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Date January 4, 1991

Effect of Plant Growth Regulators and Other Selected Compounds on the Growth of *Acremonium coenophialum* *in vitro*

A Thesis

Persented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Gregory E. Huff

May 1991

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DEDICATION

This thesis is dedicated to my mother, Janice A. Huff, and my father, Edward E. Huff and to Dr. Kimberly Gwinn, my major professor.

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I wish to thank Dr. Kimberly Gwinn for her guidance as my major professor. At times I would go off track and do things without letting her know, so in retrospect I thank her for having the patience in dealing with me. I would also like to thank Dr. Brad Reddick for his time and forgiveness, because one time I forgot to water the greenhouses and to say the least some plants died. I would also like to take time out to thank Dr. Carl Sams from whom I have borrowed numerous pieces of equipment and for some reason or another returned the items in question back a little late; o.k. a lot later than planned. I would also like to thank Ms. Anita Gavin for her help when I needed my cultures analyzed and just being a friend, and yes that goes to her husband Jim the first Tennesseean who understood what "house music" was and surprisingly he is from Tennessee. To Brett Savary who helped me in the design of some of my experimental procedures; granted he did run his mouth a little too much but that was o.k.; at least he knew the right type of beer to buy, the cheap kind. I would also extend special thanks to the Plant and Soil Science department for permitting me to conduct some of my research in their labs. Finally I would extend a special thanks to the whole department of Entomology and Plant Pathology for their support during my many times of indecision.

ABSTRACT

A mutualistic relationship exists between the endophyte Acremonium coenophialum (Morgan, Jones and Gams) and tall fescue Festuca arundenacea (Shreb). The host, tall fescue, provides protection, a source of nutrients and a means of dissemination for the endophyte. The endophyte protects tall fescue from adverse environmental conditions, overgrazing by herbivores and insect and pathogen attack. Changes in the physiology of the fescue plant causes changes to occur in the growth of the endophyte. The purpose of this study was to examine plant growth regulators and carbohydrates to determine their effect on the growth of the endophyte.

In liquid and solid media, the synthetic auxins 2,4-D (2,4-dichloro Phenoxyacetic acid) and dicamba (3,6-dichloro 2-methoxybenzoic acid), had no observable effect on growth of A. coenophialum. Abscisic acid also had no observable effect on growth of the endophyte. When A. coenophialum was exposed to an ethylene atmosphere of 9ppm, no observable difference in growth was observed when compared with the control. Gibberellic acid (GA3) had no observable effect on growth of the endophyte after 4 weeks. However, at 6 weeks, a reduction of 40 percent was observed on solid media. In liquid M102 media (Bacon et al. 1988), growth was consistent with that of the control at concentrations of 0.1, 1 and 10 μM; however, 100 percent reduction was observed at 100 μM. The synthetic

cytokinin, kinetin, reduced the growth of A. coenophialum at each concentration examined. Growth reductions ranged from 20 percent at 20 μM to 100 percent at 100 μM. Zeatin also reduced the growth of the endophyte. A 50 percent growth reduction was observed at 100 μM. In liquid M102 media, no reduction in growth of the endophyte occurred at 0.1, 1, and 10 μM kinetin, but at 100 μM kinetin, growth was reduced by 95 percent.

Growth of A. coenophialum on media containing various carbon sources was investigated. Radial growth was higher on sucrose than on other carbon sources tested. A morphological change was noted on media containing sucrose. The endophyte grown on media containing sucrose exhibited a mucoid-like appearance, while the endophyte grown on the xylose-substituted media had a cottony appearance.

In summary, none of the plant growth regulators (PGRs) examined had a stimulatory effect on the endophyte. However, a reduction in growth was noted with cytokinins and GA3. Sucrose was a better carbon source than xylose, but changes in the morphology of the endophyte occurred when grown on media supplemented with sucrose in M102 media. The endophyte took on a mucoid-like appearance when grown on M102 media supplemented with sucrose, while cultures grown on M102 media supplemented with xylose had a white cottony appearance.

TABLE OF CONTENTS

SECTION		PAGE
I.	INTRODUCTION	1
II.	LITERATURE REVIEW	3
III.	EFFECT OF NON-GASEOUS PLANT GROWTH REGULATORS ON GROWTH OF ACREMONIUM COENOPHIALUM	9
IV.	ETHYLENE AND ITS EFFECT ON ACREMONIUM COENOPHIALUM	25
V.	GROWTH OF ACREMONIUM COENOPHIALUM ON MEDIA SUPPLEMENTED WITH VARIOUS CARBOHYDRATE SOURCES	32
VI.	CONCLUSIONS	46
BIBLIOGRAPHY		47
VITA		53

I. INTRODUCTION

A mutualistic relationship exists between the endophyte Acremonium coenophialum Morgan Jones and Gams and tall fescue (Festuca arundinacea Shreb). The host, tall fescue, provides protection, a source of nutrients and a means of dissemination for the endophyte; in return the endophyte provides protection to the host that is complex and not fully understood. The presence of the endophyte permits tall fescue to survive stressful conditions. These conditions include resistance to overgrazing by herbivores, insect attack, pathogen infection and the ability to survive severe drought conditions; however, the presence of the endophyte poses serious problems in pastures. When cattle feed on endophyte-infected (E+) pastures, animals exhibit elevated body temperatures, develop a rough coat appearance and have reduced weight gain and birthing rates (Siegal et al. 1987). Since animals fed on pastures with a low incidence of endophyte (10% or less) (Stuedemann and Hoveland 1988) do not exhibit the effect of fescue toxicosis, there have been attempts to establish pastures low in endophyte. Fields containing endophyte free (E-) fescue do not survive as well as endophyte-infested (E+) pastures; this leads to problems in establishing and maintaining these fields (Siegal et al. 1984).

In nature, the entire life cycle of the endophyte is spent within tall fescue. The endophyte is found primarily in the tiller base of the host. Infloresence triggers the mycelium to grow intercellularly through the peduncle and into the seed head. Dissemination of the endophyte occurs via the dispersal of the host seed (Siegal et al. 1987).

During the transition from vegetative to reproductive state in tall fescue, many physiological changes occur. These changes include fluctuations in both the levels and composition of carbohydrate and plant growth regulators. The objective of this study is to determine if plant growth regulators present during this transition affect <u>in vitro</u> growth of the endophyte. Growth studies were conducted both on solid media and in liquid cultures. Analysis of the cell wall of tall fescue in both vegetative and reproductive stages were analyzed. The possibility of other compounds contributing to an altered growth rate was also examined. Ethylene production by the endophyte was evaluated.

II. LITERATURE REVIEW

CLASSIFICATION

Endophytic fungi are grouped in the tribe Balansiae of Clavicipitaceae (Siegal et al. 1987). The endophyte of tall fescue was first isolated and identified by Neill (1941) and reported as the anamorph of Epichloe typhina (Fr) Tulasne. Bacon et al. (1977) corroberated Neils findings and identified the endophyte as Sphacelia typhina (Sacc.). Morgan-Jones and Gams (1982) classified the endophyte of tall fescue as Acremonium coenophialum. The endophyte of tall fescue may have some relationship to the anamorph Epichloe, but they are two different genera (Morgan-Jones and Gams 1982). The endophyte of tall fescue appears to have either temporarily or permanently lost its ability to form sexual spores, but asexual spores (conidia) are observed when A. coenophialum is grown on artificial media. The appearance of the endophyte when grown on artifical media may take on a white cottony texture growing very slowly over time. At times, phialides arising solitarily from aerial hyphae may be observed. On sterile tall fescue seedlings, the endophyte sporulates prolifically with the production of synnemata (White and Cole 1985).

TALL FESCUE

Tall fescue is believed to have had its origins in Western Europe (Stuedemann and Hoveland 1988). The perennial

grass found its way to the new world as a contaminate of other seed stocks. Today, tall fescue is an important forage grass covering well over 35 million acres in the United States alone (Shelby and Dalrymple 1987). The popularity of this temperate grass is due to 1) ease and wide range of establishment, 2) wide range of adaptation, 3) long grazing season, 4) tolerance to abuse, 5) pest resistance, 6) good seed production and 7) its excellent appearance. Within the United States, areas of major use are Virginia, Kentucky, Missouri, the Carolinas, Tennessee, Arkansas and the northern parts of Mississippi, Alabama and Georgia (Walton 1983).

DISTRIBUTION

Tall fescue can be found throughout the United States; however, the incidence of endophyte-infected fescue varies greatly from western to eastern United States. Shelby and Dalrymple (1987) found that infection levels vary from 17% in the western regions to 73% in eastern regions; however, low estimates of percentage infection within the western regions may be due to the presence of various cultivars of fescue (Shelby and Dalrymple 1987.). The highest incidence of endophyte-infected fescue occurs in the eastern and southeastern United States. In the state of Tennessee 97.1% of the counties contained infected tall fescue (Long and Hilty 1985).

DETECTION METHODS

Many techniques have been developed to detect the endophyte in fescue. These methods include staining techniques, bio assays, enzyme-linked immunosorbent assay (ELISA), and endophyte grow-out tests. Bioassays involve the introduction of insects, who feed on fescue, to sections of the plant that are either infected or not infected by the endophyte. A common insect used in these experiments is Rhopalosiphum padi (Siegal et al. 1988). These aphids will feed on endophyte-free fescue while leaving the endophyte-infected fescue alone. Staining techniques (using 0.06% analine blue in 33% lactic acid) are used to determine the presence of the endophyte in the aleurone cells of the tiller base (Conger et al. 1986). Intercellular "serpentine like" hyphae are observed within the host. Estimations of viable endophyte levels in seed lots can be determined only by grow-out tests. In one procedure, fescue seeds are scarified in 60% sulfuric acid and then surface sterilized with 50% clorox solution. Seeds are then rinsed with sterile double distilled water and allowed to imbibe water over night. Seeds are plated onto SH-20 media (Schenk-Hildebrandt media augmented with 20 μm dicamba). After 4-6 weeks, the endophyte can be observed growing from callus (Conger et al 1986). The most commonly used method is to grow seedlings for 8-10 weeks then test by either PAS-ELISA or staining.

LIFE CYCLE

The endophyte of tall fescue is a true endophyte in that its entire life cycle is contained within the host. In the vegetative phase of tall fescue, the endophyte lies intercellularly in the spaces of the leaf mesophyll parynchyma and chlorenchyma of the shoot apex and leaf sheath (Bacon et al. 1977). With the onset of flowering, the endophyte grows intercellularly up the peduncle and floral apices where it penetrates the tissue of the ovary and ovule. The endophyte becomes widespread within the seedling once germination is initiated (Philipson and Christey 1986).

STRUCTURE

When observed under a light microscope, hyphae appear coarse, contorted and usually unbranched, except in areas where lateral buds appeared. The fungus is located primarily in the meristematic tissue of the shoot base and leaf sheath. Hyphae are not observed in the leaf blade (Bacon et al. 1977).

Hyphae of the endophyte grown on artificial culture appeared highly vacuolated when examined with an electron microscope (Siegal et al. 1987). These vacuoles were often seen with small amounts of electron dense material. Cytoplasmic organelles were difficult to discern. Within a natural host, the hyphae of the endophyte was not as vacuolated with very few deposits. Organelles were distinct

vacuolated with very few deposits. Organelles were distinct and were easy to identify (Siegal et al. 1987).

BENEFITS

A mutualistic relationship exists between the endophyte A. coenophialum and its host, tall fescue. The endophyte is protected from external factors and is disseminated by seed. A nutrient source is also provided by the host. The host also benefits from the presence of the endophyte. These benefits include the plant's ability to endure increased stress conditions. Stress tolerance is manifested by the plant's increased ability to resist insect attack, to survive severe drought conditions, to resist overgrazing by herbivores and protection against pathogens eg. fungi, and nematode attack. These characteristics are believed to be related to the secondary metabolites produced by the fungus and/or plant in response to the fungus. Chemical compounds known to be produced by the endophyte include ergopeptide alkaloids (predominantly ergovaline) and pyrrolizidine alkaloids which are involved in insect resistance (Siegal et al. 1987).

SIGNIFICANCE

Tall fescue is the primary grass used in many pasture lands because of its high nutritional value, easy establishment and tolerance to adverse conditions. Fescue toxicosis, which is correlated with high endophyte incidence in these pastures, results in beef cattle production losses of

200 to 500 million dollars (Stuedemann and Hoveland 1988). Symptoms of fescue toxicosis in cattle grazing on infected fescue include fescue foot, summer syndrome and poor performance associated with elevated climatic temperatures, fat necrosis and agalactia (reduced milk production) (Belesky et al. 1988). Symptoms begin with loss of weight, rough hair coat, arched back and soreness in one or both hind limbs. Summer syndrome includes elevated body temperatures and increased respiration rates, reduced reproductive rates, nervous disorders and in severe cases, sloughing off of the extremities. Currently, it is unknown if the symptoms expressed by animals feeding on infected fescue are caused by one or more toxins produced by the endophyte (Hemkin et al. 1984).

CONTROL

Treatment with some fungicides can partially control the endophyte. Since the only means of dissemination of the endophyte is via seed, the best form of control is the production of endophyte-free (E-) seeds (Siegal et al. 1985). Exposing seeds to high temperatures for a short period of time has also provided effective control of endophyte, but this method of control results in reduced seed germination rates. Storage of seeds at low temperatures with zero humidity has also proven effective in the control of the endophyte (Siegal et al. 1985).

III. EFFECT OF NON-GASEOUS PLANT GROWTH REGULATORS ON GROWTH OF ACREMONIUM COENOPHIALUM

ABSTRACT

The endophyte, A. coenophialum, remains within the tiller base of the host, tall fescue, during the vegetative state. When inflorescence occurs, the endophyte grows within the peduncle and eventually into the seeds. The purpose of this study was to determine if plant growth regulators (PGRs) affect in vitro growth of the endophyte. Dicamba, a synthetic auxin, had no effect on the endophyte at the concentrations studied. Cytokinins, kinetin and zeatin, reduced growth of the endophyte by 80% at 80 μM. No growth occurred at 100 μM. Gibberellic acid also reduced the growth of the endophyte at 100 μM.

INTRODUCTION

A mutualistic relationship exists between the endophyte, A. coenophialum, and tall fescue. The host provides an environment in which the endophyte is protected from external stresses, and a food source is provided by the host. The endophyte, in return, provides protection from overgrazing by herbivores, insect resistance, and resistance to drought.

During the vegetative state, the endophyte remains

primarily within the tiller base of the host. However, when inflorescence occurs, the endophyte grows in a serpentine-like fashion (intercellularly) up the peduncle and eventually into the seed head. The only known means of dissemination occurs via the seeds (Siegal et al. 1984).

During the transition from vegetative to reproductive states, many physiological changes occur. PGRs play an integral part in the transition of the host from vegetative to reproductive states. The major classes of PGRs to date include auxins, cytokinins, gibberellic acid, abscisic acid and ethylene. Current research on PGRs has been reviewed by Davies (1987); to date, modes of action of the PGRs are still not fully understood. Auxins are primarily involved in cell enlargement and stem growth; gibberellins are involved in stem growth (hyperelongation) and induction of seed germination. Cytokinins, in the presence of auxins, cause cell division and morphogenesis. Ethylene, a gaseous PGR, causes shoot and root differentiation, leaf and fruit abscission and flower and leaf senescence. Abscisic acid, which is exported from the leaves in the phloem, is involved in the regulation of stomatal closure and the induction of photosynthate transportation towards the developing seeds.

Little is known about the effects of PGRs on microorganisms. IAA and NAA promote cell elongation in a respiration-deficient mutant strain of Saccharomyces

ellipsoideus (Yanajishima 1965.). Kamisaka et al. (1967) suggested that yeast cells have the same cell regulation mechanisms as plants. IAA and GA promote sporulation in yeast (Elwy and Elwy 1989). Mayr et al. (1984) found that auxin, gibberellic acid (GA3) and kinetin retarded vegetative growth and the fruiting of some macromycetes. Elwy and Elwy. (1989) studied the effects of PGRs on Dipodascopsis uninucleata Batra and Milner (Biggs), and found that the number of cells increased when concentrations below 10^{-4} M IAA was added to the media. All concentrations of kinetin and 2,4-D increased the average number of cells/hyphae. GA3 at 10^{-2} M inhibited vegetative growth (Elwy and Elwy 1989).

The objective of this study was to determine the effect of PGRs on the growth of the endophyte in vitro. Studies were conducted in both liquid and solid media.

MATERIALS AND METHODS

Reagents

Dicamba, gibberellic acid (GA3), abscisic acid, kinetin and zeatin, were obtained through Sigma Chemical Corporation (St. Louis, MO.) Yeast extract, malt extract and glucose were obtained from Difco (Detroit, Michigan), and the corn meal agar was obtained from BBl Industries (Cockysville, MD.).

Media and culture conditions:

Organism. A. coenophialum was isolated from callus culture initiated from tall fescue seeds (Conger et al. 1986). Isolates were transferred to potato dextrose agar (PDA) media and permitted to grow for 6 to 8 weeks. Mycelial plugs were cut from the perimeter of the colony with a sterile 5 mm cork borer.

Solid media. Corn meal malt agar (CMM) was prepared as described by Bacon et al. (1979) and augmented with plant growth regulators in concentrations of 20, 40, 80 and 100 μM. Plant growth regulators used were dicamba, kinetin, zeatin, gibberellic acid and abscisic acid. Zeatin was added to media, after autoclaving, by filtering through a 0.45 μm nylon acrodisc (Gelman Scientific Ann Arbor, Michigan). Cultures were established by inverting a 5 mm mycelial plug onto the media and sealing with parafilm. Cultures were stored in the dark at room temperatures (ca. 25 C) for 4 and 6 weeks. Colony radii were measured in four directions from the center of the plug and averaged.

Liquid media. M102 media (Bacon et al. 1988) was used. In PGR studies, media was adjusted to a pH of 6.0. Media was augmented with plant growth regulators at concentrations of 0.1, 1, 10 and 100 μM. PGR's used include dicamba, 2,4-D, kinetin and gibberellic acid.

Establishing a growth curve. The growth rate of the endophyte in liquid M102 media was determined to establish an optimal time for culture harvest. The endophyte was grown in shake culture in M102 media (25ml) for 3 weeks in a 125 ml flask. Mycelium was macerated in a high speed blender for 2 minutes, and 2 ml of macerate was added to 50 ml of M102. Suspensions were grown for 7 to 10 days on a rotary shaker at 150 rpm. Cultures were harvested and centrifuged (3,000 rpm for 30 minutes). The pellet was suspended in sterile double distilled water to give a final concentration of 1 ml packed mycelial volume per 2 ml. A 2 ml aliquot was added to each of 20 flasks containing 50 ml of M102. Cultures were grown as before. Every three days two flasks were harvested and pH, packed cell volume (PCV) and dry weights were recorded.

PGR studies. A 2 ml aliquot of mycelial suspension (1 ml PCV/2ml) was prepared. Ten ml were added to 50 ml of M102 media in each 250 ml Erlenmeyer flasks. Flasks were covered with aluminum foil and placed on a orbit rotary shaker (150 rpm). Cultures were grown for 15-18 days. Cultures were removed from shaker and centrifuged (9,000 rpm) Pellets were placed on pre-weighed filter paper and dried at 60 C. Papers were stored in desiccator for 24 hours and then weighed. Liquid culture studies were carried out in a lab at room temperature (ca. 25 C). Radial measurements were taken by using a ruler and measuring from the center of the mycelial

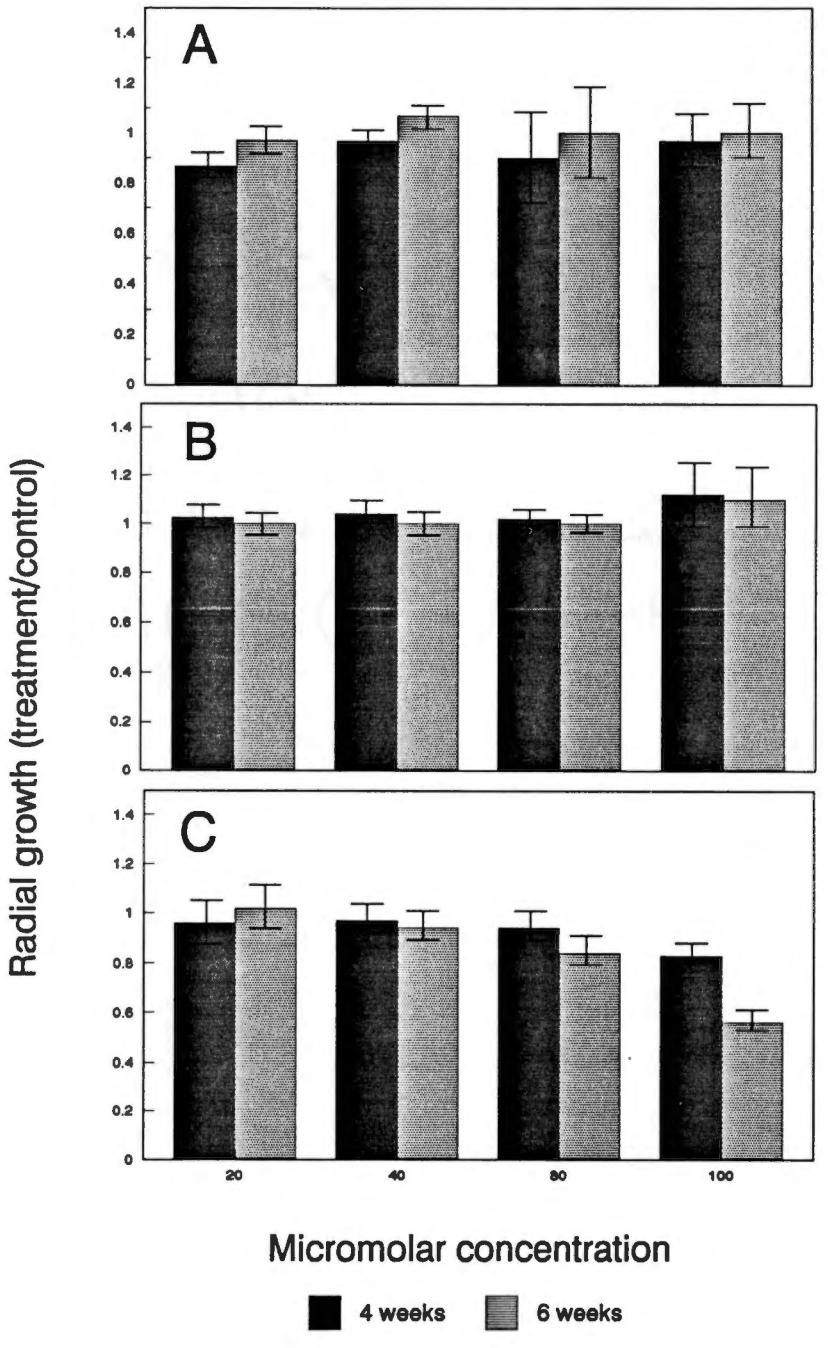
plug to the edge of the actively growing colony. Values for each treatment were calculated by dividing the average radial measurement of the treatment by the average radial measurement of the control. Treatment/control values are the averages of 3 experiments. Values were obtained for studies in liquid culture in a similar manner except that dry weight measurements were used.

RESULTS

Auxins. Mean value for the diameter of colonies grown on CMM with no added auxins was 0.063 ± 0.04 cm after 4 weeks and 1.08 ± 0.06 cm after 6 weeks. There was no observable difference in the growth of the endophyte colony on CMM media augmented with 20, 40,80, or 100 μM dicamba; i.e. treatment/ control did not significantlly differ from 1 (Fig 1A). The radial measurements of the endophyte colony at 20 μM after 4 weeks and 6 weeks were 0.6 ± 0.05 cm and 1.01 ± 0.17 cm, respectively. At 100 μM the radial measurement of the endophyte colony ranged from 0.62 ± 0.09 cm at 4 weeks and 1.08 ± 0.14. cm at 6 weeks.

Abscisic acid. In this study, mean values of radial growth for colonies grown on CMM without added abscisic acid was 0.895 ± 0.11 cm after 4 weeks and 1.2 ± 0.42 cm after 6 weeks. For all treatments, values for treatment/control, examined did not significantlly differ from 1 (Fig 1B).

Fig 1. Effect of varying μM concentrations of various PGRs on Radial growth of Acremonium coenophialum. Treatments were on CMM agar supplemented with 20, 40, 80 or 100 μM PGR. (A) dicamba, (B) abscisic acid and (C) gibberellic acid (GA3). Each treatment was replicated 6 times/ experiment. For each experiment a single value for treatment/control was determined. Average value for each treatment; values shown are the mean of three experiments. Lines represent standard error of the mean (SEM).

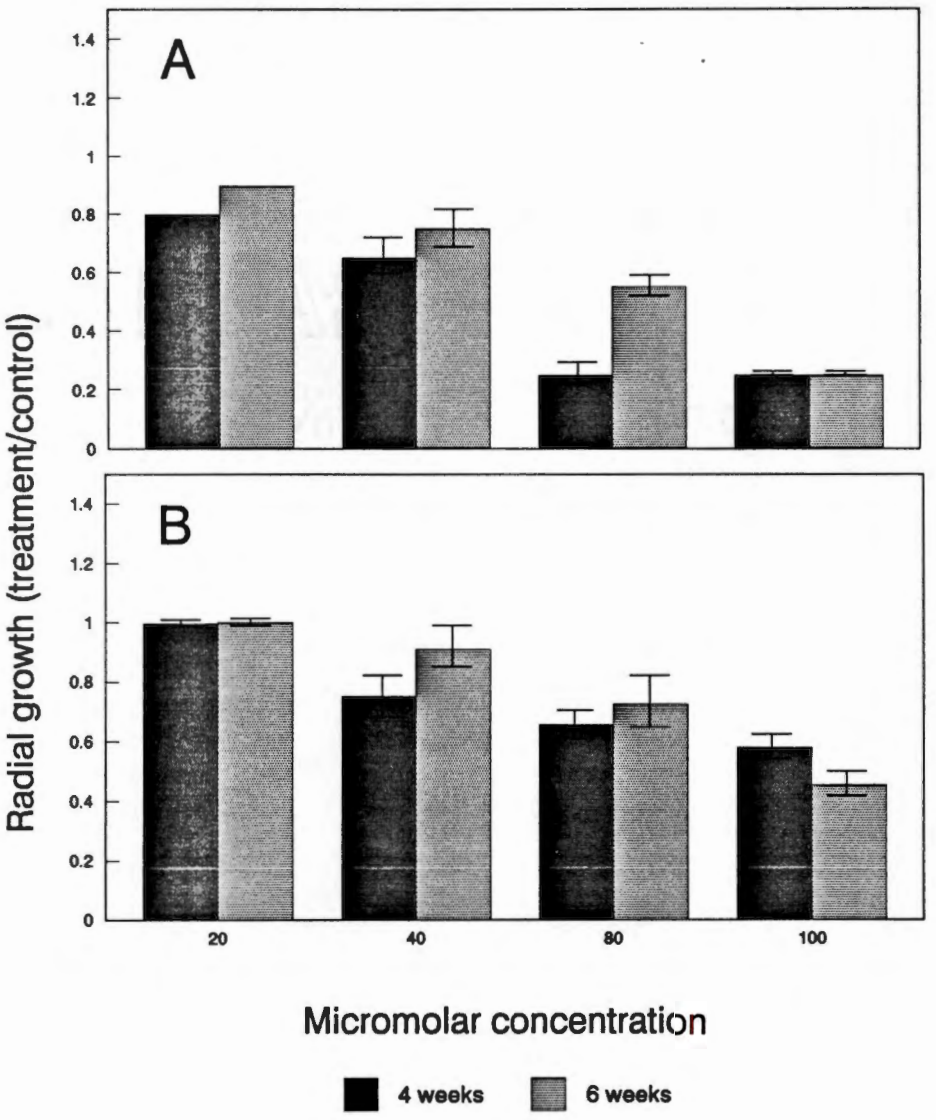

A
B
C
1.4
1.2
1
0.8
0.6
0.4
0.2
0
20
40
80
100
Radial growth (treatment/control)
Micromolar concentration
4 weeks
6 weeks

Radial measurements of the endophyte colony ranged from 0.92 ± 0.08 cm at 20 μM to 0.99 ± 0.35 cm at 100 μM after 4 weeks (Fig. 1B).

Gibberellic acid (GA3). The mean value of radial growth of colonies grown on CMM without added GA3 was 0.98 ± 0.03 cm and 1.65 ± 0.13 cm after 4 and 6 weeks, respectively. After a 4 weeks, colony radius appeared to be relatively consistant at each concentration examined. Colony size at 20 μM was 0.97 ± 0.09 cm, and 0.81 ± 0.02 cm at 100 μM GA3. In media containing 100 μM; however, at 6 weeks values for treatment/control were different from 1 (Fig. 1C). A 57 percent reduction in growth was observed when compared to the control (control= 1.65 ± 0.13 cm and 0.94 ± 0.11 at 100 μM) and the morphology of the endophyte changed to a brownish color and was mucoid-like in appearance.

Cytokinins. The mean value of the colonies grown on CMM without added kinetin was 0.88 ± 0.17 cm and 1.13 ± 0.12 cm after 4 and 6 weeks, respectively. The mean value of colonies without zeatin was 0.91 ± 0.08 cm and 1.17 ± 0.15 cm after 4 and 6 weeks, respectively. Kinetin and zeatin had a significant effect on the growth rate of the endophyte. For each concentration examined, value for treatment/control was different than 1 (Fig 2A). At four weeks, a 20 percent reduction was observed at 20 μM when compared with the control. A 30 percent reduction was observed at 40 μM and 100

Fig 2. Effect of varying μM concentrations of selected cytokinins on radial growth of Acremonium coenophialum on CMM agar. Treatments were 20, 40, 80 or 100 μM PGR. (A) kinetin and (B) zeatin. Each treatment was replicated six times/ experiment. For each experiment, a single value for each treatment/control was determined from an average value for each treatment; values shown are the mean of three experiments. Lines represent standard error of the mean (SEM).


A
B
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Radial growth (treatment/control)
20
40
80
100
Micromolar concentration
4 weeks
6 weeks

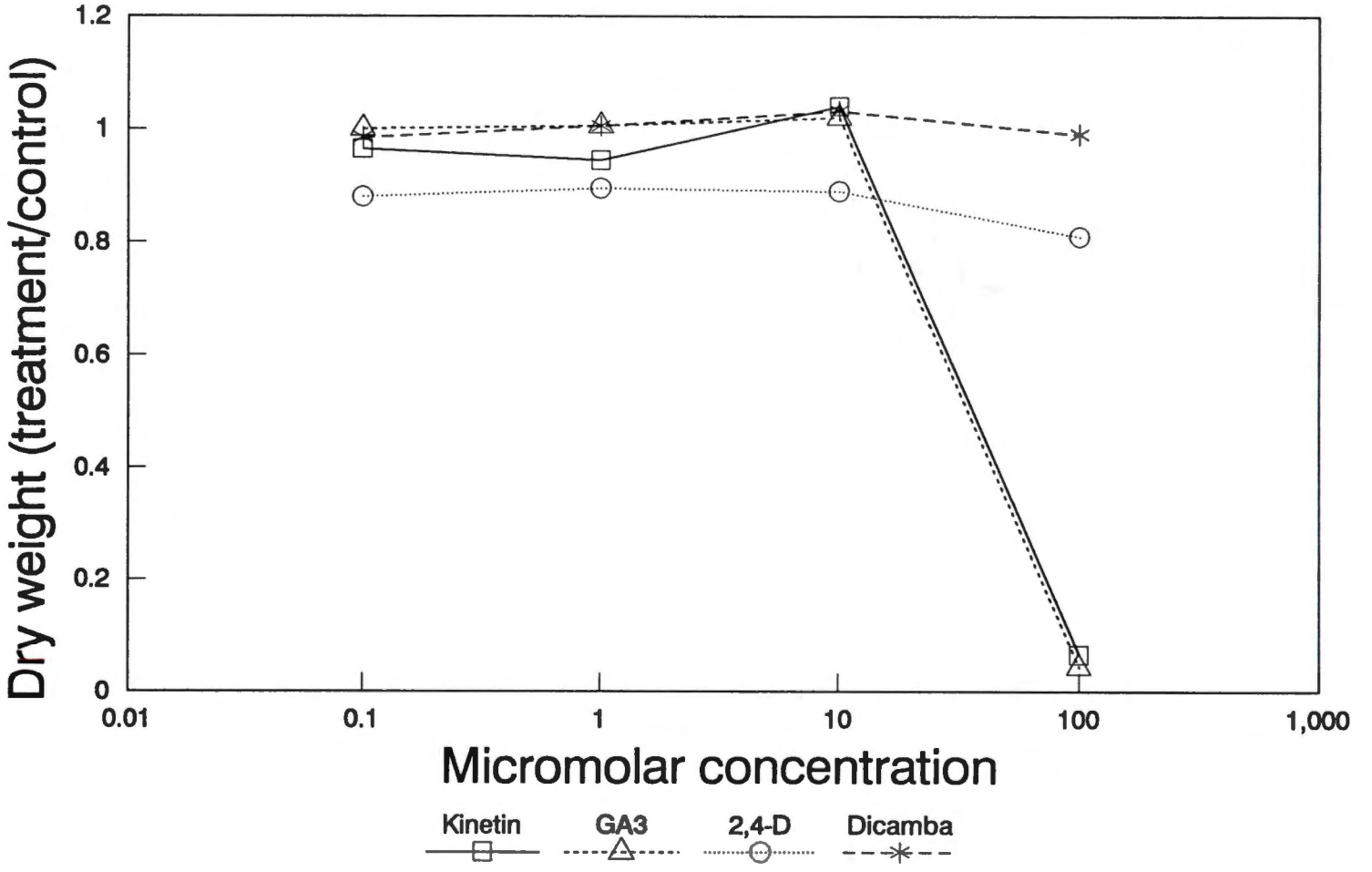
percent reduction at 80 and 100 μM. At six weeks, growth was reduced by 10 percent at 20 μM when compared to the control, by 30 percent at 40 μM and 44 percent at 80 μM. No growth occurred at 100 μM. Zeatin also reduced the growth of the endophyte (Fig 2B). The affect of zeatin was observed at 40 μM in which a 20 percent reduction was noticed, when compared with the control. At 100 μM a 42 percent reduction in growth occurred after 4 weeks, and after 6 weeks a 45 percent reduction was observed.

Liquid studies.

Growth curve. The rate at which the endophyte grows in liquid M102 media was determined by growing and harvesting cultures at 3 day intervals. Our studies revealed that our isolate had a lag phase of about 9 days prior to the log phase of growth. The log phase of growth was from 11 to 18 days. After 18 days, the endophyte growth was stationary.

Auxins. A. coenophialum grown in liquid M102 media had very little variation in dry weight of the mycelia of the endophyte. Values for treatment/control did not differ from 1 in M102. Growth of the endophyte in culture remained virtually the same when the endophyte was supplemented with 0.1, 1, 10 or 100 μM dicamba (Fig. 3). Dry weight of the control was 0.763 ± 0.4 g, whereas dry weight of the mycelium in media

Fig 3. Effect of varying concentrations of PGRs in M102 liquid media. Cultures grown in shake culture (150 rpm) for 18 days. Concentrations of kinetin, GA3, 2,4-D and dicamba were 0.1, 1, 10 or 100 μM. Each treatment was replicated five times/ experiment. For each experiment, a single value for treatment/control was determined from average value for each treatment; values shown are the mean of three experiments.


Dry weight (treatment/control)
1.2
1
0.8
0.6
0.4
0.2
0
0.01
0.1
1
10
100
1,000
Micromolar concentration
Kinetin
GA3
2,4-D
Dicamba

containing 100 μM dicamba was 0.752 ± 0.39 g. Also, 2,4-D had no observable effect on the endophyte grown in liquid M102 media. Average dry weight of the endophyte in the control media was 1.13 ± 0.11 g. Average dry weight of the endophyte exposed to 100 μM 2,4-D was 1.0 ± 0.13 g.

GA3. The mean dry weight of the endophyte grown in culture without added GA3 was 1.68 ± 0.1 cm. In liquid culture studies, mycelial dry weight of the endophyte was consistent amoung treatments less than 100 μM. However, in cultures containing 100 μM, the mycelial weight of the endophyte was 0.041 ± 0.13 g. A 97.5 percent reduction in growth occurred at 100 μM GA3.

Cytokinins. In liquid culture, kinetin had no observable effect on the endophyte at 0.1, 1, or 10 μM. At 100 μM a reduction in growth of the suspension culture was noted. Kinetin reduced the growth of the endophyte by 94.4 percent at 100 μM (0.035 ± 0.005 g) when the cultures were compared to the control cultures grown without added cytokinins (0.626 ± .055 g) (Fig 3).

DISCUSSION

A. coenophialum was tolerant of extremely high concentrations of some PGRs. Dicamba and abscisic acid had no effect on growth of the endophyte at concentrations that are equal to or higher than that found in vivo for some grasses.

In liquid culture, 2,4-D had no effect on endophyte growth. In experiments with other fungi, the synthetic auxin 2,4-D was found to be inhibitory at high concentrations. At lower concentrations either a stimulatory or no effect was observed (Elwy and Elwy 1989). Gibberellic acid reduced the growth of the endophyte at high concentrations, but had no effect at lower concentrations. Elwy and Elwy (1989) found that gibberellic acid inhibited growth of Dipodascopsis uninucleata at 10^{-2} M, but at lower concentratons, growth was increased. Reduction in growth was also induced in C. lunata at $2.9*10^{-4}$ M gibberellic acid, and growth increased with the addition of lower concentrations of gibberellic acid (Elwy and Elwy 1989). In this study, kinetin and zeatin reduced the growth of the endophyte at concentrations greater than 10 μM in liquid culture. At lower concentrations no effect was observed.

In conclusion, the PGRs examined had no stimulatory effect on the endophyte. No effect was observed with the synthetic auxins, dicamba and 2,4-D, or with abscisic acid. Reduction in growth was observed with high concentrations of the cytokinins, kinetin and zeatin, and with gibberellic acid (GA3). However, concentrations that inhibited the endophyte are not present in plants.

IV. ETHYLENE AND ITS EFFECT ON ACREMONIUM COENOPHIALUM

ABSTRACT

The endophytic fungus, A. coenophialum, was exposed to an ethylene atmosphere of 9 ppm for six weeks. Ethylene had no effect on the growth of the endophyte. Ability of the endophyte to produce ethylene was examined. ACC and l-methionine were added to CMM and M102 media, no significant amounts of ethylene were produced. Also, ACC and l-methionine had no effect on growth.

INTRODUCTION

Ethylene is an endogenous plant growth regulator produced by healthy plants as well as by some microorganisms (Axelrood et al. 1980). When plants become injured or diseased, plants produce ethylene in increasing amounts. Some fungi are also capable of producing ethylene, [e.g. Cylindrocladium floridanum (Sobers and C.P. Seymour), Cylindrocladium scoparium (Morg.), Fusarium oxysporum (Schlechtend) and Colletotrichum musae (Berk. and M.A. Curtis]. Bacterial and fungal plant pathogens produce ethylene to enable them to effectively infect and develop within a host system (Axelrood-McCarthy et al. 1981).

L-methionine is the primary compound utilized in the formation of ethylene via S-adenosylmethionine (SAM). In plants, this compound is cleaved to form 5'-S-methyl-5' thioadenosine and a cyclic amino acid, 1-aminocyclopropane 1-carboxylic acid (ACC). ACC is the immediate precursor to ethylene in plant systems (Moore 1979; Davis 1988). Microorganisms also utilize methionine in the formation of ethylene, and ethylene can also be synthesized by dehydration of ethanol (Wareing and Phillips 1978.). Other amino acids which microorganisms use to produce ethylene include beta-alanine, aspartic acid, cysteine, glutamic acid, glycine, homocysteine and serine (Arshad and Frankenberger 1989).

The purpose of this study was first to determine if ethylene affected growth of the endophyte and second, to determine if l-methionine or ACC could be used by the endophyte to produce ethylene. Effect of these compounds on the growth of the endophyte was also determined.

MATERIALS AND METHODSDS

Ethylene gas and standard air cylinders were obtained from MG Industries (Knoxville, TN). Corn meal malt agar (CMM), malt extract and yeast extract were obtained from Difco (Detroit, Michigan). ACC and l-methionine were obtained from Sigma Chemical Corp. (St. Louis, MO).

Culture conditions:

Organism. A. coenophialum was isolated from callus culture initiated from seeds (Conger et al. 1986). Isolates were transferred to PDA media and permitted to grow for 6 to 8 weeks. Mycelial plugs were cut from the perimeter of the colony with a sterile 5 mm cork borer.

Ethylene gas. Ethylene, 100 percent, was mixed in a standard air cylinder until a concentration of 9ppm ethylene was reached (Saltveit and Pilley 1977).

Mycelial plugs were inverted onto CMM media and culture plates placed into 1 liter plastic containers with two outlets cut into the top. Tank air speed was adjusted so that atmosphere within the vessel was replaced every 12 hours. This experiment was allowed to continue for six weeks. Radial measurements were taken.

ACC. To determine if ethylene could be produced by the endophyte via ACC, ethylene precursors were added to the media. A solution of 1 mg/ml of ACC was prepared by dissolving in methanol. ACC was filter sterilized, through a 0.45 μm pore size nylon acrodisc (Gelman Scientific, Ann Arbor, Mich.), and added to each flask of CMM. Final concentrations of ACC in the medium were 0.1 mM, 1 mM, 5 mM, or 10 mM. A 5 mm mycelial plug was inverted onto the media, and plates were placed into a one liter container and sealed. Cultures were incubated for six

weeks at room temperature (ca. 25 C). The presence of ethylene was determined by removing a 3 ml air sample from the container; sample was injected into an ethylene gas chromatograph (Shimadzu GC-8A, Shimadzu Inc. Columbia, MD). Growth of the endophyte was determined by recording radial measurements of the colonies with a ruler.

L-methionine. A 2 ml aliquot of A. coenophialum macerate was added to 250 ml Erlenmeyer flasks containing 100 ml M102 media. The media contained 1, 3, 5 or 7 mM l-methionine. Cultures were incubated at room temperature (ca. 25 C) for 14 days. Flasks were stoppered for 12 hours and a 3 ml sample of the air within the flask was removed and injected into a gas chromatograph (Shimadzu GC-8A, Shimadzu Inc. Columbia, MD). Samples were compared with a control and the atmosphere within the room. Cultures were harvested and centrifuged. Pellet was placed on preweighed filter paper and dried in an oven (60 C) for 24 hours. Filter papers were removed and placed in a desiccator. The dry weight for each culture was then determined.

RESULTS

Ethylene gas. After 6 weeks in a atmosphere of 9 ppm ethylene, there was no differences in radial growth measurements between control cultures and the cultures exposed to the ethylene.

ACC. The endophyte grown on media with or without ACC did not produce any ethylene. Radial measurements of the colony were also taken and found to be consistent at all concentrations.

L-methionine. Trace amounts of ethylene were detected in samples taken from the flasks containing l-methionine or control medium; however, the difference between the control and the flasks containing l-methionine was minute. Dry weights of the mycelial pellet were also recorded via the method described above. No differences in weights were found.

DISCUSSION

Ethylene is known to be functional in plants at levels as low as 40 ppb with many responses occuring at 1 ppm (Smith 1976). In our studies we used a gas mixture containing 9ppm ethylene. After 6 weeks, no effect on endophyte growth occured. We suggest that ethylene has no effect on endophyte growth.

L-methionine was introduced to shake-cultures containing M102. Concentrations resembled those administered to the shake-cultures of Acremonium falciforme in the experiment by Arshad and Frankenberger (1989). After termination of the experiment, only minute traces of ethylene could be detected. In contrast, Arshad and Frankenberger (1989) found that peak conditions for ethylene production occured at 1mM. L-

methionine, between 2-10 mM was also found to increase ethylene formation by Cylindrocladium floridanum (Sobers and Seymour; Axelrood-McCarthy et al. 1981).

Production of ethylene and the time in which ethylene analysis occurs may influence the ability to detect ethylene. It has been found that maximum ethylene production by C. floridanum and C. scoparium (Morg.) occured at 24 hrs. followed by a decline of ethylene production (Axelrood-McCarthy et al. 1981). For some microorganisms [e.g. Colletochricum musae (Berk. and Curtis) and Ceratocysstis fimbriata (Ellis and Halst)], maximum ethylene production occurs during the active growth stage (Chalutz et al. 1969; Peacock et al. 1974), but for others, maximum production occurs during the growth rate decline (e.g. Penicillium digitatum) (Sacc.) (Spalding et al. 1965; Chaltz et al. 1977). Peak production of ethylene also occured at the end of the growth phase of some Fusarium species (Swart et al. 1976).

A. coenophialum grown on solid CMM media augmented with ACC failed to produce ethylene. A. falciforme also failed to produce ethylene on media with added ACC; however, ACC was used as a carbon source (Arshad and Frankenberger 1989). It has also been found that Bipolaris sorokiniana (Sacc. and Sorok.) can produce ACC via ACC synthase, but ACC is not efficiently converted to ethylene (Coleman and Hodges 1986).

In conclusion, ethylene had no effect on the growth of the endophyte. Also, ethylene was not produced by the endophyte with the addition of ethylene the precursors l-methionine and ACC. Finally, the addition of these precursors did not stimulate growth of the endophyte.

V. GROWTH OF *ACREMONIUM COENOPHIALUM* ON MEDIA SUPPLEMENTED WITH VARIOUS CARBOHYDRATE SOURCES

ABSTRACT

The plant cell wall protects the plant from a host of detrimental environmental conditions and also serves as support. Monosaccharides found in the primary cell wall of plants include glucose, mannose, rhamnose, xylose, galactose, fucose, galacturonic acid and arabinose (Frey-Wyssling 1976).

Percent neutral sugars in the tiller base and peduncle of tall fescue were determined. The neutral sugars found were rhamnose, xylose, glucose, galactose, mannose and arabinose. The peduncle and tiller base contained 21.4% and 14.8% xylose, respectively. Other neutral sugars were present in equal ratios between the peduncle and tiller base.

The endophyte was grown on media supplemented with the carbohydrates, xylose and/or sucrose. Growth was less in the cultures containing xylose than on sucrose. Cultures grown with sucrose as the primary carbon source had a mucoid-like appearance, while those grown on medium supplemented with xylose had a cottony appearance.

INTRODUCTION

The endophyte A. coenophialum grows within the intercellular spaces of its host tall fescue. During the vegetative state, A. coenophialum is found in the tiller base of the host. Once the peduncle is produced, the endophyte grows within the peduncle and into the seeds. The cause of this response is unknown.

A. coenophialum is a true endophyte in that its life cycle is completed within the host. The endophyte is not known to produce any form of sexual reproductive structure, but conidia have been reported on several complex media (White and Cole 1985). White and Cole (1985) obtained synematenous sporulation when the endophyte was grown on autoclaved tall fescue seedlings. The only means of dissemination in nature is by seed dispersal (Siegal et al. 1984).

The plant cell wall serves as a protective barrier surrounding the protoplast. Cell wall's primary function is to protect the protoplast against injury and provide skeletal support and rigidity (Frey-Wyssling 1976). Some of the principal components of the cell wall are polysaccharides. These include cellulose, xyloglucan, xylans and beta-glucans (Varner and Lin 1989). Polysaccharides are made up of many monosaccharide units. Monosaccharides exist as pentoses and hexoses. In a study of six monocots, Burke et al. (1974) found

that the primary monosaccharides in the cell wall were rhamnose, fucose, arabinose, xylose, mannose and galactose.

The purpose of this study was to determine the neutral sugar composition of the tall fescue cell wall, and to determine the growth rate of the endophyte on media supplemented with other carbon sources.

MATERIALS AND METHODS

Chemicals. All chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.)

Plants. Two year old fescue plants were grown under greenhouse conditions. The peduncle of endophyte-free plants were harvested below the seed head. The tiller base was harvested from endophyte-free (E-) non-flowering leaf tissue.

Cell wall extraction. To 50 g of fresh tissue, 100 ml HEPES-NaOH was added. Tissue was homogenized in a blender until completely macerated, and then homogenate was filtered through Miracloth. Residue was washed with 60 ml HEPES-NaOH, then with 60 ml phenol:acetic acid:water (1:1:1) to inactivate enzymes. Homogenate was removed from filter and suspended in 60 ml chloroform:methanol (1:1) for ten minutes. During that time, suspension was homogenized with a blender for 30 sec. Suspension was filtered through coarse-grade sintered-glass filter and washed with 60 ml

chloroform:methanol (1:1). Macerate was suspended in 60 ml acetone for 10 minutes then filtered through Miracloth and washed with 150 ml acetone, then vacuum filtered for 10 minutes to remove acetone. Material was placed in a glass 250 ml beaker, and dried in vacuum oven (25-37 C). Material was kept in vacuum desiccator until a constant weight was recorded (Gross 1984).

Analysis. Neutral monosaccharide components of the cell wall were analyzed by K.C. Gross (USDA/ARS, Beltsville, MD) using a capillary gas chromatograph (GC). Cell wall material was hydrolysed with 2 N trifluoroacetic acid (TFA) as described by Jones and Albersheim (1972). Neutral monosaccharides were changed into their aldononitrile acetate derivatives via the procedure by Lehrfeld (1981) and quantified by capillary GC; myo-inositol was used as the internal standard (Gross and Sams 1984).

Endophyte growth studies. M102 media (Bacon 1988) was supplied with a carbon source of either 0.09 M sucrose, 0.04 M sucrose and 0.02 M xylose, 0.04 M sucrose and 0.04 M xylose, 0.02 M sucrose and 0.04 M xylose or 0.09 M xylose. A. coenophialum was obtained from eight week old cultures grown on CMM (Bacon et al 1979). Mycelial discs were cut using a 5 mm cork borer and inverted onto media. Radial measurements were taken at 4 and 6 weeks and averaged.

A simple procedure was adapted from Stasinopoulos and

Seviour (1989) to determine if the endophyte produced an exopolysaccharide. Approximately 2 volumes of ethanol (95%) were added to culture filtrates and percipitate formation was observed.

RESULTS

Cell wall composition. Xylose content of tall fescue cell wall preparations differed between vegetative and reproductive sections (Table 1). In both, the primary neutral sugars present were rhamnose, arabinose, mannose, glucose, galactose and xylose. However, xylose was present in higher percentages in the flowering stem than in the tiller base (21.6 percent vs 14.8 percent). Other sugars found in the cell walls of the tiller base and flowering stem include rhamnose, arabinose, mannose, glucose and galactose. Arabinose concentration was 3.65 and 3.30 percent in flowering stem and tiller base respectively, and percent glucose was 2.75 in both the flowering stem and tiller base.

The neutral sugar composition of A. coenophialum cell wall was primarily glucose, with galactose and mannose present in percentages of 3.20 and 3.25 respectively. Arabinose and xylose were also present at percentages of 0.5 and 0.1. The high percentage of glucose present in the cell walls of the endophyte may be attributed to the carbon source present in the media, which was glucose. Also, cell walls of

fungi are composed of chitin, which is a complex molecule of N-acetyl glucosamine.

Endophyte growth on media supplemented with sucrose and xylose. After four weeks, growth of A. coenophialum on M102 media supplemented with sucrose differed from that grown on xylose (Fig 4). On media containing 0.09 M sucrose, the radial dimensions averaged 1.2 ± 0.04 cm after 4 weeks and 1.7 ± 0.03 cm after 6 weeks. The endophyte grown on M102 media supplemented with xylose had a reduced radial growth when compared with the endophyte colony grown on sucrose amended M102. The radial dimension of endophyte colonies grown on xylose after 4 weeks was 1.06 ± 0.049 cm. After six weeks, the radial dimension of the endophyte colony on xylose was 1.32 ± 0.03 cm.

A. coenophialum grown on M102 media containing sucrose had a mucoid-like appearance. Growth did not appear to be affected by the change in morphology. A mucilaginous compound was also observed in liquid M102 media with added sucrose, which made it extremely hard to filter. A white precipitate that was mucoid in appearance was formed when ethanol was added to the filtrate of M102 amended with sucrose (Stasinopoulos et al. 1989). Endophyte grown on media supplemented with xylose did not produce this mucoid-like appearance. The characteristic cottony appearance was formed by the endophyte grown on M102 media augmented with xylose;

Table 1. Percent composition of the neutral sugars present in the cell wall of tall fescue and Acremonium coenophialum. Percentages are the averages of two runs.

	Rhamnose	Arabinose	Xylose	Mannose	Glucose	Galactose
Peduncle	0.15	3.65	21.65	0.10	2.75	0.80
Tiller base	0.25	3.30	14.80	0.05	2.75	0.75
Endophyte	0.00	0.50	0.10	3.25	93.50	3.20

however, the endophyte did not look as "healthy/thick" as the endophyte grown on sucrose.

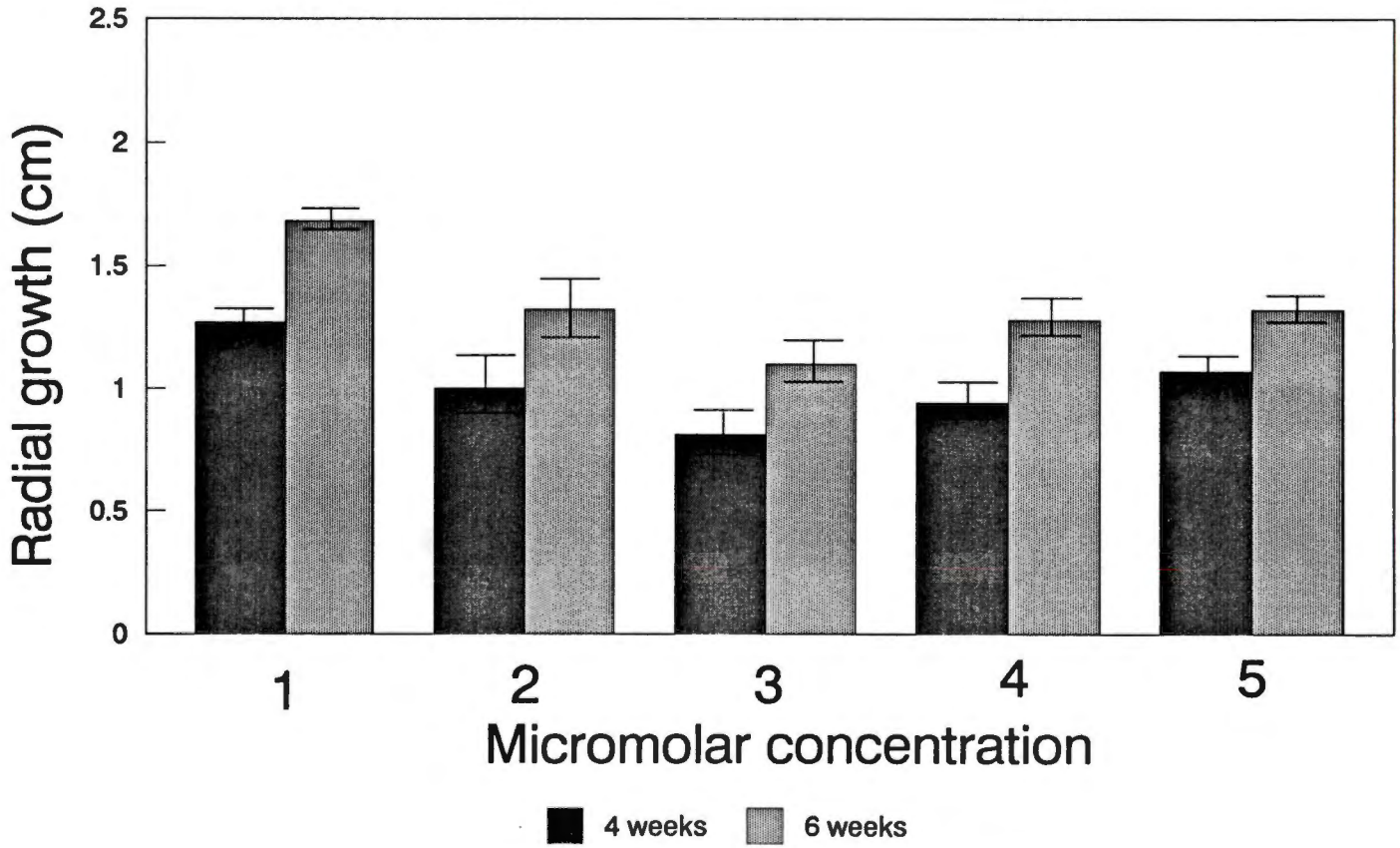
DISCUSSION

Analysis of primary cell walls of the six monocots selected by Burke et al. (1974) showed that 40 to 60% of the cell wall is made up of pentose sugars and that ratio of arabinose to xylose is 1:1. Fucose was found in the cell walls of the six monocots examined by Burk et al. (1974). In oat-coleoptile cell walls the ratio of arabinose to xylose is 1:1 (Ray 1963). In Lolium multiflorum (Lam.) the predominate monosaccharides in the endosperm cell wall are glucose, xylose and arabinose. Galactose is present in small amounts (Smith and Stone 1973).

Our studies suggest that in tall fescue xylose is the predominate monosaccharide. Only small amounts of arabinose, rhamnose, galactose, glucose and mannose were found. In A. coenophialum glucose is the predominate sugar present. This may be a result of the endophyte cultures grown on media containing glucose as the primary carbon source. Also, since many cell walls of fungi are made up of chitin, which is a complex molecule of N-acetyl glucosamine, glucose would be the predominate sugar present.

The high percentage of xylose present within the cell wall of tall fescue led us to study the ability of the

Fig 4. Effect of varying molar ratios of sucrose and xylose on the growth of Acremonium coenophialum. Treatments were M102 supplemented with either 90 μM sucrose (1) 40 μM sucrose + 20 μM xylose (2), 40 μM sucrose + 40 μM xylose (3), 20 μM sucrose + 40 μM xylose (4) or 90 μM xylose (5). Each treatment was replicated five times/ experiment. Values are the mean of two experiments. Lines represent standard error of the mean (SEM).


Radial growth (cm)
2.5
2
1.5
1
0.5
0
1
2
3
4
5
Micromolar concentration
4 weeks
6 weeks

endophyte, A. coenophialum, to grow on media containing xylose as a major carbon source. Previous studies with xylose and A. coenophialum have shown that xylose does not support growth (Kulkarni and Neilson 1986). Some fungi are capable of utilizing xylose as a carbon source. Sclerotium delphinii (Welch) can use xylose as a primary carbon source, but sucrose, glucose or fructose promoted greater growth (Perlman 1972).

Our studies show that our isolate of A. coenophialum can use xylose as a carbon source (solid M102 media supplemented with xylose); however, growth is greater on a sucrose-supplemented medium. Kulkarni and Neilson (1986) examined the growth of the endophyte in liquid non-specific media (GY media), supplemented with various carbon sources. Cultures were incubated at 23 C for two weeks on a rotary shaker set at 100 rpm. Kulkarni and Neilson found that xylose was not used by the endophyte. Glucose and sucrose served as a good carbon source for the endophyte.

A mucoid-like appearance was evident when cultures were grown on M102 media supplemented with sucrose. Culture filtrates from A. coenophialum cultures grown in M102 media produced a white mucilaginous precipitate with the addition of two volumes of ethanol Stasinopoulos and Seviour (1989). Many members of the genera, Acremonium and Cephalosporium, are

able to produce exopolysaccharides (Stasinopoulos and Seviour 1989). We believe that our isolate of A. coenophialum may be an exopolysaccharide producer, that this change in morphology may play a role in the endophytes ability to grow within the flowering stem of tall fescue.

In summary, the neutral sugars present in tall fescue include mannose, rhamninose, arabinose, galactose, glucose and xylose. Xylose was found to be the predominate monosaccharide in both vegetative and reproductive stages.

A. coenophialum grew best on media supplemented with sucrose rather than xylose; however, growth did occur on media supplemented with xylose. Finally a mucoid-like substance, which is believed to be an exopolysaccharide, was produced in media supplemented with sucrose.

VI. CONCLUSIONS

The PGRs, dicamba, 2,4-D and abscisic acid, had no effect on the growth of A. coenophialum at the concentrations examined. Kinetin and zeatin had a significant effect on growth. Reduction of growth was recorded at each concentration examined, with a 100 percent reduction recorded at 100 μM on solid media. In liquid M102 media with 100 μM kinetin 95 percent reduction was observed. Gibberellic acid also reduced the growth of the endophyte at 100 μM. Complete growth inhibition was observed when endophyte was grown in M102 liquid media supplemented with 100 μM gibberellic acid. Ethylene gas at a concentration of 9 ppm had no effect on the growth of A. coenophialum. Ethylene gas was not produced by the endophyte with the addition of the ethylene precursors l-methionine and ACC. These compounds also had no effect on growth.

Cell wall analysis of tall fescue during the vegetative and reproductive stage revealed that xylose was present in higher amounts than rhamnose, galactose, glucose, arabinose and mannose. Xylose percentages were higher in the peduncle than in the tiller base.

The endophyte was able to use sucrose as a carbon source more efficiently than xylose. Colonies of the endophyte grown

on sucrose produced a mucoid-like substance believed to be an exopolysaccharide. Cultures grown on media supplemented with xylose did not appear to produce the exopolysaccharide.

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VITA

Gregory E. Huff was born on May 9, 1964 in Great Falls, Montana. He spent most of his early years traveling throughout the United States. He attended high school in Springfield, Massachusetts at Classical High School. He was involved in many sporting activities including manager of the football team, wrestling team and track team. He later received his high school diploma in may of 1982. That same year, he entered Fisk University in Nashville, Tennessee where he earned a Bachelors degree in Biology in May of 1986. While at Fisk, Gregory became a member of Alpha Phi Alpha Fraternity and was active with the Student Government Association and also a member of the Modern Black Mass Choir.

After a two year period, Gregory was accepted at the University of Tennessee to study Plant Pathology. While attending the University of Tennessee, he met up with a diverse crowd of students with strange and interesting back grounds. A wealth of information was gained during the two year period. Gregory E. Huff finally finished all the requirements for the thesis program and received his degree in May of 1991.

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