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Examination of Plant Biotrophy in the Scale Insect Parasitizing Fungus *Dussiella tuberiformis*

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Abstract

Dussiella tuberiformis is a member of the Clavicipitaceae (Ascomycetes) associated with the perennial bamboo grass *Arundinaria tecta*. *D. tuberiformis* is one of only a few fungi that are capable of infecting insects and plants. To gain a better understanding of how the infection process is accomplished we conducted a study of the physical association of fungus and plant and the *in vitro* growth parameters of the fungus. From the structural studies we found that: 1) *D. tuberiformis* is a necrotrophic pathogen of scale insects, infecting and fully degrading the body of the scale insect; and 2) the stylet of the scale insect remains in the plant and the fungus is nourished by nutrients that leak to the surface through the stylet. The *in vitro* studies on the fungus suggest that: 1) fungal stroma development is largely fueled by non-reducing sugars, such as sucrose; 2) perithecial development on stromata may be triggered by restriction of nutrients and moisture; 3) nutritional auxotrophies, such as the inability to utilize nitrates as nitrogen sources, may be among the features of *D. tuberiformis* that limit its free-living and

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plant destructive capacity, thus relegating it to plant biotrophy rather than saprotrophy or destructive plant parasitism. We proposed that the evolution of plant biotrophy in *D. tuberiformis* may have bypassed gradual evolution from destructive pathogen to non-destructive biotroph by the intermediate infection of scale insects. Through infection of scale insects the fungus was able to gain direct access to nutrients of the plant without eliciting defensive responses, representing an evolutionary short cut route to plant biotrophy.

Keywords: *Dussiella tuberiformis*, epibiont, Clavicipitaceae, nutritional requirements, perithecial development

1. Introduction

Many plant species, particularly grasses, commonly form symbiotic associations with epibiotic or endophytic species of Clavicipitaceae. Frequently, this relationship confers benefits to both plants and fungi (Bacon and White, 2000). Endophytic or epibiotic fungi benefit primarily by obtaining nutrients from their hosts, whereas infected plants may gain protection from insect and vertebrate herbivores through the toxic or deterrent effect of alkaloids synthesized by the fungus (Clay, 1988; 1990). Other benefits of fungus association for the plant include enhanced growth and increased competitive abilities (Clay, 1990; Brem and Leuchtmann, 2001). Moreover, it has been reported that these fungi may provide increased resistance to drought, pests and fungal pathogens (Clay, 1989; Bush et al., 1997; Yue et al., 2000; Brem and Leuchtmann, 2002; Schardl, 2001). The non-destructive biotrophies in these fungi have been proposed to originate from destructive parasitisms though a series of co-evolutionary changes in the hosts and fungi, culminating in associations that are mutually beneficial (Clay, 1988).

D. tuberiformis Pat. (=*Echinodothis tuberiformis* Atk.) is associated with the warm-season perennial grass *Arundinaria tecta* (Walt.) Muhl. (Bacon and White, 2000). The developmental morphology and mating system of this fungus have been previously studied (Atkinson, 1891; White, 1993). *D. tuberiformis* appears to infect plants and somehow extract nutrients from plants as an epiphyte, however, precisely how it gets nutrients has remained unknown (White, 1993). More recently it has been shown that some species of Clavicipitaceae, including *Hyperdermium bertonii*, that were believed to be exclusively plant parasites are also scale insect parasites (Sullivan et al., 2000). These fungi attack the scale insects (Coccidae; Homoptera) to gain access to nutrients emerging from the plant through the stylet wound. White et al. (2002) reported that *D. tuberiformis* is among the clavicipitalean fungi that employ this mechanism to obtain nutrients from plant hosts. This ecological mechanism for achieving plant biotrophy has received little attention in the

scientific literature. Such a mechanism may be a way that fungi that are insect parasites transition to plant parasitism and may be important in the evolution of many plant parasites. We examined *D. tuberiformis* to better evaluate its nutritional relationship to the grass host.

2. Materials and Methods

Isolation and culture

D. tuberiformis was isolated from infected *A. tecta* located in Walterboro, North Carolina, USA, along Interstate Highway I95 in January 2001. ATCC number is MYA-2810. Conidial stromata were excised from culms and cut into pieces approximately 3 mm in diameter. Tissue pieces were surface sterilized by soaking for 3 min with occasional agitation in 50% v/v commercial bleach (Clorox®). Tissue pieces were subsequently washed three times with sterile water. The tissue was then transferred to potato dextrose agar (PDA, Difco) and incubated for 5 weeks.

Developmental observations

Plants of *A. tecta* infected by *D. tuberiformis* were examined in January, 2001 for developing stages of the fungus. Free-hand sections were made of 35 culms just beneath stromata to determine if insect stylets, wounds, or other evidence of insect involvement could be identified.

Determination of developmental conditions in vitro

To infer nutrients that the fungus obtains from the plant, experiments were conducted to determine developmental responses under culture conditions.

Seed cultures: For the inoculum culture, mycelium was grown for 10 days in a shaker, in liquid MS (Murashige and Skoog, 1962) basal medium containing sucrose (30, 100, 200, 300, 400 g/l) alone or supplemented with Proflo (Traders Protein) (10 g/l) or yeast extract (3 g/l). A combination of sucrose or sorbitol, supplemented with Proflo and ammonium citrate (3 g/l) was also tested. The pH of the media was adjusted to 5.8 with 1 N KOH before autoclaving.

Agar cultures: The medium used in carbon and nitrogen utilization studies was composed of MS basal medium containing different carbon sources (sucrose, dextrose, fructose, or sorbitol) alone or in combination with yeast extract (3 g/l) (Table 1). MS medium with sucrose or sorbitol (100 g/l) in combination with various nitrogen sources (ammonium citrate, ammonium acetate, ammonium phosphate, ammonium sulfate, ammonium nitrate, ammonium chloride,

calcium nitrate or various amino acids) were also tested (Tables 2 and 3). The pH of the media was adjusted to 5.8 before adding agar (20 g/l). All media were autoclaved except the amino acids that were filter sterilized.

One ml of seed culture was spread on sterile discs of cellophane dialysis membrane over the culture medium on agar plates. Each treatment was run in quadruplicate in total three times with all replicates fully randomized. The cultures were incubated in the dark at 24°C. After 5 weeks, individual colonies were removed from the dishes and dried in an oven at 70°C to a constant dry weight.

Table 1. Effect of carbon source on mycelial dry weight (mg) of *Dussiella tuberiformis* after 5 weeks incubation at 25°C.

Carbon source	Yeast extract (g/l)	30 g/l	100 g/l	200 g/l	300 g/l	400 g/l
Sucrose	0	64±2 qr*	101±5 o	174±7 jkl	255±6 hi	329±13 efg
	3	155±6 lm	90±6 op	774±24 a	517±73 bc	663±31 ab
Fructose	0	57±3 rs	78±4 pq	143±3 lm	152±10 lm	0
	3	145±8 lm	482±14 c	298±11 fgh	324±11 efg	0
Dextrose	0	58±2 rs	103±4 o	162±9 jklm	256±7 hi	284±8 fgh
	3	146±7 lm	222±15 ij	340±9 def	401±9 cde	486±10 c
Sorbitol	0	48±2 s	111±5 no	193±6 jk	274± 7 fghi	278±17 fghi
	3	133±6 mn	257±9 ghi	315±9 efgh	460±9 c	418±18 cd

*Mean±standard error. Means followed by the same letter are not significantly different according to LSD test at $p<0.05$.

Table 2. Growth as mycelial dry weight (mg) on MS basal medium, 100 g/l sucrose and nitrogen source.

Nitrogen source	0 g/l	2 g/l	5 g/l	10 g/l
Control	101±5 fgh*			
Ammonium citrate		623±17 a	303±30 b	179±17 c
Ammonium phosphate		0	293±12 b	367±15 b
Ammonium sulfate		159±7 de	111±14 fg	121±31 fg
Ammonium nitrate		99±13 ghi	117±4 fg	122±10 efg
Ammonium chloride		139±6 def	124±4 efg	119±2 fg
Calcium nitrate		83±5 hi	82±5 hi	187±7 cd
Ammonium acetate		0	0	0

*Mean±standard error. Means followed by the same letter do not differ statistically at $p<0.05$ according to LSD test.

Table 3. Growth as mycelial dry weight (mg) on MS basal medium with 100 g/l sorbitol and supplemented with various nitrogen sources.

Nitrogen source	0 g/l	2 g/l	5 g/l	10 g/l
Control	111±5 ef*			
Ammonium citrate		426±13 bc	496±30 ab	568±45 a
Ammonium phosphate		0	383±36 c	479±72 abc
Ammonium sulfate		183±25 d	122±8 e	126±13 e
Ammonium nitrate		78±5 h	85±8 gh	432±22 bc
Ammonium chloride		123±5 e	105±10 efgh	108±3 efg
Calcium nitrate		87±3 gh	93±7 fgh	87±2 fgh
Ammonium acetate		0	0	0

*Mean±standard error. Means followed by the same letter do not differ statistically at $p<0.05$ according to LSD test.

Table 4. Growth (mg dry weight) on MS basal medium and 100 g/l sucrose supplemented with amino acids.

Amino acid	0 g/l	2 g/l	5 g/l
Control	101 fg*		
L-Asparagine		411±39 a	330±50 b
L-Glutamine		220±16 de	0
L-Tryptophan		256±22 cd	76±28 g
L-Methionine		0	0
Glycine		158±16 e	272±17 bcd
L-Alanine		301±31 bc	272±19 bcd
Creatine monohydrate		161±7 c	0

*Mean±standard error. Means followed by the same letter do not differ statistically at $p<0.05$ according to LSD test.

For comparison of growth in different media, data were subjected to an analysis of variance (ANOVA) using the LSD procedure. Statistical differences were expressed at the 5% significance level.

Induction of perithecia in culture

To evaluate whether perithecial development is triggered by a reduction in nutrients supplied to stromata, the fungus was grown for eight weeks on 10 Petri dishes containing sucrose agar (MS salt, 3 g/l yeast extract, 200 g/l sucrose, 20

g/l agar) overlaid with cellophane sheets. Five of these were then transferred to water agar and five were transferred to fresh 20% sucrose agar; and all plates were incubated at 20°C for four weeks after which they were examined for evidence of perithecial development.

3. Results

Scale insect association

In early stages of stroma development we observed mycelium enveloping scale insect bodies (Fig. 1a). In later stages of development (Fig. 1b) the bodies of the scale insects were completely obliterated, except for the stylet that remained embedded in the plant tissues beneath the center of the stroma (Fig. 1e). The lower surface of the stromata, directly proximal to the plant epidermis, showed a central concave pink to red area centered on the stylet. This discolored part of the stroma appears to be where the stroma absorbs nutrients emerging to the surface of the plant through the stylet (Figs. 1c–e).

In vitro growth studies

Seed culture: MS basal medium with sucrose (100 g/l), proflo (10 g/l) and supplemented with ammonium citrate induced rapid growth of homogeneous mycelium (data not shown). This medium was used to produce standardized inoculum in all the treatments.

Agar cultures: Increasing the concentration of carbon source in the media enhanced growth as reflected by dry weights of the mycelia (Table 1). Maximum growth was achieved on media containing high concentrations (200 g/l) of sucrose supplemented with yeast extract. Good growth was also observed on media containing fructose, glucose (dextrose), and sorbitol (Table 1).

The highest mycelial dry weight was observed for the basal media with sucrose supplemented with ammonium citrate (2 g/l), although mycelial dry weight decreased as the concentration of ammonium citrate in the media was increased beyond that level (Table 2). Addition of ammonium phosphate significantly increased growth as concentration increased. Among nitrogen sources on basal media with sorbitol, biomass increased with an increase of ammonium citrate and ammonium phosphate in the media (Table 3).

No statistical differences were observed in mycelium grown in basal media with sucrose or sorbitol supplemented with ammonium sulfate, or ammonium chloride. The poorest growth was observed in media with calcium nitrate. Moreover, ammonium acetate inhibited growth of the fungi for all concentrations tested (Tables 2 and 3).

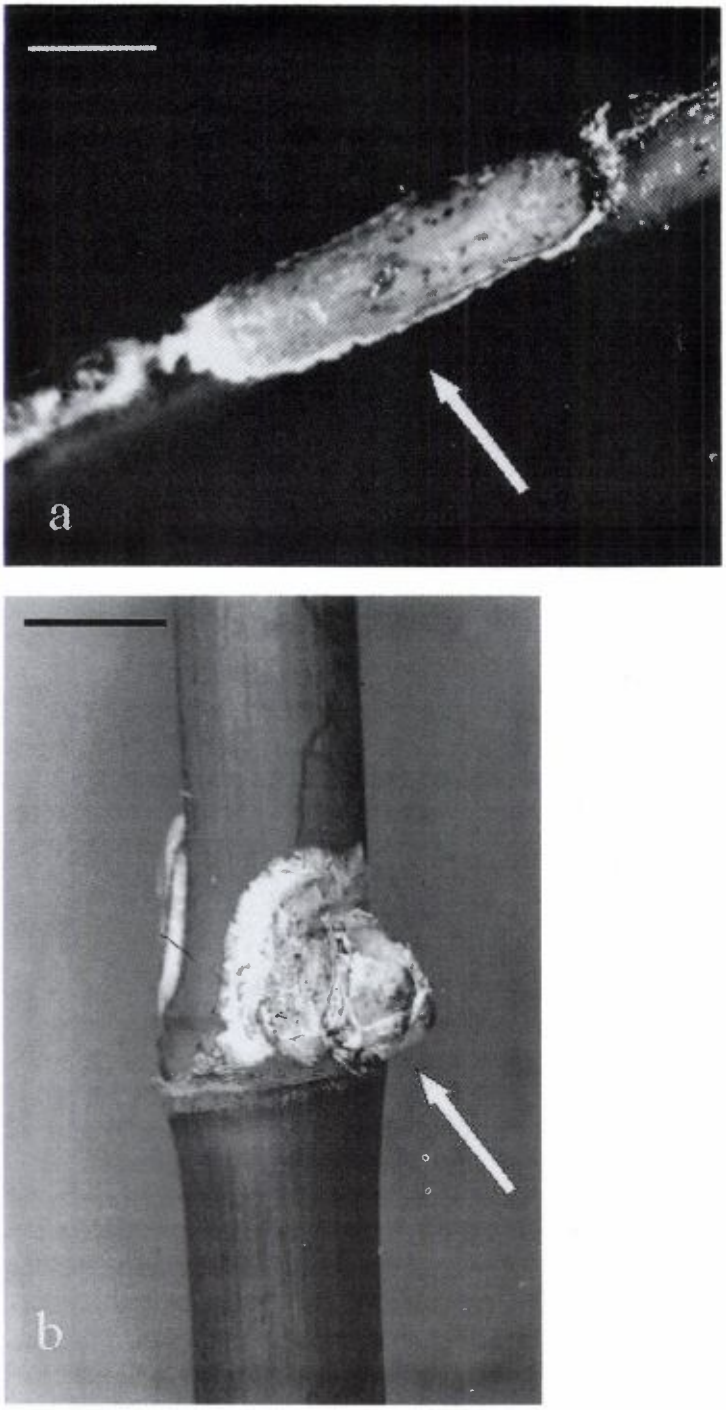

Figure 1. *Dussiella tuberiformis*. a) Scale insect with emerging mycelium; bar = 1 mm. b) Stroma on culm on plant (*Arundinaria tecta*); bar = 10 mm.

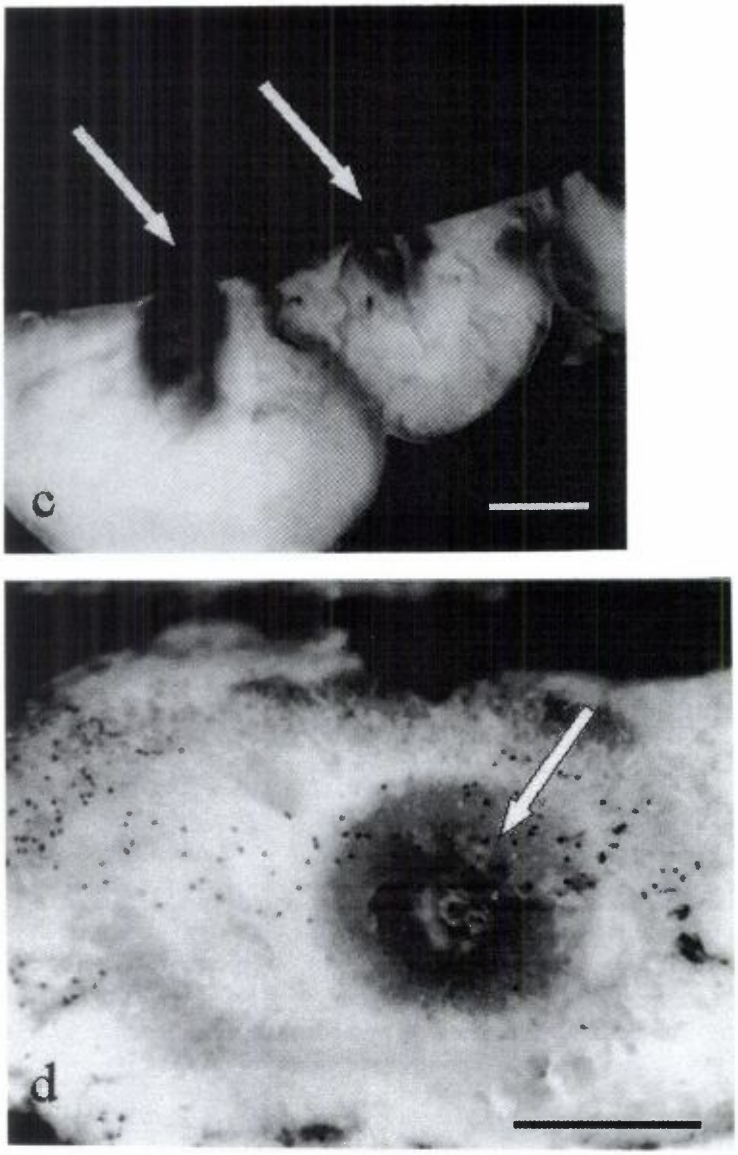

Figure 1. *Dussiella tuberiformis*. c) Section of stroma showing pigmented zone near stylet, bar = 2 mm. d) Reverse of stroma showing dark pigmented area near stylet; bar = 2mm.

Seven amino acids were tested as nitrogen sources (Table 4). The addition of (2 g/l) of asparagine to the culture media induced the highest growth rate followed by media that contained glycine or alanine. Tryptophan also stimulated biomass accumulation, but higher concentrations resulted in

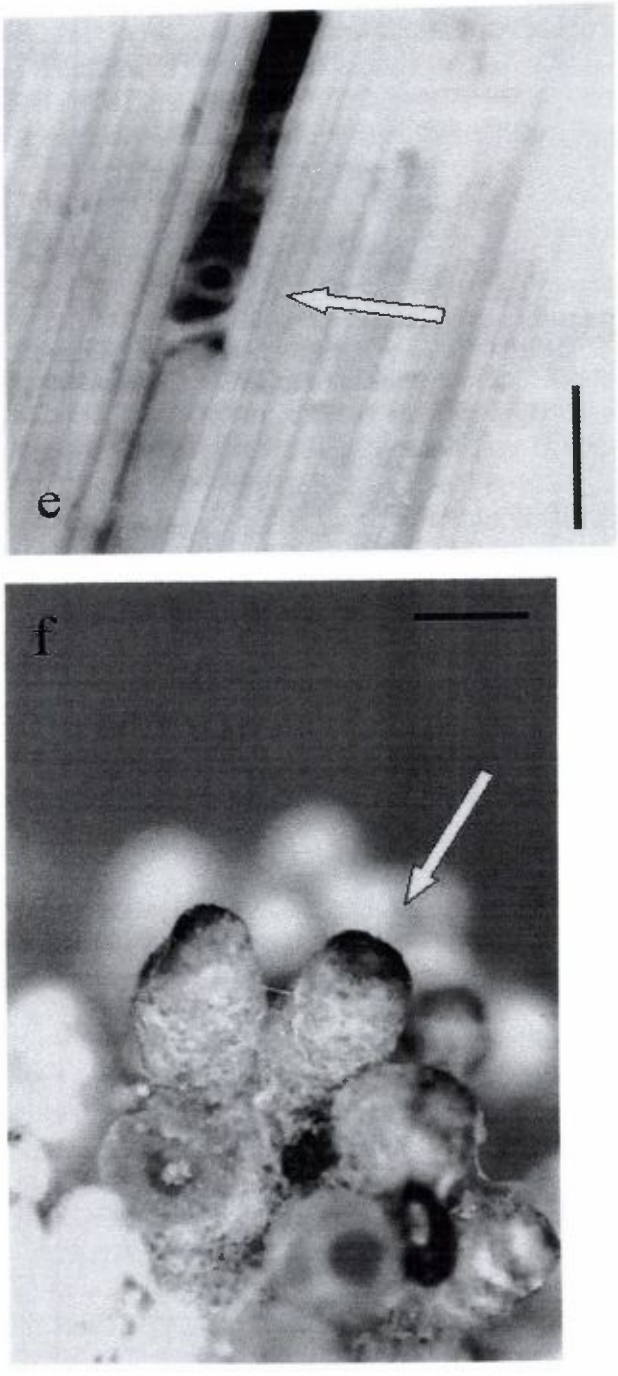

Figure 1. *Dussiella tuberiformis*. e) Cross section of stylet; bar = 10 µm. f) Mature perithecia from culture; bar = 0.4 mm.

decreased growth. *D. tuberiformis* appeared to be inhibited by methionine, and high concentrations of glutamine, or creatine monohydrate (Table 4).

Induction of perithecia in culture

Perithecial formation was observed to be most abundant on the cultures that were transferred to water agar for four weeks (Fig. 1f). Perithecia formed in dense clusters in regions of the colonies where the cellophane did not make contact with the water agar surface. We hypothesize that as moisture in the mycelium of the culture decreased, due to drying, a greater number of perithecia developed to cover the surface of the colony. Cultures that were grown exclusively on 200 g/l sucrose agar developed fewer perithecia on the colony surfaces. This suggested that restriction of moisture and nutrients from plants may play a role in triggering development of perithecia on the surface of the stroma.

4. Discussion

Scale insect association

Scale insects insert a stylet apparatus into the host plant to reach the nutrients that are transported in the phloem of the plant. *D. tuberiformis* infects the scale insect, then envelops and fully degrades it. Once the insect body is consumed, the nutrients from the phloem stream exude through the stylet to the surface of the plant. The sugars and other constituents of the phloem sap provide the nutrients that nourish the developing mycelial stroma on the surface of the plant. On the lower surface of the stroma at the interface of the plant and fungus, a pink to red discolored area is usually evident. This pink to red zone corresponds to a shallow cavity of liquid (phloem sap) that is often evident just beneath the developing stroma. This zone is apparently the site where the liquid nutrients from the plant accumulate and are absorbed by the stroma. A comparable ecology that involves parasitism of an insect followed by a biotrophic association with a plant host has previously been reported for other species. Sullivan et al. (2000) documented that *Hyperdermium* spp. are biotrophic epibionts of plant with early stages of infection that involve necrotrophy of scale insects. Similarly, Hywel-Jones and Samuels (1998) characterized three species of *Hypocrella* that infect scale insects and produce stromata on plants that are nourished from plant nutrients.

Sucrose as the primary carbon source for stroma development

The products of photosynthesis in higher plants, in the form of sucrose,

oligosaccharides, sugar alcohols, or amino acids, are highly integrated in a process involving solute transport from source to sink tissues. Sucrose, a non-reducing disaccharide, is the most common sugar translocated in the phloem sap, but sorbitol, a sugar alcohol, can also be translocated (Jennings, 1984). Because sucrose supported more mycelial growth at higher concentrations, we propose that sucrose is likely providing the majority of the carbon used by the fungus as it develops stromata on plants. The tan coloration and firm mycelial texture of the colonies produced using this medium are very similar to the color and texture of the mycelium of the stromata as they naturally develop on plants. This seems to give further support to the proposal that *D. tuberiformis* is adapted to develop its stromata using carbon in the form of sucrose. The optimal sucrose concentration was at 200 g/l concentration in the medium.

Auxotrophic dependence on the host plant

Biotrophic fungi are rarely destructive to their hosts, in part, because of their nutritional dependencies on their hosts. The slow growth on media supplemented only with MS salts and carbon sources indicates that auxotrophies may limit growth. The enriched media consisting of yeast extract supported a greater rate of growth and increased biomass accumulation. Yeast extract contains a mixture of carbon and nitrogen sources, including vitamins, which could be readily utilized in several pathways. Kulkarni and Nielsen (1986) reported that the clavicipitalean endophyte *Neotyphodium coenophialum* required thiamine and biotin. *D. tuberiformis* may have similar auxotrophies.

We observed that *D. tuberiformis* can utilize ammonium salts as nitrogen sources but could not use nitrate salts. Most pathogenic and saprophytic fungi can use nitrate as the sole or a significant source of nitrogen. The metabolic pathway for nitrate utilization involves first the reduction from nitrate to nitrite and subsequently to ammonia, with catalysis by several enzymes such as nitrate reductase, nitrite reductase and hydroxylamine reductase (Pateman and Kinghorn, 1976). *Dussiella* was not able to utilize calcium nitrate in either the presence of sucrose or sorbitol. It was previously reported that isolates of *Neotyphodium coenophialum* were able to utilize ammonium but not nitrate as a nitrogen source (Kulkarni and Nielsen, 1986). The incapacity to utilize nitrate may be due to lack of one or more enzymes in the nitrate reduction pathway (Pateman and Kinghorn, 1976). The amino acids asparagine, tryptophan, glycine and alanine supported good growth in both concentrations tested, while glutamine and creatine monohydrate supported growth at the lower concentration in the culture medium but were inhibitory at the higher concentration.

Our findings are in agreement with previous reports on amino acid utilization by other Clavicipitaceae (Kulkarni and Nielsen, 1986; Ferguson et al., 1993). Methionine is the only amino acid tested that did not support growth at either of the concentrations tested. Poor growth was observed also for *Balansia* spp. in the presence of methionine and tryptophan (Bacon, 1985). Lack of amino acid utilization could be due to an inability to uptake the nutrient, the lack of one or more enzymes in the catabolic pathway of the nutrient (Kulkarni and Nielsen, 1986), or an inability to transport specific amino acids (Ferguson et al., 1993). It is also possible that the concentration of certain amino acids, such as methionine, glutamine, and creatine, could be part of a growth regulatory system for the fungus, where the excessive concentration of these nutrients may reduce growth rate of the fungus.

Dussiella is dependent on the living plant host to provide a stream of sugars from the phloem. It is apparent that the host must also provide other nutrients to the fungus that it cannot produce itself, such as reduced forms of nitrogen (e.g., amino acids, nitrites, or ammonium compounds) and perhaps vitamins such as biotin and thiamine. These vital substances may be provided to the fungus in the stream of nutrients emerging from the plant phloem that is rich in such nutrients. Certain phloem sap nutrients (e.g., methionine, glutamine, and creatine) may not be absorbed by the stroma and might accumulate in the absorptive cavity beneath the stroma and play a role as growth regulatory compounds for the fungus. Whether such a system for control of fungal development exists in this symbiotic system requires additional investigation.

Stromal development

In nature perithecial development in *Dussiella* is a gradual process occurring over several weeks to months, after a heterothallic mating process. Here we utilized an isolate of the fungus that may have been homothallic or previously dikaryotized, having been derived from a previously fertilized stroma. The factors that are important in development of perithecia appear to be: 1) development of a thickened colony (or stroma), 2) restriction of nutrients, and 3) restriction of moisture. Maximum development of perithecia occurred when these conditions were met.

In nature high nutrients, probably mostly sucrose, would favor development of the mycelial stroma. Nutrients from the plant are expected to be highest in the spring and summer months when photosynthesis and plant growth are highest and plants are mobilizing nutrients in the phloem to fuel new growth. During this period the stromata are developing on plants. When plant nutrient production becomes restrictive, the fungal stroma may initiate its reproductive phase and develop perithecia. Perithecia form on stromata in natural

populations of plants in the late fall and early winter months. In general, late fall and winter is when plant growth and photosynthesis are reduced in bamboo. The reduced plant growth in the late fall and winter seasons means that fewer nutrients and water are mobilized in phloem to fuel shoot growth in the plant and thus fewer nutrients will be provided to the fungus. The *in vitro* observations on stroma and perithecium development appear to be consistent with the environmental conditions evident during the development and reproductive cycle of the plant and fungus in natural populations. Because of the correlation of natural and *in vitro* conditions of development of the fungus we have some confidence that the developmental determinants identified in the in vitro experiments may be the same developmental determinants that operate in natural populations of *Dussiella* on the host plant. The development and reproductive cycle of the fungus is thus determined in part by the developmental conditions of the host plant.

Biotrophy in Dussiella

It is likely that the ancestors of *Dussiella* were insect parasites. *Dussiella* may have adapted to scale insects then developed the capacity to utilize nutrients emerging form the phloem sap of the plant (White et al., 2002). We suggest that parasitism of scale insects represents one way that some epibiotic plant biotrophs may have evolved. Many other plant epibiotic clavicipitaleans, such as *Ascopolyporus, Hypocrella, Mycomalus,* may employ the same mechanism to achieve biotrophic associations with plants. Infection of scale insects may have been the intermediate step to direct parasitism of plants as exhibited in genera such as *Balansia, Epichloë* and *Myriogenospora.*

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