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Author(s): Rajiv K. Kulkarni and Barbara D. Nielsen

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## NUTRITIONAL REQUIREMENTS FOR GROWTH OF A FUNGUS ENDOPHYTE OF TALL FESCUE GRASS

RAJIV K. KULKARNI AND BARBARA D. NIELSEN

*Microbiology Division, NPI, University of Utah Research Park,  
Salt Lake City, Utah 84108*

### ABSTRACT

The nutritional requirements of *Acremonium coenophialum*, a fungus endophyte of tall fescue, were examined in a semi-defined liquid medium. The fungus utilized several carbon sources including fructose, glucose, mannose, sucrose, trehalose, raffinose, sorbitol and mannitol. Trehalose and mannitol were excellent carbon sources. Nitrogen sources utilized by the endophyte included ammonium, arginine, asparagine, cysteine, glutamine, proline and serine. Undefined complex nitrogen sources such as yeast extract, soytone and tryptone supported excellent growth. In a defined medium the fungus exhibited a requirement for thiamine.

Key Words: *Acremonium coenophialum*, nutrition, endophyte.

Fungus endophytes have been reported to occur in several species of ryegrass (*Lolium* sp.) and fescue grass (*Festuca* sp.) (Neill, 1940, 1941; Latch *et al.*, 1984; White and Cole, 1985a). The tall fescue endophyte was previously referred to as *Epichloe typhina* (Fr.) Tulasne, but has recently been renamed *Acremonium coenophialum* Morgan-Jones and Gams (Morgan-Jones and Gams, 1982). The endophyte-infected forage grasses cause fescue toxicosis (Hoveland *et al.*, 1980) or ryegrass staggers (Fletcher and Harvey, 1981) in grazing animals. Tall fescue (*Festuca arundinacea* Schreb.) is commonly used as both forage and turf grass. In the United States tall fescue is grown on 12 to 14 million hectares (Siegel *et al.*, 1984). Hence losses in animal productivity due to fescue toxicosis have been estimated as between \$50–200 million annually.

*Acremonium coenophialum* is a true endophyte in that it completes its entire life cycle within the host plant. Spores of this fungus have not been reported to occur on or in plants (Siegel *et al.*, 1985), but conidia are produced on several complex media (Latch *et al.*, 1984; Morgan-Jones and Gams, 1982). Using autoclaved tall fescue seedlings, White and Cole (1985b) obtained synnematosus sporulation *in vitro*. In nature the fungus is transmitted in seed. Fungus dissemination does not occur by pollen, wind, rain or artificial infection (Siegel *et al.*, 1984).

The endophyte-colonized tall fescue plants or perennial ryegrass plants show no external disease symptoms, hence the presence of the fungus endophyte is revealed either by microscopic observations of plant tissue stained with aniline

blue in lactic acid (Bacon *et al.*, 1977; Clark *et al.*, 1983) or by an enzyme-linked immunosorbent assay (ELISA) developed by Johnson *et al.* (1982).

Funk *et al.* (1983, 1985) have recently demonstrated a positive correlation between the presence of an *Acremonium* sp. endophyte in perennial ryegrass (*Lolium perenne* L.) and enhanced resistance to the predatory larval stage of the sod webworm (*Crambus* sp.) and the bluegrass billbug (*Sphenophorus parvulus* Gyllenhal). In addition, Johnson *et al.* (1985) observed that the presence of *A. coenophialum* in tall fescue inhibited feeding by aphids (*Rhopalosiphum padi* L. and *Schizaphis graminum* Rondani). Hence the endophyte appears to confer insect resistance upon the colonized host. This desirable characteristic may be appropriately utilized in integrated pest management practices for turfgrasses.

Although some of the effects of the endophyte–host association are well documented, e.g. fescue toxicosis and insect resistance, very little is known about the physiology of the fungus itself. In order to study the physiology and biochemistry of the fungus it is important to establish the nutritional requirements for *in vitro* growth of the endophyte. We examined, therefore, the carbon source, nitrogen source and vitamin requirements for growth of *A. coenophialum*. The results of this study are reported here.

### MATERIALS AND METHODS

*Acremonium coenophialum* was kindly supplied by Dr. E. M. Clark, Department of Botany, Plant Pathology and Microbiology, Auburn Uni-

versity, Auburn, Alabama. Cultures were maintained on GY agar, a modified medium of Clark *et al.* (1983), which consisted of (per liter of deionized water) glucose, 5 g; yeast extract, 2 g;  $\text{KH}_2\text{PO}_4$ , 3 g;  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ , 2 g;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 g; agar, 20 g; pH 6.4. To obtain inoculum, agar plugs containing a portion of the colony were transferred to 250 ml Erlenmeyer flasks containing 50 ml sterile GY medium and incubated on a New Brunswick G10 shaker at 23 C and shaken at 100 rpm for 2–4 weeks.

The basal medium used in carbon and nitrogen utilization studies consisted of (per liter)  $\text{KH}_2\text{PO}_4$ , 3 g;  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ , 2 g;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 g; yeast extract 0.05 g; trace elements, 1 ml; pH 6.4. The trace elements were added at a final concentration per liter of boric acid, 220  $\mu\text{g}$ ; cupric sulfate, 150  $\mu\text{g}$ ; potassium iodide, 120  $\mu\text{g}$ ; ferric/sodium EDTA, 300  $\mu\text{g}$ ; manganese sulfate, 130  $\mu\text{g}$ ; sodium molybdate, 110  $\mu\text{g}$ ; zinc sulfate, 110  $\mu\text{g}$ . In carbon utilization studies the basal medium was supplemented with 25 mM carbon source (TABLE I) and ammonium sulfate (2 g/l). In nitrogen utilization experiments the basal medium was supplemented with 20 mM nitrogen source (TABLE II) and glucose (5 g/l). To determine the vitamin requirements of *A. coenophialum*, yeast extract in the basal salts medium was replaced either by an individual vitamin or a combination of vitamins and the medium supplemented with glucose (5 g/l) and ammonium sulfate (2 g/l). Growth of the endophyte in a defined medium was also examined. The defined medium consisted of basal salts medium (without yeast extract) supplemented by glucose, 5 g; ammonium sulfate, 2 g; biotin, 20  $\mu\text{g}$ ; pyridoxine hydrochloride, 200  $\mu\text{g}$ ; and thiamine, 200  $\mu\text{g}$  per liter. In each case the medium was filter-sterilized using Nalgene filtration units (pore size 0.2  $\mu\text{m}$ ) and dispensed in 20 ml aliquots in sterile 50 ml Erlenmeyer flasks stoppered with cotton plugs.

For inoculum production, the mycelium was grown in liquid GY medium, harvested by centrifugation, homogenized in a sterile Dounce homogenizer with a loose fitting teflon plunger and washed twice with 50 mM potassium phosphate buffer (pH 6.4). The homogenized hyphal fragments were resuspended in phosphate buffer and used as inoculum. All flasks in each experiment received the same quantity of inoculum, but between experiments the inoculum size varied from 3 to 9 mg dry weight per 20 ml medium.

Cultures were incubated at 23 C for 2 weeks on a New Brunswick G10 shaker at 100 rpm. A 2 week incubation period was selected as growing cultures were in the middle of a rapid-growth phase. At the end of the incubation period the contents of each 50 ml Erlenmeyer flask were separately filtered through a preweighed Whatman #50 filter paper, washed with deionized water and dried at 65 C to a constant dry weight. Each nutrient variable was run in triplicate and all experiments were repeated three times. Dry weights are expressed as means  $\pm$  standard deviations of three separate determinations.

#### RESULTS AND DISCUSSION

*Carbon source utilization.*—Thirty-one different carbon sources were screened (TABLE I). Yeast extract by itself, at a concentration of 0.05 g/l, supported minimal growth of the fungus ( $3 \pm 1$  mg/20 ml) and hence served as a source of vitamins and growth factors in carbon and nitrogen utilization studies.

Four pentoses, arabinose, lyxose, ribose or xylose were not utilized by the endophyte. These four carbon sources have been previously reported to serve as sole carbon sources in other fungi (Perlman, 1965). Of the six hexoses screened, D-glucose, D-fructose and D-mannose were utilized by the endophyte for growth, whereas no growth occurred on galactose, sorbose or rhamnose (TABLE I). In fungi, D-glucose and D-fructose are readily catabolized by the cell's glycolytic pathways (Cochrane, 1976). Mannose may be catabolized by the Embden-Meyerhoff pathway, and in addition is a component of the fungal cell wall (Bartnicki-Garcia, 1968). Interestingly, D-galactose, a commonly utilizable carbon source in many fungi (Perlman, 1965), does not support growth of *A. coenophialum*.

Out of the four disaccharides screened, only sucrose and trehalose supported significant growth of the endophyte. Trehalose is generally found as a storage carbohydrate in many fungi (Thevelein, 1984). An uptake system for trehalose has been reported in *Saccharomyces cerevisiae* (Kotyk and Michaljanicova, 1979). Generally, trehalose accumulates in the cell during the sporulation stage and is mobilized during spore germination (Thevelein, 1984). It is interesting, therefore, that the endophyte can utilize extracellular trehalose. Good growth was observed on raffinose ( $61 \pm 10$  mg/20 ml), whereas, little growth occurred on soluble starch ( $14 \pm 1$

mg/20 ml). No growth occurred on pectin, cellulose, galacturonic acid or polygalacturonic acid, indicating the inability of the endophyte to utilize plant cell wall components, perhaps due to the lack of cellulases and pectinase. This result is in agreement with the electron microscopic observations of Bacon (1983), where the endophyte strictly grew in the intercellular spaces of the plant leaf and did not invade the host cells. *Acremonium coenophialum* did not grow on acetate-, citrate-, propionate- or succinate-containing media.

Initial experiments revealed that *A. coenophialum* grew very well when mannitol was used as a carbon source, hence we examined the ability of the endophyte to grow on a variety of polyols (sugar alcohols). Compared to pentoses the pentitols appeared to support a modest amount of growth, especially D-arabitol ( $20 \pm 3$  mg/20 ml) and D-ribitol ( $15 \pm 2$  mg/20 ml), but no growth occurred on L-arabitol and xylitol. The sugar alcohols of glucose and mannose, sorbitol and mannitol, respectively, were good carbon sources. Interestingly, galactitol supported a limited amount of growth ( $21 \pm 8$  mg/20 ml) whereas galactose was not utilized. It seems, therefore, that some sugar alcohols were better carbon sources than their respective sugars. Polyols have been reported in a large variety of fungi (Lewis and Smith, 1967). They may serve several functions in fungi—as carbohydrate reserves, as translocatory compounds, as agents in osmoregulation, coenzyme regulation and storage of reducing power (Jennings, 1984). The functions of polyols in *A. coenophialum* remain to be determined, but results from this study indicate that mannitol and sorbitol are good carbon and energy sources.

*Nitrogen source utilization.*—The fungus was able to utilize ammonium but not nitrate as a nitrogen source. The apparent inability to grow on nitrate could be due to lack of an enzyme in the nitrate reduction pathway (Pateman and Kinghorn, 1976). The requisite cofactors for enzymes of the nitrate reduction pathway include molybdenum/iron (nitrate reductase), copper/iron (nitrite reductase) and magnesium/manganese (hydroxylamine reductase) (Garraway and Evans, 1984). Since these metal ions were present in basal medium, lack of nitrate utilization could not be attributed to cofactor deficiency. The endophyte was not able to utilize urea (TABLE II). A specific

TABLE I  
GROWTH OF *ACREMONIUM COENOPHIALUM* ON BASAL MEDIUM SUPPLEMENTED WITH AMMONIUM SULFATE AND VARIOUS CARBON SOURCES

Carbon source	Dry weight <sup>a</sup> ± SD
<b>Pentoses</b>	
D-arabinose	0
D-ribose	2 ± 1
D-xylose	2 ± 1
D-lyxose	5 ± 1
<b>Hexoses</b>	
D-fructose	69 ± 22
D-galactose	1 ± 0.6
D-glucose	43 ± 12
D-mannose	50 ± 3.8
L-sorbose	4 ± 1
L-rhamnose	2 ± 1
<b>Disaccharides</b>	
Maltose	4 ± 0.5
Lactose	3 ± 1
Sucrose	50 ± 22
Trehalose	100 ± 18
<b>Oligosaccharide</b>	
Raffinose	61 ± 10
<b>Polysaccharides</b>	
Soluble starch <sup>b</sup>	14 ± 1
Pectin <sup>b</sup>	0
Cellulose <sup>b</sup>	0
<b>Carboxylic acids</b>	
Acetate <sup>c</sup>	0
Citrate <sup>c</sup>	0
Propionate <sup>c</sup>	4 ± 0.6
<b>Polyols</b>	
D-arabitol	20 ± 3
L-arabitol	8 ± 1
D-galactitol	21 ± 8
Glycerol	12 ± 0.6
D-sorbitol	72 ± 20
D-mannitol	111 ± 18
Ribitol	15 ± 2
Xylitol	7 ± 0.6
<b>Uronic acids</b>	
D-galacturonic acid	3 ± 1
Polygalacturonic acid <sup>b</sup>	3 ± 1

<sup>a</sup> Dry weights (mg/20 ml medium) are expressed as means ± standard deviations of three different experiments. Controls that did not have a carbon source weighed  $3 \pm 1$  mg.

<sup>b</sup> Polysaccharides and polygalacturonic acid was added at a final concentration of 5 g/l.

<sup>c</sup> The sodium salt of the carboxylic acid was used.

transport system for urea has been reported in *Aspergillus nidulans* (Dunn and Pateman, 1972). Urea is generally broken down to ammonia and carbon dioxide by the enzyme urease; hence urea

TABLE II

GROWTH OF *ACREMONIUM COENOPHIALUM* ON BASAL MEDIUM SUPPLEMENTED WITH GLUCOSE AND VARIOUS NITROGEN SOURCES

Nitrogen source	Dry weight <sup>a</sup> ± SD
Ammonium chloride	35 ± 6.9
Ammonium nitrate	40 ± 5
Potassium nitrate	9 ± 2
L-alanine	3 ± 1
L-arginine hydrochloride	34 ± 12
L-asparagine	42 ± 2.6
L-cysteine hydrochloride	31 ± 3
L-glutamine	60 ± 13
Glycine	5 ± 1
L-histidine hydrochloride	3 ± 1
L-leucine	5 ± 0.5
L-lysine monohydrochloride	5 ± 0.5
L-methionine	3 ± 1
L-phenylalanine	3 ± 1
L-proline	61 ± 5.5
L-serine	36 ± 1.5
L-tryptophan	1 ± 1
Urea	1 ± 1
Soytone <sup>b</sup>	183 ± 35
Yeast extract <sup>b</sup>	129 ± 27
Peptone <sup>b</sup>	85 ± 12
Casamino acids <sup>b</sup>	55 ± 2.5
Tryptone <sup>b</sup>	205 ± 16

<sup>a</sup> Dry weights (mg/20 ml medium) are expressed as means ± standard deviations of three separate experiments. Controls that did not have a nitrogen source weighed 13 ± 2 mg/20 ml.

<sup>b</sup> These complex nitrogen sources were added at 2 g/l.

may serve as a nitrogen source in several fungi (Pateman and Kinghorn, 1976). The lack of urea utilization in *A. coenophialum* could be due to the absence of an uptake system or absence of urease.

Amino acids that were good nitrogen sources (TABLE II) included L-arginine, L-asparagine, L-cysteine, L-glutamine, L-proline, and L-serine. These findings are in agreement with previous reports on amino acid utilization by filamentous fungi (Pateman and Kinghorn, 1976), although L-cysteine may be toxic to certain fungi. Although good growth occurred on peptone and casamino acids, soytone, tryptone and yeast extract supported excellent growth. Several amino acids did not support fungal growth (TABLE II), including L-alanine, glycine, L-phenylalanine and L-tryptophan. The amino acid transport system in fungi may have broad specificity, e.g. neutral aliphatic and aromatic amino acids in *Neurospora crassa* (Pall, 1969), or the transport system

TABLE III

GROWTH OF *ACREMONIUM COENOPHIALUM* ON MINERAL SALTS MEDIUM<sup>a</sup> SUPPLEMENTED WITH GLUCOSE, AMMONIUM SULFATE AND VARIOUS VITAMINS OR GROWTH FACTORS

Vitamin(s) and growth factors	Concentration per liter	Dry weight ± SD
Biotin	20 µg	24 ± 1
Folic acid	100 µg	22 ± 2
Inositol	5 mg	19 ± 1
Nicotinic acid	200 µg	23 ± 1
Pantothenic acid	200 µg	19 ± 2.6
Pyridoxine	200 µg	23 ± 1
Riboflavin	200 µg	25 ± 2.5
Thiamine	200 µg	48 ± 4
Vitamin B <sub>12</sub>	200 µg	19 ± 1.6
All of the above		52 ± 3.8
All of the above but thiamine		20 ± 3.0
Yeast extract	0.05 g	48 ± 5.0
No vitamins		19 ± 1.5
Combinations <sup>b</sup>		
Vitamin mixture		40 ± 3.0
All but biotin		41 ± 1.5
All but nicotinic acid		40 ± 4.6
All but pyridoxine		38 ± 6
All but thiamine		12 ± 4.9
All but vitamin B <sub>12</sub>		38 ± 4

<sup>a</sup> The mineral salts medium had the same constituents as the basal salts medium except yeast extract.

<sup>b</sup> The vitamin mixture contained biotin, nicotinic acid, pyridoxine, thiamine and vitamin B<sub>12</sub> at the respective concentrations, one vitamin was left out in some cases to determine if it was required for growth.

may be specific, e.g. L-proline transport in *Penicillium chrysogenum* (Hunter and Segel, 1971). Lack of amino acid utilization, therefore, could be due to lack of uptake, or lack of one or more enzymes in the catabolic pathway.

*Vitamin requirements.*—At first the basal salts medium containing glucose and ammonium sulfate was supplemented with individual vitamins (TABLE III) or inositol. Only thiamine significantly stimulated growth in the defined medium. The growth was comparable to that obtained by supplementing the medium with yeast extract.

To determine if there were any synergistic or additive effects of vitamins on fungal growth, a combination of vitamins was further investigated. One vitamin at a time was left out. All combinations yielded approximately equivalent amounts of growth when thiamine was included in the vitamin mixture. However, when thia-

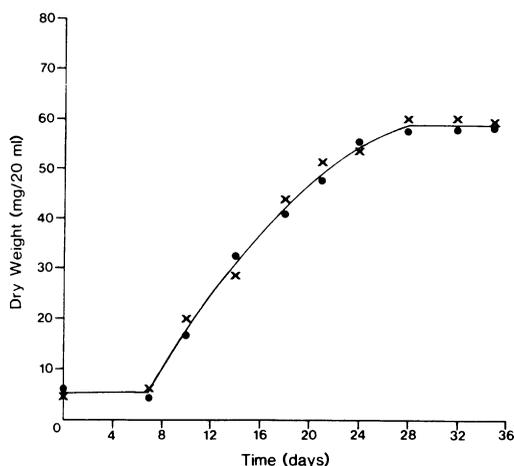


FIG. 1. Growth of *A. coenophialum* in a defined medium (●) and a semidefined medium (×).

mine was left out the amount of fungal growth was similar to that of the vitamin-free control. The results, therefore, indicate that *A. coenophialum* has an obligate requirement for thiamine.

*Growth in defined medium.*—The growth cycle of *A. coenophialum* in a defined medium is shown in FIG. 1. The lag phase was approximately 7 days, followed by a phase of rapid growth (from 7–28 days after inoculation). The dry weight doubling time was approximately 7 days. Cultures entered the stationary phase approximately 28 days after inoculation. If the vitamins in the defined medium were replaced by yeast extract (0.05 g/l), essentially similar growth characteristics were obtained (FIG. 1). These results suggest that the three vitamins (biotin, pyridoxine and thiamine) were able to replace the growth factors supplied by the yeast extract.

When ammonium sulfate in the basal salts medium was replaced by a complex nitrogen source, e.g. soytone, tryptone or yeast extract, the final mycelial dry weight was 3 to 4 times greater than that obtained in the defined medium. These undefined factors contain a mixture of carbon and nitrogen sources (as well as vitamins in the case of yeast extract), which could be readily utilized via the fungal salvage pathways. Hence further studies are needed to optimize the composition of the defined medium to obtain increased growth.

Although the nutritional requirements for *in vivo* growth of the endophyte in the host plant are not known, results of this study reveal some

of the important nutritional factors required for growth of *A. coenophialum* in pure culture. Out of the 31 carbon sources surveyed, the fungus grew on mannitol, trehalose, sorbitol, fructose, raffinose, sucrose, mannose and glucose. Soluble carbohydrates generally found in plant tissue include glucose, fructose and sucrose (Smith, 1973). Raffinose is found in trace amounts. The fungus was able to utilize ammonium and 6 amino acids (arginine, asparagine, cysteine, glutamine, proline and serine). These amino acids are commonly found as free amino acids in grass species (Hegarty and Peterson, 1973). Since only a limited number of carbon and nitrogen sources were utilized by the endophyte *in vitro*, and as some of these compounds are found as soluble components of plant tissue that may become available to the fungus plant tissue, it is tempting to hypothesize that the fungus may have undergone physiological adaptation to mutualistic growth within the host plant over evolutionary time. Adaptation of the fungus to endophytic growth appears to be highly successful as the fungus has only been isolated from the host grass plants. The results of this study provide the impetus for testing the hypothesis.

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