NEOTYPHODIUM

IN

COOL-SEASON GRASSES
This volume is dedicated to

**Dr. Charles W. Bacon**
Distinguished Senior Research Scientist
Toxicology and Mycotoxin Research Unit
USDA Agricultural Research Service

in recognition of
his seminal discovery of the link between
the tall fescue endophyte and animal toxicosis in 1977,
his vision in articulating research directions, and
his continuing output of key research on the physiology
of the *Neotyphodium/grass* symbiosis.
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Fungi belonging to the *Neotyphodium* and *Epichloë* genera are among the many types of microbes termed *endophytes* by virtue of their in planta symbiosis. *Neotyphodium* in particular is associated with several species of cool-season grasses, particularly those belonging to the *Festuca* and *Lolium* genera. The most common *Neotyphodium*-infected species of *Festuca* and *Lolium* are tall fescue and perennial ryegrass, two grasses that are widely grown in temperate climates throughout the world as forage for cattle, sheep, and horse enterprises, as well as for turf and conservation uses. The complex functions of these endophytes have received intensive study during the past three decades because of their roles in causing production losses in livestock, enhancing biotic and abiotic stress resistances in hosts, and controlling biodiversity and trophic interactions in wild populations.

This book, *Neotyphodium in Cool-Season Grasses*, presents the most recent research findings related to these endophytes. It also documents global trends in endophyte-related research and application and addresses current issues in commerce and education. *Neotyphodium in Cool-Season Grasses* was produced in conjunction with the 5th International Symposium on *Neotyphodium*/*Grass Interactions*, held in Fayetteville, Arkansas, USA, 23 to 26 May 2004. It was produced in lieu of standard proceedings, as symposium organizers attempted to provide a historical framework for the scientific advances reported in molecular genetics, mycology, toxicology, ecology, agronomy, and animal pathophysiology. Its intended audience includes both the researcher and the educator.

*Neotyphodium in Cool-Season Grasses* is divided into five sections, the first of which details *Neotyphodium* research and application in all geographical regions where these endophytes are of economic and academic importance. The next three sections present new findings while providing comprehensive research coverage from all relevant disciplines, including molecular biology, ecology and agronomy, and animal toxicoses. The final section addresses contemporary issues in commerce and public education.

The editors of *Neotyphodium in Cool-Season Grasses* are grateful to all authors for their scholarly contributions to this publication and for their unprecedented willingness to produce a book in timely fashion. We are also grateful to the associate editors who applied their expertise and volunteered their talents in reviewing the chapters. We are especially grateful to Ms. Carrie Czerwonka, Managing Editor, for her dedication in helping us honor the
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It is our hope that this publication will inform the scientific community and empower science educators with the most up-to-date knowledge and definitive reviews of the state of the science. We expect this publication to facilitate a better understanding among students and practitioners regarding the agricultural, ecological, and economic roles of Neotyphodium endophytes in cool-season grasses.

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Section I

Current Trends in *Neotyphodium*
Research and Application
NEOTYPHODIUM RESEARCH AND APPLICATION IN THE USA

Henry A. Fribourg and John C. Waller

We have been asked to summarize accomplishments and findings in the USA since the 4th International Symposium on Neotyphodium/Grass Interactions in 2000. Thus, we have limited our inclusion to publications since 1999, unless very significant and previously unreported. We have not included material or discussion that should be covered adequately in other contributions to this symposium. We have surveyed scientific publications, primarily in the USA, abstract journals, and proceedings of meetings and workshops. In particular, we have relied heavily on the annual proceedings of SERA-IEG/8, the Southern Extension and Research Activities Information Exchange Group.

Tall fescue (Lolium arundinaceum = Festuca arundinacea) toxicosis has existed in the USA ever since the ‘Kentucky 31’ (KY-31) cultivar was released in the 1940s and rapidly filled a vacant niche in the mid-South transition zone. Tall fescue toxicosis did not acquire its name until the mid-1970s, although earlier attempts at isolating the antiquality components did not recognize the existence of the endophytic fungus. Annual economic losses of $600 million to the U.S. cattle (Bos spp.) industry are probably an underestimate, covering both growth and reproduction (Allen and Segarra, 2001). In addition, the endophyte affects adversely the $60 million grass seed and hay export industries (Craig, 2003).

ENDOPHYTES AND HOST PLANTS

Recent Cultivar Developments

The most significant development in the last 4 yr has been the emergence of tall fescue cultivars in which a desired endophyte has been introduced into the host plant in the laboratory. The pioneering work in New Zealand by Latch, Easton, and co-workers was brought into the USA by Bouton and associates (Bouton et
al., 2002b) when they reinfected endophyte-free (E-) 'Jesup' and 'Georgia 5' tall fescues with different non-ergot alkaloid-producing endophyte strains (=nontoxic endophytes). The best combination, Jesup with AR542 (= MaxQ™), possessed greater yield and better survival than the E- entries and was equal to the fescue with a wild endophyte (E+) (Neotyphodium coenophialum). Animal average daily gains (ADG) were equivalent to those obtained from the E- forage and greater than those from the E+ pasture, and animals did not suffer the prolactin depression and elevated body temperature common to those grazing E+.

The AR542 endophyte was selected primarily for its lack of production of ergovaline; however, it is yet to be determined whether ergovaline is the sole or the primary cause of tall fescue toxicosis. Hill et al. (2001) investigated ergot alkaloid transport across ruminant gastric tissues, and they found that the greatest transport was for lysergic acid and lysergol rather than for other alkaloids.

A short while after Bouton et al. (2000) released JesupMaxQ in Georgia, the FFR Cooperative, Lafayette, Indiana, was licensed to market the cultivar 'Hi-Mag' (Sleper et al., 2002) in which had been inserted the nontoxic endophyte strain No. 4, developed by West et al. (2002) in Arkansas, currently marketed under the brand name ArkPlus™. The strain No. 4 came from wild tall fescue plants which had no detectable ergot alkaloids (Nihsen et al., 2004). It has been shown that, in response to natural selective forces, the genetic diversity within E+ and E- tall fescue cultivars leads to different genotypes with altered morphological and agronomic characteristics (Vaylay and van Santen, 2002).

**Nontoxic Endophytes**

It is likely that MaxQ and No. 4 are different. These two isolates are only the first releases from series of collections by New Zealand, Georgia, and Arkansas workers. Clement et al. (2001) and Hill et al. (2002a) have shown that there exists considerable diversity among Neotyphodium fungi from the Mediterranean basin, and that these strains can be distinguished morphologically and biochemically. The range of adaptation of E+ tall fescue extends west and north from the U.S. eastern Coastal Plain physiographic area, south from a short distance north of the Ohio River, and east of eastern Oklahoma. ArkPlus is expected to be well adapted to the Ozark region of the south-central USA and similar climatic zones to the east across the mid- to upper South (West et al., 2002).
In contrast, the GA-5/E+ cultivar was developed with a wild endophyte for adaptation to the severe summer climate and insect populations of the Coastal Plain (Gates et al., 1999). The developers of nontoxic endophyte tall fescue cultivars face the problem of determining whether the range of adaptation of these new associations coincides with the adaptation of E+ tall fescue. This challenge is complicated by the fact that mutualistic fungal endophytes often confer benefits to the hosts, but the physiological interactions involved so far have not been well characterized (Moy et al., 2002). The adaptation is influenced not only by climatic characteristics which affect drought tolerance and/or winter survival, but also by competition from other plants, management, and animal grazing effects.

Evaluation of Nontoxic Endophytes in Breeding Programs

Many assessments of the persistence of nontoxic endophyte cultivars have been done or are in progress. Numerous agronomic evaluations and grazing trials with both sheep (*Ovis aries*) (Bouton and Hopkins, 2003) and cattle (Bouton et al., 2001) have been conducted in Georgia, often using established bermudagrass (*Cynodon dactylon*) and continuous grazing to evaluate persistence under aggressive competition. Agronomic trials have included up to 14 location-years of data (Bouton et al., 2002a). Grazing studies (Parish et al., 2003a, 2003b) demonstrated the positive long-term advantage of using nontoxic endophytes to improve beef steer (*Bos spp.*) performance, even though there was more available forage produced in E+ pastures. The performance of cow–calf (*Bos spp.*) pairs was also evaluated in GA; cattle grazing MaxQ had greater weight gains and body condition scores than pairs grazing E+ (Watson et al., 2002). In Arkansas, agronomic evaluations at several locations and in three neighboring states, and grazing trials at three locations, have shown that steers grazing HiMag with nontoxic endophytes performed similarly to steers grazing E- and much better than those grazing E+ (Nihsen et al., 2004). HiMag with nontoxic endophytes had acceptable persistence for several years. Additional grazing evaluations have been conducted in several other locations (Nihsen et al., 2004).

Research in two states has used water stress as a tool for selecting persistent tall fescue/nontoxic endophyte combinations; the fungal endophyte may boost host stress tolerance through signals that amplify the production of membrane-protecting dehydrins (West et al., 2003). Irrigation levels have been used to determine seasonal distribution of yield and could be a logical criterion for selection of germplasm for use in arid but irrigated areas (Asay et al., 2001).
Tall fescue cultivars infected with different endophytes have been evaluated in small plots at several locations under grazing. In Tennessee, Fribourg et al. (2002) started in 1999, comparing 15 combinations of endophytes and host plants at three locations. Stand persistence under moderate grazing pressure was acceptable for most entries, but E- tall fescues lost more stand than tall fescues with wild or nontoxic endophytes. Similar results have been obtained at a location in Mississippi (Lang et al., 2001). At Lexington, KY, Henning et al. (2002) evaluated numerous public and commercial tall fescue cultivars, as well as many experimental host/endophyte associations, under heavy grazing pressure for several years. Nontoxic cultivars such as Jesup/MaxQ and GA-5/MaxQ persisted as well as KY-31/E+, but E- cultivars such as ‘Kenhy’ and Jesup were also persistent in some seedings. The Kentucky data in general indicate that both E- fescues and fescues with nontoxic endophyte may be valid alternatives to KY-31/E+ under controlled management practices. Evaluations of nontoxic endophytes in tall fescue hosts are continuing at most of these locations, and have been expanded recently to Oklahoma and Texas (Hopkins, 2003).

Seeds and Seedlings

The control and labeling for endophytic fungi in tall fescue seed are important, especially since the advent of cultivars containing nontoxic endophytes. Seed harvesting should be controlled, since more mature seed result in seedlings with greater infection by nontoxic endophytes (Hill et al., 2003). Prechilling of seed decreased germination time and uniformity (Hill et al., 2002c). The SERA-IEG/8 group was concerned about quality assurance of grass seed containing nontoxic endophytes being sold for forage (Hill et al., 2003). Diseases must be controlled when evaluating seedlings for endophyte viability; this can be done with contact fungicides (Hill and Brown, 2000). The group agreed that methods of analysis should be consistent with those of recognized state or international seed associations, that alkaloid analyses should be conducted by methods substantiated in scientific journals, and that endophyte and alkaloid analyses should be performed on statistically representative samples. Seed labels should provide information on valid grow-out analyses reflecting viable endophyte content, showing the date when the test was performed and the percentage of infected seeds which were toxin-producing off-types.
In the 1980s, after the realization that fungal endophytes were the cause of tall fescue toxicosis, some advocated the substitution of E+ tall fescue pastures with E- tall fescue prior to justification of this practice by reliable research data. This movement was doomed from the start and led many producers to discount future studies. In the late 1980s to early 1990s, and prior to the advent of nontoxic endophytes in the USA, the primary emphasis was to compare the productivity and persistence of E- with E+ tall fescue. An example of this approach was conducted in Tennessee (Fribourg et al., 2000a) starting in 1994. Steers grazing E- tall fescue plus clover (Trifolium spp.) pastures had the greatest ADG, and steers grazing E+ tall fescue had the lowest; this may be related to the reduction in forage intake and fiber digestibility observed with E+ (Humphry et al., 2002). The performance of steers grazing pastures with alternating strips of E+ and E- tall fescue was intermediate to that of steers grazing solid stands of either E- or E+. The addition of clovers improved the performance of steers on both E- and E+ pastures. Benefits obtained by renovating E+ tall fescue pastures with clover were similar to those gained by replacing half the pasture with E- tall fescue.

As seed of tall fescue cultivars containing different non-ergot alkaloid-producing endophyte strains became available for testing in the late 1990s, grazing and persistence evaluations were initiated in many states in the tall fescue belt in addition to those where the cultivars originated. An early study in Tennessee corroborated that some fungal endophyte strains were adapted and persistent in the mid-South while others were not. A few of the endophyte strains tested resulted in steer performance similar to that obtained from steers grazing E- tall fescue (Waller et al., 2001a). The performance and persistence of tall fescues with nontoxic endophytes have been evaluated in Mississippi at several locations with steers (Macoon et al., 2002) and heifers (Best et al., 2002) (Bos spp.). South of about 33° N latitude in the USA, dairy cows grazing E- tall fescue produced as much milk as cows grazing 'Marshall' annual ryegrass (L. multiflorum). Although milk production was lower for cows grazing Jesup/MaxQ than for those grazing Marshall, the economics of utilizing a perennial forage tall fescue should be attractive to dairy producers, as long as the tall fescue stand persists (Murphey et al., 2002). Winter and early spring grazing by steers in Louisiana (Alison, 2002) resulted in acceptable animal performance from Jesup/MaxQ and GA-5/MaxQ in a region marginally adapted to tall fescue, but stands of E- declined after 2 yr. Preliminary data from Oklahoma (Hopkins, 2003) indicate that nontoxic endophyte tall fescue cultivars had good persistence.
for 2 yr on a site with fertile soil and very good moisture holding capacity, and that weight gain was acceptable and superior to that of steers grazing E+ tall fescue. Steers that grazed E+ tall fescue entered the feedlot lighter and produced smaller carcass weights than those that grazed E- or MaxQ tall fescues, illustrating the lack of compensatory gain (Coblentz et al., 2003a; Duckett et al., 2001).

**Endophyte as a Modifier of Mineral Composition of Tall Fescue**

Several investigators have examined the possible relationships between the occurrence of *N. coenophialum* and minerals in tall fescue. Dennis et al. (1998) found that Cu concentrations were higher in E- than in E+ tall fescue, and increased linearly in response to N. They showed that the presence of endophyte was associated with lower Cu concentrations, possibly contributing to lowered Cu status in animals. Oliver et al. (2000b) determined that there was a relationship between Cu level and endophyte presence: Steers grazing E+ tall fescue had a serum Cu level below the generally accepted normal for cattle, but steers grazing E- tall fescue had serum Cu at the commonly accepted level. The low Cu levels could explain in part the rough haircoat usually seen on cattle grazing E+, although supplementation with Cu has not affected haircoat appearance consistently. In a 3-yr survey of Tennessee tall fescue farm pastures, presumably E+, Fisher et al. (2003) collected more than 800 plant samples. They found that Cu was marginally deficient in nearly all the samples, and that high S levels were considered marginally antagonistic to Cu utilization. Malinowski and Belesky (1999a) determined that the interaction between tall fescue genotype and endophyte status had a significant influence on mineral element uptake: Endophyte infection modified tall fescue responses to P source, suggesting that P acquisition by the plant may be determined by a specific association of fungal endophyte and tall fescue genomes. The same authors (1999b) found that Al sequestration was greater on root surfaces and in root tissues of E+ than in those of E- plants of a specific tall fescue genotype. Their results suggested that Al tolerance may contribute to the widespread adaptability and success of tall fescue/endophyte associations. Malinowski and Belesky (2000) reviewed these and other mechanisms associated with the adaptation of tall fescue to environmental stresses.
Whereas tall fescue toxicosis in cattle is characterized by lowered ADG, increased body temperature, rough haircoats, and reduced conception rates, mares (*Equus caballus*) exposed to E+ tall fescue exhibit increased gestation lengths, agalactia, foal mortality, and thickened placentas (Cross et al., 2000). Domperidone (Equidone®) therapy was used with considerable success as early as 1993 in South Carolina to treat tall fescue toxicosis in late gestation mares. It has evolved as a treatment of choice other than removal of the animals from E+ tall fescue for several weeks prior to parturition. Administering fluphenazine decanoate to pregnant pony mares grazing E+ tall fescue was effective in maintaining systemic relaxin and improving pregnancy outcome (Ryan et al., 2001a). It has been determined since, in Mississippi, that mares grazing Jesup/MaxQ exhibited no tall fescue toxicosis signs (Ryan et al., 2000, 2001b).

Numerous studies have shown that intake of E+ tall fescue increases hyperthermia during heat stress, which itself may induce oxidative stress. Heat challenges result in marked increase in body temperature of cattle, especially at night (Al-Haidary et al., 2001). The inability of the animal to dissipate heat significantly depletes blood glutathione (Lakritz et al., 2002). Burke et al. (2004) determined that the combination of heat stress and consumption of E+ tall fescue seed led to reduced diameter of the preovulatory dominant follicle and fewer large follicles during the estrous cycle in beef heifers. When E+ tall fescue seed, or E- seed supplemented with ergovaline, was fed to heat-stressed lambs, the ergovaline diet was not as effective as the E+ seed in producing tall fescue toxicosis signs (Gadberry et al., 2003), suggesting that alkaloids other than ergovaline may be also responsible.

Once confirmed pregnant, no differences in fetal losses of bred heifers grazing either E+ or E- tall fescue were observed (Waller et al., 2001b). However, calf birth weight was lower when dams grazed E+ tall fescue than when they grazed E- tall fescue. The difference in birth weight could be explained by decreased growth of bred heifers grazing E+ tall fescue. Follicular and luteal development and function were examined in mature lactating beef cows grazing E- or E+ tall fescue during the early postpartum period (Burke and Rorie, 2002); even though follicular dynamics were altered in cows grazing E+, follicular function was apparently not affected by ergot alkaloids. Pregnancy rate and embryonic losses tended to be different among cows between 30 and 60 d of gestation after environmental temperatures were high for 3 wk when grazing E- or E+ tall fescue (Burke et al., 2001). Pregnancy rate of yearling ewes may be
reduced by E+ tall fescue, but those of older ewes were not affected (Burke et al., 2002).

The D2 dopamine receptor antagonist domperidone, so successful in treating the signs of tall fescue toxicosis in gravid mares, may have beneficial effects on beef heifers grazing E+ tall fescue, including reversal of decreased ADG and increased circulating concentrations of progesterone (Jones et al., 2003), the hormone associated with maintenance of pregnancy. The gene expression of luteal tissue from heifers fed E+ diets suggests that administration of domperidone may be beneficial in treating reproductive problems associated with tall fescue toxicosis in heifers (Jones et al., 2004).

Reproductive effects on males have not been studied extensively. Blood serum prolactin levels, which are greatly depressed in cows and steers, do not seem to be affected in bulls (Bos spp.) consuming E+ tall fescue pasture (Schrick et al., 2003). Male mice (Mus musculus domesticus) susceptible or resistant to E+ tall fescue were not affected in their reproductive responses when consuming E+ seed (Ross et al., 2004).

Several species other than cattle, sheep, horses, and mice exhibit signs of susceptibility to ergot alkaloids from tall fescue. Rabbits (Oryctolagus cuniculus) were affected negatively by the endophyte and were used during the search for possible vaccination against ergot alkaloids (Filipov et al., 1998). The range of meadow voles (Microtus pennsylvanicus) and the feed consumption by prairie voles (M. ochrogaster) were influenced by E+ tall fescue (Conover, 1998; Fortier et al., 2001). Consumption by hens (Gallus gallus) of E+ tall fescue seed resulted in reduced egg production and growth (Conover, 2003).

Several genera of invertebrates are affected, in some cases differently from mammals. Growth of earthworms (Eisenia fetida) fed E+ tall fescue leaves was much greater than that of those fed E- tissues (Humphries et al., 2001). Fall armyworm (Spodoptera frugiperda) fed E+ tall fescue responded differently at different nutrient availability levels for the plants (Bultman and Conard, 1998). Plant-parasitic nematodes may contribute to the poor growth and persistence of tall fescue on sandy soils in the southeastern USA. The presence of the wild E+ conferred resistance to feeding by some of these nematodes, notably the root-knot nematode (Meloidogyne marylandi) and the lesion nematode (Pratylenchus scribneri). It has been determined that a nontoxic endophyte did not confer feeding resistance to P. scribneri equivalent to that from the wild type (Elmi et al., 2000; Timper et al., 2003).

Divergent selection for ADG response to ingestion of E+ tall fescue seed by mice resulted in a favorable correlated response in survival following exposure