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Soil nematodes mediate positive interactions between legume plants and rhizobium bacteria

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Abstract Symbiosis between legume species and rhizobia results in the sequestration of atmospheric nitrogen into ammonium, and the early mechanisms involved in this symbiosis have become a model for plant-microbe interactions and thus highly amenable for agricultural applications. The working model for this interaction states that the symbiosis is the outcome of a chemical/molecular dialogue initiated by flavonoids produced by the roots of legumes and released into the soil as exudates, which specifically induce the synthesis of nodulation factors in rhizobia that initiate the nodulation process. Here, we argue that other organisms, such as the soil nematode *Caenorhabditis elegans*, also mediate the interaction between roots and rhizobia in a positive way, leading to nodulation. We report that *C. elegans* transfers the rhizobium species *Sinorhizobium meliloti* to the roots of the legume *Medicago truncatula* in response to plant-released volatiles that attract the nematode. These findings reveal a biologically-relevant and largely unknown interaction in the rhizosphere that is

multitrophic and may control the initiation of the symbiosis.

Keywords Symbiosis · Legume · Sinorhizobium meliloti · *Caenorhabditis elegans* · Volatiles

Introduction

The nodulation of legumes mediated by rhizobium species has received considerable attention due to its biological and agronomic importance (Marx 2004). Several mechanisms have been elucidated regarding the initial steps of recognition and communication between the legume root and the symbiont. The roots of legume species release flavonoids that attract (Caetano-Anolles et al. 1988; Pandya et al. 1999) and induce the *nod* genes of the bacterium; subsequently the bacterium releases nod factors that elicit root hair curling, allowing efficient bacterial colonization and nodule formation (Firmin et al. 1986; Peters et al. 1986; Perret et al. 2000; Riely et al. 2004). Such a model adequately explains short-distance contact communication (a few millimeters) between legume roots and the infecting rhizobia, but does not explain how a rhizobium species separated from a legume root by a larger distance, reaches its target, as root exudates have only been shown to move a few millimeters from the plant root (Bais et al. 2004; Weir et al. 2004). Furthermore, it is known that leguminous plants cultivated in a field with no exogenous supplementation of rhizobium cultures, accumulate rhizobia in their rhizospheres over time (Purchase and Nutman 1957; Roviria 1961). This information prompted us to study the influence that other organisms may have in the movement of rhizobia towards a target root.

Nematodes are a diverse group of soil-dwelling organisms. Nematodes have long been regarded as

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deleterious to agriculture production, stemming from the fact that plant parasitic species are the most researched group of nematodes. The negative effects of plant parasitic nematodes are two fold; they feed on the plants and reduce economic yields (Wasilewska and Webster 1975; Williamson and Gleason 2003), and some species belonging to the genera *Xiphinema*, *Longidorus*, *Trichodorus* and *Paratrichodorus* transmit several viral diseases (Brown et al. 1995). Many nematodes are known to interfere with the symbiotic association between Rhizobia and legumes and as well as mycorrhizal associations (Haung 1987; Williamson and Gleason 2003). However, some plant pathogenic nematodes like *Meloidogyne spp.* can have positive effects on the legume-rhizobial association by enhancing the number of nodules and the amount of nitrogen fixed (Baldwin et al. 1979) by unknown mechanisms. Besides the plant parasitic forms, there are a large group of saprophytic nematodes found in the soil. Laboratory experiments and field studies have demonstrated that saprophytic nematodes play a critical role in influencing the turnover of the soil microbial biomass and thus enhance the availability of plant nutrients (Bardgett et al. 1999; Bongers and Ferris 1999; Yeates 2003). In some ecosystems nematodes contribute up to 40% of nutrient mineralization (De Ruiter et al. 1993). However, the other roles of saprophytic nematodes that might improve plant growth have largely been neglected.

In the rhizosphere, the interaction between the plant and other organisms including microorganisms and soil invertebrates like nematodes, are mostly mediated by chemicals present in the root exudates (Bais et al. 2004). Several aboveground interactions between plants and other organisms are mediated by volatile organic compounds that are released by the plant in response to biotic and abiotic cues; these cues, either attract or repel other organisms (Dicke et al. 1990a; Turlings et al. 1995; Takabayashi et al. 1996; Sabelis et al. 1999). Although plant roots are capable of releasing volatile compounds (Steeghs et al. 2004), the biological significance of these root-released volatiles has not been studied. Furthermore, the free-living soil nematode *Caenorhabditis elegans* is a well studied and genetically tractable experimental system (Kamath et al. 2003), which is thought to employ chemotaxis-mediated movement in response to bacterial volatile gradients, which are known to disperse up to a few meters through the soil (Allaire et al. 2002), to locate pockets of bacteria (*C. elegans*' food) in the soil (Troemel et al. 1997). Therefore, in the present communication, using the free-living soil nematode *C. elegans* (Kamath et al. 2003) as a model, we have studied if free-living nematodes could positively influence the *Medicago truncatula*-*Sinorhizobium meliloti* interaction by acting as potential vectors that transport *S. meliloti* to the plant root. Further, we studied the effect of root-released volatiles of *M. truncatula* on the chemotaxis behavior of *C. elegans*.

Materials and methods

Plant material, bacteria, nematodes and chemicals

Seeds of *M. truncatula* cv Gaernt were kindly provided by Dr. D. Cook (University of California, Davis), and *Arabidopsis thaliana* (Col-0) seeds were purchased from the Lehle Seeds Co. (Round Rock, Texas). The seeds were germinated on solidified Murashige and Skoog (MS) medium (Murashige and Skoog 1962) in a climate-controlled chamber (20°C, 16/8-light/dark cycles). Two- to -three-week-old seedlings were used for the experiments. Wild-type *S. meliloti* (strain 1021) and the mutant Rm1021 pHC60 (constitutively expresses green-fluorescent-protein [GFP], Chen and Walker 1998) were obtained from Dr. Sharon R. Long (Stanford University, California) and Dr. A.M. Hirsch (University of California, Los Angeles), respectively. *Escherichia coli* (OP50) were obtained from Dr. F. M. Ausubel (Harvard Medical School, Boston). The bacteria were maintained on Luria-Bertani (LB) medium with appropriate selection antibiotics. Nematodes (wt *C. elegans* N2 and the srf-3 mutant [Cipollo et al. 2004] obtained from Dr. F. M. Ausubel and Dr. C. N. B. Hirschberg [Boston University, MA] and wt *Acrobeloides maximus* obtained from Dr. D.H. Wall [Colorado State University, CO]) were grown on nematode growth medium (NGM, Sulston and Hodgkin 1998). Dimethyl sulfide was purchased from Sigma-Aldrich. This compound was diluted to the appropriate concentration in methanol and 10 µl of the cold (4°C)-diluted compound was added to sterile cotton wool, which was used for experiments.

Nematode survival assay

Five adult *C. elegans* and *A. maximus* were washed with an antibiotic cocktail (kanamycin 50 µg ml⁻¹) and transferred onto NGM plates containing either OP50 or wild-type *S. meliloti* (Rm1021) as a food source. These plates were incubated at 20°C in the dark and scored for live and dead worms every 24 h for 35 days. A worm was considered dead when it failed to respond to plate tapping or a gentle touch with a platinum wire. Worms that died as a result of getting stuck to the wall of the plate were not included in the analysis. These experiments were repeated three times.

Nematode intake of *S. meliloti* and microscope observation

Adult *C. elegans* and *A. maximus* were separately placed on NGM plates containing *S. meliloti* Rm1021 pHC60, which constitutively expresses green-fluorescent-protein (GFP), and incubated at 20°C in the dark. After 2 days, the nematodes were removed and observed under an

Olympus BX60 microscope equipped with CoolSnap imaging software (San Diego, CA) for the presence of bacteria within the guts. Fluorescence microscopy was performed to observe wild-type *S. meliloti* colonization of nematodes using the Live-dead Bac Light Bacterial Viability Kit (Molecular Probes, Eugene, OR) at room temperature in the dark for 15 min, according to the manufacturer's instructions. Bacteria attached to the nematode's cuticle were stained with propidium iodide and SYTO9 (to visualize the polysaccharides). Phase contrast and fluorescence images of *S. meliloti*-colonized nematodes were captured with a 10×–100× objective on an Olympus BX60 microscope equipped with CoolSnap imaging software (San Diego).

Survival assay of *S. meliloti* in the *C. elegans* gut

Five adult *C. elegans* worms grown on NGM media with *S. meliloti* as the food source were transferred to a sterile tube with M9 buffer (Brenner 1974) and centrifuged (500× *g*, 2 min, 21°C). The worms were resuspended in 300 µl of M9 buffer and centrifuged again to remove the bacteria that were attached to the cuticle. This wash treatment was repeated three times. The supernatant was removed, and the pellet was resuspended in 100 µl of M9 buffer. The worms were crushed with a pellet pestle. A suspension (5 µl) was plated on the surface of solidified LB medium containing streptomycin: 400 mg l⁻¹. The plate was incubated at 30°C for 1 day, and the numbers of colonies were counted. These experiments were repeated three times.

Nematode chemotaxis behavior assay

To test our hypothesis that the chemotaxis of the worms towards the plant is mediated by plant VOCs, we designed an experimental setup that is depicted in Fig. 3a. Two glass conical flasks were connected by a 10 cm diameter glass petri dish using glass tubing, and silicone rubber was used as the sealant. Three plants (*M. truncatula* or *A. thaliana*) were placed in one glass flask containing 3 ml MS liquid medium while the other flask had 3 ml of MS liquid medium without the plant. The central petri dish contained 20 ml of NGM agar medium in which four spots of 5 µl OP50 were applied 1 cm away from each flask's connection site to prevent nematode movement after their final choice. About 30 adult *C. elegans* or *A. maximus* were placed at the center of the petri dish, which was sealed with parafilm to prevent the leakage of volatiles. The whole setup was incubated at 20 ± 2°C for 1 h. At the end of the incubation period, the volatile-mediated chemotaxis behavior of the nematode was judged based on the movement of the nematode towards the conical flasks containing the plants. The nematode was deemed to have made the choice of a plant when it reached the OP50 colonies that were in proximity to the conical flask containing that plant. The

number of nematodes on each side of the petri plate was counted. The data obtained was subjected to a binomial test. Dimethyl sulfide was diluted in methanol to appropriate concentrations (2, 0.2, 0.02 M) to examine its effect on the *C. elegans* olfactory response. Different dilutions of dimethyl sulfide (10 µl total volume) were applied to cotton wool plugs and placed in one of the flasks as described above; 10 µl of methanol were used as a control. These experiments were repeated three times.

VOC analysis

Four *M. truncatula* plants were placed in a glass tube containing 1 ml MS liquid medium. The headspace volatiles were concentrated using a purge and trap instrument (Tekmar 3000 Purge and Trap Concentrator, Cincinnati, OH) with helium as the carrier gas. The headspace gas was collected for 1 h. For root VOC analysis, the roots of ten *M. truncatula* plants were cut from the stems and used for the experiments. VOCs trapped on the adsorbent material (Purge Trap C [Tenax 34%, silica gel 33%, charcoal 33%]: Supelco Co.) were desorbed at 250°C and analyzed using a GC/MS (Hewlett-Packard 5890 series II, Avondale, PA) equipped with a 30 m×0.25 mm capillary column (DB-5.625, J&W Scientific, Folsom, CA) with helium as the carrier gas. The initial oven temperature was maintained at 0°C for 8 min by cryogenic cooling. The oven temperature was increased to 70°C at rate of 7°C/min, and then to the final temperature of 300°C at the rate of 20°C/min, which was maintained for 10.5 min. The injection port temperature was 250°C, and the helium carrier gas linear velocity was maintained at 35 cm s⁻¹ with automated pressure control. Detection was achieved by mass selective detection (Hewlett-Packard 5972, Avondale, PA) in the scan mode (*m/z* 33–500). The chemical structures of VOCs were identified by using a Wiley 138 K mass spectral database (John Wiley and Sons, New York), and by comparing the mass spectra and the retention time with those of authentic chemical samples. Dimethyl sulfide, dimethyl disulfide, octane, hexanal, 1-hexanol, nonane were purchased from Sigma-Aldrich. These experiments were repeated three times. Dimethyl sulfide amounts released from roots were calculated using an approximated curve.

S. meliloti movement assay in soil

Peat pellets (Jiffy products, Canada) were soaked in water for 10 min, and then the soil from the peat pellets was placed in a plastic pot (base: 55 cm, height: 9.5 cm) and autoclaved for 15 min at 125°C. In one corner of the plastic pot, an *M. truncatula* plant was placed, with its roots surrounded by a mesh to prevent spreading of the root. For inhibition of root VOCs, a charcoal filter was

kept around the roots. The peat mixture was soaked with 100 ml of water, and excess watering was avoided to prevent passive bacterial movement by water current. The plastic pot was kept in a climate-controlled room for 2 days (18 h: light 26°C, 6 h: dark 18°C). Wild-type streptomycin-resistant *S. meliloti* was placed on a piece of cotton wool ($O.D_{600} = 0.3$, 50 μ l), which was kept in the soil at end of the pot farthest from the plant. The cotton wool was placed at a depth of 1.5 and 6 cm away from two- to-three-week-old *M. truncatula* plants with or without a charcoal filter. Fifty *C. elegans* were transferred onto the cotton wool (see Fig. 4a). This plastic pot was kept in a climate controlled room for 2 days (18 h: light 26°C, 6 h: dark 18°C). The colony-forming units of *S. meliloti* were estimated by sampling the soil with a plastic stick in the center of the pot (3 cm away from the plant) and spreading the soil suspension made with sterile distilled water on an LB plate supplemented with streptomycin (400 mg l⁻¹). The plate was incubated at 30°C for 1 day, and the numbers of colonies counted. These experiments were repeated three times. Using the same experimental setup as described, we tested the efficacy of dimethyl sulfide, the active fraction of the *M. truncatula* root volatiles. Dimethyl sulfide (2 μ mol) was dispensed onto a cotton pad and placed in the soil in lieu of *M. truncatula* plants. A cotton pad soaked with an amount of methanol equal to that used to dissolve dimethyl sulfide in the treatment

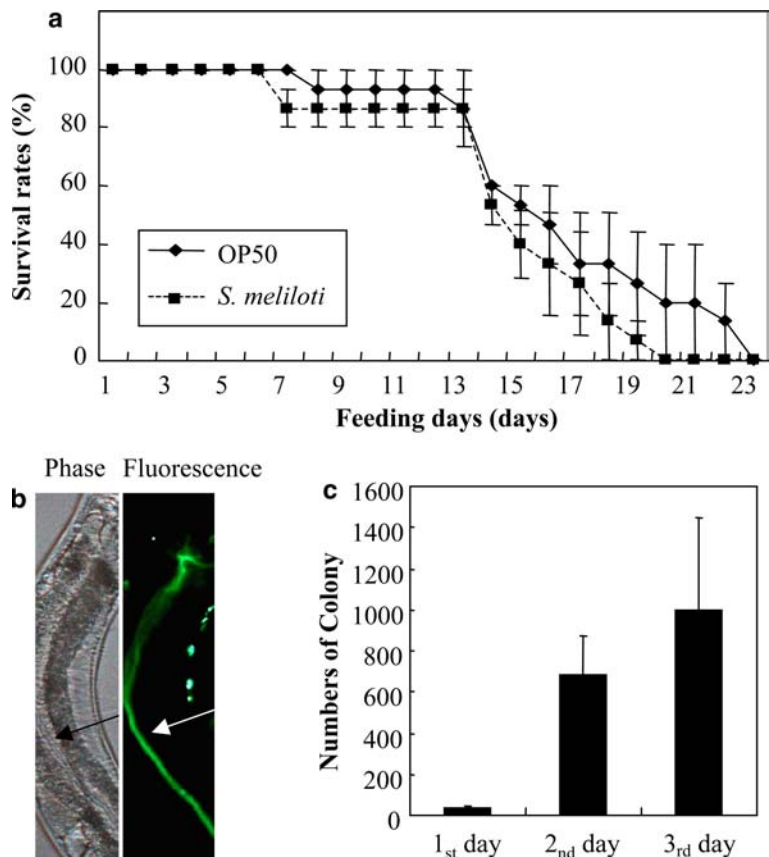
served as the control. The colony forming units were estimated as described above.

Results

Rhizobium as a food source for the nematode

To study the possible interaction between *C. elegans*, *S. meliloti* and *M. truncatula*, we first tested whether *S. meliloti* was a food source for the nematode. The survival rate is given in Fig. 1a. With *S. meliloti*, 86.7, 33.3 and 0% of *C. elegans* survived after 10, 15 and 20 days. With OP50, 93.3, 46.7 and 0% of *C. elegans* survived after 10, 15 and 22 days. With *S. meliloti*, *C. elegans* showed a similar survival curve when compared with the OP50 feeding. Furthermore, we observed ample accumulation of *S. meliloti* in the anterior part of the intestine of the worm when fed with GFP-tagged *S. meliloti* (Chen and Walker 1998, Fig. 1b). These worms were subsequently analyzed for populations of the rhizobium that survived in the gut (see Materials and methods). The number of colony-forming units (CFUs) of *S. meliloti* inside the nematode gut increased with the length of incubation (Fig. 1c). Average CFUs were 37, 684, and 998, at the first, second, and third days. These data suggest that *S. meliloti* is able to survive/multiply in the alimentary canal of *C. elegans*.

Fig. 1 **a** *C. elegans* fed on *S. meliloti* without detectable pathogenicity. Survival rates of *C. elegans* on *S. meliloti* lawns were comparable to the regular nematode feed source OP50. Vertical bars represent standard error ($n = 3$). **b** In-worm localization of GFP-expressing *S. meliloti*: the fluorescence was localized in the pharynx and gut. Arrows indicate gut. **c** *S. meliloti* survived in the nematode. *C. elegans* fed on *S. meliloti*, which were homogenized after 1–3 days and bred on the rhizobium sterile medium containing a selection antibiotic (Streptomycin 400 mg/l). The number of colony forming units (CFUs) of *S. meliloti* in the worm increased daily. Vertical bars represent standard error ($n = 3$)



Nematode as a vector of the rhizobium

Using the setup described in Materials and methods (Fig. 2a1), we rigorously tested the hypothesis that *S. meliloti* was capable of being transported by *C. elegans*. *S. meliloti* colonies with no *C. elegans* (control) failed to reach the *M. truncatula* roots under this condition, indicating that the bacteria can't move on MS media. However, when the bacteria were co-inoculated with *C. elegans*, which can move on MS media, the nematode transported *S. meliloti* to the roots and 2.3 ± 0.33 nodules per plant ($N=3$) were observed at 16 days after co-cultivation (Fig. 2a). Further, we observed *S. meliloti* communities on the cuticle of the wild-type N2 Bristol nematode (Fig. 2b), demonstrat-

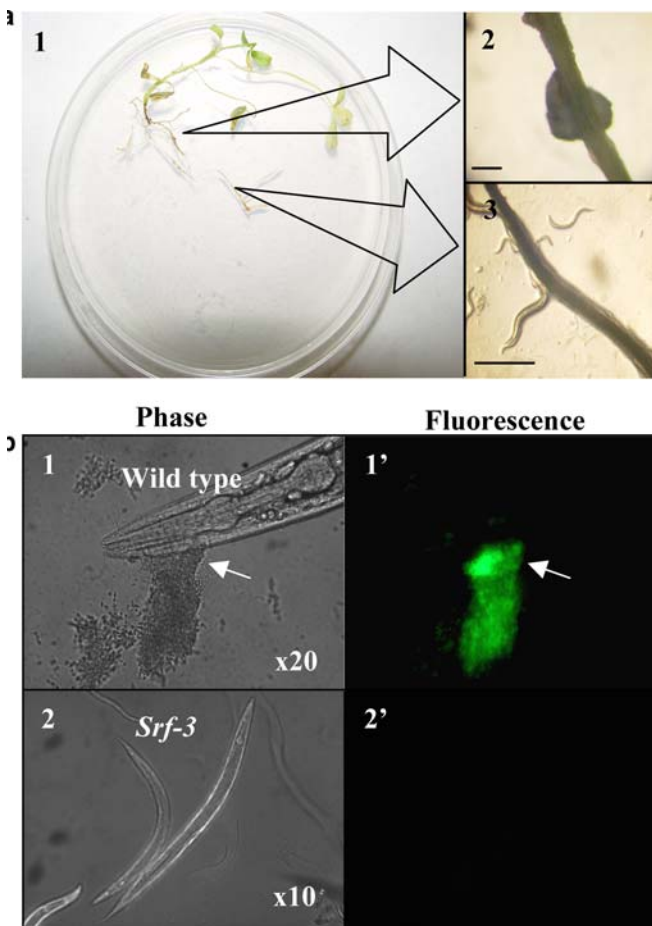


Fig. 2 a Induction of nodulation in *M. truncatula* by co-inoculating *S. meliloti* with *C. elegans*. (1) *S. meliloti* was inoculated onto a petri dish containing solidified MS medium at a distance of 4.5 cm away from *M. truncatula*. Inclusion of *C. elegans* in the system induced nodulations at after 16 days. (2) and (3) are images indicated by the arrowheads in (1) and magnified 25 times. Two to three nodules per plate were observed when *C. elegans* was co-inoculated with *S. meliloti* and *M. truncatula*. Bars in (2) and (3) indicate 1 mm. **b** Attachment of *S. meliloti* on *C. elegans* surface visualized with bacterial stain. 1-1': wild-type *C. elegans*, 2-2': *srf-3* mutant. The number in the picture represents the magnification

ing the ability of *S. meliloti* to attach to the nematode cuticle. This behavior may aid in the transport of the bacteria. It was interesting to note that a *C. elegans* mutant, *srf-3*, altered in surface antigenicity (Hoflich et al. 2004), failed to support bacterial community formation on its cuticle and thus prevented bacterial adherence (Fig. 2b).

Chemotaxis of the nematode towards the legume and VOC analysis

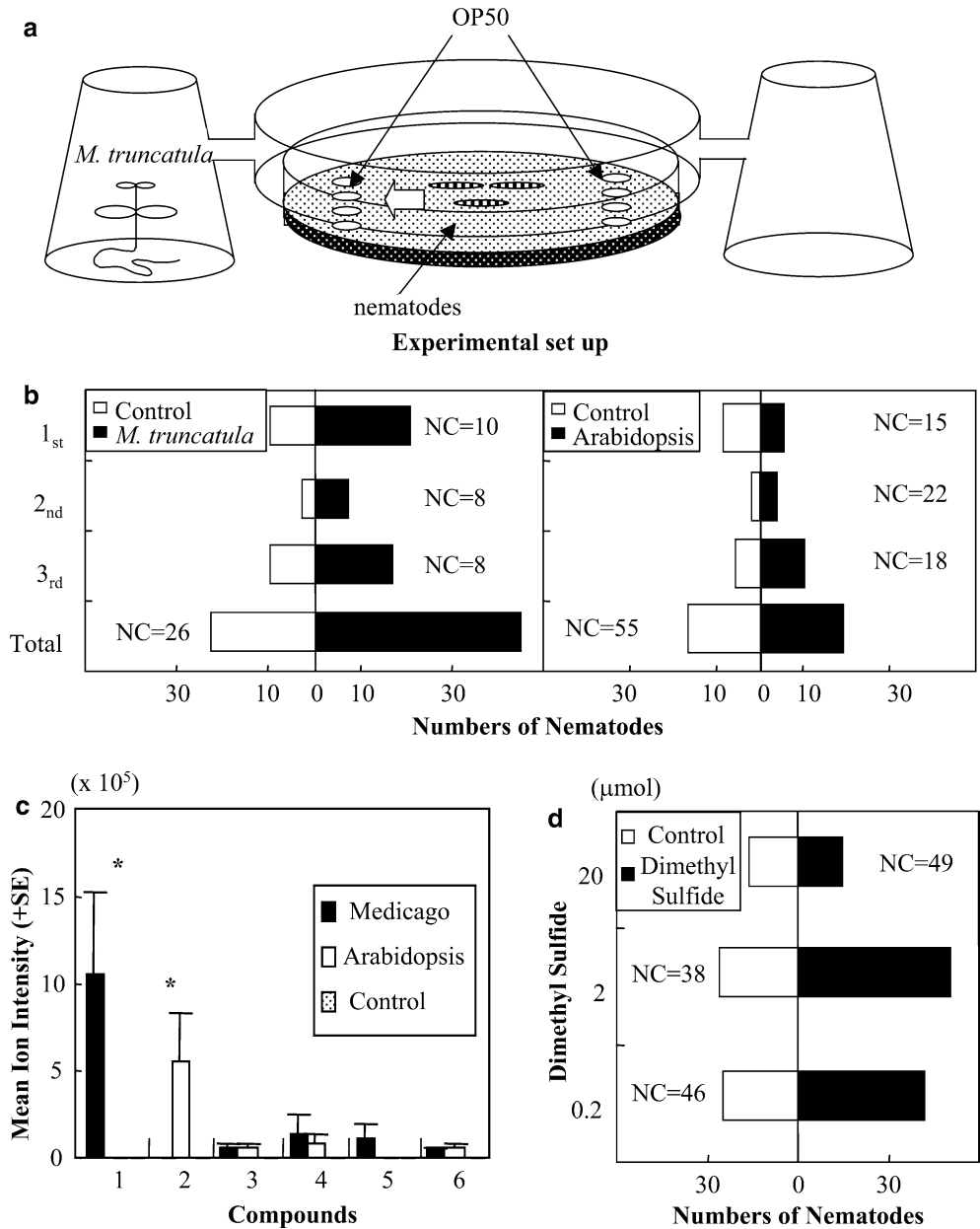
Using the device described in Materials and methods, we examined the chemotaxis of *C. elegans* towards *M. truncatula* plants (Fig. 3a). In our experimental conditions, we did not observe any significant difference in nematode preference caused by parafilm (right:left=23:19 no-choice=25 [with parafilm], right:left=28:35 no-choice=18 [without parafilm]), even though parafilm is known to emit a minimal amount of VOCs (Selby et al. 1996). Significantly more *C. elegans* moved towards *M. truncatula* than towards the control (MS only, *M. truncatula*:control=45:23, no-choice=26; binomial test, $P < 0.05$, Fig. 3b). In contrast, there was no significant difference in the movement of the nematode towards Arabidopsis plants or control (no plants, Arabidopsis:control=17:19, no-choice=55; binomial test, $P > 0.05$).

Since *M. truncatula* was able to attract *C. elegans*, we analyzed the VOCs produced by the plant using GC/MS. Under our experimental conditions, we detected dimethyl sulfide, octane, hexanal, and 1-hexanol from in vitro grown *M. truncatula*, and dimethyl disulfide, octane, and hexanal from Arabidopsis (Fig. 3c). There were significant differences in the amount of dimethyl sulfide and dimethyl disulfide released in the volatiles of *M. truncatula* versus in those of Arabidopsis (Mann-Whitney U test: $P < 0.05$). Significantly more *C. elegans* were attracted to 0.2 and 2 μmol dimethyl sulfide compared to the control (methanol, 0.2 μmol dimethyl sulfide:control=42:25, no-choice=46, χ^2 -test, $P < 0.05$, 2 μmol dimethyl sulfide:control=51:26, no-choice=38, χ^2 -test, $P < 0.01$), but there were no significant differences in the movement of the nematode towards 20 μmol dimethyl sulfide or control (20 μmol dimethyl sulfide:control=15:16, no-choice=49, χ^2 -test, $P > 0.05$, Fig. 3d). There were no significant differences in the movement of the nematode towards dimethyl disulfide (20 μmol dimethyl disulfide:control=12:10, no-choice=52, binomial test, $P > 0.05$; 2 μmol 24:28, no-choice=27, binomial test, $P > 0.05$; 0.2 μmol 19:24, no-choice=39, binomial test, $P > 0.05$). Gas chromatography combined with bioassay experiments revealed that the volatile produced by *M. truncatula* that attracted *C. elegans* was dimethyl sulfide. The roots of *M. truncatula* released $0.0994 \mu\text{mol}$ dimethyl sulfide (mean ion intensities = $136178 \pm 59085.61/10$ plant roots, $N=3$).

Fig. 3 a Experimental setup for nematode olfactory behavior.

Two glass conical flasks were connected by a glass petri dish using glass tubing. Plants were placed in one glass flask containing MS liquid. The central petri dish contained NGM agar medium, where OP50 was inoculated near each connection site to prevent nematode movement after they had made a choice. Nematodes were placed at the center of the petri dish, which was sealed with parafilm to prevent the leakage of volatiles (see Supplementary methods).

b Olfactory response of *C. elegans* to *M. truncatula* plants and Arabidopsis. The worm preferred *M. truncatula* to the control, and showed no preference for Arabidopsis. *NC* no-choice. **c** Relative amounts of induced volatile compounds released from *M. truncatula*, Arabidopsis and MS liquid (control) analyzed by GC-MS. Bars represent standard error ($n=3$). Compound names: (1) dimethyl sulfide, (2) dimethyl disulfide, (3) octane, (4) hexanal, (5) 1-hexanol, (6) nonane. * Indicates a significant difference between the amount of compound released by *M. truncatula* versus that released by Arabidopsis (Mann-Whitney U test, $P<0.05$). **d** Olfactory response of *C. elegans* to dimethyl sulfide. These experiments were repeated four times. *NC* no-choice



Rhizobial movement assay in soil

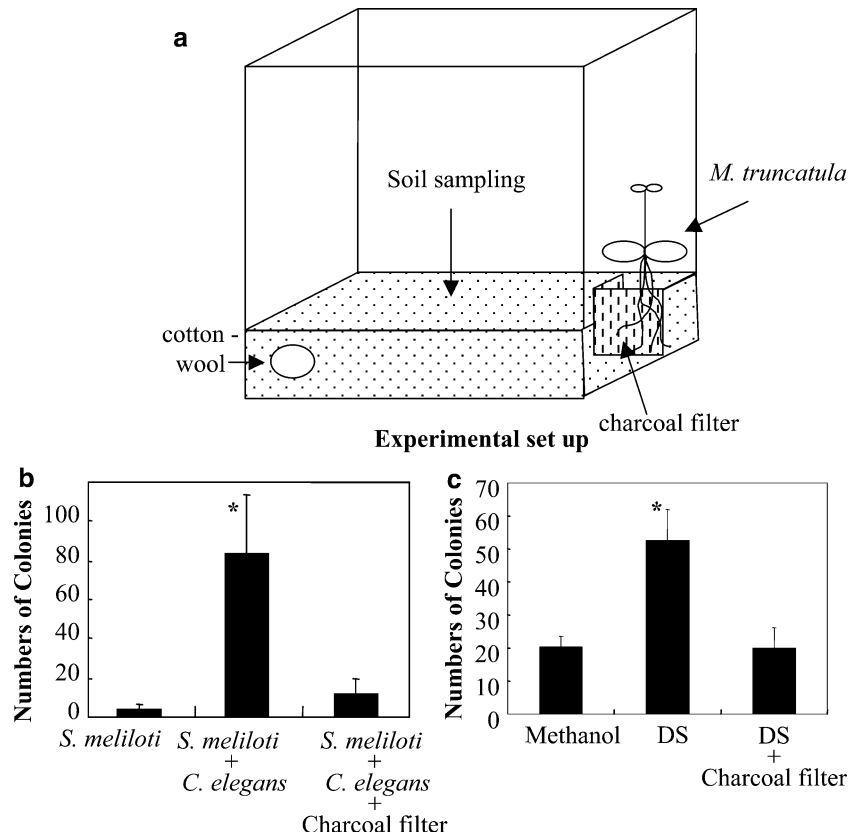
We tested if *C. elegans* carrying *S. meliloti* would be attracted to *M. truncatula* volatiles under soil conditions using the experimental prototype described in Materials and methods (Fig. 4a). After 2 days, cfu counts revealed that in the presence of *C. elegans*, more *S. meliloti* colonies (84.0 ± 29.5 cfu per stab) were observed than without the nematodes (4.3 ± 1.6 cfu per stab, Fig 4b). Concomitantly, a treatment including *C. elegans* and a charcoal filter showed only a marginal difference in the number of rhizobial colonies compared to the condition without nematodes (12.3 ± 6.3 cfu per stab). Similarly, dimethyl sulfide was able to attract *C. elegans* under soil conditions and a

concomitantly higher *S. meliloti* cfu count was observed at 2 μmol dimethyl sulfide, while the control and the condition adding dimethyl sulfide with a charcoal filter did not show any attractive influence (Fig. 4c).

Acrobeloides maximus displays similar feeding behavior as *C. elegans* on *S. meliloti*

We tested the possibility that other bacterial feeding nematodes such as *A. maximus* might use rhizobia as a food source. We observed significant accumulation of *S. meliloti* in the pharynx and midgut of the intestine when the nematode was fed with GFP-expressing *S. meliloti*

Fig. 4 a Experimental setup for soil conditions. *S. meliloti* movement assay in soil. Pieces of cotton wool with *S. meliloti* and with or without *C. elegans* were placed in a flat bed containing sterilized soil (Jiffy-7, Jiffy Ltd. Canada) at a depth of 1.5 and 6 cm away from *M. truncatula* plants or 2 μ mol dimethyl sulfide with or without a charcoal filter. Soil samples were taken at 3 cm from the plant and spread on the rhizobium sterile medium containing the selection antibiotic (streptomycin 400 mg/l). **b** and **c** Numbers of *S. meliloti* colonies 1 day after plating. Vertical bars represent standard error ($n=3$). DS dimethyl sulfide. * indicates a significant difference between the three conditions (ANOVA followed by Fisher's PLSD, $P < 0.05$)



(Fig. 5 a1-1'). *Acrobeloides maximus* grown on an *S. meliloti* lawn also exhibited bacterial attachment to the worm surface as observed in *C. elegans* (Fig. 5 a2-2'). However, this nematode showed a decreased survival rate in the presence of *S. meliloti* when compared with OP50 feeding (Fig. 5b) indicating that *S. meliloti* is not an ideal food for this nematode. When we examined the chemotaxis response of *A. maximus* towards *M. truncatula* plants, we did not find significant preferences between the control and *M. truncatula* plant volatiles (Fig. 5c, *M. truncatula*:control=2:1, no-choice=42, binomial test, $P > 0.05$). These results highlight the specificity of the chemotaxis response towards legume plants by certain soil nematodes.

Discussion

In the present experiments, the nematode *C. elegans* transfers *S. meliloti* by two mechanisms: (1) transmission of bacteria attached to the nematode surface, and (2) transmission of live *S. meliloti* in the feces. *Caenorhabditis elegans* defecates every 45 s (Liu and Thomas 1994); thus it is highly plausible that this biological process may abet the transfer of the bacteria. The results of in vitro and in vivo experiments suggest that the bacteria transferred by the nematode are physiologically active, as they were able to initiate functional nodules in the roots of *M. truncatula*.

Above ground, plant-herbivore-carnivore interactions mediated by plant volatiles are well studied (Takabayashi and Dicke 1996; Sabelis et al. 1999), and these show that plant VOCs attract specific carnivores to plants infested by herbivores. In an ecological perspective, this means that a plant can enlist the help of other phyla when it is under attack. In the present communication, we show that legume plants can also call for help by using VOCs to enlist specific soil nematodes to facilitate the transfer of rhizobia to the roots. Interestingly, *C. elegans* is also known to be affected by volatile chemicals, attracted by some volatiles and repelled by others (Bargmann et al. 1993). Our data indicate that *C. elegans* may transfer *S. meliloti* to the roots of *M. meliloti*. Tol et al. (2001) reported that the roots of a coniferous plant (*Thuja occidentalis*) release a mixture of unidentified chemicals upon attack by the larvae of *Otiorynchus sulcatus*; these chemicals attract the parasitic nematode *Heterorhabditis megidis*, which, in turn, attack the larvae. Recently, Rasmann et al. (2005) reported that maize roots release a volatile signal, (E)-beta-caryophyllene in response to feeding by larvae of the beetle *Diabrotica virgifera virgifera*, that attracts an entomopathogenic nematode *H. megidis*. These results and ours suggest that there are complex multitrophic interactions mediated by plant volatile chemicals in the rhizosphere.

Plant roots are capable of producing a suite of VOCs, the composition of which is influenced by biotic and

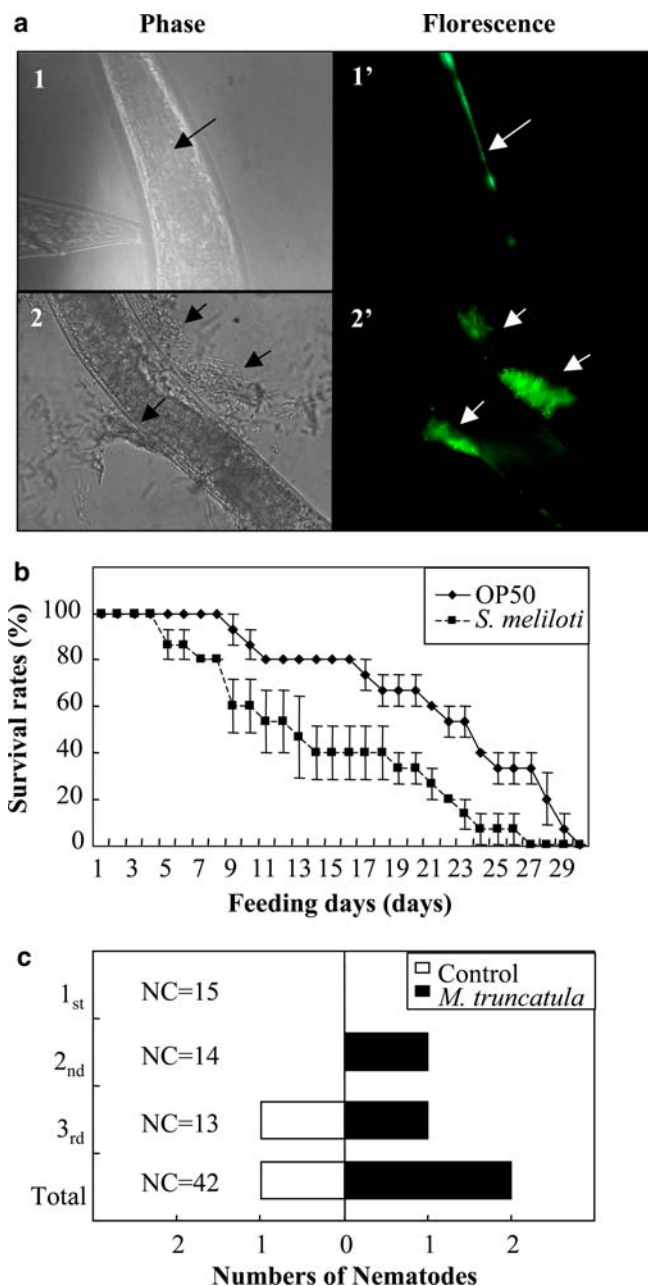


Fig. 5 **a** Microscopic pictures of *A. maximus* with GFP-tagged *S. meliloti* (pHC60). (a-a') GFP-fluorescence was observed in the pharynx and gut after 2 days. (b-b') *A. maximus* incubated with wild-type *S. meliloti*. With bacteria stain, *S. meliloti* attachment on the surface of *A. maximus* was observed. The number in the picture represents the magnification. Arrows in (1-1') and (2-2') indicate the nematode's gut and bacterial attachment. **b** Survival rate of *A. maximus* with *E. coli* OP50 or wild-type *S. meliloti*. Vertical bars represent standard error ($n=3$). **c** Olfactory response of *A. maximus* to *M. truncatula*. The worm did not show preference for *M. truncatula*

abiotic stress (Holopainen 2004). Under our experimental conditions, *M. truncatula* roots released VOCs consisting of a mixture of dimethyl sulfide, octane, hexanal, and 1-hexanol. Interestingly, one of the components, dimethyl sulfide, was a strong chemo-attractant

of *C. elegans*, suggesting its role in the biological interactions reported here. However, it should be noted that dimethyl sulfide is not unique to *M. truncatula*; several plant species produce dimethyl sulfide (Duke 1992). Some of these plants are nonlegumes, such as onion (*Allium cepa*) (Prithiviraj et al. 2004). Above ground, it is known that insects respond to not only one component of a plant's volatile "bouquet", but also to a specific blend of plant volatiles (Dicke et al. 1990b; De Moraes et al. 2001; Horiuchi et al. 2003). Therefore, it is most likely that a nematode responds to a blend of *M. truncatula* volatiles, including dimethyl sulfide. The nematodes attracted are specifically those which may carry compatible rhizobia. Once the rhizobia come within a few millimeters of the root, they are acted upon by plant-to-rhizobium signal molecules, isoflavonoids, that are secreted by legume roots.

Three-way interactions between plants, nematodes, and bacteria have largely been studied as negative interactions, in which the bacteria-carrying nematodes feed on plant roots and thus transmit disease (Williamson and Gleason 2003). In contrast, positive interactions between plants, nematodes, and bacteria have largely been overlooked. It's known that rhizobia help their host legume plants (such as peas, soybeans and alfalfa) by providing essential nutrients (Marx 2004). Furthermore, plants are known to be affected by soil nematodes; for example, bacteria-feeding nematodes affect the type and availability of nutrients by aiding decomposition (the modification of complex organic compounds into organic nutrients available for plant growth), carbon sequestration, and nutrient cycling, and plant parasitic nematodes are obligate parasites, feeding exclusively on the cytoplasm of living plant cells (Wasilewska and Webster 1975; Whitford et al. 1982; Williamson and Gleason 2003). Although many scientists are aware of the presence and importance of rhizobia and nematodes in the soil to plants, the existence of tritrophic interactions as explained here, are poorly understood.

Taken together, these results reveal a hitherto unsuspected multitrophic interaction that facilitates the long-distance communication between *M. truncatula*, *S. meliloti* and *C. elegans*, and is mediated by dimethyl sulfide. A new model of symbiotic rhizobia-root communication taking into account these data, is proposed: in response to the relatively specific signal of plant volatiles, certain nematodes are attracted to legume roots. These nematodes bring a bacterial "hitchhiker," either attached to the cuticle of the worm or surviving in its intestine. Once the nematode arrives at the legume roots, the rhizobium is close enough to the plant to be affected by other root signals, such as isoflavonoids. In other words, the plant harnesses the nematode to obtain beneficial bacteria. We suspect that the nematode also benefits, as the rich rhizosphere environment surrounding the legume roots should provide an ample bacterial food source. The model that we have proposed is critical for understanding multitrophic interactions in the rhizosphere as well as for developing new methods for

improving nodulation in intensive agricultural systems by considering soil invertebrates as key contributors to this process.

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