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Production of loline alkaloids by the grass endophyte, *Neotyphodium uncinatum*, in defined media

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Abstract

Lolines (saturated 1-aminopyrrolizidines with an oxygen bridge) are insecticidal alkaloids produced in symbioses of certain *Epichloë* (anamorph-*Neotyphodium*) species (fungal endophytes) with grasses, particularly of the genera *Lolium* and *Festuca*. Prior to the present study, it was unknown whether lolines were of plant or fungal origin. *Neotyphodium uncinatum*, the common endophyte of meadow fescue (*Lolium pratense* = *Festuca pratensis*) produced loline, *N*-acetylnorloline, and *N*-formylloline when grown in the defined minimal media at pH 5.0–7.5, with both organic and inorganic nitrogen sources and sugars as carbon sources. In contrast, lolines were not detected in complex medium cultures. GC–MS and ^{13}C NMR spectroscopic analyses confirmed the identity of the alkaloids isolated from the defined medium cultures. Lolines accumulated to ca. 700 mg/l (4 mM) in cultures with 16.7 mM sucrose and 15–30 mM asparagine, ornithine or urea. Kinetics of loline production and fungal growth were assessed in defined medium with 16.7 mM sucrose and 30 mM ornithine. The alkaloid production rate peaked after the onset of stationary phase, as is common for secondary metabolism in other microbes.

Keywords: *Neotyphodium uncinatum*; Clavicipitaceae; Epichloë endophyte; *Lolium pratense*; *Festuca pratensis*; Poaceae; Meadow fescue; Fungal fermentation; 1-Aminopyrrolizidines; Loline alkaloids

1. Introduction

Loline alkaloids (saturated 1-aminopyrrolizidines with an oxygen bridge) (Fig. 1) have been identified in some grass-endophyte associations (Siegel et al., 1990), but are absent in most other organisms. These grasses are naturally infected with symbiotic endophytic fungi, especially *Epichloë* spp. and their asexual derivatives (*Neotyphodium* spp.), which grow in the intercellular spaces (apoplast) of host plants. In these symbioses, an array of other anti-herbivore alkaloids is produced. Three of the alkaloid classes commonly found in endophyte–grass associations—peramine (a pyrrolopyrazine), ergot alkaloids, and lolitrems (indolediterpenes)—are of known fungal origin because these are reported to accumulate in fermentation cultures (Bacon, 1988; Rowan, 1993, Gurney et al., 1994; Porter, 1994). The lolines are produced in a number of grass-endophyte symbioses: *Lolium arundinaceum* (= *Festuca arundinacea*) with *Neotyphodium coenophialum* (Siegel et al., 1990), *Lolium giganteum* (= *Festuca gigantea*) with *Epichloë festucae* (Leuchtmann et al., 2000), *Lolium pratense* (= *Festuca pratensis*) with *Neotyphodium uncinatum* (Bush et al., 1993; Justus et al., 1997), *L. pratense* with *Neotyphodium siegelii* (Craven et al., 2001), and endophyte-infected *Festuca argentina* (Casabuono and Pomilio, 1997), *Lolium temulentum* (Dannhardt and Steidt, 1985), *Poa autumnalis* (Siegel et al., 1990), and *Stipa robusta* (TePaske et al., 1993). The plants, *Argyreia mollis* (Convolvulaceae) and *Adenocarpus* spp. (Fabaceae) have also been reported to contain loline alkaloids, although endophytes have not been implicated (Hartmann and Witte, 1995; Tofern et al., 1999).

The lolines are secondary metabolites and have an unusual structure consisting of a saturated necine ring with an –NRR′ substituent at C1 and an oxygen bridge

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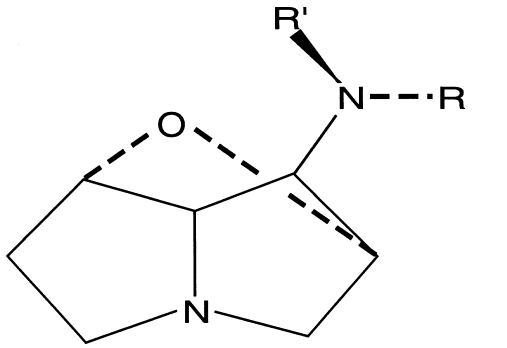

	R	R'
Loline (**1**)	H	CH_3
N-Acetylnorloline (**2**)	$COCH_3$	H
N-Formylloline (**3**)	CHO	CH_3
N-Formylloline (**3'**)	CH_3	CHO

Fig. 1. Structures of loline alkaloids.

between C2 and C7 (Yates et al., 1990; Powell and Petroski, 1992). Lolines thus differ from most plant pyrrolizidines, which are synthesized from homospermidine (Böttcher et al., 1993) and have a $-CH_2OR$ group at C1. Nothing is known about the biosynthesis of the endophyte-associated lolines, but based on this difference, the loline alkaloids have been suggested to be derived from spermidine or spermine (Bush et al., 1993). The alkaloids are found throughout the plant in these associations and have protective effects for the plant due to their anti-invertebrate and feeding deterrent activities (Patterson et al., 1991; Justus et al., 1997; Dougherty et al., 1998; Wilkinson et al., 2000). Lolines are broad-spectrum insecticides (Siegel and Bush, 1997); and in grasses symbiotic with strains of *E. festucae* segregating for loline alkaloid expression, accumulation of these alkaloids was clearly associated with activity against certain aphid species (Wilkinson et al., 2000). Thus, the loline alkaloids are of great interest as natural plant protectants. Although studies do indicate immunosuppressive effects in mice (Dew et al., 1990) and depressed feed intake has been observed in rats fed loline alkaloids (Jackson et al., 1996), it has not been conclusively shown that the loline alkaloids at in planta levels are toxic to mammalian herbivores (Siegel and Bush, 1997). Lolines do not share the same potent anti-mammalian hepatotoxicity and carcinogenicity as plant pyrrolizidines, 1-hydroxymethylpyrrolizidines, which contain C-1,2 unsaturation in the necine ring (Hincks et al., 1991); cytochrome P450 converts the 1-hydroxymethylpyrrolizidines to more bioactive forms, which to varying degrees are capable of crosslinking of DNA (Kim et al., 1999).

Loline alkaloid expression correlates with fungal genotype, and in some grass-endophyte symbiota levels can exceed 2% plant dry mass (Craven et al., 2001). Until the present study lolines have been the only grass-endophyte associated alkaloid class reported in the literature that was not observed in fungal cultures (Porter, 1994). Knowledge of the origin of lolines would be beneficial for elucidating the biosynthetic pathway and its regulation, identifying biosynthesis genes, and expanding biotechnological possibilities for these alkaloids.

Since lolines were only detected in endophyte-infected plants, we hypothesized the endophyte was responsible for their production, and that cultures that adequately mimic the internal environment of the plant may stimulate loline production. In this paper, it is demonstrated that three of the loline alkaloids found in plant tissue—loline (**1**), *N*-acetylnorloline (**2**), and both rotamers of *N*-formylloline (**3** and **3′**)—are produced by *N. uncinatum* under certain minimal medium conditions.

2. Results

The common endophyte of *L. pratense, N. uncinatum,* was examined for potential production of loline alkaloids in culture because grasses symbiotic with this species accumulate lolines at high levels. In a minimal medium modified from Chung and Schardl (1997), with mannitol as the carbon source and asparagine as the nitrogen source, *N. uncinatum* produced **1**, **2**, **3**, and **3′**. The identities of these alkaloids were confirmed by GC–MS and ^{13}C NMR spectroscopic analyses. Another loline alkaloid, *N*-acetylloline, is (together with **3** and **3′**) one of the most abundant alkaloids in *L. pratense–N. uncinatum* symbiota (Siegel et al., 1990), but was not detected in the culture. No loline alkaloids were detected from the cultures grown in potato dextrose broth (PDB).

Various carbon sources typically present in plant apoplasts were examined. Initially, mannitol was chosen because it is a good carbon source for growth of the related endophyte, *N. coenophialum* (Kulkarni and Nielsen, 1986). Fructose, glucose, and sucrose were then tested as alternative carbon sources because sucrose is a major transport sugar in the apoplast, while glucose and fructose are produced from sucrose by the action of invertase secreted by endophytes (Lam et al., 1994). As shown in Fig. 2, sucrose was the best of these carbon sources for loline production, giving a range of 500–750 $\mu g\ ml^{-1}$ (up to 4 mM) total of **2**, **3**, and **3′**. This was a 12-fold increase of production over cultures with mannitol. Glucose or fructose also tended to increase production only slightly compared to mannitol cultures, but not to the level obtained using sucrose.

Nitrogen sources tested were asparagine, arginine, ornithine, glutamine, ammonium sulfate, and urea, in cultures at pH 5.5 with sucrose as carbon source.

Nitrogen concentration was maintained at 30 mM total nitrogen atoms. In this experiment, cultures with urea as the sole nitrogen source yielded the greatest overall level of loline alkaloid production, ca. 425 μg ml^{-1} of culture filtrate (Fig. 3). Ornithine as the sole nitrogen source also gave much greater levels than expressed in cultures with asparagine or arginine. The level of loline alkaloid production at day 20 was 5-fold greater when ornithine rather than asparagine was the nitrogen source (Fig. 3).

Altering concentrations of phosphate between 6.8 and 68 mM, $MgSO_4$ between 0.2 and 4.0 mM, mannitol between 8.3 and 16.7 mM, or asparagine between 15 and 30 mM had only slight effects on loline alkaloid production levels (data not shown).

To determine how loline alkaloid production and growth may be related, a time course experiment was conducted with medium containing 16.7 mM sucrose and 30 mM ornithine (Figs. 4 and 5) (these carbon and nitrogen sources were chosen for the time course because they promote loline alkaloid production and are known substituents of plant apoplasts; Pate, 1973). The proportion of **2** to **3** with **3′** in this experiment (Fig. 4a and b) was consistent with levels obtained in similar experiments. (Levels of **1** were characteristically very low, so they are not included in Fig. 4.) The maximum rate of loline alkaloid accumulation (Fig. 4a and b) consistently occurred after cessation of the fungal growth with onset of stationary phase (Fig. 5a). A slight increase in pH occurred whether the initial pH was 5.5

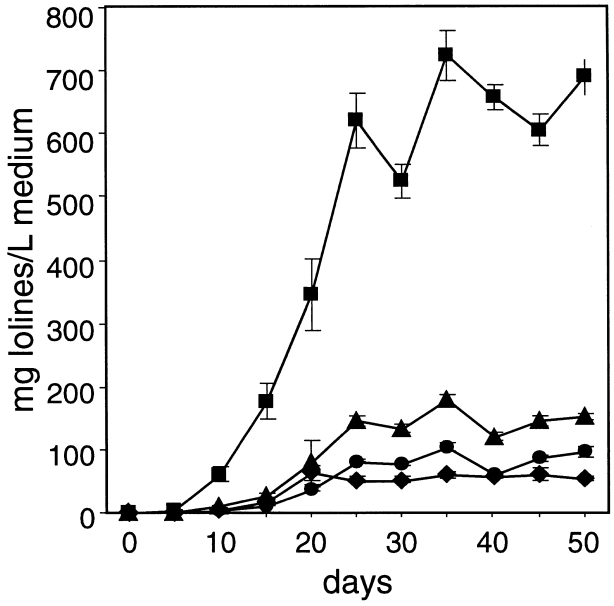

Fig. 2. Effect of carbon source on loline production. Carbon sources were ◆ 16.7 mM mannitol, ▲ 16.7 mM glucose, ● 16.7 mM fructose, or ■ 16.7 mM sucrose. Asparagine concentration was at 15 mM; pH 5.5. Means ($n=5$) are indicated with error bars equal to ± 1 S.D. S.D. smaller than the symbol are not shown.

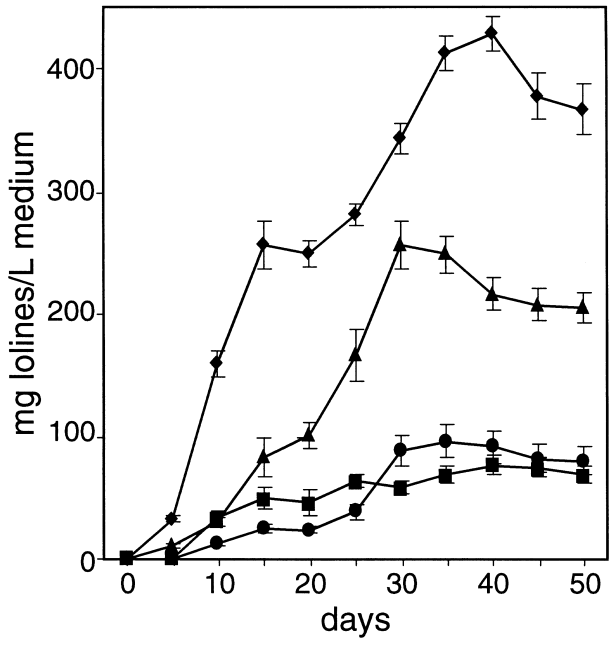

Fig. 3. Effect of nitrogen source on loline production. Nitrogen sources were ▲ 15 mM ornithine, ● 15 mM asparagine, ■ 7.5 mM arginine, or ◆ 15 mM urea. Sucrose concentration was 16.7 mM, and pH was 5.5. Means ($n=4$, except urea $n=3$) are indicated with error bars equal to ± 1 S.D.

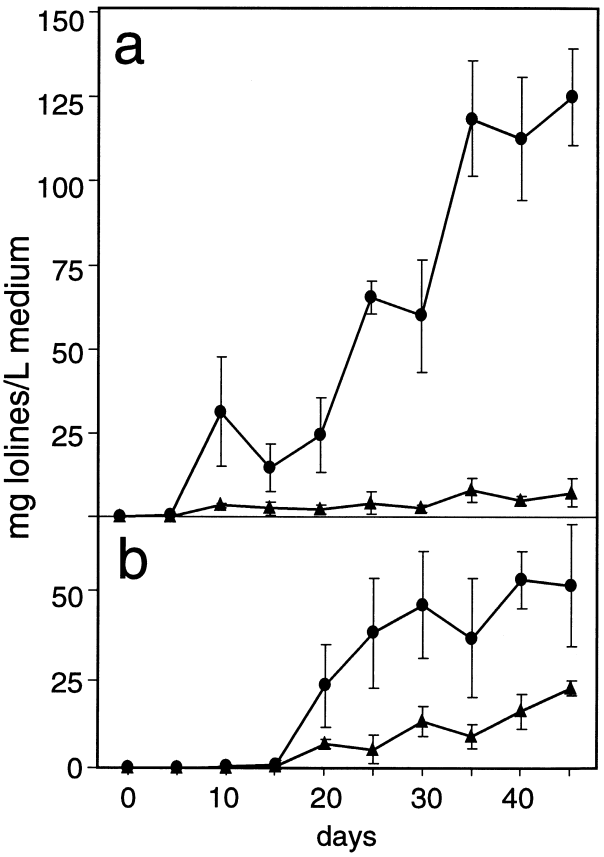

Fig. 4. Levels of ▲ *N*-acetylnorloline and ● *N*-formylloline in cultures. (a) Production at pH 5.5. (b) Production at pH 7.0. Means ($n=4$) are indicated with error bars equal to ± 1 S.D.

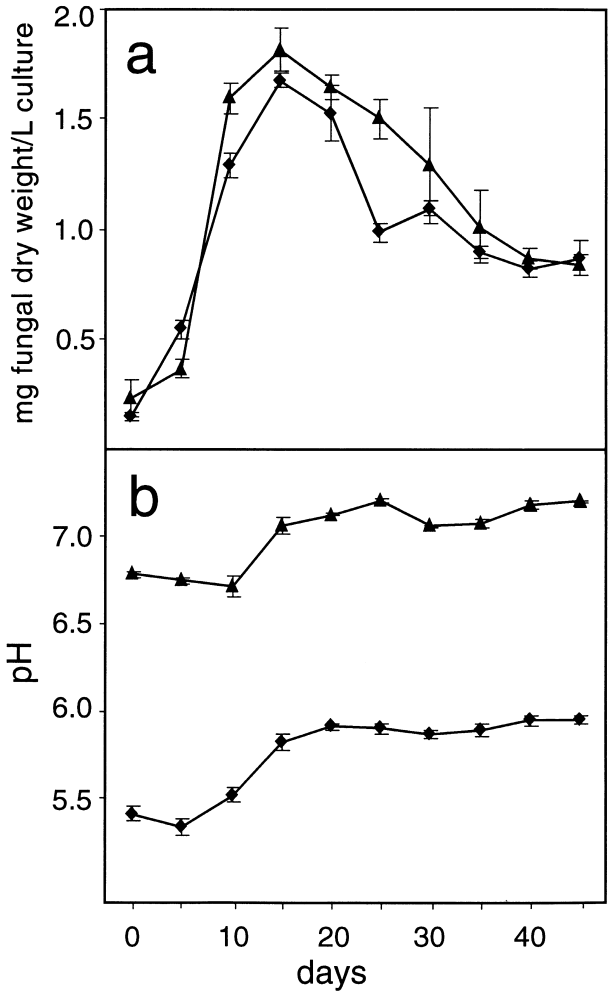

Fig. 5. Comparison of loline production at pH ◆ 5.5 and ▲ 7.0. (a) Growth of *Neotyphodium uncinatum*. (b) Change in pH of culture medium. Carbon and nitrogen sources were 16.7 mM sucrose and 30 mM ornithine, respectively. Means ($n=4$) are indicated with error bars equal to ± 1 S.D.

or 7.0 (Fig. 5b), and the change coincided with the onset of loline production (Fig. 4a and b) and slowing of fungal growth (Fig. 5a).

Other grass endophytes known to produce lolines in planta—*E. festucae* from *L. giganteum* (Leuchtmann et al., 2000), *N. coenophialum* from *L. arundinaceum* (Siegel et al., 1990), and *N. siegelii* from *L. pratense* (Craven et al., 2001)—were also tested for production. These fungi were cultured under the preferred conditions established for *N. uncinatum* (including 20 mM sucrose and 30 mM ornithine), but lolines were not detected in these cultures.

3. Discussion

The *Epichloë* and *Neotyphodium* species, closely related fungi that live as endophytes of grasses, have been implicated in the production of the ergot, lolitrem, and peramine alkaloids (Porter, 1994). Previously, lolines were never observed outside the grass-endophyte symbiota (Schardl and Phillips, 1997). Thus, prior to this study it was not established whether the plant or fungus, or the combination of both, produced the loline alkaloids. By establishing culture conditions for production of the loline alkaloids (specifically *N*-formylloline, *N*-acetylnorloline, and loline) by *N. uncinatum,* it was demonstrated that this fungus has the full biosynthetic capacity for some of the most common loline alkaloids found *in planta.* The observed correspondence of maximal loline production with the stationary phase was in keeping with secondary metabolism in other microorganisms (Campbell, 1984).

Nitrogen sources for these experiments were chosen to test organic and inorganic nitrogen and also based on the known precursors of polyamines and plant pyrrolizidines (Böttcher et al., 1993). Ammonium sulfate was used as an inorganic nitrogen source with levels of production comparable to those with asparagine, glutamine, and arginine. Cultures with urea as nitrogen source yielded the highest levels of lolines, but urea was not examined further because its levels in plant apoplast and xylem are unknown. Arginine and ornithine are apoplast substituents in grasses (Pate, 1973), and ornithine is a direct precursor in the polyamine biosynthetic pathway. It has been proposed, based on structures of the lolines, that spermidine, spermine, or both could be precursors to the lolines (Siegel and Bush, 1997). Arginine and ornithine are closely related, and removal of the urea group from arginine yields ornithine in the urea cycle. A determinant step in polyamine synthesis is decarboxylation of ornithine to form putrescine, which is converted to spermidine with the addition of a propylamine group from decarboxylated *S*-adenosylmethionine. Adding an additional propylamine group to spermidine yields spermine. These propylamine groups ultimately derive from aspartate, which is readily derived from asparagine. Preliminary feeding studies with ^{13}C-labelled ornithine and aspartate confirmed that these are likely loline alkaloid precursors (J.D.B. and C.L.S., unpubl. data).

The absence of *N*-acetylloline in culture, the other major loline alkaloid produced in planta, remains to be explained. Alternative possibilities are that the fungus does not express the necessary enzyme to convert loline (or other lolines) to *N*-acetylloline, or that the plant is responsible for the conversion. Another surprising observation is that no other fungal endophyte that produces the loline alkaloids in planta produced measurable quantities in culture, even under conditions favorable for production by *N. uncinatum*. A possibility is that only *N. uncinatum* has the full competence to produce lolines outside its host, whereas in other grass-endophyte symbiota loline alkaloid production may be accomplished by both symbiotic partners. A more likely possibility, however, is simply that the culture conditions promoting production by *N. uncinatum* were unsuitable for production by the other endophytes.

Now that it has been shown that some common loline alkaloids can be produced by cultured *N. uncinatum*, work is underway on the biosynthetic pathway for loline alkaloids. The high level of their production in culture will significantly facilitate studies of the pathway and will aid in identifying and characterizing the genes that may be responsible.

4. Experimental

4.1. General experimental procedure

For gas chromatography, all samples were run on a Hewlett Packard (Avondale, PA) 5890 Series II Plus GC equipped with a flame ionization detector and a J&W Scientific Inc. (Rancho Cordova, CA) fused silica capillary column (SE 30, 60 m×0.30 mm inner diameter with 0.25 μm film thickness of dimethylpolysiloxane). Nitrogen was used as the carrier gas at a flow rate of 20.4 ml min^{-1}. The run parameters were an initial column temperature of 110 °C, an injection temperature of 250 °C, a detector temperature of 325 °C, a 2.2-min purge time, and a 4 °C min^{-1} increase in column temperature to 188 °C. Between each sample run, the column was purged at 280 °C for 5 min. ^{13}C NMR spectroscopic analysis employed a Varian Gemini 200 equipped with VNMR 6. 1B software (Varian Inc., Palo Alto, CA). Samples of purified alkaloids in 750 μl D_2O (Cambridge Isotopes Laboratories, Inc., Andover, MA) were prepared in 5 mm×17.8 cm NMR tubes (Wilmad, Beuna, NJ) and 10 μl methanol added as an internal standard. ^{13}C NMR spectroscopic conditions were at 200 mHz, ambient temperature, and proton decoupling. Chemicals for these experiments were obtained from Sigma-Aldrich (Milwaukee, WT).

4.2. Fungal isolate isolation

Cultures were obtained by growing *Neotyphodium uncinatum* (W. Gams Petrini et Schmidt) Glenn Bacon et Hanlin (voucher specimen CBS 102646 at Centraalbureau Voor Schimmelcultures) out of an infected plant (number 167 in our plant collection) of *Lolium pratense* (Huds.) Darbysh. [*Festuca pratensis* Huds., realigned with genus *Lolium* by Darbyshire (1993).] Leaf blades were sterilized in 50-ml Costar tubes with 95% ethanol for 1 min, followed by a rinse of sterile water, then soaked for 2 min in 5.25% sodium hypochlorite. The leaf blades were then rinsed with sterile water, cut into 2-mm sections, and plated on potato dextrose agar (PDA, Difco Laboratories, Detroit, MI) with penicillin (167 units ml^{-1}) and streptomycin (76.3 units ml^{-1}). After 14 days, 5 pieces of fungal mycelium grown out (approximately 0.5 cm) of the plant material was removed from the agar and homogenized in 10 ml of the minimal medium in an Omni Homogenizer Model 17105 (Omni International, Waterbury, CT) with six 10-s bursts. The ground mycelium was drop-inoculated onto PDA and grown for 7 days, then 1.5–2 cm plugs were cut out of the agar, homogenized in minimal medium, and 1 ml used to inoculate each 30 ml minimal medium culture for study. Large differences in alkaloid expression between experiments most likely were due to unavoidable differences in inoculum density and physiological condition of the source mycelium, so employing a single source of inoculum for each experiment minimized variation between replicates. Cultures of *Epichloë festucae* Leuchtm. Schardl et Siegel (CBS 102474) from *Lolium giganteum* (L.) Darbysh. (= *Festuca gigantea* L.), *Neotyphodium coenophialum* (Morgan-Jones et W. Gams) Glenn Bacon et Hanlin (American Type Culture Collection accession ATCC 90664) from *Lolium arundinaceum* (Schreb.) Darbysh. (= *Festuca arundinacea* Schreb.), and *Neotyphodium siegelii* K. D. Craven Leuchtm. et Schardl (ATCC 74483) from *L. pratense* were obtained similarly (Craven et al., 2001).

4.3. Culture medium

The defined media used in these experiments were adapted from Chung and Schardl (1997) with modifications. The 10× stock solutions of basal salts—300 mM K_2HPO_4 and 300 mM KH_2PO_4 in water purified by Milli-Q (Nanopure, Model D4751, Barnstead/Thermolyne, Dubuque, IA)—were mixed to the desired pH (4.5–8.0 as specified). Then, 2(*N*-morpholino)ethanesulfonic acid (MES) was added to the basal salt solution to 300 mM, and pH readjusted with NaOH. MES was used to prevent the drastic pH increase that sometimes occurred in cultures with only phosphate as buffer. Final concentrations in the minimal medium were 30 mM potassium phosphate and 30 mM MES. The carbon sources were added to a conc. of 8.3 mM, and nitrogen sources were added to give 30 mM nitrogen atoms, except where otherwise specified. To the medium were added $MgSO_4$ to 2 mM, thiamine to 0.6 μM, and a solution of trace elements (from 1000×stock) to give 3.6 μM H_3BO_3, 1 μM $CuSO_4$, 0.7 μM KI, 0.8 μM FeNa-ethylenediaminetetraacetic acid, 1 μM $MnSO_4$, 0.5 μM $NaMoO_4$, and 0.4 μM $ZnSO_4$ (Kulkarni and Nielsen, 1986). Basal salts and MES in purified water were autoclaved together, and the $MgSO_4$ was autoclaved separately to prevent precipitation of salts. Carbon source, nitrogen source, thiamine, and trace elements were filter-sterilized together then combined with the basal salts and $MgSO_4$ solution. After preliminary trials with cultures in Erlenmeyer flasks proved inconsistent, 100×25 mm polystyrene petri plates (Fisherbrand, Fisher Scientific, Pittsburgh, PA) were used for the 30 ml cultures in all experiments reported here. The cultures were incubated at 21 °C with 100 rpm rotary shaking.

4.4. Sampling from cultures

Aliquots of medium from each culture were transferred into 1.7-ml Eppendorf (Brickmann Instruments, Inc., Westbury, NY) microcentrifuge tubes that had been pretreated at 100 °C for 2 days to remove extractable contaminants that otherwise interfered with the analysis. Prior to day 20 of culture, 1 ml aliquots were sampled; thereafter, aliquots were 0.5 ml. For fungal biomass and loline level determinations, 30-ml cultures were harvested for each time point and filtered through tared Whatmann no. 1 filter paper (Whatmann Limited, England). Then the mycelium on each filter was oven dried (37 °C, 2 days) and weighed, and the culture filtrate was frozen and freeze-dried for later analysis of lolines.

4.5. Alkaloid analysis

For GC analysis, freeze-dried samples were extracted using a modified procedure from Yates et al. (1990) and Robbins et al. (1972). One-tenth volume of a saturated sodium bicarbonate solution (0.25 g ml^{-1} in H_2O) was added to each dried filtrate and the tubes were vortexed. Chloroform (1 ml), with 14.2 mg l^{-1} of quinoline (RR $t=7.60$) as the internal standard was added to each tube. The suspension was vortexed, then shaken 30 min on a platform shaker. The $CHCl_3$ layer was transferred to a capped glass vial. Levels for lolines from GC analysis in these experiments are reported as the total of **2** (RR $t=16.75$) and **3** with **3′** (RR $t=17.44$), because the levels of **1** (RR $t=9.21$) were always very low.

For analysis by GC–MS and ^{13}C NMR spectroscopy, loline alkaloids were isolated using modifications of the procedure of Petroski et al. (1989). Fungal culture filtrates were freeze-dried, then extracted with 1 ml satd. sodium bicarbonate (aq.) and 30 ml $CHCl_3$. The aqueous layer was discarded and the organic solvent evaporated in a stream of nitrogen gas to yield a brown oil. One ml $CHCl_3$ was added to the oil and 500 μl of this mixture was spotted onto a K6F Silica gel 60A TLC plate (Whatmann Inc, Clifton, NJ). After drying, the plate was developed in a chromatography chamber with 140 ml of a 50:50:1 (by volume) mixture of CH_3OH, $CHCl_3$ and satd. NH_3OH. Loline alkaloids were visualized as dark brown spots after exposing the plate to iodine vapor. R*f* values for **1**, **2**, and **3** with **3′** were determined as 0.26, 0.37, and 0.49, respectively. The visible spots corresponding to the loline alkaloids (**1**, **2**, and **3** with **3′**) on the plate were scraped off individually and alkaloids eluted with purified H_2O, extracted with $CHCl_3$ and solvent removed by a stream of N_2 gas. These purified samples were then used in GC–MS and ^{13}C NMR analysis.

4.6. Alkaloid purification and identification:

Loline (**1**) was isolated by separation of the alkaloids by TLC and extracted from the silica with H_2O, and because of its volatility, conc. HCl was added to yield the salt form, which was recrystallized with EtOH: ^{13}C NMR (D_2O, 200 MHz), 29.2 (C6), 34.0 (NMe), 55.5 (C5), 61.8 (C3), 63.8 (C1), 70.1 (C8), 71.9 (C2), 80.9 (C7); EIMS *m*/*z* 154 $[M]^+$ (0), 123(58), 110 (7), 95 (75), 82 (100).

N-Acetylnorloline (**2**) was purified as a clear oil after being separated from the other alkaloids by TLC, extracted from the plate with H_2O followed by extraction with $CHCl_3$ which was removed with a stream of nitrogen gas: ^{13}C NMR (D_2O, 200 MHz), 24.2 (MeC=O), 35.1 (C6), 54.8 (C5), 58.4 (C1), 61.4 (C3), 70.1 (C8), 74.1 (C2), 81.6 (C7), 171.9 (HC=O); EIMS *m*/*z* 182 $[M]^+$ (0), 153 $[M\text{-}29]^+$ (8), 139 (0), 123 (9), 110 (0), 95 (30), 82 (100), 69 (22).

N-Formylloline (**3**) was purified as a viscous brown oil similar to purification of **2**: ^{13}C NMR (D_2O, 200 MHz), 32.0 (C6), 34.8 (NMeth), 54.1 (C5), 59.7 (C3), 65.5 (C1), 67.8 (C8), 74.3 (C2), 83.2 (C7), 165.1 (HC=O); EIMS *m*/*z* 182 $[M]^+$ (0), 154 $[M\text{-}28]^+$ (25), 123 (13), 110 (13), 95 (28), 82 (100).

N-Formylloline (**3′**) extracted with **3** as a brown oil: ^{13}C NMR (D_2O, 200 MHz), 30.4 (NMeth), 31.7 (C6), 54.3 (C5), 60.6 (C3), 63.0 (C1), 68.6 (C8), 73.5 (C2), 81.4 (C7), 167.0 (HC=O); EIMS *m*/*z* 182 $[M]^+$ (0), 154 $[M\text{-}28]^+$ (25), 123 (13), 110 (13), 95 (28), 82 (100).

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References

Bacon, C.W., 1988. Procedure for isolating the endophyte from tall fescue and screening isolates for ergot alkaloids. Applied and Environmental Microbiology 54, 2615–2618.

Böttcher, F., Adolph, R.D., Hartmann, T., 1993. Homospermidine synthase, the first pathway-specific enzyme in pyrrolizidine alkaloid biosynthesis. Phytochemistry 32, 679–689.

Bush, L.P., Fannin, F.F., Siegel, M.R., Dahlman, D.L., Burton, H.R., 1993. Chemistry, occurrence, and biological effects of saturated pyrrolizidine alkaloids associated with endophyte grass interactions. Agriculture, Ecosystems, and Environment 44, 81–102.

Campbell, I.M., 1984. Secondary metabolism and microbial physiology. Advances in Microbial Physiology 25, 2–59.

Casabuono, A.C., Pomilio, A.B., 1997. Alkaloids from endophyte-infected *Festuca argentina*. Journal of Ethnopharmacology 57, 1–9.

Chung, K.R., Schardl, C.L., 1997. Vegetative compatibility between and within *Epichloë* species. Mycologia 89, 558–565.

Craven, K.D., Blankenship, J.D., Leuchtmann, A., Hignight, K., Schardl, C.L., 2001. Hybrid fungal endophytes symbiotic with the grass *Lolium pratense*. Sydowia 53, 44–73.

Dannhardt, G., Steidt, L., 1985. Alkaloids of *Lolium temulentum*: isolation, identification, and pharmacological activity. Planta Medica 51, 212–214.

Darbyshire, S.J., 1993. Realignment of *Festuca* subgenus *Schedonorus* with genus *Lolium* (Poaceae). Novon 3, 239–243.

Dew, R.K., Boissonneault, G.A., Gay, N., Boling, J.A., Cross, R.J., Cohen, D.A., 1990. The effect of the endophyte (*Acremonium coenophialum*) and associated toxin(s) on the serum titer response to immunization and spleen cell flow cytometry analysis and response to mitogens. Veterinary Immunology and Immunopathology 26, 285–295.

Dougherty, C.T., Knapp, F.W., Bush, L.P., Maul, J.E., Van Willigen, J., 1998. Mortality of horn fly (Diptera: Muscidae) larvae in bovine dung supplemented with loline alkaloids from tall fescue. Journal of Medical Entomology 35, 798–803.

Gurney, K.A., Mantle, P.G., Penn, J., Garthwaite, I., Towers, N.R., 1994. Loss of toxic metabolites from *Acremonium lolii*, the endophyte of ryegrass, following mutagenesis. Naturwissenschaften 81, 362–365.

Hartmann, T., Witte, L., 1995. Chemistry, biology and chemoecology of the pyrrolizidine alkaloids. In: Pelletier, S.W. (Ed.), The Alkaloids: Chemical and Biological Perspectives, Vol. 9. Springer, New York, pp. 155–231.

Hincks, J.R., Kim, H.Y., Segall, H.J., Molyneux, R.J., Stermitz, F.R., Coulombe, R.A., 1991. DNA cross-linking in mammalian cells by pyrrolizidine alkaloids: Structure-activity relationships. Toxicology and Applied Pharmacology 111, 90–98.

Jackson, J.A., Varney, D.R., Petroski, R.J., Powell, R.G., Bush, L.P., Siegel, M.R., Hemken, R.W., Zavos, P.M., 1996. Physiological responses of rats fed loline and ergot alkaloids from endophyte-infected tall fescue. Drug and Chemical Toxicology 19, 85–96.

Justus, M., Witte, L., Hartmann, T., 1997. Levels and tissue distribution of loline alkaloids in endophyte-infected *Festuca pratensis*. Phytochemistry 44, 51–57.

Kim, H.Y., Stermitz, F.R., Li, J.K.K., Coulombe, R.A., 1999. Comparative DNA crosslinking by activated pyrrolizidine alkaloids. Food and Chemical Toxicology 37, 619–625.

Kulkarni, R.K., Nielsen, B.D., 1986. Nutritional requirements for growth of a fungus endophyte of tall fescue grass. Mycologia 78, 781–786.

Lam, C.K., Belanger, F.C., White, J.F., Daie, J., 1994. Mechanism and rate of sugar uptake by *Acremonium typhinum*, an endophytic fungus infecting *Festuca rubra*: evidence for presence of a cell wall invertase in endophytic fungi. Mycologia 86, 408–415.

Leuchtmann, A., Schmidt, D., Bush, L.P., 2000. Different levels of protective alkaloids in grasses with stroma-forming and seed-transmitted *Epichloë/Neotyphodium* endophytes. Journal of Chemical Ecology 26, 1025–1036.

Pate, J.S., 1973. Uptake, assimilation and transport of nitrogen compounds by plants. Soil Biology and Biochemistry 5, 109–119.

Patterson, C.G., Potter, D.A., Fannin, F.F., 1991. Feeding deterrency of alkaloids from endophyte-infected grasses to Japanese beetle grubs. Entomologia Experimentalis et Applicata 61, 285–289.

Petroski, R.J., Yates, S.G., Weisleder, D., Powell, R.G., 1989. Isolation, semi-synthesis, and NMR spectral studies of loline alkaloids. Journal of Natural Products 53, 810–817.

Porter, J.K., 1994. Chemical constituents of grass endophytes. In: Bacon, C.W., White Jr., J.F. (Eds.), Biotechnology of Endophytic Fungi of Grasses. CRC, Boca Raton, FL, pp. 103–123.

Powell, R.G., Petroski, R.J., 1992. The loline group of pyrrolizidine alkaloids. In: Pelletier, S.W. (Ed.), The Alkaloids: Chemical and Biological Perspectives, Vol. 8. Springer, Berlin, pp. 320–338.

Robbins, J.D., Sweeny, J.G., Wilkinson, S.R., Burdick, D., 1972. Volatile alkaloids of Kentucky 31 tall fescue seed (*Festuca arundinacea* Schreb.). Journal of Agricultural and Food Chemistry 20, 1040–1042.

Rowan, D.D., 1993. Lolitrems, peramine, and paxilline—mycotoxins of the ryegrass–endophyte interaction. Agriculture Ecosystems and Environment 44, 103–122.

Schardl, C.L., Phillips, T.D., 1997. Protective grass endophytes: where are they from and where are they going? Plant Disease 81, 430–438.

Siegel, M.R., Bush, L.P., 1997. Toxin production in grass/endophyte associations. In: Carroll/Tudzynski (Ed.), The Mycota V, Part A: Plant Relationships. Springer, Berlin, Heidelberg, pp. 185–207.

Siegel, M.R., Latch, G.C.M., Bush, L.P., Fannin, F.F., Rowan, D.D., Tapper, B.A., Bacon, C.W., Johnson, M.C., 1990. Fungal endophyte-infected grasses: alkaloid accumulation and aphid response. Journal of Chemical Ecology 16, 3301–3315.

TePaske, M.R., Powell, R.G., Clement, S.L., 1993. Analyses of selected endophyte-infected grasses for the presence of loline-type and ergot-type alkaloids. Journal of Agricultural and Food Chemistry 41, 2299–2303.

Tofern, B., Kaloga, M., Witte, L., Hartmann, T., Eich, E., 1999. Phytochemistry and chemotaxonomy of the Convolvulaceae part 8—occurrence of loline alkaloids in *Argyreia mollis* (Convolvulaceae). Phytochemsitry 51, 1177–1180.

Wilkinson, H.H., Siegel, M.R., Blankenship, J.D., Mallory, A.C., Bush, L.P., Schardl, C.L., 2000. Contribution of fungal loline production to protection from aphids in a grass fungus mutualism. Molecular Plant-Microbe Interactions 13, 1027–1033.

Yates, S.G., Petroski, R.J., Powell, R.G., 1990. Analysis of loline alkaloids in endophyte-infected tall fescue by capillary gas chromatography. Journal of Agricultural and Food Chemistry 38, 182–185.