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The pathogen causing Dutch elm disease makes host trees attract insect vectors

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Dutch elm disease is caused by the fungal pathogen *Ophiostoma novo-ulmi* which is transmitted by the native elm bark beetle, *Hylurgopinus rufipes*. We have found that four semiochemicals (the monoterpene (−)-β-pinene and the sesquiterpenes (−)-α-cubebene, (+)-spiroaxa-5,7-diene and (+)-δ-cadinene) from diseased American elms, *Ulmus americana*, synergistically attract *H. rufipes*, and that sesquiterpene emission is upregulated in elm trees inoculated with *O. novo-ulmi*. The fungus thus manipulates host trees to enhance their apparency to foraging beetles, a strategy that increases the probability of transportation of the pathogen to new hosts.

Keywords: Dutch elm disease; *Ulmus americana*; fungal pathogen; *Ophiostoma novo-ulmi*; *Hylurgopinus rufipes*; semiochemicals

1. INTRODUCTION

Non-motile parasites that complete one or more stages of their life cycle in intermediate or definitive hosts can manipulate these hosts to optimize transportation to new hosts (Poulin 2002). For example, protozoan parasites, *Toxoplasma gondii*, cause their intermediate rat hosts to approach and be eaten by cats, the definitive host (Berdoy *et al.* 2000). Likewise, the fungal pathogen *Ophiostoma ulmi* kills elm trees (Hubbes 1999; Brasier 2001) and then requires transportation to new elms (Agrios 1988). Since its introduction into the United States in the 1930s, it has ravaged forest and urban American elms across the north-eastern United States and Canada. With the appearance in the 1960s of the more virulent strain *Ophiostoma novo-ulmi*, elms have been severely decimated across all geographical locations. In North America, *O. novo-ulmi* relies on the smaller European elm bark beetle, *Scolytus multistriatus*, or the native elm bark beetle, *Hylurgopinus rufipes*, to be transported to new host elms (Millar *et al.* 1986; Agrios 1988; Hubbes 1999). In the prairie regions of North America, *H. rufipes* can withstand cold winter temperatures (Agrios 1988), and is the primary vector of Dutch elm disease.

Plants are known to synthesize and emit semiochemicals in response to invading or damaging organisms (Turlings *et al.* 1990) in order to recruit natural enemies of those organisms. Tobacco (*Nicotiana tabacum*), cotton (*Gossypium hirsutum*) and maize (*Zea maize*) plants each produce distinct semiochemical blends in response to damage by caterpillars of two closely related herbivore species. The specialist parasitic wasp *Cardiochiles nigriceps* exploits these differences to distinguish infestation by its host *Heliocoverpa virescens* from that by the non-host *Heliocoverpa zea* (DeMoraes *et al.* 1998). Volicitin, *N*-(17-hydroxylinolenoyl)-L-glutamine, in the oral secretion of beet armyworms (*Spodoptera exigua*) triggers the release of plant semiochemicals which attract natural enemies of the caterpillar (Alborn *et al.* 1997; Paré *et al.* 1998). Insects that feed by sucking plant sap also induce changes in plants' semiochemicals to attract parasitic wasps (Guerrieri *et al.* 1993; Du *et al.* 1996; Powell *et al.* 1998). *cis*-Jasmone has been found to attract an insect predator and parasitoid of aphids (Birkett *et al.* 2000); it may even serve as a phyto-pheromone in plant–plant communications (Powell & Pickett 2003). Trees under attack by bark beetles that carry symbiotic fungi respond by forming necrotic lesions around the infection, and by increasing the concentration of allelochemicals with fungistatic properties within the lesions (Raffa 1988). In all these cases, the plants' response helps alleviate the impact of the damage caused by insects or fungi. Here, we show that the fungal plant pathogen *O. novo-ulmi* induces change in the elm's semiochemical blend that is detrimental to the tree in that it attracts insect vectors that kill the host and carry the pathogen to new hosts.

2. MATERIAL AND METHODS

(a) *Collection of semiochemicals from elm wood*

Trunk sections of American elm wood were cut and ground into fine sawdust which was weighed and placed in a Pyrex glass aeration chamber (15.5 cm inner diameter (i.d.)× 20 cm). For 96 h, a water-driven aspirator drew purified air at 1 l min^{-1} through the chamber and a downstream Pyrex glass column (140×5 mm i.d.) filled with Porapak Q (50–80 mesh, Waters, Milford, MA, USA). Volatiles were eluted from Porapak Q with 2 ml of freshly distilled pentane.

(b) *Analyses of volatiles*

Aliquots of Porapak volatile extracts were analysed by coupled gas chromatographic–electroantennographic

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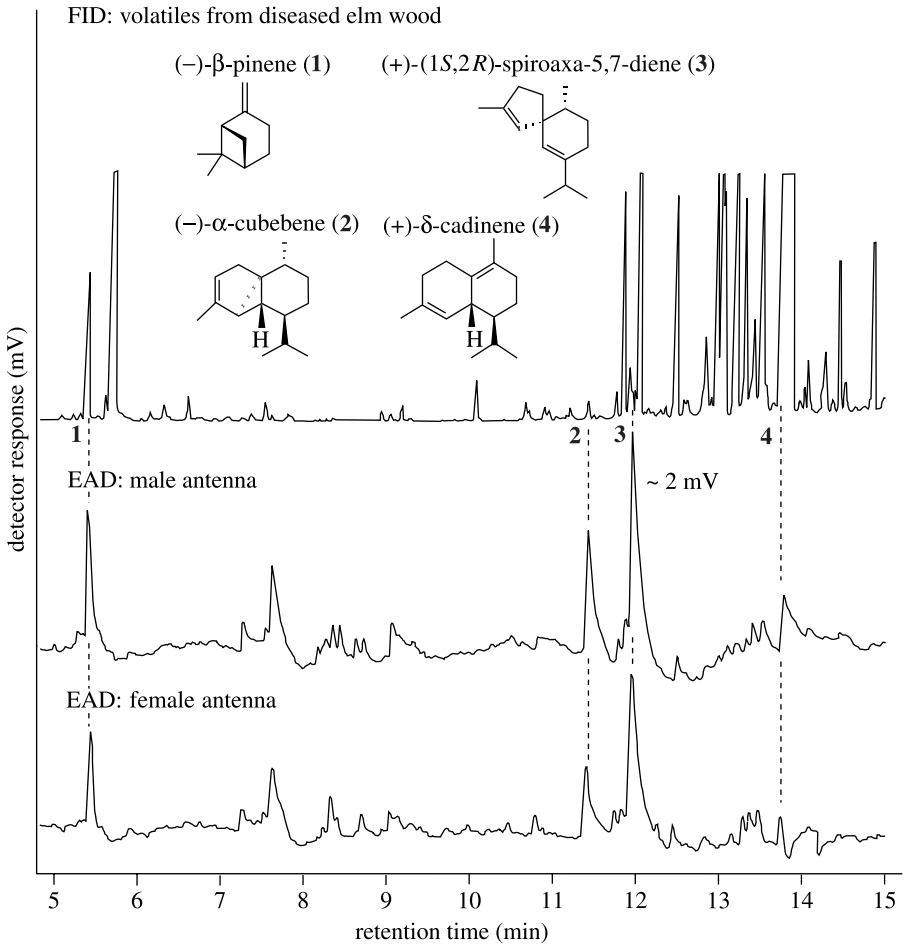

Figure 1. Gas chromatograms of volatiles (desorbed from Porapak Q) emanating from ground elm wood infected with the fungal pathogen *Ophiostoma novo-ulmi*. Hewlett Packard 5890A gas chromatograph with DB-5 column (30 m×0.32 mm i.d.; J&W Scientific, Folsom, CA 95630) with flame ionization (FID) or electroantennographic detector (EAD: male or female *Hylurgopinus rufipes* antenna); splitless injection; temperature program: 50 °C (2 min), then 10 °C min^{-1} to 280 °C.

detection (GC–EAD) (Arn *et al.* 1975; Gries *et al.* 2002). An *H. rufipes* antenna was removed and the base inserted into the tip of a glass capillary filled with a saline solution (Staddon & Everton 1980). The club of the antenna was pierced with a sharply pointed open tip of a second capillary also filled with saline. Volatiles that elicited responses from male or female antennae were analysed by GC–mass spectrometry (MS), employing a Varian Saturn 2000 Ion Trap GC–MS fitted with a DB-5 column (30 m×0.32 mm i.d.; J&W Scientific, Folsom, CA, USA).

High-performance liquid chromatography (HPLC) of samples employed a Waters LC626 and a Waters 486 variable wavelength UV/visible detector set to 210 nm, HP CHEMSTATION software (Rev.A.07.01), and a reverse-phase Nova-Pak C18 column (60 Å, 4 μm, 3.9×300 mm).

(c) *Acquisition of candidate semiochemicals*

(−)-β-Pinene (**1**) and (−)-α-cubebene (**2**) were purchased (Fluka Chemika-Biochemika, Buchs, Switzerland CH-9470; Sigma-Aldrich, Oakville, Ontario L6H 2J8). (+)-Spiroaxa-5,7-diene (**3**) was formed as a minor product by palladium-catalysed rearrangement of **2** during hydrogenation. Reduced palladium (5% on barium sulphate, 200 mg) was added to a solution of **2** (30 mg) in 10 ml pentane. While stirring, hydrogen was bubbled through the suspension. We monitored the reaction by GC analysis of aliquots and terminated it after 3–6 min when the yield of **3** reached its maximum (approx. 3%). Compound **3** was isolated from the mixture by HPLC on a reverse-phase column (see above) eluted with acetonitrile (1 ml min^{-1}). Elution with 88% aq. acetonitrile afforded (+)-δ-cadinene (**4**) which was also produced by heating a solution of **2** (20 mg) in 1,4-dioxane (1 ml) in the presence of 0.2 ml 0.1 M HCl (50 °C, 2–4 h) and extraction with pentane (Ohta *et al.* 1968).

To determine the molecular structure and absolute configuration of **3**, the dextro- and levorotatory enantiomers were synthesized by TiO_2/SO_4^{2-}-catalysed rearrangement (Polovinka *et al.* 2000) of (−)-*ent*-aromadendrene (a sesquiterpene derived from bicyclogermacrene which was isolated from the liverwort *Mylia taylorii*; von Reuβ *et al.* 2004) and (+)-aromadendrene, respectively, and analysed by GC on an octakis-(2,6-di-*O*-methyl-3-*O*-pentyl)-β-cyclodextrin column which separated them with complete baseline resolution.

(d) *Laboratory bioassay experiments*

Response of *H. rufipes* to aliquots of Porapak Q extract of diseased elm wood volatiles was tested in a Y-tube olfactometer (Delury *et al.* 1999) at 22–26 °C and 40–44% relative humidity. The olfactometer was enclosed on three sides with white poster board, and illuminated by two overhead light tubes (fluorescent GE Plant and Aquarium F40PL/AQ Wide Spectrum and Sylvania Daylight Deluxe F40DX 40W). Treatment and control odour sources were micro-pipetted onto Whatman No. 2 filter paper (13.5 mm diameter) assigned near the orifice of side arms. For each replicate, a new male or female beetle, a clean Y-tube, and new

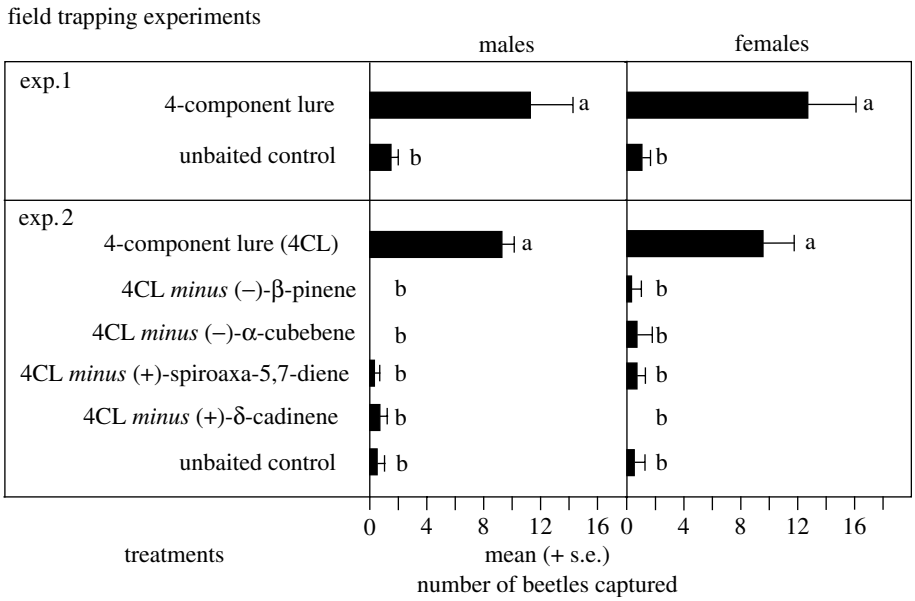

Figure 2. Results of field experiments 1 (2–5 June 2004; 12 replicates) and 2 (17–20 August 2004; 24 replicates), comparing captures of *H. rufipes* on adhesive traps baited with a blend of (−)-β-pinene, (−)-α-cubebene, (+)-spiroaxa-5,7-diene and (+)-δ-cadinene in release rates of, respectively, 25, 2, 2 and 184 μg per 24 h, or baited with lures lacking one of the four components (experiment 2). Location: near Regina, Saskatchewan, Canada. Bars with different letters are significantly different (ANOVA, Zar 1999, followed by Tukey–Kramer HSD comparison of means; JMP statistical software, $p<0.05$).

filter papers were used, with test stimuli assigned randomly to one of the side arms. Air was drawn through the olfactometer at a rate of 1 l min^{-1} with a water-driven aspirator. Thirty seconds after placement of stimuli, a beetle was released into the entrance of the olfactometer. Beetles walking up-wind that reached within 5 min a filter paper emanating host-derived odour or pentane as the control stimulus were classed as responders, and included in statistical analyses.

(e) *Field trapping experiments*

We suspended adhesive cardboard traps (45×67 cm) (Phero Tech, Inc., Delta, BC, V4G 1E9, Canada) between poles at a height of approximately 2 m and spacings of 20–25 m in randomized complete blocks separated by 2–5 km. Trap baits consisted of a piece of dental cotton roll (10×15 mm) (Richmond Dental, Charlotte, NC 28234, USA) that was impregnated with (−)-α-cubebene (**2**), (+)-spiroaxa-5,7-diene (**3**) and (+)-δ-cadinene (**4**), and affixed to a 400-μl polyethylene microcentrifuge tube containing a 5-μl capillary tube filled with (−)-β-pinene (**1**) (Sigma-Aldrich Canada Ltd, Oakville, Ont. L6H 2J8). Release rates of **1**, **2**, **3** and **4** were, respectively, 25, 2, 2 and 184 μg/24 h, approximating the ratio found in diseased elm wood (figure 1). We recorded the number of *H. rufipes* captured in traps 24 h after trap placement, replaced lures and traps, and re-randomized their location within blocks.

(f) *Inoculation of elm saplings with* Ophiostoma novo-ulmi

To determine whether *O. novo-ulmi* emits these four semiochemicals or induces host trees to emit them, we conducted the following inoculation experiment. We flooded culture plates of *O. novo-ulmi* grown on potato dextrose agar with distilled water and diluted the suspended conidia to 6×10^7 ml^{-1}. Using a sterile probe, we then punched 10 holes similar in diameter to those bored by *H. rufipes* into the stem and twig crotches of potted 2 m tall healthy elm saplings ($n=3$) maintained in a quarantine greenhouse. Into each hole, we pipetted 10 μl of *O. novo-ulmi* spore suspension. Control saplings ($n=3$) with the same number and distribution of holes received equivalent volumes of distilled water, and additional control saplings ($n=3$) received no treatment. After 12 weeks, when treatment saplings exhibited disease symptoms, we ground wood tissue from all nine saplings into separate samples of fine sawdust, weighed them, and collected volatiles on Porapak Q (see §2*a*).

3. RESULTS AND DISCUSSION

Our data show that the fungal plant pathogen *O. novo-ulmi* induces change in the elm's semiochemical blend that is detrimental to the tree in that it attracts insect vectors that kill the host and carry the pathogen to new hosts. To identify these semiochemicals, we adsorbed the volatiles from finely ground diseased American elm trunk wood on Porapak Q and eluted them with pentane. Subsequent bioassays showed strong attraction of male and female *H. rufipes* to the eluate. We analysed aliquots of the Porapak Q extract by GC–EAD (Arn *et al.* 1975; Gries *et al.* 2002), using *H. rufipes* antennae as detectors. Four compounds elicited consistent and significant antennal responses (figure 1). Comparative analyses by GC–MS of volatiles from diseased elm wood and of authentic standards revealed that these four compounds were (−)-β-pinene (**1**), (−)-α-cubebene (**2**), (+)-spiroaxa-5,7-diene (**3**) and (+)-δ-cadinene (**4**). The absolute configuration of (−)-β-pinene was determined by GC analysis on a chiral column. The presence of **1**, **2** and **4** in volatiles from diseased elm trees has been previously reported (Millar *et al.* 1986). (+)-Spiroaxa-5,7-diene (**3**), discovered as a semiochemical for the first time in this study, was present in only trace quantities but elicited the strongest response from *H. rufipes* antennae (figure 1). Stronger antennal responses to synthetic (+)-(1*S*,2*R*)-spiroaxa-5,7-diene than to its antipode (data not shown) support the absolute configuration assignment of **3**.

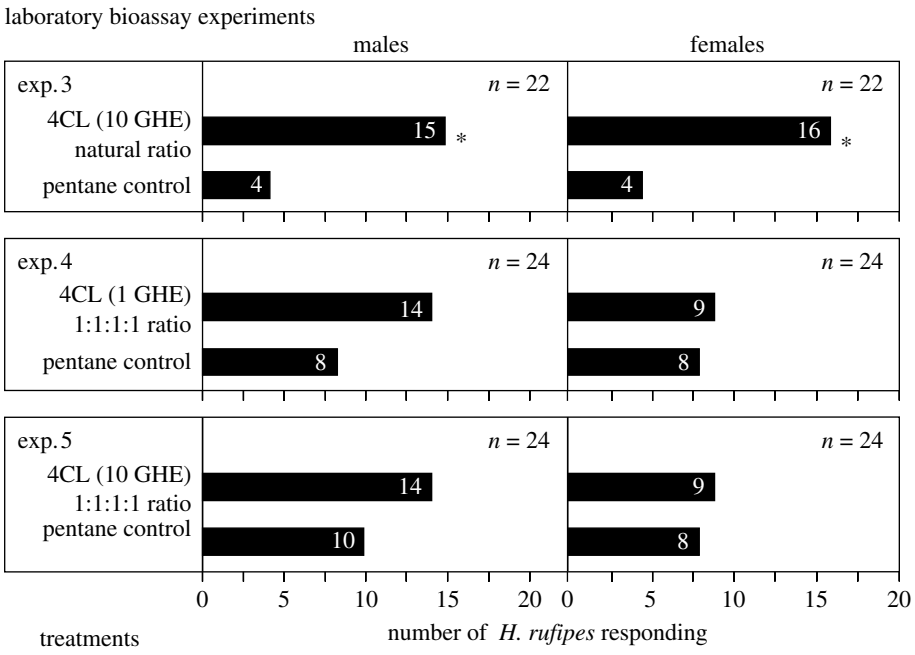

Figure 3. Number of male and female *Hylurgopinus rufipes* responding in Y-tube olfactometers to a 4-component lure (4CL) of synthetic (−)-β-pinene, (−)-α-cubebene, (+)-spiroaxa-5,7-diene and (+)-δ-cadinene at a natural ratio (20 : 1.5 : 1.25 : 175; determined in volatile blend of diseased elm (figure 1)) of components (experiment 3), or at a non-natural ratio (1 : 1 : 1 : 1) of components (experiments 4, 5). One GHE = 1 g-h equivalent = amount of semiochemicals released from 1 g of ground diseased elm wood during 1 h of volatile acquisition. Number of insects responding to each stimulus given in bars, number of insects tested given in parenthesis. For each experiment an asterisk (*) indicates a significant preference for a particular treatment; χ^2 test with Yates correction for continuity, treatment versus control; $*p < 0.05$.

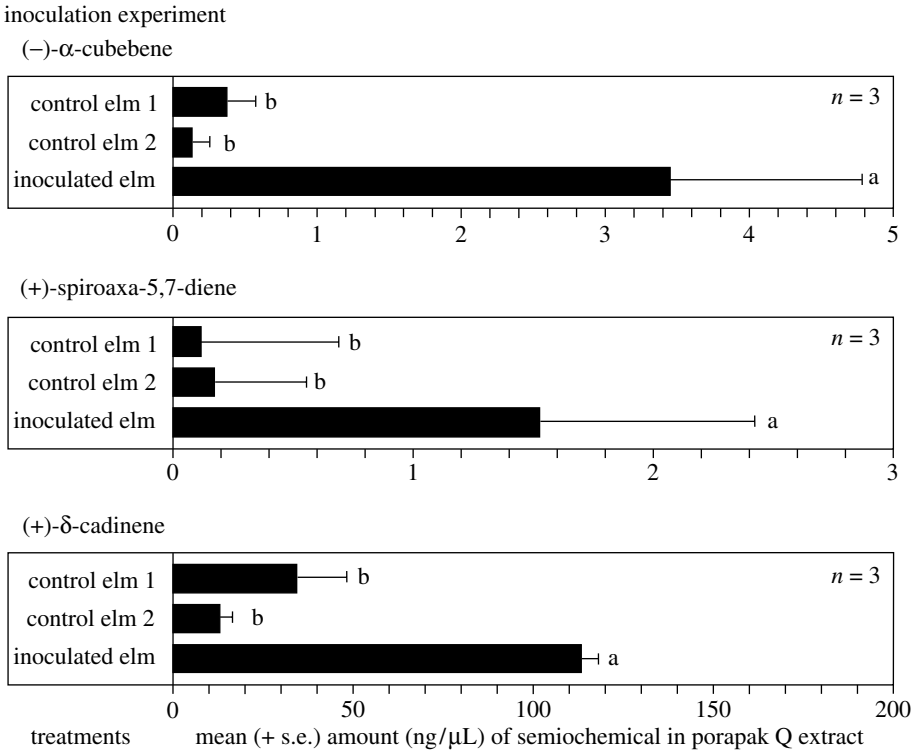

Figure 4. Results of an inoculation experiment, comparing quantities of α-cubebene, spiroaxa-5,7-diene and δ-cadinene in Porapak Q volatile extracts of wood tissue from potted elm saplings ($n = 3$) 12 weeks after inoculation with an aqueous spore suspension of *O. novo-ulmi* (inoculated elm; $n = 3$), distilled water (control elm 1; $n = 3$) or left untreated (control elm 2; $n = 3$). β-Pinene occurred in amounts too low for analysis. Statistics: see figure 2 caption.

The attractiveness of synthetic **1**, **2**, **3** and **4** was tested in field trapping experiments in elm forests near Regina, Saskatchewan, Canada. Traps baited with the 4-component blend in an approximately natural ratio captured significantly more male and female *H. rufipes* than unbaited control traps (figure 2, experiment 1), whereas blends lacking any one of the four compounds were as unattractive as unbaited traps (figure 2, experiment 2). In laboratory bioassay experiments, a synthetic blend containing equal amounts of all four components

did not elicit a behavioural response from the beetles (figure 3). Thus, the attractiveness of the semiochemical blend requires both the presence of all four components and their release at a natural ratio.

To determine whether *O. novo-ulmi* emits these four semiochemicals or induces host trees to emit them, we inoculated healthy elm saplings with *O. novo-ulmi* and analysed by quantitative GC–MS the volatiles collected on Porapak Q (as above). Spiroaxa-5,7-diene, α-cubebene and δ-cadinene were found to be significantly more abundant in pathogen-inoculated than in healthy elm saplings (figure 4), while the concentration of β-pinene, which is typically released in response to severe mechanical injury (Trapp & Croteau 2001), was not affected. None of the four semiochemicals was present in volatiles emitted by *O. novo-ulmi* grown on potato dextrose, indicating that the pathogen does not produce them. Whether other factors causing ill health in elms, such as different pathogens or herbicide poisoning, may induce similar changes in the trees' semiochemical blend has not yet been determined.

Our data provide strong evidence that *H. rufipes* uses a blend of four elm-derived semiochemicals to find a susceptible host. All four occur at low quantities in healthy elms. By an as yet unknown mechanism, *O. novo-ulmi* then upregulates the production of these semiochemicals, thus enhancing the apparency of host trees to foraging beetles, and increasing the probability of transportation of the pathogen to new hosts.

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