TOXICITY OF R,S-LINALOOL TO FOUR SPECIES OF STORAGE COLEOPTERA AS INFLUENCED BY DEGRADATION AND VOLATILIZATION

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Abstract

Linalool was present at 8.59 ± 0.92 S.D.(standard deviation) mg/g in the dried leaves of Ocimum canum Sims, an annual mint used in Rwanda to protect against postharvest insect damage. The essential oil of O. canum contains up to 90% linalool. Direct exposure of adults of Zabrotes subfasciatus Bohem. to milled, dried Ocimum leaves resulted in 100% mortality of males and 50% mortality of females after 48 hours. Dose-response curves for linalool with adult Z. subfasciatus Bohem., Acanthoscelides obtectus (Say), Rhyzopertha dominica (F.), and Sitophilus oryzae (L.) were completed. A filter paper bioassay system was used to obtain the LC_{50} values which ranged between 412 and 430 ug/cm^2. These species all have a narrow dosage between those doses causing 100% mortality and those causing 0% mortality. Concomitant chemical analyses of the treated filter papers at bioassay count times indicated time-dependent quantitative and qualitative changes in the chemical composition of the treated substrates. These results are discussed in terms of the efficacy of using O. canum for the protection against loss due to insects in the traditional food storage systems of Rwanda.

Introduction

In Rwanda, some farmers store dry edible beans, Phaseolus vulgaris L., in traditional closed structures (imboho). Sprigs of whole leaves of Ocimum canum Sims are usually added to the stored foodstuff to prevent insect damage within these structures (Dunkel et al., 1988). Linalool is a major component of the essential oil of this annual mint, representing 60% to 90% of the total volatiles collected (Ntezurubanza, 1987). Linalool (3,7-dimethyl-1,6-octadien-3-ol) is a common component of floral scents and is an olfactory cue in the seeking of host plants by numerous phytophagous invertebrates. It is an oxygenated monoterpenoid which acts as a reversible competitive inhibitor of acetylcholinesterase (Ryan and Byrne, 1988)

and has been suggested as an alternative to conventional insecticides in controlling all life stages of the cat flea, Ctenocephalides felis (Bouche), (Hink et al., 1988). The acute oral LD_{50} for rats is 2.8 g/kg and the acute dermal LD_{50} for rabbits is 5.6 g/kg (Opdyke, 1979). The LC_{50} value for adult C. felis is 39 ug/cm^2 (Hink et al., 1988). The LC_{50} value for adult red flour beetles, Tribolium castaneum (Herbst) is 2.5 X 10^4 ppm (Ryan and Byrne, 1988).

At the national government warehouses of Rwanda (OPROVIA = Office National pour le Developpement et la Commercialisation des Produits viviers et des Protections Animales), a search is underway to identify preparations (Dunkel et al. 1990a,b) or procedures that will replace their sole insect grain/bean protectant, actellic (pirimiphos methyl) which has been used prophylactically since 1983 (Dunkel et al. 1988). Populations of A. obtectus and S. oryzae with a significantly increased resistance to actellic have been identified in OPROVIA Warehouses (Sriharan et al. 1990).

This article describes the quantification of linalool from dried leaves of O. canum Sims and experiments using milled dried leaves against adults of Z.subfasciatus Bohem. This basic information is followed by an evaluation of the toxicity of linalool to adults of stored-products insect species. The degradation and volatilization of linalool from treated filter papers is subjected to insect bioassay and quantitative chemical analysis. The use of this information in the postharvest systems, on farm and in national (OPROVIA) warehouses, of Rwanda is discussed.

Materials and Methods

Insect rearing- All four species were reared at 27$\pm$1°C and 65$\pm$5% relative humidity under a photoperiodic regime of 12:12 light:dark. Z. subfasciatus and A. obtectus were reared on a diet of dried Pinto beans. R. dominica and S. oryzae were reared upon a diet of 96:2:2 (w/w) soft white wheat:whole wheat flour:brewers yeast.

Quantification of linalool from Ocimum canum- Leaves of O. canum were collected from the Butare prefecture in Rwanda, air-dried according to traditional practice, and express-shipped to Montana State University where they were stored in a -20°C freezer prior to bioassay and chemical analysis. The leaves (1g- 4 replicates) were milled in a Waring blender and extracted immediately in 1:4 isopropanol:hexane (containing decane as an internal standard)in an 125 ml Erlenmeyer flask. The flasks were covered to prevent photodegradation and occasionally agitated. An aliquot of the resulting solution was directly injected into a gas chromatograph for quantitative analysis. Narrow-bore capillary gas chromatography was performed on a Varian Model 3700 equipped with a flame-ionization detector containing a 50m X .20mm (i.d.) HP-1 column with 0.11um film thickness; carrier gas velocity was 31cm^3/s (200°C- helium). Temperature program-initial temperature 60°C, initial hold 8 min, temperature increase 4°C/ min, final temperature 260°C.

The linalool peak was tentatively identified using narrow-bore capillary GC-MS. The gas chromatograph was a Varian Model 3700 equipped with a flame ionization detector and a 30.0 m X 0.25 mm (i.d.) DB5 column with 0.25u film thickness: Column conditions used were: He carrier gas velocity- 30cm^3/s (220°C); temperature

programming- initial temperature 50°C, initial hold 4.0 min, temperature increase 5.0°C, final temperature 280°C, final hold 10 min; injector temperature 260°C; detector temperature 290°C. The electron impact mass spectra were obtained on a VG Analytical model VG 70EHF mass spectrometer operating at 70eV with a source temperature of 200°C.

Leaf exposure bioassay- Ten adult Z. subfasciatus, 5 male and 5 female (0-3 days post-adult eclosion), were added to 5.5 cm glass petri dishes in which 1.0 g of milled dried leaves of O. canum had been distributed evenly. Ten replicates of the treatment and ten replicates of a control consisting of a petri dish containing no plant material were also conducted. Bioassays were evaluated by viewing mortality at 24 and 48 hours. The bioassay conditions were 28±1°C at 65±5% R.H. with a 12:12 light:dark photoperiodic regime.

Linalool dose-response bioassays- A 9.0 cm diam. Whatman #1 filter paper was placed in a 10cm diam. glass Corning petri dish. A 1.0 ml aliquot of the appropriate dilution of R,S linalool to the filter paper in 1.0 ml of absolute ethanol. The ethanol was allowed to evaporate for 20 minutes prior to the addition of the insects. Mortality was evaluated if the insect was immobile and did not react to a probing with a blunt dissecting probe three times. Moribundity was assessed by viewing those insects that were on their backs and ambulating very weakly. These insects were subsequently righted and viewed carefully. Those that immediately fell onto their backs again as a result of intoxication were classified as moribund. At higher dosages all moribund insects subsequently died with the passage of time. Recovery occurred rarely at lower dosages. The bioassay conditions for this and the following bioassay were 27±2°C at 65±8% R.H. with a 12:12 light:dark photoperiodic regime. Z. subfasciatus were sexed with 5 males and 5 females used in each replicate; 10 adults of unknown sex were used for the other species. Counts of mortality/moribundity were conducted at 24 hours.

Bioassay of linalool with increasing duration of air exposure- The protocol was similar to that for the dose-response bioassays. The ethanol in an aliquot delivering 500 ug/cm^2 on the filter paper was allowed to evaporate for 20 minutes and ten replicates of Z. subfasciatus (5 male, 5 female; 0-1 days post-adult eclosion) were added immediately. Other replicates were covered though they contained no insects and ten Zabrotes (as above) were added at times of 0.25 hr, 6 hr, 18 hr and 24 hr post-ethanol evaporation. Mortality /moribundity were determined as above and evaluated at 24 hr after the introduction of the insects into each trial. Ten replicates of an ethanol control were conducted simultaneously for each trial. The bioassay conditions were 28±1°C at 65±5% R.H. with a 12:12 light:dark photoperiodic regime.

Quantitative chemical analysis of linalool-treated substrates with increasing duration of air exposure- The air exposure bioassay procedure (above) included four additional replicates. At the time of insect introduction each filter paper for these replicates was handled with forceps, cut into ca. 0.5 cm^2 pieces and transferred into 1:4 isopropanol:hexane (containing decane as an internal standard)in a 250 ml Erlenmeyer flask for 24 hours. The flasks were covered to prevent photodegradation

and occasionally agitated. The resulting solution was directly injected into a gas chromatograph for quantitative analysis and GC-MS for identification as per above.

Statistical analysis- The linalool dose-response bioassay data were subject to probit analysis (Matsumura, 1975) after Abbott's formula (Abbott, 1925) was used to adjust for control mortality. Since the dominant response of the moribund insects was to die, moribundity and mortality were pooled for statistical analysis.

Results

Extraction and quantitative gas chromatographic analysis indicated that linalool is present in milled, air-dried leaves of O. canum at 8.59±0.92 S.D. (standard deviation) mg/g. Figure 1 shows the gas chromatogram of the solvent extract of milled Ocimum leaves; the peak at the retention time of 11.73 is decane (internal standard) and the peak at 15.89 is linalool (Weaver et al., submitted).

The results of the leaf exposure bioassay (Table I) indicated 100% mortality of male Z. subfasciatus at 24 hours and only 50% mortality of the females at 48 hours (Weaver et al., submitted).

The linalool dose-response bioassays (Figure 2) indicated that the LC_{50} values for all four species were similar (Weaver et al., submitted) The LC_{50} values for each species were: Z. subfasciatus- 429.3 ug/cm^2; A. obtectus- 412.1 ug/cm^2;R. dominica- 430.2 ug/cm^2;S. oryzae- 426.7 ug/cm^2 (Table II).

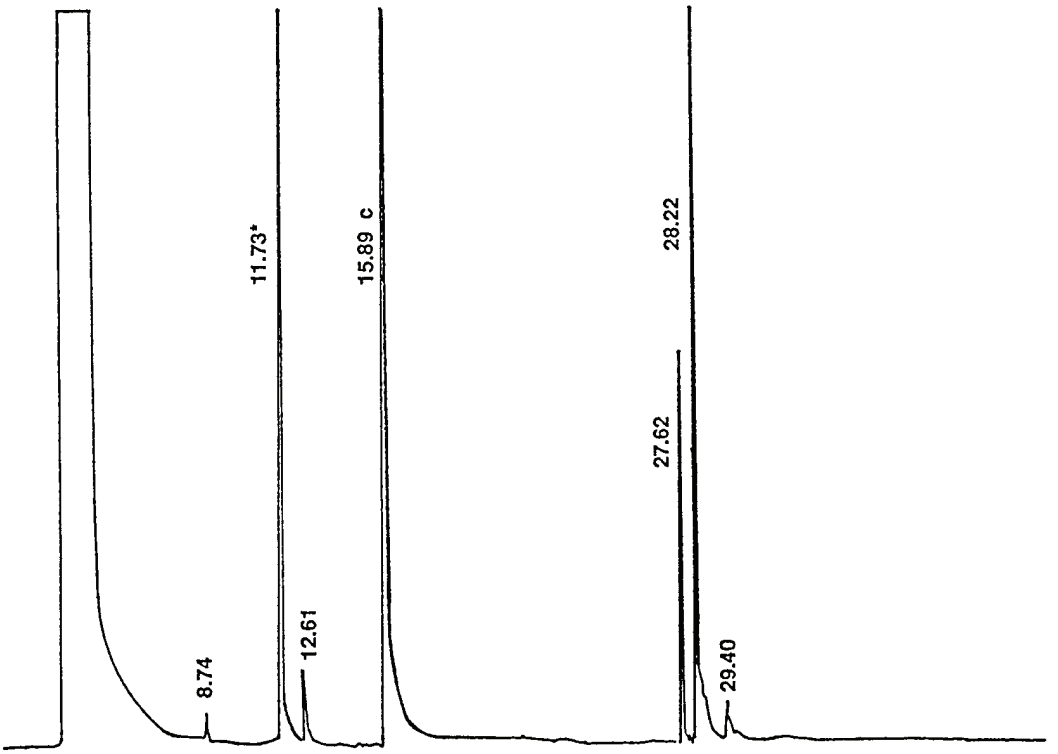
Increased duration of air exposure of linalool treated substrates had differing effects upon male and female Z. subfasciatus susceptibility (Table III). The susceptibilty of females was halved at fifteen minutes post-ethanol evaporation, whereas the susceptibity of males was not noticeably decreased until eighteen hours post-ethanol evaporation (Weaver et al., in preparation).

The quantitative analysis indicated that the decrease in susceptibity of females at 15 minutes post-solvent evaporation is correlated to a decrease of 50 ug/cm^2 (approximately 10% of the initial aliquot) of linalool at this time (Table IV). No further increase in the amount of linalool volatilized is evident, nor is any consistent change in the proportions of linalool relative to degradation products evident (Weaver et al., in preparation).

The amount of linalool in the stock solution decreases most dramatically when the stock solution is delivered on filter paper. After the introduction to filter paper, the next major decrease occurs 15 minutes after evaporation of the ethanol solvent is begun. No further changes in quantity of linalool on the filter papers occurs after 6, 18, or 24 hours of air-exposure on Whatman # 1 filter paper, either qualitatively or quantitatively.

Discussion

The large amount of linalool in the dried leaves of O. canum corroborates the earlier reported high percentage of linalool found in the essential oil of this species (Ntezurubanza, 1987). The milled leaves of this species are quite toxic to Zabrotes males, however the amount of linalool present does not correlate with the filter paper dose-response trials (Table II). This is due to two factors relating to methodology. The first factor is that the insects in the milled plant trials are rapidly coated with



c Tentative identification: R,S-linalool.

* decane — internal std.

Figure 1. Gas chromatogram of 1g of freshly-milled leaves of air-dried Ocimum canum extracted in 1:4 isopropanol:hexane for 24 hours. Retention times: 11.73- decane (internal standard); 15.89- R,S-linalool. Gas chromatography described in Materials and Methods.

Table I. Acute mean mortality (± std. dev.) of *Zabrotes subfasciatus* Bohem. (0–3 days post-adult eclosion) exposed[1] to 1g of milled dried leaves of *Ocimum canum* Sims (Lamiaceae). 10 replicates; 5 ♀, 5 ♂ per rep.

Treatment	Mortality			
	(24 hr)		(48 hr)	
	♀	♂	♀	♂
O. canum	1.8 ± 1.2	5.0 ± 0.0	2.5 ± 1.4	5.0 ± 0.0
control	0	0	0	0

[1] Temperature — 28 ± 1°C. Relative humidity — 65 ± 5%. Photoperiod — 12:12 light:dark.

Table II. Acute mean mortality (± standard deviation) and LC50 from petri dish bioassay of adult stored product insects to R,S-Linalool (3,7-dimethyl-1,6-octadien-3-ol). (n=10; 10 insects/rep; 27 ± 2° C; 65 ± 8% relative humidity; 12:12::light:dark).

Zabrotes subfasciatus Bohem. (0–1 day post adult eclosion; 5♂ + 5♀)

Dose (µg Linalool/cm² filter paper)								LC50
0	250	300	350	400	450	500	750	(95% confidence interval)
0	0	0.20± 0.42	0.40± 0.84	2.40± 2.17	5.80± 2.57	9.80± 0.63	10	428.30 (417.91, 438.94)

Acanthoscelides obtectus (Say) (0–1 day post adult eclosion; unsexed)

Dose (µg Linalool/cm² filter paper)								LC50
0	250	300	350	400	450	500	750	(95% confidence interval)
0	0.10± 0.32	0.60± 0.97	2.50± 1.18	4.00± 1.49	6.60± 2.76	9.90± 0.32	10	412.15 (401.64, 422.92)

Rhyzopertha dominica (F.) (0–6 days post adult eclosion; unsexed)

Dose (µg Linalool/cm² filter paper)								LC50
0	250	300	350	400	450	500	750	(95% confidence interval)
0.40± 0.69	0.50± 0.71	1.40± 1.27	2.30± 1.49	3.40± 1.96	5.30± 3.90	8.40± 3.06	10	430.19 (417.74, 443.02)

Sitophilus oryzae (L.) (0–6 days post adult eclosion; unsexed)

Dose (µg Linalool/cm² filter paper)								LC50
0	250	300	350	400	450	500	750	(95% confidence interval)
0.10± 0.32	0.70± 1.25	0.90± 1.60	1.80± 1.55	1.80± 0.79	5.30± 4.24	9.50± 1.27	10	426.66 (412.75, 441.05)

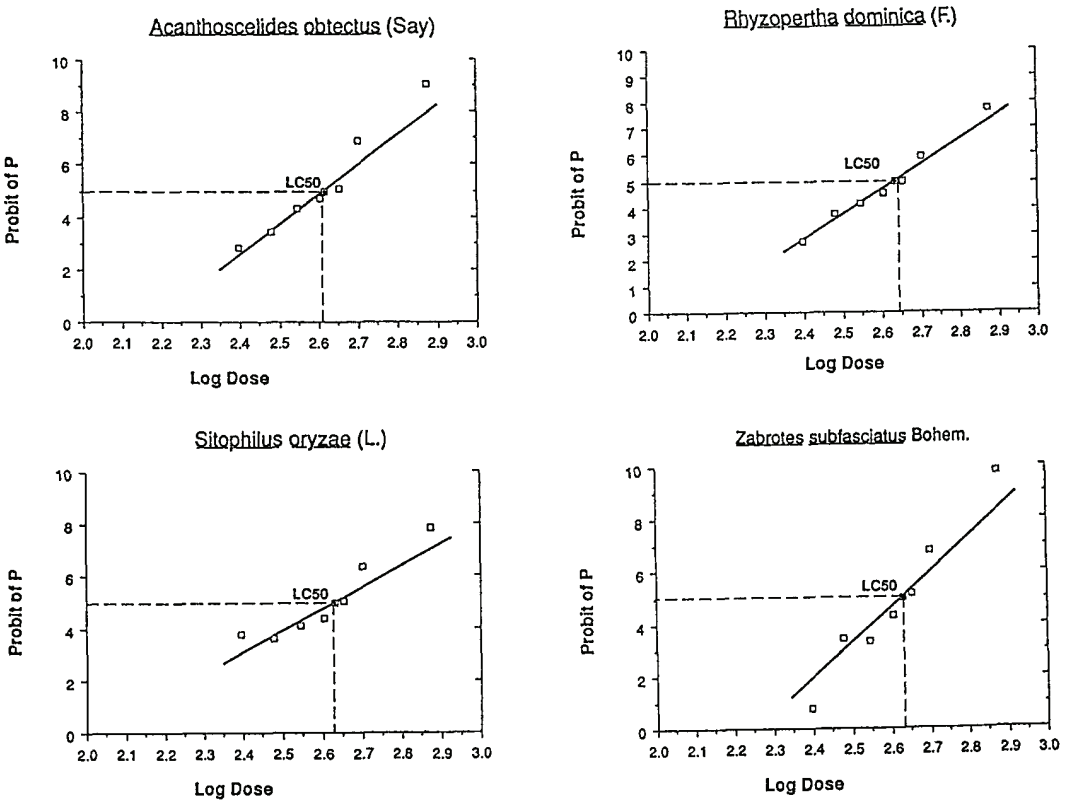


Figure 2. Linalool-induced dose-response curves for four species of stored-products Coleoptera in filter paper bioassays (n = 10; 10 insects/rep; 27 ± 2°C; 65 ± 8% relative humidity; 12L:12D). Zabrotes subasciatus and Acanthoscelides obtectus- 0-1 day post-adult eclosion; Rhyzopertha dominica and Sitophilus oryzae- 0-6 days post-adult eclosion.

Table III. Acute mean mortality (± std. dev.) of *Zabrotes subfasciatus* Bohem. (0–1 day post-adult eclosion) exposed[1] to linalool-treated Whatman #1 filter papers with increasing pre-bioassay interval duration. R,S-linalool (aliquot to yield 500 µg/cm²) applied in ethanol. 10 replicates, 5 ♀ and 5 ♂ each. Mortality and moribundity data after 24 hours of exposure. Ethanol was allowed to evaporate 20 min in all trials including the control.

Treatment	Sex	Pre-exposure interval[1]				
		0 hrs	0.25 hrs	6 hrs	18 hrs	24 hrs
linalool	♂	5.0 ± 0.0	4.9 ± 0.2	4.8 ± 0.4	0.6 ± 0.7	1.2 ± 1.2
	♀	3.8 ± 1.9	1.7 ± 1.7	2.0 ± 1.1	0.3 ± 0.5	0.1 ± 0.3
control	♂	0	0	0	0	0
	♀	0	0	0	0	0

[1] Temperature — 28 ± 1°C. Relative humidity — 65 ± 5%. Photoperiod — 12:12 light:dark.

Table IV.

Amount (µg/cm²) (± std. dev.) and proportions (± std. dev.) of R,S-linalool and degradation products extracted from a Whatman #1 filter paper with increasing duration of air exposure. Linalool delivered at 500 µg/cm²; four replicates. Extractions were concomitant with introduction of insects for bioassay of the same protocol (Table 3).

Amount (µg/cm²)

Compound[I]	Time						
	0[(1)]	0[(2)]	0[(3)]	15 minutes*	6 hours*	18 hours*	24 hours*
14.6 a	0 634 ± 0.138	0.548 ± 0.170	1.133 ± 0 234	1.181 ± 0.277	1.344 ± 0.129	1.335 ± 0.138	1 213 ± 0.382
15.2 b	1.124 ± 0.115	1.217 ± 0.329	1.648 ± 0.364	1.692 ± 0.195	1.362 ± 0.322	1.533 ± 0 316	1.664 ± 0.389
15.8 c	482.974 ± 11.699	402.528 ± 19.533	437.128 ± 37.099	387 447 ± 25.411	384.288 ± 18.080	385.114 ± 22.627	388.502 ± 20 076
17.3 d	2 990 ± 1.415	4.709 ± 0.404	2 524 ± 0.170	2.379 ± 1.455	2.798 ± 2.062	2.741 ± 1 255	1.349 ± 0.629

Proportion (≤1.0)

Compound[I]	Time						
	0[(1)]	0[(2)]	0[(3)]	15 minutes*	6 hours*	18 hours*	24 hours*
14.6 a	0.001 ± 0.000	0.002 ± 0 000	0.002 ± 0 000	0 003 ± 0 001	0 003 ± 0 000	0.003 ± 0.000	0.003 ± 0 001
15.2 b	0.002 ± 0.000	0.003 ± 0.001	0.004 ± 0.001	0.004 ± 0.001	0.003 ± 0.001	0.005 ± 0.001	0.004 ± 0.001
15.8 c	0.991 ± 0.003	0 987 ± 0.005	0.988 ± 0.001	0 986 ± 0.005	0 986 ± 0 006	0.986 ± 0 003	0 989 ± 0.001
17.3 d	0.006 ± 0.003	0 011 ± 0.000	0.006 ± 0.000	0 013 ± 0.002	0 007 ± 0.005	0.007 ± 0.003	0.003 ± 0 002

0[(1)] Aliquot delivered directly into extraction flask.
0[(2)] Aliquot delivered upon filter paper and immediately extracted.
0[(3)] Aliquot delivered upon filter and ethanol evaporated (20 min).
* After ethanol evaporation.
Air exposure at 28 ± 1°C; 55 ± 5% relative humidity; 12:12 light:dark.

a Tentative identification: beta-myrcene.
b Tentative identification: d-limonene.
c Tentative identification: R,S-linalool.
d Tentative identification: 3,7 dimethyl 1-octen-3-ol.
I Tentative identification based on gas chromatography — mass spectroscopy

sticky particles (due to their own movement) that adhere to them and receive a topical chemical and physical treatment whereas the filter paper trials provide little direct topical treatment initially. After the insects become disoriented and fall upon their dorsal surfaces frequently they may also become coated with residual linalool from the surface of the filter paper. The second factor is that the active surface area of the filter paper is much greater than its two dimensional area, so the actual amount per cm^2 is much lower than that reported.

The data indicate a surprising degree of similarity in LC_{50} values for the four species. However, the slopes of the probit lines vary, the lesser slopes indicating that the responses of R. dominica and S. oryzae are more heterogeneous (Figure 2). This may be due to greater genetic variability in the susceptibility of the populations of these species or to the greater age range of individuals used for these two species (0-6 days). The Z. subfasciatus and A. obtectus were 0-1 day old.

The LC_{50} values reported differ greatly from the 39 ug/cm^2 reported for Ctenocephalides felis adults (Hink et al., 1988). However, this cat flea bioassay involved saturated papers treated with linalool in a water/Tween 80 solution, which was not evaporated, thus allowing greater potential contact (and susequent topical coating) than the bioassay method used in the present study. The LC_{50} values do compare very favorably with the 2.5×10^4 ppm (526 ug/cm^2) reported for an insecticide-susceptible strain of Tribolium castaneum (Ryan and Byrne, 1988). This bioassay was very similar to the one used in the present study, except that the solvent was acetone and was evaporated in one minute.

The decrease in the amount of linalool extracted from the filter papers with increasing air exposure appears to be directly related to volatilization. Immediately after ethanol evaporation the odor of linalool is quite apparent, whereas after several hours it is much less apparent. The logical assumption is that the amount of linalool present has decreased below an odor threshold. The extractions indicate that this decrease may be due to the linalool molecules associating with the substrate strongly enough to prevent volatilization, but not liquid solvation. It is interesting that the males have higher susceptibility to both O. canum and air-exposed linalool, though this may be simply due to their smaller size.

In the Rwandan grain and bean storage system, insecticidal plants such as O. canum are used only at the farm level. Of the farmers surveyed throughout Rwanda, only 2.1% (n=94) use insecticidal plants in their bean storage, and 2.6% of the farmers use insecticidal plants in their sorghum storage (n=39)(Dunkel et al., 1988). Based on our results, it would be efficacious for more Rwandan farmers to grow O. canum and to use finely crushed, dried leaves of the plant for on farm protection of beans and grain. The methodology that the farmers would need to practice would make traditional baskets with open tops ineffective with this preparation. The O. canum will be more efficacious if used with the imboho, the plastic sack, or the metal drum. Other studies (Dunkel et al., 1988) indicate that these structures were used by 17% of the farmers for June harvest beans, 10 % of the farmers for January harvest beans and 23% of the farmers for sorghum. Rwandan farmers also use earthen pots and gourds for storage. If these are used with the O. canum preparation, our results suggest that these structures should be covered and used for long term storage or re-sealed immediately when beans or grain are used prior to the end of the storage period.

At national (OPROVIA) warehouses in Rwanda, there is a critical need for alternatives to actellic (pirimiphos methyl) as a long term protectant against insects.

Results of this study indicate that use of the entire plant or a preparation that contained a high percentage of linalool would be more efficacious in structures or containers that prevent reentry by insects. At OPROVIA warehouses, the plastic bags already in use would be preferable. At cooperative storehouses, the concrete silo structures would be ideal for use of this preparation. Although more efficacy testing is required before actual use of the preparation, this promises to be a useful material which does not require foreign currency to produce.

The studies on O. canum and linalool reported here indicate that freshly milled or very finely crushed leaves of O. canum have the potential for protection in closed structures such as the imboho, above ground sealed storage, individual plastic bags, metal drums, and below ground storages.

Conclusion

The efficacy of O. canum in providing protection against postharvest insect damage may be partly due to the concentration of linalool in its leaves. Linalool, as a component of O. canum, or when purchased as a synthetic preparation, appears to have its effect in a relatively short time frame, similar to that of a fumigant. Control, however, may be achieved by behavioral effects such as repellency or oviposition deterrence which require lower dosages of chemicals. We are currently investigating these phenomena.

The short effective period suggests that the leaves of Ocimum canum provide the greatest protection against insect damage when initially added to the stored foodstuff in a finely milled preparation. Protection over many months would require that the grain or other food commodity be sealed and airtight. With minor modifications in the current on farm storage system in Rwanda, farmers could make better use of this linalool-producing plant which can be produced by the farmers without the expenditure of local currency. At the national (OPROVIA) warehouses, linalool, as a component of O. canum, could be adapted as a fumigant in plastic, not jute, bags. For OPROVIA, this preparation could be obtained within the country without the use of foreign exchange. Use of the entire plant or a preparation which contained a high percentage of the linalool component would be especially useful in other areas of the world where underground and/or sealed storage is a traditional practice.

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INFLUENCE DE LA DEGRADATION ET DE LA TENSION DE VAPEUR DU R.S-LINALOOL SUR SA TOXICITE ENVERS QUATRE COLEOPTERES DES STOCKS

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Résumé

On a établi des courbes doses-réponses pour le linalool mis en contact avec des adultes de *Zabrotes subfasciatus* Bohem., *Acanthoscelides obtectus* (Say), *Rhizopertha dominica* (F.) et *Sitophilus oryzae* (L.) en utilisant la méthode du papier filtre. Les résultats indiquent que toutes les espèces sont sensibles à ce monoterpénoïde oxygéné. La DL50 s'abaisse pour toute les espèces jusqu'à des valeurs situées entre 250 et 500 microgrammes/cm2. Ces espèces réagissent toutes dans une gamme de doses très étroite entre les doses occasionnant une mortalité de 100 % et celles n'occasionnant pas de mortalité (0 %). Une analyse chimique concomitante des papiers filtres traités aux différents moments des contrôles biologiques montre que des changements qualitatifs et quantitatifs de la composition chimique des substrats traités interviennent. Ces résultats sont discutés dans le but d'évaluer l'efficacité d'une pratique de stockage traditionnelle du Rwanda utilisant *Ocimum canum* Sims. Les feuilles séchées de cette menthe annuelle (*Lamiaceae*) s'utilisent dans la protection contre les attaques d'insectes. L'huile essentielle d'*Ocimum* contient jusqu'à 90 % de linalool.