

Contribution of symbiotic mycangial fungi to larval nutrition of a leaf-rolling weevil

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Abstract Some phytophagous insects have been known to inoculate certain fungi on plant substrates. In many cases of such insect–fungi relationships it has been considered that fungi contribute to insects by decomposing lignin or polysaccharides, and that the insects feed on the decomposition products or fungi themselves. Females of the leaf-rolling weevil in the genus *Euops* (Attelabidae) store spores of symbiotic fungi in the mycangia and inoculate them on leaf rolls. To determine the effect of mycangial fungi on larval nutrition in *E. lespedezae*, the nutritional value was compared between leaves with and without mycangial fungi. Two *Penicillium* species were isolated from the mycangia. These mycangial fungi showed little effect on the decomposition of lignin and polysaccharides, and showed little effect on enhancement of soluble sugars within leaves. Thus, the mutualism between *Euops* and its mycangial fungi contrasts with the mainly nutritional mutualisms between wood-infesting insects (termites, bark/ambrosia beetles, and wood wasps) and lignin/polysaccharide-decomposing fungi.

Keywords *Euops lespedezae* · Insect–fungus symbiosis · Larval development · Nutritional enhancement · *Penicillium*

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Introduction

Symbiotic relationships with fungi have evolved in many insect taxa (Batra 1979; Wheeler and Blackwell 1984; Wilding et al. 1989). These insects transfer and inoculate fungi on plant substrates, and feed on fungi or their decomposition products (Batra and Batra 1979; Haack and Slansky 1987; Paine et al. 1997). Most of these insects, i.e., termites, bark/ambrosia beetles, and wood wasps, feed on dead plant tissue and are considered dependent on symbiotic fungi for the decomposition of lignin and cellulose (Morgan 1968; Barras and Hodges 1969; Martin and Martin 1978; Bridges 1983; Kukor and Martin 1983; Madden 1988; Watanabe et al. 1998; Hyodo et al. 2000), which are the main components of plants, but are indigestible by most insect herbivores (Talmadge et al. 1973; Abe and Higashi 1991; Martin 1991; Hochuli 1996; Schoonhoven et al. 1997). Therefore, insects feeding on nutrient-poor plant tissue gain more nutrients by feeding on fungi inoculated on the tissue or their decomposition products than by feeding on the plant tissue directly.

Leaf-cutting ants inoculate fungi on living plant tissue, mainly old leaves (Hölldobler and Wilson 1990), which contain smaller amounts of lignin and cellulose and larger amounts of non-cellulose polysaccharides than dead plant tissue. It is assumed that fungi that are symbiotic with leaf-cutting ants do not decompose lignin (Lee and Wood 1971; Maynard et al. 1979). Rather, these fungi decompose plant polysaccharides that ants cannot digest (Martin and Weber 1969; Bacci et al. 1995; Siqueira et al. 1998; D’Ettorre et al. 2002; Richard et al. 2005), although the ability to decompose cellulose may differ among species (Martin and Weber 1969; Bacci et al. 1995; Siqueira et al. 1998; Abril and Bucher 2002). Workers of these ants have been observed to ingest the juice of cut leaves prior to inoculation with symbiotic fungi (Littleddyke and Cherrett 1976). Thus, leaf-cutting ants are considered to feed on soluble sugars and other usable components from plant juice, in addition to products decomposed by fungi and fungi themselves (Quinlan and Cherrett 1979; Silva et al. 2003).

Leaf-rolling weevils of the genus *Euops* (Coleoptera: Attelabidae), which are mainly distributed in Africa, the Papuan region, and Asia (Riedel 2002), have symbiotic relationships with fungi. *Euops* females construct small leaf rolls (<5 mm in length and <3 mm in diameter) by cutting ribbon-shaped pieces from the leaf margin (Sakurai 1985; Sawada and Morimoto 1986), and inoculate the leaf pieces with fungal spores stored in the mycangia (Sakurai 1985; Sawada and Morimoto 1986; Riedel 2002). Leaf rolls are constructed from pieces of young leaves, which are considered one of the most nutrient-rich plant parts. Nevertheless, the weevils inoculate the leaf rolls with mycangial fungi. Recently, mycangial fungi of some *Euops* species were reported to belong to the genus *Penicillium* (Riedel 2002). *Penicillium* species are one of the most common soil fungi, having worldwide distribution (Domsch et al. 1993). Colonization on leaf litter and decomposition ability of polysaccharides were reported in some species (Domsch et al. 1993). The role of mycangial fungi in *Euops*, however, has not yet been determined. It is unknown whether these mycangial fungi can decompose lignin or polysaccharides in the leaf rolls.

The objective of this study was to determine whether mycangial fungi of *Euops* species enhance the nutritive value of leaf rolls by decomposing lignin or polysaccharides. First, we isolated fungi from mycangia and leaf rolls of *E. lespedezae* Sharp and monitored the succession of fungal flora on the leaf-rolls. We then calibrated the ability of mycangial fungi, epiphytes, and endophytes to decompose lignin, polysaccharides, and soluble sugars. If the mycangial fungi are good at decomposing lignin or polysaccharides, the decomposition of these indigestible substances would be accelerated in leaf rolls inoculated with

the mycangial fungi. Consequently, soluble sugar content would be high in leaf rolls inoculated with mycangial fungi. In addition, the ability of the mycangial fungi to enhance the nitrogen content of leaf rolls was also measured.

Our results suggest that there is a different important role of mycangial fungi than enhancing nutrients by decomposing lignin or polysaccharides, viz. suppressing antagonistic bacteria or fungi.

Material and methods

Insect species

Euops lespedezae is distributed in Japan and Korea, and females of this species construct leaf rolls from the leaves of Fabaceae and Polygonaceae (Suzuki and Uehara 1997). The most common host plant in Kyoto, Japan, is *Lespedeza cyrtobotrya* Miq. (Fabaceae), which has trifoliate leaves. The weevil cuts ribbon-shaped pieces of leaf from the leaflet (hereafter, leaf) margin, inoculates them with the mycangial fungi, and then forms leaf rolls (Fig. 1a–c). One egg is oviposited per leaf roll, and the hatched larva feeds on the molded leaf roll until emergence. This weevil species is univoltine in Kyoto.

Isolation of mycangial fungi

Ten female *E. lespedezae* were collected from the host shrub *L. cyrtobotrya* at Mt. Daimonji (35.2°N, 135.48°E), Kyoto, Japan, on 13 and 25 May 2005. Spores stored in the mycangia of

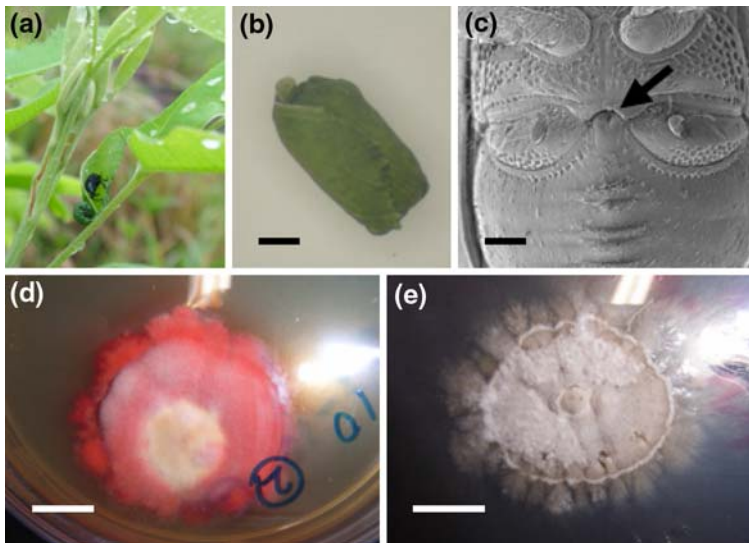


Fig. 1 Leaf rolls, adults, and mycangial fungi of *E. lespedezae*. (a) Leaf-roll construction by a female weevil. (b) Leaf roll. (c) Ventral side of female thorax and abdomen with an arrow showing the entrance to the mycangia. Colony of (d) *Penicillium* sp. 1 and (e) *P.* sp. 2 cultured on 2% malt extract agar medium for 9 months. Bars in photos represent (b) 1 mm, (c) 200 μ m, (d, e) 1 cm

females were removed using a sterile needle under a microscope and inoculated on 2% malt-extract agar (MA) medium at room temperature, and then on LCA medium (0.1% glucose, 0.1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02% KCl, 0.2% NaNO_3 , 0.02% yeast extract, and 1.3% agar (w/v); Miura and Kudo 1970) at 25°C in the dark. Sporulating colonies were used to identify the fungi.

A molecular method was also used to identify the mycangial fungi. DNA was extracted from mycelia of these isolates following the methods of Gardes and Bruns (1993). The 28S rDNA (D1 and D2 regions) was amplified using D1 (Peterson 2000) and NL4 primers (O'Donnell 1993). DNA base sequences were determined according to the method of Iwamoto et al. (2002). The accession numbers for the DNA sequences are AB253796 and AB253797. Sequenced isolates were compared to known species using a BLAST search.

Isolation of leaf-roll-associated fungi

Leaf rolls recently constructed by weevils were collected from Mt. Daimonji (ten leaf rolls) on 3 June 2005 and from weevils in the laboratory that had been collected at Mt. Daimonji (14 leaf rolls). Fungi from these 24 leaf rolls were isolated just after construction (ten each from the field and laboratory) and 1 month after construction (four from the laboratory). The leaf rolls were unrolled and eggs or larvae inside were removed. Five 2 × 2 mm pieces were cut from each leaf roll using a razor and placed on 2% MA medium. Isolated fungi were cultured at room temperature for >2 weeks in natural light, and then identified by microscopic observation. To determine the fungal composition, the dominance of each fungus within a leaf roll was determined as the number of pieces from which the fungus was isolated. One-way analysis of variance (ANOVA) with a Tukey–Kramer test at $P = 0.05$ was used to compare the dominance within a leaf roll among fungi using JMP ver. 5 software (SAS Institute, Cary, North Carolina, USA).

Contribution of mycangial fungi to the nutritional value of leaf rolls

Fungi isolated from the mycangia of *E. lespedezae* females were cultured on 2% MA medium for 1 month. Before the inoculation experiment, 78 pieces of 1 × 1 mm in size per fungal species were cut from the culture and placed separately on 1.5% plain agar plates using forceps.

As inoculation substrates, leaves were collected from one *L. cyrtobotrya* shrub at Mt. Daimonji on 23 June 2005. Ribbon-shaped pieces were cut from the leaves, and each piece was weighed within 24 h after collection. Leaf pieces were treated in three ways: sterilized by ethylene oxide gas at 40°C for 6 h to kill both endophytes and epiphytes; surface-sterilized using 70% ethanol for 1 min, 15% hydrogen peroxide for 1 min, and 70% ethanol for 1 min to kill only epiphytes; or left untreated. Each leaf piece was deposited over each fungal colony on plain agar. As a control, each leaf piece was similarly deposited on an uninoculated 1.5% plain agar plate. Leaf pieces were stored at 20°C for 3 months.

Leaf pieces were then oven-dried at 40°C for 24 h, weighed, and ground to a powder. Powdered leaves were then used to determine the lignin, total carbohydrate, soluble sugar, and nitrogen content.

To determine the amount of water, lignin, polysaccharide, soluble sugar, and nitrogen content without inoculation, 30 leaf pieces per treatment (gas sterilized, surface-sterilized, and untreated) were weighed fresh and after air-drying for 3 months, and used for chemical

analysis similar to the inoculated leaf pieces. The initial dry masses of inoculated leaf pieces were estimated using the uninoculated leaf pieces.

The decomposition abilities of fungi were determined as the residue of each chemical component divided by the initial dry weight of the leaf piece.

Chemical analysis

Lignin content was determined using hot sulfuric acid digestion according to the standard method. After the extraction of soluble compounds using alcohol-benzene at room temperature, the residue was treated with 72% sulfuric acid for 2 h. The mixture was diluted with distilled water to 2.5% sulfuric acid solution, autoclaved at 120°C for 60 min, and then filtered and washed with water. After drying at 105°C, the filtered residue was weighed. The filtrate was used to determine total carbohydrates. The ability of each fungus to decompose lignin was compared using a two-way ANOVA for untreated and surface-sterilized leaf pieces, and one-way ANOVA for gas-sterilized leaf pieces.

Total carbohydrate content was determined using the phenol-sulfuric acid method. The 5% phenol and 98% sulfuric acid were added to the filtrate obtained from the lignin analysis. The optical density was measured with a spectrometer at 490 nm, using glucose as a standard.

The soluble sugar content was determined using the phenol-sulfuric acid method. Powdered leaf pieces were extracted with 50% methanol at 75°C for 60 min and filtered, and 5% phenol and 98% sulfuric acid were added to the filtrate. The optical density of the filtrate was measured with a spectrometer at 490 nm, using glucose as a standard. The polysaccharide content was estimated as the difference between the total carbohydrate and soluble sugar contents.

Nitrogen content was measured using an automatic gas chromatograph (NC analyzer, Sumitomo Chemical Co., Osaka, Japan). Powdered leaf pieces were completely burned in a furnace carried by helium gas at 830°C for 1 min, transforming all nitrogen into N₂. N₂ was quantitatively measured in the chromatograph tube using hippuric acid as a standard.

For the comparison of the amount of polysaccharide, soluble sugar, and nitrogen content among each treatment, no statistic was done because all leaf pieces of the same treatment were unified and powdered together for the technical necessity.

Results

Isolation of fungi from mycangia

Of the ten observed females, eight had spores stored in the mycangia, but two did not. The mycangial fungus spores inoculated on MA media formed orange and white colonies (Fig. 1d, e). Both isolates sporulated 2 weeks after inoculation on LCA medium and were identified as *Penicillium* spp. by the morphological characters of the conidia, conidiogenous cells and conidiophores. Blast searches of the two isolates indicated that the two isolates represented probably different species of the genus *Penicillium*. Thus, we identified orange and white colonies as *P. sp. 1* and *P. sp. 2*, respectively. *P. sp. 1* and *P. sp. 2* were isolated from three and five females, respectively (Table 1). No fungus was isolated from the mycangia of two females. No female had both *Penicillium* species.

Table 1 Frequency of fungal species isolated from the mycangia of *Euops lespedezae*

		<i>Penicillium</i> sp. 1	
		+	–
<i>Penicillium</i> sp. 2	+	0	5
	–	3	2

Isolation of fungi from leaf rolls

A total of 22 fungal species, including *P.* sp. 1 and *P.* sp. 2, were isolated from the 24 leaf rolls. For leaf rolls constructed in the laboratory, either *P.* sp. 1 or *P.* sp. 2 was isolated from all leaf rolls (Table 2). *P.* sp. 1 or *P.* sp. 2 was isolated from 60% of leaf rolls constructed in the field. *Cladosporium* sp. 1 was isolated from all leaf rolls at the larval stage, although it was only isolated from 30% of leaf rolls at the egg stage.

The dominance within a leaf roll of eight fungal species isolated from two or more leaf rolls was analyzed, with *P.* sp. 1 and *P.* sp. 2 grouped together. The dominance within a leaf roll was significantly different among fungi (one-way ANOVA; field, egg stage, $df = 7$, $F = 5.13$, $P < 0.0001$; laboratory, egg stage, $df = 7$, $F = 68.01$, $P < 0.0001$; laboratory, larval stage, $df = 7$, $F = 5.85$, $P < 0.001$). The dominance within a leaf roll of *P.* sp. 1 or *P.* sp. 2 in leaf rolls at the egg stage was significantly higher than that for other fungi (Tukey–Kramer test, Fig. 2). The dominance within a leaf roll of *Penicillium* at the egg stage (mean \pm SE) was 4.3 ± 0.3 in leaf rolls constructed in the laboratory and 1.7 ± 0.6 in leaf rolls collected in the field. The dominance within a leaf roll of *Penicillium* in leaf rolls at the larval stage was 3.3 ± 0.9 (mean \pm SE), which was highest among the fungal species, but not significantly different from that of *Cladosporium* sp. 1 (3.3 ± 0.9) and hyphomycete sp. 1 (0.8 ± 0.8 ; Tukey–Kramer test; Fig. 2).

Decomposition ability of mycangial fungi

Lignin content decreased only slightly after 3 months (Table 3). The final lignin content did not differ among inoculation (*P.* sp. 1, *P.* sp. 2, and uninoculated) or sterilization (untreated and surface-sterilized) treatments, nor was the inoculation \times sterilization interaction significant (two-way ANOVA; Table 4). The decomposition ability of *P.* sp. 1, *P.* sp. 2, epiphytes, and endophytes were similarly low. Among gas-sterilized leaf pieces, those inoculated with *P.* sp. 1 or *P.* sp. 2 had lower lignin content than uninoculated leaf pieces (mean \pm SE, 411.15 ± 16.52 mg/g), although the difference was not significant (one-way ANOVA, $df = 2$, $F = 6.02$, $P > 0.05$; Table 3).

Some polysaccharide appeared to be decomposed by both endophytes and epiphytes (Table 3). Among the gas-sterilized leaf pieces, those inoculated with *P.* sp. 1 or *P.* sp. 2 had a slightly lower polysaccharide content than those uninoculated leaf pieces (Table 3), suggesting that *P.* sp. 1 and *P.* sp. 2 decomposed only small amounts of polysaccharide. Among the unsterilized and surface-sterilized leaf pieces, those inoculated with *P.* sp. 1 or *P.* sp. 2 did not show greatly different polysaccharide content from uninoculated leaf pieces, suggesting that the *Penicillium* fungi did not decompose polysaccharides more than epiphytes and endophytes.

Table 2 Number of leaf rolls collected from the field (egg stage, $n = 10$) and constructed in the laboratory (egg stage, $n = 10$; larval stage, $n = 4$) from which each fungal species was isolated

Source of leaf rolls	Weevil stage	Number of leaf rolls		Fungal species							Total number of fungal species isolated				
		Fungal species		<i>Penicillium</i>		<i>Cladosporium</i>		<i>Pestalotiopsis</i>				Hyphomycete			
		sp. 1	sp. 2	sp. 1	sp. 2	sp.	sp. 1	sp. 2	sp. 3	sp. 4		Others ^a			
Field	Egg	10		0	6	3	1	1	1	0	1	2	0	7	13
	Egg	10		7	3	3	0	1	1	1	1	0	2	3	10
	Larva	4		3	1	4	1	1	1	1	1	0	0	3	10

^a The fungal species that were isolated from only one leaf roll are included here

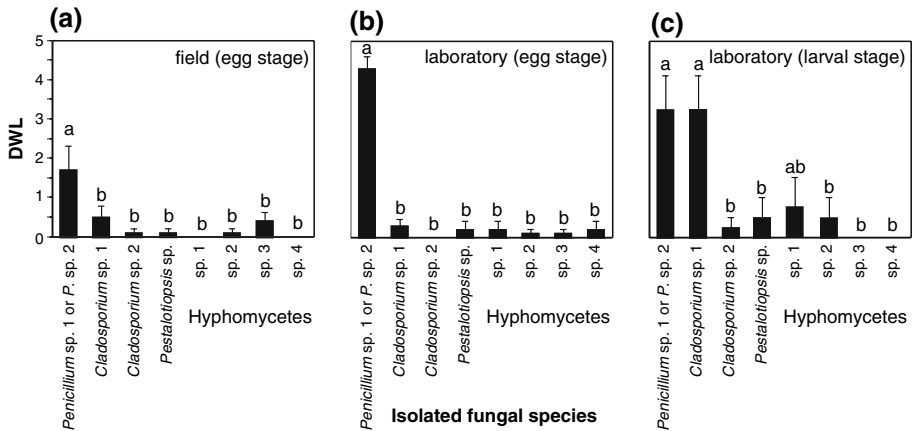


Fig. 2 Mean \pm SE of dominance within a leaf roll of each fungal species isolated from three types of leaf rolls of *E. lespedezae*. **(a)** Leaf rolls at the egg stage collected in the field ($n = 10$). **(b)** Leaf rolls at the egg stage constructed in the laboratory ($n = 10$). **(c)** Leaf rolls at the larval stage constructed in the laboratory ($n = 4$). Different letters indicate significant differences between fungal combinations (Tukey–Kramer test; $P < 0.05$)

Although soluble sugar was consumed dramatically in unsterilized leaf pieces, the decrease in soluble sugars was not so dramatic in gas-sterilized and surface-sterilized leaf pieces (Table 3). Among the unsterilized and surface-sterilized leaf pieces, those inoculated with *P. sp. 1* or *P. sp. 2* did not show greatly different soluble sugar content than uninoculated leaf pieces. Among the gas-sterilized leaf pieces, those inoculated with *P. sp. 1* or *P. sp. 2* had a slightly higher soluble sugar content than uninoculated leaf pieces, suggesting that some of the soluble sugars that disappeared were not decomposed by fungi.

No distinct changes in nitrogen content were detected in the three treatments (Table 3). The presence of *Penicillium* species did not affect the nitrogen content.

Discussion

Two *Penicillium* species were isolated from both mycangia of females and leaf rolls of *E. lespedezae*. Either *P. sp. 1* or *P. sp. 2* was isolated from 80% of females, and they were the most dominant fungal species in leaf rolls, especially at the egg stage, similar to results for *E. splendidus* (Takabe 2004). These results suggest that *Penicillium* fungi potentially affect the performance of early-instar larvae. The number of insect samples used in this study for isolation of fungi was small, and there is a possibility that another fungal species is found from mycangia of *E. lespedezae*. At least, however, it is evident that mycangial fungi of *E. lespedezae* have not been selected into one species within a population. Isolation of more than two fungal species from a symbiotic insect species was also reported in bark/ambrosia beetles (Paine et al. 1997) and fungus-growing termites (Aanen et al. 2002). In these cases, several fungal species may be maintained within an insect species because these fungi have similar function to symbiotic insects and fitness of insects does not differ among individuals with different fungal species.

Table 3 Residue content (mean \pm SE) of each chemical component in leaf pieces of *Lespedeza cyrtobotrya* before and after the inoculation experiment

Leaf Treatment	Experimental timing	Inoculated fungus	Lignin (mg/g)	Polysaccharides (mg/g)	Soluble sugar (mg/g)	Nitrogen (mg/g)
Untreated (epi ^a + endo ^b)	Before experiment	–	391.1 \pm 16.9	n.d.	24.39 \pm 0.05	n.d.
	After experiment	<i>Penicillium</i> sp. 1	376.8 \pm 7.5	104.4	1.07	19.4
		<i>Penicillium</i> sp. 2	366.5 \pm 11.7	90.4	0.38	18.7
Surface sterilized (endo)	Before experiment	Uninoculated	366.1 \pm 11.9	88.8	0.28	18.7
	After experiment	–	456.4	199.6	20.41	n.d.
		<i>Penicillium</i> sp. 1	366.1 \pm 3.5	131.6	7.57	24.8
Gas-sterilized (fungus free)	Before experiment	<i>Penicillium</i> sp. 2	371.6 \pm 10.3	124.7	8.01	24.9
	After experiment	Uninoculated	388.7 \pm 15.1	116.9	6.60	24.2
		–	421.6	173.0	19.20	23.2
	Before experiment	<i>Penicillium</i> sp. 1	360.1 \pm 9.6	114.4	10.76	26.5
	After experiment	<i>Penicillium</i> sp. 2	350.2 \pm 13.0	86.9	12.88	24.3
		Uninoculated	411.2 \pm 16.5	127.9	9.41	26.8

^a epiphyte, ^b endophyte

n.d. = no data

Table 4 Effects of inoculated fungal species and leaf treatment on lignin decomposition

Source	df	MS	F	P
Inoculated fungal species	2	73.958	0.351	0.72
Leaf treatment	1	95.592	0.453	0.53
Fungal species × treatment	2	275.30	1.31	0.34
Error	6	210.90		

While only *Penicillium* fungi dominated on the leaf roll at the egg stage, *Cladosporium* sp. 1 and hyphomycete sp. 1 were isolated as frequently as *Penicillium* fungi from leaf rolls at the larval stage. This differences in the species of fungi and frequency of isolation with age of leaf rolls may be explained by the change in chemical component of the leaf roll through decomposition by fungi. It was reported that fungal species on freshly fallen leaves were different from those on decomposing leaves (Osono 2002). How *Penicillium* fungi influence the fungal succession on leaf rolls and how fungal succession influence the larval survival will be needed to study to reveal the role of mycangial fungi.

The mycangial fungi seldom decomposed lignin and polysaccharides compared to other epiphytes and endophytes of leaf rolls. Further, soluble sugar contents of leaves were not enhanced by mycangial fungi. These suggest that the role of mycangial fungi is not to improve the nutritional value of leaf rolls by decomposing indigestible lignin and polysaccharides. As is the case in some leaf-cutting ants (Abril and Bucher 2002), the symbiosis of the weevil with the mycangial fungi differs from other insect-fungus symbioses in which the fungi contribute to decomposition of lignin or cellulose (Martin and Martin 1978; Kukor and Martin 1983; Watanabe et al. 1998).

Another considerable contribution of the fungus to the fungus-insect symbiosis is to provide antimicrobial activity against pathogenic or toxic microbes (Evans 1989; Kendrick 2000; Martin et al. 2005). An antimicrobial effect of mycangial fungi against harmful fungi has been reported in bark beetles (Bridges and Perry 1985). Because *Penicillium* species were reported to produce antifungal or antibacterial compounds (Domsch et al. 1993; Yamaji et al. 1999; Kendrick 2000), *P. sp. 1* and *P. sp. 2* may improve their survival rate by protecting larvae from harmful fungi. Further, *Penicillium* species were reported not to have harmful effect on fly larvae although other sympatric fungi such as *Aspergillus* and *Alternaria* species inhibited larval growth (Rohlf's et al. 2005). This harmless nature of *Penicillium* for insect larvae may be one of the reasons that they are selected as mycangial fungi of *Euops* weevils.

Although the main role of the mycangial fungi was not clear, our results suggest that the mycangial fungi do not accelerate the decomposition of lignin or polysaccharides, despite the expected advantages. Further studies of the antagonistic relationships between mycangial fungi and other microbes in leaf rolls will be needed to reveal the benefits gained by the weevil from the symbiosis with mycangial fungi.

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