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Host nutrient adaptation in two symbiotic fungi: *Balansia henningsiana* and *Hypocrella phyllogena* (Clavicipitaceae; Ascomycetes)

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Abstract

To better understand how clavicipitalean fungi have adapted to their hosts, cultural studies of the plant biotrophic endophyte *Balansia henningsiana* and the scale insect parasite *Hypocrella phyllogena* (Clavicipitaceae; Hypocreales; Ascomycota) were conducted using chemically defined media supplemented with various carbon and nitrogen sources. Although we used a limited number of substrates, this study shows that both fungi exhibit a restricted range of similar nutrient sources that may support growth. The nutrients supporting growth are those that are expected to be derived from plants. This study suggests that the scale insect pathogen *Hypocrella phyllogena* obtains at least part of its nutrients from the host plant rather than exclusively from the insect hosts. Based on its nutritional use *Hypocrella phyllogena* more closely resembles plant biotrophs than it does insect parasitic clavicipitalean fungi.

Keywords: Clavicipitaceae, growth requirements, *in vitro* growth, fungal physiology

1. Introduction

Symbiotic interactions between plants and fungi are widespread in nature. The family *Clavicipitaceae* (Hypocreales; Ascomycota) includes pathogenic and mutualistic fungi that infect grasses and sedges. Over the past 25 years, research has focused on symbiotic interactions with economic or ecologic importance (Schardl and Phillips, 1997; Bacon and White, 2000; Schardl, 2001; Schardl et al., 2004). Most fungal endophytes of plants show no external symptoms of infection with the vegetative mycelia of these fungi growing in the above ground organs of plants (White, 1994). Symbiotic endophytic and epibiotic species of the *Clavicipitaceae* extract nutrients from hosts without use of haustoria. Instead, host tissues are modified or permeated so that nutrients move across them freely (White and Camp, 1995).

The associations between fungal endophytes and hosts are frequently considered to be mutualistic (Clay, 1988; Schardl, 2001). The benefits for endophytes and some epibionts are access to nutrients from hosts; and benefits for hosts include drought tolerance, greater field persistence, resistance to nematodes and fungal pathogens, and protection from mammalian and insect herbivory (Clay, 1990; Bush et al., 1997; Azevedo et al., 2000; Schardl, 2001; Schardl et al., 2004). The anti-herbivore properties of endosymbiotic fungi are largely attributable to production of various biologically active compounds, usually alkaloids, which accumulate within infected host tissues (Bush et al., 1997; Azevedo et al., 2000). *Balansia henningsiana* (Moell) Diehl infects only warm-season grass species (Diehl, 1950; White, 1994). Sections through leaves bearing stromata show abundant mycelia permeating vascular bundles, apparently to facilitate the transfer of nutrients and moisture from living leaf tissues to the fungal stroma (White, 1994).

Hypocrella phyllogena (Mont.) Speg. (anamorph *Aschersonia basicystis* Berk. & Curt.) is a pathogen of scale insects on *Citrus* species, and characterized by a convex stroma that is superficial on leaves (Petch, 1921; Mains, 1959). *Hypocrella* species generally infect immature stages of scale insects belonging to two families of Homoptera (*Aleyrodae* and *Coccidae*) (Hywel-Jones and Samuels, 1998). It has long been acknowledged that *Hypocrella* spp.

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are destructive parasites of scale insects (Petch, 1921; Mains, 1959) but it is not clear whether they are also adapted to obtain nutrients from plant hosts (Koroch et al., 2004). In biotrophic fungi closely adapted to hosts, nutrients supporting growth *in vitro* should reflect the nutrients supplied by hosts (Pateman and Kinghorn, 1976; Bacon, 1985; Kulkarni and Nielson, 1986; Ferguson et al., 1993; and White et al., 2002). The acquisition of information on growth requirements *in vitro* may provide a better understanding of the nature of the symbiotic interactions between symbionts in the association (White and Chambless, 1991). The objective of the research reported herein was to evaluate the *in vitro* growth requirements of a known plant biotroph *B. henningsiana* and the scale insect parasitic *H. phyllogena* using defined media supplemented with various carbon and nitrogen sources.

2. Materials and Methods

The fungi

The *Balansia henningsiana* isolate was obtained from stromata on *Andropogon virginicus* collected in Patuxent wildlife Research Refuge in Maryland in 1999. Isolates of *Hypocrella phyllogena* were obtained from stromata on leaves of lemon plants collected in Xalapa, Mexico in 2001. Representative isolates of *B. henningsiana* and *H. phyllogena* were submitted to the American Type Culture Collection.

Inoculum preparation

For the inoculum medium, mycelium was grown in liquid MS (Murashige and Skoog, 1962) basal medium (4.32 g l^{-1}) containing sucrose (30 to 400 g l^{-1}) alone or supplemented with 10 g l^{-1} proflo (Traders Protein®, Fermentation nutrients TN, USA) or 3 g l^{-1} yeast extract. A combination of sucrose or sorbitol, supplemented with proflo and 3 g l^{-1} ammonium citrate was also tested. The pH of the media was adjusted to 5.8 with 1 N KOH before autoclaving.

In vitro *cultivation in agar*

One ml of seed culture was spread on sterile discs of cellophane dialysis membrane over the culture medium on agar plates. The culture media were composed of MS basal medium (4.32 g l^{-1}) containing various concentrations (30, to 300 g l^{-1}) of a carbon source (sucrose, dextrose, fructose, or sorbitol) alone or in combination with 3 g l^{-1} of yeast extract. MS medium with sucrose or sorbitol (100 g l^{-1}) in combination with various nitrogen sources (ammonium citrate, ammonium acetate, ammonium phosphate, ammonium sulfate, ammonium nitrate, ammonium chloride, calcium nitrate or various amino acids) in several concentrations (0.5 to 10 g l^{-1}) were also tested. The pH of the media was adjusted to 5.8 before adding agar (20 g l^{-1}) and autoclaving. All amino acids were filter sterilized.

Experimental design

Each treatment was run in quadruplicate and repeated twice. After 5 weeks, individual colonies were removed from the dishes and dried in an oven at 70°C to a constant dry weight. The treatments and replicates were fully randomized. For comparison of growth in different media, data were subjected to an analysis of variance (ANOVA) using the LSD procedure. Statistical differences were determined at the 5% significance level.

3. Results

In vitro *growth of* B. henningsiana

Of the carbon sources tested, sucrose supported the highest growth rates as evidenced by the accumulation of biomass expressed as dry weight (Table 1). There was poor growth in all media supplemented with monosaccharides. *Balansia henningsiana* was able to metabolize dextrose, fructose or sorbitol in lower concentrations, but growth reduced when concentrations increased to 200 g l^{-1}. In media supplemented with yeast extract growth improved in all sugars (Table 1). Mycelial dry weight increased when the concentration of ammonium citrate in the media was increased (Table 2). Good growth was also observed on ammonium phosphate, whereas, little growth occurred on ammonium nitrate or ammonium chloride. Seven amino acids were also tested (Table 3). Among the amino acids tested, only glutamine and alanine increased biomass production at 2 g l^{-1}. Higher concentrations of amino acids tested inhibited growth. The growth response to amino acids did not show correlation to the number of nitrogen atoms contained in the amino acids. Although creatine contains 3 nitrogen atoms and most others one or two nitrogen atoms, no growth was recorded on creatine.

In vitro *growth of* H. phyllogena

The ANOVA (Table 4) shows differences in mycelial dry weight production cultured in carbon sources alone or in combinations with yeast extract. Increasing sucrose concentration in the medium to 200 g l^{-1} enhanced mycelial growth rate. Similar results were observed when the concentration of dextrose was increased to 200 g l^{-1}, while, increase in concentrations of fructose or sorbitol resulted in poor growth. When media were supplemented with yeast extract maximum growth was observed in 300 g l^{-1} of

Table 1. Effect of carbon source and yeast extract supplementation on mycelial dry weight (mg) of *B. henningsina* after 5 weeks incubation at 25°C.

Carbon sources	Yeast extract (g l^{-1})	30 g l^{-1}	100 g l^{-1}	200 g l^{-1}	300 g l^{-1}
Sucrose	0	210 ± 10 g*	327 ± 30 cd	414 ± 15 b	249 ± 8 efg
	3	299 ± 20 de	443 ± 138 b	595 ± 59 a	618 ± 10a
Fructose	0	168 ± 4 e	253 ± 3 efg	0	0
	3	243 ± 9 fg	398 ± 9 bc	0	0
Dextrose	0	201 ± 12 d	268 ± 7 ef	169 ± 3 e	0
	3	255 ± 9 efg	451 ± 10 b	391 ± 4c	206 ± 7 g
Sorbitol	0	131 ± 4 f	312 ± 12 d	135 ± 12 ef	0
	3	238 ± 7 fg	341 ± 36 cd	271 ± 21ef	0

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

Table 2. Growth of *B. henningsiana* as mycelial dry weight (mg) on MS, 100 g l^{-1} sucrose and nitrogen source.

Nitrogen sources	0 g l^{-1}	2 g l^{-1}	5 g l^{-1}	10 g l^{-1}
Control	327 ± 24 e*			
Ammonium citrate		278 ± 34 ef	701 ± 110 ab	793 ± 28 a
Ammonium nitrate		275 ± 13 ef	275 ± 8 ef	236 ± 20 f
Ammonium phosphate		483 ± 41 d	547 ± 30 cd	614 ± 69 bc
Ammonium chloride		293 ± 15 ef	296 ± 8 ef	232 ± 12 f

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

Table 3. Growth (mg dry weight) on MS basal media and 100 g l^{-1} sucrose supplemented with amino acids in *B. henningsiana*.

Amino acid	0 g l^{-1}	2 g l^{-1}	5 g l^{-1}
Control	327 ± 33 b*		
L-Asparagine		153 ± 11 c	0
L-Glutamine		449 ± 52 ab	0
L-Tryptophan		0	0
L-Methionine		0	0
Glycine		0	0
L-Alanine		459 ± 62 a	0
Creatine monohydrate		0	0

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

sucrose. There were no statistical growth differences at the 200 g l^{-1} level of sucrose and dextrose. For nitrogen source analysis, the greatest mycelial dry weight was obtained when ammonium citrate was present in the medium (Table 5), showing a significant increase in biomass accumulation as concentrations increased. The poorest growth was observed in media with ammonium nitrate or ammonium chloride. There were also seven amino acids tested as N sources (Table 6). Asparagine and glycine in the culture medium increased fungal growth when compared to the control. *Hypocrella* was not able to utilize glutamine, tryptophan, methionine or alanine in the concentrations tested. Higher concentration of all the amino acids tested in the media inhibited growth. The pattern of enhanced growth was not seen to correlate with the number of nitogen atoms in each amino acid. The highest growth was recorded on glycine, containing a single nitogen atom in its structure; poor growth was recorded on creatine with three nitrogen atoms in its structure.

4. Discussion

The scale insect parasite *H. phyllogena* demonstrates similarities to the plant biotrophic fungus *B. henningsiana* in that it shows a restricted capacity to grow on specific plant available nutrients. Our data (Tables 1–6) supports the hypothesis that *H. phyllogena* is a plant biotrophic symbiont even though it is also a parasite of scale insects. Sucrose is the most common sugar translocated in plant phloem tissues (Taiz and Zeiger, 2002). Sucrose was the carbohydrate that supported the most rapid growth (Tables 1 and 4) in both *H. phyllogena* and *B. henningsiana*. This seems to suggest that both fungi are adapted to growth on this carbohydrate and likely possess membrane invertases and other enzyme systems for the active absorption and processing of sucrose. Lam et al. (1995) demonstrated the presence of membrane invertases in genus *Epichloë*, another endophytic biotroph in the family *Clavicipitaceae*. Growth of both species on all carbohydrates was enhanced when yeast extract was added to media (Tables 1 and 4).

Table 4. Effect of carbon source and yeast extract supplementation on mycelial dry weight (mg) of *Hypocrella phyllogena* after 5 weeks incubation at 25°C.

Carbon sources	Yeast extract (g l^{-1})	30 g l^{-1}	100 g l^{-1}	200 g l^{-1}	300 g l^{-1}
Sucrose	0	304 ± 9gh*	998 ± 25 cd	1648 ± 340 b	526 ± 110 f
	3	240 ± 20 ij	829 ± 29 de	1996 ± 381 b	3836 ± 128 a
Fructose	0	197 ± 12 j	322 ± 41 gh	194 ± 13 j	139 ± 10 k
	3	230 ± 18 ij	1037 ± 20 cd	1029 ± 166 cd	549 ± 18 f
Dextrose	0	219 ± 17 ij	980 ± 155 cd	1587 ± 280 b	280 ± 8 hi
	3	220 ± 14 ij	844 ± 54 de	2122 ± 259 b	992 ± 88 cd
Sorbitol	0	190 ± 12 j	363 ± 53 g	544 ± 12 f	355 ± 10 g
	3	254 ± 13 if	760 ± 44 e	960 ± 31 cde	485 ± 23 g

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

Table 5. Growth of *H. phyllogena* as mycelial dry weight (mg) on MS, 100 g l^{-1} sucrose and different nitrogen source.

Nitrogen sources	0 g l^{-1}	2 g l^{-1}	5 g l^{-1}	10 g l^{-1}
Control	998 ± 25 c*			
Ammonium citrate		1047 ± 56 c	1310 ± 65 b	1465 ± 102 a
Ammonium nitrate		189 ± 10 f	142 ± 6 f	119 ± 2 f
Ammonium phosphate		348 ± 35 e	340 ± 6 e	618 ± 40 d
Ammonium chloride		177 ± 3 f	162 ± 5 f	139 ± 8 f

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

Table 6. Growth (mg dry weight) of *H. phyllogena* on MS basal media and 100 g l^{-1} sucrose supplemented with amino acids.

Amino acid	0 g l^{-1}	2 g l^{-1}	5 g l^{-1}
Control	998 ± 25 b*		
L-Asparagine		1098 ± 86 ab	0
L-Glutamine		0	0
L-Tryptophan		0	0
L-Methionine		0	0
Glycine		1204 ± 56 a	0
L-Alanine		0	0
Creatine monohydrate		119 ± 13 c	0

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

These enhancements in growth on addition of yeast extract may be due to auxotrophies in these two fungi. It has previously been demonstrated that *Epichloë* and asexual species in genus *Neotyphodium* are auxotrophic for vitamins biotin and thiamine (Kulkarni and Nielsen, 1986). Auxotrophies are common in biotrophic fungi where nutrients are directly supplied from the host (Griffin, 1994). The plant biotrophic fungus becomes dependent on its host that provides not only carbon but certain key vitamins. We have not evaluated specific auxotrophic vitamin requirements in this study.

Our results suggest that the form in which nitrogen is supplied is important in both *B. henningsiana* and *H. phyllogena* (Tables 2, 3, 5, 6). Ammonium citrate supported the greatest mass accumulation (Tables 2 and 5). Ammonium chloride and ammonium nitrate support growth of many saprophytic fungi but are utilized poorly or not at all by many plant biotrophic fungi (Pateman and Kinghorn, 1976). *Balansia henningsiana* and *H. phyllogena* do not appear to utilize these compounds (Tables 2 and 3). Ammonium citrate was an effective nitrogen source for both species (Tables 2 and 5). This compound is available to the fungus in the stream of nutrients from the phloem and is a natural component of the plant apoplast (Tejera et al., 2006). Citrate may also affect growth by altering carbon allocation to favor mycelial development over other forms of metabolism (Griffin, 1994). Amino acids that were good nitrogen sources in *B. henningsiana* included L-glutamine and L-alanine. This result agrees with previous reports of amino acid utilization in other plant biotrophic *Clavicipitaceae* (Pateman and Kinghorn, 1976; Kulkarni and Nielsen, 1986; Ferguson et al., 1993; Koroch et al., 2004).

In *Hypocrella* only asparagine and glycine were metabolized to support growth (Table 6). Higher concentrations (5 g l^{-1}) of all the amino acids tested were inhibitory to both species. This result agrees with previous reports on the clavicipitalean plant biotrophic epibiont *Dussiella tuberiformis* that also grew only on a restricted suite of amino acids (Koroch et al., 2004). Specific amino acids, including aspartate and glutamate and their respective

amides, glutamine and asparagines, are abundant in phloem sap (Taiz and Zeiger, 2002). It is not unexpected that plant biotrophic *B. henningsiana* would utilize one or more of these amino acids. A similar restrictive pattern of growth by *H. phyllogena* on amino acids common in plant phloem suggests that it is similarly adapted to nitrogen provided by the plant in the phloem sap. The nutrient growth capacities of *B. henningsiana* and *H. phyllogena* are as expected for fungi that have coevolved with plant host species. These species appear to grow on a narrow range of nutrients that is provided by their plant hosts. Fungal saprophytes and more generalist insect pathogens demonstrate a much broader range of growth substrates (Pateman and Kinghorn, 1976; M.S. Torres and J.F. White, unpublished data).

While *Hypocrella phyllogena* is a necrotrophic parasite of scale insects, it has cultural features of a plant biotrophic fungus. *Hypocrella* infects the scale insect, degrades its body completely, then likely nourishes itself biotrophically on plant nutrients that flow from the phloem to the surface of the plant through the stylet or stylet wound left by the scale insect. A similar life cycle has been demonstrated for *D. tuberiformis* (Koroch et al., 2004). Evolutionarily adapting to growth on a limited range of plant host available nutrients may have been the mechanism by which clavicipitalean insect parasites became non-destructive plant symbionts. Increasingly adapting to nutrients provided by the host increases dependence of the fungus on the host. The *H. phyllogena* life cycle may be compared to life cycles of plant biotrophic species such as *Claviceps* and *Epichloë* (White et al., 2002). Species such as *H. phyllogena* replace the scale insect and develop on the nutrient stream emerging from the plant pholem. In *Claviceps* the fungus replaces plant ovaries and develops its sclerotium using the nutrients that flow into the infected floret. In *Epichloë* the fungus replaces the inflorescence with the fungal stroma. The fungal stroma develops on the primordial inflorescence and takes nutrients from the plant that would normally be used by the plant to fuel inflorescence and seed development (White and Chambless, 1991; White and Camp, 1995).

In essence *H. phyllogena* and many of the plant biotrophic species are employing similar plant biotrophic nutritional strategies. What differs between these species is the mechanism by which they gain access to the plant nutrients. In *H. phyllogena* a scale insect is needed in the life cycle in order for the fungus to gain access to plant nutrients; while in species of *Balansia*, *Claviceps*, and *Epichloë* the insect intermediary is not necessary and the fungi gain access to plant nutrients by direct infection of meristematic plant tissues (White, 1994). While the mechanism used by species of *Hypocrella* to access plant nutrients involves scale insects, it is clear that it is a plant biotroph comparable to other plant-infecting biotrophic members of the family *Clavicipitaceae*.

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