Pheromone study on acarid mites IV* Citral : composition and function as an alarm pheromone and its secretory gland in four species of acarid mites

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Abstract : Through bioassay and biological investigations on the alarm pheromone of five species of acarid mites, *Aleuroglyphus ovatus*, *Carpoglyphus lactis*, *Lardoglyphus konoi* and *Dermatophagoides farinae*, following results were obtained.

Citral and neryl formate were more or less active as an alarm pheromone and not strictly species-specific within five species. Along with *T. putrescentiae*, two more acarid mites, *C. lactis* and *L. konoi*, were concluded to possess the alarm pheromone and citral was identified as their pheromone.

Citral was emitted to the environment and the quantity of this discharge was 10 to 50 times larger when mites were disturbed than when undisturbed. Furthermore the composition of citral differed significantly between the disturbed (74-86 % of neral) and undisturbed conditions (54-66 % of neral), that is, the percentage of neral increased when disturbed.

The original composition of extracted citral under 1-min soaking consisted of more than 90 % of neral in the three species except *L. konoi* (75 % of neral). Therefore the initial composition of citral when discharged from glands is supposed to consist of more than 90 % of neral in three species, *L. konoi*, *A. ovatus* and *D. farinae*.

Secretory gland of citral was identified as the latero-abdominal gland or the oil gland in all species of mites.

INTRODUCTION

As the alarm pheromone of the mold mite, *Tyrophagus putrescentiae*, neryl formate, (Z)-3,7-dimethylocta-2,6-dien-1-ol formate, was isolated, and its function and the possible excretory glands were identified (Kuwahara *et al.*, 1975, 1979). The related monoterpenoid, citral, mixture of (Z)- and (E)-3,7-

dimethylocta-2,6-dienal, was also identified against *T. putrescentiae* as the active principle of an alarm pheromone contained in four species of taxonomically related acarid mites, *Aleuroglyphus ovatus*, *Carpoglyphus lactis*, *Lardoglyphus konoi* and *Dermatophagoides farinae* (Kuwahara *et al.*, 1980). To examine the species-specificity of alarm pheromones and to elucidate the possible function of citral on these acarid mites, the present study was undertaken.

MATERIALS AND METHODS

Mite species : Following five species of

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mites were used; *Tyrophagus putrescentiae*, *Aleuroglyphus ovatus*, *Lardoglyphus konoi*, *Carpoglyphus lactis* and *Dermatophagoides farinae*. All species of these mites were bred by methods as reported (Kuwahara *et al.*, 1980).

Bioassay method: Each species of mites was freed from culture medium by the saline flotation method (Matsumoto, 1965), washed thoroughly with tap water, and kept at room temperature at least 2 hr to remove moisture from body surface. Each of thus-prepared mites of appropriate number was transferred and scattered evenly on a glass cover slip (24×36 mm) underside of which was painted black by a marking-pen to ease observation of mite's behaviour. After 30 min when mites were evenly distributed on the surface, a small piece of filter paper (5×5 mm), impregnated with 0.05 ml of known concentration (ppm) in hexane of candidate compounds, or with hemolymph from about 100 crushed mites, was placed at the center of plate. Observation of mites escaping from the filter paper were made for 10 min at 23–25°C, 60–70% RH. When all of mites around the filter paper were repelled and clear no-mite-zone was observed (Fig. 1a), the alarm pheromone activity was designated as ++. In the case that the barely enough clear zone was made (Fig. 1b), it was indicated as +. If the evacuated zone was observed underside of the paper only when the paper was moved after 10 min (Fig. 1c and 1d), it was regarded as ±.

Compounds and the crude pheromone preparation: Neryl formate, the alarm pheromone of *T. putrescentiae*, was prepared from nerol (Nakarai Chemical Co., Ltd., Kyoto) by the treatment of formic acid-acetic anhydride mixture in dry pyridine at 0°C (85% yield, bp. 102–104°C/15 mmHg, IR: C=O, $\nu_{\text{film}}^{\text{max}}$ 1724 and 1155 cm⁻¹). Citral was purchased from Nakarai Chemical Co., Ltd., Kyoto, which consisted of 35% neral and 65% geranial by gas-liquid chromatography (GLC).

Crude extracts of mites were prepared by soaking 1 g each of five species of mites in 2 ml hexane and the supernatant layer was employed at least 24 hr after solvent introduction, unless otherwise mentioned.

Identification of excretory gland of citral:

A commercially available and highly sensitive aldehyde-detecting reagent, Purpald, 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Aldrich Chem. Co., Ltd.), which gives reddish purple color with citral at basic condition (Dickinson and Jacobson, 1970), was applied for the present purpose. Following procedure was used for color development of mites. Alive mites of each species were placed in a small petridish (5.6 cm in diameter, 1.8 cm in height), and mixed with about 3 ml of freshly prepared solution of 3% aqueous agar (Special Agar-Noble, Difco) containing 1% Purpald around 50°C. After cooling to room temperature, a cotton plug soaked with 3–4 drops of 28% aqueous ammonia were added and kept overnight at room temperature. Mites embedded in agar sol were dug out and observed under a binocular microscope to examine the body surface.

Effect of disturbance: Air extraction method by Porapak Q was employed (Kuwahara *et al.*, 1979), and the absorbed volatiles was analyzed by GLC using a 15% PEG-20M column (4 mm id × 75 cm) at 100°C, 30 ml/min He flow rate.

Relative proportion of neral and geranial in the volatiles was measured from peak-area integration by a triangular method, that is, the peak height multiplied by width of the peak at half of its height. Absolute quantity of citral on each peak-area on the chromatogram was also calculated by comparing with peak-area of the known quantity of citral at the same condition. By the above-mentioned extraction and quantitative determination system, two species of mites were investigated. In the case of *A. ovatus*, about 50 ml of mites with 50 ml of culture medium was introduced to the conical flask (300 ml volume) and disturbance was provided in moderate shaking by hand for one hour. In the case of *C. lactis* total of 80 ml of mites and medium mixture was employed, when the maximum level of mite's population had built up in the colony. As the culture medium of *C. lactis* at this time was so wet and sticky that the hand shaking seemed to be inefficient for disturbance, the disturbance was provided by mechanical stirring with a spatula twice for 1 min at 30 min

interval when airtation started.

Determination of initial composition of citral: About 1 ml of alive mites of each species was soaked in 1 ml of hexane and after 1 min the supernatant hexane layer of extract was subjected to GLC, of which analytical condition was stated above. From thus-obtained chromatogram the relative proportion of neral and geranial was determined by the method described above.

RESULTS AND DISCUSSION

Alarm pheromone activity of neryl formate: Using five species of acarid mites, the alarm pheromone activity of neryl formate was assayed. The result, as shown in Table 1, indicated that three species of mites, *L. konoï*, *T. putrescentiae* and *C. lactis* had high sensitivity to neryl formate, in which *L. konoï* was the most sensitive and was

Table 1 Alarm pheromone activity of acarid mites to neryl formate

Concentration of neryl formate	<i>T.p.</i>	<i>A.o.</i>	<i>L.k.</i>	<i>C.l.</i>	<i>D.f.</i>
10,000 ppm					+
1,000	+	+	++	+	—
100	+	—	+	±	—
10	—	—	+	—	—
1	—	—	+	—	—

++: totally repelled, +: almost totally repelled, ±: partially repelled, —: unaffected. *T.p.*: *Tyrophagus putrescentiae*, *A.o.*: *Aleurogyphus ovatus*, *L.k.*: *Lardoglyphus konoï*, *C.l.*: *Carpoglyphus lactis*, *D.f.*: *Dermatophagoides farinae*

even repelled from the filter paper impregnated with 1 ppm of neryl formate. The sensitivity of *A. ovatus* was low and 1,000 ppm solution was required to develop the activity. *D. farinae* showed the lowest sensitivity to neryl formate and only active to 10,000 ppm test solution. The sensitivity of the mites to neryl formate was shown to be in the following decreasing order: *L. konoï* > *T. putrescentiae* > *C. lactis* > *A. ovatus* > *D. farinae*. Therefore four species of acarid mites except *T. putrescentiae* indicated the

alarm pheromone activity to the related but different compound, neryl formate, which was originally not contained in their bodies.

Alarm pheromone activity of citral: In Table 2, the biological activity of citral to

Table 2 Alarm pheromone activity of acarid mites to citral

Concentration of citral	<i>T.p.</i>	<i>A.o.</i>	<i>L.k.</i>	<i>C.l.</i>	<i>D.f.</i>
1,000 ppm	+	+	++	+	+
100	±	—	++	±	—
10	—	—	+	—	—
1	—	—	+	—	—
0	—	—	±	—	—

All marks and signs refer to Table 1.

five species of mites was summarized. *L. konoï* was the most sensitive among five species and showed the escaping behaviour from the filter paper impregnated with 1 ppm solution of citral. *D. farinae* and *A. ovatus* were so insensitive that they only responded to 1,000 ppm. *T. putrescentiae* and *C. lactis*, both showed the same level of activity to 100 ppm of citral. It was noted that the repellent activity of citral was variable considerably ranging from 1 ppm to 1,000 ppm, even though citral was discharged from their own species. The sensitivity of mites to citral was in the following order: *L. konoï* > *C. lactis* = *T. putrescentiae* > *A. ovatus* = *D. farinae*. Here again it was noted that *T. putrescentiae* showed the alarm pheromone activity to a different compound from its own.

It is well admitted that the alarm pheromone of the social insects is the least species-specific among insect pheromones and the same compounds are shared in several taxonomically related species (Blum, 1969), and a related but different compound possess the same biological activity. The present bioassay results of neryl formate and citral against five species of acarid mites were also identical to those known phenomena of the alarm pheromone observed in social insects. As a result, there was no species-specificity on alarm pheromones of acarid mites, not only chemically but also biologically. The fact,

however, that 1,000ppm of citral was required for mites, such as *A. ovatus* and *D. farinae*, to release pheromone behaviour, was the least conceivable as an alarm pheromone, because of its unusually high concentration and of the dischargeable quantity from their minute body sizes. Therefore, hemolymph from each species or the crude extract was assayed against five species of mites to demonstrate the validity of the pheromonal function.

Alarm pheromone activity of hemolymph and crude extracts of each species: The result is shown in Table 3. Three species,

Table 3 Alarm pheromone activity of acarid mites to hemolymph (from about 100 mites per paper) and to crude hexane extract (1g of mites per 2 ml)

Reactant	Reactor				
	<i>T.p.</i>	<i>A.o.</i>	<i>L.k.</i>	<i>C.l.</i>	<i>D.f.</i>
<i>T.p.</i>	+(+)	—*	++	±	—
<i>A.o.</i>	—*(—)	—*(—)	++	+(±)	—
<i>L.k.</i>	—*(—*)	—*(—)	++(++)	—*(—*)	—
<i>C.l.</i>	—*(±)	—*(±)	++	++(+)	—
<i>D.f.</i>	—*	—*	++	+	—

(): Results with hexane extract, — : none of repellency or aggregation have occurred, —*: Aggregation tendency to the tested paper was observed and no repellency.

All other marks and signs refer to Table 1.

L. konoi, *C. lactis* and *T. putrescentiae*, reacted sensitively to their own species, but the other two, *A. ovatus* and *D. farinae* did not. *L. konoi* reacted to all species of hemolymph and of extracts. Even though *D. farinae* did not react to their own species, it was a powerful repellent to other species. Based on the number of reactants from which each species of mite was repelled, sensitivity to the alarm pheromone was assumed as following decreasing order: *L. konoi*>*C. lactis*>*T. putrescentiae*>*A. ovatus*>*D. farinae*. The result that the three species of mites, *L. konoi*, *C. lactis* and *T. putrescentiae*, showed alarm pheromone activity to their own species was also supported by the behavioural observation. On *T. putrescentiae*,

the escaping behaviour of aggregating mites evoked with the disturbance by patting with a soft drawing brush or squashing one or several mites at the center of the aggregation was reported (Kuwahara *et al.*, 1975), the same observation was reproducible on *L. konoi* and *C. lactis*, but not on *A. ovatus* and *D. farinae*. Therefore it was concluded that the alarm pheromone was present in these three species in a strict sense, and that citral or neryl formate was discharged outside for its function. On the other two species of mites, *A. ovatus* and *D. farinae*, the function of citral was not known, however, citral is more likely to be a defensive substance against fungal infestation as stated by Wilson (1971), because of the antifungal property of citral (Cole *et al.*, 1975; Okamoto *et al.*, 1978; Kurita *et al.*, 1979) and of the fact that in the healthy culture of these mites molds did not develop.

In the course of this experiment, aggregation behaviour of mites upon the filter paper impregnated with hemolymph or crude extracts was observed, especially after the alarm pheromone activity being faded out. *T. putrescentiae*, *A. ovatus*, *C. lactis* and *L. konoi*, were attracted to the filter paper and covered the paper (Fig. 2). This phenomenon may suggest the existence of some attractant, in which hemolymph from *L. konoi* was the most powerful.

Identification of excretory gland of citral: Using Purpald as a color-developing reagent specific to aldehyde detection, the site of pheromone production was investigated. As shown in Fig. 3, it was found that all four species of mites, *L. konoi*, *A. ovatus*, *C. lactis* and *D. farinae*, were stained from purple to black in their latero-abdominal gland or oil gland in binocular-microscopic observation. Therefore, on four species of mites, which possessed citral, the excretory gland of citral was identified as this gland. The same result has been obtained on *T. putrescentiae* (Kuwahara *et al.*, 1979). Other carbonyl reagent such as 2,4-dinitrophenylhydrazine and *o*-dianisidine were also tried to get poor results.

The fact that, from isolation study (Kuwahara *et al.*, 1980), no other aldehyde as a major extractable was noticed, was another

Fig. 1 Bioassay method

Observation was made 10 min after introduction of filter paper impregnated with solution of candidate materials.

a: Reaction of *C. lactis* to the paper with 2,000 ppm solution of neryl formate. The result was indicated as ++. b: Reaction of *C. lactis* to 1,000 ppm solution of neryl formate, which was indicated as +. c: Apparently no repellency was observed to 100 ppm solution of citral, before removal of the paper. d: Just after removal of paper, if no mites-zone underside of the paper was observed as shown, the result was indicated as ±.

Fig. 2 A typical example of attraction or aggregation observed in the case of *C. lactis* against hemolymph of *L. konoï*

a: 2 min after introduction of paper. Aggregation around the paper was observed. b: 10 min after introduction of paper, the paper was almost covered up by aggregating mites.

Fig. 3 Identification of excretory gland of citral by Pulpald
 Note that all species of citral-producing mites, from upper to bottom, *A. ovatus* (a, b), *C. lactis* (c, d), *L. konoi* (e, f), and *D. farinae* (g, h) were stained in their latero-abdominal gland or oil gland.

Table 4 Effect of disturbance on volatiles from acarid mites

Mite spp.	Exp. No.	Mite*** volume (ml)	Disturbed citral		Undisturbed citral		Ratio of citral disturbed by undisturbed
			Yield (μ g)	% Neral (%)	Yield (μ g)	% Neral (%)	
<i>A.o.*</i>	1	50	37.3	77	3.5	59	10.7
	2		22.5	77	1.3	56	17.3
	3		40.1	86	1.9	61	20.9
<i>C.l.**</i>	1	40	43.1	82	1.2	66	35.9
	2		76.2	78	1.4	56	54.4
	3		25.9	74	0.48	54	54.0

*: Disturbance was provided by moderate shaking by hand.

**: Two times of disturbance for one minute each were provided by stirring up with spatula.

***: Mites were kept about the same volume of culture medium in both species.

evidence of citral present in those glands.

Effect of disturbance: Effect of disturbance was examined using *A. ovatus* and *C. lactis*, both of which contained citral. Results are summarized in Table 4. In both species, the disturbed mites increased discharge of citral 10 to 50 times more than the undisturbed control did. The percent composition of neral and geranial from the mites both disturbed and undisturbed rather fluctuated even in the same species. The undisturbed mites emitted lesser quantity of citral and lesser content of neral (54–66%), whereas the disturbed mites released larger quantity of citral and higher percentage of neral (74–86%). From the present experiment, it was concluded that citral was actually discharged when mites were disturbed in both species, moreover, the composition of discharged volatiles differed significantly from that of undisturbed control. The composition of citral was also differed from the results obtained during isolation (Kuwahara *et al.*, 1980), that is, the isolated citral from *C. lactis* and those of crude extracts from other acarid mites, were almost constant around 40% neral and 60% geranial as observed in the commercially available citral (35% neral and 65% geranial). When mites were disturbed, the composition of citral was changed and the percentage of neral in citral was increased to 74–86% from 54–66% of undisturbed control. Even the value of the undisturbed control was noticed much higher than 35% neral of commercially available citral or 40% neral of isolated citral. The

present result seems to indicate that the initial composition of citral may be neral, which is transformed to geranial by some unknown reasons and purposes. To obtain evidences on this hypothesis, the initial composition of citral was determined.

Initial composition of citral: The initial composition of citral was determined on four species of mites. As shown in Table 5,

Table 5 Relative abundance of neral and geranial found at one minute after mites were soaked in hexane

Mite spp.	Exp. No.	Neral (%)	Geranial (%)
<i>A. ovatus</i>	1	94.9	5.1
	2	94.8	5.2
<i>L. konoï</i>	1	74.7	25.3
	2	75.0	25.0
<i>C. lactis</i>	1	92.7	7.3
	2	90.4	9.6
<i>D. farinae</i>	1	92.6	7.4
	2	92.1	7.9

three species, *A. ovatus*, *C. lactis* and *D. farinae*, discharged citral in which more than 90% consisted of neral. In the case of *L. konoï*, citral was composed of 75% neral and 25% geranial. If the period of solvent-soaking for extraction became longer, the percentage of neral was noticed to decrease quite rapidly. Therefore it was supposed that a part of neral originally present in the glands was transformed to geranial during solvent extraction and also a part of discharged neral was changed to geranial

during the process of air extraction, though the mechanism and function of this geometrical isomerization was not clear. Further study of this phenomenon will be published elsewhere.

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摘 要

コナダニ類のフェロモン研究 IV
4 種のコナダニにおける組成、警報
フェロモン機能および分泌腺

シトラールを分泌する 4 種のコナダニ類 (サトウダニ *Carpoglyphus lactis*, ムギコナダニ *Aleuroglyphus ovatus*, コオノホシカダニ *Lardoglyphus konoi*, コナヒョウヒダニ *Dermatophagoides farinae*) について、シトラールの組成、機能、分泌腺について検討した。ケナガコナダニ *Tyrophagus putrescentiae* を加えて、5 種のダニにつき、蟻酸ネリルとシトラールのフェロモン活性を調べた。両化合物共に種により同様の傾向で感受性が異なり (1-10,000 ppm, 1-1,000 ppm), フェロモン機能を特定できなかった。そこで同種の体液 (約 100 頭相当) または抽出物 (ダニ 1 g/2 ml ヘキサン) に活性を示す種を調べ、ケナガコナダニ以外にサトウダニとコオノホシカダニがフェロモンを持つと判断した。

Porapak Q による空気捕集法で、サトウダニとムギコナダニについて刺激の効果を調べた。刺激により 10-15 倍分泌発散が多くなり、ネラルルの混合率が増加した。そこで各ダニを生きた状態でヘキサンに投入し、1 分後にその上澄液を分析して、シトラール組成を調べると、結果は従来と異なり、コオノホシカダニ (ネラルル 75 %, ゲラニアル 25 %) を例外として、他の 3 種は 90 % 以上がネラルルであった。このことからシトラールはネラルルとして分泌されると判断した。

分泌腺をケナガコナダニの場合と同様に、発色剤による発色で調べ、4 種のダニ共に側胞腺 (または油腺) から分泌すると結論した。