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RAPID COMMUNICATION

Phytopathogen Lures Its Insect Vector by Altering Host Plant Odor

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Abstract Many phytopathogens that cause worldwide losses of agricultural yield are vectored by herbivorous insects. Limited information is available about the interactions among phytopathogens, host plants, and insect vectors. In this paper, we report that the cell wall-lacking bacterium *Candidatus* Phytoplasma mali can alter both the odor of its host plant (apple) and behavior of its vector, the univoltine psyllid *Cacopsylla picta.* Apple trees infected by this phytoplasma emitted higher amounts of β-caryophyllene when compared to uninfected ones. Psyllids that had no previous contact with *Ca.* P. mali, as well as infected pyllids, are more attracted by volatiles emitted from phytoplasma-infected apple plants than from uninfected ones. Psyllids that had developed on infected plants without getting infected showed the opposite behavior. These results suggest that the pathogen modifies host plant odor that lures its vector to infected plants. This may result in higher numbers of transmitting vector insects within the population.

Keywords Apple proliferation · Vector–plant–pathogen interaction · β-Caryophyllene · *Candidatus* Phytoplasma mali · *Cacopsylla picta* · *Malus domestica*

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Introduction

The cell wall-lacking bacterial phytopathogen *Candidatus* Phytoplasma mali causes the apple proliferation disease (Seemüller and Schneider 2004) and is responsible for severe economic losses by inducing "witches brooms" and tasteless dwarf fruits in apple trees (*Malus domestica*), for which no curative approaches are available (Seemüller et al. 2002). The univoltine psyllid species *Cacopsylla picta* (Foerster) (Hemiptera: Psyllidae) was discovered as a vector of this phytoplasma that infects both the host plant and insect vector (Frisinghelli et al. 2000). Information on orientation and host finding by these phloem-feeding insects vectoring phytoplasmas is lacking (Weintraub and Beanland 2006). The olfactory response of two psyllid species suggested that plant kairomones may play a role in host-finding behavior (Gross and Mekonen 2005), but the influence of the vectored phytoplasma in this multitrophic system has not been examined in detail.

In this study, we investigated the behavior of both infected and uninfected vector insects toward the odors of uninfected and phytoplasma infected plants in a Y-shaped olfactometer. The infection status of psyllids and plants was confirmed by polymerase chain reaction (PCR) with primers specific to *Ca.* P. mali. Further, we collected the headspace of uninfected and infected apple plants for a period of 45 d and analyzed the volatile components by gas chromatography/mass spectrometry (GC/MS).

Materials and Methods

Cultivation of Plants Two-year-old apple trees (cultivar Gala Royal on rootstock M9) were either infected with

Ca. P. mali by grafting shoots of already infected apple trees (from the previous years experiments) or left untreated as uninfected controls. All trees were checked for apple proliferation prior to experiments by extracting deoxyribonucleic acid (DNA) of the rootstock's phloem followed by PCR with specific primers (fAT/rAS) as described below. Trees were transferred from a cooling chamber to a greenhouse chamber 5 wk before the headspace sampling was started. The trees were maintained under natural light and temperature conditions (day/night: 16/8 hr, 20/15°C, 60% relative humidity [RH]) until flowering was completed (phenology stage 68). Subsequently, trees were transferred into a climate chamber (day/night: 12/12 hr, 20°C constant, 60% RH) for headspace sampling (phenology stages 69–79).

Rearing of Insects for Bioassays Mature adults of *C. picta* were collected from apple trees (*M. domestica*) in early spring after having returned from their overwintering host plants. In rearing cages (60×60×90 cm), they were maintained on uninfected or *Ca.* P. mali-infected apple plants where they could feed and oviposit. The rearing cages were located in a tempered greenhouse chamber under natural light conditions and a day/night temperature program (20/15°C). For behavioral trials, test insects were collected from the boxes 1 d before testing in groups of ten in Eppendorf vials at 4°C. All insects were tested within 2 wk after emergence.

Behavioral Bioassays Behavioral tests were carried out by using a dynamic olfactometer consisting of a Y-shaped glass tube (entrance arm: 12.5 cm, test arms=21.0 cm, inner diameter=0.6 cm) mounted on an angular board. Charcoal cleaned air (granulated 4–8 mm, Applichem GmbH, Darmstadt, Germany) was pumped (75 ml/min) through two glass jars (vol=2 l) containing the volatile sources. A twig of uninfected or phytoplasma infected apple plants (length 10–15 cm, phenology stage 69–71) was placed in a water-filled glass vial in each jar. The odors of infected and uninfected twigs were offered simultaneously. According to the method of Soroker et al. (2004), for each test, ten females were put together into the entrance arm. Every individual that passed a final marking (10.0 cm after the branching) on one of the test arms within 15 min was counted and placed into separate vials filled with ethanol (70%) for later determination of their infection status (see below). Both experiments were repeated 29 times. The numbers of psyllids were analyzed statistically after log (x+0.5) transformation by dependent paired t test using Statistica 5.5.

DNA Extraction: Plant Material Phloem tissue from cut roots was abraded. Between 1.0 and 1.5 g of phloem tissue was subjected to DNA extraction following the procedure described by Doyle and Doyle (1990). Instead of 0.2% β-mercaptoethanol, 2% sodium metabisulfide was used. The resulting DNA pellet was resuspended in 50 µl of sterile water and stored at −20°C.

Insect Material Psyllids that were used in bioassays (see above) were subjected to DNA extraction by using the same protocol as described above for plant material. Single insects were homogenized with a conical pestle (polypropylene, 1.5 ml, Eppendorf, Hamburg, Germany) in a 1.5-ml tube containing 150 µl extraction buffer and a pinch of silicon carbide (carborundum, Sigma-Aldrich, Munich, Germany) as grinding additive. The resulting DNA pellet was resuspended in 20 µl sterile water and stored at −20°C.

PCR Analysis DNA was purified by using the primer pair fAT/rAS specific for *Ca.* P. mali (Smart et al. 1996) and amplifying a 400-bp sequence in the 16S–23S ribosomal ribonucleic acid spacer region. PCR reactions were performed in a thermal cycler (Robocycler 96, Stratagene, La Jolla, CA, USA) with a reaction volume of 25 µl containing 2.5 µl extracted DNA, 0.5 pM of each primer, 250 µM of each nucleotide, 0.5 U polymerase, and 1× polymerase buffer (Invitrogen, Karlsruhe, Germany). Samples were subjected to 35 cycles that consisted of 1 min denaturation at 95°C, 1 min annealing at 51°C, and 1.5 min at 70°C. The products of each PCR were electrophoresed on a 1% agarose gel containing ethidium bromide (0.3 µg/ml) in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0). DNA was visualized and photographed while exposed to UV light (302 nm).

Headspace Sampling Single branches of apple plants (see above) were carefully wrapped in polyethylene terephthalate bags (20 cm diameter, Melitta, Minden, Germany). A stream of purified air (250 ml/min; controlled by a flowmeter) was pumped through the bag for 4 hr. For each treatment, two trees were sampled every second or third day over a period of 45 d. Volatiles from plant headspace were trapped in collection filters (charcoal 5 mg, Gränicher+Quartero, Daumazan, France) and eluted by rinsing the filter with 25 µl of dichloromethane containing 50 ng/µl of tridecane (Sigma-Aldrich, Munich, Germany) as internal standard (IS).

Analysis of Headspace Samples by GC/MS Each sample (0.5 µl) was injected splitless into a gas chromatograph (Shimadzu GC 17A, injector temperature 300°C) equipped with a 30 m×0.32 mm×0.25 µm HP-5 column (J & W, Santa Clara, CA, USA). The temperature program started at 40°C, was held for 3 min, and then

raised by 10°C/min to 250°C. The final temperature was held for 6 min. Helium (Air Liquide, Germany) was used as carrier gas (inlet pressure 3.2 kPa). The gas chromatograph was coupled to a quadrupole mass spectrometer (Shimadzu QP 5000). Electron ionization mass spectra were recorded at 70 eV with scanning from mass 35 to 500 at 0.5 scan/sec. Solvent delay was adjusted to 5 min. Identification of volatiles was determined by comparing retention times with commercially available standards (Sigma-Aldrich, Munich, Germany) and by comparison with the NIST 02 mass spectral database and NIST MS search 2.0 (National Institute of Standards and Technology, USA). The relative amounts of volatiles compared to IS were calculated as previously described by Gross et al. (2008).

Results and Discussion

Phytoplasmas infecting apple trees alter the odor of this host plant that lures their vector. *C. picta*, reared on uninfected plants without any contact with the phytoplasma during their ontogenesis (inexperienced), was attracted by the odor of infected plants (AP^+; Fig. 1a; dependent paired *t* test, $P<0.05$). By contrast, *C. picta* that were reared on infected plants (experienced) showed the opposite behavior depending on their infection status when exposed to the different odor sources. The odor of infected apple was highly attractive for psyllids that had been infected by phytoplasma ($P<0.001$), while adults of *C. picta* that had not been infected were attracted by the odor of uninfected plants or repelled by the odor of infected plants, respectively ($P<0.05$). The motivation of *C. picta* females to enter an olfactometer arm was 51% for experienced and 67% for inexperienced individuals. These are high activities for psyllids in olfactometer bioassays (Soroker et al. 2004). Interestingly, breeding of *C. picta* on infected apple trees resulted only in 29% infection of the potential vectors by phytoplasmas, as shown by PCR analysis of whole-body extracts, using specific primers for detection of *Ca.* P. mali. As all infected trees used for conducting experiments and rearing purposes showed apple proliferation symptoms, we assume that the phytoplasmas were present in the tissue of all parts of these plants. Thus, a much higher infection rate of *C. picta* reared on these trees is expected. Why some females of *C. picta* are susceptible to phytoplasma infection while others are not remains to be elucidated. The psyllids that apparently were not able to transmit the disease (experienced, but uninfected) were attracted by the odor of uninfected apple trees.

Analysis of the plants' headspace by GC/MS revealed about 25 different volatile compounds during a sampling period of 45 d. While there were no significant differences among all other volatiles emitted by uninfected and infected plants (data not shown), only apple plants infected by *Ca.* P. mali emitted the sesquiterpene β-caryophyllene in higher amounts (Fig. 1b). Emission was highest from the beginning of headspace sampling (phenology stage 69; BBCH scale) and decreased during 29 d to 0 (phenology stage 72). After this time, nearly no β-caryophyllene was emitted until the end of the sampling time (45th day, phenology stage 79).

C. picta spends only a short period of its life (2–3 mo) exclusively on apple for reproduction (phenology stages 53–59) and juvenile development (reproduction host). Soon after hatching (phenology stages 69–71), newly emerged adults migrate onto conifers, spending the next 8–9 mo including winter time there (overwintering host). During the relatively short period on apple, the newly hatched adults of *C. picta* are able to ingest *Ca.* P. mali from infected plant phloem (acquisition feeding). Following a latent period, ingested phytoplasmas leave the intestinal tract of their vector, invade the hemolymph, multiply, and infect further tissues of the insect (Christensen et al. 2005). Finally, they intrude into the salivary glands, from which they can infect a new host plant (transmission feeding). Interestingly, the phytoplasma induces emission of β-caryophyllene from apple plants exactly between hatching and migration of its vector (Fig. 1b). According to our behavioral studies, vector insects that developed on uninfected plants were attracted by the odor of infected plants. The phytoplasma-induced change in odor composition may be the attracting signal for its vector to change orientation from uninfected to infected plants, and promote the uptake of phytoplasmas from the phloem. Additionally, infected psyllids are also attracted by infected plants, and that may ensure a longer insect feeding period from these plants, thus acquiring a higher titer of *Ca*. P. mali before shifting to their overwintering host.

In summary, the presented results support our hypothesis that the apple proliferation phytoplasma manipulates both odor of infected plants and behavior of vector insects to promote its propagation. Because experienced but uninfected psyllids show the opposite behavior to infected psyllids, the phytoplasma within the insect may affect behavior as well. These abilities are certainly of epidemiological relevance and will be studied further to understand the complex interactions between pathogens reproducing in distinct hosts. Thus, both laboratory and field experiments are in progress in order to confirm a direct attraction of β-caryophyllene to *C. picta*.

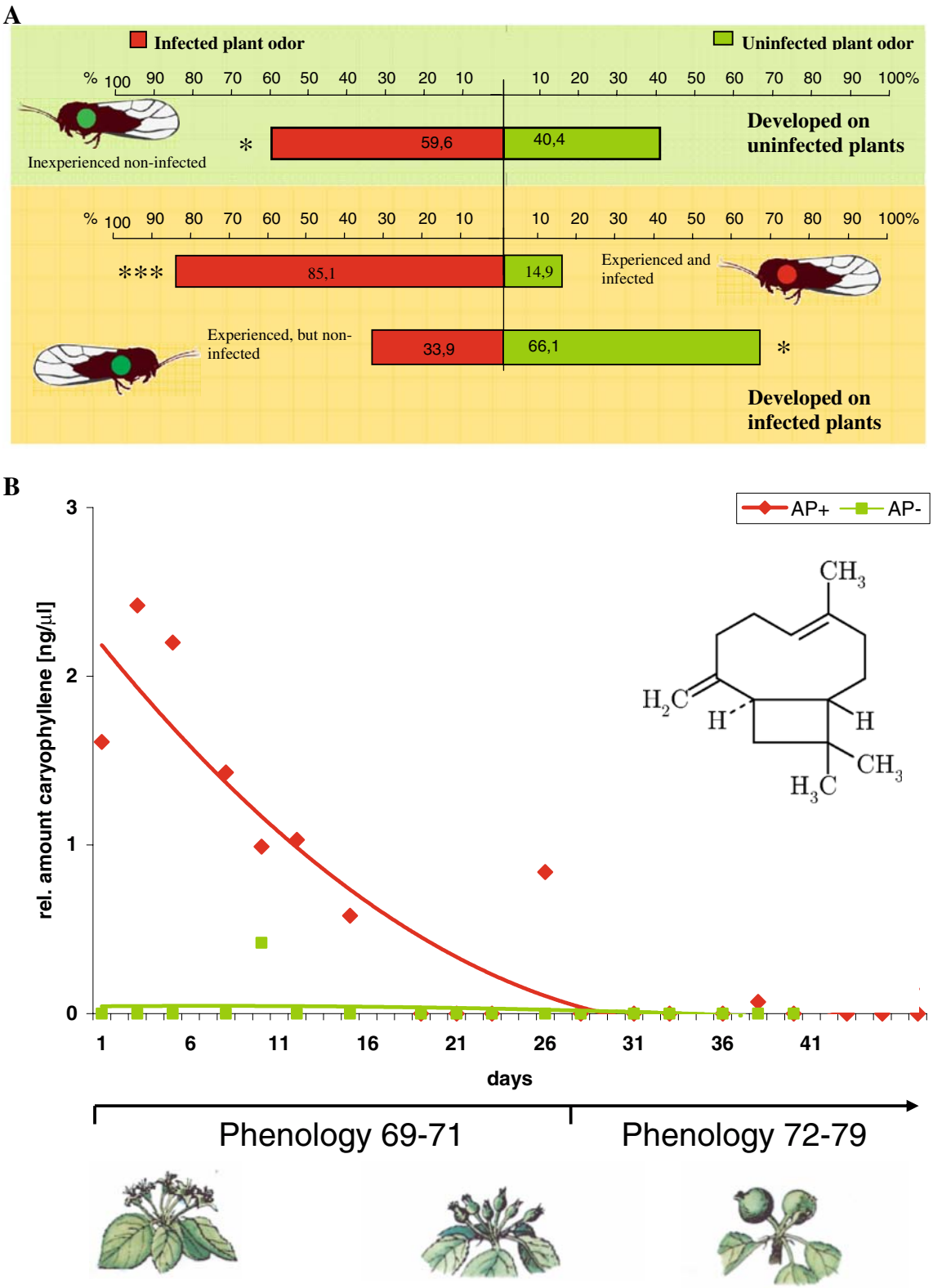

Fig. 1 **a** Olfactory preferences (%) of young adult females of *C. picta* to odors of phytoplasma infected apple vs. odors of uninfected apple. All preferences are statistically significant (dependent paired *t* test, *triple asterisk*, $P<0.001$; *asterisk*, $P<0.05$). **b** Means of relative amounts of β-caryophyllene emitted by infected plants (*red rhombs–darker gray*) compared to uninfected plants (*green squares—lighter gray*). The main emission occurs during the phenology stages 69–71 (BBCH scale: end of blooming [stage 69] until fruit diameter is about 10 mm [stage 71]) until day 28. *AP+* Apple infected by *Ca.* P. mali, *AP−* uninfected apple plant

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