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I am submitting herewith a thesis written by R. Wayne Thompson entitled "Sampling intensity and timing for estimating acremonium coenophaialum incidence in fescue pastures." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Plant, Soil and Environmental Sciences.

Henry A. Fribourg, Major Professor

We have read this thesis and recommend its acceptance:

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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We have read this thesis
and recommend its acceptance:

Bradford B Reddick

John C Waller

James B. McLaren

Accepted for the Council:

Vice Provost
and Dean of The Graduate School

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SAMPLING INTENSITY AND TIMING FOR ESTIMATING *ACREMONIUM COENOPHIALUM* INCIDENCE IN FESCUE PASTURES

A Thesis

Presented for the

Master of Science

Degree

The University of Tenneessee, Knoxville

R. Wayne Thompson

March 1988

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DEDICATION

This thesis is dedicated to my Uncle, Charles E. Johnston, for his support, love, and vigil that I succeed as a man in life. Thanks for being a father when I needed one the most in my life.

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ABSTRACT

Acremonium coenophialum Morgan-Jones and Gams has been associated with several animal disorders known collectively as fescue toxicosis. Reestablishment of infected (E+) pastures with seed not containing the fungus (E-) is beneficial for eliminating the symptoms of fescue toxicosis. However, since management decisions must be based on knowledge of *A. coenophialum* incidence, appropriate sampling methods should provide information about fungal incidence with accuracy. Two sampling studies were conducted to determine an effective sampling method. In the first, eight 4-ha research pastures that had been established for a grazing trial having E+ incidence ranging from near 0 to more than 70% were sampled in June 1986 using a transect method (TM) and a stratified random sampling design (SR) at an intensity of 23 tillers ha^{-1}. In the second, four 2-ha pastures were sampled at monthly intervals from November 1985 through October 1987, using SR at 41 samples ha^{-1}. Samples were assessed for E+ status using Protein A enzyme-linked immunosorbent assay (PAS-ELISA). In the first study, observed variability in E+ incidence of two pastures increased from 15 or 30% to about 60% during the 27-month period; six other pastures, with original 0, 45, 60, and 75% E+ incidence, had only small increases in E+ incidence. In the second study, only small fluctuations in fungal incidence were observed in four pastures wiht 60% E+ incidence. Significant E+ incidence could be detected at any time during the year using PAS-ELISA. The

distribution of the E+ and E- plants was random in the first study because they had been seeded uniformly, but was highly aggregated in the older (>11 y) pastures of the second study. Therefore, dispersal of sampling sites across the entire field is important when sampling older pastures that may have an aggregated fungal distribution. It appears that relatively few samples are required for assessment of E+ status in pastures. Eight or more stratified random samples ha^{-1} might be an adequate sampling intensity for producers; for research purposes, sampling intensity should approach one sample 250 m^{-2} in a stratified random sampling. The TM and SR gave similar estimates, but SR could better detect spatial relationships.

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I. THE TALL FESCUE TOXICOSIS PROBLEM

THE IMPORTANCE OF TALL FESCUE

Tall fescue (*Festuca arundinacea* Schreb.), a perennial cool-season grass, is one of the most widely grown forage species in the U.S., occupying over 14.5 million ha. (Hoveland et al., 1982). It is grown primarily in the transition zone between the northern and southern areas of the eastern U.S. where many cool-season and warm-season grasses are at the limits of their best range of adaptation (Buckner and Cowan, 1973). Other important agronomic attributes include the ability to grow on a wide range of soil types, tolerance of both wet and droughty conditions, and the ability to withstand abusive grazing management. Fescue also produces high yields (8 to 10 $Mg \cdot ha^{-1} \cdot yr^{-1}$) allowing a lengthy grazing season for the incorporation of a backgrounding program into the farm operation (Buckner and Cowan, 1973).

Indices of forage quality such as crude protein, digestible dry matter, cell wall content, and mineral concentration suggest tall fescue is of high quality and should result in good animal performance (Bush and Buckner, 1973). Best animal performance is achieved when a legume such as *Trifolium sp.* is present as a companion crop, and when close grazing has reduced competition, sustaining the grass in a vegetative state (McLaren et al., 1983).

Expanded livestock production in the mid-South during the past 35 years would not have been possible without the use of tall fescue. The least-cost approach to pastures will continue to

depend on adapted quality forages like tall fescue to increase livestock performance. As the need for human consumption of cereals increases, and as a health-conscious public continues to demand leaner meat products, the desirability of higher proportions of roughage used to feed ruminant animals also will increase in the future. Thus, it is apparent that tall fescue will continue to be a forage essential for livestock agriculture.

FESCUE ENDOPHYTE DISCOVERY AND ANIMAL DISORDERS

As fescue use became more widespread in the 1940's, farmers began to observe animal production problems. Beef producers recognized the problem as a lack of production and as an occasional loss of extremities (switch and tail) known as 'fescue foot'. Dairy producers thought fescue was unpalatable because of small milk production.

In 1973, researchers at the R.B. Russell Agricultural Research Center in Athens, Georgia noticed two herds of Angus cattle which performed differently on two separate pastures. One herd performed adequately while the other herd continually had poor average daily gains (ADG) and reproductive problems. In 1976, Bacon et al. (1977), discovered that a high percentage of fescue tillers sampled from the pasture noted for poor performance were highly infected (E+) with an endophytic fungus labelled *Epichloe typhina* (Pers.) TUL. The productive pasture contained less than 10% fungus incidence. The implication was that the fungus could be the agent responsible for poor animal performance. The fungus was

reclassified later as *Acremonium coenophialum* Morgan-Jones and Gams (Morgan-Jones and Gams, 1982). The following is a brief classification of the animal disorders strongly associated with livestock ingesting E+ fescue.

a) Summer syndrome or summer slump- This livestock disorder is by far the more prevalent of all fescue endophyte disorders. It occurs primarily during the high ambient temperatures of summer. Clinical symptoms of summer syndrome include rapid respiration, slightly elevated body temperatures, rough hair coat, poor ADG, excessive salivation, and nervousness. In addition, animals stand in shade and/or water, presumably to feel more comfortable. Economic losses attributed to summer syndrome are in excess of 360 million dollars yr^{-1} in the U.S. based on animal performance data collected to date (Evans, 1986).

b) Fescue foot- Fescue foot causes lameness and possible losses of feet, tail and ear tips. This non-infectious, gangrenous condition closely resembles ergot toxicity. Fescue foot usually begins with livestock showing an arched back, sore hooves, and rough hair coat. A vasoconstricter that may be produced by the fungus *in vitro* appears to cause swelling and inflamation of the coronary band (area between dewclaws and hooves) (Greighton and Garner, 1980). Fescue foot occurs during late winter, but fortunately is rare.

c) Bovine fat necrosis or lipomatosis- Bovine fat necrosis is defined as the presence of hardened or necrotic fat masses primarily in the adipose tissue of the abdominal cavity (Wilkinson et al.,1983). Symptoms include digestive disturbances, scanty

feces and bloating, dystocia, urine retention, and most of the symptoms of 'summer syndrome'. The hard masses of fat result in constriction of the colon and strangulation of the intestines (Wilkinson et al., 1983).

d) Reproductive problems and hypoagalactia- Calving percentages have been known to be poor for cows pastured on E+ tall fescue. Horseman have noted longer gestation periods, abortion, and rebreeding problems of mares ingesting E+ fescue prior to foaling (Garret et al., 1980). Hypoagalactia, a decrease in the amount of mammary secretion or flow of milk (Reece, 1981) is common in cows, ewes, and mares ingesting the endophtye. Hypoagalactia is thought to occur due to the depression in the level of serum prolactin, the hormone that begins and sustains lactation (Hurley et al., 1981).

e) Tall fescue toxicosis or tall fescue toxicity- This term is used collectively for all the previous animal disorders, which in some unknown way are related to a toxin(s) produced either by the fungus, by fescue tissue, or in the digestive tract of herbivores.

CHARACTERISTICS OF THE ENDOPHYTE

Fungus endophytes have been reported in several species of *Festuca sp.* (Latch, Christensen, and Samuels, 1984; White and Cole, 1985). However the tall fescue endophyte, *Acremonium coenophialum* possesses certain distinctive characteristics. *A. coenophialum* is a true endophyte, since completion of its entire life cycle is within the host plant. Unlike some spore-producing

endophytes, spores of this fungus have not been found on plants (Siegel et al., 1985). Therefore, the only known mode of natural dissemination of the endophyte is in the host seed; the fungus is not transmitted by pollen, rain, or wind (Siegel et al., 1984). Conidia and ascospores have been produced on several media (Latch et al., 1984; Morgan-Jones and Gams, 1982).

Endophyte-colonized tall fescue plants show no external symptoms. The presence of fungal mycelium is found by miscroscopic examination of plant tissue stained with aniline blue in lactic acid (Bacon et al., 1977; Clark et al., 1983), as presented in Appendix C, or by the highly sensitive and specific enzyme-linked immunosorbent assay (ELISA) developed by Johnson et al., 1983, and revised by Reddick and Collins (1988) and as presented in Appendix C. In seed, however, neither method can detect living from non-living endophyte. In order to detect viable endophyte, E+ embryos must be plated on agar or the seed must be germinated and fungal presence determined by ELISA or staining of plant tissues when plants are 3 to 5 weeks old (Siegel et al., 1985; Conger et al., 1986).

In the seed, the fungus is sequestered between the embryo and the aleurone layer. As the seed germinates, so grows *A. coenophialum;* strengthening tissue, sclerenchyma, and surrounding air spaces develop, allowing the fungus to grow intercellularly up the shoot (Bacon et al., 1983). The movement of the endophyte from seed to seedling is usually accomplished after 3 weeks in culture (Welty et al., 1986). Most of the hyphae are found in leaf sheaths

and seeds, but only small amounts are found in the blades and none in the roots (Fribourg et al., 1988). The fungus content probably depends on the development of precise tissue types. *A. coenophialum* growth may await development of unoccupied areas, the intercelluar spaces (Bacon et al., 1983). Nutrients supplied to the fungus are those available in the intercellular spaces. In storage, the endophyte slowly dies as the seed ages unless E+ seed is stored at cold temperatures and low humidity.

TOXIC ALKALOIDS DISCOVERED IN INFECTED FESCUE

Cattle disorders seem to occur due to a complex relationship between the plant, the bovine, the environment (soil type, temperature, and nutrients supplied to the plant and animal), and *A. coenophialum.* Peptide and clavine ergot alkaloids have been isolated recently from E+ tall fescue leaf blades and sheaths (Lyons et al., 1986; Yates et al., 1985). These ergot alkaloids are presumed to be synthesized by *A. coenophialum*, since E+ plants produce them whereas E- plants do not. The ergot alkaloids found in leaf blades probably are produced elsewhere and translocated to the blades, because little mycelium is found there (Lyons et al., 1986). Extensive research is being conducted to further isolate these toxic compounds in order to understand their effect on livestock ingesting E+ fescue.

ACREMONIUM INCIDENCE AND MANAGEMENT ALTERNATIVES

Current estimates from field surveys indicate over 80 to 90% of all tall fescue pastures in Alabama, Kentucky, Missouri, and Tennessee are infected (E+) with *A. coenophialum* (Fribourg et al., 1988). High fungal incidence is due primarily to the extensive use of the Kentucky 31 cultivar (Fergus and Buckner, 1972). The original seedlot was obtained from a pasture in Kentucky known now to be severely E+. The high demand for obtaining fresh seed of this popular cultivar caused the vast dissemination of *A. coenophialum* Laboratories throughout the southeast indicate that most E+ pastures range between 60 and 90% incidence (Fribourg et al., 1988). Dr. D. P. Belesky, Univ. of Georgia, (personal communication) indicated that pastures virtually free of the fungus (0 to 5%) or highly E+ pastures (> 90%) remain at constant incidence levels for several years. On the other hand, those pastures ranging from 20 to 60% are 'creeping upwards to higher fungal levels'.

Fortunately, renovation of E+ pastures with fungus free seed (E-) or the incorporation of legumes directly into the sward has been beneficial (Fribourg et al., 1988). The exact threshold level of E+ fescue that is toxic to bovine has not been determined. Present recommendations for beef production suggest that those pastures with 30% or more fungal incidence should be reestablished, and those with lesser incidence levels may be improved with legumes (Fribourg et al., 1988). Dairy cows or pregnant mares should not be allowed to ingest E+ fescue that has a greater incidence than 10% (Mueller, 1986).

II. SAMPLING TECHNIQUES AND THEORY FOR DETECTING PATHOGENS

The endophytic fungus, *Acremonium coenophialum* is a major concern to beef, dairy, and horse producers throughout the transitional zone. Fortunately, management practices can be used to result in productive pastures. However, since management decisions must be based on knowlege of *A. coenophialum* incidence, appropriate sampling procedures should provide information about infection levels with a known accuracy. To date, an effective sampling technique for identification of endophyte incidence (the number of E+ samples expressed as the percentage of the total number assessed) has not been defined. An experiment to monitor endophyte incidence over a two-year sampling period was designed, using a stratified random sampling scheme (Cochran, 1977) in four 2-ha pastures. The objectives were: 1) to determine the effects of sample size and intensity on accuracy of endophyte incidence estimates; 2) to examine the variability in *A. coenophialum* incidence over time; and 3) to determine the within-field variance and spatial relationships of endophyte occurrence. Another sampling experiment was done on eight 4-ha. pastures ranging from 0 to 75% fungal incidence, using two sampling procedures: a Transect method (Garner, Univ. of Missouri, personal communication) and the stratified random design, to compare the results and the precision obtained with the two procedures. The techniques and theory of sampling were reviewed and summarized.

The review is limited to those techniques and theory used to identify pathogens in plants, animals, and soils, and to the current information on sampling for the endophyte. All discussion pertains to probablity sampling from a binomial distribution, because the observed response variable is dichotomized as 0 or 1 (E+ or E-) (Couey and Chew, 1986). If n experimental units (e.u.) act independently, *i.e.*, the response of an e.u. does not influence the response of another e.u., and if the probability (p) of success (1) is the same for all e.u.'s, the total number of successes (x) is a random variable that follows the binomial distribution (Couey and Chew, 1986).

SAMPLING THEORY

In scientific research, our knowledge and our actions are based largely on information obtained from sampling. It is impractical to measure the entire population, so inferences must be made from a sample. Unfortunately, sampling has the disadvantage of providing only an estimate of the information required (Samford, 1962). Therefore the ultimate problem of the investigator is to design a sampling scheme in such a manner that the total error in the results is minimized (Hansen, Hurwitz, and Madow, 1953). As long as the measurement or response in the sample is not in error, any difference between the expected value of the sample statistic and the parameter being estimated is due only to the bias in the sample and the resultant statistic is an accurate estimate of the population parameter (Hansen et al., 1953). The population to be

sampled should coincide with the population about which information is needed (Cochran, 1977).

REDUCING THE ERROR ASSOCIATED IN A SAMPLE

The amount of error above the minimum amount needed to estimate the population for which information is desired can be reduced by a well planned sampling procedure. Several statistical texts discuss the appropriate steps in planning a sample survey, and they should be reviewed thoroughly before conducting a sample survey (Cochran, 1977; Deming, 1950; and Hansen et al., 1953). Provided these steps have been followed with great care, the best method to reduce error associated with a sampling survey is to select the optimum sample size and/or sampling system.

CHOOSING THE SAMPLE SIZE

The number of samples to collect is ultimately a subjective decision and represents a compromise between the amount of effort which is feasible and the desired accuracy of the estimate. The sample must not be so small that the estimate is inaccurate, or so large that the estimate is more precise than needed. This decision often cannot be made easily due to lack of sufficient information to determine optimum sample size.

The first step in selecting the optimum sample size (critical density) is to determine how much error one can tolerate in estimating the parameter, μ of the population (Snedecor and Cochran, 1967). This requires careful thought about the use to be

made from the sample estimate, and the consequences of associated error. The next step is to express the allowable error in terms of confidence limits (Cochran, 1977; Snedecor and Cochran, 1967). For example, two entomologists believed that asparagus should be sprayed with diazinon when the density of aphids reached 10 per primary branch. A sample mean of 10 would require 141 samples for a 33% error (confidence interval from 6.7 to 13.3 aphids per primary branch) at a 90% C.L. Therefore, a sample size must be chosen with a degree of accuracy (a level of precision expressed in C.L.'s) that is sufficient for control decisions (Wright and Cone, 1986).

Several probability formulas have been used to determine the appropriate sample size (Snedecor and Cochran, 1967; Cochran, 1977; Deming, 1950). One formula to determine the size of sample needed to attain a given limit of error is: $n = 4pqL^{-2}$, where p = the probability of successes, q = 1-p, and L = the allowable error (Cochran, 1977).

For example, if it is assumed that 30% of 81 samples are infected with *A. coenophialum*, the sample size needed to determine p, the per cent fungal incidence, to within ±10% is: $n = 4(0.30)(0.70)/ 0.10^2 = 84$ samples. If p is supposed to be 90%, then $n = 4 (0.90)(0.10)/0.10^2 = 36$ samples.

The main drawback of these formulas is that p is assumed to be known. In scientific research, p is unknown and can only be estimated. If s successes occur out of n e.u.'s, the estimate of p is s/n. This is called a point (or single-valued) estimate of p (Couey

and Chew, 1986). Unfortunately, point estimates are always wrong (i.e. $p \neq s/n$ even if n is as large as 1,000,000) (Couey and Chew, 1986). On the other hand, if p is between 35 and 65%, a rough estimate of p can be made, because the product pq varies little when p lies within these limits (Snedecor and Cochran, 1967). If p is near 0 or 100%, then a close guess about p is required for precise determination of n (Snedecor and Cochran, 1967). Whether or not p or q is < 30%, if np and nq are > 35, the coefficient of variation [$CV = (q/np)^{-.5}$] will be < 10% (Hansen et al., 1953).

Many estimates of sample size have been determined on the basis of some assumptions that have not yet been confirmed. Rather, they were chosen arbitrarily so that the estimates might be within manageable ranges. For example, if a simple random sample is to be drawn from an original population that is approximated by the normal distribution, then 50 observations are enough to yield a reasonably reliable estimate of the standard error of the mean or total (Hansen et al., 1953).

Presently, sampling programs for estimating the true incidence of *A. coenophialum* have relied on such reasoning. Investigators at Auburn University believe that 30 samples per 20 ha. provide a reliable estimate of fungal incidence. Two or more sample sets are taken if the field appears to be non-uniform. The University of Missouri uses a procedure where 3 equidistant transects are established in a field. Each transect, has 10 sampling sites. Three samples are collected per site, for a total of 90 samples. The fungal incidence status of one sample from each

site per transect is assessed. If the first sample is found to be E+, then the remaining two samples are not used. However, if the first sample was E-, then the other two samples are assessed for presence of *A. coenophialum.* A 0% field would require that all 90 samples be assessed. The rationale behind the Auburn and Missouri programs is that fungal incidence is enumerated very conservatively.

The use of sampling theory is not limited entirely to a subjective decision concerning sample size. Statistical inferences regarding sample size can be made through the repeated sampling of several independent populations that possess wide ranges of p. For example, a study at Auburn University (Peques et al., 1985) determined sample size using six, 148 m^2 plots of tall fescue that ranged from near 0 to almost 100% endophyte incidence. Plots were established by mechanically mixing specific quantities of E- seed with nearly 100% viable E+ seed. Each plot was sub-divided into 144, 0.835 m^2 sampling sites. Thirty random sampling sites were computer generated per plot. One sample was collected from each sampling site on April 19, August 7, and December 17, 1984. Random sampling sizes of 6, 9, 12, 15, 18, 21, 24, and 27 tillers were computer generated from the original 30 sites to determine the optimum sample size per plot. Twenty independent runs of the program for each sample size were made. Plot means and variances were calculated for all sample sizes.

There was no significant difference among sampling dates ($P>.05$), although the mean fungal incidence was slighty higher in

December. However there were significant differences in the actual and proposed E+ levels ($p > .01$). For example, the actual percentage for the proposed 17% E+ level was 34%; the actual percentage for the proposed 35 and 52% levels were 42 and 61%, respectively. Averages of all nine sample sizes resulted in similar estimates of infection incidence, but the s^2 of the estimates decreased as sample size increased. The sample size of 6 had an average s^2 of 285. As the sample size was increased to 9, the s^2 made a steep decline. This trend continued to 18 samples where the rate of s^2 decreased slowly.

Peques et al., 1985, subjectively decided, using statistical inferences about the population studied, that 10 tillers was acceptable for a uniform, 4 to 8 ha. pasture. If different seed was used to establish the pasture, smaller areas should be sampled. For research purposes, at least 20 tillers per 2-ha. should be collected in order to provide a less variable estimate of infection incidence (Peques et al., 1985).

It does appear that statistical inferences concerning sample size can be made by using the s^2 (s or C.V. could also be used) of infection percentages. It is a rational approach to determining a sample size with an acceptable level of precision. Unfortunately, the exact area for sampling pastures cannot be described until further research can be done (Peques et al., 1985).

SAMPLING DESIGNS

The previous discusion was based on the use of unbiased estimates obtained from random samples for the entire population. For this type of sampling, the size of sampling error can be modified only by changing the sample size (Hansen et al., 1953). A change in the sampling design with no accompanying change in the size of the sample can increase the precision of the results (Samford, 1962).

The stratified random sampling design (SR), in which the population is divided into uniform groups or strata, is one design that decreases the amount of error associated with a sample without changing the sample size (Cochran, 1977; Deming, 1950; Samford, 1962). The strata must not overlap and should cover the entire population. Random samples are then collected within each stratum.

For disease detection, the SR is the best design because it disperses sampling sites better than other sampling designs, which cover only a portion of a field using a predetermined path (Delp, Stowell, and Marois, 1986). Disease detection must be biased (or stratified) in favor of locating the pathogen (Seem et al., 1985). The SR is the only design which insures that every individual sample has an equal likelihood of being included in the entire sample. The other designs sample only a subpopulation that contains a finite amount of information about the entire population. Therefore, stratification is extremely useful when the population is non-uniform or aggregated (Cochran, 1977; Deming, 1950;

Samford, 1962). Sampling designs used to identify plant pathogens are illustrated in Figure 1. (Delp et al., 1986). Various degrees of aggregation within a field are illustrated in Figure 2. (Delp et al., 1986). Sampling designs that use a predetermined path (X, W, diagonals, partial X or W) cannot help to detect spatial relationships nor to determine the within-field variance unless the potential bias is accounted for in advance (Nicot, Rouse, and Yandel, 1984). The SR permits variance analysis, because the strata are uniform and independent (location of each sample for the entire population is known), (Delp et al., 1986). Several statistical methods, such as Lloyd's indices of mean crowding and patchiness, Fisher's variance to mean ratio, David and Moore's index of clumping, Greig-Smith's method, the nearest neighbor and the spatial autocorrelation methods, have been used for analysis of spatial patterns (Nicot et al., 1984). For example, if Fisher's mean to variance ratio, (V/m) is ≤ 1, than the response variable (infection) is randomly distributed in the field. If $V/m > 1$, the entire population is aggregated. Thus, when precise knowledge of *A. coenophialum* distribution is needed, (i. e. experiments that monitor grazing habits of bovine on aggregated pastures or sampling large pastures that have been sown with different seed sources), stratification of samples may prove to be beneficial. On the other hand, stratification will be advantageous over random sampling only if the strata are more homogeneous than the whole population (Samford, 1962). Therefore, stratification will not be advantageous if the pasture to be sampled is uniform (*i.e.* sown

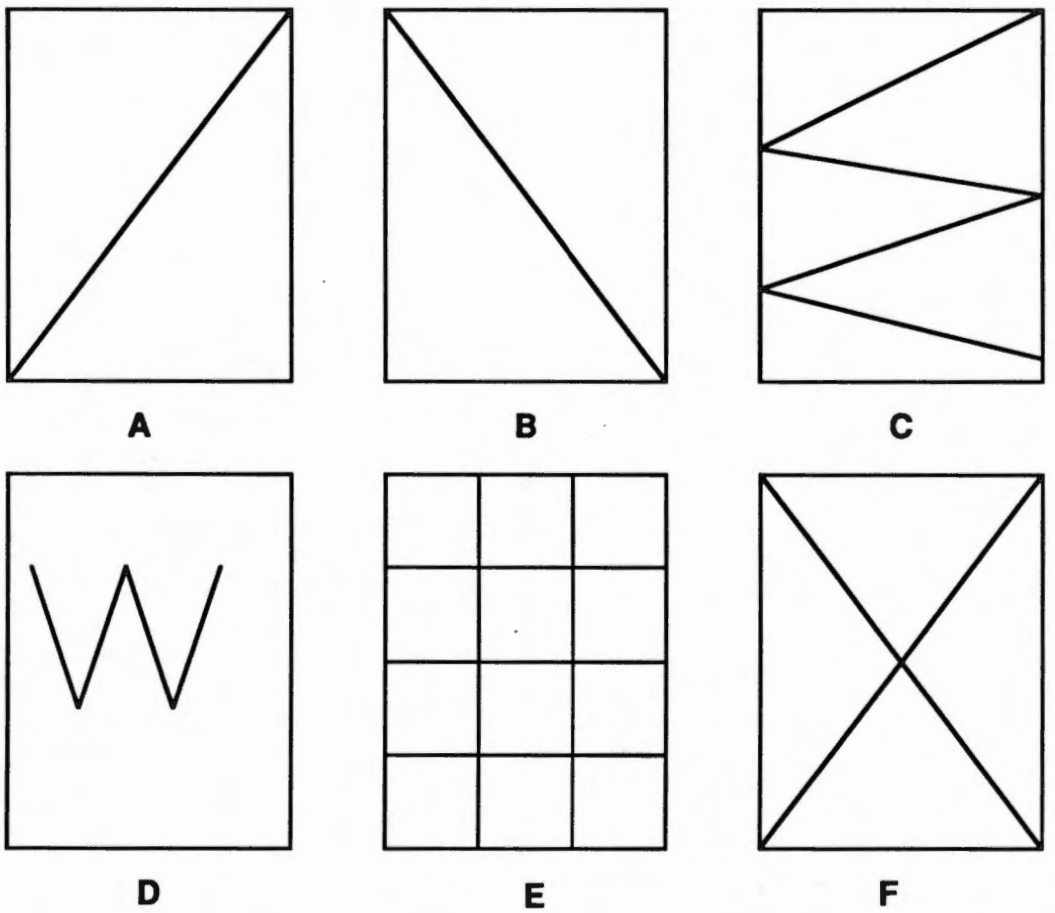

Figure 1. Sampling designs for detection of plant pathogens. (A) right diagonal (B) left diagonal (C) right 'W' (D) partial 'W' (E) stratified random, and (F) 'X'.

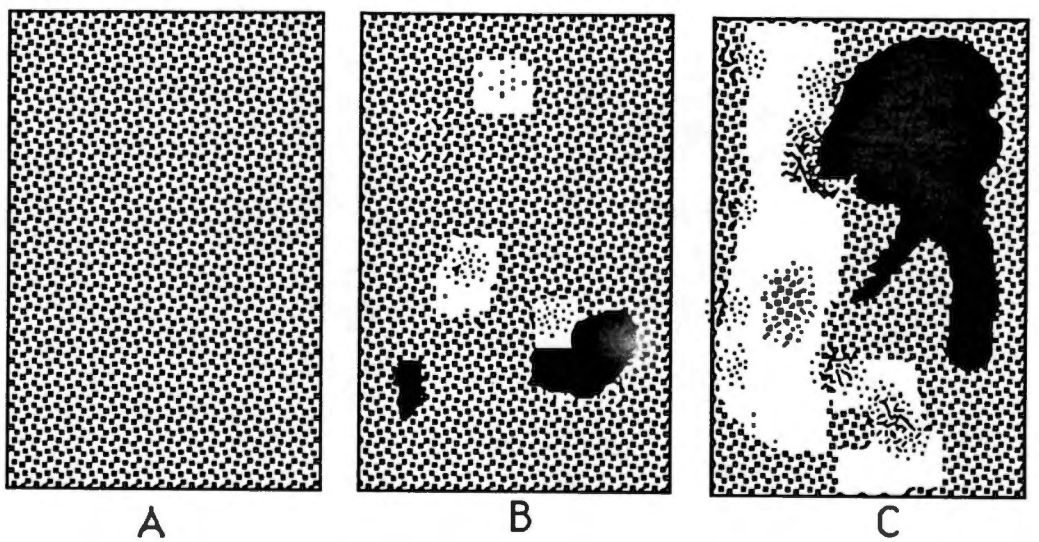

Figure 2. Example of various degrees of aggregation from A, random to C, highly aggregated.

with the same seed source, and having uniform soil conditions).

Cluster sampling is another sampling design used to reduce the error associated in estimating the response variable (Deming, 1950; and Hansen et al., 1953). In cluster sampling the whole population is divided into groups, and representative samples are drawn. The groups are the sampling units (frames). All the individual elementary units within a group can be selected, or smaller clusters of individual units can be drawn from the sampled clusters. The design is called two-stage (or more) sampling or subsampling when only a portion of individual units is selected from each frame (Hansen et al, 1953).

Provided the location of individual units within a frame is known, cluster sampling differs from stratified sampling in that the clusters must be heterogeneous. The concepts that allow for efficient stratified sampling allow for inefficient cluster sampling. The more alike individual units are within a cluster, the better results will be if a cluster is used as a stratum in stratified sampling and the worse results will be if used as a frame (Hansen et al., 1953).

CONCLUSIONS

The uniform dispersal of sampling sites across the entire population appears to be the best design to detect the response variable (p). The problem is: how is an appropriate sampling intensity determined for p values that have unknown incidence and distribution? Estimation of p is more difficult when p is small and

aggregated (Delp et al., 1986 (a,b); Lin et al., 1979; and Nicot et al., 1984). One approach may be to establish an incidence threshold defined as the lowest level of p that must be estimated accurately (Delp et al., 1986 (b)). Once the approximated p distribution and incidence threshold are established, a sampling intensity with an acceptable allowable error can be decided. This sample intensity could be used for subsequent populations.

III. ESTIMATION OF *ACREMONIUM COENOPHIALUM* INCIDENCE USING TRANSECT AND STRATIFIED RANDOM DESIGN SAMPLING METHODS

INTRODUCTION

The endophytic fungus, *Acremonium coenophialum* Morgan -Jones and Gams (1982) has been associated with agalactia, fat necrosis, fescue foot, and summer syndrome (Garner, 1984; Garret et al., 1980; Hoveland et al., 1983). Economic losses attributed to summer syndrome are in excess of 360 million dollars yr^{-1} in the U.S. based on animal performance data (Mueller, 1986). Current estimates indicate over 80 to 90% of all tall fescue (*Festuca arundinacea* Schreb.) pastures in the eastern U.S. have the endophyte (E+) (Fribourg, Wilkenson, and Rhodes, 1987). Reestablishment of E+ pastures with seed not containing the fungus (E-), or the incorporation of legumes into the sod is beneficial (Fribourg et al., 1987). However, since management decisions must be based on knowledge of *A. coenophialum* incidence, appropriate sampling methods should provide information about incidence levels with an acceptable level of confidence and effort.

Estimates of *A. coenophialum* incidence and distribution depend on data obtained from field samples. The accuracy of these estimates, and the expenditure of time needed to obtain them, are affected by sample intensity and by sampling design. Previous research on the assessment of crops for pathogens indicates that sample intensity was more critical than sampling design if the

pathogen was distributed randomly in the field (Delp, Stowell, and Marois, 1986; Lin, Poushinsky, and Mauer, 1979). Sampling design was more important if the disease was aggregated. Maximum dispersal of sampling sites over the entire population was the critical factor.

The stratified random sampling design (SR) (Cochran, 1977) is the best design for disease detection because it disperses sampling sites more than other designs, which cover only a finite portion of a field (Delp et al., 1986). Disease detection must be biased or stratified in favor of locating the pathogen (Seem et al., 1985). The SR is the only design which insures that every individual sample has an equal likelihood of being included in the sample survey (Cochran, 1977; Samford, 1962). The SR also allows the detection of spatial relationships and determination of the within-field variance, because the strata are uniform and independent (Cochran, 1977). Therefore, when precise knowledge of *A. coenophialum* distribution is needed, stratification of samples may be beneficial. On the other hand, stratification will be advantageous over random sampling only if the strata are more homogeneous than the whole population (Samford, 1962).

Uniform dispersal of sampling sites across the entire population appeared to be the best design to detect disease incidence and distribution (Delp et al., 1986). The problem faced by researchers in the past was the determination of an appropriate sampling intensity for populations of unknown incidence and distribution. Estimation of disease incidence is more difficult for

populations that have small disease incidences and/or a large degree of aggregation (Delp et al., 1986, Lin et al., 1979; and Nicot, Rouse, and Yandell, 1984). One approach has been to establish an incidence threshold, defined as the lowest level of disease incidence that must be estimated accurately (Delp et al., 1986). Once the approximated disease distribution and incidence threshold are established, an intensity with an acceptable allowable error can be selected (Delp et al., 1986).

The objective of this study was to assess the variability in estimates of *A. coenophialum* incidence and to compare the transect method (TM) described for Missouri (Garner, personal communication, 1986) and a SR sampling design. The two sampling methods were used to determine: 1) whether the incidence of E+ in pastures changed over time; 2) the within-field variance of incidence and its effect on sampling design; and 3) the effects of sample size and intensity on accuracy of incidence determination.

MATERIALS AND METHODS

Pasture Establishment

Eight 4-ha pastures at the Highland Rim Experiment Station, Springfield, TN were used for this study. Fescue with different levels of *A. coenophialum* incidence were established in pastures in March 1985. Nominal incidence levels were obtained by mixing specific quantities of E- seed with 80% E+ seed, adjusted for germination and fungal incidence (Appendix A-1).

Prior to seeding, existing sods had been destroyed with an application of glyphosate, [N-(phosphonomethyl) glycine], in Sep. 1984, and one application of paraquat, [1,1'-dimethyl-4, 4'-bipyridinium ion] in early March, at rates of 1.12 and 0.28 a.i. kg ha^{-1}, respectively. Remaining broadleaf weeds were spot-treated with dicamba [3,6-dicloro-2 methoxybenzoic acid]. Pastures were under grazing management for concurrent studies by the Animal Science and the Plant and Soil Science Departments.

Sample Collection

Forty-five samples were collected at random from each pasture on 10 June 1986 and assessed for fungal incidence. Each pasture was sampled again on 11 June 1987 using each of two sampling methods: TM and SR. On 23 July 1987 three pastures were resampled because infection estimates for both sampling methods were much higher than the nominal levels of 45, 30, and 15%. The authors assumed either that the estimates were incorrect or that nominal levels had changed 27 months after being established. Thirty random samples were collected from each pasture. Individual samples were assayed for presence of *A. coenophialum* using both the microscopic staining technique and the Protein-A ELISA procedure (Edwards and Cooper,1985) revised by Reddick and Collins (1988) and as presented in Appendix C.

Sample collection for the TM consisted of dividing each pasture into 3 transects. Each transect had 10 sampling sites about 18 m apart, at which 3 samples were collected.

For the SR, 10 x 10 (100 adjacent 405 m^2 strata) grids were established in each pasture; in a few cases, the grid was 9 x10 or 8 x 10 because of obstructions. Sample collection was done by walking down each column and sampling at each row.

For both sampling methods, a sample consisted of one tall fescue tiller from which the lowest 6 cm above ground level were used. Roots, soil, and leaf blades were excluded from each sample. Samples were labelled individually and frozen immediately upon collection in liquid N.

Fungal Determination

A. coenophialum presence was determined using a modified, indirect Protein A enzyme-linked immunosorbent assay (PAS-ELISA) (Edwards and Cooper, 1985) as revised by Reddick and Collins (1987) and as presented in Appendix C. Field samples known to be E+ or E- from microscopic examination of sheaths from individual fescue spaced clumps (Conger et al., 1986) were collected as needed for use as standards. Three E- and two E+ standards were included in each 96-well medium binding polystyrene ELISA plate (Platic Injectors, Spartanburg, S. C.). Four adjacent wells of PBS-Tween 20 were included also in each plate to determine the range of the E- background absorbance interval. Two times the average absorbance of E- standards was the minimum positive-negative threshold level used to decided whether a sample was E+. Samples were considered as missing data if the absorbance was near the threshold level. A histogram for data

from each plate was constructed to determine if a bimodal distribution of the two populations existed. Data were not used if the two populations were not separated by a large interval (Sutula et al., 1986). The 60% nominal pasture was resampled on 19 July 87 using the TM, because the enzyme that had been used for one plate of 44 samples was too old for good color development.

Statistical Analysis

Statistical analysis was based on the binomial distribution, since each observation was dichotomized as 0 (E-), or 1 (E+) (Couey and Chew, 1986) and the array of samples could be considered to constitute such a distribution. *A. coenophialum* incidence, the proportion of E+ samples (p), was calculated for each pasture and sampling method. Fisher's variance to mean ratio, V/m, was calculated to determine if the distribution of *A. coenophialum* was random or aggregate in each pasture (Nicot et al. 1984). A value > 1 implied that the disease was distributed aggregately. A value ≤ 1 implied that distribution was random. The associated sampling error or bias was calculated to determine the precision of the estimates of both methods. The sample statistics used to measure the degree of sampling error were the standard deviation [$s = (pq/n)^{-0.5}$] and the standard error of the mean [$s_{\bar{x}} = s/(n)^{-0.5}$] (Snedecor and Cochran, 1963). Sample variation was described by the coefficient of variation [$CV = (q/np)^{-0.5}$]. A one sample z-test for sample proportions was used to test the null hypothesis that estimates of *A. coenophialum* incidence from each sampling

method were not significantly different from the 1985 nominal infection levels. Acceptance of the null hypothesis meant that p was distributed approximately with mean p and $s = pq/n^{-0.5}$. Values of z for each pasture and sampling method were calculated by $z = (\hat{p}-\overset{o}{p})/s^{-0.5}$ (Snedecor and Cochran, 1963), where $\hat{p}$ = the observed value of fungal incidence, and $\overset{o}{p}$ = the expected nominal fungal incidence. An additional test by means of the normal deviate z was done to determine the size of the difference between $\hat{p}$ estimates from the TM and the SR methods. The formula was $z = p_1 - p_2 / [pq\ (1/n_1 + 1/n_2)]^{-0.5}$ (Snedecor and Cochran, 1963), where p_1 and p_2 were the estimated fungal incidence of the TM and the SR, n_1 and n_2 were the sample sizes, $\hat{p} = (p_1 n_1) + (p_2 n_2) / n_1 + n_2$, and q = 1-p.

Several subsampling arrangements were selected to determine if smaller sample intensities might have given acceptable estimates of *A. coenophialum* incidence. The allowable error of each arrangement was expressed in terms of confidence limits for p approximations. The approximate 90 percent confidence limits for p were $p \pm z_{1-\alpha/2}\ [\ (pq/n\text{-}1\ (1\text{-}n/N))]^{-0.5}$, where p = the estimated fungal incidence of each arangement, q = 1-p, n = sample size of each arrangement, N = original sample size of each sampling method, and 1-n/N = finite population correction factor for each sampling arrangement (Stoodely, Lewis, and Stainton, 1980). The standard deviation (s) and standard error of the population mean $[s_{N\bar{x}} = \{(N\ s/(n^{-0.5})((1\text{-}n/N))^{-0.5})\}]$ (Snedecor and Cochran, 1963) were calculated also to determine the accuracy of the sample mean

(p) of each arrangement as an estimator of the mean of all observations. The mean (μ) of each sampling method was estimated using only the results of subsampling arrangements drawn randomly from each method. The amount of variation of each arrangement was described by the CV.

For samples collected using the TM, the first arrangement was done according to the specifications used at the University of Missouri (Garner, personal communication, 1986). The fungal incidence status of one sample from each site per transect per pasture was determined. If the first sample was found to be E+, then the remaining two samples were not used. However, if the first sample was E-, then the other two samples were included into the sample set. A 0% infected field would require that all 90 samples be included.

The second arrangement from TM consisted of sequentially sampling 3 independent stages or groups of 30 samples. Samples from each site were trichotomized as A, B, or C. Only samples with the same classification were included into each stage. The last selected sampling arrangement for the TM consisted of grouping samples independently by each of the three transects per pasture (30 samples per transect).

For samples obtained using the SR, a computer program was written to select the following arrangements of random samples from each pasture:

Subsampling arrangements	% of total sample size
a. 1 sample out of 2 adjacent samples col^{-1}	50
b. 1 sample out of 2 adjacent samples row^{-1}	50
c. 1 sample out of 3 adjacent samples col^{-1}	33
d. 1 sample out of 3 adjacent samples row^{-1}	33
e. 1 sample out of 4 adjacent samples col^{-1}	25
f. 1 sample out of 4 adjacent samples row^{-1}	25
g. 1 sample out of each 2 rows X 2 columns square	25
h. 1 sample out of each 3 rows X 3 columns square	16
i. 1 sample out of each 4 rows X 4 columns square	4
j. all cols out of every three rows starting with row 2	30

RESULTS AND DISCUSSION

Infection Status Over Time

The change in *A. coenophialum* incidence from 1985 to 1987 may be dependent upon the initial nominal level in a pasture (Table1). Incidence in pastures with 1985 nominal levels of 75 and 60% had not changed appreciably 15 and 27 months after establishment. On the other hand, the status of two pastures with 1985 nominal levels of 30 and 15% changed substantially. On the average, incidence in the 30% pasture had doubled, and that in the 15% pasture had tripled by June 1986 and almost quadrupled by July 1987. Results obtained from a subsequent 1987 sampling of the same pastures, using both the staining technique and PAS-ELISA for

Table 1. *A. coenophialum* incidence estimates (p) 15, 27, and 28 months after establishment of fescue with nominal levels of fungal incidence and the average daily gains of calves and cows Springfield, TN, 1985-1987.

Fungal incidence (p)					
20 March 85	10 June 86	11 June 87		1986 Spring gain/d	
Nominal incidence at establishment	45 random samples	Transect Method	Stratified Random	Calves	Cows
--%--	--%--	-------%-------		----kg·d^{-1}----	
75	88	74	81	0.75	-0.08
75	83	*83	*86	0.75	-0.07
60	63	66	67	0.85	-0.13
45	35	*72	*74	0.79	-0.13
30	62	*63	*49	0.77	-0.07
15	47	*59	*55	0.79	-0.01
0	--	*12	*12	1.03	0.15
0	2	*5	*9	0.94	0.00

* estimates (p) significantly deviated (p>z) from expected 1985 nominal fungal incidences.

detection of *A. coenophialum*, confirmed the July estimates: the 30% nominal pasture had 60 and 68% E+ for the staining and PAS-ELISA techniques, respectively. The 15% pasture had fungal estimates of of 60 and 81%, respectively.

The 45% nominal pasture had variable incidence. Incidence decreased 10% after 15 months and increased almost 30% after 27 months for both sampling methods. A sampling at 28 months gave estimates that were close to the 45% nominal level, 42% and 50% for the staining technique and PAS-ELISA, respectively.

One of the two E- nominal pastures was infected slightly with *A. coenophialum* 15 months after establishment. Data from the other E- nominal pasture were not used because E- and E+ samples were not separated by a large interval of absorbance. Levels of 5 to 12% E+ *A. coenophialum* were found in both E- pastures 27 months after establishment.

Performance of cattle (*Bos taurus* L.) grazing theses pastures during 1986-87 correlated well with observed *A. coenophialum* estimates (Keltner et al., 1988) (Table 1). Calves grazing the 60 and 75% nominal pastures gained an average of 0.2 kg day^{-1} less than calves on the 0% nominal pastures during spring. On the other hand, calve gains on the 15 and 30% nominal pastures were similar to those of calves on the 60 and 75% pastures. Gains of cows grazing the 15 and 30% nominal pastures were also similar to those of cows grazing the 60 and 75% E+ pastures. Cows grazing the 0% E- pastures gained 0.15 kg day^{-1} more than did those grazing the E+ nominal pastures. It was apparent that cow-calf performance on

the 15 and 30% pastures was similar to performance on the 60 and 75% pastures, because the fungal incidence increased. Cow and calf gains were sastisfactory on the E- pastures because the fungal incidence changed little.

Other research has indicated that the fungal level incidence of pastures established with endophyte free seed (95 to 99%), increases in succeeding years to much higher levels, because of the seed-borne nature of the fungus (Bacon et al., 1986) Depending on the prevailing environmental conditions, E+ plants produce more seed than E- plants. Preliminary results from sample assessment of research paddocks sown with the E- cultivars 'Forager' and 'Johnstone' support the observations of Bacon et al., (1986). Fungal infection levels ranged from 10 to 20% over all paddocks (Fribourg, McLaren, and Gwinn, unpublished data, 1987).

Another factor that may contribute to this dilemma is that the fungus may remain dormant until favorable environmental conditions allow it to grow into seed. For example, a breeder's seed field sown with the fungus free cultivar 'AU Triumph' was monitored for E+ incidence over a 5-yr period (Pedersen et al., 1984). The field produced essentially clean seed the first 4 years (1978-1981). However, seed were 31% E+ in the fifth year. The seed field was found to have been established from seed that was 59% E+, producing plants that were 57% E+. It was assumed that the fungus did not invade the seed until 1982 when environmental factors were favorable for hyphal growth.

In summary, the fungal incidence of the 60 and 75% nominal pastures did not change appreciably over a 2-yr period. The other pastures had significant changes in fungal incidence 15 and 27 months after establishment. The fungal estimates of the E- nominal pastures were still small by the end of the second year. The fungal incidence of pastures starting out with moderate levels of E+ increased greatly. These increases may be due to the competitive advantage of E+ plants over E- plants and/or the occurrence of unknown environmental conditions favorable for growth of the fungus. It is possible that some sample bias may have been introduced in the sampling of these pastures, because large, vigorous E+ tillers may have been selected inadvertently over smaller, stressed E- tillers. It is often easy to overlook the human error associated with obtaining a representative sample. Therefore, in the future, each random tiller should be selected blindly, *i.e.*, throwing a piece of flagging material behind one's back and collecting the closest tiller. In conclusion, reestablished pastures may have higher fungal incidence in succeeding years and should be sampled periodically (yearly or once every two years) to determine whether *A. coenophialum* is present and, if so, to what extent.

A. coenophialum Spatial Distribution

Random occurence of *A. coenophialum* was expected, since the pastures had been sown uniformly. Fisher's variance to mean ratio test for disease distribution for all pastures sampled using both the TM and the SR gave V/m ratios that were less than 1. A variance to

mean ratio that is less than or equal to 1 implies that distribution is random in the field.

However, there was some variation in estimates of *A. coenophialum* among transects of the TM, indicating some within-field variance, especially among pastures having low incidence levels (Table 2). Pastures possessing variable degrees of aggregation need to be sampled to determine if the TM could detect consistently within-field variance. It is logical to assume that the distribution of *A. coenophialum* could be random or aggregate depending on environmental conditions prevailing within a pasture and/or whether or not the field was sown uniformly using the same seed source. The detection capability of partial designs like the TM may be enhanced by increasing the number of transects among fields without an associated increase in sample size (Lin et al., 1979).

Comparison of the Transect Method and the Stratified Random Sampling Design

Observed sample proportion estimates were not significantly different for seven of eight pastures, when the normal deviate z test was applied to data from both sampling methods (Table 3). Similar degrees of sampling error or bias (s) and variation (CV) were observed for both TM and SR. Sample estimates were similar because *A. coenophialum* was distributed randomly in all pastures, negating the sampling design effect, and sampling intensities also were approximately equivalent. Stratification of samples will be

Table 2. Estimates of *A. coenophialum* incidence (p) and associated sampling error (s) among transects of the transect method used to characterize eight pastures, Springfield, TN, 1987.

All samples	Transect								
	1			2			3		
p	p	n	$s*10^2$	p	n	$s*10^2$	p	n	$s*10^2$
--------%--------				%			%		
83	83	29	7.0	81	30	6.9	86	28	6.6
74	77	30	7.7	62	29	9.0	80	30	7.3
72	73	22	8.1	*87	26	6.2	*57	30	9.0
71	72	29	8.3	77	30	7.7	*44	9	16.5
63	70	30	8.4	57	28	9.3	60	30	8.9
59	50	30	9.1	*70	30	8.4	59	29	9.1
12	*23	30	7.7	8	25	5.4	0	18	0.0
5	11	26	6.3	0	29	0.0	4	29	3.4

* Estimates deviated significantly from observed estimates of all samples of the TM.

Table 3. Estimates of A. coenophialum incidence (p), the normal deviate z test of sample proportions (p>z), associated sample error (s), and variation (CV) for the transect method (TM) and a stratified random (SR) sampling procedure, Springfield, TN 1987.

Nominal fungal incidence	*A. coenophialum* incidence, p		sample size		probability of p > z	$s*10^2$		CV	
	TM	SR	TM	SR		TM	SR	TM	SR
--%--	----%----		-----n-----						
75	74	81	89	77	0.133	4.7	4.5	6.4	5.6
75	83	86	89	90	0.288	4.0	3.7	4.8	4.3
60¶	61	67	46	71	0.255	7.2	5.6	11.8	8.5
	71		68		0.305	5.5	.	.	7.8
45	72	74	90	97	0.378	4.7	4.4	6.5	6.0
30	63	49	88	86	0.031*	5.2	5.4	8.3	11.0
15	59	55	89	100	0.291	5.2	4.9	8.7	9.0
0	12	12	73	74	0.500	3.8	3.8	.	.
0	5	9	84	89	0.152	2.3	3.0	.	.

¶ Identical pasture resampled using the MT method due to small n.
* significant deviation between estimates (p) of the MT and SR sampling procedures.

advantageous over other designs which cover a finite portion of a pasture (i.e. three equidistant transects) only if the strata are more homogeneous than the whole population. Sample intensity is more important than sampling design if the disease is distributed randomly (Delp et al., 1986; Lin et al., 1979).

Since sample proportion estimates deviated significantly in the 60% pasture a second sampling was done 45 days later, using both the PAS-ELISA and the microscopic staining technique. Estimates of *A. coenophialum* incidence were similar to those obtained from the TM. Whether or not the sample estimate observed from the SR was biased is not known.

Precision of Subsampling Arrangements of the Transect Method and the Stratified Random Design

Appropiate sampling methods must provide information of *A. coenophialum* incidence with known accuracy. The precision of a sampling method is affected by the sampling intensity and design. Unfortunately, selection of optimum sample intensities for pastures having unknown fungal incidence is difficult, since some prior knowledge of the incidence level is needed in order to select precise sample intensities. The apparent dilemma is that prior knowledge of incidence levels requires a subsequent sample. Therefore, appropriate sampling methods must be selected subjectively from statistical inferences drawn from independent samples. Selected subsampling arrangements from each sampling method were assessed from four pastures that encompassed the range of fungal

incidence to determine whether smaller sample intensities than the entire array would have given acceptable estimates of *A. coenophialum* (Appendix A-2 and Appendix A-3).

For sampling arrangements selected from the TM, a sequential stage or group of thirty samples (3-stage sampling) gave estimates that were less variable than the other arrangements. (Appendix A-2 and Appendix A-3). Twenty-six of 27 independent sample stages gave estimates that did not deviate significantly from the expected estimates from all samples (Table 4). On the other hand, pastures sampled according to the specifications of the University of Missouri gave estimates that were often biased: 5 estimates out of 9 deviated significantly from expected estimates of all samples (Table 4). Sample bias or error was observed because E- samples have a higher probability of being included in the sample survey than do E+ samples, which caused E+ incidence levels to be consistently underestimated. Therefore, biased estimates often may be observed when moderately E+ pastures are assessed according to Missouri specifications. Unbiased estimates usually will be obtained from pastures with low levels of E+, because the population tends to be enumerated.

In general, estimates obtained from individual transects were accurate (19 of 24 transects gave nonsignificant estimates), since *A. coenophialum* distribution was random. The assessment of pastures by individual transects is not advocated in view of the importance of dispersing sampling sites.

Table 4. Estimates of *A. coenophialum* (p) and associated 90% confidence limits (CL) using n subsamples of all samples from the transect method, Springfield, TN, 1987.

Nominal level	All samples		Missouri criteria¶		Sample Stage£ 1		2		3	
p	p	no.	p±CL	no.	p±CL	no.	p±CL	no.	p±CL	no.
---%	----	n	--%	n	--%	n	--%	n	--%	n
75	83	89	*73±09	41	83±09	30	83±09	30	83±10	29
75	74	89	*60±09	43	79±10	29	73±11	30	67±12	30
45	72	90	66±09	44	77±11	30	67±12	30	73±11	30
30	63	88	*51±08	51	63±12	30	60±12	30	64±13	28
#60	61	46	*46±10	28	63±17	16	67±17	15	53±18	15
#60	71	68	73±10	33	78±12	23	*56±17	23	77±13	22
15	59	89	*49±07	53	62±12	29	60±12	30	57±12	30
0	12	73	11±02	65	14±08	29	9±08	23	14±11	21
0	5	84	5±00	84	0±00	28	4±05	28	11±08	23

¶ If the first sample of each site was E+ then the other two samples were discarded. If the first sample was E- then all three samples per site were included into the sample set.

£ Three stages of 30 were sampled sequentially with one sample being drawn independently from each site per stage.

Identical pasture resampled due to small n.

* significant deviation observed between estimated incidence and the expected incidence (all samples).

In summary, estimates obtained from individual stages of 30 samples were more accurate than those obtained from individual transects. Associated 90% confidence limits (CL's) and standard error's of the means ($S_{\bar{x}}$) were smaller for samples arranged according to the Missouri specifications (Appendix A-2 and Appendix A-3). This was expected, because the size of the band of each C.L. and the degree of sampling error are both based largely on sampling intensity. Sampling intensities were larger for arrangements selected according to the Missouri specifications giving smaller confidence bands and standard errors. Estimates drawn from sampling arrangements assessed according to the Missouri specifications often were biased even though sampling sizes were always larger than those selected using three stage or individual transects sampling.

Observed estimates of *A. coenophialum* deviated little from the expected estimates for most of the sampling arrangements selected from the SR with 25 or more samples per 4 ha (Appendix A-2 and Appendix A-3). It appears that few samples are required for assessment of endophyte status in randomly distributed fields.

Nine samples per 4 ha (3 X 3 square arrangement) often gave adequate estimates of fungal status in each pasture, but this intensity resulted in wide 90% confidence limits that were not acceptable. Estimates from 4 samples per 4 ha (4 X 4 square arrangement) were consistently biased because this sampling intensity is too small. All other sampling arrangements tried should give adequate estimates of *A. coenophialum* incidence for

production purposes since over 90% of all fescue pastures are E+ and most of these fields are severely infected.

Twenty-five samples per 4 ha or a 5 X 5 grid of 25 adjacent 1620 m^{-2} stata (2 X 2 square arrangement) should be an acceptable sampling method for producers, because few samples are needed to determine incidence levels accurately in random fields. Dividing fields into three equidistant rows (10 samples per row) (Appendix A-2 and Appendix A-3) also gave accurate estimates. This sampling arrangement is equivalent to sequentially selecting one stage or group of 30 samples per 4 ha from TM.

For research purposes, a more precise estimation of *A. coenophialum* incidence is required than for producer's fields, especially when knowledge of spatial patterns is desired. For sample collection, fields should be divided into grids for stratified random sampling with a sampling intensity approaching at least one sample per 250 m^2. Collected samples shoud be assessed by selecting at random 1 out of 3 adjacent samples col^{-1} or row^{-1}. The remaining samples should be saved as backup samples for possible analysis in case the original ones do not provide a clear-cut assessment.

IV. SAMPLING INTENSITY AND TIMING FOR DETECTING INCIDENCE OF *ACREMONIUM COENOPHIALUM* IN FESCUE PASTURES

INTRODUCTION

Current estimates indicate over 80 to 95% of all tall fescue (*Festuca arundinacea* Schreb.) pastures are infected (E+) with the endophytic fungus *Acremonium coenophialum* Morgan-Jones and Gams (1982) (Fribourg, Wilkenson, and Rhodes, 1988). The fungus, either by itself or because of some interaction with fescue tissue, produces a toxin(s) causing livestock disorders like fescue foot, fat necrosis, agalactia, and summer syndrome (Stuedemann and Hoveland, 1988; Mueller, 1986). Summer syndrome is by far the more prevalent disorder observed when bovines (*Bos taurus* L.) ingest E+ fescue. Symptoms include reduced average daily gains (ADG), small milk production, low conception rates, rough hair coats, and slightly higher rectal temperatures and respiration rates. (Stuedemann and Hoveland, 1988). Economic losses attributed to summer syndrome in the U.S. are as much as 360 million dollars$\cdot yr^{-1}$, based on animal performance data (Mueller, 1986). Fortunately, reestablisment of E+ pastures with non-infected (E-) cultivars or the incorporatation of legumes into the sod is beneficial (Fribourg, Wilkenson, and Rhodes, 1988). However, since management decisions must be based on the knowledge of *A. coenophialum* incidence (the number of E+ samples expressed as the percentage of the total number assessed), appropriate sampling

procedures should provide information about E+ levels with known accuracy.

To date, sampling procedures for identification of *A. coenophialum* incidence have been based on random sample intensities that were chosen so that estimates might be within manageable ranges. For example, researchers at Auburn University (Auburn Univ. Fescue Toxicity Diagonostic Center, circa 1986) subjectively concluded that 30 random samples per 20 ha would provide a reliable estimate of E+ incidence. Two or more sample sets are collected if the field appears to be non-uniform, was sown using two or more seed sources, or is larger than 20 ha. The same sampling procedure is used by the Plant and Pest Diagnostic Center of the University of Tennesse (Windham, 1986).

The University of Missouri uses a procedure where 3 equidistant transects are established in a field (Garner, personal communication). Each transect has 10 sampling sites. Three samples are collected per site, for a total of 90 samples. One sample from each site is assayed for presence of *A. coenophialum*. If the first sample is E+ then the remaining two samples are not examined. However, if the first sample is E-, then the other two samples are included into the sample survey. A 0% E+ field requires that all 90 samples be assayed. Results are recorded on a map to determine if there is aggregation within a field. The rationale behind the Auburn and Missouri sampling procedures is that *A. coenophialum* incidence is enumerated conservatively.

Some research has been done to estimate sample intensities that predict *A. coenophialum* incidence with an acceptable level of confidence. At Auburn Universtiy (Peaques et al., 1985) it was determined that 10 random samples constituted an acceptable sampling size for a uniform 4 to 8-ha pasture. For research purposes, at least 20 tillers per 2-ha should be collected to provide a more precise estimate of fungal incidence (Peaques et al., 1985). To the author's knowledge, this procedure is not used currently.

The purpose of this experiment was to monitor *A. coenophialum* incidence over a 2-year sampling period using a stratified random sampling design (Cochran, 1977) on four 2-ha pastures. The objectives were to determine: 1) the seasonal distribution of *A. coenophialum* incidence; 2) the within-field variance and spatial relationships of E+ incidence; and 3) the effects of sample size and intensity on accuracy of fungal incidence estimates.

MATERIALS AND METHODS

Sample Collection

Four 2-ha tall fescue pastures at the Blount Animal Science Farm, Knoxville Experiment Station, were selected for use in this study. Two of the pastures, established in 1969, were made up of a fescue clover mixture. The other two, established in 1977, were solid fescue stands. Pastures were under grazing management for concurrent studies by the Animal Science Department.

Permanent 9 x 9 grids (81 adjacent 232 m^2 two-dimensional strata) were established on each pasture for sample collection. One tiller, cut 4-6 cm directly from the crown of the plant, was collected at random within each of the 81 sampling sites. Roots, soil, and leaf blades were removed. Samples were collected at monthly intervals starting from November 1985 through October 1987, and frozen in liquid N after collection.

Fungal Incidence Determination

A. coenophialum presence was determined using direct enzyme-linked immunosorbent assay (DAS-ELISA), (McLaughlin et al., 1981) (Appendix C) from November 1985 through April 1986. Later samplings were assayed with a modified, indirect Protein A enzyme linked-imunosorbent assay method (PAS-ELISA) (Edwards and Cooper, 1985) as revised by Reddick and Collins (1988) (Appendix C). Detection methods were changed because PAS-ELISA was more specific for *A. coenophialum* mycelia, reducing adjacent well and background variability. Absorbance readings for positive standards also were consistently higher with PAS-ELISA.

Field samples, known to be E+ or E- from microscopic examination of sheaths were collected from individually spaced fescue clumps (Conger et al., 1986) (Appendix C) as needed for use as standards. Two E- and two E+ standards were included in each 96-well medium binding polystyrene ELISA plate (Plastic Injectors, Spartanburg, SC). Four adjacent wells of PBS-Tween were included also in each plate to determine the range of the healthy background

absorbance interval. Three times the average background absorbance of E- standards was the minimum threshold level used to conclude that a sample was E+. Samples were considered as missing data if the absorbance was near the positive-negative threshold level. Histograms for data from each plate were constructed to determine if a bimodal distribution of the two populations existed. Data were not used if the E+ and E- populations were not separated by a large interval of absorbance (Sutula et al., 1986).

Statistical Analysis and Data Presentations

E+ incidence over time

Data were digitized as either 0 (E-) or 1 (E+). Since the observed response was dichotomized, the appropriate statistical treatment was based on the binomial distribution (Couey and Chew, 1986). Sample intensity (N=81) was the number of tillers collected per pasture. Sample size (n=1) was the number of tillers examined at each sample site. *A. coenophialum* incidence, the proportion of E+ tillers (p) was calculated for each pasture and sampling date. Bar graphs of the estimated p's were constructed to present the seasonal distribution of *A. coenophialum* incidence. Data from the fescue-clover pastures (group A) and the solid fescue stands (group B) were merged into two individual data sets, because both pastures within each group had been sown with the same seed source, received identical mangement, and were separated only by a driving range and/or fence rows. Monthly estimates of E+ incidence were

also merged into one data set for each pasture, and the associated sampling error ($S\bar{x}$), variation (CV), and departure from normality (kurtosis ratios) were calculated.

Spatial distribution within pastures

The degree of within-field variance was determined by calculating the variability among three transects of 3 rows or columns. Fisher's mean to variance ratio, V/m (Delp and Marois, 1986), was used to quantify the distribution of E+ estimates among transects within each pasture. A value > 1 implied that the fungus was aggregated within the field and a value ≤ 1 implied that the distribution was random.

Contour plots were constructed depicting the weighting or steepness of E+ incidence over all contiguous strata of equal size. A third order, saturated, polynomial model was fitted using the independent variables row and column (location) to predict the variability in E+ distribution. The model used was: $Y = BX \cdot Eij$, where $B = B_0 \times B_{row} \times B_{column} \times B_{row^2} \times B_{column^2} \times B_{rowxcolumn} \times$ ..... B_{col^3}. The general linear model (GLM) procedure (SAS, 1985) was used for analysis of data from each sampling date per pasture. A preliminary GLM analysis was performed on data containing the saturated polynomial model. A final GLM analysis was done on models comprised of only the significant ($p < 0.10$) row and column effects. Contour plots were then constructed to describe the steepness or weighting of E+ incidence across individual strata.

Sampling intensity

To determine whether smaller sample intensities might have given acceptable estimates of *A. coenophialum* incidence the following arrangements were selected from each 9 x 9 grid per pasture per sampling date:

Subsampling arrangements	% of total sample size
a. even row and columns	80
b. 1 sample out of 2 adjacent samples col^{-1}	44
c. 1 sample out of 2 adjacent samples row^{-1}	44
d. 1 sample out of 3 adjacent samples col^{-1}	33
e. 1 sample out of 3 adjacent samples row^{-1}	33
f. 1 sample out of 4 adjacent samples col^{-1}	22
g. 1 sample out of 4 adjacent samples row^{-1}	22
h. 1 sample out of each 2 rows X 2 columns square	20
i. 1 sample out of each 3 rows X 3 columns square	11
j. 1 sample out of each 4 rows X 4 columns square	5
k. all cols out of every three rows starting with row 2	33

The allowable error of each arrangement was expressed in terms of confidence limits for p approximations. The approximate (1-alpha) percent confidence limits for p were $p \pm z_{1\text{-alpha}/2} [(pq/n\text{-}1\ (1\text{-}n/N))]^{-0.5}$, where p = the estimated fungal incidence of each arrangement, q = 1-p, n = sample size for each arrangement, N = original sample size, and 1-n/N = finite population correction factor for each sampling arrangement (Stoodely, Lewis, and Stainton, 1980). The standard deviation ($s = (pq/n)^{-0.5}$ (Snedecor and Cochran,

1963) was calculated to determine the accuracy of the sample mean (p) of each arrangement as an estimator of the mean of all samples per pasture per sampling date. The amount of variation for each arrangement was described by the coefficient of variation [CV = $(q/np)^{-0.5}$]. A one sample z-test for sample proportions was also used to test the null hypothesis that estimates of *A. coenophialum* incidence from each sampling arrangement were not significantly different from the expected estimate of all samples per pasture per sampling date. Acceptance of the null hypothesis meant that p was distributed approximately with mean $\mathring{p}$ and s = pq/n -0.5. Values of z for each pasture and sampling date were calculated by z = $(\hat{p}-\mathring{p})$ s^{-1} (Snedecor and Cochran, 1963), where $\hat{p}$ = the observed value of fungal incidence for each sampling arrangement, $\mathring{p}$ = the expected nominal fungal incidence of all samples.

RESULTS AND DISCUSSION

Occurence of *A. coenophialum* Over Time

The seasonal occurence of E+ incidence was homogeneous when data from the two mixed and the two solid fescue pastures were merged into separate data sets, groups A and B respectively (Figure 3 and Figure 4). Simple regression analysis to describe the variability in E+ incidence relative to season indicated that incidence was linear for both groups , y = 59.6 + 0.39X (R=0.21) for Group A, and y = 62.4 + 0.23X (R=0.19) for Group B, respectively.

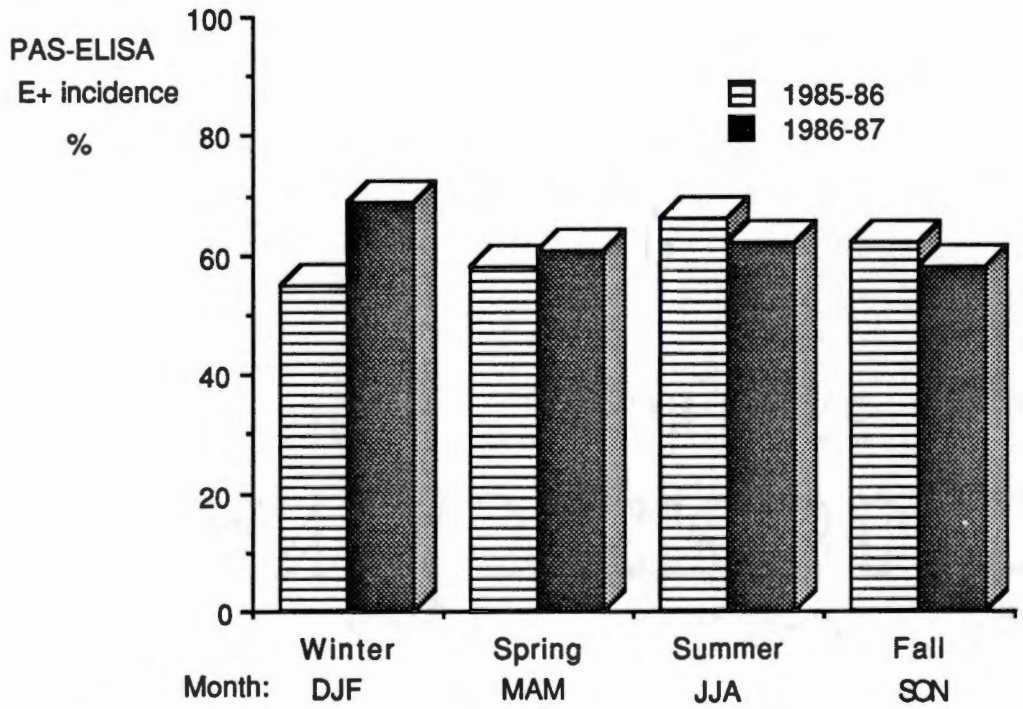

Figure 3. Seasonal E+ incidence for Pasture Group A (fescue + clover).

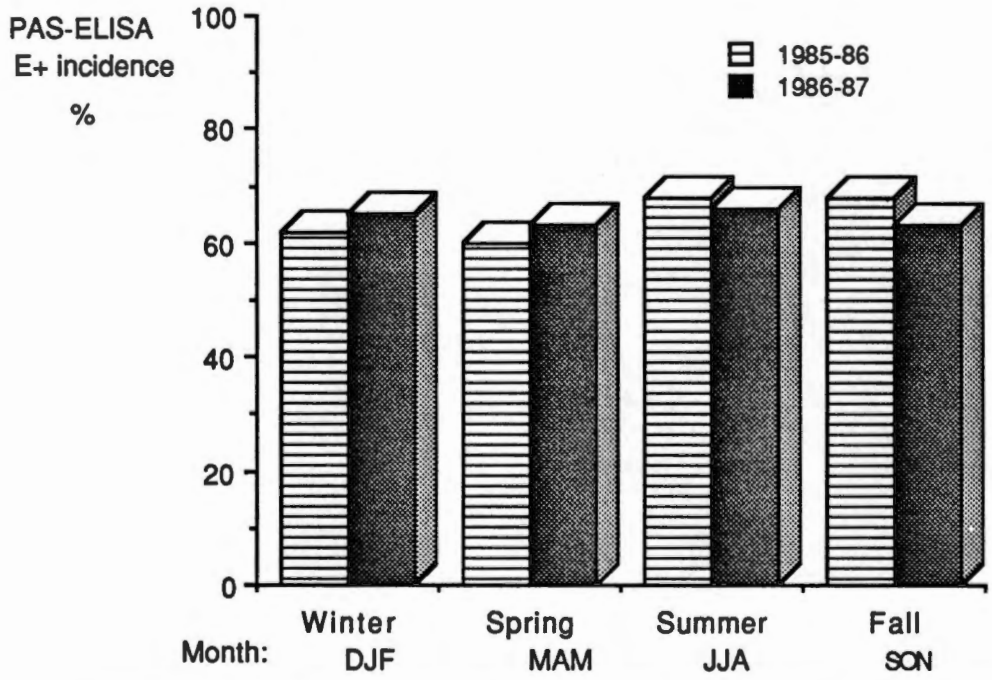

Figure 4. Seasonal E+ incidence for Pasture Group B (fescue).

The E+ incidence for each pasture varied only slightly from month to month (Table 5). Kurtosis ratios were less than 1, indicating the departures from normality were very small for all pastures. A kurtosis ratio that is less than +3 implies that the distribution is flat (Snedecor and Cochran, 1963). The associated sampling error ($S_{\bar{x}}$) and variation (CV) were also small for all pastures.

There have been differences reported in the literature concerning the E+ incidence of a pasture over time (Bush and Burrus, 1988). Most sample surveys have indicated that the E+ incidence of a pasture reflects the percentage of E+ seed planted. On the other hand, a few surveys have indicated that E+ incidence can be heterogeneous over time. The apparent ability of E+ plants to grow more vigorously than E- plants under less than optimum environmental conditions may be a cause for the disparity among fungal incidence over time.

Surprising results were obtained in a concurrent sampling experiment at another location where 90 tillers were collected from eight, 4-ha pastures that had been established with nominal E+ levels ranging from near 0 to 75%. The moderate E+ pastures of 15 and 30% at seeding increased to about 60% 27 months after establishment (unpublished data). On the other hand, the 0 and the 45, 60, and 75% nominal pastures had only small increases in E+ incidence. All pastures were sampled in summer when stressful environmental conditions were prevalent. It may be that pastures with moderate E+ increase in E+ incidence over time, because E+

Table 5. Variability of monthly E+ incidence estimates for four 2-ha fescue pastures sampled from November 1985 to October 1987 using a stratified random sampling design, Blount County, TN.

	E+ incidence					
	Mean	Minimum	Maximum	Kurtosis	$S_{\bar{x}}$	CV
	---------- % ----------					%
Fescue + clover						
Pasture 1	61	45	73	0.41	0.02	11
Pasture 2	62	51	74	-1.06	0.02	12
Fescue						
Pasture 3	61	52	70	-1.01	0.01	10
Pasture 4	67	50	79	-0.28	0.02	12

plant tend to be more tolerant of stressful conditions. Pastures which are slightly E+ or are severely E+ probably change little, because the ratio of E+ to E- plants within a field is highly skewed. This assumption may explain why the four 2-ha pastures had small changes in their E+ incidence of about 60%. Cattle have also been noted to preferentially graze E- fescue over E+ fescue. It is possible also that some sample bias may have been introduced in the sampling of the pastures because large, vigorous E+ tillers may have been selected inadvertently over smaller, stressed E- tillers. It is often easy to overlook the human error associated with obtaining a representative sample. Therefore, in the future, each random tiller should be selected blindly, *e.g.*, by throwing a piece of flagging material behind one's back and collecting the closest tiller. In summary, the degree of variability of E+ incidence may be a function of the initial incidence. Further monitoring of these pastures needs to be done to support this hypothesis.

Since each tiller was classified as being E+ or E- , it can be concluded only that the presence of *A. coeonophialum* was consistent. The observed consistency of A. *coenophialum* presence may have been due to the sensitivity of the PAS-ELISA technique. PAS-ELISA can detect as little as 40 ng of homogenized hyphae (Reddick and Collins, 1988). It is possible that the quantity of *A. coenophialum* hyphae within each pasture may have been variable over time. Monthly mean counts of hyphae mm^{-1} breadth of leaf sheaths were highly variable from season to season at several locations for a similar perennial ryegrass endophyte, *Acremonium*

loliae Latch, Christensen, and Samuels (Menna and Waller, 1986).

An important conclusion is that significant E+ incidence could be detected at any time during the year when tillers were assessed using PAS-ELISA (Figure 3 and Figure 4). Other research has suggested that tiller collection be timed to coincide with active growth of fescue tissue (Bacon and Siegel, 1988) when the aniline blue microscopic staining technique is performed. Tiller collection must be timed because *A. coenophialum* becomes dormant and hyphae may disintegrate under stressful conditions resulting in assessment difficulties. Freezing samples for later assessment of E+ incidence also would cause disintegration of hyphae, making microscopic examination difficult. On the other hand, freezing samples does not affect the sensitivity of PAS-ELISA detection; significant estimates of *A. coenophialum* were detected in one sample set that had been frozen for four months. Thus, PAS-ELISA seems to be a detection technique which permits detection of *A. coenophialum* presence at any time during the year and with samples stored for several months. PAS-ELISA also is more reliable than the staining technique in estimating the presence of E+ fescue (Reddick and Collins, 1987). Larger numbers of samples can be tested in less time with PAS-ELISA than with the stain technique. Unfortunately, initial ELISA laboratory setup costs are large.

Spatial Distribution of *A. coenophialum*

Data from 12 of 16 transects gave variance to mean ratios that were < 1, indicating the departure from randomness generally was not evident (Table 6). Thus, mean E+ estimates were similar among transects in groups of 3 rows or columns of 27 samples each. Only data from two pastures containing one sampling month within each season are shown, due to the large number of sampling dates. Data from other sampling dates did indicate that a state of randomness usually occurred within all pastures when the variability among transects was determined.

On the other hand, contour plots, depicting the weighting or steepness of E+ incidence, indicated the pattern of dispersion of E+ fescue was aggregated over contiguous strata of equal size (Figures 5, 6, 7, and 8). Frequency distributions, such as the associated variability among 3 transects of 27 samples, often are biased because the precise location of each sample is ignored (Nicot, Rouse, and Yandell, 1984). Thus, consideration of the location of each sample must be made when precise knowledge of spatial distributions is desired.

One evident problem is that E+ incidence did not remain in the same proportion of the pasture area, over time. Some consistency was observed when the same predicted ranges of E+ distribution were examined, but the trend was not pronounced. Positive determinations of spatial patterns were not possible, because only one tiller was collected from each 232 m^2 area. Each sample may not have been representative of its particular stratum. How similar

Table 6. Variability of E+ incidence estimates (%) among transects grouped by 3 rows or 3 columns of the stratified random sampling design for two pastures sampled monthly in 1985 and 1986, Blount County, TN.

	Monthly E+ incidence (%) for Pasture 2, 1985-86			
Transect	December	March	June	October
Rows 1,2,3 of 9	60	50	56	62
Rows 4,5,6 of 9	50	52	64	58
Rows 7,8,9 of 9	58	77	74	68
¶* V/m ratio	0.17	1.70	0.90	0.06
Cols 1,2,3 of 9	54	60	50	74
Cols 4,5,6 of 9	65	67	59	63
Cols 7,8,9 of 9	50	54	86	48
¶* V/m ratio	0.10	0.30	1.20	1.31

	Monthly E+ incidence (%) for Pasture 4, 1986			
Transect	January	April	August	November
Rows 1,2,3 of 9	78	52	89	64
Rows 4,5,6 of 9	74	60	73	91
Rows 7,8,9 of 9	50	56	76	77
¶* V/m ratio	0.44	0.07	0.40	0.52
Cols 1,2,3 of 9	50	60	81	77
Cols 4,5,6 of 9	84	48	78	83
Cols 7,8,9 of 9	67	61	80	71
¶* V/m ratio	1.2	.07	.02	0.23

¶ Ratio of within-field variance to mean number of E+ tillers observed monthly for each pasture.

* A value ≤ 1implies randomness and values > 1 imply aggregation of E+ incidence within each pasture.

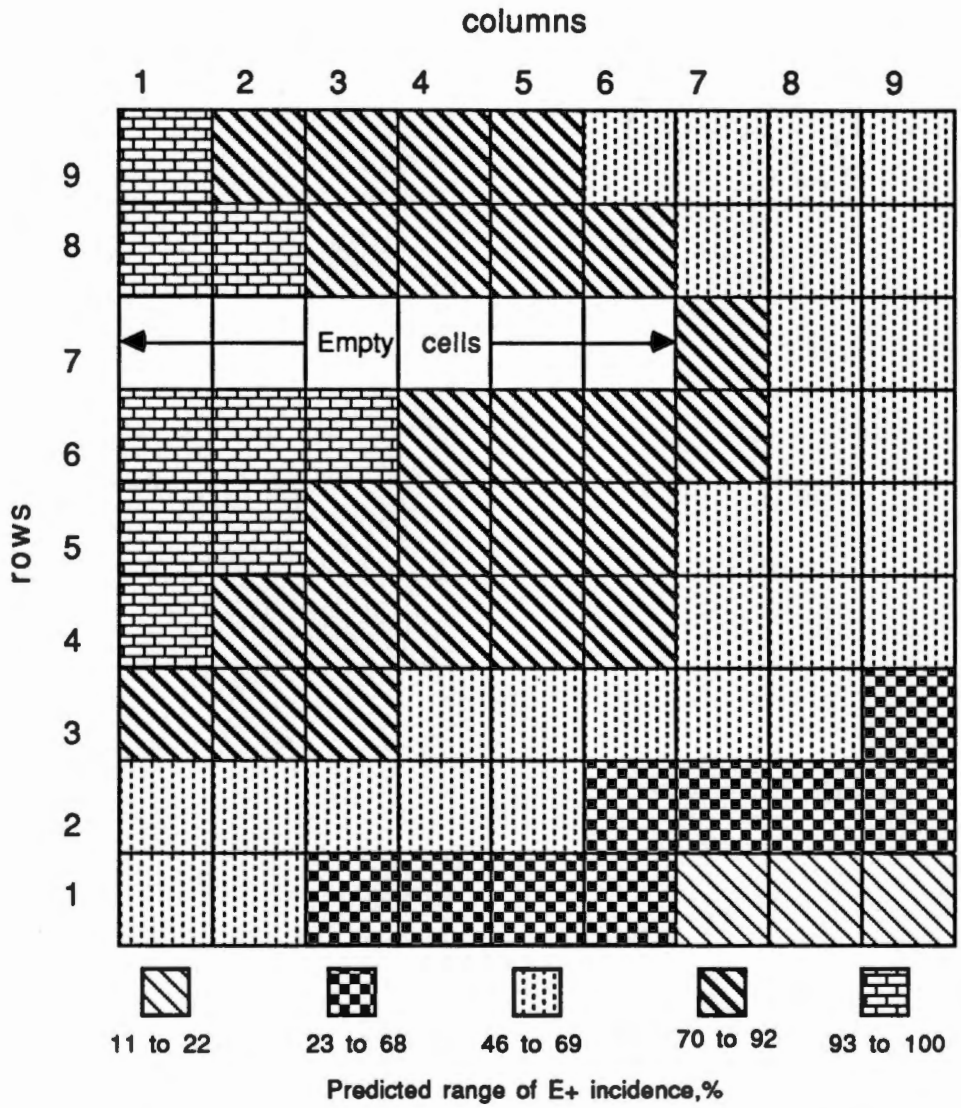

Figure 5. Within-field variance of E+ incidence for Pasture 4 sampled using a stratified random design in January, 1986, Blount County, TN.

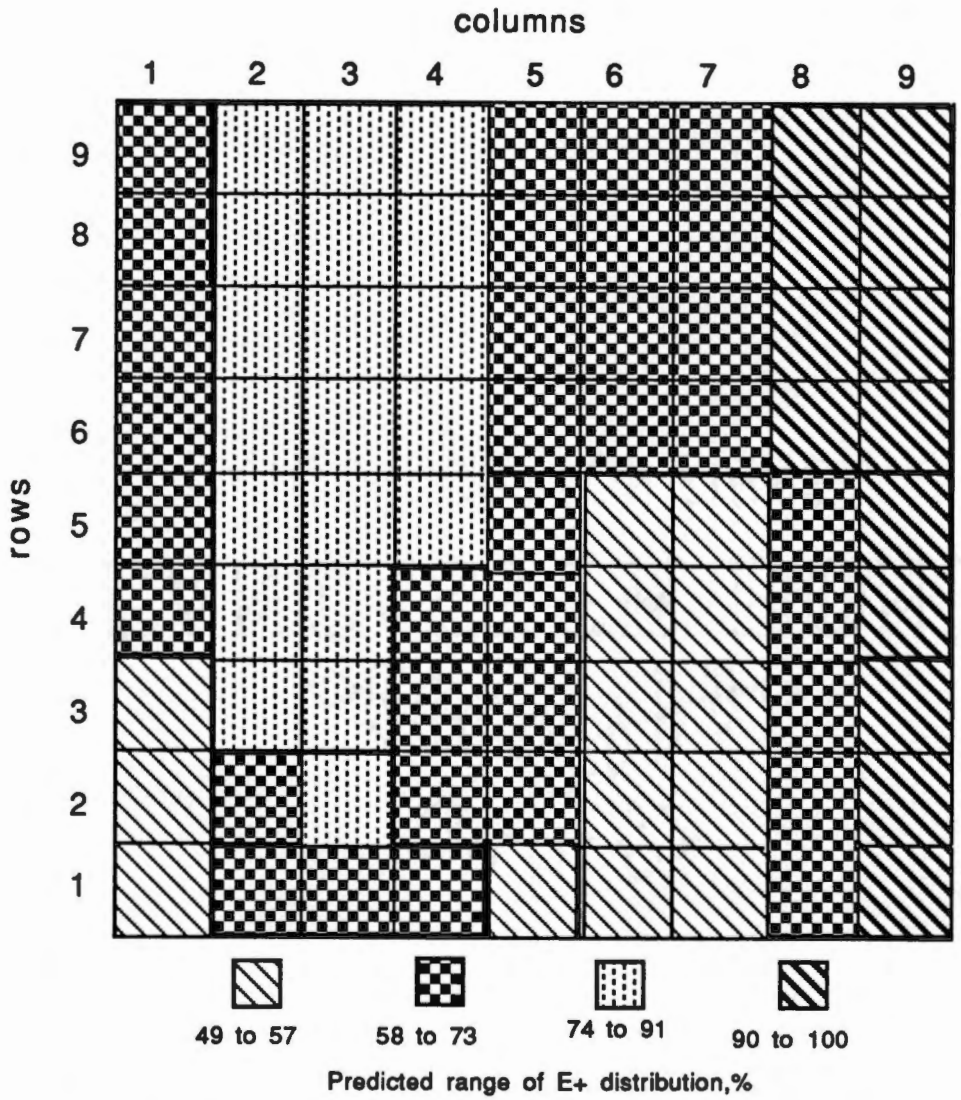

Figure 6. Within-field variance of E+ incidence for Pasture 4 sampled using a stratified random design in April, 1986, Blount County, TN.

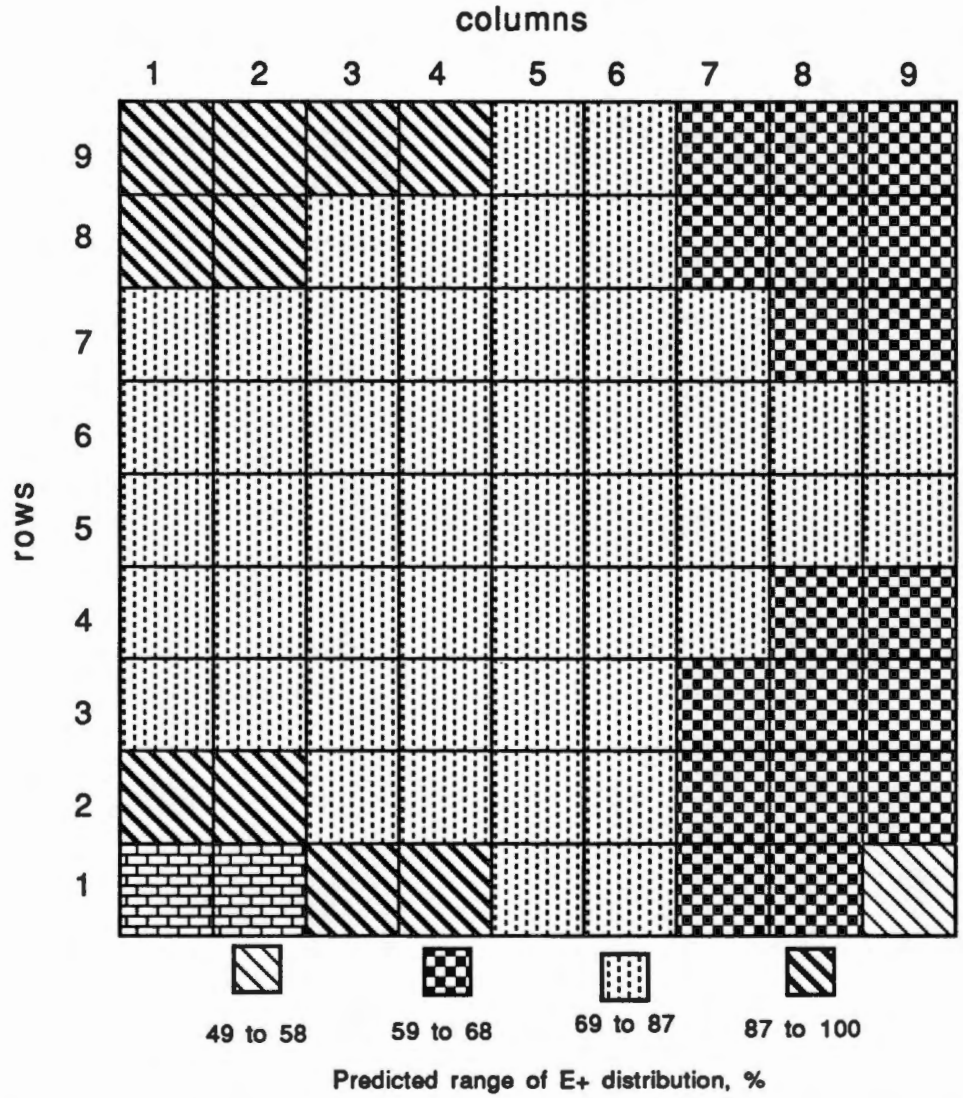

Figure 7. Within-field variance of E+ incidence for Pasture 4 sampled using a stratified random design in August, 1986, Blount County, TN.

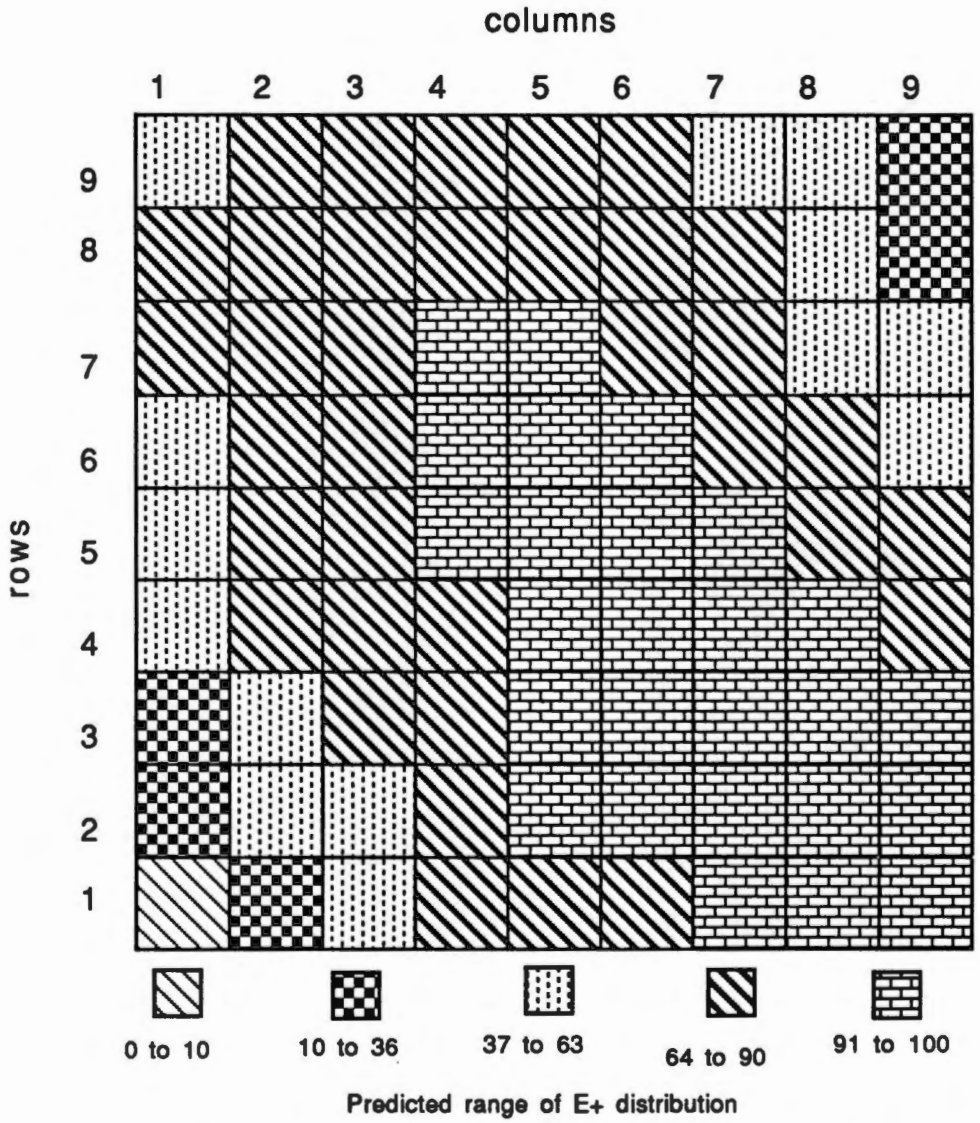

Figure 8. Within-field variance of E+ incidence for Pasture 4 sampled using a stratified random design in November, 1986, Blount County, TN.

would the E+ estimates have been if two or more tillers had been collected within each stratum is not known. On the other hand, the labor involved in collecting and analyzing 81 samples per 2-ha was demanding enough that additional sampling would not be realistic. Thus, the only positive conclusion that can be drawn is that the sample surveys were comprised of aggregated data; this could have affected the precision of subsample sets drawn at random from the entire population.

Estimation of Sampling Intensity

Appropriate sampling methods must provide information of *A. coenophialum* incidence with known accuracy and a reasonable level of effort. The precision of a sample survey is affected by the sampling intensity and design. Estimation of disease incidence is more difficult for populations that have small disease incidence and or a large degree of aggregation (Delp, Stowell, and Marois, 1986; Lin, Poushinsky, and Mauer, 1979; and Nicot, Rouse, and Yandell, 1984). The stratified random sampling design (Cochran, 1977) has been demonstrated to be the best design for disease detection because it disperses sampling sites more than other designs which cover only a finite portion of the population (Delp, Stowell, and Marois, 1986). Unfortunately, selection of optimum sampling intensities for pastures having unknown E+ incidence is difficult regardless of the design used, since some prior knowledge of the incidence level is needed in order to select precise sampling intensities. The apparent dilemma is that prior knowledge of

incidence levels requires a subsequent sample. Therefore, appropriate sampling methods must be selected subjectively from statistical inferences drawn from independent samples. Selected subsampling arrangements were drawn at random from monthly data from Pastures 2 and 4 to determine whether smaller sample intensities than the entire array collected would have given acceptable estimates of *A. coenophialum* (Appendices B-1, B-2, B-3, and B-4).

Observed estimates of *A. coenophialum* deviated little from expected estimates for most sampling arrangements selected from the stratified random design with 8 or more samples ha^{-1} (Appendices B-1, B-2, B-3, and B-4). It appears that few samples are required for assessment of endophyte status in pastures that are severely E+ (greater than about 60%). In some cases, some sampling arrangements having sample intensities larger than 8 samples ha^{-1} gave biased E+ estimates, while those having smaller intensities did not. The geometry of these arrangements may have caused the random selection of more E+ or E- samples from apparent aggregate data.

Sampling intensities larger than 5 samples ha^{-1} (3 X 3 square arrangement) always gave high E+ estimates. Estimates from 5 samples ha^{-1} gave adequate estimates of E+ incidence in 10 of 14 sampling dates, but the wide 90% confidence limits were not acceptable. Estimates from 2 samples ha^{-1} (4 x 4 square arrangement) were biased consistently. All other sampling arrangements tried should give adequate estimates of *A.*

coenophialum incidence for production purposes. This is particularly true since over 90% of all fescue pastures are E+ and most of these fields are severely infected. The author recommend a second sample survey if the initial survey gives E+ estimates that are less than 20%, especially if symptoms of fescue toxicosis have been observed in that pasture.

For research purposes, a more precise estimation of *A. coenophialum* incidence is required than for producer fields. For sample collection, fields should be divided into grids for stratified random sampling, with a sampling intensity approaching at least one sample per 250 m^{-2}. Collected samples should be assessed by selecting at random 1 out of 3 adjacent samples col^{-1} or row^{-1}. The remaining samples should be saved as backup samples for possible analysis in case the original ones do not provide a clear-cut assessment.

IV. CONCLUSIONS FOR SAMPLING FOR FESCUE ENDOPHYTE

Eight or more stratified random samples ha^{-1} should be an adequate sampling intensity for fungal determination in producers fields (Figure 9). Only fields seeded at the same time and from the same seed source should be sampled together. Areas not characteristic of the entire pasture should not be sampled (*i.e.* sinkholes, spots where winter hay has been fed, or along fence rows). A second sample survey should be done if the initial one gives fungal estimates that are less than 20%, especially if symptoms of fescue toxicosis have been observed in that pasture.

For research purposes, fields should be divided into grids for stratified random sampling, with a sampling intensity approaching at least one sample per 250 m^{-2}. Collected samples should be assessed by selecting at random 1 out of 3 adjacent samples col^{-1} or row^{-1}. The remaining samples should be saved as backup samples for possible analysis in case the original ones do not provide a clear-cut assessment.

Samples can be collected at any time during the year provided that the Protein A enzyme-linked immunosorbent assay (PAS-ELISA) is used for sample assessment. Unfortunately, all endophyte diagnostic testing centers in the southeast use the microscopic staining test, which should be performed only on actively growing fescue tissue. Therefore producers should collect samples in the spring or fall when samples are assessed using the staining test.

TWO HECTARE SECTION OF A PASTURE

16 samples per 2 ha
8 samples per ha
5 samples per acre

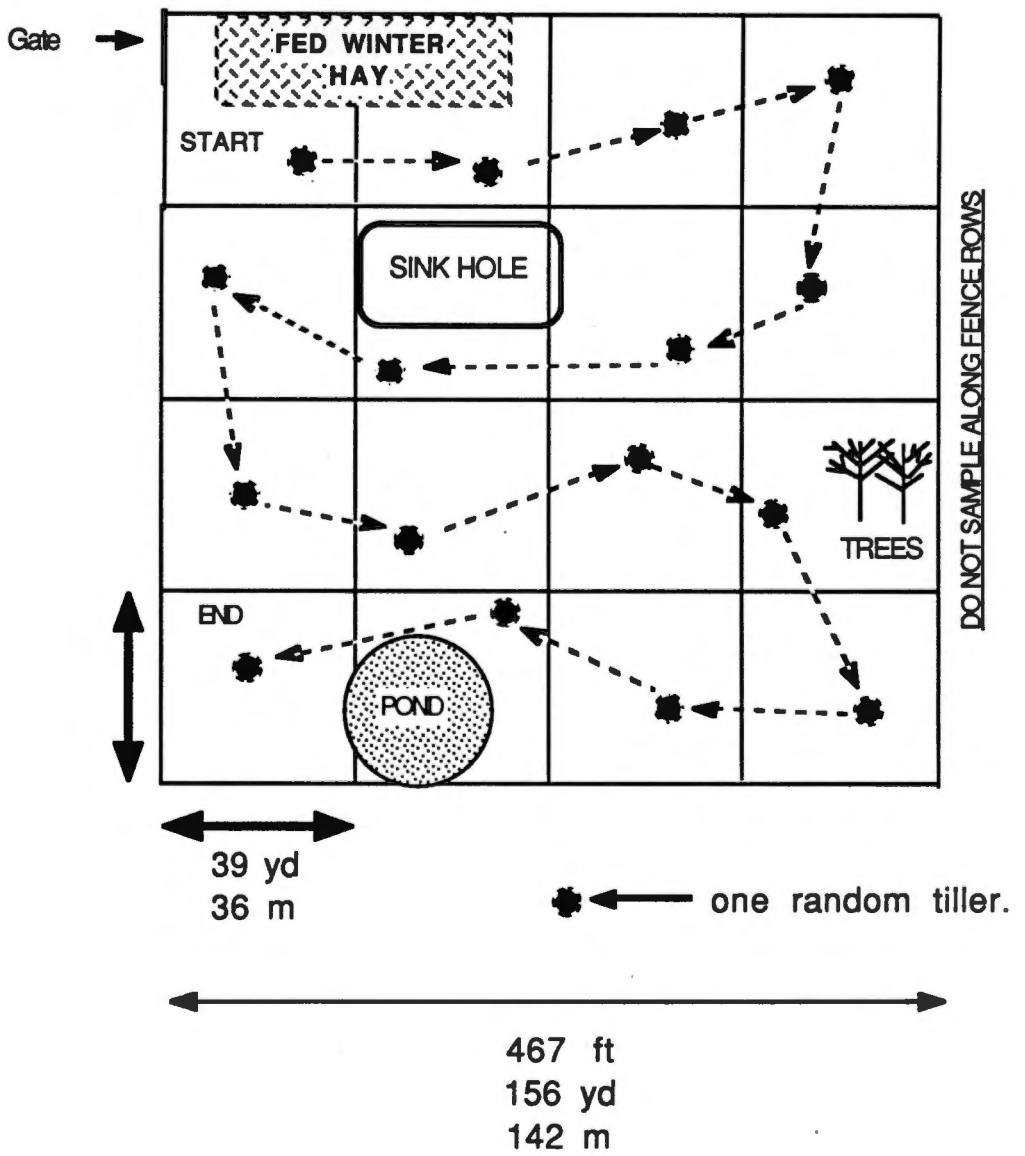

Figure 9. Stratified random grid for sampling for fescue endophyte.

Newly established low endophyte (E-) pastures should be sampled once every year or two years to determine if the endophyte is present, and if so, to what extent. It also appears that low endophyte pastures will require more stringent management practices than the E+ KY-31 cultivar.

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LITERATURE CITED

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APPENDICES

APPENDIX A

SEEDING RATES, SAMPLING ARRANGEMENTS AND DATA COLLECTED FROM THE HIGHLAND RIM EXPERIMENT STATION.

Appendix A-1. Seeding rates adjusted for germination and fungal incidence used to establish eight 4-ha. pastures with different levels of *A. coenophialum* incidence, Springfield, TN, 1985.

Nominal level of infection	80% germination Fungus infected E-	60% germination Fungus infected E+	seeding rate
-- % --	-- kg --	- - kg --	- - $kg \cdot ha^{-1}$ --
£0	158.9	0	21.6
15	65.8	24.9	24.7
30	43.1	47.7	24.7
45	31.8	77.2	26.9
60	15.9	93.1	26.9
£75	0	181.6	22.4

£ Two pastures of each.

Appendix A-2. Estimates of *A. coenophialum* (p) incidence and associated 90% Confidence Limits (CL), normal deviate z test for sample proportions (p>z), and sample error ($s_{\bar{x}}$) from selected sampling arrangements of the transect method and a stratified random design for two pastures seeded with 75% E+ seed.

	75% nominal fungal incidence					
	Pasture 1			Pasture 5		
Arrangements	sample fraction n/N	p±CL	$s_{\bar{x}}$	sample fraction n/N	p±CL	$s_{\bar{x}}$
		--%--			--%--	
Transect Method						
all samples	100	74±00	0.0	100	83±00	0.0
stage 1	33	79±10	7.5	34	83±09	6.8
stage 2	34	73±11	8.1	34	83±09	6.8
stage 3	34	67±12	8.6	33	83±09	6.9
transect 1	34	77±11	7.7	33	83±09	7.0
transect 2	33	62±11	9.0	33	81±09	6.9
transect 3	34	80±10	7.3	32	86±09	6.6
MO criteria	48	*59±09	7.5	46	*73±08	6.9
Stratified random						
all samples	100	81±00	0.0	100	86±00	0.0
1 out of 2 col^{-1}	52	80±07	8.4	44	90±06	4.7
1 out of 2 row^{-1}	40	81±09	9.7	39	*97±04	3.8
1 out of 3 col^{-1}	35	85±09	10.3	34	90±08	7.5
1 out of 3 row^{-1}	30	70±14	9.9	27	85±09	6.8
rows 2,5,8	25	80±12	10.0	34	80±10	5.6
rows 3,6,9	25	84±12	10.1	33	86±09	8.4
1 out of 4 col^{-1}	25	89±10	10.2	22	90±10	8.9
1 out of 4 row^{-1}	20	87±13	12.9	17	83±13	11.1
1 out of 2X2 squares	21	88±12	12.8	17	86±14	9.7
1 out of 3X3 squares	8	83±26	19.2	11	89±17	11.5
1 out of 4X4 squares	5	*100±00	35.3	5	*100±00	0.0

* significant deviation observed between the estimated incidence (p) and the expected incidence for all samples.

Appendix A-3. Estimates of *A. coenophialum* (p) incidence and associated 90% Confidence Limits (CL), normal deviate z test for sample proportions (p>z), and sample error ($s_{\bar{x}}$) from selected sampling arrangements of the transect method and a stratified random design for two pastures seeded with 15 and 0% seed.

Arrangements	Pasture 2 15% nominal incidence			Pasture 6 0% nomial incidence		
	sample fraction n/N	p±CL	$s_{\bar{x}}$	sample fraction n/N	p±CL	$s_{\bar{x}}$
		--%--			--%--	
Transect Method						
all samples	100	60±00	0.0	100	5±00	0.0
stage 1	33	62±12	9.0	33	0±00	0.0
stage 2	34	60±12	8.9	33	4±05	3.5
stage 3	34	57±12	9.1	33	11±08	5.8
transect 1	34	50±12	9.1	31	11±09	6.3
transect 2	34	*70±11	8.4	34	0±00	0.0
transect 3	33	59±13	9.2	34	3±05	3.4
MO criteria	60	*49±07	6.9	100	5±04	2.3
Stratified random						
all samples	100	56±00	0.0	100	9±00	0.0
1 out of 2 col^{-1}	40	54±10	8.0	45	10±06	4.8
1 out of 2 row^{-1}	41	50±10	7.9	41	5±05	3.4
1 out of 3 col^{-1}	30	48±13	9.3	34	10±08	7.2
1 out of 3 row^{-1}	30	52±13	9.3	30	11±09	7.6
rows 2,5,8	29	54±13	9.9	34	6±06	5.2
rows 3,6,9	30	67±12	9.6	34	13±08	8.0
1 out of 4 col^{-1}	20	50±17	11.2	23	10±10	11.1
1 out of 4 row^{-1}	20	65±16	10.7	20	5±08	10.2
1 out of 2X2 squares	19	59±17	11.2	18	6±09	10.5
1 out of 3X3 squares	8	63±19	17.1	10	22±23	19.6
1 out of 4X4 squares	4	*75±40	21.6	5	0±00	0.0

* significant deviation observed between the estimated incidence (p) and the expected incidence for all samples.

APPENDIX B

SELECTED SAMPLING ARRANGEMENTS AND DATA COLLECTED FROM BLOUNT COUNTY, TN.

Appendix B-1. Estimates of *A. coenophialum* (p) incidence and associated 90% Confidence Limits (CL) determined from selected sampling arrangements of the stratified random sampling design, Pasture 2, 1985-86, Blount County, TN.

Sampling arrangements	Sampling fraction	Month			
		Dec	Mar	Jun	Oct
	n/N	p±CL	p±CL	p±CL	p±CL
		%			
all samples	100	57±00	60±00	64±00	63±00
even rows and cols	79	60±05	58±05	61±06	63±04
1 out of 2 col^{-1}	45	63±10	58±11	59±12	58±11
1 out of 2 row^{-1}	46	49±10	59±10	61±12	58±10
1 out of 3 col^{-1}	33	58±13	60±13	60±15	68±13
1 out of 3 row^{-1}	34	*42±13	63±14	73±13	65±13
rows 2,5,8 out of 9	34	64±13	72±12	*77±12	56±14
rows 3,6,9 out of 9	33	58±13	63±14	60±15	54±13
1 out of 4 col^{-1}	23	65±17	53±18	57±20	69±18
1 out of 4 row^{-1}	22	61±17	71±20	*40±19	67±17
1 out of 2X2 squares	21	*73±17	63±18	58±22	67±19
1 out of 3X3 squares	11	56±27	*38±18	71±29	57±32
1 out of 4X4 squares	5	*25±40	*100±--	50±81	50±46

¶ CV ranged from 5.5 to 25.0%.

£ S$\bar{x}$ ranged from 0 to15.7.

* significant deviation observed between the estimated incidence (p) and the expected incidence for all samples.

Appendix B-2. Estimates of *A. coenophialum* (p) incidence and associated 90% Confidence Limits (CL) determined from selected sampling arrangements of the stratified random sampling design, Pasture 2, 1987, Blount County, TN.

Sampling arrangements	Sampling fraction	Month			
		Jan	May	Aug	Fall
	n/N	p±CL	p±CL	p±CL	p±CL
		%			
all samples	100	63±00	53±00	71±00	- -
even rows and cols	79	66±05	50±05	72±05	- -
1 out of 2 col^{-1}	44	64±10	56±10	79±19	- -
1 out of 2 row^{-1}	44	66±11	*64±10	71±10	- -
1 out of 3 col^{-1}	32	68±13	63±13	63±14	- -
1 out of 3 row^{-1}	31	50±15	59±13	64±13	- -
rows 2,5,8 out of 9	32	63±14	48±13	79±12	- -
rows 3,6,9 out of 9	34	62±13	56±13	62±13	- -
1 out of 4 col^{-1}	20	60±19	*39±17	69±18	- -
1 out of 4 row^{-1}	21	75±16	*72±16	80±16	- -
1 out of 2X2 squares	21	63±19	50±19	93±09	- -
1 out of 3X3 squares	11	67±26	67±26	75±25	- -
1 out of 4X4 squares	4	*100±--	*25±40	50±46	- -

¶ CV ranged from 5.1 to 25.0%.
£ $S\bar{x}$ ranged from 0 to 9.3.
* significant deviation observed between the estimated incidence (p) and the expected incidence for all samples.

Appendix B-3. Estimates of *A. coenophialum* (p) incidence and associated 90% Confidence Limits (CL) determined from selected sampling arrangements of the stratified random sampling design, Pasture 4, 1986, Blount County, TN

Sampling arrangements	Sampling fraction	Month			
		Jan	Apr	Aug	Nov
	n/N	p±CL	p±CL	p±CL	p±CL
		%			
all samples	100	67±00	56±00	79±00	77±00
even rows and cols	77	68±05	50±05	81±04	78±06
1 out of 2 col^{-1}	44	75±10	51±10	83±08	72±12
1 out of 2 row^{-1}	43	61±10	56±11	83±08	83±10
1 out of 3 col^{-1}	31	64±11	64±13	81±11	79±17
1 out of 3 row^{-1}	32	64±11	67±13	74±12	78±14
rows 2,5,8 out of 9	33	56±15	62±13	*88±09	86±11
rows 3,6,9 out of 9	33	57±14	60±13	*62±13	79±11
1 out of 4 col^{-1}	22	*50±19	47±18	72±16	*92±11
1 out of 4 row^{-1}	21	75±16	56±17	88±12	78±22
1 out of 2X2 squares	20	67±18	50±19	69±18	82±18
1 out of 3X3 squares	10	*44±27	44±27	*100±--	50±46
1 out of 4X4 squares	4	*33±54	50±46	*100±--	*100±--

¶ CV ranged from 4.6 to 27.2%.
£ $S\bar{x}$ ranged from 0 to11.1.
* significant deviation observed between the estimated incidence (p) and the expected incidence for all samples.

Appendix B-4. Estimates of *A. coenophialum* incidence (p) and associated 90% Confidence Limits (CL) determined from selected sampling arrangements of the stratified random sampling design, Pasture 4, 1987, Blount County, TN.

Sampling arrangements	Sampling fraction	Month			
		Jan	May	Aug	Fall
	n/N	p±CL	p±CL	p±CL	p±CL
		%			
all samples	100	55+00	68±00	64±00	- -
even rows and cols	79	60±03	67±05	61±05	- -
1 out of 2 col^{-1}	43	56±10	61±11	67±10	- -
1 out of 2 row^{-1}	45	65±10	68±10	71±10	- -
1 out of 3 col^{-1}	32	60±13	72±12	50±13	- -
1 out of 3 row^{-1}	31	58±14	71±13	70±12	- -
rows 2,5,8 out of 9	33	54±14	72±12	54±13	- -
rows 3,6,9 out of 9	34	48±13	*80±11	72±12	- -
1 out of 4 col^{-1}	20	63±18	75±16	71±17	- -
1 out of 4 row^{-1}	21	60±19	*39±17	71±17	- -
1 out of 2X2 squares	21	86±14	56±19	73±11	- -
1 out of 3X3 squares	11	*80±32	75±25	75±25	- -
1 out of 4X4 squares	5	75±40	75±40	25±40	- -

¶ CV ranged from 5.5 to 22.0%.
£ $S\bar{x}$ ranged from 0 to 8.1.
* significant deviation observed between the estimated incidence (p) and the expected incidence for all samples.

APPENDIX C

METHODS USED TO DETECT THE
THE ENDOPHYTE

Microscope Staining Test: Tall fescue culms that were cut 6cm above the crown of the plant were split longitudinally. Lower epidermis was peeled from an interior leaf sheath and placed in a drop of stain (0.06% aniline blue in 33% lactic acid solution). After excess stain was removed by blotting, stained material was crushed, placed on a microscope slide, and a cover glass applied. Tissue was observed with a compound microscope(200X). Scrapings were recorded as (-) when no fungus was found and as (+) when at least a small fragment of mycelium was detected.

Protein A Sandwich ELISA: The PAS-ELISA method was performed according to Edwards and Cooper (1985), as revised by Reddick and Collins (1988). Medium binding polystyrene ELISA plates (Plastic Injectors, Spartanburg, S.C.) were rinsed with tap water. Protein A (1 μ ml^{-1}) (Sigma Chemical Co., St. Louis, M.O.) in 0.05 M sodium carbonate buffer (pH 9.6 containing 0.02% NaN_3) was added to wells and incubated at 28-30°C for 2 h. Nonbounded protein A was removed by 4 successive rinses with PBS-TWEEN (phosphate buffered saline, pH 7.3 + O.O5% Tween 20) after each step. Antiserum induced in New Zealand white rabbits, *Oryctolagus cuniculus,* by partially purified mycelial proteins, was diluted 1:200 in PBS-TWEEN and added to each well. Plates were then incubated for 2h. at 28-30°C. Fescue stems were macerated in a leaf squeezer with PBS-TWEEN and sap (200 µl) from each culm was pipeted into two adjacent wells and incubated overnight at 5°C. Antiserum (1:200 dilution as above) was added to plates and incubated for 2 h.

at 28-30°C. Protein-A-alkaline phosphate was diluted 1:1000 in PBS-TWEEN and added to each plate. Plates were then incubated for 2h at 28-30°C. Substrate [p-nitrophenylphosphate (1mg/ml) in 10% diethanolamine] was added to wells at room temperature. ELISA plates were then placed on a white surface to periodically observe color development. Abosorbance (A_{405} nm) was recorded after 45-90 min. on a Mini Reader II (Dynatech Lab. Inc., Alexandria, V.A.). See text for determination of E+ or E- tillers.

Direct Double Antibody Sandwich ELISA: The DAS-ELISA method was performed according to McLaughlin and Barnett (1978). Medium-binding polystyrene ELISA plates (Dynatech Laboraties, Alexandria, V.A. or Plastic Injectors, Spartanburg, S.C.) were rinsed with tap water. Antiserum (as obtained in PAS-ELISA) was diluted 1:200 in sodium carbonate buffer (pH 9.6 containing 0.02% NaN_3) and added to ELISA plates and incubated for 1 h. at 5°C. Plates were rinsed 3 times (3 minute intervals) with distilled deionized water. Two-hundred µl of emacerated sap (fescue tissue, PBS-TWEEN, and NaDIECA) from each culm was added to two adjacent wells and incubated overnight at 5°C. Plates were rinsed as above and alkaline phosphatase linked antibody diluted 1:200 in PBS-TWEEN was added to wells and incubated overnight at 5°C (McLaughlin et al. did not incubate overnight). Plates were rinsed and substrate (p-nitrophenylphosphate, 1mg/ml 10% diethanolamine) was added to wells at room temperature. Plates were read as in PAS-ELISA.

VITA

Robert Wayne Thompson was born in Roncerverte, West Virginia on May 4, 1961 to Mr. and Mrs. Robert C. Thompson. He was raised in Hampton, Virginia where he attended primary and secondary schools. He was graduated from Albemarle High School, Charlottesville, Virginia in May 1980. The following fall he entered Berea College and completed the Bachelor of Science degree in Agribusiness in December of 1984. After working as a landscape foreman in Dallas, Texas, he accepted an assistantship to pursue a Master's degree in Plant and Soil Science at The University of Tennessee, Knoxville. He received his degree in March 1988. Mr. Thompson plans to continue his graduate studies either at Clemson University or The University of Tennessee.