Olfactory Responses of the Asiatic Citrus Psyllid (*Diaphorina citri*) to Mineral Oil-Treated Mandarin Leaves

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Abstract: Problem statement: Evidence of rejection or acceptance by phytophagous pests of citrus plants treated with mineral oils suggests that impacts of oils may be related to detection by olfactory and/or contact chemoreceptors of repellent oils or plant volatiles. Alternatively, the oils may mask or suppress attractant host plant volatiles. To date, no studies have reported that oils elicit an olfactory response in *Diaphorina citri* Kuwayama [Hemiptera: Psyllidae]. Therefore, we undertook two studies to determine how oil deposits on citrus leaves influenced the behaviour of *D. citri*. **Approach:** The attractiveness of citrus leaves treated separately with an *n*C21 horticultural mineral oil and an *n*C24 agricultural mineral oil to *D. citri* was evaluated under laboratory conditions. In the first of two studies, the oils were applied as 0.5% aqueous emulsions to mandarin leaves and responses of adults determined after they walked on surfaces saturated with either distilled water or 2% aqueous emulsions of the oils. In the second study, responses of adult psyllids to host-leaf volatiles, mineral oil volatiles emanating from leaves dipped in 2% v/v aqueous emulsions of each oil and similarly treated filter paper paired with water-dipped leaves were determined in single, linear-tube olfactometers. **Results:** In the first study, deposits of both oils significantly reduced the proportion of *D. citri* attracted to citrus leaves. Prior exposure of the insects to oil deposits did not influence this response. However, prior contact with the HMO slightly reduced the proportion of psyllids alighting on the water-dipped leaves. In the second study, oil volatiles emanating from filter paper significantly reduced the attractiveness of water-dipped mandarin leaves. **Conclusion:** These results indicate that application of the oils to the mandarin leaves may have: (a) suppressed release of attractant host plant volatiles; (b) masked attractant host plant volatiles; (c) led to the release of repellent volatiles from leaves and/or (d) to adults being repelled by oil volatiles. The results from the study using the olfactometer suggested that detection of mineral oil volatiles by olfactory receptors of adult *D. citri* contributed to their response to oil deposits on the mandarin leaves.

Key words: Citrus Vein-Phloem Degeneration (CVPD), Horticultural Mineral Oil (HMO), Agricultural Mineral Oil (AMO), prior exposure, olfactometer suggested

INTRODUCTION

In Asia, the Asiatic citrus psyllid, *Diaphorina citri* Kuwayama [Hemiptera: Sternorrhyncha: Psyllidae] is the only known vector of huanglongbing, a devastating disease of citrus that is known as Citrus Vein-Phloem Degeneration (CVPD) in Indonesia and more widely as citrus greening (Soelaeman, 1981; Aubert, 1990; da Graca, 1991; Halbert and Manjunath, 2004). In Asia, the putative pathogen causing huanglongbing is 'Candidatus' Liberibacter asiaticus' [α-Proteobacteria] (Bove, 2006).

The impact of the disease has led to the heavy use of pesticides for reducing populations of *D. citri* and, hence, slowing the spread of the pathogen. Most emphasis has been placed on contact and systemic synthetic insecticides to kill psyllid eggs, nymphs or adults, but use of these and other chemicals merely slows the inevitable death of trees from the disease. Infected adult psyllids can also transmit the pathogen

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while acquiring lethal doses of insecticide (Beattie and Barkley, 2009; Frederic *et al*., 2010). Limited emphasis has been placed on strategies such as the use of mineral oils (petroleum spray oils) (Rae *et al*., 1997; Huang *et al*., 2005) and guava interplants (Cen *et al*., 2008; Hall *et al*., 2008; Zaka *et al*., 2010) to reduce feeding, oviposition and ingress of psyllids into orchards by altering the behaviour of adults. However, Huang *et al*. (2005) make detailed recommendations for the application of oil sprays applied to flush growth to discourage egg laying by adults and to kill young nymphs. Oils have been successfully used in southern Viet Nam for several years as part of integrated pest management programs developed through Australian Centre for International Agricultural Research-funded research projects led by GACB.

Evidence of rejection or acceptance by phytophagous insect and mite pests of citrus plants treated with mineral oils suggests that reported impacts of oil deposits on citrus red mite (*Panonychus citri* (McGregor) [Acari: Tetranychidae]) (Beattie, 2002), greenhouse thrips (*Heliothrips haemorrhoidalis* Bouche [Thysanoptera: Thripidae]) (Beattie, 2002), citrus leafminer (*Phyllocnistis citrella* Stainton [Lepidoptera: Gracillaridae]) (Beattie *et al*., 1995; Liu *et al*., 2001) and *D. citri* (Rae *et al*., 1997) may be related to detection by olfactory and/or contact chemoreceptors of repellent oil or plant volatiles. Alternatively, the oils may mask or suppress attractant host plant volatiles. To date, no studies have reported that oils elicit and olfactory response in *D. citri*. Therefore, we undertook two studies to determine how mineral oil deposits on citrus leaves may influence the behaviour of *D. citri*. In the first study, we determined the response of adult psyllids with oil-contaminated tarsi to deposits on leaves of an *n*C21 Horticultural Mineral Oil (HMO) and an *n*C24 Agricultural Mineral Oil (AMO). In the second study, we used single linear-track olfactometers to determine responses of adult psyllids to oil-treated leaves and oil-impregnated filter paper.

MATERIALS AND METHODS

Psyllid cultures: Disease-free cultures of *D. citri* were maintained on the common ornamental form of orange jasmine (*Murraya exotica* L. [Sapindales: Rutaceae: Aurantioideae: Aurantieae] in nylon mesh cages in a controlled-temperature greenhouse at 28±2°C and 60% relative humidity.

Mineral oils: Two oils were used, an *n*C21 HMO (Sunspray UltraFine®, Amtrade Pty Ltd, Melbourne, Victoria, Australia) with an Unsulfonated Residue (UR) value≥92% and an *n*C24 AMO (SK Enspray 99®, Oilblend Pty Ltd, Sefton, New South Wales, Australia)

with a UR value of≥99.8%. The glossary in Beattie (2002) defines relevant spray oil terms used in this study and more widely. Beattie (2002) described the chemistry of petroleum-derived spray oils and Beattie (2002) summarised classifications, standards and nomenclature.

Responses of adults with oil-contaminated tarsi: Capitalise "Ten" freshly excised mandarin (*Citrus reticulata* Blanco [Sapindales: Rutaceae: Aurantioideae: Aurantieae]) leaves were dipped in water or in 0.5% aqueous emulsions of either the HMO or AMO. Petioles of each pair of water- and oil-treated leaves were then inserted into 15×50 mm bottles containing water and held in position with a thin piece of sponge so that the leaves did not touch each other. The tubes and leaves were then placed in a 120 mm diameter×150 mm high cylindrical cage covered by nylon mesh for 24 h. ten, 10-day-old adult psyllids were then released into each cage and allowed to choose to settle on either the water- or oil-treated leaves; this was repeated on 15 occasions. Four sets of experiments were performed using both oils. In the first two, immediately prior to release, the psyllids were allowed to walk or stand for 10 min on water-saturated filter paper before being placed in the cylindrical cage. In the second two, the psyllids were allowed to walk or stand on a deposit of 2% oil emulsion immediately prior to release. In all instances, the oil on which the psyllids were allowed to alight was the same as the one used to dip the leaf. The position of the psyllids was recorded 24 h after they were released.

Olfactometer responses of adults to citrus and oil volatiles: Each single, linear-track olfactometer was constructed from a 31.5 mm diameter (internal), 260 mm long, transparent glass tube, sealed at the proximal end with a rubber plug connected by a 2.3 mm diameter (internal) plastic tube to a 3 W aquarium aerator (Sunshine 2001, Sunshine Co. Ltd., China) that was used to pump air into the olfactometer at 141 mL min^{-1}, as measured with a flowmeter (Model N 112-02G, Cole-Parmer Instrument Company, Illinois, USA) (Fig. 1). Air entering each olfactometer was filtered through activated charcoal and humidified by passing it through distilled water before it was passed through a transparent plastic container (80 mm long×80 mm wide×100 mm high) housing the odour source (treatment). Responses of adult psyllids of mixed gender were determined for the paired treatment comparisons listed in Table 1. Healthy, turgid leaves used in the treatments were excised from mandarin seedlings. The petiole of each leaf was wrapped immediately in wet cotton wool that was then inserted into a water-filled, 20×50 mm specimen tube.

Table 1: Paired, single-tube olfactometer comparisons: for each comparison, responses of 10 adult *D. citri* per replicate (n = 15) to odours from sources listed in the left hand column were recorded over 15 min intervals then, after an interval of 30 minutes in the absence of odours, for 15 min to odours in the right hand column. Treatment codes used in Fig. 1 and 2 are given in parentheses

Comparison	First test		Second test
i	Filtered Air: no odour source (FA)	Vs	mandarin leaf dipped in water (WL)
ii	mandarin leaf dipped) in 2% HMO (2HL	Vs	mandarin leaf dipped in water (WL)
iii	mandarin leaf dipped in 2% AMO (2AL)	Vs	mandarin leaf dipped in water (WL)
iv	mandarin leaf dipped in 2% HMO (2HL)	Vs	mandarin leaf dipped in 2% AMO (2AL)
v	mandarin leaf dipped in water + filter paper dipped in 2% HMO (WL 2HFP)	Vs	mandarin leaf dipped in water (WL)
vi	mandarin leaf dipped in water + filter paper dipped in 2% AMO (WL 2AFP)	Vs	mandarin leaf dipped in water (WL)
vii	mandarin leaf dipped in 2% HMO (2HL)	Vs	mandarin leaf dipped in water + filter paper dipped in 2% HMO (WL 2HFP)
viii	mandarin leaf dipped in 2% AMO (2AL)	Vs	mandarin leaf dipped in water + filter paper dipped in 2% AMO (WL 2AFP)
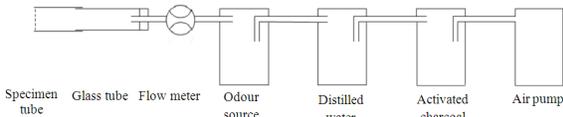
Fig. 1: Diagram of lactometer used to determine the responses of adult psyllids to citrus and oil volatiles

The leaves were then dipped in either 2% aqueous oil emulsions or distilled water for 5 s and then left to air-dry for 60 min. They were then placed vertically in the transparent plastic container. Strips of filter paper (20×20 mm) used in some comparisons (Table 1) were also dipped in 2% aqueous oil emulsions, or distilled water for 5 s and then left to air-dry for 60 min. They were placed vertically beside leaves and held in place by Styrofoam.

Adult psyllids were collected in specimen tubes (31.5 mm internal diameter and 50 mm long). The specimen tubes were open at one end and covered by fine mesh at the other. The open end was immediately covered with fine mesh after the psyllids were collected. The psyllids were then starved for 60 min before they were released into an olfactometer by connecting the open end the specimen tube to the distal end of the olfactometer used for the first of two tests (Table 1). The adults were left within this olfactometer for 15 min, then removed, kept within a covered specimen tube for 30 min and then released into a second olfactometer for 15 min for the second test. Ten adult psyllids were used for each replicate (n = 15 for each paired treatment). Responses to the odour sources were recorded as the proportion of adults observed during a 15 min period moving beyond the specimen tube at the distal end of the olfactometer. All observations were undertaken in laboratory at 27-29°C. Both olfactometers were washed with soapy water and then rinsed in tap water and 90% ethanol between replicates.

Statistical analysis: In both the experiments undertaken to determine the effect of oil-contaminated tarsi and in the olfactometer experiments, data from each set of replicates were compared using contingency χ^2 tests (including Yates' correction) to determine if data within the replicate sets were homogenous. All data within the sets of replicates were found to be homogenous; therefore, data from the replicates were combined. Combined data were then compared by contingency χ^2 to determine differences between treatments.

RESULTS AND DISCUSSION

Studies by Rae *et al*. (1997) suggested that responses of *D. citri* to mineral oils may be due to detection by olfactory and/or contact chemoreceptors of oil molecules or plant volatiles. In their study, Rae *et al*. (1997), the authors determined the effects on oviposition of foliar deposits of 0.1-1.0% (v/v) aqueous emulsions of an *n*C22 mineral oil and found a highly significant dose response. In this current study, the oil concentrations used in the choice tests and olfactometer studies were based on Rae *et al*. (1997) ia's work such that they would be likely to elicit a behavioural response.

In all choice tests, where adult *D. citri* were given the option of alighting on mandarin leaves dipped either in water or in 0.5% aqueous emulsions of HMO or AMO (Fig. 2), significantly larger proportions of psyllids alighted on leaves dipped in water only. In the two AMO treatments and in the HMO treatment where the psyllids were allowed to alight on water prior to the choice test (Fig. 2A, B and D), there was no difference (χ^2 = 3.13; p = 0.21) in the ratio of psyllids on the control and treatment leaves, with approximately 50% of psyllids being found on the water-dipped leaves as opposed to 4-8% on oil-dipped leaves. The remainder of the psyllids were elsewhere inside the cage. Prior tarsal contact with the AMO had no effect on the response to the dipped leaves (χ^2 = 1.26; p = 0.53) (Fig. 2B and D). In contrast, with the HMO, where the tarsi had been in contact with the HMO before the choice test (Fig. 2C), significantly (χ^2 = 76.20; p<0.0001) fewer psyllids alighted on the water-dipped leaves compared to the other treatments. However, there were still proportionately more psyllids (22.6%) on the water-dipped leaves than on the oil-dipped leaves (4.7%). These results show that, by some means, psyllids respond to plant surfaces treated with mineral oils.
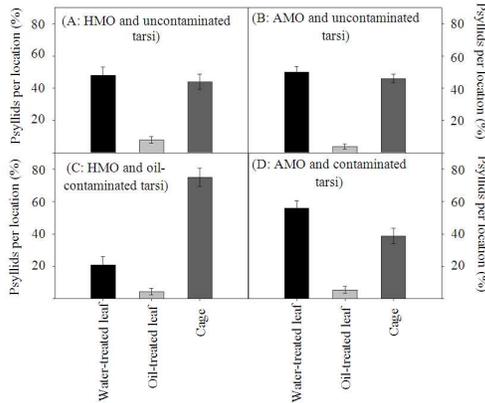
Fig. 2: Mean (±SE) proportions (%) of D. citri adults observed on mandarin leaves dipped in water or 0.5% v/v HMO (A and C), or in water or 0.5% v/v AMO (B and D). In panels A and B, immediately prior to release, the psyllids were allowed to walk or stand for 10 min on water-saturated filter paper. In panels C and D, the psyllids were allowed to walk or stand on a deposit of 2% oil emulsion immediately prior to release. In these two instances, the oil on which the psyllids were allowed to alight was the same as the one used to dip the leaf. In each panel, the proportions of psyllids on the control or treatment leaves or on the cages were significantly different from each other as determined by χ^2 tests (p<0.0001 in all instances)

The results of the olfactometer experiments are shown in Fig. 3. Significantly higher proportions (80.7% compared to 33.3%) of adults observed in comparison (i) moved towards the source of host plant volatiles than towards the source of clean air (Fig. 3i). This outcome showed that the olfactometer was an effective means of testing responses of D. citri adults to volatiles. Application of HMO (Fig. 3ii) or AMO (Fig. 3iii) to leaves significantly reduced (50 and 46%, respectively) proportions of psyllids attracted to mandarin leaves. Responses to both oils in paired comparisons (Fig. 3iv) were similar, with 30.7% and 31.3% of adults moving towards the volatiles from HMO- and AMO-dipped leaves, respectively. This result indicates that both oils had similar impacts on psyllid behaviour.

Comparisons (v) and (vi) (Fig. 3v and vi) showed that oil volatiles emanating from oil-dipped strips of filter paper significantly reduced psyllid movement towards mandarin leaf volatiles. Movement towards the source of leaf volatiles in the absence of oil-treated filter paper was 22 and 20% higher, respectively, for the HMO and AMO treatments, than towards leaves in the presence of oil volatiles.
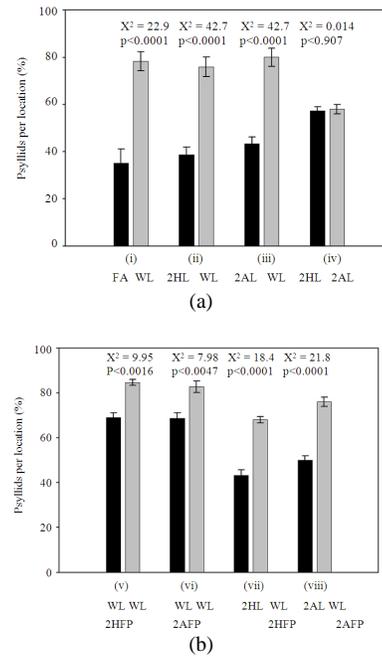

Fig. 3: Mean (±SE) percentage of D. citri adults that moved towards volatiles entering single tube olfactometers: (i) response to clean filtered air versus response to volatiles from a normal mandarin leaf dipped in water (FA Vs WL); (ii) responses to a mandarin leaf dipped in 2% aqueous horticultural mineral oil (HMO) emulsions versus response to volatiles from a normal mandarin leaf dipped in water (2HL Vs WL), (iii) responses to a mandarin leaf dipped in 2% aqueous agricultural mineral oil (AMO) emulsions versus response to volatiles from a normal mandarin leaf dipped in water (2AL Vs WL) and (iv) response to mandarin leaves dipped separately in 2% HMO or 2% AMO (2HL Vs 2AL); (v) response to mandarin a leaf dipped in water + filter paper dipped in 2% HMO Vs response to a normal mandarin leaf dipped in water (WL 2HFP Vs WL); (vi) response to a mandarin leaf dipped in water + filter paper dipped in 2% AMO Vs response to a normal mandarin leaf dipped in water (WL 2HFP Vs WL); (vii) response to a mandarin leaf dipped 2% HMO Vs a mandarin leaf dipped in water + filter paper dipped in 2% HMO (2HL Vs WL 2HFP) and (viii) response to a mandarin leaf dipped 2% AMO Vs a mandarin leaf dipped in water + filter paper dipped in 2% AMO (2HL Vs WL 2HFP). For each comparison the significance of the different proportions moving towards the odour source was determined by χ^2 tests

CONCLUSION

These results suggest that oil volatiles: repel adult psyllids; interfere with psyllid recognition of attractant leaf volatiles and/or influence the release of attractant or repellent plant volatiles from the water-dipped leaves. The results again suggest that the impact of the oils was similar.

Comparisons (vii) and (viii) showed that volatiles from oil-treated leaves were less attractive and/or more repellent than volatiles emanating simultaneously from water-dipped leaves and oil-treated filter paper (Fig. 3vii and viii). These outcomes may have been related to: (a) possibly different concentrations of oil volatiles emanating from treated leaves and filter papers if oil deposits or volatilisation rates on the treated substrates varied; or (b) if oil deposits suppressed, masked or increased release of plant volatiles as suggested above.

Detection and processing of plant odours plays an important role in host plant detection by herbivorous arthropods (Moran and Brown, 1973; Todd *et al.*, 1990; Bullas-Appleton *et al.*, 2004; Bichao *et al.*, 2005) and even in complex environments, an insect's central nervous system is capable of receiving information from volatiles that permits fine-scale spatio-temporal separation of odour sources (Bruce *et al.*, 2005). Such odours vary substantially, depending on species, cultivar, growing conditions, age and plant parts (Takabayashi *et al.*, 1994). Plants under herbivorous insect attack also produce volatiles that attract natural enemies of the insects (Takabayashi *et al.*, 1994; Pallini *et al.*, 1997; Henneman *et al.*, 2002; Lou and Cheng, 2003). Herbivorous insect may also use such cues to refrain from visiting plants that are already infested, thereby avoiding competition for food, or, alternatively, to visit plants with defences weakened by earlier attacks (Pallini *et al.*, 1997). Some psyllids are known to discriminate between the host and non-host, or between the susceptible and the resistant species, when presented with olfactory stimuli (Moran and Brown, 1973; Lapis and Borden, 1993).

Applications of mineral oils can also lead to the production of volatiles that are naturally released in response to feeding by phytophagous arthropods (Xue, 2007) and responses of Queensland fruit fly (*Bactrocera tryoni* Froggatt [Diptera: Tephritidae]) to mineral oil deposits have been attributed to several possible modes of action. These include physical disruption of epicuticular lipids, masking of oviposition stimulants and repellency, either directly or through mimicry of C6 and C8 green leaf volatiles (Beattie, 2002; Nguyen *et al.*, 2007).

We did not measure volatile concentrations or determine levels of oil deposits on treated surfaces.

However, the series of comparisons showed that the mineral oils repelled adult *D. citri* by: (a) suppressing the release of attractant host plant volatiles; (b) masking attractant host plant volatiles; (c) causing release of repellent volatiles from leaves and/or (d) oil molecules directly repelling psyllid adults. Both sets of experiments suggest that detection of mineral oil volatiles by the antennal olfactory receptors, probably antennal, *D. citri* contributed to their response to oil deposits on the mandarin leaves. Further studies using electroantennograms are required to determine if the response is solely related to the detection of oil volatiles or enhanced by effects of deposits on the release of attractant or repellent plant volatiles.

ACKNOWLEDGMENT

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