities (*e.g.* peroxidases, polyphenoloxidases, lipoxygenases) or induction of cell death to limit attachment, and iv) blocking of vascular continuity to delay parasitic development and in some cases cause death [74–76]. Several studies support that host plant defense mechanisms against broomrape species could be induced by soil bacteria such as plant growth promoting rhizobacteria [77–81]. For instance, in addition to HIF degradation, Kawa et al. [68] showed that soil microbiota induced changes in the cellular anatomy of sorghum roots, toward a more prominent aerenchyma thus limiting chemical signaling and a suberized endodermis limiting *Striga* penetration. As for fungi, they seem to rather directly attack and colonize post-attachment stages of broomrape species [25, 27, 82, 83].

Although we cannot properly describe the mechanisms occurring in the suppressive soil, established broomrape tubercles developing necrosis is an additional evidence that microbe-mediated resistance takes place after penetration and vascular connection, *i.e.* during tubercle development, and may include direct and indirect mechanisms. Since soil-sterilization reduced the incidence of necrotic tubercles in both CN and SN soils, we propose at least one microbial origin for necrosis. However, it is unclear whether necrosis is the result of a direct biotrophic or necrotrophic pathogenic infection or whether the consequence of saprotrophic colonization following parasitic plant attachment failure.

Rhizosphere microbial dynamics associated with suppression of plant parasitism

The description of microbial community dynamics during parasitism allowed us to conjecture possible scenarii of microbial suppression in the soil.

From the onset of parasitism, bacterial and fungal richness converged in SN and CN soils, fungal communities were no longer different, while parasitism remained contrasted. In addition, fungal richness exhibited a sharp decrease in the number of observed ASVs. This effect is related to the high incidence of *Olpidium brassicae*, which might have been present in the soil or in rapeseed seeds, and then selected in the rhizosphere. However, this taxon was expected in the samples, as several authors found that *Olpidium brassicae* is extremely abundant in rapeseed rhizosphere, and constitutes the only species of rapeseed core microbiome, *i.e.* fungi always associated with their host [84–86]. Nevertheless, the incidence of *O. brassicae* was similar in the rhizosphere of the conducive and suppressive studied soils and is therefore not associated to parasitism suppression. Instead, this may indicate that suppression of parasitism operates independently of the dynamics of the overall community, and may result from specific fungal groups.

Indeed, there are two types of disease-suppressive soils, depending on whether it relies on collective competitive and antagonistic activities from the whole microbial community (general suppression) or on selected individuals or group of microorganisms acting specifically against a pathogen (specific suppression) [39, 87, 88]. Soil suppression usually comes from a combination of general and specific suppression [89]. Therefore, we suggest that the overall initial bacterial and fungal communities also participate in the immunity of the soil, so-called general suppression, and should not be overlooked [90]. Indeed, high community diversity is sometimes associated with disease suppression [65, 71, 91], which is consistent with the higher bacterial and fungal richness found in the suppressive soil. However, relationship between diversity and suppression varies according to context and community functioning, as in some cases greater species richness might also enhance trophic competition and disadvantages suppressive communities [66, 92]. In addition, general effect might be secondary compared to specific suppression, as microbial richness was not as high as other studies based on 16S or ITS sequencing, especially fungal richness [65, 67, 71, 93].

According to Weller et al. [39], specific suppression is often induced after severe pathogen outbreaks, during long-term monocultures [94, 95]. Possibly, such a phenomenon happened in the present suppressive study soil, since parasitism reduction was first observed after repeated and shortened rotations of rapeseed (2-years), infested with broomrapes, as opposed to the conducive neighbor field, cultivated more heterogeneously (personal communication). Specific suppression is usually confirmed by its elimination (pasteurization or biocides) and especially by the transfer of 1-10% of the suppressive soil into a conducive soil, which is sufficient for causal populations to establish and restore suppression [65, 96]. While we did not test the transferability of soil suppression due to lack of material, we suggest that repeated cultivation of rapeseed eventually led to assemblages of both stable communities, enriched in specific fungal consortia targeting broomrape [97].

Detailed descriptions of the microbial populations were not sufficient to clearly identify the causal agents of parasitism suppression. Nevertheless, the following strategies aim to propose key groups potentially involved in general and specific suppression in the study soil.

Characterization of key groups involved in general parasitism suppression

As general microbial-mediated suppression usually induces host plant resistance and does not target specific pathogen traits [88], our functional approach was not adapted to detect the causal organisms. Instead, more appropriate approaches propose to assess the effect of specific community disruption on pathogen incidence, which can be conducted by selective sterilization [98] or by diluting soil diversity [99]. Nevertheless, we suggest that microorganisms that were more abundant in the initial SN soil relatively to the CN soil, could exert a positive influence on overall soil immunity, and proposed bacterial and fungal families.

For bacterial ASVs that were more present in the suppressive soil, three ASVs of the order *Vicinamibacterales*, two of which belong to *Vicinamibacteraceae* family (genus *Luteitalea* and unknown), one *Lechevalieria* sp. (*Pseudonocardiaceae* family) and one *Streptomyces* sp. (*Streptomycetaceae* family), are potentially involved the general broomrape suppression. However, other than Xu et al. [100], who showed that the proportion of the *Vicinamibacteraceae* was increased during incorporation of organic mulch and suppression of litchi downy blight, no other studies have reported this family, nor the genus *Luteitalea*, as involved in soil disease suppression. This is probably due to the recent classification of this family [101]. Conversely, genera *Lechevalieria* and *Streptomyces* are both from *Actinomycetes* class [102, 103]. This gram-positive filamentous group of bacteria are ubiquitous in the rhizosphere and are considered as PGPRs and antagonists of many pathogens, mainly due to their ability to secrete numerous bioactive compounds and plant hormones [104–106]. In addition, *Streptomyces* species have been shown to suppress germination, radicle elongation and late development of *Phelipanche aegyptiaca* for *S. pactum* [80] or *O. cumana* for *S. enissocaesilis* [79] and *S. rochei* D74 [107].

For fungal ASVs that were differentially abundant in the suppressive soil, the three ASVs from genus *Aspergillus* (*A. caesiellus* and two *A. penicillioides*) as well as species *Fusarium domesticum* and *Wallemia topicalis*, are potentially involved in the general broomrape suppression. Indeed, genus *Aspergillus* represented around 70% of total genera in SN soil compared to 11% in CN soil. These results are consistent with the fact that *Aspergillus* spp. are common soil filamentous fungi, often considered beneficial to plants in many ways, especially through auxin phytohormones and antimicrobial metabolites production or P-solubilization capabilities [108, 109]. In addition, an *Aspergillus* species, *A. alliaceus*, has also been proposed as a biocontrol agent against broomrapes [29]. As for the other ASVs, *Fusarium domesticum* has been identified as a foodborne rather than a soilborne fungus [110]. Similarly, the genus *Wallemia* is known to comprise xerophilic and extremophilic species, mostly associated with food spoilage [111, 112]. Therefore, their contribution to plant immunity should be interpreted with caution.

Description of key groups involved in specific parasitism suppression

As for specific microbial-mediated suppression, our decisive approach was based on the description of correlations between ASV abundance and reduction of parasitism. We suggest that clustered ASVs that were both differentially abundant in the SN soil and correlated with parasitic suppression may be potential candidates for biocontrol. Given the resemblances between bacterial community in CN and SN initial soils and absence of relevant correlation with parasitic traits, it is very likely that bacterial species are not the main players in this specific suppression. Results from parasitic plant phenotype in soil and WGCNA on fungal abundance (Blue, Turquoise and Pink clusters) both suggested that suppression specifically targeted the number of attached and necrotic tubercles.

Following this strategy, seven ASVs were proposed as inversely correlated to broomrape attachment success. Among these ASVs, there were 3 members of the *Hypocreales* order (*Monocillium mucidum*, *Fusarium domesticum* and one species from *Nectriaceae* family), while the other four ASVs belonged to *Bartaliniaceae* family, *Tetracladium* sp., *Holtermanniella takashimae* and *Pleosporales* order. Interestingly, the order *Hypocreales* is known to be the richest source of biocontrol fungi. It contains many families with potent plant pathogens such as *Fusarium* spp. (family *Nectriaceae*). Besides, it also contains plant growth promoting fungi such as *Trichoderma* spp. (family *Hypocreaceae*), of which species *T. harzianum* have been reported to effectively inhibit germination of *P. ramosa* [113], or plant symbionts [114]. Similarly, *Bartaliniaceae* family (syn. *Sporocadaceae*) contains pestalotioid fungi, also known as coelomycetous fungi. These pestalotioid fungi are important plant pathogens [115–117], but their pathogenicity on broomrape is not yet characterized. For the other fungal candidates, no information was available on possible suppressive properties [118] or broomrape pathogenicity.

As for tubercle development, one ASV was positively correlated to the induction of necrosis, and was remarkably described as a necrotrophic fungus. Indeed, this ASV belongs to the genus *Berkeleyomyces* either *B. basicola* or *B. rouxiae* (syn. *Thielaviopsis basicola*) of *Ceratocystidaceae* family, known as serious plant pathogens responsible for black root rot in several crops. [119, 120]. Upon tissue penetration and colonization, the species *B. basicola* enters a necrotrophic phase during which it lyses and consumes plant root cells [121]. We thus suspect that this species could also be pathogenic to broomrapes. Given the candidate we propose, pathogenic assays on will be necessary to conduct on rapeseed and on potential successive crops.

In all cases, metabarcoding analyses are increasingly valuable to monitor biodiversity and assess potential shifts in diverse environmental samples, without requiring steps of cultivation and taxonomic identification in the lab [122]. Nevertheless, they are strictly descriptive and still carry limits (e.g. low resolution for species assignment, lack of representative DNA sequence in reference databases, rDNA gene copy numbers, DNA extraction or PCR amplification [123, 124]). Furthermore, pathogenicity is not always correlated with the abundance of the corresponding pathogen, thus searching for active microbial genes in suppressive soils should be more informative [125, 126].

Conclusions

Validation of parasitism soil suppression and characterization of microbial dynamics associated with the phenomenon constitute original and relevant results, both from a fundamental and applied point of view. We suggest that initial diversity of the microbial community as well as presence of specific groups of fungi were probably responsible for the reduction of broomrape root parasitism. Our results suggest that this suppressive effect results from the influence exerted by soil microbiota, that dampens the broomrape post-attachment developmental stages and stimulate broomrape necrosis. Interestingly also, the influence of microbial community does not seem to modulate the earliest stages of broomrape infestation (*i.e.* seed germination and prehaustorium) nor the well documented semiochemical mechanisms involved in these processes (*i.e.* glucosinolate derivatives). Altogether, these findings reveal the importance and the complexity of tri-partite interactions, especially in a system where host and bio-aggressors are plants which share the same rhizosphere. Furthermore, these results improve the already well-documented literature on microbial-mediated defense mechanisms against broomrape. The comprehension of naturally occurring suppressive soils arises a number of perspectives for crop pest control; as for instance the formulation of specific consortia of broomrape antagonists or targeted agronomic practices for maintaining or transferring parasitism soil suppression or detection techniques based on microbial patterns associated to soil suppression.

Declarations

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Authors' 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 and Lucie Poulin. UPLC-MS and GC-MS analyses were conducted by Nathalie Marnet and Solenne Berardocco under the supervision of Antoine Gravot and Alain Bouchereau. Analyses were conducted by Lisa Martinez with the supervision of Lucie Poulin, Jean-Bernard Pouvreau, Gregory Montiel and Philippe Simier. The first draft of the manuscript was written by Lisa Martinez and Lucie

Poulin, Philippe Simier and Christophe Jestin commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Availability of data and material

The datasets generated and/or analyzed during the current study are available in the NCBI repository, <https://dataview.ncbi.nlm.nih.gov/object/PRJNA967171?reviewer=nut35p6c3tmc1bk5kdn66p0n35>

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1
Comparisons of fungal and bacterial richness, α-diversity indices and differentially abundant ASVs between the conducive (CN) and suppressive (SN) soils at initial time.

Community	Soil	Microbial richness ^a	Shannon's index ^b	Eveness index ^c	Number of differentially abundant ASVs ^d
Bacteria	CN	352±35.40 b	5.26±0.08 a	0.89±0.01 a	12
	SN	447±116.49 a	5.39±0.28 a	0.88±0.02 a	12
Fungi	CN	176.50±15.40 b	2.92±0.23 a	0.57±0.04 b	69
	SN	220.00±22.21 a	2.21±0.42 b	0.41±0.07 a	63

Diversity indices are calculated after rarefaction with a threshold of 16,487 reads for bacteria and 22,865 reads for fungi. Medians were calculated from six replicates per soil (CN, SN). Standard deviations of the means are indicated in parentheses. Significant differences among soils are indicated by letters (a,b) after a test.

^a Microbial richness is equal to the number of ASVs in the sample

^b Shannon indice is defined as $H = -\sum p_i \log p_i$ where p_i is the proportional abundance of species i and b is the base of the logarithm

^c Eveness indice is defined as $\text{Shannon}/\log(\text{richness})$

^d Number of differentially abundant ASVs were obtained with DEseq2 by comparing ASVs abundance between CN and SN at initial time

Table 2
Description of fungi potentially involved in the suppression of plant parasitism

ASV differentially abundant in initial SN soil ^a	Taxonomic assignment	Parasitic fixation	Cluster ^b
		Correlation	Ajdusted P-value
ASV_321	<i>Ascomycota</i> phylum	-0.341	0.011
ASV_95	<i>Sclerotiniaceae</i> family	-0.392	0.003
ASV_365	<i>Periconia macrospinos</i>	-0.336	0.013
ASV_476	<i>Aspergillaceae</i> family	-0.338	0.013
ASV_756	<i>Holtermanniella takashimae</i>	-0.374	0.005
ASV_844	<i>Aspergillus penicillioides</i>	-0.277	0.043
ASV_853	<i>Aspergillus</i> sp.	-0.346	0.010
ASV_951	<i>Nectriaceae</i> family	-0.495	0.000
ASV_1166	<i>Wallemia tropicalis</i>	-0.288	0.035
ASV_1193	<i>Darksidea</i> sp.	-0.272	0.046
ASV_1474	<i>Penicillium bilaiae</i>	-0.276	0.044
ASV_1542	<i>Fusarium</i> sp.	-0.373	0.006
ASV_1649	<i>Wallemia tropicalis</i>	-0.311	0.022
ASV_1804	<i>Aspergillus protuberus</i>	-0.296	0.030
ASV_1832	<i>Sordariales</i> order	-0.366	0.007
ASV_2040	NA	-0.365	0.007
ASV_2191	<i>Cercophora</i> sp.	-0.270	0.048
ASV_2450	<i>Filobasidium</i> sp.	-0.282	0.038
ASV_2460	<i>Monocillium mucidum</i>	-0.474	0.000
ASV_2712	<i>Talaromyces purpureogenus</i>	-0.396	0.003
ASV_2920	<i>Fusarium domesticum</i>	-0.312	0.022
ASV_2949	<i>Tetracladium</i> sp.	-0.326	0.016
ASV_2992	<i>Gibberella acuminata</i>	-0.350	0.010
ASV_3498	<i>Tetracladium</i> sp.	-0.414	0.002
ASV_3513	<i>Pleosporales</i> order	-0.302	0.026
ASV_3836	<i>Bartaliniaceae</i> family	-0.425	0.001
		Necrotic symptoms	Cluster
	Taxonomic assignment	Correlation	Ajdusted P-value
ASV_2609	<i>Ceratocystidaceae</i> family	0.321	0.018

^a ASVs differentially abundant in SN soil correspond to ASVs found with DEseq2, which have significant log2foldchange value after comparisons between initial SN and CN soils

^b Clusters Blue, Pink and Turquoise correspond to the three cluster of co-abundant ASVs significantly correlated with parasitic traits total attachments and relative proportion of necrotic symptoms.

Figures

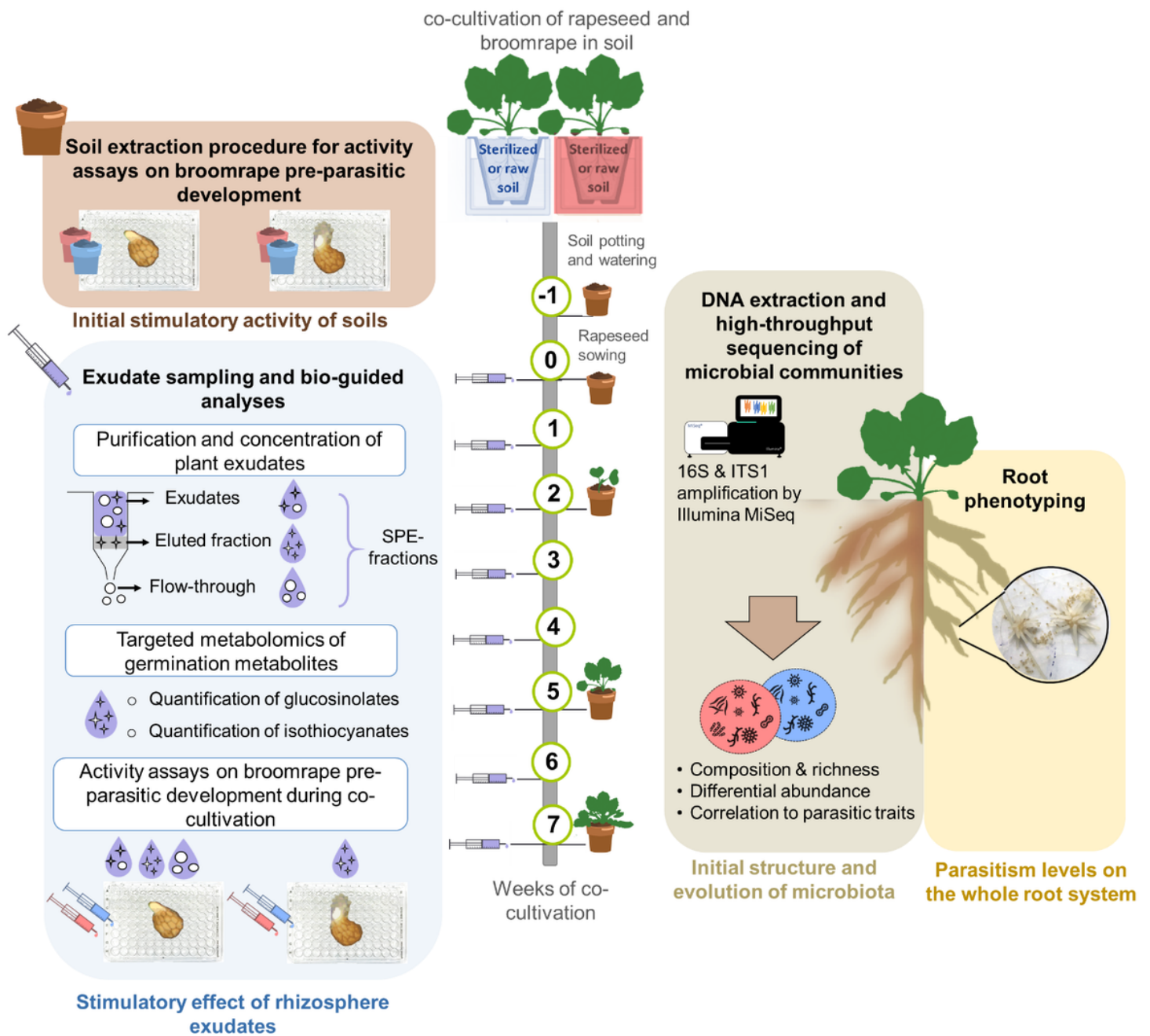


Figure 1

Experimental co-cultivation set-up used to simultaneously compare parasitism in rapeseed at the phenotypic, metabolomic and metagenomic level.

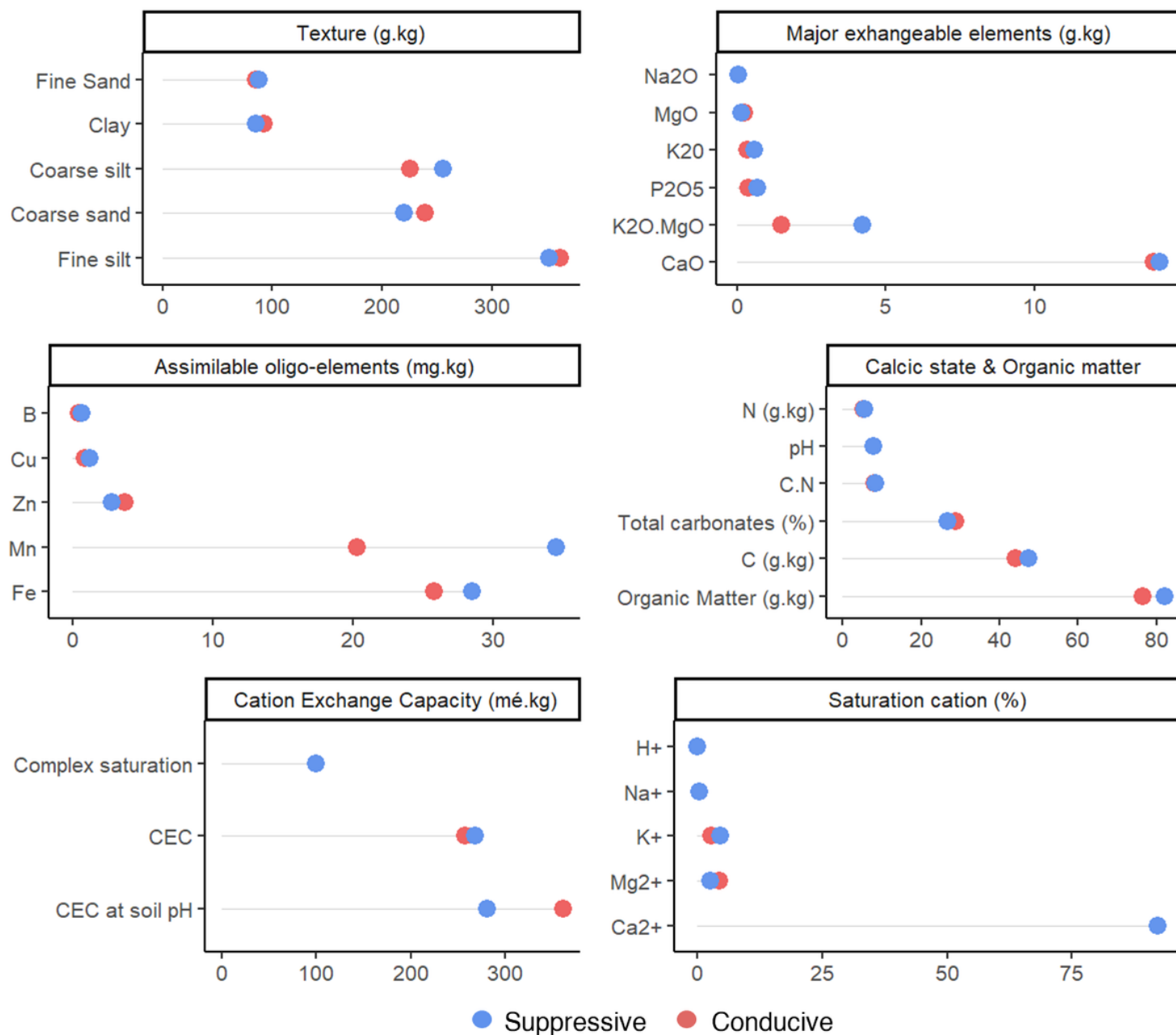


Figure 2

Physicochemical profiles of the conducive and suppressive native soils

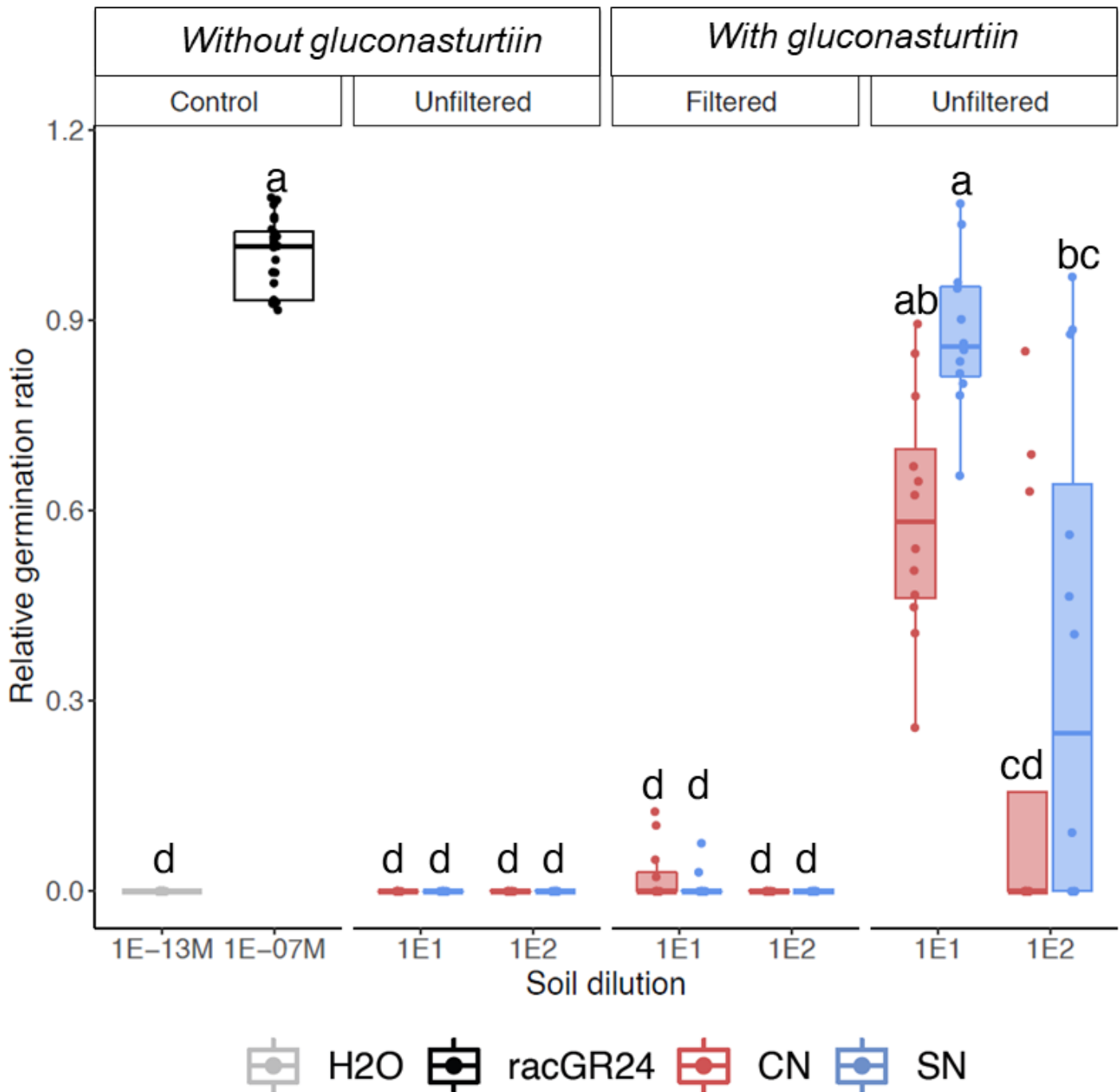


Figure 3

Influence of soil extracts on the stimulatory effect exerted by exogenous gluconasturtiin on broomrape seed germination

Relative germination ratios of broomrape seeds in presence of conducive (CN) or suppressive (SN) soil extracts either unfiltered or 0.22 μm -filtered at 10^1 or 10^2 -fold dilutions and in presence, or not, of gluconasturtiin (10^{-6} M). 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 giving maximum germination ratio of $1 \approx 65.20 \pm 6.32\%$ (median $\pm$ sd). Significance was determined using

a non-parametric Kruskal-Wallis rank test and Dunn's Kruskal-Wallis Multiple Comparisons (p value ≤ 0.05) and is indicated with different letters.

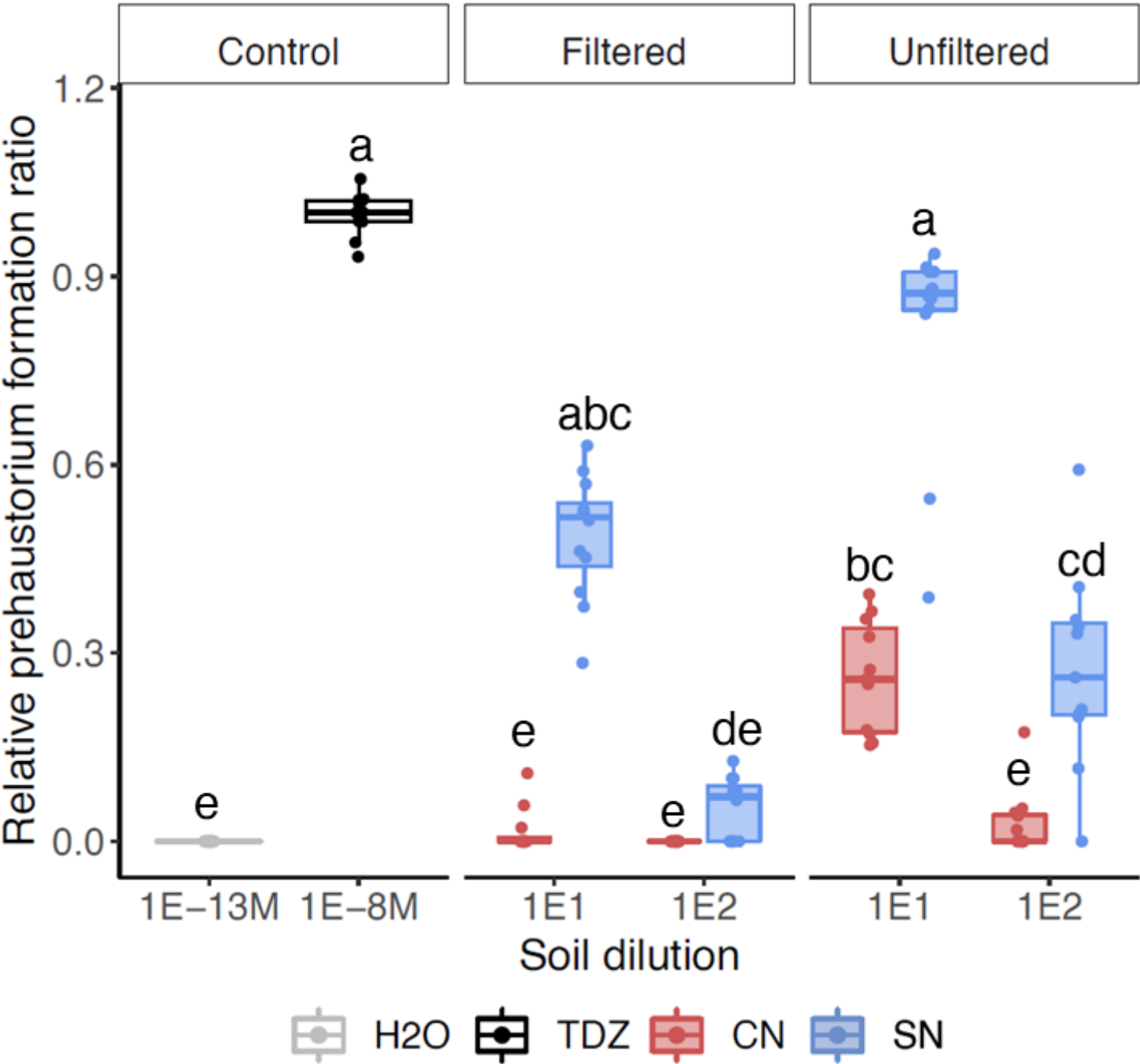


Figure 4

Influence of soil extracts on the stimulation of broomrape seed prehaustorium formation

Relative prehaustorium formation ratio of broomrape seeds in presence of conducive (CN) or suppressive (SN) soil extracts either 0.22 μ m-filtered or unfiltered and at 10^1 or 10^2 dilutions. Ratios are expressed as the number of germinated seeds presenting prehaustoria on total germinated seeds, relatively to the average ratio obtained with the positive control TDZ 10^{-8} M, a synthetic analog of cytokinin, giving

maximum prehaustorium formation ratio of $1 \approx 95.33 \pm 5.33\%$ (median $\pm$ sd). Significance was determined using a non-parametric Kruskal-Wallis rank test and Dunn's Kruskal-Wallis Multiple Comparisons (p value ≤ 0.05) and is indicated with different letters.

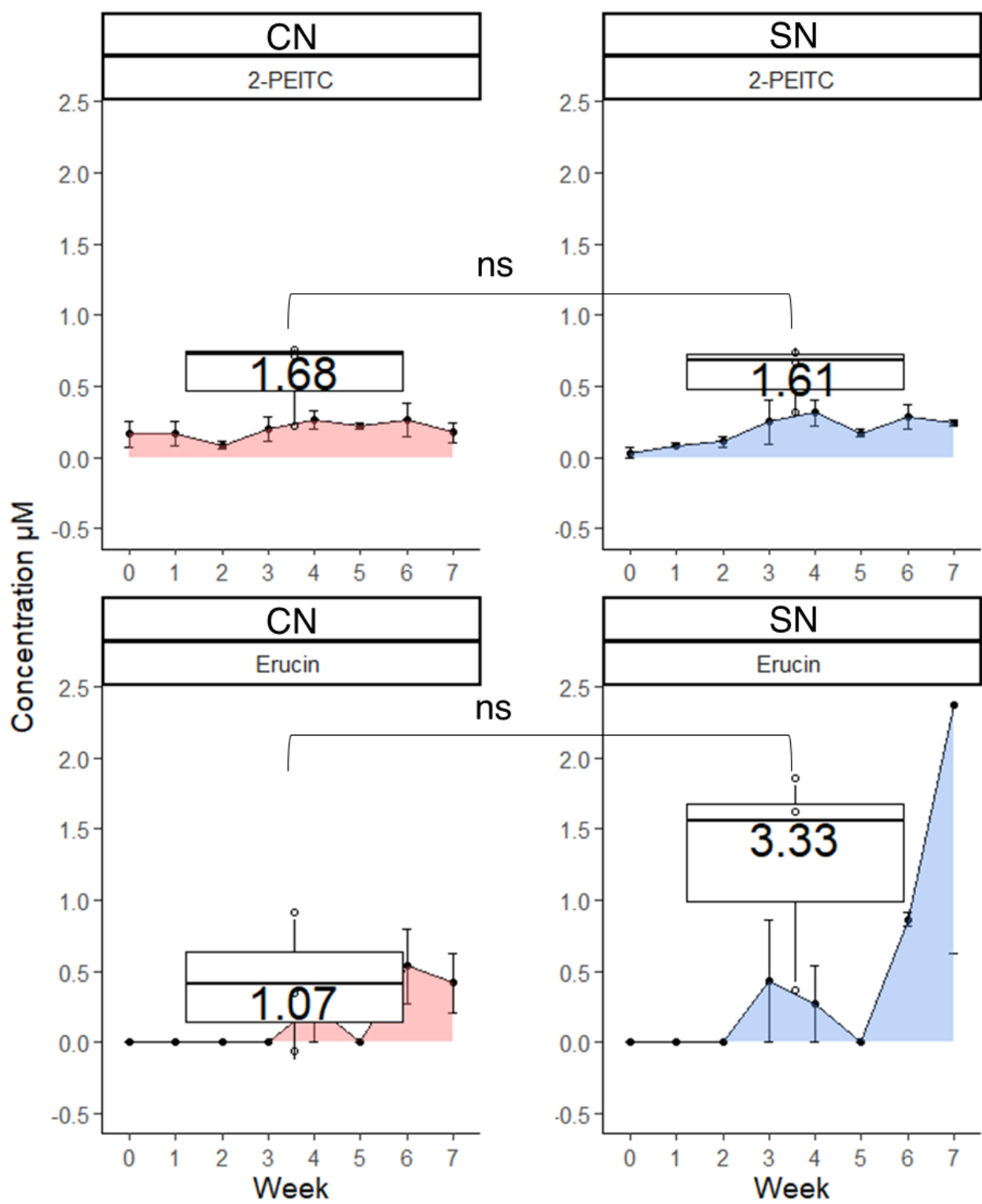


Figure 5

GC-MS bio-guided quantification of the two isothiocyanates detected in the eluted fractions from rhizosphere exudates

Global stimulatory effects were compared through the area under the curve (AUC), measured from the 7-week kinetic (Concentration.week⁻¹). Significance between AUC was determined thanks to a non-parametric Kruskal-Wallis rank test and Dunn's Kruskal-Wallis Multiple Comparisons (ns: non-significant; *p < 0.05; ** p < 0.01; *** p < 0.001). Values displayed are means with standard errors as error bars.

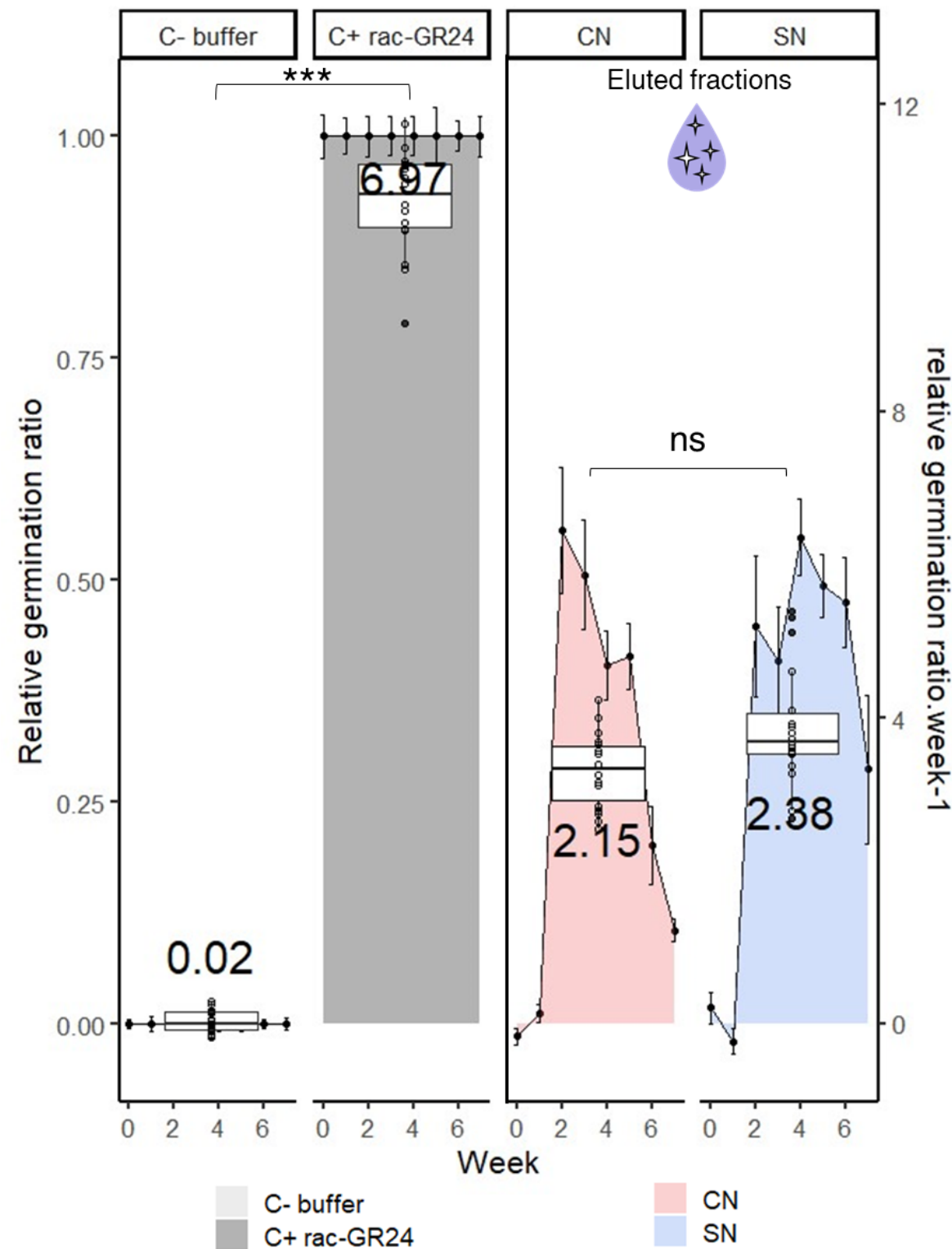


Figure 6

Stimulatory effect of soil type on the germination activity of rapeseed rhizosphere exudates

A Germination kinetics of broomrape seeds treated with positive control rac-GR24 (10^{-7} M), negative control buffer (HEPES 1 mM; Plant preservative mixture 0.1% pH 7.5) and SPE-eluted fraction (10^3 -fold dilution) of rhizosphere exudates from native conducive (CN) and suppressive (SN) soils during 7 weeks of co-cultivation with rapeseed. Relative germination ratios are approached via absorbance ($A_{570-690\text{nm}}$) measured on MTT-colored seeds well. Global stimulatory effects were compared through the area under the curve (AUC), measured from the 7-week kinetic (Relative germination ratio. week^{-1}). Significance between AUC was determined thanks to a non-parametric Kruskal-Wallis rank test and Dunn's Kruskal-Wallis Multiple Comparisons (ns: non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Values displayed are means with standard errors as error bars.

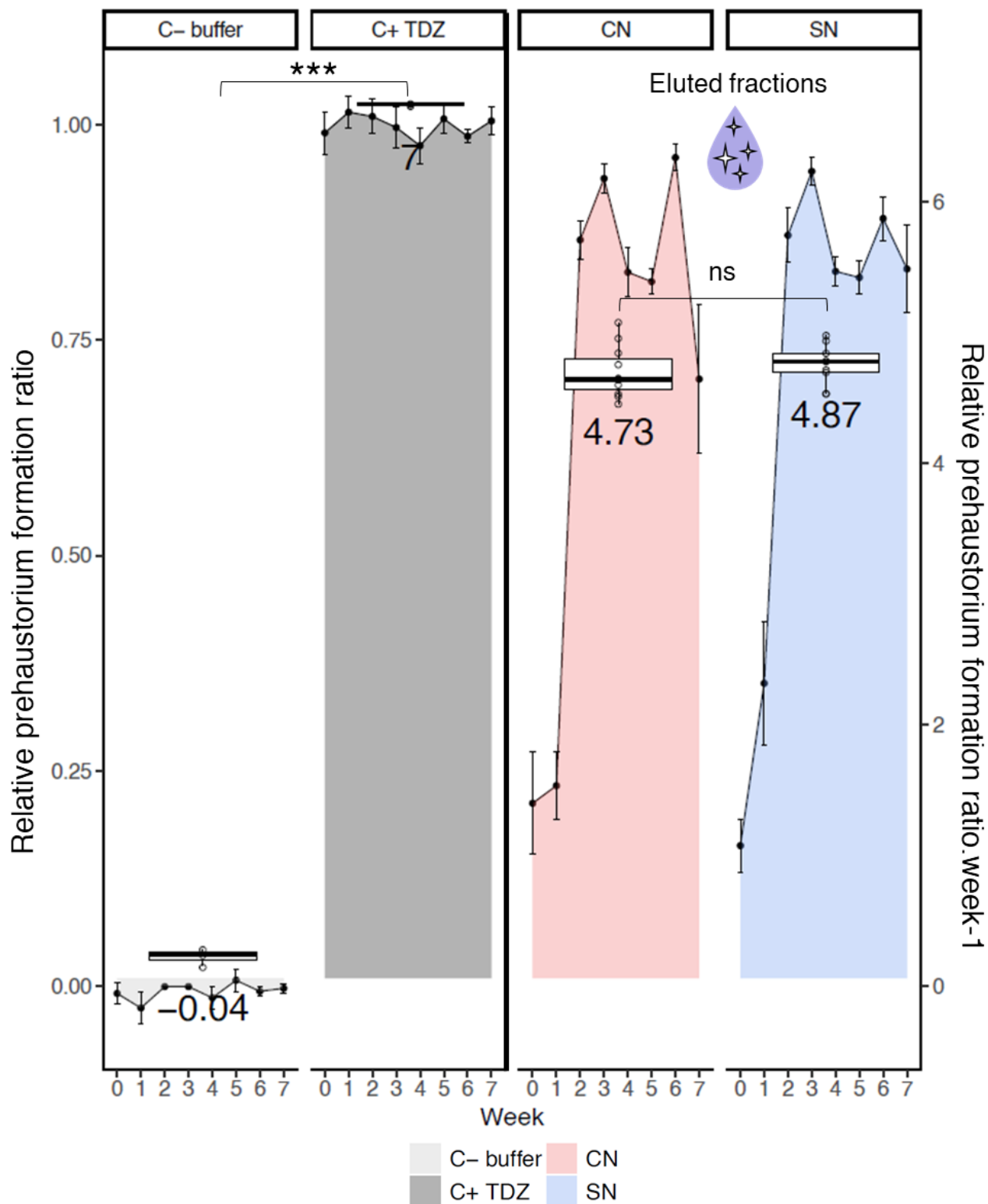


Figure 7

Stimulatory effect of soil type on the prehaustorium formation activity of rapeseed rhizosphere exudates

Prehaustorium formation kinetics of germinated broomrape seeds treated with positive control TDZ (10^{-8} M), negative control buffer (HEPES 1 mM; Plant preservative mixture 0.1% pH 7.5) and SPE-eluted fraction (10^3 -fold dilution) of rhizosphere exudates from native conducive (CN) and suppressive (SN)

soils during 7 weeks of co-cultivation with rapeseed. Prehaustorium formation ratios were measured by counting MTT-colored seeds presenting papillae, and reported as relative ratios according to the minimum and maximum ratios obtained with the controls.

Global stimulatory effects were compared thanks the area under the curve (AUC), measured from the 7-week kinetic (Relative prehaustorium formation ratio.week⁻¹). Significance between AUC was determined thanks to a non-parametric Kruskal-Wallis rank test and Dunn's Kruskal-Wallis Multiple Comparisons (ns: non-significant; *p < 0.05; ** p < 0.01; *** p < 0.001). Values displayed are means with standard errors as error bars.

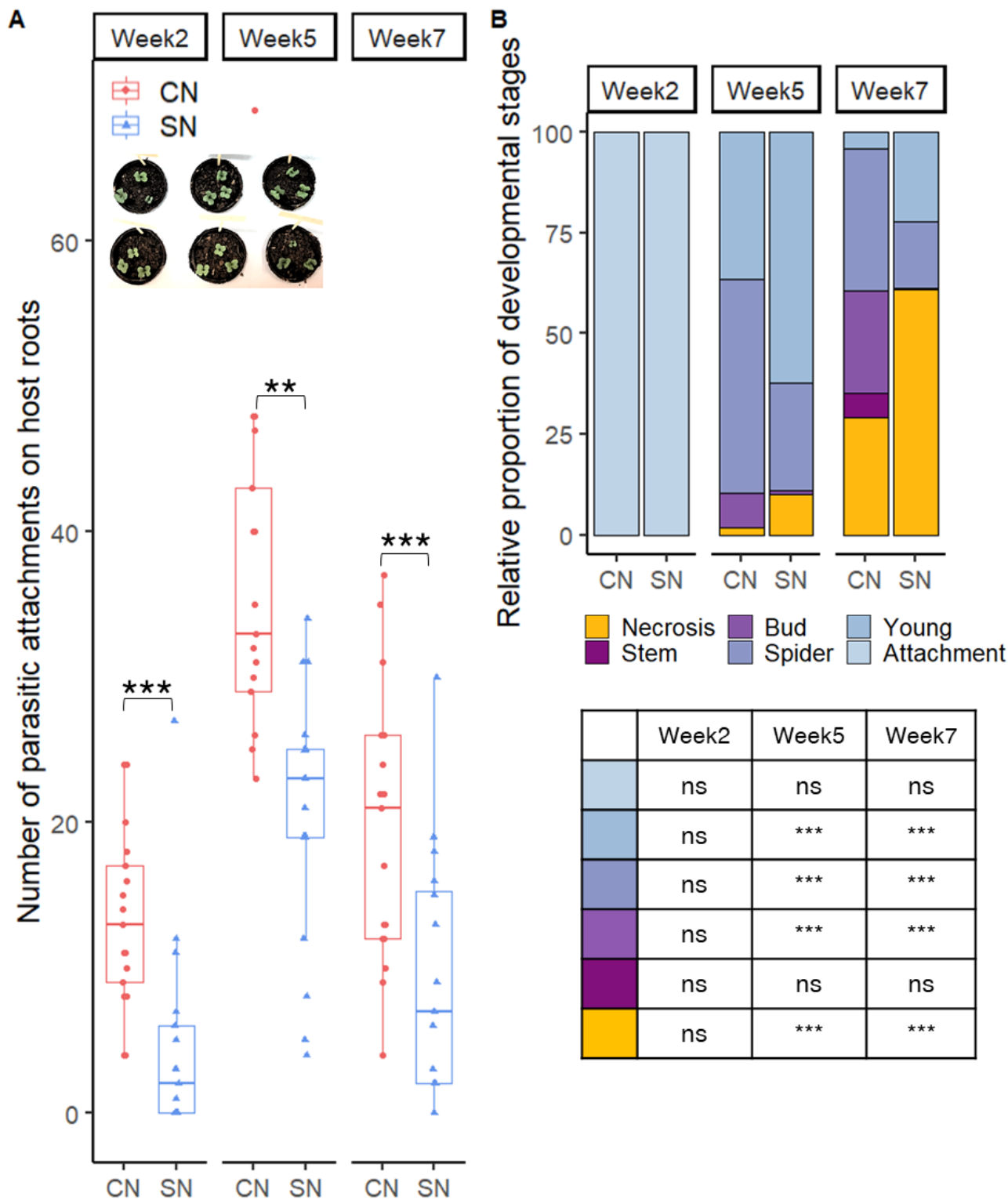


Figure 8

Native soil effects on parasitic plant attachment and development

A Total number of *Phelipanche ramosa* attachments on roots of *Brassica napus* growing in co-cultivations conducted in the native conducive (CN) or suppressive (SN) soils on week 2, 5 or 7 after rapeseed sowing. Significance was determined using a pairwise comparisons on a generalized linear mixed model

according to a Poisson distribution (ns: non-significant; *p < 0.05; ** p < 0.01; *** p < 0.001). **B** Relative proportion of *P. ramosa* developmental stages for the counted attachments. Significance was determined using a pairwise comparisons on generalized linear mixed model via quasi-likelihood for each stage, and are reported in the table (ns: non-significant; *p < 0.05; ** p < 0.01; *** p < 0.001).

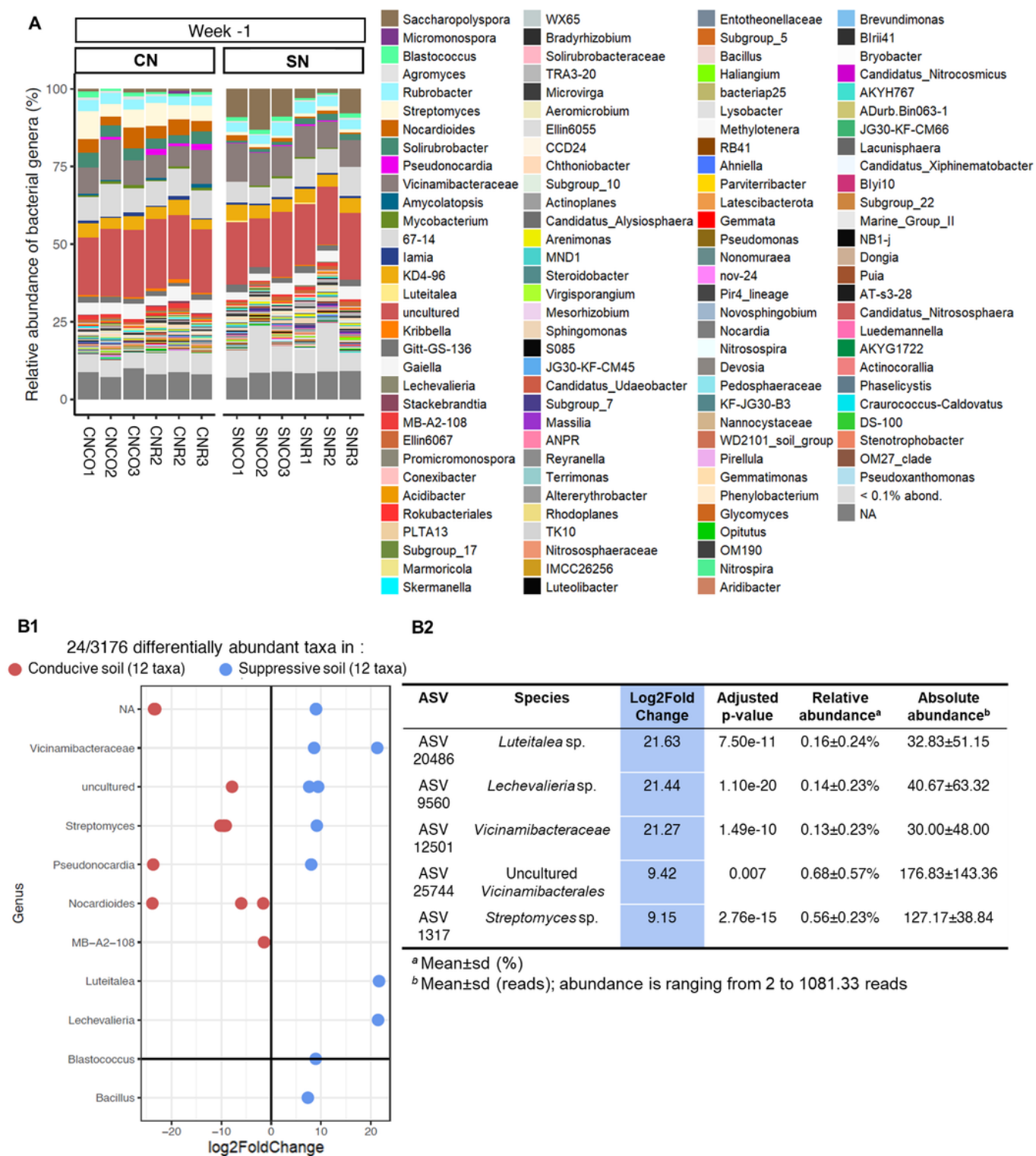


Figure 9

Comparative description of bacterial communities between the two initial soils

A Relative abundance of bacterial families in the conducive soil (CN) and suppressive soil (SN) at the initial time (R1, R2, R3) and before every co-cultivation (CO1, CO2, CO3). Taxonomic assignments of 16S rRNA amplicons were conducted on SILVA database. Taxa less than 0.1% abundant were grouped into a common class named "<0.1% abund.", and taxa with no genus attribution were grouped into "NA", not available. **B1** Differential abundant taxa between the initial CN and SN soils. **B2** Five differentially abundant taxa with higher Log2FoldChange value among the 12 over abundant taxa in SN soil.

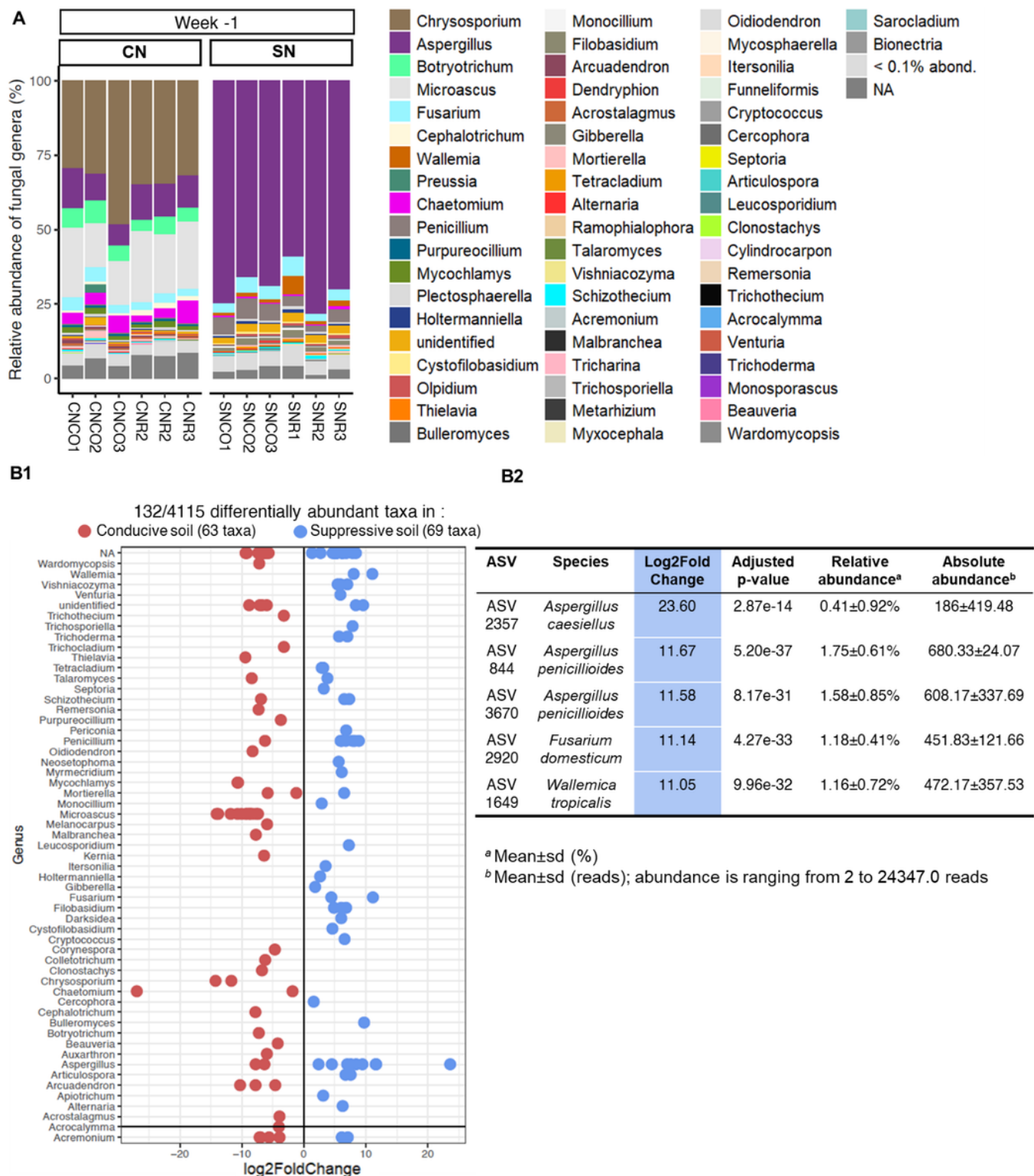


Figure 10

Comparative description of fungal communities between the two initial soils

A Relative abundance of fungal taxa at the Genus level in the conductive soil (CN) and suppressive soil (SN) at the initial time (R1, R2, R3) and before every co-cultivation (CO1, CO2, CO3). Taxonomic assignments of ITS amplicon were conducted on UNITE database. Taxa less than 0.1% abundant were

grouped into a common class named “<0.1% abund.”, and taxa with no genus attribution were grouped into “NA”, not available. **B1** Differential taxa abundance between the soil. Analysis were performed via DESeq2 package on geometrical means with CN and SN as the two tested conditions. **B2** Five differentially abundant taxa with higher Log2FoldChange value among the 69 over abundant taxa in SN.

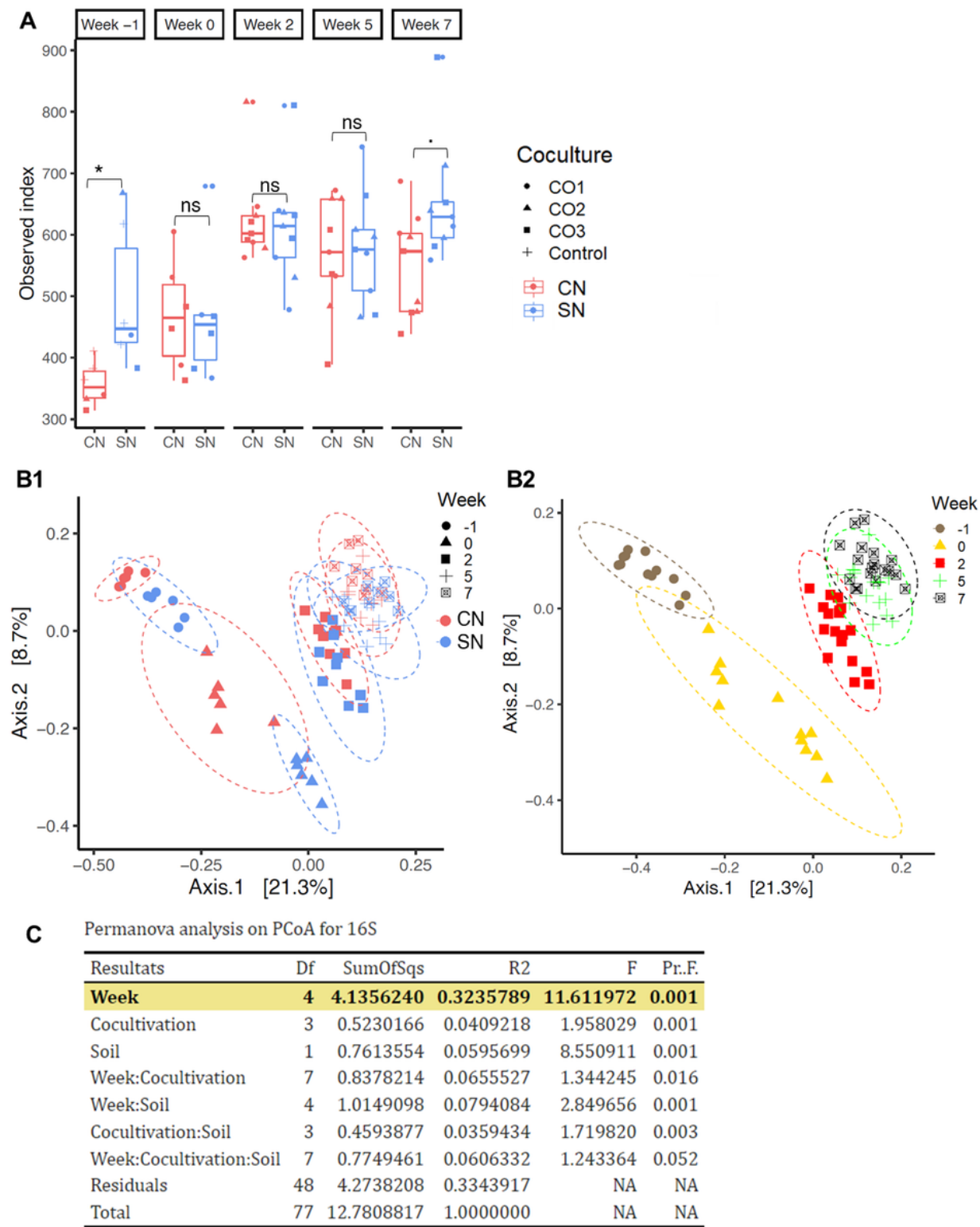


Figure 11

Comparative description of bacterial diversity between the conducive and suppressive soils during plant parasitism

A Estimation of bacterial richness approached by the observed indice. Significance is estimated using two-sided Wilcoxon Rank Sum tests (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns non-significant). **B** Dissimilarities of bacterial composition between the two CN and SN soils measured with the Bray-Curtis indice and analyzed on principal coordinate analysis (PCoA). Dissimilarities are projected on axes 1 and 2 with total of 30% of inertia. **B1** Ellipses calculated on soil effect **B2** Ellipses calculated on week effect. **C** Factors analyzed thanks to Permutational Multivariate Analysis of Variance. High values of R^2 (R-square) are highlighted in yellow and indicate the proportion of variation which is explained by the factor.

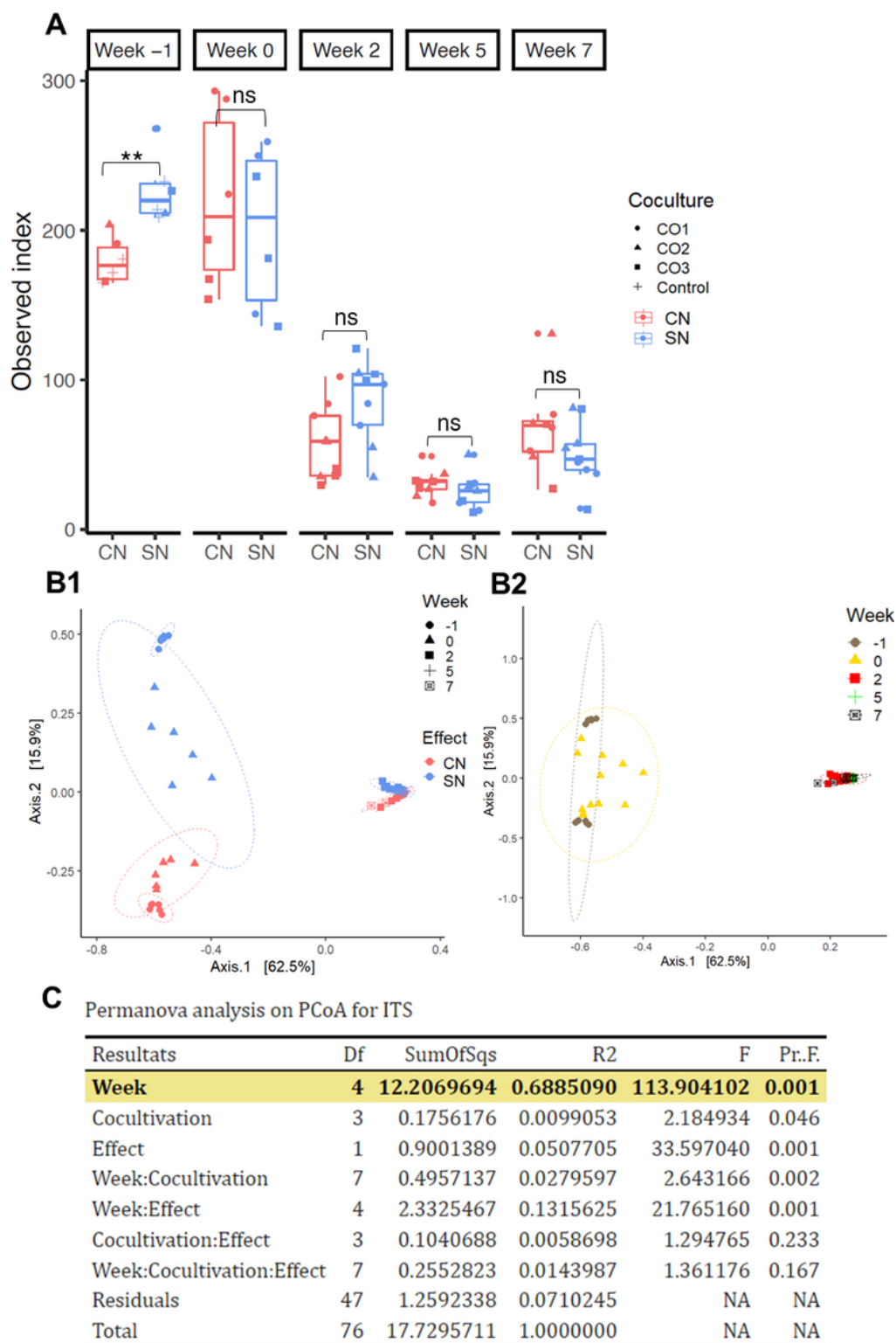


Figure 12

Comparative description of fungal diversity between the two conductive and suppressive soils during plant parasitism

A Estimation of fungal richness approached by the observed indice. Significance is estimated using two-sided Wilcoxon Rank Sum tests (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns non-significant). **B** Dissimilarities

of fungal composition between the two CN and SN soils measured with the Bray-Curtis indice and analyzed on principal coordinate analysis (PCoA). Dissimilarities are projected on axes 1 and 2 with total of 78.4% of inertia. **B1** Ellipses calculated on soil effect **B2** Ellipses calculated on week effect. **C** Factors analyzed thanks to Permutational Multivariate Analysis of Variance. High values of R2 (R-square) are highlighted in yellow and indicate the proportion of variation which is explained by the factor.

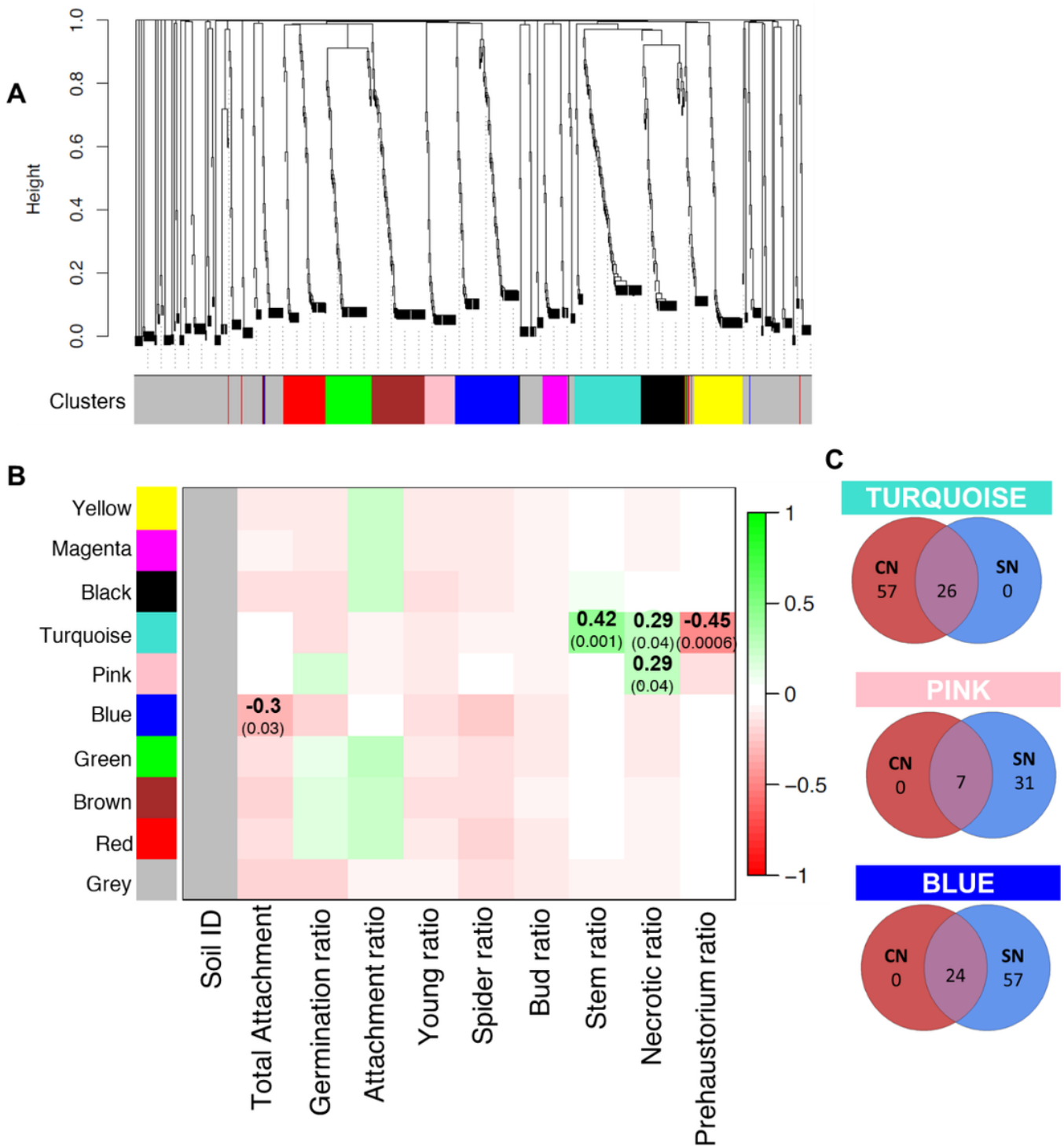


Figure 13

Weighted Correlation Network Analysis (WGCNA) of the abundance of fungal ASV correlated with parasitic traits

A Dendrogram based on abundance clustering of fungal ASV. Each color corresponds to a cluster. **B** Correlation between clustered-ASV and parasitic traits measured during co-cultivations: Total attachments (number of parasite attachments on rapeseed roots), Germination ratio (broomrape seeds treated with eluted fraction; 10^3 -fold dilution), Attachment, Young, Spider, Bud, Stem and Necrotic ratios (relative proportion of the development stages; see methods) and Prehaustorium ratio (germinated broomrape seeds treated with eluted fraction; 10^3 -fold dilution). Pearson's correlation tests are displayed in bold and p values between parenthesis (Student's tests). Turquoise, Pink and Blue clusters were identified as correlated to suppressive traits. **C** Venn's diagram showing number of ASVs found in the three correlated clusters, both unique and shared between CN and SN soils.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementarymaterials.pdf](#)
- [TableS216S.csv](#)
- [TableS3ITS.csv](#)