An Ecological Role of Volatiles Produced by *Lasiodiplodia theobromae*

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One function of the volatiles produced by the fungus *Lasiodiplodia theobromae* was supposed to be ecological, as an insect attractant, which could get the attracted insects to be a vector of the fungal spores. *L. theobromae*, a mellein-producing fungus, was isolated from the outer surface of a danaid butterfly, *Idea leuconoe*. Ingestion by the butterfly of mellein on some plant tissues that contained mellein from 0.3 to 11 ppm was observed in the field and also in an insectarium. All these plant tissues were infected with *L. theobromae*, therefore, the mellein was concluded to be produced by the fungi. These observation suggests the presence of a kind of mutualistic relation between *Idea leuconoe* and *L. theobromae* associated with mellein, which is a suspected sex pheromone of the butterfly, accumulated in its hairpencil.

We previously reported some strains of *Fusarium solani* which produced (R)-decano-δ-lactone (massolactone; 1), and *Lasiodiplodia theobromae*, fungi that produce (R)-2-octeno-δ-lactone (designated as lasiolactone; 2) and mellein (3). This paper deals with an ecological role of these compounds, especially mellein.

\[
\text{C}_3\text{H}_{11}O_\text{O} \quad \text{C}_3\text{H}_7O_\text{O} \quad \text{Me}
\]

\[
\begin{align*}
1 & : \text{Massolactone} \\
2 & : \text{Lasilactone} \\
3 & : \text{Mellein}
\end{align*}
\]

In general, secondary products can have certain positive functions for the producing (or mother) organisms. We presumed that one of the supposed positive function of all described δ-lactone compounds which were produced by fungi of the genera *Fusarium* and *Lasiodiplodia*, would be ecological, as insect attractants. The δ-lactones including mellein, which have a slightly fruity odor, could attract some insects (or mites), and could make the insects touch the spores of the producing fungi. The spores that attached to the mobile insects would be disseminated to new places that also attracted these insects, and these new places could be, in many cases, flowers, fruits, or some edible substances containing the same or similar aroma compounds. Consequently, we can probably obtain the fungi that have the ability to produce these aroma substances from insects as well as from fruits or flowers containing compounds with sweet aromas like the δ-lactones. This was the reason we selected the coconut as the isolation source when we attempted to isolate the δ-lactone-producing fungi, *Fusarium solani* and *L. theobromae* described in the previous two reports.

Regarding the relation between the isolated fungus *Lasiodiplodia theobromae* and insects, Filer suggestively reported that the fungus causing sycamore canker were probably spread by crawling insects, primarily ants, because insecticide treatments of the trees were effective to prevent the infection of the fungi, and large numbers of ants were observed on untreated trees.

A fungal strain of *L. theobromae*, IFO 30028, which had been isolated from *Pandanus boninensis* (takonoki, in Japanese) was also confirmed to produce lasiolactone (2) and mellein (3) in this laboratory. Therefore, *L. theobromae* was expected to be also isolated from the places *Pandanus odoratissimus* (screw-pine; adan, in Japanese) grows, where we could have a chance to investigate the genus *Pandanus*.

In the meantime, Nishida et al. discovered that many males of the giant danaid butterfly, *Idea leuconoe* Ericson, grown outdoors, had accumulated mellein in the hairpencils as a suspected sex pheromone, and mellein was characterized as the specific attractant and exhibited potent phagostimulant activity for the male butterflies. The male effectively accumulated mellein in its hairpencil (few μg/male), when the butterfly was compulsively fed with the compound. (R. Nishida, personal communication). The details of the significance of mellein for the life cycle of the butterfly are still unknown, however, they suggested that the origin of the mellein might be microorganisms.

We presumed that one of these microorganisms would be *L. theobromae*, because the giant danaid butterfly inhabits the coastal areas of tropical and subtropical regions, in which also the genus *Pandanus* and coconut palm etc., possible host plants for the fungi *L. theobromae*, are growing.

In this study, we selected some ants and butterflies as the isolation sources of the δ-lactone-producing fungi to obtain evidence concerning this supposition.

Materials and Methods

*Isolation of microorganisms from ants.* Hundreds of individuals of ants, *Anoplolepis longiceps* Jerdon which were crawling on the young fruit of the screw-pine, were caught at the coast of Kinowan (Okinawa island) in October using a sterile milk bottle and some sugar as an attractant. Six of them were placed on a Potato-Dextrose-Agar (PDA) plate, and incubated for 5 days at 25 °C. All the colonies broken out from the outer surface of these ants were isolated.

*Isolation of fungi from outer surface of giant danaid butterfly.* A butterfly net was washed with 80% ethanol solution before each butterfly was caught. One male each of giant danaid butterfly was caught at Okanzaki (Ishigaki island) and Maedamisaki (Okinawa island) respectively in October 1991. One male and female each of the butterfly was caught at Haemidianohama (Iriomote island) in February 1993. The whole dead body of each butterfly was placed on the PDA medium in a large petri dish (18 cm) before being
incubated at 25°C for 10 to 20 days. Dominant colonies of the fungi broken out from the outer surface of each butterfly were isolated and identified.

Collection of the suspected host plants of *L. theobromae* involved in the danaid butterfly, and isolation of the fungi from these plants. Five kinds of plants that were suspected to be the host plants of *L. theobromae* were selected in the habitats of the giant danaid butterfly in Iriomote, Ishigaki, and Okinawa islands. Each collected plant tissue, leaf, flower, involucrum and seed pod, was placed on the PDA medium, and incubated at 25°C for 20 days. The *L. theobromae* broken out on the PDA palate was isolated and identified by its characteristic hypheae and conidia, and also by the volatile compounds produced in the culture.

Qualitative analyses of δ-lactone compounds and mellein contained in fungal cultures, plant tissues, and insects. Culture conditions for all fungi isolated from ants, butterflies, and plant tissue, and methods for identification of the volatiles produced by these fungi were the same as described in our previous report. All mellein detected from various sources described in this report was identified by gas chromatography (GC) and GC-MS compared to the authentic sample obtained from the culture of *L. theobromae* GK-1.

Measurement of mellein in plant tissues that were naturally infected with *L. theobromae*. A green fruit of coconut palm as described in our previous paper was again purchased from a local market, and without artificial inoculation, it was kept for 3 weeks, until it was covered by the hypheae of *L. theobromae* over about one-fifth of the surface. After being tested for insect attraction in an insectarium, about 40 g of the fruit flesh was cut off circularly with about 1 cm thickness from just the outer part of the fungal colony of *L. theobromae*. In the case of screw-pine and bottle palm, a part of blackened leaf or leaf sheath obtained in Maedaminsaki (Okinawa island) was cut in to pieces of 2 to 4 cm², respectively, before being steam-distilled and analyzed for mellein by GC. The GC was operated using an OT-FS column (CBP-1) under the same conditions as described in our previous paper. Mellein was measured compared to the peak area of undecano-γ-lactone. A sound part of each plant tissue that had kept green was also analyzed in the same way.

Mellein content in the hairpencil of giant danaid butterfly. The males of the giant danaid butterfly were caught in February 1992 in Chinen (Okinawa island) and Haemidannahama (Iriomote island), one and two individuals, respectively. Each hairpencil cut out from the butterfly body was steam-distilled. Mellein in the distillate was measured by the same way as that in the plant tissue described above.

The butterfly-attractivity test. The butterfly-attractivity test was done in the Minoo-Koon Insectarium (Osaka) in which more than two hundred of the giant danaid butterflies were released in a space of 2600 m² (200 m² x 13 m) together with some other kinds of butterflies. (1) Purified mellein, which was obtained from the culture of *L. theobromae* was further purified by silica-gel thin-layer chromatography. About 5 mg of the mellein which was put in a mini-vial (2 ml) was opened in the space of the insectarium for about 5 min. (2) Plant tissues. Blackened tissues of plants which were infected with *L. theobromae* were brought into and set at the center of the ground of the space, together with the sound part of each plant tissue as a control.

Results and Discussion

Isolation of δ-lactone-producing fungi from ants

Six individuals of ants, *Anoplolepis longipes* Jerdon (ashinagakari in Japanese) that were crawling on a young fruit of the screw-pine were caught in Okinawa island, and placed on PDA medium. Only a few kinds of filamentous fungi grew on the medium, one strain each of the genera *Fusarium*, *Trichoderma*, and *Aspergillus*. These strains were cultured and the volatiles produced were investigated as before. Massoialactone and 6-pentyl-γ-pyrone (2,4-decadieno-δ-lactone) were identified from the cultures of *Fusarium* and *Trichoderma*, respectively.

The expected strain of *L. theobromae* was not isolated from ant bodies; possibly the isolation of δ-lactone-producing fungi suggests the presence of a significant ecological relation between ants and δ-lactone-producing fungi.

*L. theobromae* isolated from outer surface of the body of a danaid butterfly

A male giant danaid butterfly was caught on Ishigaki island in October, and placed on the PDA medium in a large petri dish (diameter, 18 cm). Twenty days after inoculation, about half of the medium was blackened as shown in Fig. 1. On these black stains, characteristic gray hypheae were observed, and the isolated fungal strain, named GD-1, was identified as *L. theobromae* with its characteristic unisepate conidia, as described in our previous paper. We could not isolate other mellein-producing fungi than *L. theobromae* from the same source. *L. theobromae* was also obtained from a male giant danaid butterfly that was caught at Maedaminsaki (Okinawa island) in the same month.

Three other individuals of *Idea leucconoe* (two males and one female) were caught in February on Iriomote and Okinawa islands. These butterflies did not carry any spores of *L. theobromae*, however, a lot of colonies of the genus *Aureobasidium* that were unwittingly discovered to be massoialactone-producing fungi, were broken out from almost every part of the butterflies on the PDA medium. This is probably because of the cold season when *L. theobromae* and *Idea leucconoe* are inactive, although the two males had mellein in their hairpencils as described later. These suggest the need of further study on the seasonal changes of this topic.

Butterfly-attractivity of the mellein obtained from *L. theobromae* GD-1

*L. theobromae* GD-1 was cultured on PDA medium for 7 days, and volatiles were obtained as before. By using GC and GC-MS, lasio lactone and mellein were identified in the volatiles, compared to those obtained from the strain GK-1. About 5 mg of mellein, which was finally purified by thin-layer silica gel chromatography, was obtained from the culture mats (300 g) from 15 petri dishes, and it was used in the butterfly-attractivity test. As shown in Fig. 2, males of *Idea leucconoe* were attracted and ingested the mellein from the fungi attached to the butterfly.

The host plants of *L. theobromae* found on the giant danaid butterfly

The suspected host plants of the fungi were searched for and selected in the surroundings of the habitat of the butterfly. The parts of selected plants were placed on PDA plates and incubated at 25°C for 20 days. *L. theobromae* was isolated from some plants: (1) a seed pod of *Parsonisia lavigata* (Apocynaceae) on which the larvae of the butterflies feed exclusively; (2) flowers of *Anodendron affine* (Apocynaceae); (3) a involucrum of *Hernandia ovigera* L., which the butterflies fly around. All strains of *L. theobromae* isolated from these plants were also confirmed to be mellein-, and lasio lactone-producing ones.

These facts suggest that rather many varieties of plants could be involved with the *L. theobromae* rather than a single plant as the mellein source of the butterflies.
An Ecological Role of Volatiles of *L. theobromae*

Fig. 1. Microorganisms Broken out on Potato-Dextrose-Agar Medium Inoculated with a Naturally Grown Giant Danaid Butterfly, *Idea leuconoae*. Photo was taken from the bottom side. Black stains were mostly caused by *L. theobromae*.

Fig. 2. Ingesting Behavior of Male Giant Danaid Butterflies for Purified Mellein Produced by *L. theobromae*. The butterflies were attracted to the vial and its cap (right), which held purified mellein (5mg), and attempted to ingest the chemical, however, the blank vial and its cap (left) were ignored.

**Ingesting behavior of male giant danaid butterfly on plant tissues in the field and also in an insectarium**

We made efforts to observe the mellein-ingesting behavior of *Idea leuconoae* outdoors on Okinawa islands, and in the insectarium in which about two hundreds of the giant danaiid butterflies were released in the space of 200 m² × 13 m together with other butterflies under sub-tropical conditions.

A case was observed on a leaf of the wild-orange-tree, *Taddalia asiatica* (sarukakemikan, in Japanese) at a habitat of the butterfly, in the Maedamisaki district. The butterfly extended the proboscis, and reciprocally spat saliva and ingested it for something on the leaf as shown in Fig. 3. The same behavior was also observed on the leaf of screw-pine, *Pandanus odoratissimus*. This behavior is probably for mellein ingestion, because it is quite the same as the behavior shown on a filter paper containing synthesized chemicals of methyl o-hydroxybenzoate and some other similar compounds to mellein as observed by Nishida et al.41

The leaf of each plant which the butterfly reacted to was brought into the laboratory and a part was placed on the plate of PDA medium. More than 90% of the surface of each PDA medium plate was covered in 3 days with the hyphae of *L. theobromae*, and both strains that were isolated from the wild-orange tree and the screw-pine were also confirmed to produce both lasiolactone and mellein. *L. theobromae* was also isolated from a part of the leaf of the wild-orange tree which the butterfly reacted to, even after being washed with 80% ethanol solution for about ten seconds, this suggesting the leaf was deeply invaded by the *L. theobromae*.

Fig. 3. Ingesting Behavior of a Male Giant Danaid Butterfly on a Leaf of the Wild-orange-tree in the Field (Maedamisaki, Okinawa Island). The butterfly extended the proboscis, and then reciprocally spat saliva and ingest it. The leaf was infected mostly with the fungus *L. theobromae*. The picture was transcribed from a video-tape.

Fig. 4. Ingesting Behavior of Male Giant Danaid Butterflies in an Insectarium on a Fruit of Coconut Palm. The fruit of coconut palm was naturally infected with *L. theobromae*. 
*L. theobromae* was also obtained by transfer from the leaf of the screw-pine which the butterfly reacted to, to a sterile tissue paper. The paper was first pressed to the surface of the leaf with a finger touch, and then the transferred fungal spores were made a replica on a plate, from which a lot of colonies with characteristic hyphae of *L. theobromae* broke out. This fact demonstrates the fungal spores could be easily transferred to the butterfly body when the butterfly reacts to the surface of the plant leaf infected with the fungi. In these experiments on the leaves of wild-orange tree and screw-pine, we could not isolate *L. theobromae* from sound parts of these leaves.

Furthermore, a green fruit of coconut palm, which was the same as just described as the isolation source of the *L. theobromae* 31 was again purchased from a local market, and without artificial inoculation, it was kept for 3 weeks until it was covered by the hyphae of *L. theobromae* over about one-fifth of the surface. The moldy fruit of coconut palm was then brought into the insectarium. In a few minutes after the sample was set down, the males of the giant Danaid butterfly came flying on to the moldy coconut by turns, and each butterfly showed the ingesting behavior for about 0.5 to 2 min as shown in Fig. 4. In 10 min, about 20 individuals of male of *Idea leuconoe* came and showed this behavior, however, no other kinds of butterfly nor the females of *Idea leuconoe* showed any interest in the moldy coconut. As seen in Fig. 4, the males of *Idea leuconoe* were interested especially in the peripheral part of the colony of the *L. theobromae* which was growing from the stem-end of the fruit. The same behavior of the male *Idea leuconoe* in the insectarium was also observed on both blackened leaf of the screw-pine and leaf sheath of bottle palm which were brought from Okinawa island. We also confirmed that sound parts or whole fruit of these samples did not attract any butterflies in the insectarium.

Mellein contents in the hairpencils of the butterflies and in the plant tissues infected with the *L. theobromae*, and the butterfly-attractivity of these plant tissues

Nishida et al. detected significant mellein from many outdoor-grown males of *Idea leuconoe*, 31 although indoor-grown males originally lacked mellein. (R. Nishida, personal communication) We also analyzed the mellein contained in the hairpencils of *Idea leuconoe* which were caught on Okinawa and Ishigaki islands in February. The results, which were obtained from three individuals collected in February, were; by mellein content/hairpencil (locality of the *Idea leuconoe*), 110 ng (Haemidanoahama, Iriomote island), 110 ng (Haemidanoahama, Iriomote), 210 ng (Chinen, Okinawa island).

Mellein contents of the plant tissues which were positive in the insect-attractivity test in the insectarium were measured. These tissue samples were cut to pieces of 2 to 10 cm², and then steam-distilled without homogenization, and the analytical results obtained by the same ways as to the fungal culture are shown in Fig. 5 and Table, in which the results of insect-attractivity test are also shown.

In Fig. 5-A, which is the chromatogram of volatiles obtained from the infected fruit of coconut palm, other peaks than mellein did not appear, therefore, strong insect-attractivity of the infected fruit shown in the Fig. 4 and Table definitely indicate that the male of giant Danaid butterfly was attracted only to mellein. The normal part of these plants, fruit flesh of coconut palm and leaf of screw-pine, were confirmed not to contain mellein, therefore, the compound in these infected plant tissues with *L. theobromae* was concluded to be produced by the fungi in these plants. The mellein peak 3 in the GC of the infected

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**Fig. 5.** Gas Chromatographic Profiles of Volatiles Obtained from the Plant Tissues Invaded Mostly by *L. theobromae*.

(A): From fruit flesh of coconut palm with fungi. (B): From leaf of screw-pine with fungi. Extraction conditions were described in Materials and Methods. GC column; 0.2 mm i.d. x 50 m OT-FS column bonded with CBP-1 (Shimidzu). The column temperature; programmed from 80°C at a rate of 10°C/min. The inlet pressure of the carrier gas (nitrogen); 4 kg/cm².

**Table** Mellein Content and Butterfly-attractivity of Infected Plant Tissues with *L. theobromae*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Part of plant</th>
<th>Weight of sample (mg/cm²)</th>
<th>Mellein content (ppm)</th>
<th>Butterfly-attractivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Screw-pine</td>
<td>Leaf and leaf sheath</td>
<td>96⁴</td>
<td>26</td>
<td>(+)</td>
</tr>
<tr>
<td>Coconut palm</td>
<td>Surface part of the fruit</td>
<td>650⁴</td>
<td>7000</td>
<td>(+ + + + +)</td>
</tr>
<tr>
<td>Bottle palm</td>
<td>Leaf sheath</td>
<td>70⁴</td>
<td>136</td>
<td>(+)</td>
</tr>
<tr>
<td>Papaya</td>
<td>Whole fruit</td>
<td>—d</td>
<td>—</td>
<td>(-)</td>
</tr>
</tbody>
</table>

⁴ Samples were those naturally infected with *L. theobromae*, except for papaya which was inoculated with *L. theobromae* GK-1. The sound part or whole fruit of each sample was also analyzed for mellein, which showed no content of it, and attractivity test, which had a negative result.

⁵ Weight of leaf and/or leaf sheath with full thickness of them.

⁶ Surface part just outside of the fungal colony of *L. theobromae* was cut out in about 1 cm thickness.

⁷ Not determined.

⁸ (+): (Poor) A settled butterfly showed the ingesting behavior to the sample when the sample was laid in front of it. (+ +): (Good) A butterfly came flying onto the sample and showed the ingesting behavior. (+ + + + +): (Excellent). In 10 min, about 20 individuals of the male butterfly came flying onto the sample, and each butterfly showed the ingesting behavior for about 30 s to 2 min. (-): (Negative) The butterflies completely ignored the sample.
leaf of screw-pine (Fig. 5-B) was rather small among many miscellaneous peaks, therefore, there might be other attractive compounds for the butterfly in the infected leaf of screw-pine, but the weak insect-attractiveness of the leaf probably disproves this possibility. The mellein content of 26 ng/cm² in the infected leaf of screw-pine makes it not unreasonable for a male of the giant danain butterfly to accumulate 110 to 210 ng of the compound in its hairpencil.

All these facts indicate that the mellein compound stored in the hairpencils of male giant danain butterflies comes from the mellein produced by L. theobromae in these plants infected with the fungi.

Papaya fruit artificially inoculated with L. theobromae, which was described in detail previously,31 contained 12 ppm of mellein, the same level as the fruit of coconut shown in the table. The moldy papaya was also brought into the insectarium, however, the butterlies paid no attention to it. The reason for this negative result has not been clarified, however, it may be due to the strong aroma of other compounds than mellein, e.g., benzyl isothiocyanate and some monoterpene derivatives.6)

**Mutualistic relation of microorganisms with insect**

Many examples of fungus-insect mutualism have been reported and reviewed, including the case of the genus Ceratoctis associated with bark beetles as the vectors of fungal pathogens.7–15) Fungi, mostly Ceratoctis, were isolated from adult bodies of the bark beetles.14,16) There are very few cases of fungi to be reported from outer bodies of insects.

Some cases of insect attractant produced by microorganisms have been reviewed.17) However, cases involving the relation between fungus and butterfly have not appeared in preceding references so far as we are aware. Regarding the relation between L. theobromae and insects, besides the case of ants on sycamore trees described already, Thapa observed that L. theobromae had been introduced by ambrosia beetles into majau trees and developed within the tunnels, spreading to all parts of the trees.23)

Our observation described above suggested the presence of a kind of mutualistic relation between the fungus L. theobromae and the giant danain butterfly associating with the compound mellein, which was suggested to be a sex pheromone of the butterfly,3–5) although the mutualistic relation might be rather weak, because fungal spores also can be disseminated by wind and water.24)

Kunesch et al. reported a similar case of a mutualistic relationship between aumblebee wax moth and a mellein-producing fungus. They discovered mellein as the major component of the male wing gland pheromone of theumblebee wax moth, Aphomia sociella, and a mellein-producing fungus, Aspergillus ochraceus, was detected in the intestines of last-instar larvae.25) They argued that these facts might suggest a biosynthesis of mellein by the fungi present in the intestines of the larvae. In the case of the danain butterfly described here, however, most of the mellein would be extrinsic, because the butterflies were attracted to and showed ingesting behavior to extrinsic mellein as shown in Fig. 2.

The male of Grapholitha molesta, a injurious insect for fruit trees, also has mellein in its hairpencil together with methyl 7-iso-jasmonate and others.26) This insect might be also associated with the L. theobromae or other mellein-producing fungi.

In the reported fatty-acid-metabolites from the culture of L. theobromae, mellein (carbon number, 10; C10), (+)-7-isojasmonic acid and its related compounds (C12), and lasiodiplodin and its derivatives (C16), except for lasiolactone (C8), have shown to have plant growth regulatory effects.27–29) This may suggest the presence of a certain association between the L. theobromae and the host plant.

Regarding the chemical-ecological relation of α,β-unsaturated lactones to insects, some suggestive papers have been published, e.g., Cavill et al. obtained massoilactone from one of the formicine ants of the genus Camponotus collected in Western Australia.30) This is also suggestive for the presence of an ecological relation between ants and δ-lactone-producing fungi, however, we have no more data to support this contention so far.

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