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Stimulation of the Biosynthesis of the Antibiotics Lambertellols by the Mycoparasitic Fungus *Lambertella corni-maris* under the Acidic Conditions Produced by Its Host Fungus *in Vitro*

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The filamentous fungus, *Lambertella corni-maris* (*L. corni-maris*), a mycoparasite on *Monilinia fructigena*, produces the antibiotics, lambertellols A (1), B (2), and lambertellin (3), in a substantial amounts under acidic conditions, whereas these antibiotics were hardly detected when the fungus was cultured on a potato-sucrose (PS) medium without added acids. Our investigations also revealed that the host, *M. fructigena*, changed its surroundings into acidic conditions, suggesting that the acidic conditions acted as kairomones that stimulated the production of 1–3.

Key words: lambertellols; pH-dependent production; mycoparasite

Hori reported in 1912 the discomycete, *Seclerotinia phaeospora* Hori, in apples as a causal fungus of fruit rot, the so-called "Natsu-Nenju" disease in Japanese.[1)] Based on his bionomical observations, he mentioned that this fungus had two stages in its lifecycle: anamorphic (asexual) and teleomorphic (sexual). The fungus was then raised to a new genus and species, *Phaeosclerotinia nipponia* Hori, in 1916.[2)]

One of the present authors (Y.H.) reported in 1990 that *Phaeosclerotinia nipponica* Hori is a misapplied name in reality, and that Hori's observation can be explained by a mycoparasitic development[3)] of *Lambertella corni-maris* (*L. corni-maris*) on *Monilinia fructigena* (*M. fructigena*), which is a common pathogenic fungus in apples in Japan. Simultaneous culturing of *L. corni-maris* and *M. fructigena* on a potato-sucrose-agar (PSA) medium for a couple of months resulted only in *L. corni-maris* being isolated, even from the region where *M. fructigena* had developed independently. Harada has recently identified *Lambertella* sp. 1346 (*L.* sp. 1346) as a novel and similar mycoparasite on *M. fructigena*. Under similar conditions, *L.* sp. 1346 interfered with host growth during competitive culture on a PSA medium, whereas *L. corni-maris* did not at a glance.

During an investigation of the chemical substances responsible for the mycoparasitism of apples, we have studied the metabolites produced by *L.* sp. 1346 and found lambertellols A (**1**) and B (**2**)[4,5)] as the major antifungal substances (Fig. 1), together with minor amounts of lambertellol C,[6)] neolambertellin,[7)] and known lambertellin (**3**).[8)] In order to elucidate the behavioral difference against host *M. fructigena* between *L. corni-maris* and *L.* sp. 1346, we studied the relationship between the secondary metabolites of these fungi and the culture conditions.

Our investigations disclosed that *L. corni-maris* could produce **1**, **2** and **3** in large amounts by culturing it under acidic conditions. The PSA medium around the hyphal surroundings of host *M. fructigena* was found to become acidic. This indicated that the acidic conditions around the host hyphae stimulated *L. corni-maris* to initiate biosynthesis of these antifungal substances. Since the acidic region was limited only to that where the host hyphae were observed, *L. corni-maris* did not produce these compounds in a competitive culture on PSA until its hyphae had developed to reach the host.

Materials and Methods

The discomycetes used for our experiments were isolated by one of the present authors (Y.H.). *Lambertella corni-maris, L.* sp. 1346, and *M. fructigena* were collected in Aomori prefecture in Japan in 1988, 1989, and 2003, respectively. They were stored on a PSA medium (*ca.* 2-mm thickness) in 9-cmϕ Petri dishes at 20 °C. The PSA medium was prepared from sucrose (20 g), an aqueous potato extract (1.0-liter, 200 g from potato), and agar (20 g).

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Abbreviations: PSA, potato-sucrose agar; PS, potato-sucrose; ODS, octadecyl silyl

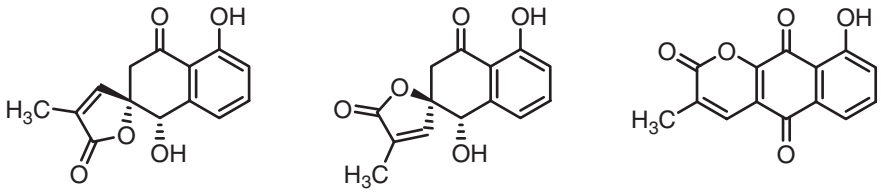


Fig. 1. Structures of the Secondary Metabolites Isolated from *Lambertella* sp. 1346.

Lambertellols A (**1**) and B (**2**) were prepared by following our established procedure.[4,5] The HPLC analysis was performed with a Merck LiChrospher® 100 RP-18e 5-μm column (250 × 4.0 mm I.D.) and a Waters 626 pump equipped with a Waters 600S controller, Waters 474 scanning fluorescent detector, and 996 photodiode array detector. The ^{1}H-NMR data for **1** and **2** thus obtained were identical to those reported.[4]

Lambertellin (**3**) was prepared by chemical conversion from lambertellols.[4] A solution of a mixture of **1** and **2** (20 mg, **1**:**2** = 2:3 at UV 330 nm) in a mixture of MeOH (10 ml) and H_2O (10 ml) was allowed to stand in the ambient atmosphere at room temperature for 1 week. The dark reddish precipitate was collected by filtration, followed by recrystallization from acetone to give pure lambertellin (**3**, 12 mg). The ^{1}H-NMR data were identical to those reported.[8]

Moniliniol and 3-Cl moniliniol[9] were isolated from the culture broth of *M. fructigena* after culturing in the PS medium (200 ml × 20 flasks) at 26 °C for 2 weeks without shaking. The broth after filtration was extracted with EtOAc (2.0-liter). The extract was concentrated under reduced pressure to give a residue. Silica gel column chromatography of this residue using hexane–acetone (80:20) as the eluent afforded moniliniol and 3-Cl moniliniol. The structures of these compounds were fully assigned by comparing with the reported data.[9]

Competitive culture of Lambertella species with the host M. fructigena. The parasite, *L.* sp. 1346, and the host, *M. fructigena*, were seeded on a freshly prepared PSA medium in a Petri dish (9 cmϕ), leaving *ca.* 4 cm of space. The Petri dish was kept at 25 °C for two weeks (Fig. 2, left). A similar experiment was performed using *L. corni-maris* used instead of *L.* sp. 1346 (Fig. 2, right).

Detection of lambertellols from L. corni-maris on the PSA medium by culturing in the presence of M. fructigena. Both *L. corni-maris* and *M. fructigena* were seeded on a Petri dish (9-cmϕ), leaving *ca.* 6 cm of space. After cultivating at 20 °C for 18 days, five sections (8-mmϕ, the points where *L. corni-maris* and *M. fructigena* were transplanted as well as three equidistant points) were sampled with a cork borer. Each section was put in CH_3CN (3.0 ml). After 12 h, these were independently filtered through cotton pads and concentrated *in vacuo*. Each residue was diluted with 30% aqueous CH_3CN (150 μl), and 10 μl of the solution was injected into the HPLC instrument. Compounds **1**–**3** were detected by their fluorescence at 550 nm, with excitation at 350 nm and UV 430 nm, as described in the text.

Cultivation of L. corni-maris in the presence of Monilinia broth. The *Monilinia* broth was prepared by culturing *M. fructigena* in a potato-sucrose (PS) medium (200 ml) for 14 days without shaking at 25 °C, and then filtering. *Lambertella corni-maris* was seeded on another PS medium (150 ml baffled flasks), and cultured for 7 days at 25 °C on a rotary shaker (110 rpm). Into the medium, the *Monilinia* broth (100 ml) was added through a cotton filter, and then resulting mixture was further cultured under the same conditions for an additional 7 days. The supernatant of the culture medium was directly analyzed by HPLC. This series of experiments was triplicated, and the mean amounts of **1**–**3** are used in the discussion.

Cultivation of L. spp. under acidic conditions. The PS medium (200 ml) was adjusted to pH 3.8 by adding a 1.0 M citric acid solution (*ca.* 1.0 ml). After sterilization by autoclaving, *Lambertella* species were seeded and cultured under the same conditions as these already mentioned. After 8 days, the supernatant (1.0 ml) was collected and directly analyzed by HPLC. Experiments using *L. corni-maris* with hydrochloric acid instead of citric acid were also performed. This series of experiments was duplicated, and the means of the data are used in the discussions.

Cultivation of L. spp. under neutral conditions. Sodium citrate dehydrate (290 mg) was added when the PS medium (200 ml) was prepared. *Lambertella corni-maris* was cultured in this medium under the same condition as those just mentioned. After culturing for 8 days, the supernatant was sampled and analyzed by HPLC. A similar experiment using *L.* sp. 1346 was also performed. This series of experiments was duplicated, and the means of the data are used in the discussions.

Results and Discussion

Lambertella corni-maris is the initially identified mycoparasite on *M. fructigena*. In contrast with *L.* sp.

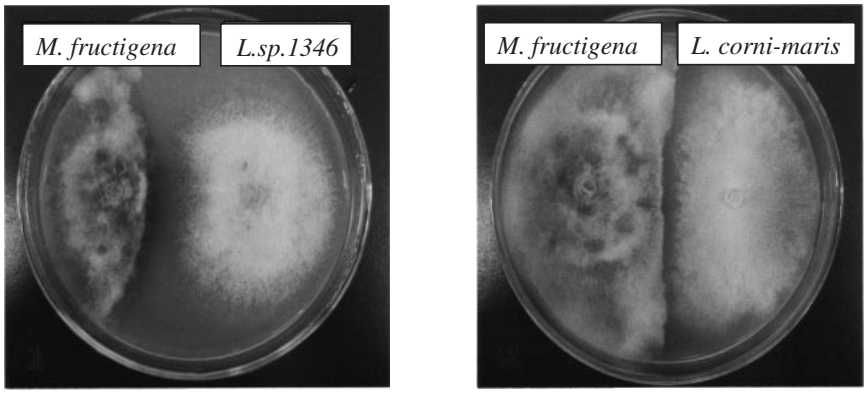


Fig. 2. Competitive Cultures of the Mycoparasite *Lambertella* spp. with the Host *Monilinia fructigena* on PSA Medium.

1346, *L. corni-maris* did not suppress the host *M. fructigena* at a glance under competitive culture on PSA medium as shown in Fig. 2. However, only *L. corni-maris* was isolated from the medium with *L. corni-maris* and *M. fructigena* after storing for a couple of months. Our preliminary experiments showed that *L. corni-maris* produced **1**–**3**, but at quite low levels (near the detection limit) under the same conditions used for *L.* sp. 1346 which provided several milligrams of the antibiotics from 4-liter of the culture broth.

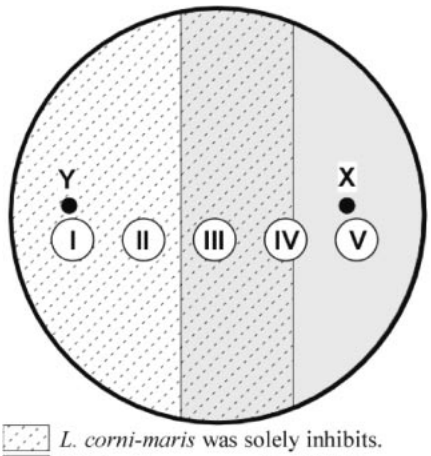
We assumed that *L. corni-maris* also produced **1**–**3**, but this production was limited only in the presence of the host. Based on this assumption, we investigated the influence of this production by *L. corni-maris* on the host metabolites.

Prior to these experiments, we developed sensitive and specific analytical conditions for identifying **1**–**3**. Lambertellin (**3**; λ_{max} 430 nm, ε 4800) could be identified by HPLC at UV 430 nm. The limit of detection was estimated to be 100 ng/injection. This condition was specific enough for quantifying **3** from a crude extract. However, the conditions were much less sensitive for **1** and **2**. The λ_{max} values of **1** and **2** were both 261 nm (ε 9500 for both), but this wavelength was applicable only for pure samples. The crude extract gave many signals due to the impurities involved, which decreased the signal-to-noise ratio. We found that these compounds could be specifically quantified by fluorescence at 550 nm (excitation at 350 nm), which sensed around 100 ng/injection for both. These methods enabled us to analyze **1**–**3** directly from the PS medium.

When *L. corni-maris* and *M. fructigena* were competitively cultured for 18 days on the PSA medium, the fungi were intermixed and three zones were found by visual observation: one solely inhabited by the parasite, one solely inhabited by the host, and another one inhabited simultaneously by both the parasite and host (Fig. 3). Five small sections (8 mmϕ) were sampled from the medium, extracted with acetonitrile, and analyzed by HPLC under the conditions described.

Interestingly, **1**–**3** were detected in considerable amounts in the regions inhabited by both *L. corni-maris* and *M. fructigena* (sections **III** and **IV**). In contrast, **1** and **2** were below the detection level in sections **I**, **II** and **V**. In these regions, the parasite or the host existed independently. Lambertellin (**3**) was observed in region **II**, but the amount was quite small. These results may support our assumption that *L. corni-maris* exuded these antibiotics by detecting the host *M. fructigena*. Two explanations are possible for this phenomenon: chemical stimulation so-called allelopathy and inducement by physical contact.

These possibilities were investigated by culturing *L. corni-maris* in a liquid medum in the presence of the host *M. fructigena* culture broth. It was found that *L. corni-maris* provided **1** (18 μg/ml), **2** (52 μg/ml), and



zone	I	II	III	IV	V
1 (μg/cm^2)	n.d.	n.d.	0.41	0.22	n.d.
2 (μg/cm^2)	n.d.	n.d.	1.74	0.41	n.d.
3 (μg/cm^2)	n.d.	0.39	4.36	0.31	n.d.

n.d., not detectable (less than limit of detection)

Fig. 3. Production of Lambertellol A (**1**), Lambertellol B (**2**), and Lambertellin (**3**) by *L. corni-maris* in the Presence of the Host *M. fructigena*.

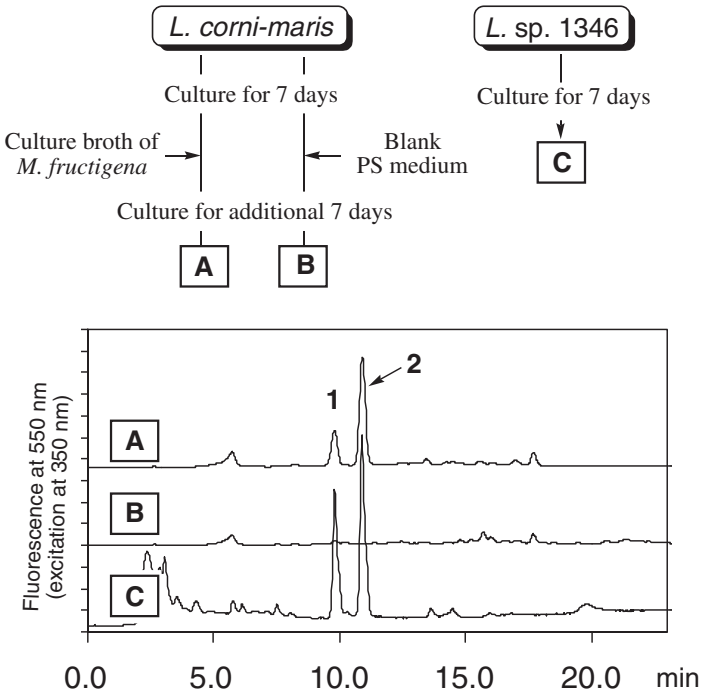


Fig. 4. HPLC Chromatograms of the Culture Broth of *L. corni-maris* with the *M. fructigena* Culture Broth (A), with the Blank PS Medium (B), and with the Culture Broth of *L. corni-maris* Obtained under the Usual Conditions (C).
Lambertellin (**3**) was not detected under these conditions.

3 (9.0 μg/ml) as shown in Fig. 4 (chromatogram **A**, chromatograms at UV 430 nm detecting **3** are not shown) when it was cultured with the host broth for 7 days. These compounds were within the detection limits (chromatogram B: **1**, <0.1 μg/ml, **2**, <0.1 μg/ml, **3**, 0.9 μg/ml) in the control experiment for which the blank PS medium was added instead of the culture broth. In these experiments, *L. corni-maris* had been cultured for 10 days before adding the host broth or the blank PS medium. Notably, *L.* sp.1346 provided **1** (44 μg/ml) and **2** (75 μg/ml) after 7 days of culture without any addition of the host broth (chromatogram **C**). Lambertellin (**3**) was also found (0.3 μg/ml) in this sample, but in a relatively low quantity. These experiments proved that chemical conditions stimulated *L. corni-maris* to produce these antibiotics, and not physical contact.

However, all our attempts to fractionate the active substance(s) failed. The activity could not be extracted by organic solvents. Fractionation by ODS was not reproducible. Neither of the major secondary metabolites of *M. fructigena*, moniliniol and 3-Cl moniliniol, induced the biosyntheses of **1**–**3**.

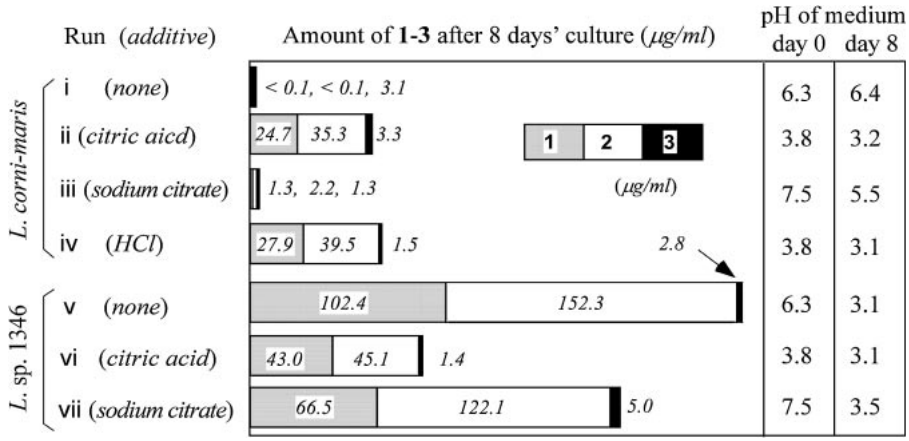
Interestingly, the PS medium was found to become strongly acidic (pH 4.2) by culturing the host *M. fructigena* for a week. The original pH value was 6.3 before the culture. This result suggested that the acidic conditions might be the stimulant. To confirm this assumption, we cultured *L. corni-maris* in a liquid medium under acidic conditions. When the pH value of the medium was adjusted to 3.8 with citric acid before seeding, *L. corni-maris* afforded **1**–**3** in considerable amounts without a *Monilinia* extract after culturing for 8 days (Table 1, run ii).* Under regular conditions, the compounds were hardly found (run i). These results may indicate citric acid to be responsible for this; sodium citrate, however, did not increase the production of **1**–**3** (run iii). We found HCl also induced this productions (run iv), which suggests acidic conditions were responsible.

In contrast, *L.* sp. 1346 produced considerable amounts of **1**–**3** under all the conditions examined (runs v–vii). The addition of either citric acid or sodium citrate seemed to decrease the levels of **1**–**3** compared with the regular condition. This may have been due to the slightly lower growth rate of *L.* sp. 1346 under these conditions.

We found that *L.* sp. 1346 also dramatically decreased the pH value of the culture medium. Neutral conditions could not be maintained even with sodium citrate (run vii). After culturing for a week, the pH value of the culture medium with sodium citrate became 3.5, whereas the initial pH value was 7.5. In contrast, *L. corni-maris* did not markedly affect the pH value of the medium. These results suggest that *L.* sp. 1346 stimulated the production of **1**–**3** by acidifying the environment by itself.

* Our preliminary experiments revealed that the production of **1**–**3** was synchronized with the logarithmic growth phase of the fungi, especially for *L. corni-maris*. Since acidic conditions slightly decreased the growth rates of both *L. corni-maris* and *L.* sp. 1346, HPLC analyses were performed after culturing for 8 days in these experiments.

Table 1. Production of Lambertellol A (**1**), Lambertellol B (**2**), and Lambertellin (**3**) by *L. corni-maris* and *L.* sp.1346 after Culturing for 8 Days on a PSA Medium under Various Conditions



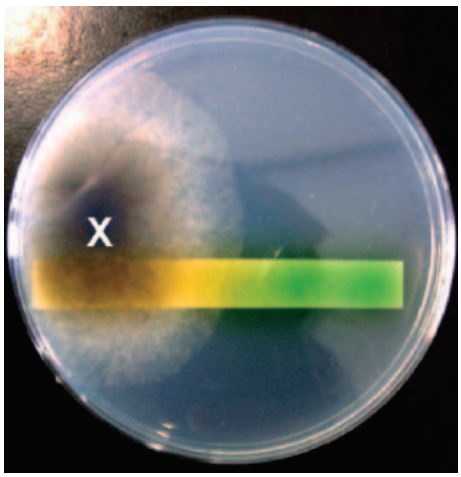


Fig. 5. pH Test Strip with *M. fructigena* on the PSA Medium. X, the point *M. fructigena* was seeded.

The decrease in pH value by the host *M. fructigena* was also observed on PSA gel. Since it is difficult to identify the acidity of gels by pH, we used a pH test strip as the indicator. When the pH test strip was placed on the PSA culture medium, only the area with the host *M. fructigena* was found to be acidic, while the region without *M. fructigena* was neutral as shown in Fig. 5. This result indicates that the spreading rate of the acidic region was almost identical to the growth rate of the host hyphae on the PSA medium.

Conclusion

The present experiments disclosed that *L. corni-maris* exuded **1–3** in considerable amounts only when the host *M. fructigena* co-inhabited. We found that the host *M. fructigena* markedly acidified the environment, and that the acidic conditions stimulated *L. corni-maris* to produce **1–3**. The spreading rate of the acidic area was found to be almost identical to the growth rate of the host hyphae on the PSA medium. Since *L. corni-maris* did not produce **1–3** independently on PSA, the host could grow freely until its hyphae reached the parasite. On reaching the host, *L. corni-maris* would then begin to produce these antibiotics to deteriorate the host.** This might be the reason why only *L. corni-maris* was isolated after simultaneous cultivation with *M. fructigena*. On the other hand, *L.* sp. 1346 was found to markedly acidify the environment. This fungus might stimulate itself to this production by acidifying its environment.

Acknowledgment

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** There is another possible candidate. Both *L.* sp. 1346 and *L. corni-maris* constantly produced **1–3**, but in the case of *L. corni-maris*, we did not see them because of immediate digestion. The latter fungus could turn off this system by detecting the acidic conditions of the host.

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