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Recruitment of entomopathogenic nematodes by insect-damaged maize roots

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Plants under attack by arthropod herbivores often emit volatile compounds from their leaves that attract natural enemies of the herbivores. Here we report the first identification of an insect-induced belowground plant signal, (*E*)-β-caryophyllene, which strongly attracts an entomopathogenic nematode. Maize roots release this sesquiterpene in response to feeding by larvae of the beetle *Diabrotica virgifera virgifera*, a maize pest that is currently invading Europe. Most North American maize lines do not release (*E*)-β-caryophyllene, whereas European lines and the wild maize ancestor, teosinte, readily do so in response to *D. v. virgifera* attack. This difference was consistent with striking differences in the attractiveness of representative lines in the laboratory. Field experiments showed a fivefold higher nematode infection rate of *D. v. virgifera* larvae on a maize variety that produces the signal than on a variety that does not, whereas spiking the soil near the latter variety with authentic (*E*)-β-caryophyllene decreased the emergence of adult *D. v. virgifera* to less than half. North American maize lines must have lost the signal during the breeding process. Development of new varieties that release the attractant in adequate amounts should help enhance the efficacy of nematodes as biological control agents against root pests like *D. v. virgifera*.

Plants are not simply passive victims of attacking herbivores; they have evolved an arsenal of physical and chemical defences to protect themselves. Often these defences are mobilized only in response to herbivory[1,2]. Among the proposed inducible defences is the production and release of volatile chemicals that could serve as signals to attract natural enemies of the herbivores[3–5]. Manipulating these signals can help increase the effectiveness of these natural enemies as control agents[6–8]. The induced emission of chemical signals is not limited solely to aboveground plant parts. The entomopathogenic nematode *Heterorhabditis megidis* was found to be attracted to exudates emitted by plant roots after damage by weevil larvae[9,10], but the nature of the attractants involved is unknown. Here we show that maize roots damaged by larvae of the economically important coleopteran pest *Diabrotica virgifera virgifera* LeConte are attractive to entomopathogenic nematodes, and we identify the chemical compound responsible for the attraction. *D. v. virgifera* or Western corn rootworm (WCR) is a voracious pest of maize that is responsible for the use of the bulk of pesticides applied in the cultivation of this crop in the USA[11]. The recent introduction and rapid spread of WCR into Europe has caused major concern for maize production on this continent and has stimulated the search for new methods of maize protection[12,13]. The use of nematodes to control WCR is an ecologically sound option[14,15], especially if researchers can optimize their efficacy at finding and killing WCR.

Attraction of nematodes by WCR-damaged roots

To determine whether or not WCR-infested maize plants would attract nematodes, three glass pots each containing one 10-day-old maize plant (var. Delprim) were attached to the arms of a custom-made six-arm olfactometer filled with moist (10% water) sand (Fig. 1a). The plants had been grown on clean sand in the pots, starting 5 days after seed germination. Three additional pots, containing only sand, were attached to the remaining three arms of the olfactometer. Four such olfactometers, each containing three plants plus three sand controls, were prepared on a given day. One plant of each set of three received four second-instar or third-instar WCR larvae, the roots of a second plant were damaged daily by stabbing them five times with a metal corkborer 7 mm in diameter, and the third plant was left unharmed. On day 3 after initial damage, about 2,000 *Heterorhabditis megidis* nematodes were released in the centre of each olfactometer, where they were free to enter the arms until their passage was blocked by an ultra-fine metal screen (see Fig. 1a and Methods). One day after release, the number of nematodes in each arm was recorded. Significantly more nematodes were recovered from arms connected to the pots with the WCR-damaged plants than from the arms connected to the other treatments or controls (Fig. 1b), indicating that damage by WCR induces maize roots to release a nematode attractant.

Identification of the attractant

Maize leaves had previously been shown to emit a mixture of volatile compounds in response to damage by caterpillars[4]. To determine whether WCR damage induces similar changes in plant volatiles, the leaves and roots from WCR-damaged (3 days) and healthy maize plants were ground and volatiles collected by solid-phase micro-extraction (SPME) were analysed by gas chromatography–mass spectrometry (GC–MS). A marked difference between the treatments was that the sesquiterpene (*E*)-β-caryophyllene was present in roots damaged by WCR but was completely absent from undamaged roots (Fig. 1c). The damaged roots contained small amounts of α-humulene and caryophyllene oxide as well. To a smaller extent, the WCR-induced increase in (*E*)-β-caryophyllene content was also apparent in the leaves (Fig. 1d). To test whether (*E*)-β-caryophyllene was indeed attractive to *H. megidis*, an authentic standard (Sigma-Aldrich, more than 98% pure) was tested in the olfactometer. For this purpose the system was entirely filled with clean moist sand and a 0.2-μl dose of (*E*)-β-caryophyllene was

injected in the centre of one of the pots, whereas the five remaining pots received no such treatment. Nematodes were released in the middle of the olfactometer and on the next day nematodes were recovered from the six arms. The arm attached to the pot that had received (*E*)-β-caryophyllene contained almost three times as many nematodes as the average control arm (Fig. 2a). Using a much lower dose of three injections with 200 ng of (*E*)-β-caryophyllene in pentane produced very similar results. In an additional experiment, a 10-day-old healthy maize plant (var. Delprim) was placed in each of two opposing pots of the olfactometer and the other four pots contained sand only. One of the pots with a plant was spiked with a 0.2-μl dose of (*E*)-β-caryophyllene and nematodes were released in the olfactometer centre. On the next day, the arm with the caryophyllene-spiked plant contained on average almost fourfold as many nematodes as the control arms, whereas there was no statistical difference between the plant without (*E*)-β-caryophyllene and the control pots (Fig. 2b). We tested several other synthetic compounds that are commonly released from caterpillar-damaged maize leaves in three choice tests, always including (*E*)-β-caryophyllene as one of the choices (data not shown). These compounds either were not attractive (linalool) or were significantly less attractive than (*E*)-β-caryophyllene ((*Z*)-3-hexenyl acetate, methyl salicylate, (*E*)-β-farnesene, α-humulene and (*E*)-nerolidol) at a 0.2-μl dose per pot.

Loss of signal in North American maize genotypes

The very limited number of compounds in the volatile blend obtained from WCR-induced roots is in striking contrast to what is emitted from maize leaves in response to caterpillar feeding, a complex mixture of different terpenoids, aromatic compounds and green-leaf volatiles[4,16,17]. Many of the maize lines that we have screened in the past for caterpillar-induced leaf volatiles do not emit (*E*)-β-caryophyllene in detectable amounts. This is particularly characteristic for most varieties that originate from North American breeding programmes[18]. We tested whether this difference also holds true for the roots by measuring WCR-induced (*E*)-β-caryophyllene in six inbred lines selected from a study on caterpillar-induced leaf emissions[18]: three that emitted large amounts (*E*)-β-caryophyllene from their leaves (Du101, F2 and F268) and three lines that released no or very little (*E*)-β-caryophyllene (F584, A654 and F7001). In this experiment we also included the closest wild ancestor of maize, teosinte (*Zea mays parviglumis*[19,20]), which is known to release relatively large amounts of (*E*)-β-caryophyllene from its leaves in response to caterpillar feeding[17]. Ten-day-old plants were subjected to 3 days of WCR feeding, after which (*E*)-β-caryophyllene levels were measured in the roots as above. Teosinte roots were found to release moderate amounts of (*E*)-β-caryophyllene in response to WCR damage (Fig. 3a). The experiment also confirmed a correlation between the levels of (*E*)-β-caryophyllene induced in the leaves and the roots (Fig. 3a): Du101, F2 and F268 emitted considerable amounts of (*E*)-β-caryophyllene from the roots after WCR attack and F584, A654 and F7001 emitted barely detectable amounts. These differences offered an excellent opportunity to test whether (*E*)-β-caryophyllene is a key compound for nematode attraction, because non-emitting varieties should be far less attractive than emitting varieties. This was tested with representative lines of commercial maize for which we had information on caterpillar-induced (*E*)-β-caryophyllene releases[17].

Pactol is a commercial maize variety that releases no detectable amounts of (*E*)-β-caryophyllene from its leaves in response to caterpillar feeding, whereas Graf releases relatively large amounts, significantly more than the variety Delprim, which was used in the first experiments[17]. Root extracts from WCR-damaged plants confirmed the presence of (*E*)-β-caryophyllene in Graf and Delprim

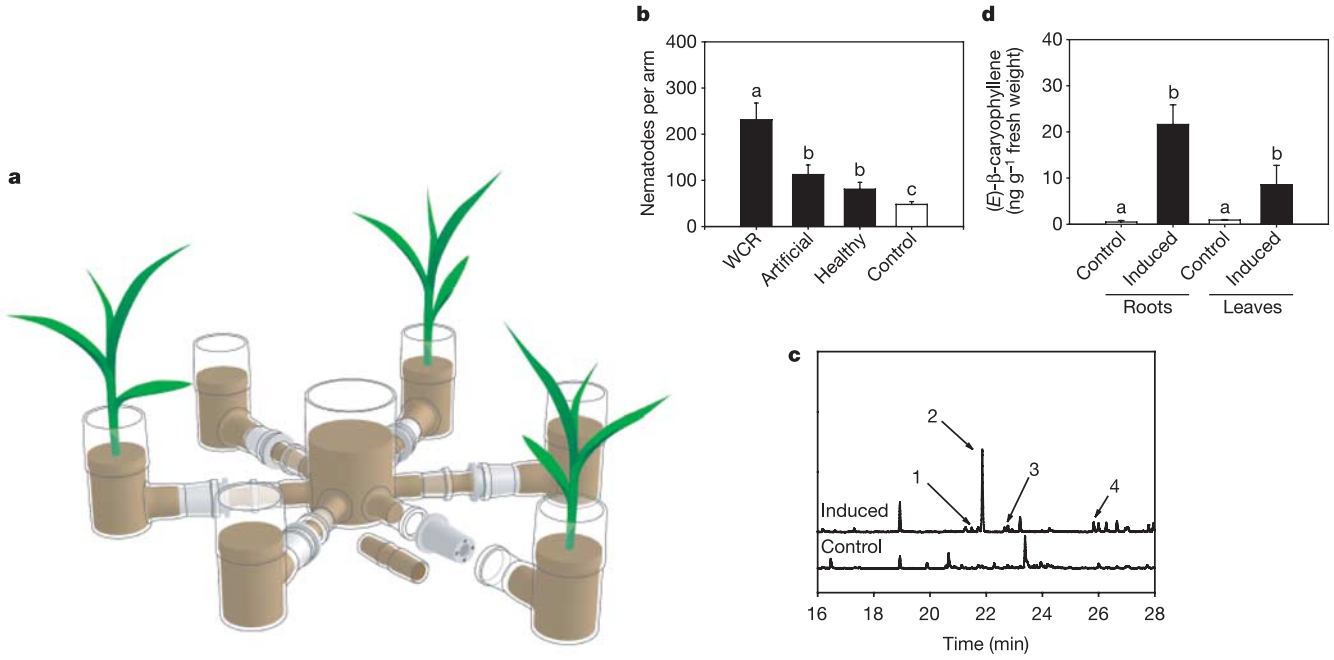

Figure 1 Attraction of entomopathogenic nematodes to a WCR-induced root signal. **a**, Drawing of a newly designed belowground six-arm olfactometer in which nematode attraction was tested. **b**, Choices between plants: the average number of nematodes recovered from olfactometer arms that were connected to pots holding either a maize plant with WCR-damaged roots, mechanically damaged roots or undamaged roots ($n = 12$). For each replicate, the total number of nematodes that went to the three control pots (only moist sand) were summed and divided by three. **c**, Typical chromatographic traces obtained from the roots of a healthy plant and of a WCR-damaged plant. The labelled peaks are as follows: 1, unknown sesquiterpene; 2, (*E*)-β-caryophyllene; 3, α-humulene; 4, caryophyllene oxide. **d**, Quantification of (*E*)-β-caryophyllene in roots and leaves from healthy and WCR-damaged maize plants ($n = 6$). Letters above bars indicate significant differences. Error bars indicate standard errors.

roots and its absence from Pactol roots (Fig. 3b). Next, individual plants of these three varieties were tested simultaneously in the olfactometer by letting four third-instar WCR larvae feed on their roots for 3 days and then releasing nematodes from the olfactometer centre as before. The numbers of nematodes recovered from the olfactometer arms revealed strong attraction to Graf and Delprim and no attraction to Pactol (Fig. 3c). The importance of (*E*)-β-caryophyllene for this difference in attractiveness was confirmed in a nearly identical experiment with the three varieties, except that on the third day of WCR feeding 0.2 μl of (*E*)-β-caryophyllene was added to the sand in the pot with the Pactol plant. After this treatment the Pactol plant was as attractive to the nematode as the two other plants (Fig. 3d).

Attractiveness in the field

To verify the importance of (*E*)-β-caryophyllene as an attractant for *H. megidis* under realistic conditions, we conducted two types of field experiment in Hungary, where WCR is already an established pest. For each experiment, six maize plants were planted at an equal distance from each other in circles 1 m in diameter. For the first experiment, three of the plants in each of 33 circles were of the variety Graf, alternated with three plants of the variety Pactol (Fig. 4a). Eight weeks after planting each plant was infested with six second-instar WCR larvae. Seven days after this infestation we released about 10,000 *H. megidis*, three times at 2-day intervals, in the centre of each circle. Larval infection rate by nematodes was determined by collecting the roots with larvae for 15 circles at 3 days after the last nematode release. For the remaining 18 circles, larvae were left to pupate and sleeve cages were placed around the plants at least 1 week before expected adult emergence. In circles with nematode release, the infection rate for larvae on Graf (43.6% of the recovered larvae) was more than fivefold that for larvae on Pactol (8.3% of the recovered larvae; Fig. 4b). This nematode effect was also evident from a significantly lower emergence of adults from Graf roots (Fig. 4c).

More direct evidence for the importance of (*E*)-β-caryophyllene was obtained with a second experiment with only the Pactol variety planted in the six-plant circles. Again, all plants were infested with six WCR larvae. The soil directly next to three of the plants per circle was spiked on a daily basis with 2 μl of (*E*)-β-caryophyllene for 5 days (Fig. 5a). One day after the first spiking (7 days after WCR infestation), about 10,000 nematodes were released in the centre of each circle; this was repeated twice at 2-day intervals. We recovered relatively few larvae (18% as opposed to 40% for the Pactol–Graf experiment) from the 12 circles that had been reserved to measure infection rates. This was probably due to poor irrigation of these circles, which could also explain why we did not observe a difference in infection rate between treatments. However, the results from the 24 circles that were left to measure adult emergence showed a significant effect of (*E*)-β-caryophyllene, with a more than twofold decrease in adult emergence for the plants that had been spiked with the signal (Fig. 5b).

The possibility that there could have been a direct effect of (*E*)-β-caryophyllene on the WCR larvae or on the quality of the plant was tested in subsequent laboratory experiments. Equal amounts of (*E*)-β-caryophyllene to those in the field experiments were injected in 15 0.5-litre pots each containing a maize plant and five WCR larvae, whereas 15 other pots each containing a plant and five larvae

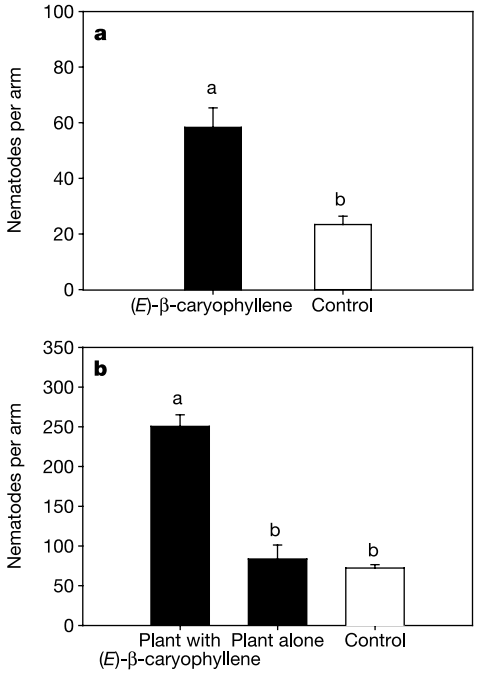

Figure 2 Attraction of *H. megidis* to authentic (*E*)-β-caryophyllene. **a**, Average number of nematodes recovered from olfactometer arms connected to a pot spiked with 0.2 μl of (*E*)-β-caryophyllene compared with those recovered from arms connected to untreated pots ($n = 12$). **b**, Average number of nematodes recovered from olfactometer arms connected to a pot with a healthy maize plant and spiked with 0.2 μl of (*E*)-β-caryophyllene, an arm connected to a pot with a healthy plant only, and four control pots with moist sand only ($n = 12$). For each replicate, the results for control pots were summed and divided by the number of control pots. Different letters above bars indicate significant differences. Error bars indicate standard errors.

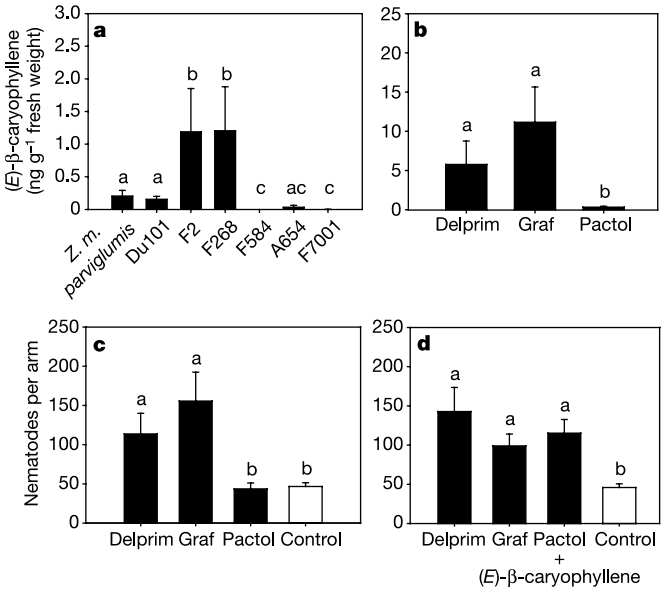

Figure 3 The absence of the (*E*)-β-caryophyllene signal in certain maize genotypes renders these plants unattractive to the nematode. **a**, Average amounts of (*E*)-β-caryophyllene detected from the WCR-damaged roots of *Zea mays parviglumis* (teosinte), of three lines (Du101, F2 and F268) that are known to release (*E*)-β-caryophyllene from their leaves in response to caterpillar damage and of three lines (F584, A654 and F7001) that release no detectable amounts of (*E*)-β-caryophyllene from their leaves[18]. **b**, Average amount of (*E*)-β-caryophyllene extracted from WCR-damaged roots of the commercial maize varieties Delprim, Graf and Pactol ($n = 6$). **c**, Average number of nematodes recovered from olfactometer arms connected to pots holding WCR-damaged maize plant of the varieties Delprim, Graf and Pactol ($n = 12$). **d**, Average number of nematodes recovered from olfactometer arms connected to pots holding WCR-damaged maize plant of the varieties Delprim, Graf and Pactol, after the pot with Pactol was spiked with 0.2 μl of (*E*)-β-caryophyllene ($n = 12$). Statistical differences are indicated with different letters above the bars. Error bars indicate standard errors.

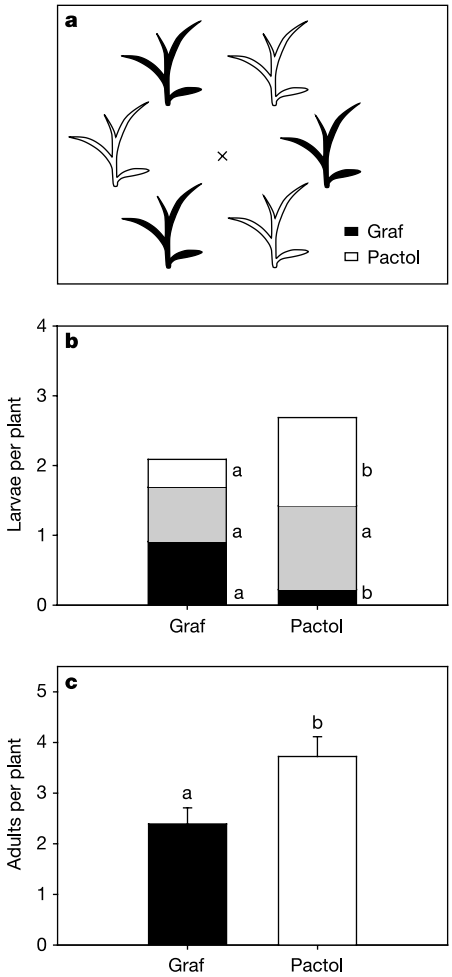

Figure 4 More WCR larvae were infected with nematodes and fewer adults emerged near Graf plants than near Pactol plants. **a**, Design of field circle experiment for which maize plants of the varieties Pactol and Graf were alternated. The cross marks the spot at which nematodes were released. **b**, Mean numbers of larvae per plant that were healthy (white areas), infected by fungi (grey areas) or infected by nematodes (black areas). Statistical differences between the proportions of the three larval types are indicated with different letters. **c**, The mean number of adults that emerged for each plant was significantly different for the two varieties ($P < 0.01$). Error bars indicate standard errors.

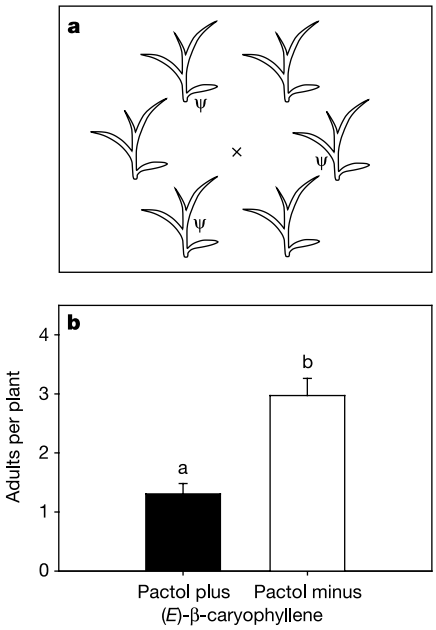

Figure 5 Fewer WCR adults emerged near Pactol plants that were spiked with (*E*)-β-caryophyllene than near Pactol plants that received no (*E*)-β-caryophyllene. **a**, Design of field circle experiment with only plants of the variety Pactol. The ψ signs mark the sites at which five times 2 μl of (*E*)-β-caryophyllene was injected into the soil; the cross marks the spot at which nematodes were released. **b**, The mean number of adults that emerged near the spiked plants was significantly lower than for the unspiked plants ($P < 0.0001$). Error bars indicate standard errors.

served as controls. No difference was found in the total number of adults that emerged from these pots (data not shown), supporting the hypothesis that nematode attraction to (*E*)-β-caryophyllene was responsible for the difference observed in the field.

Suitability of (*E*)-β-caryophyllene as a belowground signal

(*E*)-β-Caryophyllene is a common secondary plant compound that is also emitted from the silk of mature maize plants and has been shown to be weakly attractive to adult WCR females[21]. This sesquiterpene is probably not the only attractant for *H. megidis*, because some degree of nematode attraction was also found to healthy and mechanically damaged plants (Fig. 1b), even though emission of (*E*)-β-caryophyllene from maize leaves and roots has been detected only after herbivory. Indeed, several plant metabolites, including CO_2, are known to be attractants for entomopathogenic nematodes[22]. Cues that come directly from host larvae might also guide nematodes[23–26], but these have been shown to be attractive only over short distances. The overriding importance of (*E*)-β-caryophyllene as a long-range attractant is best indicated by its abundance in the root extracts of the most attractive varieties and the fact that supplementing sand with (*E*)-β-caryophyllene renders an otherwise unattractive variety highly attractive (Fig. 3d).

To test the ability of (*E*)-β-caryophyllene to diffuse in moist sand, 2 μg of this sesquiterpene were pipetted into one spot in a sand-filled glass dish. At a distance of 10 cm from this spot a SPME fibre was inserted into a hole in the sand (see Methods). Every half hour the compounds adsorbed on the fibre were desorbed and analysed by GC–MS, starting with the half hour before the addition of (*E*)-β-caryophyllene. (*E*)-β-Caryophyllene travelled rapidly through the sand and was already trapped on the fibre during the first half hour after it had been introduced to the sand. The amount trapped increased steadily for 2 h, after which it decreased sharply (Fig. 6a). A similar experiment in a sand-filled olfactometer, with an arm modified to permit the introduction of a SPME fibre, revealed the presence of (*E*)-β-caryophyllene in the centre part of an arm 2 h after injecting 0.2 μl into a pot connected to that arm (not shown). To determine whether the rapid decrease in (*E*)-β-caryophyllene detection was due to evaporation from the sand, an additional experiment was performed by which a drop containing 1 μg of (*E*)-β-caryophyllene was placed on the bottom of a beaker, which was immediately covered by 5 cm of moist sand. The beaker was placed in a closed-loop volatile-collection system where the headspace above the sand was continuously sampled at intervals of 30 min. A very similar time course of (*E*)-β-caryophyllene diffusion was obtained, with the first detection after 30 min and a peak after 2 h (Fig. 6b), indicating rapid evaporation. Recovery was more than 90%, which implies that the degradation of (*E*)-β-caryophyllene or its immobilization to sand particles is not significant under these conditions. The rapid diffusion of (*E*)-β-caryophyllene in moist sand and its chemical stability seem to make it exceptionally suitable as a belowground signal. In the olfactometer assays described above, the nematodes were released 25 cm from the treatment pots. Therefore, after detecting the signal they move a distance of more than 250 times their body length within a day.

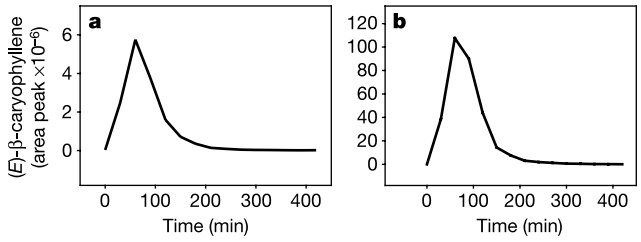

Figure 6 (*E*)-β-Caryophyllene diffuses readily through sand and then evaporates rapidly without breakdown or irreversible adsorption. **a**, Detection of authentic (*E*)-β-caryophyllene with a SPME fibre in moist sand at 10 cm from a release point, every half hour after release. **b**, Detection of (*E*)-β-caryophyllene in the headspace above a beaker containing 5 cm of moist sand after (*E*)-β-caryophyllene had been placed at the bottom of the beaker.

Discussion

The failure of most North American maize lines to release (*E*)-β-caryophyllene suggests that the ability to produce this compound has been lost during breeding. Indeed, the closest wild ancestor of maize, *Zea mays* ssp. *parviglumis*[19,20], was also found to release (*E*)-β-caryophyllene from its roots in response to WCR damage (Fig. 3a). The loss of direct defences to herbivores during plant domestication has been amply documented[27]. However, to our knowledge this is the first example of the loss of a signal involved in indirect defence.

WCR has already caused large economic losses to maize in Central Europe. Since 2003 it has been detected in almost all European countries south of Scandinavia, and will inevitably become a major threat to maize cultivation throughout Europe[28]. Effective, ecologically sound control methods are needed. Entomopathogenic nematodes could be an option[14,29–31], but they have not yet been employed with sufficient efficacy. The results of this study lead us to speculate that the absence of an attractive signal in many American maize lines could explain why attempts to control WCR with nematodes have yielded only mixed results on the North American continent[32,33]. Reintroduction of this signal in newly developed maize varieties might aid in effective control of this voracious pest.

This first identification of an inducible belowground plant signal that attracts enemies of root-feeding herbivores underscores the breadth and sophistication of indirect plant defences. With a growing interest in belowground plant-mediated interactions and their effects on various trophic levels[34,35] our results should prompt new studies into the evolutionary history and ecological consequences of multitrophic-level interactions and should lead to the exploitation of the signal for crop protection. □

Methods

Olfactometer assays

The attraction of nematodes to plant-produced substances was tested in a belowground olfactometer consisting of a central glass chamber (8 cm in diameter, 11 cm deep) with six equally distributed side arms at 0.5 cm height with a female (24 mm diameter × 29 mm long) connector (Fig. 1a). These arms connected the central chamber with six glass pots (5 cm in diameter, 11 cm deep) in which plants or other sources of attractants could be placed. Each pot also had a female connector (29/32) at 0.5 cm height. The connecting arms consisted of two detachable parts; one was a glass tube with ground-glass connectors (male, 24/29) on both sides, and the second part, a Teflon connector (24/29 to 29/32) was used to attach the glass tube to the odour source pot. The custom-made Teflon connectors (Analytical Research Systems) contained an ultra-fine metal screen (2,300 mesh; Small Parts Inc.) preventing the nematodes from reaching the odour source pots (Fig. 1a). For each experiment, the entire system was filled with sterilized white sand (Migros) to about 5 cm from the rim of the pots. Nematodes were released in a drop of water in the centre of the central pot. One day after nematode release, the olfactometer was disassembled and the sand in each detachable glass tube was placed on a separate cotton filter disk 19 cm in diameter (Hoeschele GmbH). The disk with the sand was placed in a Bearmann extractor[36,37], and nematodes in the collection tube were counted on the next day.

Statistical differences in choices made by the nematodes were determined with log-linear models on the basis of the assumption that the nematodes would disperse equally among the arms in the absence of any attraction. The models were adapted to account for possible overdispersion due to directional biases[38].

Root analyses

For the analysis of volatile terpenes, roots of WCR-damaged and undamaged maize plants were washed with water and frozen in liquid nitrogen; they were then pulverized in a mortar and 0.4 g of root powder was placed in a glass vial with a septum in the lid. A 100-μm polydimethylsiloxane (PDMS) SPME (Supelco) fibre was inserted through the septum and exposed for 60 min at 40 °C. The compounds adsorbed on the fibre were analysed by GC–MS with an Agilent 6890 Series GC system G1530A coupled to a quadrupole-type mass-selective detector (Agilent 5973; transfer line 230 °C, source 230 °C, ionization potential 70 eV). The fibre was inserted manually into the injector port (230 °C) and desorbed and chromatographed on an apolar column (DB5-MS, 30 m, 0.25 mm internal diameter, 0.25 μm film thickness; J & W Scientific). Helium at a constant pressure of 18.55 lb in^{-2} (127.9 kPa) was used for carrier gas flow. After fibre insertion, the column temperature was maintained at 50 °C for 3 min and then increased to 180 °C at 5 °C min^{-1} followed by a final stage of 3 min at 250 °C. Approximate quantification was performed with an external standard by performing analyses on 0.4 g of powdered root tissue from maize line B73 (which produces only traces of (*E*)-β-caryophyllene)[39] spiked with known amounts (4.5, 9.0, 45 and 90 ng) of this compound.

The (*E*)-β-caryophyllene in the roots was provisionally identified as the (−)-enantiomer by chromatography on a chiral column using published procedures for the separation of the two enantiomers[40]. However, the lack of a standard for the (+)-enantiomer prevented final confirmation.

Field experiments

Field experiments were conducted at the Plant Health Station in Hodmézövásárhely, in southern Hungary (46° 15.554′ N, 20° 09.743′ E), from April to October 2004. Six plants were grown from seed in 1-m-diameter circles and with a 1-m distance between circles.

Two types of circle were formed: one contained the two varieties *Zea mays* var. Pactol (Syngenta) and *Z. mays* var. Graf (Landi) and the other contained only the Pactol variety. Eight weeks after planting, each plant was infested with six WCR larvae by digging out 5 cm of soil near the base of the plant and dropping the larvae with some potting soil into the hole. The larvae came from a laboratory colony that had been established with field-collected adults the year before. At 7, 9 and 11 days after infestation, about 10,000 *H. megidis* nematodes were released in the centre of the treatment circles at a depth of about 10 cm. Additionally, half of the plants in the circles with only the Pactol variety were spiked daily with 2 μl of (*E*)-β-caryophyllene (more than 98% pure; Sigma-Aldrich) for 5 days, starting on the sixth day after infestation with WCR larvae (1 day before nematode release).

Two measurements were taken to determine the effect of the treatments on nematode effectiveness. For 15 of the Pactol–Graf circles and 12 of the Pactol–Pactol circles, the aerial part of the plants was removed and with a 1-litre core sample the roots and soil around it were collected. Larvae were extracted by crumbling the soil over a black plastic sheet and dissecting the roots. Each recovered larva was placed on a moist filter paper in a plastic Petri dish (5 cm in diameter, 2 cm deep) and stored at 17 °C for 1 month. They were checked weekly under a microscope for nematode infection, characterized by red pigmentation resulting from symbiotic bacteria, and for nematode emergence. Infections by other pathogens were also noted.

In addition, adult emergence was measured in another 18 Pactol–Graf circles and 24 Pactol–Pactol circles. For this, cylindrical sleeve cages (30 cm × 70 cm, MegaView Science Education Services Co. Ltd) were fixed on plastic cylinders 20 cm in diameter and 25 cm deep that were placed about 10 cm in the soil around each plant. The upper part of each sleeve was tightly attached around the stem of the plant to prevent adults from escaping. Once a week, from the beginning of July until the end of August, adults in the emergence cages were counted and collected until no more adults were found. The same log-linear models as employed for the olfactometer data were used to determine differences between treatments[38].

Diffusion measurements

A glass dish (15 cm in diameter, 8 cm deep) was filled with a 5-cm layer of moist (10% water) sand. With a micropipette, 2 μg of authentic (*E*)-β-caryophyllene (98% pure; Aldrich) in 10 μl of pentane was placed 3 cm deep in the sand at 2 cm from the dish side, immediately after which the hole was covered. At a distance of 10 cm from this spot, a hole 2 mm wide and 3 cm deep was made with a metal rod and a 100-μm PDMS SPME fibre was placed in the hole. Every 25 min the compounds adsorbed on the fibre were analysed by GC–MS essentially as described above, except that the mass selective detector was operated in the selective ion mode, scanning only for the characteristic ions at molecular masses 204, 133 and 93. After the 5-min desorption period, the fibre was placed back in the hole in the sand for a further 25-min collection. The first collection started 30 min before the (*E*)-β-caryophyllene sample was added to the sand, and the last collection was 7 h later.

To measure the time course of evaporation from sand, a 10-μl drop of dichloromethane containing 1 μg of (*E*)-β-caryophyllene was placed on the bottom of a 25-mm diameter glass beaker and was immediately covered by a 5-cm layer of 30 ml moist sand. The beaker was placed in a closed-loop volatile collection system consisting of a 1-litre desiccator in which the headspace above the sand was continuously collected by pulling air through a 75-mg activated charcoal filter at a rate of 2 l min^{-1}. The filter was extracted with dichloromethane at intervals of 30-min and the eluate was analysed by GC–MS as described above.

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