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In Vitro Growth of *Acremonium coenophialum*, an Endophyte of Toxic Tall Fescue Grass†

N. D. DAVIS, E. M. CLARK, K. A. SCHREY, AND U. L. DIENER*

Department of Botany, Plant Pathology, and Mircrobiology, Alabama Agricultural Experiment Station, Auburn University, Auburn, Alabama 36849

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***Acremonium coenophialum*, an endophytic fungus present in toxic tall fescue grass and seed, grew very slowly or not at all with conventional media and cultural practices. However, a considerable increase in growth was achieved in a relatively dilute medium consisting solely of glucose and yeast extract. The optimal levels of glucose and yeast extract were 3 to 6% and 0.35% (wt/vol), respectively. The addition of salts which lowered the pH suppressed growth. Even when the pH was controlled, the addition of KH_2PO_4 at a level of 3.2% or more greatly inhibited growth. *A. coenophialum* grew better in shake culture than in stationary culture. The optimal temperature was 23°C, and the optimal pH was 6.5.**

Acremonium coenophialum Morgan-Jones & Gams (6) was originally isolated from toxic tall fescue grass by Bacon et al. and reported as *Epichloë typhina* (Fries) Tulasne (1). Hoveland et al. (5) first documented the fact that the endophyte is highly detrimental to steer performance on infected tall fescue pastures. Yates et al. (10), using mass spectrometry-mass spectrometry, recently detected ergopeptide alkaloids in endophyte-infected tall fescue and reported an absence of such alkaloids in noninfected tall fescue. Porter et al. (7, 8) had previously reported that *E. typhina* (probably *A. coenophialum*) produces ergopeptide alkaloids in vitro. Davis et al. (3) reported that *A. coenophialum* produces several steroids, including ergosterol peroxide, a toxic derivative of ergosterol, in both toxic tall fescue and in in vitro mycelium. Thus, *A. coenophialum* is an important fungus (9) that has been implicated as a cause of the fescue foot and summer syndrome forms of fescue toxicosis. The endophyte grows very slowly (2), greatly hindering basic research on the growth and metabolism of the fungus. The purpose of this research was to initiate studies on cultural and environmental factors that influence the growth and development of *A. coenophialum*.

MATERIALS AND METHODS

Organisms. *A. coenophialum* ET-26 was used throughout this investigation. This strain was isolated from toxic tall fescue and had been used in previous investigations (2, 3). *Aspergillus terreus* was isolated from the rumen fluid of cattle, and *Aspergillus flavus* was obtained from the U.S. Department of Agriculture Northern Regional Research Center, Peoria, Illinois. Cultures of *A. coenophialum* were maintained on medium 9 (2), consisting of 0.5% dextrose, 0.2% yeast extract (Difco Laboratories, Detroit, Mich.) 0.5% KH_2PO_4, 0.05% $MgSO_4$, and 2% agar. The *Aspergillus* species were maintained on a medium containing 5% glucose, 0.5% KH_2PO_4, 0.05% $MgSO_4$, and 0.7% yeast extract. Liquid and solid media were inoculated directly with the *Aspergillus* species by transfer of conidia. Other liquid and solid media were inoculated with 4-mm disks cut with a sterile cork borer from the margins of sporulating cultures of *A. coenophialum*.

TABLE 1. Influence of glucose concentration on growth of *A. coenophialum* in 0.7% yeast extract broth

Glucose concn (%)	Mycelial dry wt (g/100 ml)[a]
0	0.089
1	0.064
3	1.125
6	1.058
12	0.778

[a] The data represent the averages of three replications incubated for 6 weeks at pH 6.0 in stationary culture.

Media. The basal liquid medium consisted of yeast extract and glucose. Glucose levels were evaluated in 100 ml of 0.7% yeast extract broth in 250-ml Erlenmeyer flasks, and yeast extract levels were similarly evaluated in 6% glucose broth. Experiments with added salts were conducted in 1% glucose–0.35% yeast extract broth. The influence of pH and temperature on the radial growth of *A. coenophialum* was determined in petri dishes containing medium of 0.5% glucose, 0.5% KH_2PO_4, 0.05% $MgSO_4$, 0.2% yeast extract, and 2% agar. The effects of various levels of KH_2PO_4 was determined in the same medium.

Culture conditions. Liquid cultures were incubated at 23°C for 4, 5, and 6 weeks in stationary culture in a Labline Incubator-Shaker Model 3598-3. Three flasks of cultures were used per treatment for the glucose and yeast extract experiments at a pH of 6.0 and for the added salt experiments, in which the intial pH varied with the treatment. Five flasks of cultures per treatment were incubated for 2 weeks

TABLE 2. Influence of yeast extract concentration on growth of *A. coenophialum* in 6% glucose broth

Yeast extract concn (%)	Mycelial dry wt (g/100 ml)[a]
0	0.081
0.35	0.163
0.70	0.162
1.40	0.130
2.80	0.079

[a] The data represent the averages of three replications incubated for 6 weeks at pH 6.0 in stationary culture.

* Corresponding author.
† Alabama Agricultural Experiment Station journal 6-86954.

TABLE 3. Influence of added salts on growth of *A. coenophialum* in 1% glucose–0.35% yeast extract broth

Added salt (0.04 mol/flask)	pH		Mycelial dry wt (g/100 ml)[a]
	Initial	Final	
None	6.4	5.9	0.287
KH_2PO_4	5.4	5.4	0.198
KCl	6.5	5.9	0.355
$(NH_4)_2HPO_4$	7.7	6.3	0.324
NH_4Cl	6.4	4.0	0.245

[a] The data represent the averages of three replications incubated for 4 weeks in stationary culture.

TABLE 4. Influence of added KH_2PO_4 on growth of *A. coenophialum* in 1% glucose–0.35% yeast extract broth

KH_2PO_4 concn (%)	pH		Mycelial dry wt (g/100 ml)[a]
	Initial	Final	
0	6.5	6.0	0.413
0.5	5.6	5.7	0.348
1.0	5.3	5.4	0.260
2.0	5.1	5.0	0.238
4.0	4.9	4.8	0.075
8.0	4.7	4.6	0.060

[a] The data represent the averages of three replications incubated for 6 weeks in stationary culture.

in the shake culture versus stationary culture experiment. Cultures on solid media were incubated at 23°C, except those for the anaerobiosis study, which were incubated at 25 to 27°C. The radial growth of 14 to 42 colonies of *A. coenophialum* in petri dishes was averaged to measure the effects of pH, temperture, and KH_2PO_4 levels; the initial pH in the last two studies was 6.5, and in the pH study it was the variable. In the anaerobiosis studies, the cultures were incubated in an Analab-6 anaerobic chamber (Bio-Safe Corp., Miami, Fla.) with a gas mixture of 85% nitrogen, 10% hydrogen, and 5% carbon dioxide.

Growth determinations. Determinations of mycelial dry weight were made after the cultures were filtered onto tared filter papers, dried at 50°C for 12 h, and stored in a desiccator for 24 h. Determinations of growth on agar plates were made by measuring the radial increase in diameter from the edge of the 4-mm plugs. In the anaerobiosis studies, growth was measured after 4 and 6 days for the aspergilli and after 18 days for *A. coenophialum*.

TABLE 5. Influence of various levels of KH_2PO_4 on growth of *A. coenophialum*

KH_2PO_4 concn (%)	No. of colonies	Radial growth (mm)[a]
0	20	11.98
0.05	29	12.41
0.1	26	12.48
0.2	26	12.44
0.4	17	13.71
0.6	30	13.40
0.8	30	13.25
1.2	30	13.40
1.6	21	12.64
3.2	24	11.00
6.4	14	10.07
12.8	21	5.26

[a] The data represent the averages for plate colonies incubated for 6 weeks in media adjusted to pH 6.5.

TABLE 6. Influence of pH on radial growth of *A. coenophialum*

pH	Radial growth (mm)[a]
5.0	3.40
5.5	6.10
6.0	7.65
6.5	8.10
7.0	7.15
7.5	7.25

[a] The data represent the averages for 16 to 24 petri dish colonies (on potato dextrose agar) incubated for 5 weeks at 23°C.

RESULTS

The effects of various levels of glucose on the growth of *A. coenophialum* are shown in Table 1. In 6 weeks, a biomass of only 0.089 g per flask was produced in stationary cultures of glucose-free basal medium consisting of 0.7% yeast extract plus traces of nutrients introduced with the inoculum. An average mycelial dry weight of 1.125 g per flask was obtained with 3% glucose, compared with 1.058 g per flask with 6% glucose and only 0.778 g per flask with 12% glucose.

The effects of various yeast extract levels are shown in Table 2. In a 6% glucose basal medium without added yeast extract, 0.081 g of mycelium per flask was produced. Growth increased to 0.163 and 0.162 g per flask, respectively, when 0.35 and 0.70% yeast extract were added to the basal medium but declined in increments when 1.4 and 2.8% yeast extract were added.

When various salts were added to a glucose and yeast extract medium, growth was increased 24 and 13% by KCl and $(NH_4)_2HPO_4$, respectively, but was decreased 13 and 31% by NH_2Cl and KH_2PO_4, respectively (Table 3). The effects of various levels of KH_2PO_4 were examined further. The results of a study on the effect of added KH_2PO_4 on the growth of *A. coenophialum* are presented in Table 4. Both growth and pH were clearly suppressed by added KH_2PO_4.

The influence of KH_2PO_4 levels on the radial growth of *A. coenophialum* on agar medium is shown in Table 5. In all treatments, the medium was adjusted initially to pH 6.5. The results are similar to those presented in Table 4 except that no apparent reduction in colony growth occurred until the KH_2PO_4 level reached 3.2% or higher.

The data in Table 6 show that growth of *A. coenophialum* was quite sensitive to pH. The most growth occurred at a pH of 6.5. A pH either greater or less than 6.5 resulted in significant reductions in the radial growth of the fungal colonies.

Temperature also greatly influenced the growth of *A. coenophialum* (Table 7). Optimum growth occurred in a range of 20 to 26°C, with less growth occurring at tempera-

TABLE 7. Influence of temperature on radial growth of *A. coenophialum*

Temp (°C)	Radial growth (mm)[a]
11	1.01
14	3.70
17	4.94
20	9.25
23	10.51
26	9.06
29	3.87
32	0.00

[a] The data represent the averages for 17 to 42 petri dish colonies (on potato dextros agar) incubated for 6 weeks at pH 6.5.

TABLE 8. Growth of *A. coenophialum*, *Aspergillus terreus*, and *Aspergillus flavus* under aerobic and anaerobic conditions

Fungus	With oxygen		Radial growth (mm)	Without oxygen		
	Days	No. of colonies		Days	No. of colonies	Radial growth (mm)
Acremonium coenophialum	18	56	6	18	50	0[a]
Aspergillus terreus	6	48	28	6	60	0[b]
Aspergillus flavus	4	11	30	9	50	0[b]

[a] No growth when returned to ambient O_2.
[b] Normal growth when returned to ambient O_2.

tures above and below 23°C. The fungus grew more rapidly at 20 than at 26°C.

The data for comparisons of shake cultures with stationary cultures showed a five-flask average growth of only 0.04 of *Acremonium* mycelial dry weight per flask in stationary cultures versus 0.37 g of mycelium per flask in cultures shaken at 100 rpm.

Under aerobic conditions, *Aspergillus flavus* and *Aspergillus terreus* had an average radial growth of 28 to 30 mm in 4 to 6 days compared with 6 mm in 18 days for *A. coenophialum* (Table 8). None of these fungi grew under the anaerobic conditions of the experiment; the two aspergilli initiated normal growth when they were returned to ambient O_2, whereas *A. coenophialum* did not survive anaerobiosis.

DISCUSSION

Because of the slow growth of *A. coenophialum*, major difficulties are encountered in maintaining pure cultures for the extended periods required in most investigations with this organism. Any contaminant, however small the initial cell or spore load, inevitably outgrows *A. coenophialum*. Thus, extreme caution must be used to prevent contamination when working with this fungus, especially when scaling up fermentations to obtain large quantities of secondary metabolites or mycelium for various analyses. Therefore, for practical as well as academic reasons, it is important to understand the parameters that affect the growth of the fungus.

The results of our investigation indicate that *A. coenophialum* ET-26 differs considerably from fungi such as *Aspergillus flavus*. For example, in sucrose and yeast extract media, the growth of *Aspergillus flavus* increased with each increment of sucrose and yeast extract and growth did not decline at higher levels (4). On the other hand, *A. coenophialum* produced notably less biomass if carbohydrate was increased above 6% and yeast extract levels exceeded 0.70%. Growth (mycelial dry weight) was about the same at 3 and 6% glucose and at 0.35 and 0.70% yeast extract in stationary cultures of liquid media (Tables 1 and 2). *A. coenophialum* appeared to grow optimally in dilute media, whereas *Aspergillus flavus* and *Aspergillus terreus* grow optimally in enriched media.

When KH_2PO_4 was added to the basic glucose and yeast extract medium, the growth of *A. coenophialum* declined when levels reached or exceeded 3% in both liquid and solid media. The substitution of other salts for KH_2PO_4 resulted in an increase of mycelial dry weight in the case of KCl and $(NH_4)_2HPO_4$ but a decrease in the case of NH_4Cl. This seemed to be due to the effects of these salts on the pH of the medium. The data in Table 6 show that growth fell off rapidly as the pH decreased below 6.0. In the case of added KCl and $(NH_4)_2HPO_4$, the pH of the medium was higher than that of the basal medium and growth was somewhat greater. Potassium phosphate lowered the medium pH, and growth was decreased. The pH remained approximately the same when NH_4Cl was added, and consequently growth was not impaired. Thus, the decrease in the yield of mycelium shown in Table 4 may have been due primarily to a pH effect since there was relatively little inhibition of *A. coenophialum* growth at KH_2PO_4 concentrations between 0 and 6.4% at pH 6.5 (Table 5), whereas growth decreased rapidly between pH 5.5 and pH 5 (Table 6).

A. coenophialum appeared to have slightly lower and narrower temperature range than do many mesophilic fungi. It grew comparatively well over a range of 20 to 26°C but preferred a temperature near 23°C. Also, considerably more growth occurred in shake culture than in stationary culture. Surprisingly, considering its endophytic natural habitat, *A. coenophialum* appeared to be less tolerant of anaerobic conditions than either *Aspergillus flavus* or *Aspergillus terreus* (Table 8). Although none of the cultures grew under anaerobic conditions, the *Aspergillus* cultures grew out when they were restored to aerobic conditions. The *A. coenophialum* cultures, however, did not survive. Thus, unlike *Aspergillus terreus*, which can survive in the rumen of cattle, it is unlikely that *A. coenophialum* could do so.

We concluded from this investigation that *A. coenophialum* is a unique fungus with respect to its environmental and nutritional requirements, as might be expected given its restricted natural habitat. It is not known to occur naturally except in association with toxic tall fescue grass and seed. It apparently prefers a medium with a relatively low osmotic concentration, a pH near 6.5, and a temperature of about 23°C. In laboratory media, shaken flasks are essential to obtain significant biomass production.

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