Occurrence of an *Ephelis* Fungus on Ishigaki Island and Observations on its Epiphytic Association with Host Grasses

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Abstract

Distribution of *Ephelis*-infected grasses on Ishigaki, and the frequency at which infected plants were present, were observed over a three year period. Sites with infected plants were most commonly wet areas that had been undisturbed for many years. The frequency of infection varied widely between grass species. In three of the fifteen grasses found to be hosts to this fungus, infected plants were present at many sites, while in other species, infected plants were present at only one or two sites. Ephelis infection of three of the grasses was found in only one plant. The number of infected tillers at each site was typically low with apparently a random distribution, and no evidence of spread from sites of infection. Infected plants were characterized by stromata that covered the inflorescence with dense mycelial growth, and in most grasses, by hyphae on the surface of the leaf blades. Infected plants typically showed both infected and *Ephelis*-free tillers. Examination of infected plants of 10 species revealed that the *Ephelis* fungus was essentially an epiphyte, with hyphae in vegetative tillers being located on the surface of the stem apex, including primordial leaves. Hyphae on the leaf blades were also apparently confined to epiphytic growth. Possible mechanisms for infection, the ecological and economic significance of Ephelis infection on Ishigaki, and areas for future research are outlined.

Key words : Clavicipitaceae, Ephelis, epiphyte, grasses

Introduction

Fifteen species of grasses growing on Ishigaki Island are hosts to a fungus forming a stromata which envelop the inflorescence $^{11,12,13)}$. The black appearance of the stromata, and the sterilization of infected tillers, have given rise to the descriptive name "black choke". Leaves of some species infected with this fungus are covered with white hyphal growth. No sexual stage has been observed, but the fungus on all the grass species has been identified as belonging to the anamorphic genus *Ephelis* from the presence of characteristic elongate narrow conidia¹³⁾. Information from conidial morphology and sequence analysis of the ribosomal DNA ITS region in Ephelis spp. obtained from different hosts and locations indicate strongly that all the grasses are infected with the same species¹⁰⁾. In 1904 an *Ephelis* species was reported on Miscanthus tinctorius and Paspalum thunbergii in Japan and given the name E. japonica Hennings⁶⁾. As the description of conidia and stromata of E. japonica was similar to that observed in *Ephelis*-infected grasses in Japan in recent years, this may be the correct name to use for the *Ephelis* fungus present in Ishigaki and other locations in Japan. This paper reports on the distribution of Ephelis-infected grasses on Ishigaki Island and the frequency at which they were found. Also reported are the results of a study that assessed the nature of the association between the *Ephelis* fungus and host grasses.

Materials and methods

1) Survey of Ephelis-infected grasses in Ishigaki

The presence on Ishigaki Island of grasses with *Ephelis* infection stromata on inflorescences and/or white hyphal growth on leaves was recorded over a period of three years.

2) Study of the nature of the Ephelis/grass association

During April 2-8 1999, *Ephelis*-infected plants of 10 species were identified at previously located sites. The distribution of the infected plants at each site was observed, and the plants sampled for examination. Appearance of stromata was noted, as

were the presence and location of hyphae on leaves. The presence or absence of *Ephelis* stromata on each reproductive tiller of individual plants was also noted.

Hyphal distribution within infected plants was noted. Stems of reproductive tillers were slit open, some internal tissue removed, placed in a drop of aniline blue stain on a microscope slide, covered, heated to boiling, and examined using a compound microscope for the presence of hyphae. Epidermal strips were removed from the leaf blades and sheaths and similarly examined for the presence of intercellular hyphae. The presence of hyphae in stem apices was assessed by dissecting these from terminal portions of non-reproductive tillers, staining with aniline blue, and examining with a compound microscope.

Results

1) Survey of Ephelis-infected grasses in Ishigaki

The number of sites where *Ephelis*-infected plants were observed varied markedly for each grass species (Table 1). Ephelis infection occurred at many sites in three grasses; Chrysopogon aciculatus, Digitaria violascens and Paspalum scrobiculatum, but only in *P scrobiculatum* were infected plants common at each site. Ephelis infection was recorded at only two sites in three of the 15 grasses (Brachiaria mutica, Chloris barbata, Imperata cylindrica var. koenigii), and the number of infected plants was low. The remaining nine species were found only at a single site. In three grasses, (Cynodon dactylon, Eriochloa procera, P. urvillei), Ephelis infection was observed only on a single plant. Sites with infected plants were most commonly wet areas that had remained undisturbed for many years. Forest was the least likely habitat where Ephelis-infected grasses could be found, with only one species, Leptochloa panicea confined to one small site, being located, in spite of intensive surveys conducted over a threeyear period. Stromata were observed on 14 of the species, but not on Cynodon pletostachyrus, a grass that does not flower in Ishigaki. Leaf infection was observed for 13 of the grasses, with infection being particularly conspicuous on B. mutica and C. pletostachyrus (Table 1).

Grass species	Subfamily	Tribe
Brachiaria mutica (Forsk.) Stapf (Paragrass) ^{a,c,e)}	Panicoideae (Panicodae)	Paniceae
<i>Chloris barbata</i> Swartz. (Purple top chloris) ^{a,c)}	Chloridoideae	Chlorideae
Chloris divaricata R. Br. ^{a,c)}	Chloridoideae	Andropogoneae
<i>Chrysopogon aciculatus</i> (Retz.) Trin. ^{a,b)}	Panicoideae (Andropogonodae)	Andropogoneae
Cynodon dactylon (L.) Pers. (Bermudagrass) c,d,f)	Chloridoideae	Chlorideae
<i>Cynodon pletostachyrus</i> (K. Schm.) Pilger ^{a,c,e)} (Giant star grass)	Chloridoideae	Chlorideae
Digitaria eriantha Steud. (Pangola grass) ^{c)}	Panicoideae (Panicodae)	Paniceae
Digitaria violascens Link. ^{a,b)}	Panicoideae (Panicodae)	Paniceae
<i>Eriochloa procera</i> C. H. Hubb. ^{c,d)}	Panicoideae (Panicodae)	Paniceae
<i>Imperata cylindrica</i> (L). Beauv. var. <i>koenigii</i> (Retz). Durand et Schinz (Needlegrass) ^{a,c,d,f)}	Panicoideae (Andropogonodae)	Andropogoneae
<i>Leptochloa panicea</i> (Retz.) Ohwi ^{a,c,d)}	Chloridoideae	Chlorideae
<i>Panicum crus-galli</i> , Beauv. var. <i>praticola</i> Ohwi ^{c)}	Panicoideae (Panicodae)	Paniceae
Panicum repens L. ^{a,c)}	Panicoideae (Panicodae)	Paniceae
Paspalum scrobiculatum G. Forst ^{a,b)}	Panicoideae (Panicodae)	Paniceae
Paspalum urvillei Steud. ^{c,d)}	Panicoideae (Panicodae)	Paniceae

 Table 1. Grass species with Ephelis present on Ishigaki island, their frequency of infection, and symptoms

a) examined in April 1999 study

b) present at many sites

c) present at just 1 or 2 sites

d) very rare

e) leaf blades conspicuously colonized

f) leaf blades not colonized

2) Study of the nature of the Ephelis/grass association

Ten species of grasses with Ephelis were identified and examined in the April 1999 survey. Several Ephelis-infected plants were examined for each species except I. cylindrica, for which only a single tiller with a stroma was found. Frequency of infection of Ephelis-infected plants at each site, with just two exceptions, was typically low, with perhaps only 1% or less of tillers showing evidence of infection. The first exception was an area of mown turf that had been established for over twenty years, and where many tillers of C. aciculatus were infected with *Ephelis.* The second exception was with P. scrobiculatum where infected tillers were widespread at several sites. Distribution of infected plants within sites was apparently random, with no evidence of spread from a focal point.

3) Stromata characteristics

Stromata in all the grasses were initially comprised of dense white hyphal growth covering the developing seeds, later becoming black (Plate 1). Hyphal growth was absent below the basal branches of inflorescences. Numerous narrow, elongate conidia were present on the stromata. No perithecia were observed.

4) Leaf colonization

Hyphae were observed on the leaf blades of 8 of the 10 Ephelis-infected grasses examined in the April 1999 survey. In two grass species, B. mutica and C. pletostachyrus, hyphal growth was conspicuous, and infected plants could be readily detected. Hyphal growth on leaves of C. barbata and D. violascens was sparse and difficult to detect without the use of a stereo dissecting microscope. No hyphae were observed on the leaves of C. divaricata and I. cylindrica. Hyphal distribution on leaves was noted for the two grasses that were the most conspicuously colonized, B. mutica and C. pletostachyrus (Plate 2). Hyphae were confined almost entirely to the blades; occasionally, sparse hyphae were also present on the sheath, located in close proximity to the ligular zone. Hyphae were never seen on the inner surface of the sheath adjacent to the stem. Hyphae on blades were present as dense, longitudinally orientated masses. On the upper side of the blades they were located between the ridges containing the vascular bundles. Numerous elongate narrow conidia were produced by hyphae on the leaf blades. The hyphae appeared confined to the surface of leaves, with no hyphae detected in the mesophyll of the leaf blades or sheaths. Leaves of infected tillers of these two heavily colonized grasses were conspicuously shorter than those of *Ephelis*-free tillers.

5) Stem apex and stem infection

Hyphae were observed on the surface of stem apices, including primordial leaves, of all nine of the *Ephelis*-infected grass species for which nonreproductive tillers were available for examination. Short hyphae apparently growing intercellularly within young leaf blades were occasionally observed. Hyphae were not seen in tissues from within hollow stems of reproductive tillers, or in tissues removed from nodes of these tillers.

6) Distribution of Ephelis infected tillers within plants

Ephelis-infected plants typically had both infected and healthy tillers. In the stoloniferous grasses *B. mutica* and *C. pletostachyrus*, *Ephelis* infection was often confined to just a few tillers arising from one part of the branching network of stolons. In nonstoloniferous grasses, infected tillers appeared to be randomly distributed. In contrast, all the leaves of the infected tillers, with one exception, were colonized by hyphae. The exception was a tiller of *B. mutica* where the four youngest leaves were heavily colonized by epiphytic hyphae, the fifth was sparsely colonized, while the two oldest leaves had no visible hyphae.

Discussion

In this study it was found that the frequency of *Ephelis* infection for each grass species, both in terms of the number of sites located, and the frequency at which infected plants were infected within sites varied widely. It is certain that there are more sites where *Ephelis*-infected grasses are present than those found in this survey. However, the study provides a good indication of the frequency



Plate 1. Stroma on Brachiaria mutica inflorescence



Plate 2. Hyphae on Cynodon pletostachyrus leaves

of infection of grasses in Ishigaki. In particular, it seems certain that the occurrence of *Ephelis* infection of forest grasses is very rare as these grasses were subjected to extensive surveys over a period of three years. The most common sites were areas of grassland that had remained undisturbed for many years, with few infected plants found in recently established paddocks. However, no infected plants were detected in close proximity to the sea, even in grasslands established for a long period of time. Within sites, infection was typically very low, and no sites were found at which the majority of the tillers were infected.

All of the host grasses were C4 species, and representatives of two sub-families, and three tribes (Table 1). The general appearance of the host species varied markedly, ranging from small-leaved plants that could persist under turf management to tall broad-leaved types. The relationship of the *Ephelis* fungus to all 10 species of grass examined was essentially that of an epiphyte. Hyphae were located on the surface of the stem apices from where they can colonize the developing leaves and inflorescences, and the axillary buds which give rise to new tillers. The epiphytic nature of the association was also apparent by the dense covering of hyphae present on the surface of leaf blades of some grass species. This epiphytic relationship was similar to that reported for other Clavicipitaceous fungi which have Ephelis conidia, belonging to the genera Balansia and Atkinsonella^{9,17)}.

The epiphytic relationship between the *Ephelis* species and the grasses it colonizes on Ishigaki was illustrated by a comparison of the distribution of hyphae on leaves and reproductive tillers with the hyphal distribution of the endophytic *Epichloë/ Neotyphodium* fungi. In leaves, hyphae of these endophytic fungi were typically most concentrated in the sheaths, while *Ephelis* hyphae were almost entirely associated with blades. In the blades of *Epichloë/Neotyphodium*-infected leaves, the hyphae, if present, were mainly concentrated in the basal, oldest, region⁷). In *Ephelis*-infected plants the hyphae tended to be uniformly located along the blades. Hyphae were abundant in the stem of reproductive tillers of *Epichloë/Neotyphodium*-infected plants, and in the swollen nodes of these tillers. In *Ephelis*infected plants, hyphae were either apparently absent in these tissues or at least present at low concentrations. The efficiency of colonization of axillary buds was typically high in *Epichloë/ Neotyphodium* associations, with endophyte-free tillers being seldom observed. In contrast, healthy tillers were typically present on the *Ephelis*-infected plants examined.

This distribution of infected grasses within sites provides clues as to how plants become systemically infected. One possible mechanism for infection is the penetration of leaves or crowns by hyphae from germinating conidia. Such a mechanism is unlikely, as for systemic infection to occur hyphae would have to penetrate through the densely packed layers of leaf sheaths and then colonize the stem apex. The sparse distribution of infected plants in natural populations of B. mutica and C. pletostachyrus, species with very high concentrations of conidia being produced on the surface of leaves, provides evidence that this is not the mechanism of infection. It is unlikely that infection would occur from *Ephelis* within the soil as Clavicipitaceous fungi are in nature obligate biotrophs¹⁷⁾. Transmission through the seed appears to provide the most likely explanation of how plants infected. become systemically Vertical seed transmission has been reported for a symptomless Ephelis sp. found in association with Danthonia $spicata^{8}$. During the vegetative growth of the plant, the fungus inhabits only the external surfaces of the shoot apex and adjoining rudimentary leaves. However, with the change of the host to the reproductive phase, the fungus becomes incorporated into the developing ovary and ovule, penetrating the microphyle to the megagametophyte wall. Hyphae enter the embryo sac at early embryogenesis and remain there during normal embryo development. Since hyphae, however, invade the surface of the embryonic shoot apex before it is enclosed by the coleoptile rim, as the case of in Neotyphodiuminfected plants, infection of the next generation had already occurred before seed maturation. Following germination, hyphae from the seedling apex may colonize the stem apex of each new tiller. However, vertical seed transmission generally seems unlikely

in grasses infected with the *Ephelis* species present in Ishigaki as developing inflorescences of *Ephelis*infected grasses in Ishigaki became heavily colonised by hyphae, preventing seed development and dispersal. A possible exception is *P. scrobiculatum*, where stromatal development did not always result in complete colonisation of inflorescences. This is one of the two species of grasses where the incidence of *Ephelis* infection was relatively high at some sites.

Another possible mechanism for new infections with Ephelis is horizontal transmission. With this form of transmission, infection might occur by hyphal penetration of stigmata of neighboring uninfected plants, and subsequent growth into developing ovaries and ovules. Hyphae would invade the developing embryo and colonize the surface of the shoot apex, as in the case of the *Ephelis* of *D. spicata*. Horizontal infection via stigmata occurs in other Clavicipitaceous fungi forming associations with grasses⁹⁾. With *Claviceps* species, infection following stigmata penetration is confined to the developing seed and gives rise to fungal sclerotia. Horizontal infection with Balansia obtecta, in contrast, produces viable seeds which give rise to systemically infected seedlings. A further example is Epichloë typhina, in which, as in *Ephelis* infection of grasses in Ishigaki, stromatal development results in sterilization of the host plant²⁾. The relatively low incidence of *Ephelis*infected grasses on Ishigaki indicates that if horizontal transmission does occur it is either a rare occurrence, or that germination of infected seeds and subsequent growth of infected seedlings are adversely affected. and few plants become established. A complicating factor is that in other Clavicipitaceous fungi which form systemic seedborne infections with grasses following horizontal transmission, hyphae from ascospores and not conidia, penetrate into the stigmata^{2,3,9}. To date, no perithecia have been observed on Ephelis-infected grasses present in Japan. One possibility is that the dark hyphal masses which develop as stromata age may function as sclerotia, and germinate to produce apothecia and perithecia as occurs with *Claviceps* spp. and B. obtecta.

Factors other than the frequency of infection may

influence the incidence of *Ephelis*-infected plants. It is possible that in some grasses Ephelis infection gives them a competitive advantage. This may result from enhanced tolerance to insect pests. This hypothesis is supported by insect feeding trials which have revealed the existence of insect feeding deterrence in Ephelis-infected grasses in two grasshoppers, Oxya yezoensis¹⁴⁾, and Aiolopus thalassinus (Takahashi, unpubl. data), and an armyworm, Mythimna (Pseudaletia) separata (Takahashi, unpubl. data). Further evidence of Ephelis-induced insect feeding deterrence is by the higher incidence of a mealy bug observed on Ephelis-free than on Ephelisinfected Digitaria eriantha growing in a glass-house. Over time the incidence of infected perennial plants is likely to increase, even in the absence of fresh infection. In long-lived perennial grasses such as C. aciculatus and P. scrobiculatum, infected plants increase in size, either from growth of stolons in the case of C. aciculatus, or from increases in the number of tillers as in the case of P. scrobiculatum. Freestanding plants with enhanced competitive advantage relative to non-infected plants, would arise from the death of segments of stolons, and from the fragmentation of the crowns of non- stoloniferous grasses, increasing the frequency of occurrence of infected plants.

Conversely, the low incidence of Ephelis infection in most of the host species suggests that infection is not beneficial to the host plant or may even have a detrimental effect on plant survival and persistence. The growth of epiphytic hyphae on leaf blades may cause stress to host plant. Nutrients are required for extensive epiphytic hyphal growth to occur on blades, and these hyphae must receive a continuous supply of water to prevent desiccation. A possible mechanism to ensure a supply of nutrients and water is that the hyphae modify the cuticle and epidermal cells of leaves, allowing movement of nutrients and water from the apoplast to the hyphae where they will be absorbed. Disruption of the cuticle has been reported in D. spicata plants infected with the symptomless, seed-transmitted Ephelis species, associated with hyphal colonization of the internal surfaces of ovaries and ovules⁸⁾. Modification of epidermal cells and cuticle has also been reported in

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grasses infected with the Clavicipitaceous fungi *Myriogenospora atramentosa* Atk. and *B. epichloë* (Weese) Diehl¹⁵); and *E. amarillans*¹⁶). Water loss associated with increased permeability of the cuticle and epidermal layer may increase the susceptibility of *Ephelis*-infected plants to drought, and thus provide an explanation why infected grasses are found mainly in wet areas where the effects of water loss will be less damaging.

Infection of grasses with *Ephelis* appears to have little ecological or economic significance in Ishigaki. The typically low incidence of infected plants indicates a lack of a competitive advantage over noninfected plants, even though the presence of the fungus may confer protection against some insects. It is not known if grasses infected with this Ephelis species contain alkaloids that are toxic to grazing animals, as are grasses infected with other Clavicipitaceae fungi. These alkaloids have been extensively studied in *C*. $purpurea^{5}$, and in Neotyphodium-infected L. perenne, F. arundinacea, and F. pratensis¹⁾. Their presence has important implication for the health of grazing animals⁴⁾. It seems unlikely that even if such alkaloids were present in Ephelis-infected grasses on Ishigaki the concentrations would reach harmful levels as the incidence of infected plants is low.

The findings of this study point to areas requirng furthur investigations as follows : First, is the Ephelis fungus transmitted through vertical and/or horizontal transmission? A second area of research is the presence of alkaloids in Ephelis-infected plants. Do all of the grass species contain the same alkaloids? The findings may contribute to understanding of the evolution of biotrophic Clavicipitaceae fungi. It is also possible that infected plants may contain secondary metabolites of pharmacological importance. A third area for research is the effect of epiphytic hyphae on the cuticle and epidermal layer of leaves and the developing inflorescence. Finally, there exists within the graminicolous Clavicipitaceae fungi, a continuum in the nature of their association with host grasses, ranging from infection being confined entirely to the seed resulting in seed death, to associations in which the fungus grows as a symptomless seed-transmitted endophyte. More detailed information on the Ishigaki

Ephelis should contribute to the international effort to better understand this continuum of association and how these associations may have evolved.

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Ephelis 属菌の石垣島における発生および寄主植物との表生関係

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摘 要

Ephelis sp. はバッカク菌科 Balansiae 族に属する 糸状菌の一種である。1997 年から 1999 年の3年 間にわたり、石垣島において本菌の分布状況、お よび個々の草での感染生態を調査した。結果の大 要は以下の通りである。

1) 本菌の感染は次に示すイネ科 10 属 15 草種 において認められた。 すなわちパラグラス Brachiaria mutica (Forsk.) Stapf 、シマヒゲシバ Chloris barbata Swartz.、ヒメヒゲシバ Chloris divaricata R. Br.、オキナワミチシバ Chrysopogon aciculatus (Retz.) Trin.、バミューダグラス Cynodon dactylon (L.) Pers.、ジャイアントスターグラス Cynodon pletostachyrus (K. Schm.) Pilger、パンゴラ グラス Digitaria eriantha Steud.、アキメヒシバ Digitaria violascens Link. 、ムラサキノキビ Eriochloa procera C. H. Hubb.、チガヤ Imperata cylindrica (L). Beauv. var. koenigii (Retz.) Durand et Schinz、イ トアゼガヤ Leptochloa panicea (Retz.) Ohwi、イヌ ビエ Panicum crus-galli, Beauv. var praticola Ohwi, ハイキビ Panicum repens L.、スズメノコビエ Paspalum scrobiculatum G. Forst、タチスズメノヒ 工 Paspalum urvillei Steud.。

2) 感染植物は長年にわたって不耕起状態にあ る湿った場所に多くみられた。感染株の頻度は草 種によって以下のように大きく異なった。すなわ ちオキナワミチシバ、アキメヒシバ、スズメノコ ビエの3草種での感染は島内各所で観察され、特 にスズメノコビエでの感染率は高かった。パラグ ラス、シマヒゲシバ、チガヤは2カ所で確認され、 残りの9草種での感染はそれぞれ1カ所のみであっ た。特にバミューダグラス、ムラサキノキビ、タ チスズメノヒエの感染株はそれぞれ1株ずつのみ であった。ジャイアントスターグラスを除いて残 りの14草種でミイラ穂症状が確認された。なお、 ジャイアントスターグラスは日本では開花しな い。葉上への感染は13草種で確認され、その病 徴は特にパラグラス、ジャイアントスターグラス で著しかった。

3)子座ははじめ種子を覆う濃い白い菌糸から 成り、やがて黒化する。穂の最も下の小枝より下 では菌糸の伸長は認められなかった。子座上には 多数の細長い分生子が存在するが、子のう殻は観 察されなかった。

4) 1999 年 4 月に観察した 10 草種のうち 8 草 種で葉上に菌糸が認められた。パラグラスおよび ジャイアントスターグラスでの菌糸の生育は特に 著しく、発見は容易であった。シマヒゲシバおよ びアキメヒシバ葉上での菌糸の生育は悪く、肉眼 では観察が困難であった。ヒメヒゲシバおよびチ ガヤでは葉上での菌糸の生育は認められなかっ た。最も病徴のはげしいパンゴラグラスおよび ジャイアントスターグラスの2 草種で葉上の菌糸 の分布を観察した。菌糸の発育はほぼ葉上に限ら れているが、時には葉鞘にもみられた。しかし葉 鞘の茎に接する内側には菌糸はまったく観察され なかった。葉上の菌糸は長軸に沿って高密度で存 在し、葉表面の菌糸上には多数の細長い分生子が 形成されていた。菌糸は葉上に限られ、葉あるい は葉鞘の内部では観察されなかった。本菌に感染 した葉は、健全葉に比較してはなはだ短かった。

5) Ephelis 感染植物のうち、供試可能な感染茎 が得られた9草種について観察した結果、すべて の草種で葉原基および茎頂部表面に菌糸が観察さ れた。若い葉身では、まれに菌糸のごく一部が細 胞間隙を伸長していた。非感染茎の茎および節内 部では菌糸は観察されなかった。

6) Ephelis 感染株内には、感染茎と非感染茎が

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混在していた。匍匐茎型植物であるパラグラスお よびジャイアントスターグラスでは、感染茎は感 染部位から第2ないし第3分けつ茎までに限られ た。非匍匐型草種では、感染茎は株内にランダム に分布していた。これに対し、パラグラスを例外 として感染茎ではすべての葉表面で菌糸が伸長し ていた。パラグラスでは、感染茎の最も若い葉が 上面を厚い菌糸叢に覆われ、第5葉では菌糸がま ばらだったが、第1および第2葉では菌糸はまっ たく観察できなかった。