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Highly Potent Extracts from Pea (*Pisum sativum*) and Maize (*Zea mays*) Roots Can Be Used to Induce Quiescence in Entomopathogenic Nematodes

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Abstract Root exudates can play an important role in plant-nematode interactions. Recent studies have shown that the root cap exudates obtained from several plant species trigger a state of dormancy or quiescence in various genera of nematodes. This phenomenon is not only of fundamental ecological interest, but also has application potential if the plant-produced compound(s) could be used to control harmful nematodes or help to prolong the shelf-life of beneficial entomopathogenic nematodes (EPNs). The identification of the compound(s) involved in quiescence induction has proven to be a major challenge and requires large amounts of active material. Here, we present a high-throughput method to obtain bioactive root extracts from flash-frozen root caps of green pea and maize. The root cap extract obtained *via* this method was considerably more potent in inducing quiescence than exudate obtained by a previously used method, and consistently induced quiescence in the EPN *Heterorhabditis megidis*, even after a 30-fold dilution. Extracts obtained from the rest of the root were equally effective in inducing quiescence. Infective juveniles (IJs) of *H. megidis* exposed to these extracts readily recovered from their quiescent state as soon as they were placed in moist soil, and they were at least as infectious as the IJs that had been stored in water. Excessive exposure of IJs to air interfered with the triggering of quiescence. The implications of these results and the next steps towards identification of the quiescence-inducing compound(s) are discussed from the perspective of applying EPN against soil-dwelling insect pests.

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Introduction

Plants produce a wide range of chemical organic compounds. When released into the environment, these metabolites mediate interactions with surrounding organisms (Barber et al. 1976), and can play major roles in defense, communication, attraction, and repellency (Vining 1990). Aboveground interactions that are mediated by plant compounds generally are well documented, but it is only recently that the role of root-produced compounds in belowground interactions is receiving equal attention (Hartmann 2007; Hiltpold et al. 2011; Rasmann et al. 2012; Turlings et al. 2012; van Dam 2009). This seems pertinent because at least 20 % of the photosynthetically assimilated carbon is released by the roots (Barber et al. 1976; Kumar et al. 2006). Several effects of root-released chemicals on nematodes, bacteria, and fungi have been described (Bais et al. 2006), and evidence is accumulating that they also serve an important function in belowground tritrophic interactions among plants, herbivores, and entomopathogenic nematodes (Ali et al. 2010, 2012; Hiltpold and Turlings 2012; Rasmann et al. 2005).

Initial research on soil-dwelling nematodes and root exudates focused on plant-parasitic nematodes, due to their importance as pests of crops. Plant parasitic nematodes use constitutively released root exudates to locate their host plant (Curtis et al. 2009; Prot 1980; Reynolds et al. 2011; Rolfe et al. 2000). Root exudates also are known to trigger egg hatching in several plant-parasitic nematodes (Den Nijs and Lock 1992; Gaur et al. 2000; Khokon et al. 2009). Zhao et al. (2000) were the first to observe the intriguing phenomenon that root cap exudates induce a state of dormancy (quiescence)

in a plant-parasitic nematode (*Meloidogyne incognita*), and they proposed it to be a defense mechanism against root tip penetration. Following up, Hubbard et al. (2005) found that the root cap exudates of a wide variety of plants can trigger quiescence in several species of plant parasitic nematodes, animal parasitic nematodes, and free living nematodes. At the time, only one species of entomopathogenic nematode (EPN), *Steinernema glaseri*, was tested and found to be susceptible to the exudates.

It also is known that roots under attack by herbivores release compounds that attract EPN as a secondary defense mechanism. For instance, when maize roots are damaged by the western corn rootworm, *Diabrotica virgifera virgifera*, they release (*E*)-β-caryophyllene, a volatile sesquiterpene that attracts the EPN *Heterorhabditis megidis* (Rasmann et al. 2005). This apparent defense mechanism has been confirmed for other plants and EPN systems (Ali et al. 2010, 2012; Van Tol et al. 2001). More recently, several species of EPN also have been shown to be susceptible to root cap exudates. The exudates were found to induce quiescence in all tested EPN species, and their activity could be restored by diluting the exudate with water (Hiltpold et al. 2014).

Control of soil-dwelling insect pests mainly relies on pesticides causing environmental concerns (Köhler and Triebskorn 2013). Hence, there is a clear need for sustainable alternatives that are based on ecologically sound crop management solutions. Biological control with EPNs could offer a sustainable alternative to chemical pesticides, and thus have been under intensive research in this context (Lewis et al. 2006). The genera Steinernema and Heterorhabditis are particularly promising, as these obligate parasites of insects rapidly kill their host after initial infection (Lewis et al. 2006). The infective juveniles (IJs), the free-living stage of EPNs, have evolved various strategies to locate and enter an insect host. Once inside, they release their symbiotic bacteria in the homeocel, which produce toxins and cause a lethal septicemia within 2 to 3 days (Adams and Nguyen 2002; Dillman et al. 2012). The EPNs feed on the bacteria and reproduce into the cadaver. When the resource is depleted, a new generation of IJs is produced, and they leave the carcass and find new hosts (Dillman et al. 2012; Kaya and Gaugler 1993).

Despite their efficiency in killing insect hosts, the use of EPNs as biocontrol control agents for soil insect pests remains challenging. One of the primary constraints is their short shelf life. Infective juveniles survive about a month in refrigerated vermiculite formulations, which is the prevailing storage method of commercially available EPN (Shapiro-Ilan et al. 2006). Prolonged storage diminishes EPN quality and their ability to reach a good level of control of the target pest (Grewal 2002). Thus, storage limitation has been a critical aspect in EPN formulations. In addition, EPNs usually are sprayed on top of the soil, exposing them to UV light and desiccation (Lello et al. 1996). These constraints make the use of EPNs costly and only marginally effective in large scale application (Georgis et al. 2006). We propose that triggering a state of quiescence, to prolong shelf life, in combination with novel application methods may render EPNs much more effective as biological control agents.

The state of quiescence is characterized by a straight shape and non-motile state of nematodes. Quiescence normally is triggered by unfavorable environmental conditions, such as extreme temperature, lack of oxygen, a lack of moisture, and/or an osmotic stress (Barrett 1991). During this state of dormancy, the metabolism of nematodes is strongly reduced, allowing them to conserve energy, which can significantly prolong their lifespan and infectiousness (Hiltpold et al. 2014). Quiescence is reversible when the conditions turn more favorable. Quiescence also can be chemically triggered, for example with glycerol (Chen and Glazer 2005) or with compound(s) in exudates of root caps (Hiltpold et al. 2014; Hubbard et al. 2005; Zhao et al. 2000). The advantages of glycerol and the so-called quiescence factors (QFs) are that they provide full control of the nematodes quiescence by dose regulation, as well as of the recovery by simple dilution. Hiltpold et al. (2014) showed that exposure to pea root cap exudates conserves EPN motility, infectiousness, and lipid content, which implies that including QFs in EPN formulations has potential to enhance their efficacy. It therefore is worthwhile to identify the QF. However, collecting exudate is a tedious and highly time-consuming process. Moreover, the QF concentration contained in the exudate is low and its activity is quickly lost after a few dilutions (Hiltpold et al. 2014). It is for this reason that we set out to develop a high-throughput collection method that allowed us to obtain root extracts from root cap of green pea and maize, which were flash-frozen in liquid nitrogen.

To demonstrate that the root cap extract contains high concentrations of the QF, we incubated the EPN *Heterorhabditis megidis* in different dilutions of root cap extract and measured quiescence levels. To further improve the collection of QF, we also tested the possibility of using an extract of the entire root germinate instead of only the root cap. We compared quiescence of *H. megidis* IJs induced by the two different extracts. Furthermore, we determined whether quiescent IJs can recover from their inactive state and still efficiently penetrate and kill a target host. Two hosts were tested, larvae of the highly susceptible wax moth, *Galleria mellonella*, and larvae of the more resistant mealworm, *Tenebrio molitor* (Grewal and Peters 2005). In these tests, we compared the infectiousness of *H. megidis* IJs that had recovered from quiescence after storage in different root cap extract concentrations for 24 hr with the infectiousness of IJs that had been stored in water. Finally, because we hypothesized that quiescence is a response to oxygen deprivation, we also evaluated the effect of aeration of the storage solutions on the induction of quiescence in *H. megidis*.

Methods and Materials

Plants, Nematodes and Insects Resources Pea (*Pisum sativum* L.) seeds (variety Lancet, Wyss Samen und Planzen AG, Switzerland) were first sterilized in 95 % ethanol for 5 min, then rinsed and immerged in distilled water for 12 hr. Soaked seeds were placed in plastic boxes (15×13.5×5 cm^3) containing phytoagar 1.0 % (Duchefa Biochemie, Haarlem, The Netherlands) and incubated at 25 °C in the dark for 3 days.

Maize (*Zea mayz* L.) seeds (cultivar Delprim, DSP SA, Switzerland) were sterilized in distilled water with 10 % bleach for 12 hr. Soaked seeds were placed in plastic boxes (30×30×10 cm^3) kept moist on paper towels, and incubated at 25 °C in the dark for 3 days.

Heterorhabditis megidis was obtained from Andermatt Biocontrol SA, Switzerland. The EPNs were reared in the laboratory by infecting *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae, and new generations of IJs were recovered in White traps (White 1927) and stored at 10 °C before use. All experiments were performed with new, fresh IJs that were not more than 2-week-old.

One to 2-week-old larvae of the wax moth *Galleria mellonella* were used to rear the EPNs and for the infectiousness bioassays. These insects were obtained from Au Pêcheur SARL Neuchâtel (Switzerland). For the infectiousness test, we also used mealworm larvae (*Tenebrio molitor*), which were obtained from the University of Lausanne (Switzerland).

Extraction of Root Caps We collected 19.8 g (for maize) and 18.6 g (for pea) of the terminal 10 mm of the roots. This material was ground under liquid nitrogen in a pre-cooled mortar. The obtained extract was split equally into two 50 ml Falcon tubes with 45 ml of MilliQ water, and subjected to ultrasonication for 1.5 hr. The root cap extract then was centrifuged at 4500 rpm for 10 min (Universal 16, Hettich Zentrifugen). The supernatants were pooled per plant species and filtered over a filter paper (Whatman 90 mm Ø, 4–7 μm particle retention) *in vacuo* and lyophilized, which resulted in 1.49 g (maize) and 1.52 g (pea) of dry root cap extract. The dry extracts were stored at −80 °C until further use. For maize, we repeated the entire procedure with the remaining parts of the root (root extract), thus excluding the ten first millimeter of the root cap.

Collection of Root Cap Exudate Following Hubbard et al. (2005) and Hiltpold et al. (2014), the root cap exudate was collected from 15 pea germinates by placing the terminal 10 mm of the root caps in a 1 ml drop of MilliQ water on a Teflon plate for 2 min. Then, the water drop was collected and centrifuged at 14,000 rpm for 10 min at 18 °C (eppendorf AG, centrifuge 5424), and the supernatant was stored at −20 °C (root cap exudate).

Quiescence Induction Tests with *H. megidis* The sensitivity of *H. megidis* to the QF in the root cap extract and the root cap exudate at different concentrations was assessed following a previously described method (Hubbard et al. 2005; Hiltpold et al. 2014). A suspension containing 30 IJs per 50 μl of distilled water was prepared. Additionally, pea root cap extract was adjusted to seven different dilutions in MilliQ water: 10 mg (not diluted), 2 mg (×5), 1 mg (×10), 0.66 mg (×15), 0.5 mg (×20), 0.4 mg (×25), 0.33 mg (×30). Root cap exudate already in solution was adjusted to obtain the same seven dilutions (not diluted, ×5, ×10, ×15, ×20, ×25, and ×30). The control contained only MilliQ water. Suspensions (50 μl) with *H. megidis* IJs were poured into each well of a 96-well tissue plate (Sigma-Aldrich). Then, 175 μl of the different solutions of root cap extract or exudate were added, resulting in a total volume of 225 μl per well. After 12 hr, we counted the number of quiescent IJs in the wells (N=10/dilutions) under a microscope. Immobile and straight-shaped IJs were considered to be quiescent. The experiment was repeated three times with different batches of IJs. The same bioassay was used to assess the induction of quiescence by maize germinate root caps (0–10 mm, N=12) compared to the rest of the maize germinate root (>10 mm, N=12). For the maize extract, we tested a reduced number of dilutions (not diluted, ×5, ×10, ×20, and ×30). The experiment was replicated twice.

The percentages of quiescent EPN were compared using *Wilcoxon Signed-Rank test* performed in R version 2.15.2 (http://www.r-project.org/). Bonferroni correction was applied on the P-values to overcome multiple comparisons.

Recovery and Infectiousness of *H. megidis* The ability of *H. megidis* to infect and kill a host depending on storage (for 24 hr) in different concentrations of root cap extract was compared to *H. megidis* stored in water only. We tested the infectiousness using highly susceptible *G. mellonella* larvae, as well as more resistant *T. molitor* larvae. Suspensions (1.5 ml) of water with *H. megidis* at a concentration of 50 IJs in 10 μl were centrifuged in a 1.5 ml Eppendorf tube at 8000 rpm for 5 min (Eppendorf AG, centrifuge 5424), and the supernatant was replaced by different concentrations of root extracts (pea and maize). The final solutions were transferred into 4 ml glass tubes (BGB analytik, AG) for 24 hr to trigger quiescence before inoculation. The different concentrations of extract were: 10 mg (not diluted), 1 mg (×10), 0.5 mg (×20), 0.33 mg (×30), and water as control (N=15/concentration). Following Hiltpold et al. (2012), 50 ml Falcon tubes were filled with 60 % moist potting soil. One *G. mellonella* or one *T. molitor* larva was placed in an individual plastic specimen tube (1.5 ml Eppendorf tube pierced with 12 holes). The tubes containing the larvae then were each placed inside a 50 ml Falcon tube, 3 cm below the soil surface. They were placed at the edge of the tube so that the larvae could be observed. To each Falcon tube we added 50 μl of one of the dilutions, or water as control,

containing IJs (ca. 250). Each solution was poured onto the soil, and a 1 cm soil layer was added to cover the drop. The tubes were not fully closed to allow gas exchange, and they were stored in the dark at room temperature. Every treatment was replicated 15 times. Every day, the success of *H. megidis* in infecting the larvae was evaluated for each tube by visual inspection, looking for red larvae, which is indicative of infection (Forst and Clarke 2002). Immobile larvae also were checked for mortality. Overall larval survival was evaluated 7 days after the start of an experiment. Parametric survival analyses were performed to evaluate significant differences in larval survival using the *survival* package of R program version 2.15.2 (http://www.r-project.org/). The Weibull distribution of error produced the minimum error deviance and was therefore selected for further analyses.

Quiescence Factor Aeration To verify if an excess availability of oxygen interferes with the triggering of quiescence, a set of three experiments was performed. The general setup involved ca. 1000 IJ's in 50 ml of water that were poured into a 4 ml glass tube (BGB analytik, AG). After application of the treatments described below, tubes were covered with one layer of parafilm to avoid evaporation, but allowing gas exchange. The tubes were stored at room temperature, and after 24 hr of exposure, quiescence was assessed.

In the first experiment, two different volumes of a solution of 1 mg of maize root cap extract were added to the tubes, a large volume of 1 ml (N=20) or a smaller volume of 200 μl (N=20).

In a second experiment, 1 ml of a solution of maize root cap extract at a concentration of 1 mg was added, half of the tubes were placed on an agitator to oxygenate the solution at 400 tr/min (Edmund Bühler Compact Mixer Shaker KL-2) for 24 hr (N=20) and the other tubes (N=20) were just placed on a shelf next to the agitator. All tubes were kept at room temperature.

In the third experiment, maize root cap extract was prepared in a 250 ml Falcon tube and oxygenized for 3 hr with an aquarium air pump, before IJs were placed in the solution. In parallel, a similar solution was made without pump aeration. One ml of the oxygenized solution (N=20) and non-oxygenized solution (N=20) were added to the tubes with the IJs. Twenty vials containing only 1 ml of water served as controls. The purpose of this latter experiment was to specifically test if the QF would become inactive after exposure to excess oxygen.

Every experiment was replicated twice. Binomial GLM performed in R version 2.15.2 (http://www.r-project.org/) was used to assess difference between treatments.

Results

Extract *Versus* Exudate Root cap exudate was efficient only in inducing quiescence in *H. megidis* when not diluted, resulting in 90.34 % of quiescent EPNs, whereas there was no quiescence in the water controls (Bonferroni corrected P-value<0.001, W=900, P<0.001). All other root cap exudate dilutions were not significantly different from the water control (α<0.001; ×5: W=309.5, P=0.03; ×10: W=240.5, P=0.001; ×15: W=337.5, P=0.09; ×20: W=374, P=0.2; ×25: W=278.5, P=0.01; ×30: W=408.5, P=0.54). In contrast, root cap extract induced 100 % quiescence even at a 15× dilution, and quiescence induction was not significantly different from the undiluted root cap extract up to the 25× dilution (α<0.001; ×1, ×5, ×10 and ×15 induced 100 % quiescence, ×20: W=525, P=0.02; ×25: W=585, P=0.001; ×30: W=780, P<0.001). The undiluted root cap exudate was less effective in inducing quiescence than the undiluted root cap extract (α<0.001; W=765, P<0.001), this difference persisted until the×20 dilution of the extract (α<0.001; 25×: W=609, P=0.012; Fig. 1).

Maize Root Cap Extract *Versus* the Rest of Root Extract Extract obtained from maize roots also were efficient in inducing quiescence in *H. megidis*. Root cap extract and the "rest" of the root extract were equally efficient in inducing

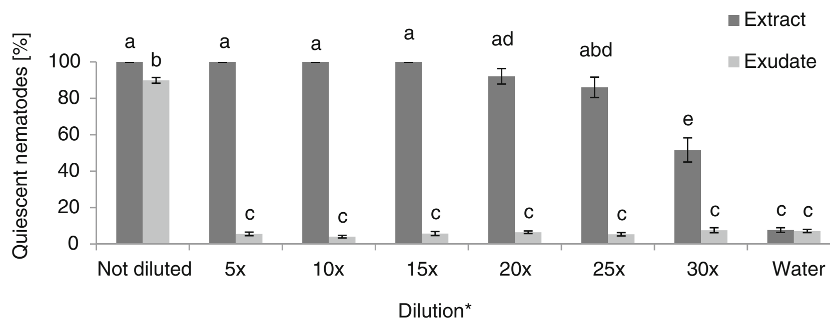

Fig. 1 Differences between pea root cap extract and root cap exudate in inducing quiescence in *Heterorhabditis megidis*. Overall, extract obtained by grinding the roots was more efficient in inducing quiescence than exudate. Only undiluted exudate induced more quiescence than water, whereas all of the tested extract dilutions induced more quiescence than water. *Different small letters* indicate statistical differences. *Bars* indicate standard error. *The values indicate dilutions of the root cap extract and root cap exudate. They do not include the additional 50 μl of infective juveniles (IJs) suspension that was added

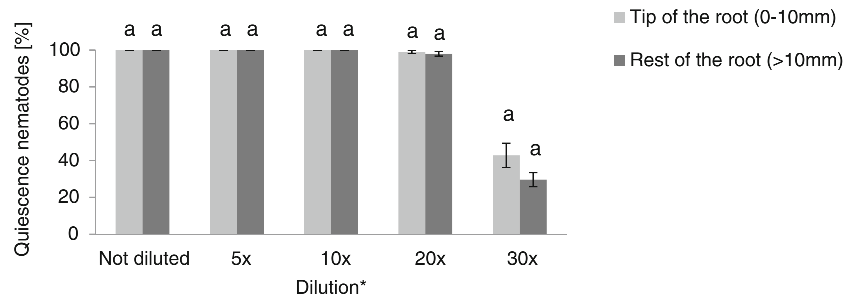

Fig. 2 Comparison of quiescence induction between extract from the first 10 mm of maize roots with extract from the "rest" of the root. The root extracts induced 100 % quiescence until the 1 mg dilution. *Small letters* indicate statistical differences. *Bars* indicate standard error. *The values indicate dilutions of the root cap extract and the rest of the root extract. They do not include the additional 50 µl of infective juveniles (IJs) suspension that was added

quiescence. The extracts induced 100 % quiescence until a dilution of 10× for both the root cap and the rest of maize roots. There also was no difference in QF activity between the extracts of the root cap and the extract of the rest of the root for the ×20 and ×30 dilutions (Bonferroni corrected *P*-value=0.002; not diluted and ×10 induced 100 % of quiescence; ×20: *W*=274, *P*=0.68; ×30: *W*=387, *P*=0.04; Fig. 2).

Recovery and Infectiousness of *H. megidis* Pea extract: *H. megidis* exposed to the different pea germinate root cap extract dilutions and water differed in their ability to kill *G. mellonella* larvae after recovery from quiescence (Weibull model: *Chisq*=13.21, *P*=0.001; Fig. 3). Overall, 81.3 % of *H. megidis*-exposed larvae were dead at the end of the experiment, and 96.7 % of the dead larvae displayed a red color. Except for the undiluted extract, IJs from all pea germinate extract dilutions were able to kill the host just as well as IJs that were kept in the control condition (water) (not diluted: *Z*=2.993, *P*=0.002; ×10: *Z*=0.114, *P*=0.9; ×20: *Z*=1.469, *P*=0.1; ×30: *Z*=−0.453, *P*=0.6, respectively; Fig. 3).

Maize extract: *H. megidis* exposed for 24 hr to the different maize germinate root cap extract dilutions or to just water did not significantly differ in their ability to kill *G. mellonella* larvae after recovery from quiescence (Weibull model: *Chisq*=6.09, *P*=0.19). Overall, 92 % of the larvae were dead at the end of the experiment, and 89.70 % of the dead larvae displayed a red color. In all maize germinate extract dilutions, the IJs were able to kill *G. mellonella* larvae just as well as the ones that were kept in the control condition (water) (not diluted: *Z*=1.212, *P*=0.2; ×10: *Z*=−1.31, *P*=0.1; ×20: *Z*=−0.068, *P*=0.9; ×30: *Z*=−0.564, *P*=0.5; Fig. 4). Nevertheless, as for the IJs exposed to pea root extract, the "undiluted" treatment was the one with the lowest number of dead larvae (24 % of larvae remained alive at the end of the experiment compared to 0, 7.6, 6.66, and 6.66 % for the ×10, ×20, ×30 dilutions and water, respectively, Fig. 4).

The results were similar when we used *T. molitor* larvae. Infective juveniles exposed for 24 hr to the different maize germinate root cap extract dilutions, or to just water did not significantly differ in their ability to kill *T. molitor* larvae after recovery from quiescence (Weibull model: *Chisq*=2.86, *P*=0.58). Overall, 85.3 % of the larvae were dead at the end of the experiment and 90.6 % of the dead larvae displayed a red color. Infective juveniles from all extract concentrations were able to kill *T. molitor* larvae just as well as the ones that were kept in the control condition (water) (not diluted: *Z*=0.794,

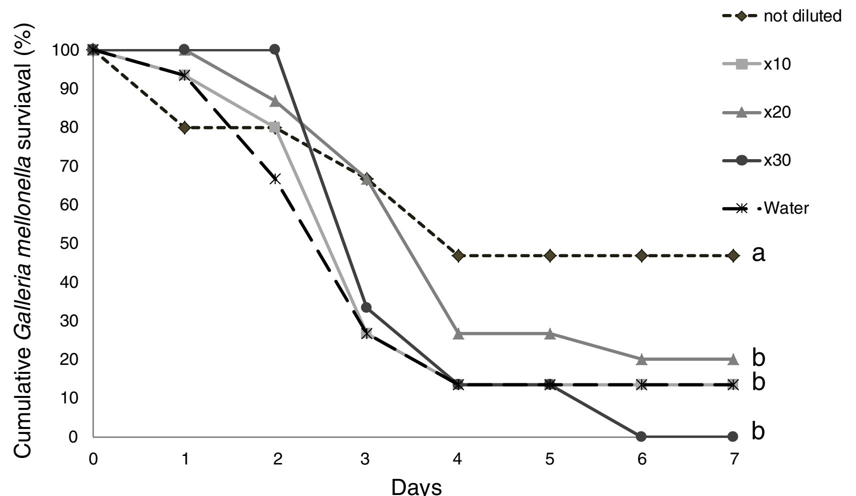

Fig. 3 Ability of *Heterorhabditis megidis* infective juveniles (IJs) to kill *Galleria mellonella* larvae after incubation in pea root extract (24 hr), as compared to incubation in just water. Nematodes incubated in all extract dilutions except the undiluted extract were as efficient as nematodes kept in water in killing host larvae. *Different small letters* indicate statistical differences

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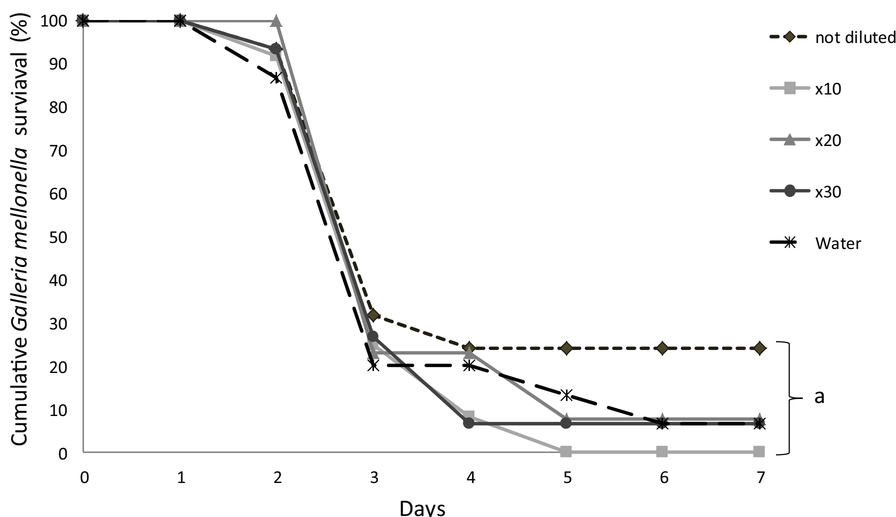

Fig. 4 Ability of *Heterorhabditis megidis* infective juveniles (IJs) to kill *Galleria mellonella* larvae after incubation in maize root extract (24 hr), as compared to incubation in just water. Nematodes incubated in all extract dilutions were as efficient as nematodes kept in water in killing host larvae. *Different small letters* indicate statistical differences

P=0.4; ×10: *Z*=1.33, *P*=0.1; ×20: *Z*=1.005, *P*=0.3; ×30: *Z*= 0.021, *P*=0.9). Although at the end of the experiment the numbers of *G. mellonella* and *T. molitor* larvae killed were comparable, we found that the infection was slower for *T. molitor* (Weibull model: *Chisq*=15.27, *P*=0.019) (Fig. 5).

Aeration of the Quiescence Factor All the *H. megidis* IJs in the vials exposed to a large volume of solution of root cap extract were quiescent, contrary to all the vials filled with the smaller volume of extract, which only had a few quiescent IJs (*DF*=78, *P*<0.001). Moreover, none of the IJs exposed to root cap extract in vials that were shaken were quiescent, contrary to those of the unshaken vials (*DF*=78, *P*<0.001). This appeared not to be due to inactivation of the QF after exposure to oxygen, as IJs in oxygenated root cap extract showed the same rate of quiescence as IJs exposed in non-oxygenated root cap extract (*DF*=78, *P*=1). Finally, the IJs in the control vials with a larger volume (1 ml) of water showed no quiescence, unlike IJs in the same volume of root cap extract (*DF*=78, *P*<0.001) (Table 1).

Discussion

For an optimal EPN formulation, especially in the context of commercialization, a prolonged infectiveness of EPNs is decisive (Grewal 2002). The possibility to induce quiescence in nematodes is a highly promising way to prolong the shelf life of EPNs and to maintain infectiveness for an extended period of time (Hiltpold et al. 2014). Quiescent factor is found in various genera of plants, and triggers a state of quiescence in all types of nematodes. Normally, the QF is collected from the exudate of root caps (Hiltpold et al. 2014; Hubbard et al. 2005). This collection method has several drawbacks, as it is time consuming and a tedious process, and only small quantities can be obtained. In this paper, we present a new and efficient way to collect QF

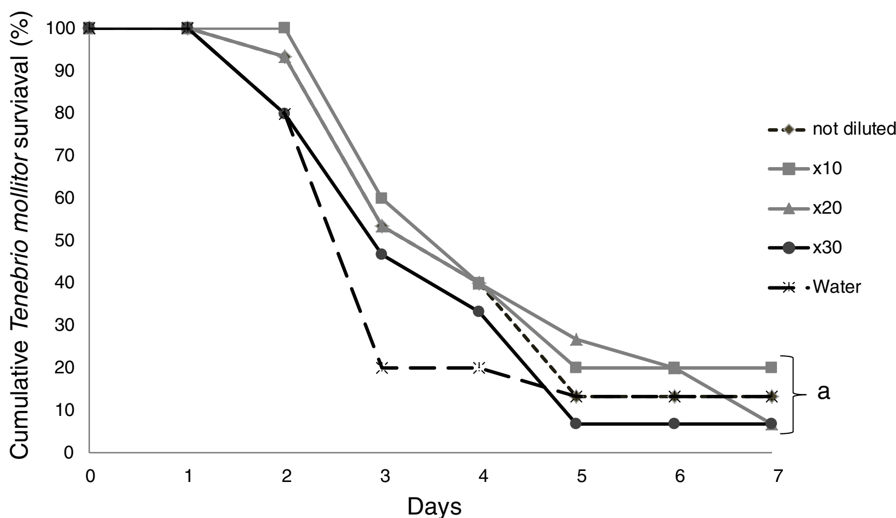

Fig. 5 Ability of *Heterorhabditis megidis* infective juveniles (IJs) to kill *Tenebrio molitor* larvae after incubation in maize root extract (24 hr), as compared to incubation in just water. Nematodes incubated in all extract dilutions were as efficient as nematodes kept in water in killing host larvae. *Different small letters* indicate statistical differences

Table 1 Effect of volume, agitation and aeration of extract on *Heterorhabditis megidis* quiescence

Parameter	Volume		Agitation		Aeration		Control
Treatment	1 ml extract	200 μl extract	No agitation	Agitation	No aeration	Aeration	1 ml water
H. megidis quiescence	+	−	+	−	+	+	−

from plants by using extraction of flash-frozen roots in liquid nitrogen. The root extracts that we obtained had high concentrations of QF and in all cases triggered 100 % quiescence at dilutions as low as 0.5 mg for pea root cap and 0.66 mg for maize root cap (0.5 mg, was not tested for maize). In contrast, root cap exudate had only low levels of QF and quickly lost its activity once diluted, which is consistent with the results obtained by Hiltpold et al. (2014).

Previous research on the QF focused on the root cap, and it was not known that the QF also is present in other parts of the root. Here we showed that the QF is constitutively produced in the entire root. The root cap extract and the extract of the "rest" of the root were equally efficient in triggering quiescence in *H. megidis*. This finding implies that considerably more plant material can be used to collect the QF. Whether the other root parts also release the QF into the rhizosphere remains to be determined, and we cannot exclude the possibility that the quiescence inducing compound(s) present in the rest of the root are different from those in the root cap.

It is important to note that a highly concentrated extract might contain too much QF for EPNs to recover from quiescence. This is important to consider when extracts are used in EPN formulation. We therefore tested the efficiency of the *H. megidis* IJs to recover from the state of quiescence, as well as their infectiousness after quiescence. For this, we used the relatively resistant host species *T. molitor*, as well as the very susceptible model species *G. mellonella*, and found that, after recovery, all the IJs exposed to the different dilutions of pea and maize extract for 24 hr were able to kill both hosts efficiently. The results imply that EPNs, even after exposure to high doses of QF, can be released in crop fields in a state of quiescence and still be able to kill a target pests.

In previous assays, we had noticed that root cap extract did not always trigger quiescence in *H. megidis* IJs. After further investigation, it was found that overexposure to air was possibly responsible for this absence of quiescence. We subsequently showed that quiescence induction by maize root cap extract is efficient only under relatively low oxygen conditions. Indeed, IJs kept in a small volume of root cap extract, representing a thin layer of solution and thus considerable exposure to air, did not turn into a state of quiescence, contrary to IJs that were kept in a larger volume (thick layer). Importantly, IJs kept in a larger volume of root cap extract that was shaken also failed to become quiescent. Aeration of the root cap extract by means of an aquarium pump extract did not have any effect; IJs that were placed in extract that had first been aerated (thick layer) all became quiescent. As aeration did not change the proprieties of the QF in the root cap extract, it is clearly not that the QF is sensitive to exposure to air, but the exposure of EPNs to air interferes with the triggering of quiescence. A possible explanation is that a slightly weakened condition due to lack of oxygen may facilitate the triggering of quiescence. For the identification of the QF, we plan to use liquid chromatography to fractionate the exudate and bioassay guided isolation of the active compound(s) from these fractions. From the current results we learned that for these bioassays it is essential that we maintain the right conditions to ensure that a loss of activity is due only to a loss of active compound(s).

For now, we can only speculate on the ecological role of QFs in nature. It may well be a root defense against phytopathogenic nematodes (PPNs). Indeed, PPNs are considerable more sensitive than EPNs and may not recover from quiescence when exposed to high concentrations of QF (Hiltpold et al. 2014). Even if they recover, "drugging" PPNs may be an effective way for root tips to escape infection (Hiltpold et al. 2014). The eventual identification of QF should shed more light on its function and importance in shaping plant-nematode interactions.

In summary, we demonstrated that flash-frozen pea and maize roots extracts are highly effective in inducing quiescence in *H. megidis,* and are far more concentrated in QF than root exudates. At all concentrations, the state of quiescence was reversible after placing the IJs in soil with a high water content, and the IJs were still highly infectious after recovery. However, we did find that there is a limitation of the use of root cap extracts to induce quiescence: induction works only under conditions of slightly reduced oxygen availability. By using the presented method to obtain high concentrations of QF, we hope to be able to identify the key compound(s) that is (are) for quiescence induction.

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