Terpene-Induced Morphological Changes to Exoskeleton of Formosan Subterranean Termites (Isoptera: Rhinotermitidae): Toxic Effects of *cis*-nerol[1]

Betty C. R. Zhu,[2,4] Gregg Henderson,[3,4] Huixin Fei,[4] Xiao Ying[5] and Roger A. Laine[2,4,6]

Louisiana State University and the LSU Agricultural Center, Baton Rouge, LA 70803 USA

J. Entomol. Sci. 38(2) 225-233 (April 2003)

Abstract The terpenoid, *cis*-nerol was found to be highly toxic to the Formosan subterranean termite, *Coptotermes formosanus* Shiraki. Morphological abnormalities were observed in the exoskeleton including the spiracles and trichoid sensilla using scanning electron microscopy after exposure of termites to *cis*-nerol for 2 h. Proteins were found in a water-soluble fraction collected from the whole termite body exposed to *cis*-nerol for 60 min. It appears that terpenoids cause damage to cell membranes resulting in a significant loss of proteins.

Key Words *cis*-nerol, Formosan subterranean termite, termite morphology, protein leakage, toxicant

Terpenoids, with a diverse array of natural structures, are synthesized from C5 isoprene units. Mono- (10 carbons) and sesqui- (15 carbons) terpenoids are the principal components of essential oils that can be extracted from the leaves, roots, fruit, and flowers of higher plants (Templeton 1969). Several terpenoids exhibit toxic, repellent and attractive properties to insects (Osborne and Boyd 1974, Brattsten 1983, Siegfried 1987, Chantraine et al. 1998). More complex terpenoids and their derivatives are effective on a variety of insects that attack stored products (Amos et al. 1982, Singh et al. 1989), and a few are reported to be repellents and toxicants to termites (Shama et al. 1994, Cornelius et al. 1997, Zhu et al. 2001). These natural compounds have potential use as insect repellents and insecticides because of their low mammalian toxicity. However, little is known about the mechanism of their toxic action to insects.

Terpenoids are typically lipophilic, therefore they can potentially interfere with the biochemical and physiological functions of insect membranes (Gershenzon and Croteau 1991). Some effects of terpenoids on biological membranes have been reported (Tamir et al. 1984, de Smet et al. 1978, Sikkema et al. 1992, 1994, Uribe et al. 1985, 1990). These interactions affect the structure and function of the cellular membrane

[1]Received 30 March 2002; accepted for publication 24 August 2002.

[2]Department of Biological Sciences, Biochemistry and Molecular Biology Division.
[3]To whom requests are to be directed (Department of Entomology; email: grhenderson@agctr.lsu.edu).
[4]Department of Entomology.
[5]Socolofsky Microscopy Center of Biological Sciences.
[6]Department of Chemistry.

by hydrophobic interaction with the lipid bilayer, or hydrophobic protein domains, because of their lipophilicity. However, structural specificity predicts that certain protein binding sites are important for the effects.

Cis-nerol is an acyclic terpenoid having activity as a termiticide (Cornelius et al. 1997). We employed scanning electron microscopy to investigate the effects of *cis*-nerol on Formosan subterranean termites. Significant morphological changes were observed at the outer cuticular layer of termites including the exoskeleton, spiracles and trichoid sensillum. In addition, we examined the water-soluble fractions collected from terpenoid-treated termites and found proteins in the extracellular space. We present evidence that these compounds cause damage to termite cell membranes, resulting in a significant loss of proteins.

Materials and Methods

Termites. Two carton nests (colonies A and L) of Formosan subterranean termites (*Coptotermes formosanus* Shiraki) were collected from infested trees. Colony A was collected in Algiers, LA, in November 1997 in an oak tree; colony L was collected in

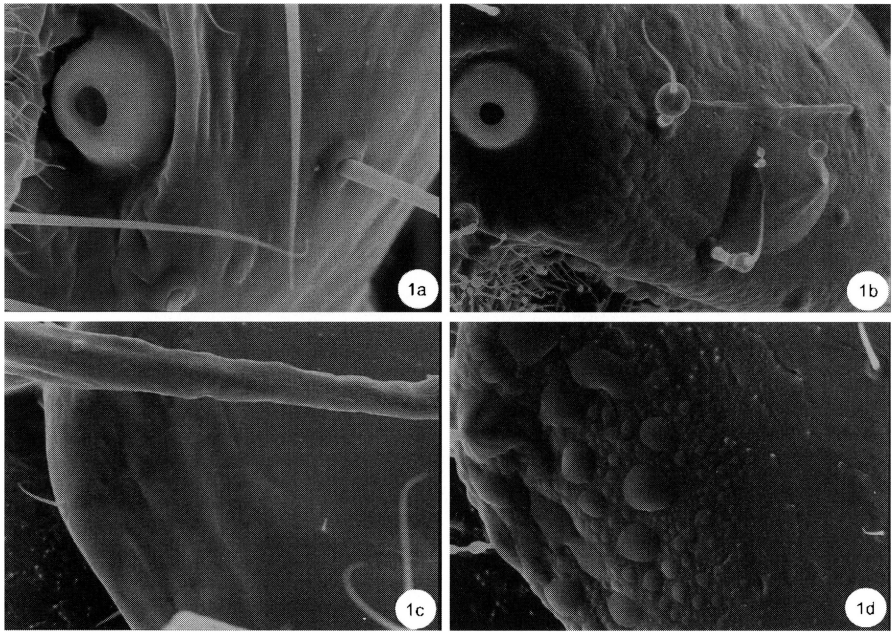


Fig. 1. Scanning electron micrographs of *C. formosanus* exoskeleton. (a) Exoskeleton of an untreated termite from colony A showing 2nd abdominal spiracle (1540×); (b) Exoskeleton of a termite from colony A which was exposed to *cis*-nerol (8 mg/dish) for 2 h showing bleb-modified cuticle and hairs (1000×); (c) Exoskeleton of an untreated termite from colony L. Photograph showing section of postmentum of labium (1010×); (d) Exoskeleton of a termite from colony L which was exposed to *cis*-nerol (8 mg/dish) for 2 h showing bleb-modified exoskeleton located at the postmentum of the labium (1010×).

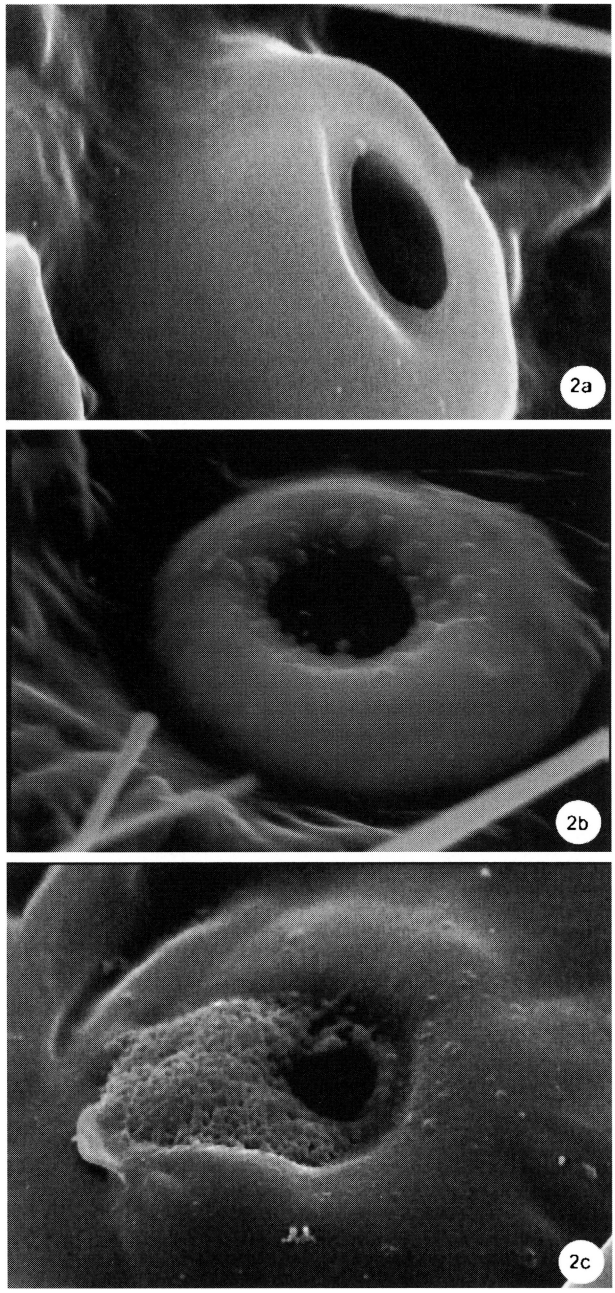


Fig. 2. Scanning electron micrographs of abdominal spiracles of *C. formosanus*. (a) Third abdominal spiracle of an untreated termite from colony L (4700×); (b) Spiracle with bled-modified surface from a termite of colony L which was exposed to *cis*-nerol (8 mg/dish) for 2 h; (c) Fourth abdominal spiracle from a termite from colony A exposed to *cis*-nerol (8 mg/dish) for 2 h (4460×).

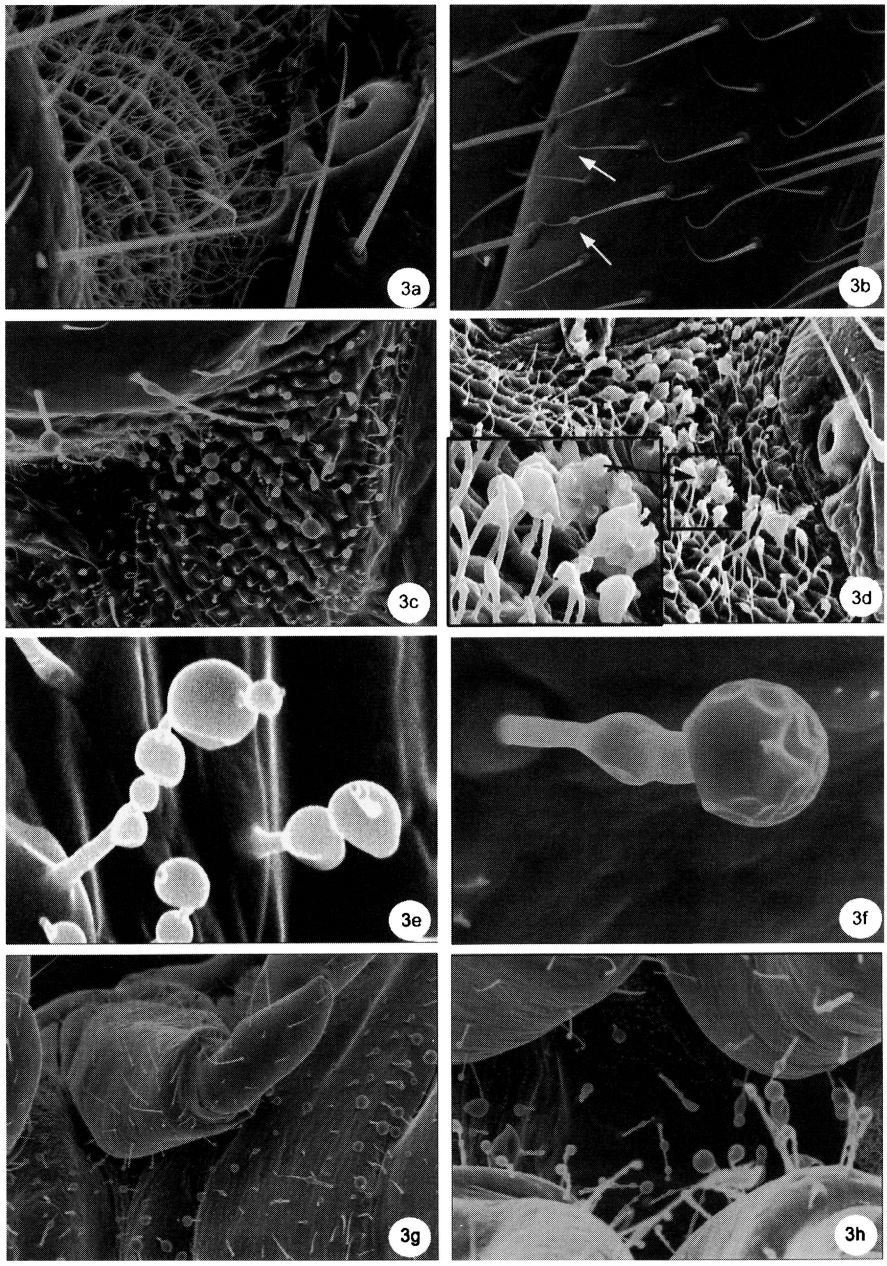
New Orleans in 1999 from a tree limb of undetermined species. Termites were held in 250-liter cans with pine as a food source and kept at 24 to 26°C. Moistened corrugated cardboard rolls were used to retrieve termites from the cans (La Fage et al. 1983). Termites were gently knocked from the cardboard rolls into clean plastic trays (40 × 50 cm) and isolated from debris by allowing them to climb on moistened paper towels.

Acute toxicity of *cis*-nerol. Plastic Petri dishes (10 × 50 mm) were used for testing the acute toxicity of *cis*-nerol against workers of the Formosan subterranean termite from colony A and colony L. Forty mg of *cis*-nerol was dissolved in 1 ml ethanol as stock solution. Each filter paper disk (5.5 cm diam) was treated with 200 L of ethanol solution and five concentrations (1, 2, 4, 8 and 16 mg/dish) were tested. Ethanol alone (200 L) was negative control and each filter paper was air-dried for 10 min. The *cis*-nerol and ethanol treated filter papers were placed in the bottom of each dish. Ten worker termites with 3 replicates were used for each test. The toxicity tests were observed at 2 and 4 h for both colonies.

Assay of protein leakage from termite body. Fifty worker termites from colony A and 50 from colony L were placed on filter papers treated with 8 mg of *cis*-nerol (acute toxicity) and *trans*-nerolidol (not acute toxicity) in a Petri dish (10 × 50 mm) for 60 min. Termites with no exposure to these chemicals served as control. Termites were transferred to micro-centrifuge tubes, and 1 mL distilled H_2O was added to each tube. The tubes were shaken for 10 min using an orbital shaker operating at 120 to 180 rpm. The supernatant fraction (0.8 mL) was removed and lyophilized. Proteins of body fluids (hemolymph) were obtained by a 10-µL Hamilton syringe (Reno, NV) inserted into the abdomen of the worker termite, and the debris removed by centrifugation. The proteins putatively leaking through termite cell membranes as measured from whole body extracts and proteins of the body fluids were examined using 12% SDS-PAGE. The gel was stained with Coomassie Blue R. The molecular standards were M.W. 205 KD (myosin), 116 KD (β-galactosidase), 97 KD (phosphorylase), 66 KD (albumin, bovine), 45 KD (albumin, egg) and 29 KD (carbonic anhydrase).

Scanning electron microscopy (SEM). Thirty worker termites from the negative control, 30 workers exposed to *cis*-nerol (8 mg/dish) for 2 h and 30 workers exposed to *cis*-nerol (8 mg/dish) for 4 h were fixed with a solution comprised of 45% ethanol, 5% acetic acid and 2% paraformaldehyde at room temperature for 24 h. They were

Fig. 3. Scanning electron micrographs of *C. formosanus* sensillae. (a) Hair-shaped sensillum from an untreated termite (colony L) showing pleural membrane near the 2nd abdominal spiracle (1030×); (b) Ampoule-shaped sensillum from an untreated termite (colony L), located at 8th abdominal tergum (624×); (c) Balloon-shaped sensillum located at the pleural membrane near 2nd abdominal spiracle (628×); (d) Irregularly shaped sensillum located at the pleural membrane near spiracle 2 from termites of colony A treated with *cis*-nerol (8 mg/dish) for 4 h; (e) Bead-shaped sensillum located at the pleural membrane near 3rd abdominal spiracle (8250×); (f) Mushroom-shaped sensillum from a treated termites of colony A, located at the pleural membrane near 3rd abdominal spiracle (3640×); (g) Balloon-shaped sensillum of a treated termite (colony L), located near the cerci (201×); (h) Balloon-shaped sensillum of a treated termite (colony L), located near the coxae (571×).



dehydrated in an ethanol series and dried in carbon dioxide using a Denton DCP-1 critical-point-drying apparatus. Ten randomly-selected termites from each treatment were mounted on stubs and coated with 25-nm of gold-palladium using a Hummer II Sputter Coater. The legs were dissected just below the coax prior to sputter coating

to provide a better view of the sternum. A Cambridge S-260 scanning electron microscope was used for examination of the termite exoskeleton. The head, thorax and abdomen were examined by SEM.

Results

Termite mortality. Twenty-five of the 30 termites (83%) from colony A and 18 of the 30 from colony L (60%) died within 2 h of exposure to *cis*-nerol at 8 mg/dish, mortality in both colonies reached 100% within 4 h. None of the termites from the negative control were dead at 2 and 4 h.

Morphological abnormalities. Most changes were found in *cis*-nerol treated termites on the exoskeleton, the spiracles and the trichoid sensillum. Bleb-shaped morphological abnormalities were observed in the exoskeletons of termites after exposure of termites to *cis*-nerol for 2 h. These abnormalities were found near abdominal spiracles 2, 3, 5, 7 (Fig. 1b) in 25% of the treated termites. A blebbed exoskeleton also was observed on the postmentum of the labium from a termite from colony L (Fig. 1d). Morphological abnormalities of the spiracle appeared in 50% of the termites exposed to *cis*-nerol (Fig. 2b). The observed abnormality is shown to its extreme in Fig. 2c. A few ampoule-shaped sensilla were found in untreated termites from colony A at the 8th and 9th abdominal tergum (Fig. 3b). Trichoid sensilla with ballooned hairs were observed in 50% of the treated termites. Many balloon-shaped, mushroom-shaped and bead-shaped sensilla were found at the pleural membrane near abdominal spiracles (Fig. 3c, 3e, 3f). The shapes of trichoid sensilla became irregular after exposure to *cis*-nerol for 4 h (Fig. 3d). Balloon-shaped sensilla also were found near cerci (Fig. 3g) and on the coax (Fig. 3h) after exposure of termites to *cis*-nerol for 2 h. No morphological abnormalities were observed on the untreated termites from either colony (Fig. 1a, 1c, 2a, 3a).

Protein analysis. Proteins on an SDS-PAGE are shown in Fig. 4. Lane 1 was the molecular weight standard. Lanes 2 and 5 are "leaked" proteins collected from colony A (lane 2) and colony L (lane 5) without chemical treatment. Lanes 3 and 6 show the leaked proteins collected from colony A (lane 3) and colony L (lane 6) treated with 8 mg/dish of *trans*-nerolidol for 60 min. Lanes 4 and 7 show the leaked proteins from colony A (lane 4) and colony L (Lane 7) treated with *cis*-nerol for 60 min. Proteins of the body fluids are shown in Fig. 4, lane 8. The molecular weight of a major protein in the body fluid was near 70 KD. The natural occurrence of several protein bands in the termite rinse shown in the controls and the *trans*-nerolidol treatment samples (Fig. 4, lane 2, 3, 5, and 6) and their apparent molecular weights were 66-70 KD, 45 KD and 25-30 KD. Protein bands with a broad range of molecular weights were found in the *cis*-nerol treated termites (Fig. 4, lanes 4 and 7).

Discussion

In the 1930s, researchers reported that disruption of the insect cuticle might be an effective way to manage pest insects (Zacher and Kunicks 1931, Wigglesworth 1933). As a result, investigations were conducted on the routes of cuticular penetration of some insecticides (Kühnelt 1939). Klinger (1936) reported structures like setae sockets, dermal glands and various conjunctivae to be particularly vulnerable routes of penetration through the cuticle. Increased cuticle water permeability resulting from the action of some organics was reported by Wagner and Ebeling (1959). Surface lipids

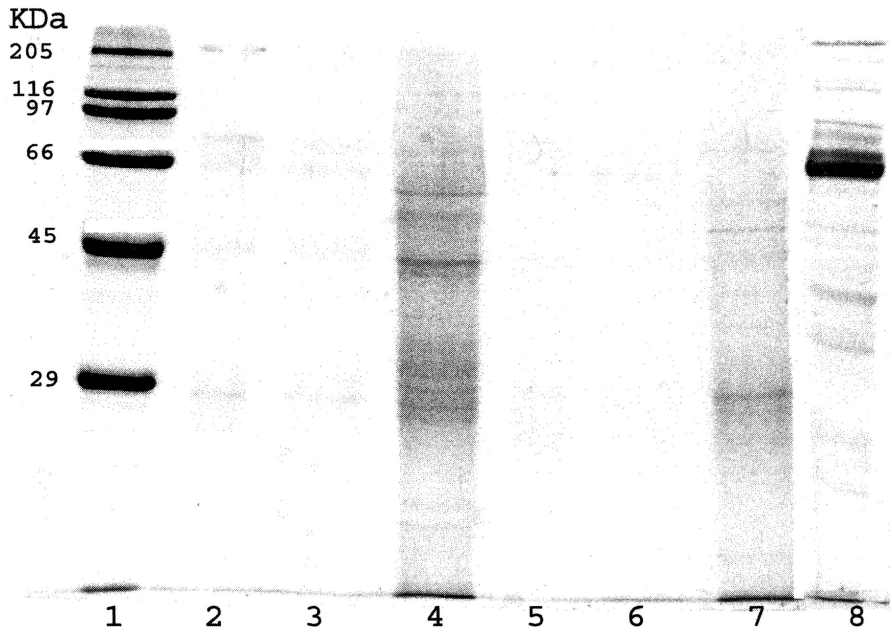


Fig. 4. SDS-PAGE separation of *C. formosanus* proteins: Lane 1—a molecular weight standard; Lane 2—proteins "leaked" from untreated Colony A termites; Lane 3—proteins "leaked" from termites from Colony A treated with *trans*-nerolidol; Lane 4—proteins "leaked" from termites from Colony A treated with *cis*-nerol; Lane 5—proteins "leaked from untreated Colony L termites; Lane 6—proteins "leaked" from termites from Colony L treated with *trans*-nerolidol; Lane 7—proteins "leaked" from termites from Colony L treated with *cis*-nerol; Lane 8—proteins from body fluids of termites.

may be removed by some organics and the cuticle rendered more water permeable. Chattoraj and Sharma (1964) concluded that insecticide-induced water loss takes place through the general body surface as well as through openings such as mouth, anus and spiracles. In the present study, the trichioid sensilla were modified after being treated with *cis*-nerol for 2 h. Most abnormal sensilla observed were on the intersegmental membranes near the 2nd and 3rd abdominal spiracles. This observation agrees with the results reported by O'Kane et al. (1933). They found that when droplets of insecticide were applied to many groups of sensilla and spines and to all articular and intersegmental membranes of cockroaches, the membrane regions became more permeable. We observed the irregular forms of sensilla after exposure of termites to *cis*-nerol for 4 h. The abnormally shaped hairs appeared ready to burst.

We hypothesize that damage of the cell envelope (of as-yet undefined cell types) may be one cause of the acute toxicity of *cis*-nerol to termites. We believe that a greater number and amount of proteins "leaked" through the exoskeletons after exposure of termites to *cis*-nerol (lane 4 and 7) were from cuticle and membrane proteins or cells. A major protein band of molecular weight near 70 KD was shown in the

body fluids (lane 8) but not in lane 4 or lane 7 supporting the hypothesis that the proteins in lane 4 and 8 were from different internal sources. The leakage may also depend on the extent of the damage to the cuticle and other structures, including the epidermis, or the basement membrane. We observed that more protein leaked if termites were exposed to *cis*-nerol for longer times (data not shown). This may explain the greater number and amount of proteins leaked from termites from colony A (lane 4) than colony L (lane 7). The natural occurrence of several protein bands in the controls is a new finding which is being pursued. The exact origins of the control and experimental protein bands are not yet known.

Acknowledgments

We thank Vadake R. Srinivasan of the Department of Biological Sciences, Louisiana State University, for very helpful advice and discussions. This paper was approved for publication by the Louisiana State University Agricultural Center and Louisiana Agricultural Experiment Station as manuscript #0-17-0586. Partial funding was provided through a specific cooperative agreement with the USDA/ARS-New Orleans (58-6435-8-084).

References Cited

Amos, T. G., R. L. Semple, P. Williams and M. Schwarz. 1982. Compounds related to juvenile hormone: activity of selected terpenoid compounds on *Rhyzopertha dominica* (Coleoptera: Bostrichidae), *Sitophilus oryzae* and *S. granarius* (Coleoptera: Curculionidae). Gen. Appl. Entomol. 14: 63-68.

Brattsten, L. B. 1983. Cytochrome P-450 involvement in the interactions between plant terpenes and insect herbivores, Pp. 173-195. *In* P. A. Hedin [ed.], Plant resistance to Insects. Am. Chem. Soc. Washington, DC.

Chantraine, J. M., D. Laurent, C. Ballivian, G. Saavedra, R. Iba ez and L. A. Vilaseca. 1998. Insecticidal activity of essential oils on *Aedes aegypti* larvae. Phytotherapy Research 12: 350-354.

Chattoraj, A. N. and V. P. Sharma. 1964. as reference cited by Ebeling W., permeability of insect cuticle, Pp. 271-343. *In* M. Rockstein [ed.], The physiology of Insecta, VI, 2nd ed. Academic Press, New York, 1974.

Cornelius, M. L., J. K. Grace and J. R. Yates, III. 1997. Toxicity of monoterpenoids and other natural products to the Formosan subterranean termites (Isoptera: Rhinotermitidae). J. Econ. Entomol. 90: 320-325.

de Smet, M. J., J. Kingma and B. Witholt. 1978. The effect of toluene on the structure and permeability of the outer and cytoplasmic membranes of *Escherichia coli.* Biochim. et Biophys. Acta 506: 64-80.

Gershenzon, J. and R. Croteau. 1991. Terpenoids, Pp. 65-291. *In* G. A. Rosenthal and M. R. Berenbeum [eds.], Herbivores: their interactions with secondary plant metabolites. vol. 1: the chemical participants. Academic Press, New York.

Klinger, H. 1936. as reference by Ebeling W., permeability of insect cuticle, Pp. 271-343. *In* M. Rockstein [ed.] The physiology of Insecta, VI, 2nd ed. Academic Press, New York, 1974.

Kühnelt, W. 1939. as reference cited by Ebeling W., permeability of insect cuticle, Pp. 271-343. *In* M. Rockstein [ed.], The physiology of Insecta, VI, 2nd ed. Academic Press, New York, 1974.

La Fage, J. P., N-Y. Su, M. J. Jones and G. R. Esenther. 1983. A rapid method for collecting large number of subterranean termites from wood. Sociobiology 7: 305-309.

O'Kane, W. C., G. L. Walker, H. G. Guy and O. J. Smith. 1933. as reference cited by Ebeling W., permeability of insect cuticle, Pp. 271-343. *In* M. Rockstein [ed.], The physiology of Insecta, VI, 2nd ed. Academic Press, New York, 1974.

Osborne, G. O. and J. F. Boyd. 1974. Chemical attractants for larvae of *Costelytra zealandica* (Coleoptera: Scarabaeidae). N. Z. J. Zool. 1: 371-374.

Sharma, R. N., V. B. Tungikar, P. V. Pawar and P. H. Vartak. 1994. Vapour toxicity and repellency of some oils and terpenoids to the termite, *Odontotermes brunneus.* Insect Sci. Applic. 15: 495-498.

Siegfried, B. D. 1987. In-flight response of the pales weevil, *Hylobius pales* (Coleoptera: Curculionidae) to monoterpene constituents of southern pine gum turpentine. Florida Entomol. 70: 97-102.

Sikkema, J., B. Poolman, W. N. Konings and J. A. M. de Bont. 1992. Effects of the membrane action of tetralin on the functional and structural properties of artificial and bacterial membranes. J. Bacteriol. 174: 2986-2992.

Sikkema, J., J. A. M. de Bont and B. Poolman. 1994. Interactions of cyclic hydrocarbons with biological membranes. J. Biol. Chem. 269: 8022-8028.

Singh, D., M. S. Siddiqui and S. Sharma. 1989. Reproduction retardant and fumigant properties in essential oils against rice weevil (*Coleoptera: Curculionidae*) in stored wheat. J. Econ. Entomol. 82: 727-733.

Tamir, I., A. Abramovici, I. Milo-Goldzweig and R. Segal. 1984. The hemolytic activity of citral: evidence for free radical participation. Biochem. Pharmacol. 33: 2945-2950.

Templeton, W. 1969. An introduction to the chemistry of terpenoids and steroids. Butterworths and Co. Ltd, London, Pp. 1-70.

Uribe, S., J. Ramirez and A. Pela. 1985. Effects of β-pinene on yeast membrane functions. J. Bacteriol. 161: 1195-1200.

Uribe, S., P. Rangel, G. Espinola and G. Aguirre. 1990. Effect of cyclohexane, an industrial solvent, on the yeast *Saccharomyces cerevisiae* and isolated yeast mitochondria. Appl. Environ. Microbiol. 56: 2114-2119.

Wagner, R. E. and W. Ebeling. 1959. Lethality of inert dust materials to *Kalotermes minor* Hagen and their role as preventives in structural pest control. J. Econ. Entomol. 52: 208-212.

Wigglesworth, V. B. 1933. as reference cited by Ebeling W., permeability of insect cuticle, Pp. 271-343. *In* M. Rockstein [ed.], The physiology of Insecta, VI, 2nd ed. Academic Press, New York, 1974.

Zacher, F. and G. Kunicke. 1931. as reference cited by Ebeling W., permeability of insect cuticle, Pp. 271-343. *In* M. Rockstein [ed.], The physiology of Insecta, VI, 2nd ed. Academic Press, New York, 1974.

Zhu, B. C. R., G. Henderson, F. Chen, L. Maistrello and R. A. Laine. 2001. Nootkatone is a repellent for Formosan subterranean termite (*Coptotermes formosanus*). J. Chem. Ecol. 27: 523-531.