Effects of Nootkatone and a Borate Compound on Formosan Subterranean Termite (Isoptera: Rhinotermitidae) and Its Symbiont Protozoa[1]

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Abstract Wood treated with disodium octaborate tetrahydrate, with nootkatone, a natural extract isolated from vetiver oil, or with both nootkatone and disodium octaborate tetrahydrate was tested for effects on *Coptotermes formosanus* Shiraki and its hindgut flagellates. Results demonstrated that after 7 d disodium octaborate tetrahydrate-treated wood induced high termite mortality and almost complete loss of flagellates, confirming the toxicity of borates to these termites. Wood treated with nootkatone alone or with the nootkatone-borate mix was consumed in significantly lower amounts than the control, and termite survival was comparable to results obtained for starved termites. A significant progressive reduction in the total number of protozoa was observed for all groups, including the controls. We conclude that nootkatone acts as a feeding deterrent, inducing starvation that results in almost a complete loss of *Pseudotrichonympha grassii* Koidzumi, the most important flagellate species for cellulose digestion in this termite.

Key Words *Coptotermes formosanus,* nootkatone, borates, natural products, insect repellent, protozoa, *Vetiveria zizanioides*

Formosan subterranean termites are a major wood pest in the southern USA. Since the banning of organochlorine pesticides, control of these imported subterranean termites should rely on methods and chemicals that guarantee best efficacy while being environmentally safe. The study of natural compounds, like extracts from plants, is acquiring growing interest for possible application in the development of selective, efficacious and toxicologically safe pesticides (Duke 1990). Nootkatone (5,6 Dimethyl-8-isopropenylbicyclo[4.4.0]dec-1-en-3-one) is a sesquiterpene ketone that has recently been isolated from vetiver (*Vetiveria zizanioides* Lynn Nash) oil and has shown strong repellent effects against *Coptotermes formosanus* Shiraki (Zhu et al. 2001).

Among the chemicals already in use as wood preservatives, borate compounds have gained acceptance for their diffusibility in wood (Barnes et al. 1989) and low mammalian toxicity (Krieger et al. 1996). Borates are odorless, non-volatile, slow-acting, non-repellent toxicants (Grace 1991), and their efficacy against termites has been well-documented (Barnes et al. 1989, Drysdale 1994, Grace 1997). Although poorly understood, the toxicity of borate compounds to termites seems to depend on

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severe interference with digestive processes (Williams et al. 1990, Nunes and Dickinson 1995). As wood-feeding insects, termites rely on their symbiont microbial community, consisting of anaerobic protozoa and bacteria, for the utilization of their food (Breznak 1982). Chemical compounds, introduced into the gut system by feeding, might affect the amount of hindgut protozoa inducing a loss that will cause the death of termites (Mannesmann 1972).

We investigated the effects of a food source treated with nootkatone or a borate compound (disodium octaborate tetrahydrate) or both nootkatone and disodium octaborate tetrahydrate on *C. formosanus* survival and feeding behavior, with a detailed analysis of the consequences on the symbiont microfauna.

Materials and Methods

Termites. One hundred *C. formosanus* workers were placed in clear plastic boxes (5.6 × 8 × 4 cm) with one hundred grams of #4 fine blasting sand, 5 ml distilled deionized water and a thin slice (3 × 2.5 × 0.1 cm) of balsa wood (*Ochroma lagopus* Swartz). The experiment was performed in February 2000 with termites from two large colonies, named colony "A" and colony "L," collected in New Orleans (LA) in January 1998 and December 1999, respectively.

Wood treatment. Before the beginning of the experiment, wood slices were air-dried for 24 h and then weighed. Three treatment solutions were prepared: "N," a 1% ethanol solution of nootkatone (Lancaster Synthesis Inc., Windham, NH); "T," a 1% water solution of disodium octaborate tetrahydrate (TIM-BOR®, U.S. Borax Inc., Valencia, CA; from heretofore referred to as "borate"); and "NT" a solution obtained by mixing the 1% ethanol solution of nootkatone to the 1% water solution of borate. Wood slices were soaked for 1 h in treatment solutions, dried in a ventilated hood for 24 h and reweighed. Retention of the solutions was calculated by mean weight gain. Containers were closed with lids and kept for 1 wk in thermostatic chambers (with non-treated units separated from treated ones) at 29°C. Three replicates for each colony (total = 6 replicates) received non-treated wood ("C," control units), or treated wood ("N," "T," "NT" units) or no wood at all ("S," starved units).

After 7 d from the beginning of the experiment, the units were dismantled, live termites were counted and wood slices were brushed clean and left to air dry for 24 h in a ventilated hood and weighed.

Protozoa counting. On the day before the beginning of the experiment (day 0) and on day 3, 5 and 7, three termites from each unit were removed and used for protozoa counting, following a modification of Mannesmann (1972) technique. The gut contents from the three randomly chosen termites were dissected in 40 µl of saline solution in which neutral red had been dissolved (0.5 ml of 1% aqueous neutral red solution into 10 ml salt solution), and the protozoa count in 0.4 µl was determined with a hemacytometer (improved Neubauer, Hausser Scientific, Horsham, PA) under a phase-contrast microscope. Protozoa species found in *C. formosanus* were identified following (Koidzumi 1921) and (Grassé 1952) as *Pseudotrichonympha grassii* Koidzumi, *Holomastigotoides hartmanni* Koidzumi, and *Spirotrichonympha leidyi* Koidzumi. Counts were made for each protozoan species and totaled. The number of individuals for each flagellate species per hindgut (X_F) was calculated as: $X_F = (G \times n)/(V \times 3)$, where G = volume (µl) of the solution containing the three hindguts; n = mean of the two counts within hemacytometer, V = volume (µl) of the counted area.

Data analysis. Response variables measured were wood consumption, mean number of protozoa per termite, and percentage of surviving individuals. Wood consumption and survival were compared with a two-tailed MANOVA followed by Newman-Keuls post-hoc test (Statistica 5.5, '99 edition, Statsoft Inc., Tulsa, OK). The factors analyzed were "wood treatment" and "colony." Numbers of protozoa were compared with two-tailed MANOVA for repeated measures followed by Newman-Keuls post-hoc test. The factors analyzed were "wood treatment" and "day." Although percentage values were transformed to arc sine of the square root for data analysis, untransformed means are reported.

Solution uptake. We used an aqueous borate solution with a concentration of active ingredient that was far lower than the one suggested for wood treatment with TIM-BOR® (10 to 15%). Previous trials performed using wood slices soaked for 1 h in solutions of 15%, 10% and 5% borate resulted in total death of all termites after 1 h (Maistrello, unpubl. data). The mean ± SD uptake for each one of the solutions was the following: nootkatone = 7.2 ± 1.4 mg, nootkatone-borate mix = 10.0 ± 1.5 mg and borate = 8.8 ± 1.1 mg. The mean ± SD uptake for the borate solution corresponds to a retention of 0.157% boric acid equivalents.

Results

Wood consumption. Termites exposed to borate were observed actively feeding on wood only until day 3, after which our observations showed they stopped feeding, and over the length of the experiment, their abdomens appeared progressively more flattened. Termites from nootkatone or nootkatone-borate groups did not show appreciable modifications in their feeding behavior or general activity during the week of the experiment. Termites consumed significantly different amounts of wood depending on wood treatment ($F = 31.18$, df = 3, 16; $P < 0.001$). The two colonies differed significantly in wood ingested ($F = 18.85$, df = 1, 16; $P = 0.0005$), however, in both colonies, the consumption of wood treated with borate alone or with nootkatone-borate was the lowest (3.6 ± 2.0 mg and 10 ± 4.0 mg, respectively), corresponding to an average wood mass loss of 2.36% and 4.40%, respectively. Consumption of wood treated with nootkatone alone was double that treated with the nootkatone-borate mix (corresponding to a wood mass loss of 9.77%) but was significantly lower than the ingestion of non-treated wood, which was the highest detected (34.0 ± 9 mg), corresponding to a wood mass loss of 21.6% (Fig. 1).

Termite survival. Survival of Formosan subterranean termites was significantly different among treatments ($F = 68.62$; df = 4, 20; $P < 0.001$) but similar for both colonies ($F = 1.89$, df = 1, $P = 0.183$). The lowest survival detected was with borate-treated wood (16%); mortality of starved termites was significantly greater than termites fed nootkatone and nootkatone-borate treated wood (Fig. 2).

Protozoa. MANOVA for repeated measures on the total number of protozoa per termite gut showed a highly significant effect for the variable "day" ($F = 69.27$; df = 3, 60; $P < 0.001$) and significant effects for the variables "colony" ($F = 17.48$; df = 1, 20; $P = 0.0004$) and "wood treatment" ($F = 3.000$; df = 4, 20; $P = 0.043$); however, none of the interactions was found significant. The variation over time in the mean total number of flagellates for each treatment group is shown in Fig. 3.

A progressive decrease in the total number of protozoa was observed in all groups, including the control units. Flagellate numbers in the treated units followed the same trend as controls but were significantly lower (Fig. 3) on d 3 ($F = 2.78$; df = 25; $P = 0.049$), on d 5 ($F = 4.17$; df = 4, 25; $P = 0.010$) and on d 7 ($F = 3.76$; df = 4, 25;

Table 1. Mean number (±SD) of different species of protozoa per gut of *C. formosanus* detected before wood treatment (d 0) and at the end of the experiment (d 7) in different groups

	Pseudotrichonympha grassii	*Holomastigotoides hartmanni*	*Spirotrichonympha leidyi*
Day 0	524.44 ± 100.37	572.22 ± 645.23	1493.33 ± 718.23
Day 7			
Control	40.00 ± 23.09 a	230.00 ± 162.80 a	681.11 ± 331.26 a
Nootkatone	2.22 ± 3.44 b	141.11 ± 161.79ab	504.45 ± 293.26ab
Noot-Bor mix	0.00 ± 0.00 b	63.33 ± 45.75 b	390.00 ± 137.84ab
Borate	3.33 ± 3.65 b	35.55 ± 35.19 b	170.00 ± 76.68 b
Starved	0.00 ± 0.00 b	26.66 ± 27.33 b	404.44 ± 392.32ab

Control = non-treated wood, Nootkatone = wood treated with nootkatone, Noot-Bor mix = wood treated with nootkatone-borate mix, Borate = wood treated with borate, Starved = no wood. In each column values marked by the same letter are not significantly different at the 0.05 level determined by the Newman-Keuls test performed for d 7.

$P = 0.016$). After 7 d, the number of flagellates present in termites fed on non-treated wood was reduced to one third of the initial amount, and in groups fed borate, this was 15× lower than in the beginning of the experiment. Considering the differences among treatments, wood treated with the nootkatone-borate mix induced a reduction in the total protozoa that was significantly lower than the controls and similar to that shown by starved termites (Fig. 3).

Considering each protozoa species, only *P. grassii* showed a significant difference (on a two-way MANOVA for repeated measures) both for the variable "wood treatment" ($F = 6.08$; df = 4, 20; $P = 0.002$) and the variable "day" ($F = 504.45$; df = 3, 60; $P < 0.001$). Considering the day effect (one-way MANOVA, variable "wood treatment"), *P. grassii* numbers were always significantly lower than the controls on d 3 ($F = 3.23$; df = 4, 25; $P = 0.029$), on day 5 ($F = 10.23$; df = 4, 25; $P < 0.001$) and on d 7 ($F = 16.16$; df = 4, 25; $P < 0.001$). The greatest reduction in the number of this species was observed with the nootkatone-borate mix. On day 7, this species was totally absent in termites fed the nootkatone-borate treated wood and in starved termites, and heavily reduced with nootkatone- and borate-treated wood. *H. hartmanni* and *S. leidyi* numbers showed highly significant differences only in the variable "day" (two-way MANOVA for repeated measures, $F = 61.16$; df = 3, 60; $P < 0.001$ and $F = 36.64$; df = 3, 60; $P < 0.001$, respectively) and did not show significant differences in the variable "wood treatment" (two-way MANOVA for repeated measures, $F = 2.80$; df = 4, 20; $P = 0.054$ and $F = 1.99$; df = 4, 20; $P = 0.133$, respectively). Considering day effect (one-way MANOVA, variable "wood treatment") both species showed a significant difference from the controls only on d 7 ($F = 3.89$; df = 4, 25; $P = 0.014$ and $F = 2.77$, df = 4, 25; $P = 0.045$, respectively).

Discussion

The lowest wood mass loss was detected in units treated with borate. Our observations showed that most of the consumption took place during the first 3 days, after

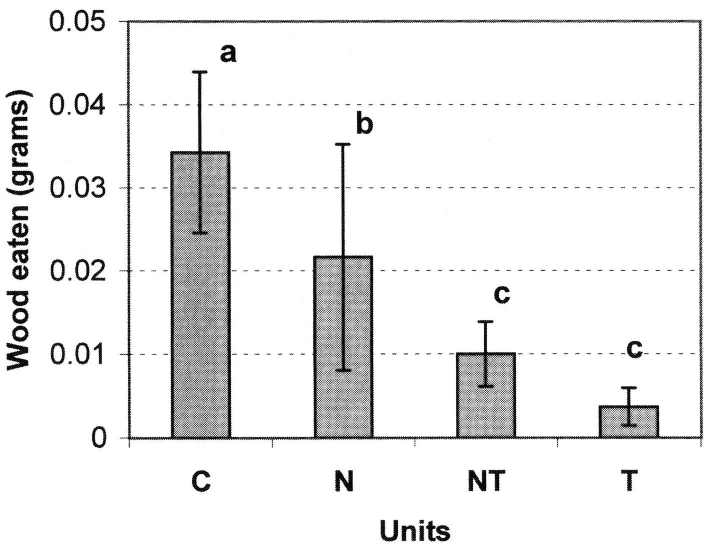

Fig. 1. Mean wood consumption ($\bar{X} \pm$ SD) by *C. formosanus* during 7 d (data from combined colonies). Bars marked by the same letter are not significantly different at the 0.05 level determined by the Newman-Keuls test. C = non-treated wood, N = wood treated with nootkatone, NT = wood treated with nootkatone-borate mix, T = wood treated with borate.

which termites appeared inactive and ailing, probably as a consequence of the effects of borate toxicity. As Grace (1997) indicated, due to the non-repellent nature of borates, termites will feed on borate-treated wood, but the ingestion of this substance will show its toxic effects soon after. Williams et al. (1990) reported that exposure for 10 d to 0.05% boric acid equivalents severely interfered with the digestive processes of termites. In our experiment, termites were exposed to 0.1571% boric acid equivalents, and the decrease in the number of protozoa was not significantly different from that of starved termites until d 7. By the seventh d, 84% of the termites in borate-treated wood group were dead, compared with 36.5% among starved termites. This supports the hypothesis that borates do not act immediately as protozoacidals; rather, their toxic action is more likely to occur at a cellular level, and the effects on the symbiont flagellates are a consequence of the alterations induced on the intestinal microenvironment (Nunes and Dickinson 1995).

Nootkatone is a mildly pungent sesquiterpene ketone found in the oil of Alaskan yellow cedar, *Chamaecyparis nootkatensis* (Lamb) Spach (Erdtman and Hirose 1962), and was recently isolated from vetiver oil (Zhu et al. 2001). Vetiver oil is extracted from the roots of vetiver grass (*Vetiveria zizanioides,* Linn Nash), a fast-growing native plant of India and is known to have insect repellent properties (National Research Council 1993). Nootkatone is widely used in the perfumery and flavor industries to add a grapefruit aroma and earthy fragrance, being safe to humans (Erdtman and Hirose 1962). Tests showed that nootkatone-treated sand repelled *C. formosanus* (Zhu et al. 2001).

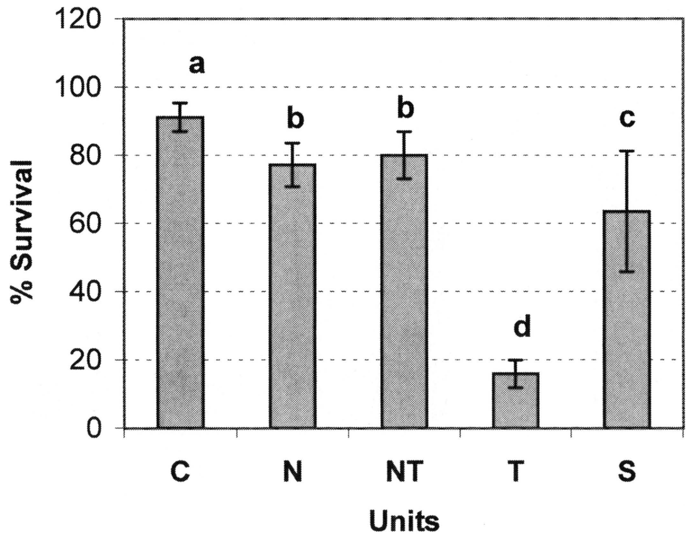

Fig. 2. *Coptotermes formosanus* % survival ($\overline{X} \pm$ SD) of survival after 7 d (data from combined colonies). Bars marked by the same letter are not significantly different at the 0.05 level determined by the Newman-Keuls test. C = non-treated wood, N = wood treated with nootkatone, NT = wood treated with nootkatone-borate mix, T = wood treated with borate, S = no wood.

Results from our experiment showed that termites in nootkatone and nootkatone-borate treated groups consumed significantly less wood than the controls, inducing a mass loss of 9.77% and 4.40%, respectively, against the 21.6% obtained in the non-treated wood group. This supports the feeding-deterrent action of nootkatone on Formosan subterranean termites. Considering the effects of nootkatone on the total number of protozoa, for both termite groups, a decrease significantly different from the controls became evident beginning on the fifth day, and this reduction was comparable to the one shown in starved termites. The decrease in the number of protozoa in the control units could be explained as a “laboratory effect” in which termites are severely limited to food choices available (Esenther 1977). When living in natural conditions, lower termites such as *C. formosanus* perform frequent exchanges of pre-digested food and protozoa through proctodeal trophallaxis with many other colony members (McMahan 1969) coming from different food sources. To optimize survival of their symbiont community, proper nutrients are essential (Mannesmann 1972). Feeding on various food sources with continuous food exchange among nest-mates may help. In our experiment, termites were provided with only a slice of balsa wood, and this may be responsible for the decline in protozoa abundance in the control group. Because this was the only food source, termites and their protozoa would lack the multiplicity of nutrients coming from various edible materials.

Nootkatone concentration used in our bioassay (7 to 8 mg nootkatone/slice of wood) was sufficient to deter termites from consuming enough wood to maintain their microbial community alive and healthy. This also may explain why termites in nootkatone-borate group showed a survival similar to the nootkatone group and much

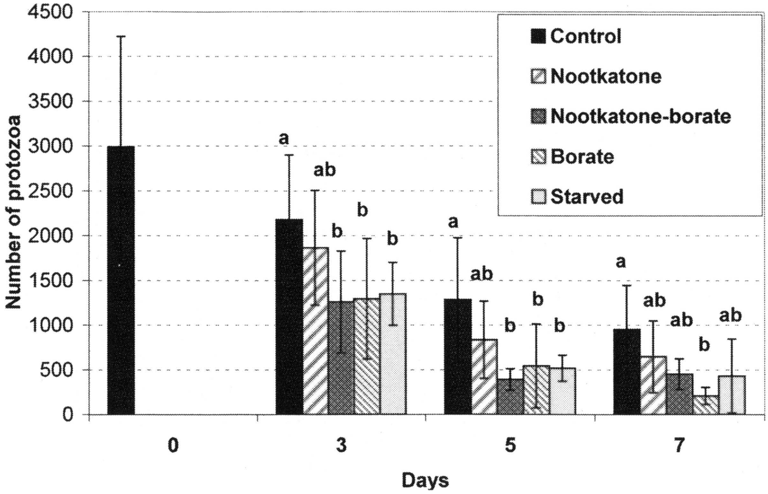

Fig. 3. Number ($\overline{X} \pm SD$) of protozoa per gut of *C. formosanus* detected on different d for different groups (data from combined colonies). Bars marked by the same letter within a d group are not significant different at the 0.05 level determined at the 0.05 level determined by the Newman-Keuls test.

higher than borate group, being intermediate between the starved group and the controls. Termites in nootkatone-borate group did not consume enough wood to acquire a sufficient quantity of borate to induce toxic effects. Further evidence of the "starved situation" induced by nootkatone is found in the almost complete loss of the flagellate *P. grassii* in all groups except the controls. Without *P. grassii,* Formosan subterranean termites cannot survive; this protozoan seems to be the most important in cellulose metabolism. As Mauldin et al. (1972) reported, *C. formosanus* artificially deprived of *P. grassii* could metabolize only 5.5% of cellulose that it otherwise could metabolize. As in our results, others have found that this protozoan is eliminated in *C. formosanus* workers that have been starved about 1 wk (Mauldin et al. 1972, Beal et al. 1974, Carter et al. 1975, Carter et al. 1981). The feeding-deterrent action shown by nootkatone on Formosan subterranean termites and the consequent negative effects on its symbiont fauna, together with its repellency suggest that this natural product might be considered a promising alternative for the control of this pest. Studies on the efficacy and longevity of this natural product are being conducted.

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