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Source: Florida Entomologist, 99(1) : 12-20

Published By: Florida Entomological Society

URL: https://doi.org/10.1653/024.099.0104

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Trichoplusia ni (Lepidoptera: Noctuidae) survival, immune response, and gut bacteria changes after exposure to *Azadirachta indica* (Sapindales: Meliaceae) volatiles

*Gricelda Nuñez-Mejía, José A. Valadez-Lira, Ricardo Gomez-Flores, Cristina Rodríguez-Padilla, and Patricia Tamez-Guerra**

Abstract

Volatile organic compounds (VOCs) produced by the neem tree (*Azadirachta indica* A. Juss.; Sapindales: Meliaceae) are known to alter growth and development of several insects. Additionally, the insect's gut microbiota and immune response are key components in insect development and have been linked to increased resistance to insecticides. In the present study, larval mortality, immune response, and intestinal bacteria changes in the cabbage looper, *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae), induced by VOCs of dried and milled neem leaves, stems, and bark were investigated. Exposure of neonates to 10 g of mixed dried leaves and stems resulted in 79 and 63% mortality of laboratory and field *T. ni* strains, respectively. In addition, differences were observed in larval weights and pupal sizes during 30 d of incubation. Further studies included transcript amplification of enterobacteria genes and genes related to immune responses and chemical synthetic insecticide resistance. Midguts from the VOC-exposed laboratory strain of *T. ni* larvae over-transcribed *cytochrome P450* (*CYP4L4*), *PGRP*, *lysozyme, attacin*, *cecropin*, *defensin*, *gallerimycin*, and *lebocin*, compared with unexposed control larvae, whereas *ribosomal protein S5* (internal control) and *caspase* transcripts did not show changes. These samples revealed reduced enterobacteria transcript amplification (27%), suggesting bacterial repression. The effect of neem VOCs on the immune response may in part explain the inhibitory effect on the molting process, in which chitin is broken down to release the old cuticle between instars. Because neem leaves, stems, and bark are widely used as fertilizers, their potential application in pest management programs for controlling *T. ni* on host crops (cauliflower, broccoli, or cabbage) is discussed.

Key Words: biological insecticide; chemical insecticide resistance marker; enterobacteria; immune marker; neem VOC

Resumen

Se tienen reportes donde mencionan que los compuestos orgánicos volátiles (COVs) producidos por el árbol de neem (*Azadirachta indica* A. Juss.; Sapindales: Meliaceae) pueden alterar el crecimiento y desarrollo de algunos insectos. Además, la microbiota intestinal del insecto y la respuesta inmune son componentes clave en su desarrollo y se han relacionado con una mayor resistencia a algunos insecticidas. Al investigar la susceptibilidad y alteraciones fisiológicas de una plaga Lepidoptera, se observó la mortalidad de larvas de dos cepas del gusano falso medidor *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae) después de su exposición a COVs de hojas, corteza y tallos (secos y molidos) del neem. Se observó una mortalidad del 79% y 63% de neonatas de las cepas NL (laboratorio) y Gto (campo), respectivamente, después de exponerlas a los COVs producidos por 10 gramos de hojas secas o tallos, además de diferencias en el peso de larvas y tamaño de pupas después de un periodo de incubación máximo de 30 días. Al analizar la amplificación de transcritos de genes de microbiota intestinal y genes de respuesta inmune relacionados con resistencia a insecticidas químicas, los resultados mostraron que el intestino de larvas de *T. ni* de la cepa NL expuestas a COVs sobre-traducían *citocromo P450* (*CYP4L4*), *PGRP, lisozima, atacina, cecropina, defensinas, galeromicina* y *lebocina* al compararla frente a larvas no expuestas (control), mientras que no se observaron diferencias con la *proteína ribosomal S5* (control interno) y la *caspasa*. Mismas muestras revelaron una amplificación reducida (27%) de transcritos para enterobacterias, lo que sugiere una represión bacteriana. El efecto de COV del neem en la respuesta inmune podría explicar en parte algún bloqueo en la muda larvaria o pupación. Debido a que hojas, tallos y corteza de neem se utilizan principalmente como fertilizante, se discute su uso potencial en programas de manejo de plagas para el control de *T. ni* en cultivos hospederos (coliflor, brócoli o repollo).

Palabras Clave: insecticida biológico; insecticida químico; marcadores de resistencia; enterobacterias; marcadores inmunológicos; COV del neem

The use of chemical insecticides to control insect pests has had a major impact on the natural and social environment (Soares & Porto 2007). Of particular concern is the development of insect resistance to insecticides (Weddle et al. 2009). Botanical pesticides represent an alternative for pest control because of their effectiveness, low cost of production, and easy preparation, and because they generally do not pollute the environment (Ignacimuthu & Jayaraj 2005). Several botanical pesticides are volatile and effective against insect pests. In this sense, the toxicity of volatile organic compounds (VOCs) could affect the insect's survival, similar to the effects observed after exposure to volatile chemical synthetic insecticides (Lighthart 1988).

The neem tree (*Azadirachta indica* A. Juss.; Sapindales: Meliaceae) has been used for hundreds of years, mainly in India, for medicinal purposes, and through the years, other properties of neem have been

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found, including its suitability as bioinsecticide against lepidopteran larvae for crop protection (Schmutterer 1985). As a bioinsecticide, mostly neem seeds have been used for this purpose (Tang et al. 2002), but it would be valuable to measure foliage and stem insecticidal activities, because they may contain various types and concentrations of bioinsecticidal VOCs. Furthermore, limited information is available on the insecticidal effect of neem VOCs. As shown by Balandrin et al. (1988) and Koul (2004), VOCs from neem seeds are mainly di-N-propyl disulfides (75.7%). These authors suggested that these disulfides may be plant bio-genetic precursors to organosulfur compounds, which could be related to repellency and biological activity. When Paranagama et al. (2002) tested VOCs from neem leaves for repellent activity and toxicity against *Sitophilus oryzae* L. (Coleoptera: Curculionidae), they observed repellency effects at doses above 100 mg and 100% mortality at the concentration of 32.5 mg/mL after 3 d of exposure to VOCs. The main insecticidal constituent of neem in seed kernels is the tetranortriterpenoid azadirachtin, of which low concentrations are found in other tree parts (Ascher 1993). Neem VOCs reported as insecticidal components include 6-beta-hydroxygedunin, nimbin, and salannin triterpenoids (22,23-dihydronimocinol and desfurano-6-alpha-hydroxyazadiradione) from fresh leaves; and meliacin (7-alpha-senecioyl-(7-deacetyl)-23-o-methyl-nimocinolide) and meliacinol [24,25,26,27-tetranorapotirucalla-(apoeupha)-1-alpha-trimethylacryloxy-21,23-6-alpha,28-diepoxy-16-oxo-17-oxa-14,20,22-trien-3-alpha,7-alpha-diol] from stems and bark (George et al. 2014). In the dried neem foliage storage room of our research laboratory, no insect infestations have been observed, which suggests that neem VOCs might have acted as repellents or affected insect viability, particularly that of cockroaches (P. Tamez-Guerra, unpublished observations).

The most sensitive insects to neem foliar applications are lepidopteran larvae (Schmutterer 1985). Neem VOCs may affect the larvae's viability and/or lead to somatic changes after larvae's exposure in a closed environment. From the evaluated VOCs produced by plants, monoterpenes have been reported to be toxic to insects and fungal pathogens (Holopainen & Blande 2013). VOCs produced by *A. indica* include the monoterpene α-pinene (Padhy & Varshney 2005), but there is limited information on how insect pathogens are affected. Other effects of neem on insects include partial or complete inhibition of larval development and of molting between instars, particularly in the prepupal stage (Ascher 1993). Insect growth hormones such as ecdysone can affect signaling pathways and antimicrobial peptide production (James & Xu 2012). However, there is no information with regard to neem foliage VOC-induced immune response in susceptible Lepidoptera. Therefore, we considered it to be relevant to evaluate such effects.

In addition, the insect's gut bacteria are known to play an important role in insect development, because they help in digestion, nutrition, pheromone production, and reproduction (Moran et al. 2008). Symbiotic relationships between insects and their gut bacteria have been studied extensively in various systems, particularly in wood-eating termites (Dillon & Dillon 2004). However, few reports on bacteria associated with other insects, especially those that feed on foliage, are available (Robinson et al. 2010). Species of Enterobacteriaceae have been reported in lepidopteran defoliators (Broderick et al. 2004; Matsuda et al. 2007). Furthermore, knowledge of the enterobacteria in Lepidoptera and the roles they can play in the biology of larvae could lead to new targets for pest control (Moran et al. 2008).

In previous studies performed in our laboratory, related to testing VOCs from neem extracts and dried material against *T. ni, Spodoptera exigua* Hübner*, Spodoptera frugiperda* Smith & Abbot (Lepidoptera: Noctuidae), *Plodia interpunctella* Hübner (Lepidoptera: Pyralidae), and *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) neonates, only *T. ni* showed significant mortality rates (P. Tamez-Guerra, unpublished observations). Because seeds are the commercial product of this plant and their efficacy in controlling *T. ni* has been proven (Akhtar et al. 2008), we hypothesized that leaves, stems, and bark could be used as biofumigants for insect pest control in certain host crops during postharvest storage. It has been reported that neem VOCs successfully eliminated a toxicity problem associated with the aflatoxin production by *Aspergillus* sp. (Eurotiales: Trichocomaceae), which is related to the damage caused by insect pests in stored grains (Zeringue et al. 2001), suggesting the potential use of neem as a grain bioinsecticide. Thus, we found it essential to investigate changes in the susceptible insect's immune response, in addition to the impact on its enterobacteria, after neonates were exposed to VOCs of neem leaves, stems, and bark.

Materials and Methods

All enzymes, substrates, and chemicals used in this study were obtained from Sigma-Aldrich (St. Louis, Missouri), unless otherwise specified.

PLANT AND INSECTS

Neem Samples

Neem tree samples were obtained from northwest Nuevo Le**ó**n State, M**é**xico, and they were confirmed in the Herbarium of the Facultad de Ciencias Biológicas at Universidad Autónoma de Nuevo León with voucher specimen # 025534. Unless otherwise specified, all bioassays used neem samples within 45 d after field collection during Aug, and samples were between 67 and 75 inches (between 170.2 and 190.5 cm) long. Tree branches were separated into leaves, stems, and bark, dried on newspaper in the laboratory at 25 ± 2 °C and 55 ± 10% RH, and stored in sealed double plastic bags in the refrigerator (3–5 °C) until used. In the bioassays, neem samples were separately crushed in a blender. To evaluate the effect of neem VOC monoterpene activity against selected insects (Choi et al. 2006), 35 g of either leaf, bark, or stem neem samples were placed in Erlenmeyer flasks with 350 mL of hexane or methyl chloride, and left for 40 h on a shaker (Orbit™ 1900 Labnet International Inc., Woodbridge, New Jersey). Next, solvent was filtered using Whatman #1 filter paper and placed in a rotary evaporator (Büchi R-200/R-205, New Castle, Delaware), and the extract was left standing for 10 min in a fume hood (Fisher Alder S.A. de C.V., Querétaro, Mexico) to complete the solvent evaporation and dry the extract. The extract was then diluted with Mazola® corn oil.

Insect Strains Tested

Trichoplusia ni was reared in our laboratory under controlled conditions (26 ± 2 °C, 65 ± 10% RH), and neonate larvae were used in bioassays as reported by Tamez-Guerra et al. (2006). Larvae were reared on artificial diet containing soy meal (1.0 g), corn meal (31.1 g), corn oil (10.6 g), sucrose (13.6 g), sorbic acid (1.0 g), methyl-p-hydroxybenzoate (4.26 g), agar (15.7 g), formaldehyde at 10% (4.4 mL), clorox at 15% (7.3 mL), acetic acid at 25% (12 mL), vitamin solution (3.5 mL), and distilled water (1 L). The vitamin solution contained calcium pantothenate (12.0 mL), niacin (6.0 mL), riboflavin (3.0 mL), folic acid (3.0 mL), thiamine (3.0 mL), pyridoxine (1.5 mL), biotin (0.12 mL), and vitamin B12 (2.5 mL); the volume was brought to 1 L with distilled water. Two strains of *T. ni* larvae were used, one obtained from the field that was exposed to *Bacillus thuringiensis* Berliner (Bacillales: Bacilaceae) (*Bt*) in Guanajuato, Mexico, coded Gto, and a laboratory-reared strain, coded NL. *Trichoplusia ni* strains were selected because in previous studies the NL strain showed higher susceptibility to *Bt* Cry toxins and higher

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antimicrobial peptide transcription after *Bt* exposure, compared with the Gto strain (Tamez-Guerra et al. 2006, 2008). Neonates were used in all VOC exposure bioassays.

NEEM VOC BIOASSAYS

VOC Toxicity

In order to determine if neem VOCs were toxic to *T. ni* Gto and NL strains, 11 L plastic containers with airtight lids were used (Mercier & Jiménez 2004) (Fig. 1A). Bioassay treatments were done in 3 replicate experiments, each testing 90 neonates from each insect strain. Neonates from new generations were used for each replicate. The experimental design consisted of placing inside the plastic container trays with 30 cups (1 oz = 29.6 mL) with 5 mL of artificial diet each, infested with 3 neonate larvae per cup covered with a cardboard lid (Tamez-Guerra et al. 2008), for a total of 90 neonates per tray (Fig. 1B). Insects were exposed to neem VOCs produced by either 1 or 10 g of plant material or by 1 or 10 mL of plant extract (Fig. 1C). Evaluation of neem VOCs from plant material against *T. ni* NL and Gto strains included 7 treatments testing either dry material (1 or 10 g of leaves, stems, or bark) or hexane or methyl chloride extracts (1 or 10 mL of leaf, stem, or bark extract) and the untreated control.

Containers with all tested insects were incubated at 25 ± 2 °C and 75 ± 5% RH, keeping the cup's cardboard lid (which allowed the VOC exchange) in place during all the bioassays and opening them just to evaluate mortality. Neonates for the untreated control group were placed inside similar plastic containers and incubated as described above, but the plant samples or extracts were not placed inside the plastic container. Mortality was recorded after 5 and 7 d incubation, after which plant or extract samples were removed from the containers and the larvae were kept inside under constant observation to record their development until pupation. When larvae reached the pupal stage, about 30 females and 25 males were placed in 8 L plastic containers as reproductive chambers. Paper towels were placed surrounding the container wall and on the bottom, then the container was covered with muslin. Both paper towel and muslin were used by females to lay their eggs. Cotton soaked in 10% sugar solution was provided as food for the adults (Blanco et al. 2009). The chambers were used to evaluate adult emergence and fertility, as well as oviposition and larval emergence from NL or Gto *T. ni* individuals that had survived each treatment (Tamez-Guerra et al. 2006). Larvae were kept to evaluate any anti-oviposition or anti-feeding effect. After larvae reached the pupal stage, 50 ± 3 pupae were transferred to 1 oviposition chamber. Emerged adults from these pupae were not sexed.

Lethal Doses of VOCs

In order to determine the neem sample amount that would produce VOCs needed to kill half of the population of each *T. ni* strain (LD50), 7 doses were used. The experiment tested 60 Gto and 60 NL neonates, placed in 1 L containers with 50 g of artificial diet and exposed to VOCs from 0, 10, 20, 30, 40, 50, and 60 g of dried neem leaves. Exposure was done by placing a tray with the 1 L container and 60 neonates, inside an 11 L container for each tested dose (Fig. 1). Bioassays were done in duplicate, testing neonates from different generations. For controls, bioassays were performed in the same way but without the test samples inside the 11 L container. The 11 L containers were incubated as described for the VOC toxicity test. Mortality rates were recorded after 7 d incubation, and after this, the plant samples were removed from the tray and larvae were kept to continue their life cycle as previously described.

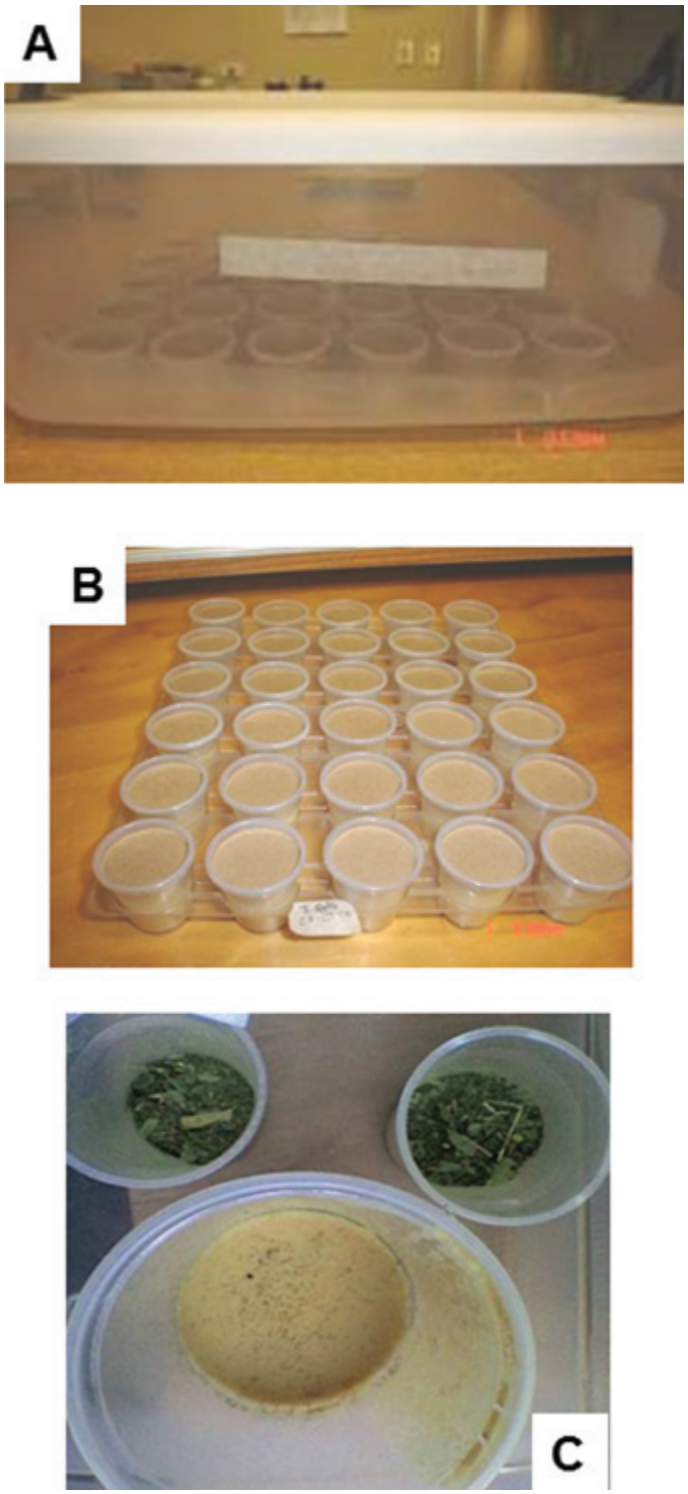

Fig. 1. Setup of the bioassay container for neem VOC exposure of *Trichoplusia ni* neonates. A) View of tray with 30 cups placed inside the 11 L plastic container with airtight lid for VOC exposure; B) view of tray with 30 cups with artificial diet infested with 3 neonates each and cardboard lid to allow VOC exchange; C) view of 1 L container with artificial diet (bottom) and 1 oz (29.6 mL) cups (top) with 1 g milled dried neem stems or leaves.

In addition, to compare the efficacy of neem VOCs after storage of plant material, leaves stored 6 mo after field collection were used to perform a dose-response bioassay against the *T. ni* NL strain, using 0, 30, 40, 50, and 60 g dried leaves per treatment. Experiments were

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done testing 90 neonates (3 larvae per cup on artificial diet) with 3 replicates per treatment. Incubation conditions and evaluations were done as previously described.

Physiologic Effects

To record physiologic changes upon neem VOC exposure, 30 surviving 4th instars, exposed and unexposed, were taken randomly from each bioassay and replicate. Larvae were weighed on an analytical scale after removal of the food particles from their guts by using dissecting forceps with blotting paper. Once the larvae reached the pupal stage, 30 pupae from each strain were placed on brown paper and their size was recorded by using a 6 inch (15.24 cm) digital Vernier (Mitutoyo, Grainger S.A. de C.V., Apodaca, Nuevo León, México).

To determine if neem VOCs induced changes in transcription of genes related to immune responses and chemical resistance, 11 markers were selected to evaluate differences among 4th instars of the NL strain that had been exposed to VOCs of dried neem leaves or unexposed as neonates. The innate humoral immune response of *T. ni* based on antimicrobial peptide gene transcription was determined by testing *attacin*, *cecropin*, *gloverin*, *lebocin*, *lysozyme*, peptidoglycan recognition protein (*PGRP*), *gallerimycin*, and *defensin* (Tamez-Guerra et al. 2008; Ericsson et al. 2009; James & Xu 2012). In addition, the *phenoloxidase* amplification as marker for innate immune response (Valadez-Lira et al. 2012), as well as *caspase* for apoptosis and *cytochrome P450* (*CYP4L4*) for chemical insecticide resistance (Hebert et al. 2009; Gu et al. 2012), were evaluated. The amplification of the *ribosomal protein S5* gene (F- ATGGCTGAAGAAAACTGGAAT, R-TAACGGTTGGACTTGGACTTAGCAAC) (Wang et al. 2005), which is a constitutive gene of *T. ni*, was used as an internal control (reference gene).

The transcription of the 11 markers was analyzed with reverse transcriptase polymerase chain reaction (RT-PCR). Larvae were surface sterilized for 5 s in 70% ethanol before dissections to obtain the midgut. The midguts from 10 VOC-exposed or unexposed larvae were homogenized with a pestle in 1 mL Trizol, and total RNA was extracted with the TRIzol® Plus RNA Purification kit (Invitrogen, Accesorios para Laboratorios, S.A. de C.V. Mexico, D.F.) according to the protocol of the manufacturer. To remove any traces of genomic DNA, samples were treated with DNase (RQ1 RNase free DNase, Promega) following the manufacturer's protocol. Total RNA was quantified with a spectrophotometer (GeneQuant, Amersham) at 230 nm. Each RNA sample was stored in 50 mL diethylpyrocarbonate water at −20 °C until use.

The RT-PCR was performed in 2 steps. In the 1st step, the extracted RNA was used to produce complementary DNA (cDNA). In the 2nd step, cDNA was used as template for transcript amplification (Valadez-Lira et al. 2012). For cDNA synthesis, the ImProm-II™ Reverse Transcription System Promega kit was used according to the manufacturer's protocol. For transcript amplification, 5 µg RNA were mixed with 1 µL of each 3› primer from the selected target gene (Table 1), 3 µL oligo dT in nuclease-free water, 3 µL reaction buffer, 2 µL magnesium chloride, 1 µL dNTP mix, 1 µL reverse transcriptase, and 0.5 µL RNasin inhibitor. Fifty µL of this mixture were incubated at 42 °C for 1 h and then the enzyme was inactivated by incubation at 65 °C for 10 min.

The PCR amplification conditions were 1 cycle at 94 °C for 4 min, followed by 30 cycles at 94 °C for 60 s, 60 °C for 90 s, 72 °C for 90 s, and then 1 cycle at 72 °C for 5 min. The amplicons were electrophoresed through 1.5% agarose gels and stained with ethidium bromide. Density of the DNA bands in agarose gels was estimated using the image software Image J (Image Processing and analysis in Java, http://rsbweb.nih.gov/ij/). DNA band amplification values of selected immune and chemical resistance markers were compared with the *ribosomal protein S5* band within the same gel, using the latter as reference for value estimation by assuming its value as one unit for comparison purposes. Analysis was performed using samples from 3 larvae per gene.

Antimicrobial Effect

To determine if neem VOCs induced changes in the enterobacteria in the exposed larvae, the *T. ni* NL strain was selected again because it had shown the highest mortality after exposure to stem and leaf treatments. For this assay, the midguts from 10 VOC-exposed (dried neem leaves) or unexposed 4th instars were dissected and subjected to RNA extraction and 2-step RT-PCR as described in the previous subsection. The *23S* ribosomal subunit of the Enterobacteriaceae group was used as a positive control and amplified with GoTaq Green Master Mix (Promega Corp., Madison, Wisconsin) using the primers En-lsu3F (TGCCGTAACTTCGGGAGAAGGCA) and En-lsu3R (TCAAGGCTCAATGTTCAGTGTC) (Matsuda et al. 2007). Reference gene, reaction mix,

Table 1. Selected primers of gut immune and chemical insecticide resistance markers for NL strain (laboratory) *Trichoplusia ni* 4th instars.

Gene	GenBank accession no.	Oligonucleotide (3′ to 5′)	Size (bp)	Tm (°C)
ribosomal protein S5	AY837869	F- ATG GCT GAA GAA AAC TGG AAT R- TTA ACG GTT GGA CTT AGC AAC	782	54
attacin	U46130	F- ATG TTC ACC TAC AAA TTG ATT R- CTA CCA CTT ATT ACC AAA AGA	764	50
cecropin	U38645	F- ATG AAT CTC GTG AAG ATT TTG R- CTA TTT TCC AGT GAT AGA GGC	189	50
lebocin	AF233589	F- ATG AGT AAA TAC ATT CTG GTG R- TTA AAC GTA GAT AGG GAA CCG	432	50
lysozyme	U38782	F- ATG CAA AAA CTC AGA GTA TTT R- TTA GCA TTT GCT GAT GTC GCA	426	60
PGRP	AF076481	F- ATG GAA ATA CTA TTT GTG CTA R- TCA GTT ATC TAG GAA ATG GTC	549	54
gallerimycin	EU016388.1	F- ATG AAG GCT TGC TTA GTT TT R- CTA TCG CAG ACA TTG ACA TC	225	50
defensin	EU016385.1	F- ATG GAG TTG AAA ACG GTG AA R- TTA AAT ACA ACT GCA TGT G	309	50
caspase	FJ266474.1	F- ATG CTG GAC GGT GAA TCG CA R- TTA CTT TTT ACC AAA TAC AA	885	50
cytochrome P450 (*CYP4L4*)	DQ768220.1	F- GGT CAA AGA TGG AAG TCT CAC R- CGT CTT GCA TTG ATC ACT TTG	267	63

Tm = melting temperature

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and temperature conditions were the same as described in the previous section. The *23S* gene was cloned in plasmid pGEM-T (pGEM-T Easy Vector System I, Promega). The amplicons were electrophoresed through 1.5% agarose gels and stained with ethidium bromide. Density of the DNA bands in agarose gels was estimated using the image software Image J. DNA density values of Enterobacteriaceae DNA amplicons (presence or absence) were compared with the *23S* amplicon within the same gel, using the latter as reference for value estimation by assuming its value as one unit for comparison purposes.

STATISTICAL ANALYSES

Statistical analysis to determine significant differences in the rates of mortality from insects subjected to neem VOC treatments was carried out by chi-square tests using SPSS (Version 13 MAC). Toxicity of neem VOCs as LD50 was calculated by the Probit method using the program Polo-PC plus (LeOra 2007). Data recorded for larval weight and pupal size were analyzed by the Student's *t*-test and Mann–Whitney test among treatments where mortality occurred and $n < 30$ individuals. Significant changes in immune and chemical resistance marker amplification were compared by ANOVA and Tukey test (SPSS, Version 2008) at $P < 0.05$.

Results

TOXICITY AND LETHAL DOSES

Results of the neem toxicity test against the 2 cabbage looper strains (NL and Gto) showed that the VOCs released by leaves, stems, and bark, as well as their hexane or methyl chloride extracts, were toxic against *T. ni* NL neonates. Significant differences in mortality were detected and the most toxic treatment was neem VOCs from dried leaves, where the calculated mortality risk was significantly higher (2.6 times, $P < 0.001$) compared with that of the control larvae. Mortality of larvae was 77% after exposure to 1 g and 79% after exposure to 10 g, whereas that of untreated control larvae was 17% (Fig. 2). Similarly, the mortality risk of *T. ni* NL neonates exposed to VOCs from 10 g of dried stems was significantly higher (2.5 times, $P < 0.001$) compared with that of untreated control larvae. Exposure to VOCs from 1 and 10 g of neem stems resulted in 24 and 63% mortality, respectively, whereas mortality was 13% in the control larvae (Fig. 3). Other treatments, including hexane or methyl chloride extracts from dried neem leaves, stems, and bark, did not show significant differences between the larval mortality at the used doses.

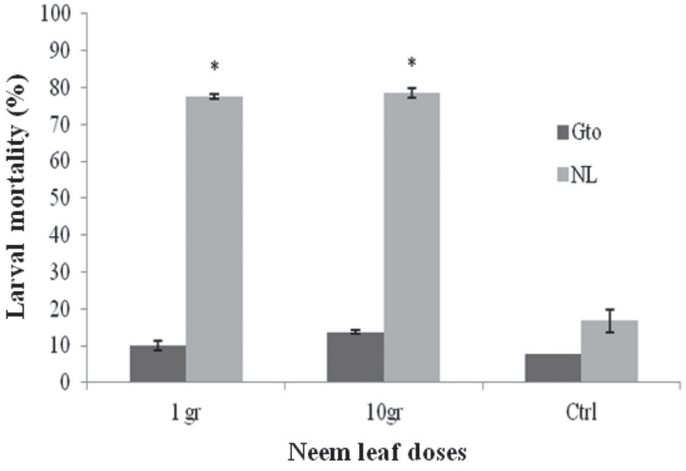

Fig. 2. Mortality for NL and Gto strains of *Trichoplusia ni* exposed as neonate larvae for 7 d in sealed containers to VOCs from 1 or 10 g of dried neem leaves compared with the unexposed controls. Data represent the mean ± standard deviation of 3 replicate experiments per treatment (90 larvae per replicate were tested).

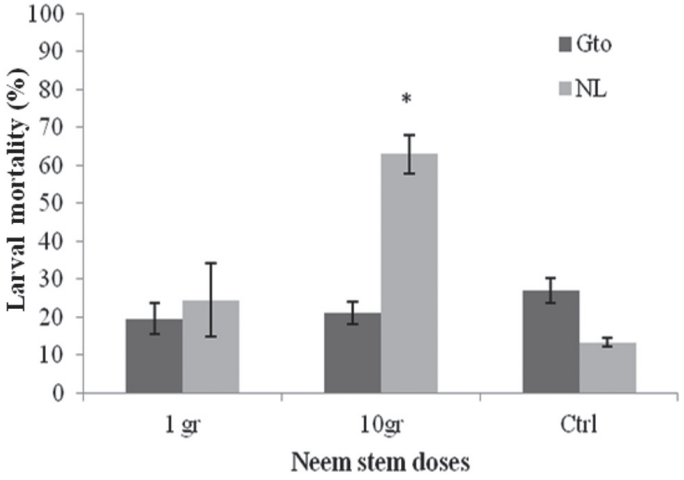

Fig. 3. Mortality for NL and Gto strains of *Trichoplusia ni* exposed as neonate larvae for 7 d in sealed containers to VOCs from 1 or 10 g of dried neem stems compared with the unexposed controls. Data represent the mean ± standard deviation of 3 replicate experiments per treatment (90 larvae per replicate were tested).

Because mortality after exposing neonates to VOCs of up to 10 g of neem samples did not reach 100%, we performed bioassays to evaluate VOC effects using higher doses (up to 60 g) of dried leaves. Results showed a dose-response effect in the 2 strains, with mortality rates reaching 100% in the NL strain and 79% in the Gto strain after exposure to 60 g of dried leaves (Fig. 4). Estimated lethal doses were LD10 = 0.75 g, LD50 = 5.6 g, and LD90 = 10.2 g for *T. ni* NL larvae, and LD10 = 0.9 g, LD50 = 24.8 g, and LD90 = 40.2 g for *T. ni* Gto larvae. Because VOCs from dried neem leaves produced the highest mortality when we tested 1 or 10 g on the *T. ni* NL strain (77 and 79% mortality, respectively), this strain was selected to compare the efficacy of neem VOCs after 6 mo of storage of leaves. Results were 9, 64, 78, 80, and 100% mortality after exposure to 0, 30, 40, 50, and 60 g of stored dried leaves, respectively.

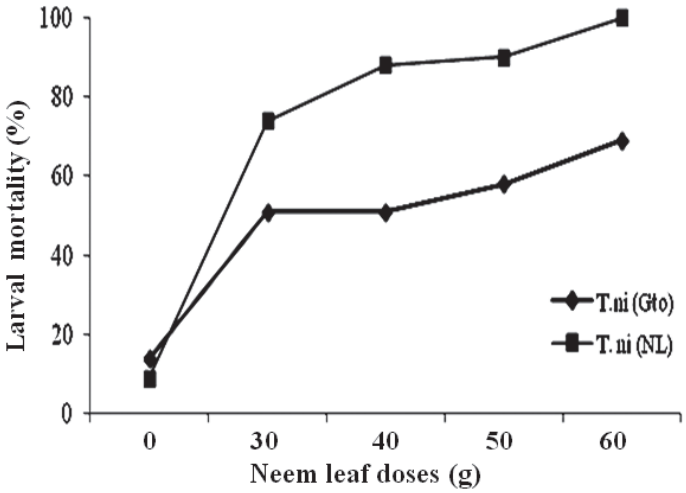

Fig. 4. Dose-response test using dried neem leaves and 5 doses. In brief, 60 neonates of the NL or Gto strain in a 1 L container with artificial diet were placed aside the dried neem leaves inside an airtight plastic box, and were examined after 7 d incubation. Data represent the mean ± standard deviation of 2 replicate experiments per treatment.

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PHYSIOLOGIC EFFECTS

After *T. ni* neonates had been exposed to dried bark, leaves, and stems, larval weights of 4th instars and pupal sizes showed differences ($P < 0.05$) compared with unexposed insects. In the Gto strain, exposure to neem VOCs of 1 g dried bark resulted in higher larval weight, whereas all other treatments with dried plant material resulted in reduced larval weight or pupal size, compared with unexposed control insects. In the *T. ni* NL strain, the methyl chloride extract from neem leaves was the only extract that caused lower larval weight, as compared with the unexposed controls (data not shown). In this strain, larval weight was reduced after exposure to dried stems, but no effect was observed after exposure to dried bark (Table 2).

Immune and chemical insecticide resistance marker amplification analysis was performed with the NL strain to determine if neem VOCs induced changes in antimicrobial peptide and chemical insecticide resistance markers between exposed and unexposed larvae. Results revealed an over-transcription of 8 of the 11 selected markers when related to the *ribosomal protein S5* internal control (Fig. 5A). ANOVA of the innate humoral immune response markers showed significant differences between exposed and unexposed larvae. VOC-exposed *T. ni* larvae over-transcribed *cytochrome P450* (*CYP4L4*) ($F_{3,8} = 13.366$; $P = 0.002$), *PGRP* ($F_{3,8} = 10.14$; $P = 0.004$), *lysozyme* ($F_{3,8} = 12.224$; $P = 0.002$), *attacin* ($F_{3,8} = 10.701$; $P = 0.004$), *cecropin* ($F_{3,8} = 15.946$; $P = 0.001$), *defensin* ($F_{3,8} = 12.199$; $P = 0.002$), *gallerimycin* ($F_{3,8} = 11.875$; $P = 0.003$), and *lebocin* ($F_{3,8} = 12.87$; $P = 0.002$) compared with unexposed (control) larvae, whereas *ribosomal protein S5* (internal control) and *caspase* transcripts showed no differences (Fig. 5B). *Gloverin* and *phenoloxidase* transcripts were not detected in our analysis.

ANTIMICROBIAL EFFECT

The transcription of the Enterobacteriaceae group marker in *T. ni* NL larvae exposed to 10 g of leaves is shown in Fig. 6. Results showed that transcription of the internal control (the *ribosomal protein S5* from *T. ni,* product of ~782 bp) was similar between unexposed and VOC-exposed larvae (Fig. 6, lane 3 for unexposed; lanes 5 and 7 for exposed), whereas the transcription of the Enterobacteriaceae marker (product of ~428 bp) was higher in guts of unexposed (Fig. 6, lane 3) compared with exposed larvae (Fig. 6, lanes 5 and 7). The same results were observed with all replicate samples (data not shown). PCR products of DNA-free RNA (used as control template in the 2nd RT-PCR step) from each treatment produced no transcripts (lanes 4, 6, and 8 in Fig. 6).

Density analysis of the Enterobacteriaceae DNA amplification band gave a relative value of 0.44 (using the given value of 1.0 for the *ribosomal protein S5* gene for comparison) in midguts of unexposed larvae (Fig. 6, lane 3), whereas the average value in midguts from VOC-exposed larvae (lanes 5 and 7) was 0.12, representing only about 27% of relative amplification.

Discussion

VOCs produced from plants are not just metabolic waste products but represent important plant adaptations for survival (Jenke-Kodama et al. 2008). Leaves, flowers, stems, bark, and seeds are primarily used as biological insecticides, and products are relatively inexpensive (US$ 4.5 per kg), are recommended as fertilizer, are biodegradable, and represent a good alternative to chemical insecticides (Weathersbee & Tang 2002). There are reports that demonstrate the mortality caused by neem VOCs (mainly from seeds) on a variety of insects (Balandrin et al. 1988; Koul 2004), but little is known about VOCs from foliage, especially after it has dried. In this regard, the potential of plants to release volatiles after being dried has been reported by others (Dube et al. 2011).

In the present study, we hypothesized that neem leaves, stems, and bark could be used for insect pest control as biofumigants. Holopainen & Blande (2013) discussed that specific VOCs produced by a plant, particularly monoterpenes, can be absorbed by neighboring plants that "borrow" those compounds to become protected against herbivores. In this study, we noticed that VOCs produced by either 1 or 10 g of neem leaves were toxic to *T. ni* neonates; VOCs were probably absorbed in the artificial diet surface as previously reported (Padhy & Varshney 2005), although their production will decrease over time. When 6 mo stored dry leaves were tested in a dose-response assay against the *T. ni* NL strain, results were different compared with those obtained with fresh material (79% mortality with 10 g of fresh against 64% with 30 g of 6 mo stored sample). Nevertheless, as previously observed, 60 g samples of material were required to produce 100% mortality by VOCs of either fresh or 6 mo old neem samples. This indicates that even if these VOCs are being reduced over time, their insecticidal activity remains active against susceptible insects. These results may encourage new trials in confined areas such as warehouses, especially relevant under certain storage conditions to reduce aflatoxin production (Zeringue et al. 2001).

Stems and bark had an effect on the metabolism of larvae of *T. ni* and there were significant differences depending on the strain. Changes in insect physiology have been reported for Lepidoptera (Nisbet 2000). Furthermore, Mitchell et al. (2004) observed an anti-feeding effect, which affected the development of *Clavigralla scutellaris* (Westwood) (Hemiptera: Coreidae) nymphs that had been submerged in water, hexane, and methanolic extracts of neem seed kernels. In our study, neem extracts obtained with solvents such as methyl chloride and hexane did not show the activity observed with fresh material, as no mortality was detected, and only methyl chloride extract from leaves reduced the weight of 4th instars in the laboratory strain. A similar case was reported by Sutherland et al. (2002), who demonstrated that the aqueous extracts of neem seeds showed very low toxicity against *Oebalus poecilus* (Dallas) (Hemiptera: Pentatomidae) by contact, compared with neem-based products.

Table 2. Average larval weights and pupal sizes of *Trichoplusia ni* strains after exposure to neem VOCs.

			Size (cm)[a]			Weight (g)[b]		
T. ni strain	Treatment	Dose	Unexposed	VOC-exposed	*P*	Unexposed	VOC-exposed	*P*
NL (laboratory)	Leaves	1 g	15.08	12.57	0.001			
	Stems	1 g	15.55	16.31	0.012	0.1716	0.1123	0.014
	Stems	10 g				0.1716	0.0882	0.002
Gto (field)	Bark	1 g	16.11	15.51	0.035			
	Bark	10 g	16.11	15.49	0.014			

[a]Differences in pupal size were calculated with Student's *t*-test and Mann–Whitney test, using 30 surviving pupae from 3 replicates.
[b]Differences were calculated using 30 surviving larvae from 3 replicates.

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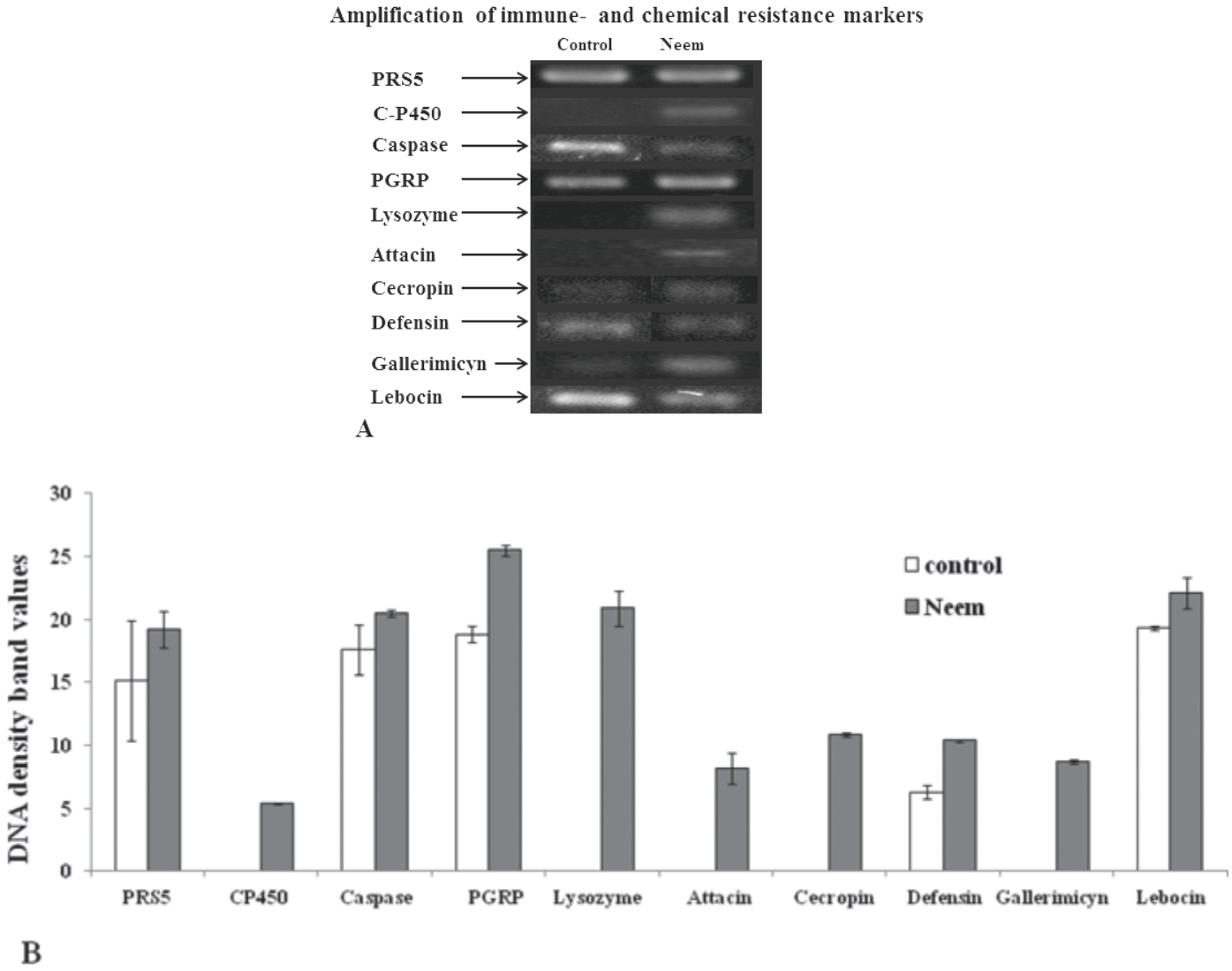

Fig. 5. Transcription of selected immune and chemical resistance markers and the *ribosomal protein S5* gene from rRNA samples of *Trichoplusia ni* NL strain larval midguts, dissected from unexposed (control) larvae or larvae exposed to 10 g of neem leaves, determined by reverse transcriptase polymerase chain reaction (RT-PCR). A) DNA amplification bands as detected by RT-PCR in agarose gels; B) DNA density estimated values in units compared with the *ribosomal protein S5* band using the image software Image J, where bars on each column represent the standard error value from 3 replicate samples.

In insects, antimicrobial peptides (AMPs) and lytic enzymes are activated after pattern recognition proteins identify invading microbes or other internal non-self objects via humoral response. It has been reported that several organic and inorganic insecticides stimulate lytic enzymes and the production of AMPs, which leads to the formation of reactive oxidative species through the phenoloxidase cascade, whereas botanicals such as pyrethrins also activate the phenoloxidase via serine protease (James & Xu 2012). In the present study, in an attempt to better understand the effect of VOCs of neem foliage on the *T. ni* larval immune response, we selected surviving insects and collected their midguts to evaluate 11 genetic markers reported as related to immune response, chemical insecticide resistance, and apoptosis (James & Xu 2012). We were unable to detect the AMP *gloverin* or the general immune response marker *phenoloxidase*, but most of the AMPs tested (*PGRP, attacin, cecropin, defensin, gallerimycin*, and *lebocin*) were over-transcribed, as were the humoral immune response lytic enzyme *lysozyme* and the chemical insecticide resistance marker *cytochrome P450* (*CYP4L4*) (Fig. 5A). The apoptosis-related marker *caspase* (Hebert et al. 2009) showed no differences among unexposed or VOC-exposed larvae (Fig. 5B). Because insect growth hormones such as ecdysone could alter the AMP production pathways (James & Xu 2012), and neem components may lead to reductions or inhibitions in larval growth and pupal molting (Ascher 1993), our results indicate that neem VOCs may be involved in this process.

In addition, we evaluated the amplification of Enterobacteriaceae genes among *T. ni* strains (exposed or unexposed to neem VOCs). A previous study reported differences in susceptibility to the bacterium *B. thuringiensis* among these *T. ni* strains (Tamez-Guerra et al. 2006). Because the insecticidal activity of *B. thuringiensis* is mostly related to toxicity by crystalline proteins (Cry toxins), and some changes in the enterobacteria have been related to susceptibility changes (Broderick et al. 2009; Hernández-Martínez et al. 2010), this may be related to an internal response in an attempt to protect the insect from the toxic compounds, as reported by Senderovich & Halpern (2012). Nevertheless, our results showed that higher susceptibility observed by the laboratory strain was associated with lower enterobacterial load (gene amplification), whereas in response to *B. thuringiensis*, higher susceptibility was associated with higher bacterial load (Broderick et al. 2009; Hernández-Martínez et al. 2010).

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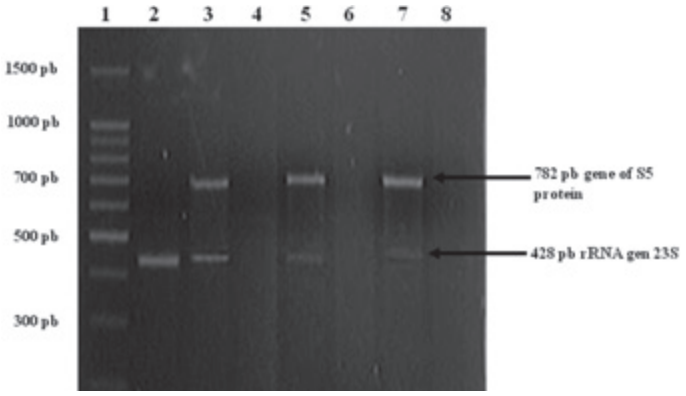

Fig. 6. Transcription of the *23S* gene of Enterobacteria (428 bp) and *ribosomal protein S5* gene (782 bp) from rRNA samples of *Trichoplusia ni* NL strain larval midguts, after exposure to 10 g of neem leaves, determined by reverse transcriptase polymerase chain reaction (RT-PCR). PCR product of RNA not subjected to RT-PCR was taken as a negative control. Lane 1, DNA ladder 100 bp; lane 2, PCR product of plasmid DNA with the Enterobacteria insert as positive control; lane 3, PCR products of the *23S* gene of Enterobacteria and the *ribosomal protein S5* gene of *T. ni* from unexposed larvae; lanes 4, 6, and 8, PCR of control RNA; lane 5, RT-PCR products in gut from VOC-exposed *T. ni* larva, showing both *23S* and *ribosomal protein S5* gene amplification (1st replication); lane 7, RT-PCR products in gut from VOC-exposed *T. ni* larva, showing both *23S* and *ribosomal protein S5* gene amplification (2nd replication).

In addition, microorganisms play important roles and often are essential to the growth and development of many insect species (Moran et al. 2008). It has been reported that these microorganisms could help in protecting insects from toxic compounds present in the environment (Senderovich & Halpern 2012). The endosymbionts help by contributing to insect reproduction, digestion, nutrition, and pheromone production (Broderick et al. 2004). In this regard, there are reports that show that neem has antimicrobial activity, but these reports studied neem seed extracts or oils with microbicidal effects, mostly against pathogenic bacteria in humans (SaiRam et al. 2000; Polaquini et al. 2006). However, to our knowledge, there are no reports related to a reduction in intestinal bacterial load induced by neem VOCs in susceptible insects, as observed in the present work (Fig. 6).

Taken together, our results indicated that neem VOCs from as little as 1 g had larvicidal activity against the laboratory *T. ni* strain. In addition, in this strain, neem VOC exposure stimulated the transcription of several immune response markers. Such transcription may be related to insect hormone changes and reductions in enterobacteria load in the surviving VOC-exposed larvae. The insecticidal activity of neem VOCs opens a new possibility, which is to "fumigate to kill" *T. ni* larvae in infested crops such as cabbage, cauliflower, broccoli, zucchini, etc., if dried neem leaves, bark, and stems are added as biofertilizer (Zheng et al. 2013). Nevertheless, more studies are needed to demonstrate the observed effects with other *T. ni* strains and lepidopteran pest species.

Acknowledgments

We thank Facultad de Agronomía–UANL personnel for providing fresh neem samples during this study. We are grateful to C. Blanco for reviewing a draft of this manuscript. This study was supported by Laboratorio de Inmunología y Virología, Facultad de Ciencias Biológicas–UANL, PAICYT–UANL grant CA760-11. No. 138 to P. Tamez-Guerra, and by CONACyT scholarship No. 228983 to G. Nuñez-Mejia.

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