Ergosterol as an Indicator of Endophyte Biomass in Grass Seeds

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Ergosterol can be used as a marker to estimate fungal biomass in soil and plant−fungal tissue samples. The following experiments assess the application of ergosterol analysis to endophyte determinations in grass seeds. Ergosterol was present in all endophytic fungi tested, ranging from 185.2 μg g^{-1} in *Epichloë typhina* to 1225.5 μg g^{-1} in *Neotyphodium lolii*. Significant differences in ergosterol content occurred both between and within species of endophytic fungi. Ergosterol content of grass seeds was highly correlated ($r^2 = 0.99$) to the endophyte content of the seeds. Several extraction techniques were tested and found to yield variable results, but the method that gave the most complete extraction in seeds was methanol extraction under refluxing conditions. The endophyte content of fine fescue seed samples was predicted from ergosterol analysis and found to be well-correlated with microscopic analysis. It is concluded that ergosterol analysis can be used in both diagnostic and research applications to predict endophyte content in seed samples.

Keywords: *Endophyte; ergosterol; grass seed; sterol*

INTRODUCTION

Endophytic fungi continue to interest researchers of both amenity and forage grasses due to the beneficial and detrimental effects of these organisms. Recent reviews indicate that many of the current turfgrass varieties of perennial ryegrass (*Lolium perenne* L.), tall fescue (*Festuca arundinacea* Schreb.), and fine fescue (*Festuca rubra* L.) have high levels of endophyte infection (Funk et al., 1994). The economically important endophytes complete their entire life cycle within the host plant and are maternally transmitted to offspring through the seed (Bacon and De Battista, 1990). An endophyte can remain viable in the seed for an extended period, but longevity is influenced by temperature and humidity of storage conditions (Welty et al., 1987).

The determination of endophyte content in seed lots is important for both seed producers and consumers. Microscopic analysis of stained tissues can be used to identify endophyte-infected seeds, but this technique requires specialized training and can be very time-consuming (Bacon and White, 1994). In addition, staining does not give any indication of viable endophyte. Viability can only be tested by analyzing seedlings for the presence of endophytic hyphae (Belanger, 1996). This process takes a minimum of 3 weeks, requires a heavy input of labor and equipment, and presents logistical problems for seed traders. In addition, results of these grow-out tests are only valid for short periods. Immunoblot assays have also been used to determine endophyte content in grass seed (Gwinn et al., 1991), but this technique is not readily available to all laboratories and is currently cost-prohibitive.

Biochemical markers such as glucosamine (West et al., 1987), extracellular lacase (Matcham et al., 1985), and adenosine 5′-triphosphate (ATP) (Suberkropp et al., 1993) have been used to quantitate fungal biomass in soils and other plant−fungal systems. While applicable to specific circumstances, these methods suffer from either poor reproducibility or the presence of the marker compound in other components of the sample. Ergosterol (Figure 1) is a triterpene sterol that is highly specific to fungi and generally absent in higher plants (Gessner and Newell, 1997). Ergosterol has been used as a marker to quantitate fungal biomass in soils (West et al., 1987), leaf litter (Newell et al., 1988), seeds (Seitz et al., 1977), and other plant−fungal systems (Martin et al., 1990). Although most techniques to determine ergosterol have employed final separation of sterols by HPLC with UV detection, a wide range of extraction and purification steps have been reported in the literature. These include room temperature extraction with various alcohols (Padgett and Posey, 1993), extraction under high-temperature refluxing conditions (Seitz et al., 1977), solid-phase extraction (Gessner and Schmitt, 1996), microwave-assisted extraction (Young, 1995), and supercritical fluid extraction (Young and Games, 1993). Each method has shown to be effective, but the variability in results and necessity for specialized equipment suggests that new applications of the technique require a preliminary assessment of methodology.

Ergosterol was previously identified in *Neotyphodium coenophialum* (Davis et al., 1986), the endophyte of tall fescue (*F. arundinacea* L. Schreb), but has not been studied any further in this group of organisms. On the basis of the widespread application of this technique to fungal determinations in mixed samples, the following research investigated ergosterol analysis as a tool to determine the endophyte content of grass seeds.

MATERIALS AND METHODS

Endophyte Culture. Endophytes were isolated (Bacon and White, 1994) from fine fescue (*F. rubra* L.), tall fescue (*F. arundinaceae* Schreb.), big blue grass (*Poa ampla* Merr.), bentgrass (*Agrostis gigantea* Roth), and perennial rye grass (*L. perenne* L.) clones maintained in the endophyte collection at Rutgers University. A detailed account of host and endophyte species is reported in Table 1. After a 3-week establishment period, isolates were subcultured on potato dextrose agar medium overlaid with sterile cellophane. After the cultures developed to a diameter of 5−6 cm, mycelium was harvested and analyzed for ergosterol content as described below.

Within-species variability of ergosterol content was tested using different isolates of *Epichloë festucae* obtained from fine fescue breeding nurseries of Dr. C. R. Funk, Rutgers Univer-

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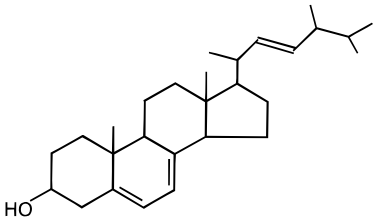

Figure 1. Ergosterol.

Table 1. Ergosterol Content of Several Endophyte Species Cultured on PDA Medium and Extracted with 100% Ethanol[a]

grass host	endophyte species	ergosterol content (μg g^{-1})
Poa ampla	*Epichloë typhina*	185.2
Festuca rubra	*Epichloë festuceae*	574.2
Festuca arundinacea	*Neotyphodium coenophialum*	834.4
Agrostis sp.	*Epichloë amarillans*	998.6
Lolium perenne	*Neotyphodium lolii*	1225.5
LSD (0.05)		243.1

[a] Each value represents the mean of three replications.

sity. Isolation and culture methods were as described in the previous paragraph. Within that experiment, the effects of mycelial age were also tested by sampling mycelium from the outer, middle, and center of the colony. All experiments on culture materials were conducted a minimum of two times, and data represent the results from a single, representative experiment. Endophyte species, isolate, and mycelial age treatment effects were tested by analysis of variance (Proc Anova) of a completely randomized design (Statistical Analysis Systems, Inc., Cary, NC). Treatment means were separated using Fisher's Protected LSD ($P < 0.05$).

To assess the correlation between endophyte mass and ergosterol content, various weights of *E. festuceae* mycelium, ranging from 15 to 105 mg, were extracted for ergosterol content as described below. Ergosterol content was regressed against fungal weight using linear regression techniques (Proc Reg procedure, Statistical Analysis Systems).

Ergosterol Analysis. Mycelium was gently lifted from the cellophane, weighed accurately in a 1.5-mL microfuge tube, and extracted for ergosterol using the method of (Martin et al., 1990). Mycelium was extracted for 5.0 min with 0.5 mL of cold (4 °C) absolute ethanol. Extraction was facilitated by grinding the sample with a power drill fitted with a custom homogenizing probe (Pellet Pestle Blue Polypro). After extraction, the probe and sides of the microfuge tubes were rinsed with an additional 0.5 mL of ethanol, and the mixture was centrifuged at 12 000 rpm for 5 min. The supernatant was filtered through a 0.45-μm nylon filter and assayed directly for ergosterol using HPLC. The HPLC system was a Shimadzu LC-10AT pumping system connected to a photodiode array (PDA) detector (Shimadzu SPD-M10A). The analytical column was a Microsorb (C_{18}) reversed phase column (Rainin, 5 μm, 150 × 4.6 mm) protected by a C_{18} guard column (Rainin, 5 μm, 15 × 4.6 mm). Samples were eluted isocratically with 100% methanol at a flow rate of 1.0 mL min^{-1}, and peaks were monitored at 280 nm.

Ergosterol eluted at ~9.0 min and was quantitated by the external standard method using a pure compound (UV absorption in MeOH, $\lambda_{max} = 282$ with shoulders at 269 and 293) obtained from a commercial source (Sigma Chemical). The presence of ergosterol in the endophyte extracts was confirmed by several methods, including comparison of HPLC retention times between the unknown peak and a pure standard, peak enrichment of the unknown with pure standard, and comparison of UV absorption between the unknown peak and pure standard. In addition, "on-the-fly" UV spectral characteristics of peaks were continuously monitored with the PDA detector.

Ergosterol Assay of Endophyte-Infected Seeds. Seeds of endophyte-infected SR5000 Chewings fescue were provided by Seed Research of Oregon (Corvallis, OR), and endophyte-free Jamestown II Chewings fescue seeds were provided by Lofts Seed Co. (Bound Brook, NJ). The two seed lots were mixed to yield samples with endophyte content ranging from 0% to 100% in 10% increments. Prior to extraction, seeds were surface-sterilized for 5 min with 5.25% sodium hypochlorite and washed four times in sterile water. Sterile seeds were air-dried overnight in a laminar flow hood to their original moisture content and milled for 1 min using an electric coffee grinder (Braun, Model KSM2).

Two methods were used to extract ergosterol from ground seeds. In the first method, seeds were extracted twice at 25 °C with absolute ethanol (1 g of seed:5 mL of ethanol) on a reciprocating shaker for 30 min. Each extract was centrifuged for 10 min at 10 000 rpm, and the supernatants from the two extractions were combined. The combined supernatants were evaporated to dryness under vacuum and redissolved in 0.5 mL of ethanol for HPLC analysis. In the second method, 1.0 g of seed was extracted with 20 mL of 100% methanol at 80 °C for 2 h under refluxing conditions. Ten milliliters of KOH in methanol (0.04 g/mL) was added to the original extract and refluxed for an additional 30 min at 80 °C. After cooling and filtering, 15.0 mL of water was added to the alcoholic base and the sample was partitioned three times against hexane. Hexane fractions were combined, reduced to dryness under vacuum, and resuspended in methanol for HPLC analysis. HPLC methods were identical to those used for culture extracts.

To assess the validity of the method against an unknown group of seed samples, 12 samples of Chewings and Hard fescue from unknown origin were collected from various sources. All samples were less than 1 year old at the initiation of the experiment. One gram of seeds was milled and extracted for ergosterol using the methanol refluxing method described above. An additional 24 seeds were analyzed microscopically for the presence of endophyte using aniline blue stain (Bacon, 1992). Percent endophyte estimated by ergosterol was regressed against percent endophyte determined microscopically using the Proc Reg procedure (Statistical Analysis System).

RESULTS AND DISCUSSION

Preliminary ergosterol investigations of mycelium of grass endophytes revealed that there were no significant differences between the ethanol extraction method reported by Martin et al. (1993) and earlier methodologies using methanol refluxing (data not shown). Therefore, all studies with endophyte cultures were conducted using the more efficient ethanol method. All Balansiae species examined in these studies contained detectable levels of ergosterol (Table 1). Ergosterol levels varied significantly between species, ranging from 185.2 μg g^{-1} in *E. typhina* to 1225.5 μg g^{-1} in *Neotyphodium lolii*. Although the ergosterol content of *N. coenophialum* was reported earlier (Davis et al., 1986), other endophytic fungi have not been tested. On the basis of comparative studies in other fungal species (Newell et al., 1987), the differences in ergosterol content between species (Table 1) are not surprising. Newell et al. (1987) reported that ergosterol content of filamentous *Phaeosphaeria typharum* could vary as much as 4−fold depending on the culture medium, suggesting that much of the variation between isolates was due to adaptability to substrate. *In vitro* growth of endophytes is also known to vary considerably depending on substrate (White et al., 1991) and may have contributed partially to our results. Unfortunately, very little is known about the growth and physiology of grass endophytes on a host substrate. Nonetheless, the between-species differences observed in this study suggest that a preliminary ergosterol determination should be conducted before the method is utilized to quantitate mycelial mass in different host/endophyte combinations.

Table 2. Ergosterol Content (Micrograms per Gram) of Several Isolates of *E. festucae*[a]

	tissue age			
isolate	young	medium	old	mean
RF2	327.7	372.3	292.6	330.9
RF13	320.5	438.9	300.5	353.3
RF21	393.6	586.7	742.4	574.2
RF26	585.4	587.0	516.1	562.8
RF52	971.5	784.4	600.4	785.5
mean	519.8	553.9	490.4	521.4

ANOVA Results

treatment	df	*F* statistic	Pr > *F*	LSD (0.05)
isolate	4	3.03	0.03	308.9
age	2	0.15	0.86	ns[b]
age × isolate	8	0.50	0.85	ns

[a] Ergosterol was extracted using 100% ethanol. [b] ns, not significant.

Within a single species of endophyte (*E. festucae*), ergosterol levels were also variable, with isolates differing by as much as 400 μg g^{-1} of mycelium (Table 2). Although these isolates all originated from U.S. collections (C. R. Funk, Rutgers University, personal communication, 1996), taxonomic data suggest various degrees of heterogeneity within the species (White and Huff, 1996). Fungal age had no effect on the amount of ergosterol in endophytes, and no interacting effects were found between isolate and age (Table 2). Compared to other fungal biochemical markers, such as glucosamine and chitin, ergosterol content is less affected by tissue age than either of the other techniques (Matcham et al., 1985). Although studies with an ectomycorrhiza (*Pisolithus tinctorius*) found that ergosterol decreased as mycelium aged (Martin et al., 1990), other studies are consistent with our work, in that ergosterol content is not significantly influenced by mycelium age (Newell et al., 1987).

Before ergosterol was used to quantitate endophytic mycelium in a seed sample, it was first necessary to determine if ergosterol content was correlated to the actual amount of fungal mass present, as some studies have shown no correlation between ergosterol and fungal mass (Bermingham et al., 1995). A highly significant correlation ($r^2 = 0.97$ and 0.99) was found between ergosterol and fungal weight for two isolates of *E. festucae* (Figure 2), indicating that ergosterol will be a good marker for endophyte biomass in seeds. Although ergosterol has been used previously to estimate fungal colonization of seed (Seitz et al., 1977), those studies were focused on potentially pathogenic or mycotoxin-producing fungi. The use of ergosterol to detect seed-borne, endophytic fungi presented several problems. Most importantly, ergosterol is common in almost all fungi (Weete, 1974), and grass seeds serve as a host for other pathogens and saprophytes (Makela, 1972). Fortunately, one of the most common fungi found on grass seeds, *Puccinia* spp., does not contain ergosterol (Weete, 1974). Surface sterilization of seeds for 5 min with 5.25% sodium hypochlorite (Bacon and White, 1994) successfully eliminated all fungal contaminants from the seed (Table 3). As sterilization times were increased from 0 to 5 min, a steady decrease in extractable ergosterol was also observed (Table 3), confirming the elimination of fungi on the surface of the seed. Seeds that had been surface-sterilized for 5 min were maintained on the potato dextrose agar medium for an additional 2 weeks and *E. festuceae* endophytes eventually grew from the seeds (data not shown), demonstrating that the endophyte remained viable in the seeds after sterilization.

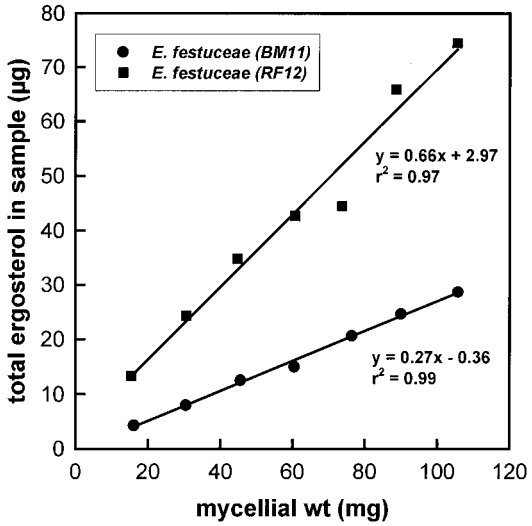

Figure 2. Ergosterol content of two isolates of *E. festucae* as influenced by the amount of mycelium extracted.

Table 3. Percentage of Seeds Contaminated with Nonendophytic Fungi and Ergosterol Content of Endophyte-Infected Seeds Receiving Three Pre-extraction Sterilization Procedures

seed treatment	seeds with contamination[a] (%)	ergosterol content[b] (μg g^{-1})
no sterilization	100	2.86 ± 0.41
5.25% NaOCl, 1 min	100	2.10 ± 0.19
5.25% NaOCl, 5 min	0	1.19 ± 0.32

[a] Expressed as a percentage of 20 seeds placed on PDA medium for 1 week. [b] Extracted with methanol under refluxing conditions at 80 °C. Each value represents the mean of four replications ± the standard deviation.

In plant and soil samples, the extraction method most often used for ergosterol analysis has been high-temperature (80 °C) refluxing with 100% methanol, followed by saponification with an alcoholic base and subsequent partitioning of the lipid fraction into a nonpolar solvent such as hexane or pentane (Newell et al., 1988; Seitz et al., 1977; Padgett and Posey, 1993). However, work by Martin et al. (1990) with root mycorrhizae indicated that the refluxing, saponification, and partitioning steps could be eliminated and replaced by extraction into cold ethanol. These extraction techniques were compared to determine the method most suitable for endophyte-infected seeds. In addition, whether seeds needed to be milled or could be extracted intact to quantitate ergosterol was investigated. Extraction of endophyte-infected seeds with both alcohols yielded ergosterol (Figure 3), but the methanol method generally yielded 3−5 times more ergosterol than ethanol. The purpose of the saponification step in the methanol extraction is to hydrolyze ergosterol esters, which can represent up to 40% of the total ergosterol (Newell et al., 1988). Due to the large increases in ergosterol using the methanol method, this extraction procedure contributed more to the total ergosterol than simple hydrolysis of esters. A detailed comparison of extraction techniques by Padgett and Posey (1993) also found ethanol extraction techniques to yield 2−5-fold less ergosterol than methanol refluxing. These combined data indicate that ethanol extraction, although appropriate for fungal mycelium, should not be used when working with complex tissues such as grass seed. Grinding of the grass seeds was also an important factor contributing to ergosterol

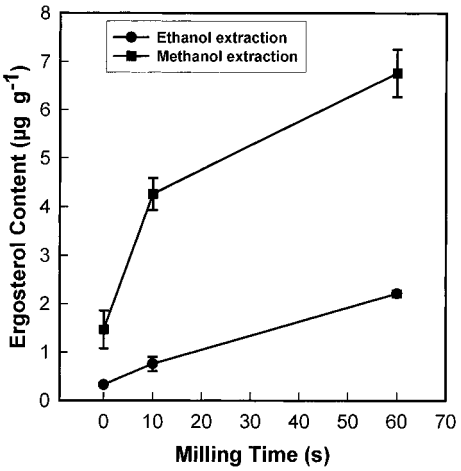

Figure 3. Ergosterol yields from an endophyte-infected fine fescue seed sample as influenced by extent of grinding (milling time) and method of extraction.

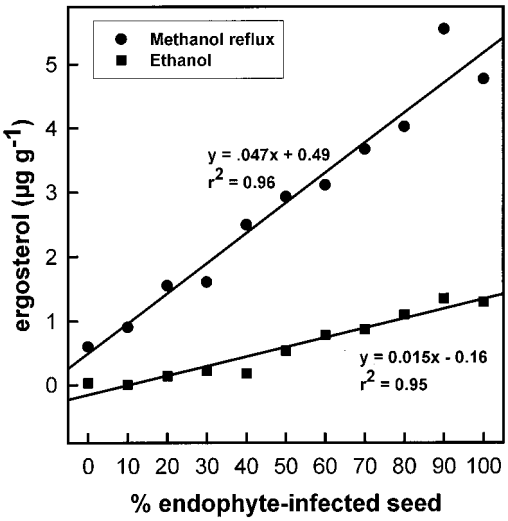

Figure 4. Ergosterol content of fine fescue seed samples containing a defined range of endophyte levels.

levels, as even a 10-s milling time improved recovery of ergosterol by almost 3-fold (Figure 3). Milling times were not extended past 1 min, as this time was sufficient to grind the seeds to pass a 0.5-mm mesh screen.

Ergosterol content was highly correlated to the percentage of endophyte-infected seed in a sample (Figure 4), suggesting that the technique can be used to estimate endophyte content in an unknown seed sample. Ergosterol yields were again very low for ethanol compared to methanol (Figure 4), but the correlation was still highly significant (r^2 = 0.95). The main advantage of ethanol compared to methanol refluxing/saponification is that only free ergosterol is released with ethanol, therefore giving an indication of living mycelium (Martin et al., 1990). The association of ergosterol with living cytoplasm has not been firmly established for plant−fungal tissue samples, even though ergosterol is susceptible to both photodegradation and oxidative degradation (Gessner and Newell, 1997). In soil samples, free ergosterol released during the senescence of a mycelium is rapidly mineralized in the system (West et al., 1987). However, to what extent these processes would affect ergosterol released by an endophyte as it senesces in a grass seed is unclear. Further studies are needed to determine whether free ergosterol can be used as a test of endophyte viability in a seed sample.

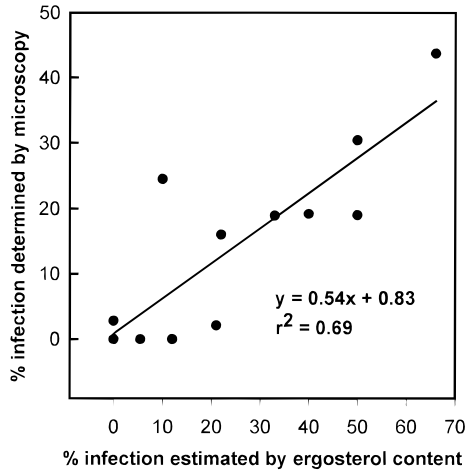

Figure 5. Comparison of percent endophyte infection of fine fescue seed estimated from ergosterol content against percent infection determined by microscopy of seeds stained with aniline blue stain. Ergosterol was extracted using the methanol refluxing method (see text).

To further test the method as a diagnostic tool, an unknown group of fine fescue seed samples was analyzed for ergosterol content using the methanol extraction method and also stained for endophytic fungi. A positive, significant correlation was observed between the estimates (Figure 5), although the correlation coefficient was not as high (r^2 = 0.69) as for the defined set of samples (Figure 4). Unlike the correlation based on the single endophyte-infected seed lot (Figure 4), the endophytes in this experiment were not the same isolate and were likely from very diverse origins. Therefore, deviations from a straight line would be expected on the basis of the differences in endophyte isolates observed in Tables 1 and 2. However, the estimates were able to identify differences in endophyte content between a group of samples that were predominantly <50% infected. This is encouraging, as both forage and turfgrass seed traders are generally concerned with high levels of endophyte (>50%), and the ergosterol assay will easily identify those samples.

The actual degree to which an endophyte colonizes a grass seed has never been reported. The data collected in these studies was also used to calculate the amount of endophyte biomass present in a fine fescue seed. The ergosterol content in the 100% endophyte-infected seed sample (Figure 4) was determined to be 5.18 μg g^{-1}. Using an ergosterol content of 574 μg g^{-1} of mycelium (*E. festucae*, Table 1), endophytes occupy slightly <1% of the seed on a weight basis. This quantitative measure of endophyte biomass correlates well to visual estimates of endophyte density in the aleurone layer made by microscopy (J. F. White, Jr., Rutgers University, personal communication, 1996). Further studies are being conducted to apply this technique to vegetative tissues, such that growth and physiology of endophytes *in planta* can be more fully understood.

In conclusion, ergosterol analysis proved to be a viable alternative for the detection of endophytes in grass seeds. In laboratories that routinely use HPLC, seed samples can be screened for endophytes in as little time as 4 h. On the basis of the results from these studies, seed extraction with methanol under refluxing conditions yielded the highest quantity of ergosterol and subsequently the most reliable results. Traditional microscopic staining generally requires an 8-h seed-softening step, followed by the extensive time required

to examine individual seeds for endophyte. With the ergosterol technique, a single technician in our laboratory could easily analyze up to 20 seed lots per day depending on the number of refluxing units available in the laboratory.

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