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# LEVELS AND TISSUE DISTRIBUTION OF LOLINE ALKALOIDS IN ENDOPHYTE-INFECTED *FESTUCA PRATENSIS*

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**Key Word Index**—*Festuca pratensis*; Poaceae; *Acremonium uncinatum*; Clavicipitaceae, Balansieae; grass–endophyte association; loline alkaloids; pyrrolizidine alkaloids.

**Abstract**—*Festuca pratensis* (meadow fescue) infected with the endophyte *Acremonium uncinatum* produces loline alkaloids (1-aminopyrrolizidines) that are not found in the uninfected grass or the fungus alone. Five alkaloids were identified by capillary GC and GC–MS: *N*-formylloline as the major compound, followed by *N*-acetylloline, *N*-acetylnorloline and trace amounts of loline and *N*-methylloline. A routine procedure for the extraction and sensitive quantitative analysis of loline alkaloids by capillary GC is described. The loline alkaloid levels and concentrations were followed quantitatively over the growing season of the grass–endophyte association. A detailed analysis of the tissue distribution of the alkaloids is given. The total alkaloid level per plant increases from almost zero in early spring and reaches its highest level during seed maturation. It drops to almost zero with seed dispersal and stalk senescence but increases again during the subsequent period of vegetative growth in late summer. The highest alkaloid concentrations were found in young leaves in early spring, and in panicles (spikelets, seeds) and leaf pseudostems during the period of vegetative growth in late summer and autumn. During seed germination loline alkaloids are not degraded, however, a significant proportion (about 20%) are lost by leaching, mainly during seed imbibition. Within a seed the embryo was found to contain a two-fold higher alkaloid concentration than the remaining seed tissue. Copyright © 1996 Elsevier Science Ltd

## INTRODUCTION

The loline alkaloids represent a small group of 1-aminopyrrolizidine derivatives [1]. They are biosynthetically unrelated to the approximately 360 known pyrrolizidine alkaloids (PAs), which represent ester alkaloids containing a 1-hydroxymethylpyrrolizidine moiety as the common structural element [2]. The PAs are particularly well known as toxic principals of certain genera of the Asteraceae (e.g. *Senecio*), Boraginaceae (e.g. *Heliotropium*) and Fabaceae (e.g. *Crotalaria*) [2, 3]. The lolines have only been reported from grasses (Poaceae; e.g. *Festuca*, *Lolium*), *Adenocarpus* species (Fabaceae) [1, 3] and, most recently, *Agyrea mollis* (Convolvulaceae) [4].

The occurrence of lolines in grasses is closely associated with the presence of endophytic fungi [5–7]. Tall fescue (*Festuca arundinacea* Schreb.) infected with the ascomycete *Acremonium coenophialum* Morgan-Jones & Gams (tribe Balansieae, Clavicipitaceae) produces three types of alkaloids, lolines, ergot alkaloids and pyrrolopyrazine alkaloids (e.g. peramine), all of which are absent in uninfected grasses [8, 9]. Loline alkaloids are found only in host–endophyte associations, whereas the two other groups of alkaloids are also produced by the fungus alone. It is well documented that the endophyte infection is the cause of the fescue toxicosis in cattle that is frequently observed in the transition zone of the U.S.A. [10]. Uninfected tall fescue is non-toxic. The livestock toxicosis seems to be caused mainly by the ergot alkaloids [11].

In an ecological context the host–endophyte association is a mutualistic one, with each partner gaining substantial benefits from the other [12]. Endophyte-infected tall fescue shows an improved grass drought resistance [13] and exhibits a better resistance to herbivorous insects and nematode and fungal attack [3, 9, 14]. The lolines were shown to be efficient feeding deterrents and toxins for a number of herbivorous insects [3, 9].

We became interested in the loline alkaloids for biosynthetic reasons. It is well established that homospermidine, the first specific intermediate in PA biosynthesis [3], is formed by the enzyme homospermidine synthase [15, 16]. It is still unclear whether spermidine is the specific precursor of the 1-aminopyrrolizidine moiety of the loline alkaloids, as has been suggested [9] but never proven. Most studies on lolines have been performed with endophyte-infected tall fescue. Little is known about other associations. We selected the as-

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sociation of meadow fescue (*Festuca pratensis* Huds.) and *Acremonium uncinatum* Gams, Patrini & Schmidt. *Festuca pratensis* is an important pasture grass in Europe and its host–endophyte association has not been analysed chemically in detail. Furthermore, in contrast to the endophyte-infected tall fescue, the meadow fescue–endophyte association does not produce detectable amounts of other alkaloids (e.g. ergot alkaloids or peramine) [9, 17].

We report here the loline patterns of the *F. pratensis–A. uncinatum* association, its quantitative evaluation and tissue-specific distribution at various developmental stages during the growing-season.

## RESULTS

### *Identification and determination of lolines in endophyte-infected meadow fescue*

Analysis of *A. uncinatum*-infected seeds of *F. pratensis* revealed the presence of five loline alkaloids (Fig. 1). The structures were unequivocally identified by gas chromatography–mass spectroscopy (Table 1) and compared to reference data [1, 18, 19]. In addition to the two major alkaloids **4** and **5**, which are already known to occur in endophyte-infected meadow fescue [9, 17], **3** was identified as a third major alkaloid, together with trace amounts of **1** and **2** (Table 1; Fig. 2).

Capillary gas chromatography has already been applied to the analysis of loline alkaloids in tall fescue

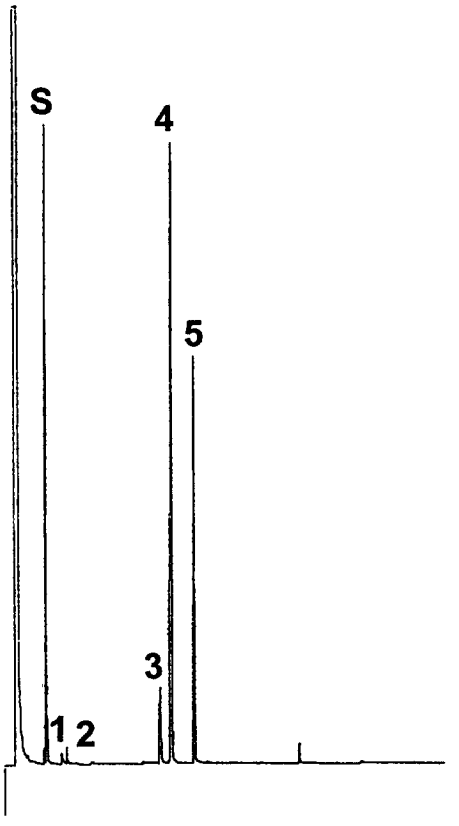
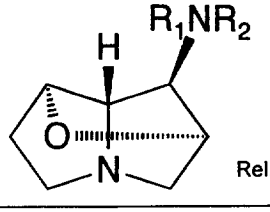



Fig. 2. Capillary gas chromatographic analysis of an extract of endophyte-infected *Festuca pratensis* seeds. Detection: FID. For numbering of compounds see Fig. 1.




| $R_1$ | $R_2$ | | Rel. abundance (%) |
|---|---|---|---|
| H | $CH_3$ | Loline (**1**) | < 3 |
| $CH_3$ | $CH_3$ | *N*-Methylloline (**2**) | < 3 |
| H | Ac | *N*-Acetylnorloline (**3**) | 13-18 |
| $CH_3$ | CHO | *N*-Formylloline (**4**) | 54-60 |
| $CH_3$ | Ac | *N*-Acetylloline (**5**) | 27-30 |

Fig. 1. Structures and relative abundance of loline alkaloids identified from endophyte-infected *Festuca pratensis*.

Table 1. GC–MS analysis: retention indices and mass spectra of the lolines detectable in endophyte-infected *Festuca pratensis* seeds

| Alkaloid (GC peak) | $R_I$ | $[M]^+$ *m/z* | Characteristic ions *m/z* (% rel. abundance) |
|---|---|---|---|
| Loline (**1**) | 1250 | 154(5) | 123(17), 110(47), 95(33), 82(100), 68(19), 55(14), 44(33) |
| *N*-Methylloline (**2**) | 1275 | 168(2) | 123(49), 111(6), 95(70), 82(100), 58(56), 55(16) |
| *N*-Acetylnorloline (**3**) | 1575 | 182(0.3) | 153(4), 123(7), 110(3), 95(24), 82(100), 69(31), 55(10) |
| *N*-Formylloline (**4**) | 1603 | 182(0.3) | 154(15), 123(10), 110(12), 95(25), 82(100), 69(21), 55(12) |
| *N*-Acetylloline (**5**) | 1657 | 196(2) | 167(5), 153(8), 123(26), 101(16), 95(42), 82(100), 69(19), 56(12) |

seeds and forage [19]. As a convenient method for sample preparation and quantification of the lolines by capillary gas chromatography, the routine methods developed in our laboratory for PA analysis [20] could be used with minor modifications. For sample preparation, an aqueous acid extraction of powdered dry material was found to be the most convenient. Extracts could then be applied directly to solid-phase extraction, prior to gas chromatographic analysis. The method allows the routine analysis of plant samples of appoximately 0.5 g dry weight. The lolines were recovered quantitatively and remained stable throughout the assay procedure. Detection limits were 2 (PND) and 20 ng (FID). Quantification (FID) with tropanol as internal standard (Fig. 2) gives reproducible results with a standard deviation of 5–10%. The method has been applied successfully to the analysis of various grass tissues and forage samples.

### *Changes in total loline alkaloids during the growing season of endophyte-infected* Festuca pratensis

Tissue contents and concentrations of the three major lolines (**3**, **4**, and **5**; Fig. 1) were followed quantitatively during the growing season between April and October 1995 (Figs 3 and 4). The development of *F. pratensis* shows two characteristic stages: (1) development of reproductive stalks, beginning in early spring and lasting until seed dispersal and senescence at the end of July (Figs 3 and 4, samplings 2–9); and (2) the reproductive stage is followed by vigorous vegetative growth from August until leaf senescence at the end of October (Figs 3 and 4, samplings 10–14). These two periods show typical fluctuations. The hibernated tufts contain only trace amounts of lolines (Figs 3 and 4, sampling 1). Alkaloid formation begins with the occurrence of new leaves and continues to increase until beginning of seed dispersal (Figs 3 and 4, samplings 2–7); with senescence of the stalks the loline content decreases to almost zero (Figs 3 and 4, samplings 8 and 9). During the following period of vegetative growth the loline alkaloid level increases with leaf growth and decreases again during leaf senescence (Figs 3 and 4, samplings 10–14). The highest loline alkaloid content per individual was found during the period of flowering and seed ripening (Fig. 3, samplings 6 and 7; Fig. 5). However, the highest concentrations were found in young leaves during the onset of growth in early spring (Fig. 4, sampling 2) and the period of vegetative growth after seed dispersal in late summer (Fig. 4, samplings 12).

In a detailed analysis the total amount of loline alkaloids and the alkaloid concentrations of successive nodes/internodes of a stalk and its inflorescence were compared (Figs 3 and 4, S-1 to S-4). Within the stalks there is a clear tendency for the lolines to concentrate in

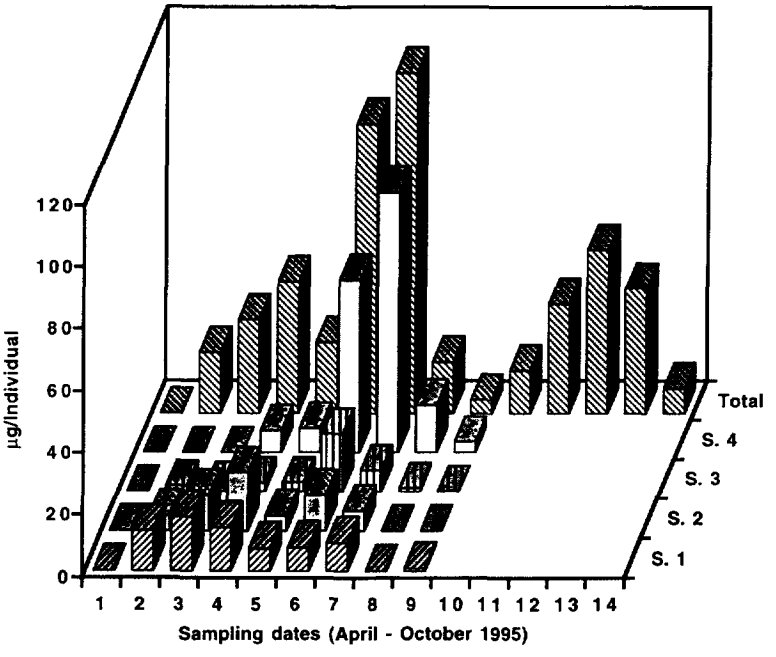



Fig. 3. Total amounts of loline alkaloids in single stalks or vegetatively growing leaves of endophyte-infected *Festuca pratensis* harvested at intervals during the vegetation period 1995. The values represent means of 50 individuals (samplings 1–3), 30 stalks (samplings 4–9), or 30 individuals (pseudostems + blades; samplings 10–14). Month/day of sampling (developmental stage): (1) 4/10 (hybernated leaves); (2) 4/25 (beginning stark growth); (3) 5/5 (first panicles became visible); (4) 5/26 (panicles visible); (5) 6/7 (flowering begins); (6) 6/21 (fully flowering); (7) 7/4 (mature seeds); (8) 7/20 (seeds mostly dispersed); (9) 8/2 (stalks senescent); (10) 8/17 (beginning vegetative regrowth); (11) 8/31 and (12) 9/21 (vigorous vegetative growth); (13) 10/10 (beginning senescence); (14) 10/26 (leaves senescent). S1 to S4, Successive internodes/inflorescence of stalks). See Fig. 5 for an illustration.

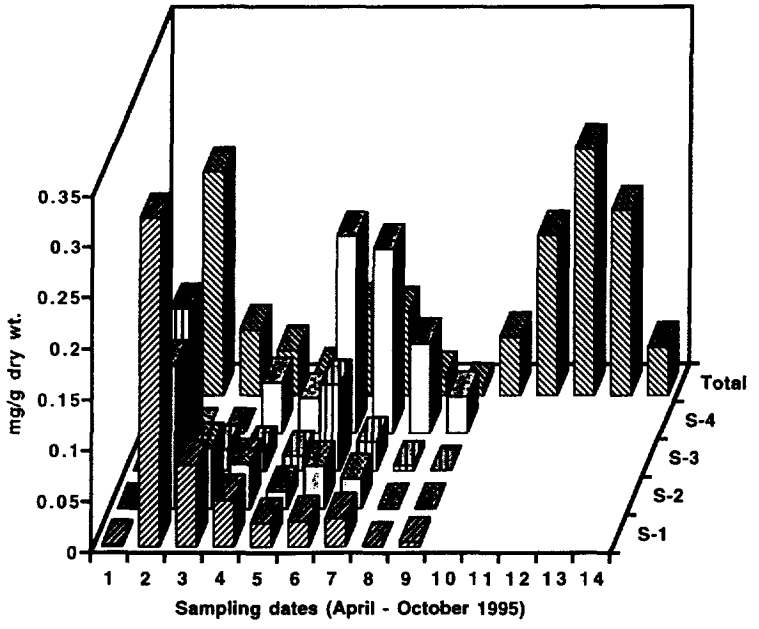



Fig. 4. Concentrations of loline alkaloids in single stalks or vegetatively growing leaves of endophyte-infected *Festuca pratensis* harvested at intervals during the vegetation period 1995. See legend to Fig. 3 for details.

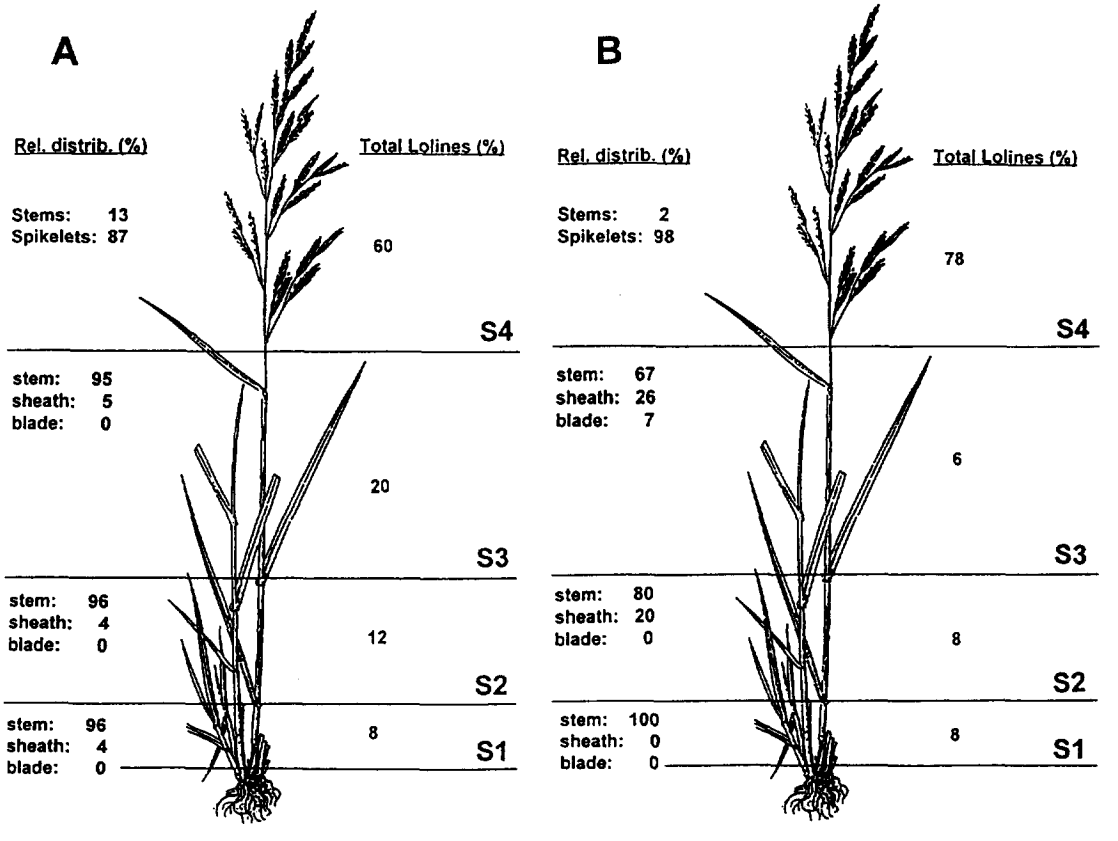



Fig. 5. Tissue-specific distribution of loline alkaloids in endophyte-infected *Festuca pratensis* stalks in two selected samples: (A) sampling 6, fully flowering specimens; (B) sampling 7, specimens with mature seeds (see legend to Fig. 3 for details). S1 to S4, successive internodes (segments) which were analysed separately.

the inflorescences, which during flowering and seed development contain 60–80% of the total loline alkaloids (Fig. 5). There is a considerable increase in total loline alkaloids during flowering and seed ripening, which is accompanied by a decrease in the amount of alkaloid in the vegetative tissues.

Analysis of the alkaloid distribution within the different plant organs revealed the following results. (1) Only trace amounts of loline alkaloids were detectable in roots during the various developmental stages (results not shown). (2) In successive nodes/internodes the highest alkaloid contents are found in stems, followed by leaf sheaths; whereas blades of the stalk leaves contain trace amounts only or became devoid of alkaloids during the beginning of senescence (Fig. 5). In panicles the lolines were localized in the spikelets where they reach the highest concentration in the seeds (Fig. 5). During the period of vegetative growth, at least until the beginning of senescence, the leaf blades were found to contain the same levels of total alkaloids as the pseudostems but, due to their higher proportion of biomass, at considerably lower concentrations (Table 2).

Table 3. Distribution of lolines between pseudostems and leaf blades in vegetatively growing endophyte-infected *Festuca pratensis*

| Sampling date (No.)* | Total lolines ($\mu$g/individual) | Concentration of lolines ($\mu g\ g^{-1}$ dry wt) |
|---|---|---|
| 17 August (10) | | |
| Pseudostem | ND | ND |
| Leaf blade | ND | ND |
| Total | 13.7 | 56.8 |
| 31 August (11) | | |
| Pseudostem | 17.8 | 295.3 |
| Leaf blade | 17.3 | 106.2 |
| Total | 35.1 | 157.3 |
| 21 September (12) | | |
| Pseudostem | 28.5 | 495.7 |
| Leaf blade | 24.0 | 152.6 |
| Total | 52.6 | 243.6 |
| 10 October (13) | | |
| Pseudostem | 14.7 | 225.6 |
| Leaf blade | 31.3 | 166.0 |
| Total | 46.0 | 182.2 |
| 26 October (14) | | |
| Pseudostem | 6.3 | 99.2 |
| Leaf blade | 1.8 | 29.1 |
| Total | 8.1 | 47.2 |

ND, not determined.
* See legend of Fig. 3 for further explanation.

*Changes in loline alkaloid content during seed germination and its distribution between seed tissues*

There is a significant loss of loline alkaloids during the first day of seed imbibition. This loss is mainly due to leakage of alkaloids into the aqueous medium (Table 3). During subsequent seed germination this leakage is no longer significant. Lolines are found in all tissues of the young seedling, but preferentially in the developing leaves. The increase in the alkaloid contents in older seedlings may indicate the beginning of *de novo* synthesis.

Dissection of caryopses, which previously had been kept in water for 24 hr, revealed the presence of loline alkaloids in the embryo as well as in the remaining seed tissues (i.e. endosperm plus testa) and almost nothing in

Table 2. Changes in total loline alkaloids during soaking and germination of endophyte-infected *Festuca pratensis* seeds (caryopses) and the tissue distribution of lolines within a caryopsis

| Sample/preparation | Age (days) | Total lolines per individual | | Conc. of lolines (mg/g dry wt) |
|---|---|---|---|---|
| | | $\mu$g | % | |
| Dry caryopsis*† | 0 | 4.17 | 100 | 1.801 |
| Soaked caryopsis | 1 | 2.90 | 70 | 1.253 |
| Soaking water | | 0.91 | 22 | |
| Beginning germination | 2–3 | | | |
| Seedling | | 2.57 | 62 | 1.114 |
| Germination water | | 0.18 | 4 | |
| Progressed germination | 7–10 | | | |
| Seedling (4 cm) | | 3.80 | 91 | 1.777 |
| Dry caryopsis‡ | 0 | 3.98 | | 1.682 |
| Soaked caryopsis§ | 1 | | | |
| Bracts | | 0.03 | | 0.045 |
| Embryo | | 0.29 | | 3.229 |
| Remaining seed | | 2.69 | | 1.680 |
| Total | | 3.01 | | 1.387 |

* cv. 41 (seed harvest 1993, 83% fungal infestation).
† The data given for the germination experiment represent means of 50 individuals analysed in three replicates.
‡ cv. 11 (seed harvest 1995, 89% fungal infestation).
§ Mean values of 50 caryopses analysed.

the bracts (Table 3). Although only 10% of total seed alkaloids are bound to the embryo, it contains a two-fold higher alkaloid concentration than the remaining seed tissues.

Ten individuals of uninfected (i.e. endophyte free) *F. pratensis* were analysed for loline alkaloids. Trace amounts ranging from <0.1 to 3.4 $\mu g\, g^{-1}$ dry weight could be detected employing the current method. These concentrations account for less than 1% of the levels found in corresponding endophyte-infected grass samples.

## DISCUSSION

Five loline alkaloids (Fig. 1) were detected in *A. uncinatum*-infected *Festuca pratensis*. This is comparable to the *A. coenophialum–F. arundinacea* association which, however, lacks **1** [19]. The relative abundance of the alkaloids is similar in the two associations, with **4** being the major alkaloid followed by **5** and **3** and trace amounts of **1** and **2** or just **2**, respectively. In the *F. pratensis*–endophyte association the loline alkaloids are found at almost the same proportions in the various plant organs. In contrast, considerable differences in the quantitative alkaloid composition, especially between seeds and forage samples, were described for the tall fescue association [19].

The quantitative distribution of the loline alkaloids in the different plant tissues seems to be similar in the two endophyte associations. The highest concentrations of loline alkaloids are found in mature seeds (caryopses), the first leaves in early spring and the leaf pseudostems during the period of vegetative regrowth following stalk senescence in late summer. Similar distribution patterns are described for the endophyte–*F. arundinacea* association [9]. Unfortunately, the endophyte infection frequency in the seed material of the cultivar used to establish our experimental paddock decreased considerably over two growing seasons (from 84% of seed harvest in 1993 to 36% in 1995). This change was positively correlated with the loline alkaloid concentrations (i.e. 1.801 $mg\, g^{-1}$ dry weight in 1993 and 0.345 $mg\, g^{-1}$ dry weight in 1995). The decrease in the endophyte infection frequency observed in this cultivar at 10 different locations (M. Pfannmöller and S. Eggestein, personal communication) explains the relatively low alkaloid levels found in our 1995 survey (see Fig. 4). Much higher levels (up to 6.9 $mg\, g^{-1}$ dry weight) have been reported for a different cultivar [9, 17]. As known for the tall fescue–endophyte associations [9, 21] we always found a strict positive correlation between endophyte infection frequency and the amount of loline alkaloids in seeds and forage (Eggestein, Justus, Pfannmöller, Schöberlein and Hartmann, in preparation).

The observation that lolines, although in extremely low concentrations, were always detectable in uninfected individuals of *F. pratensis* may indicate, but not prove, that the alkaloids are produced by the plant upon induction by the fungal endophyte.

The quantitative changes in the loline alkaloid distribution between vegetative and reproductive tissues during flowering and seed maturation suggests long-distance translocation of loline alkaloids from the vegetative plant parts into the developing seed. Specific transport and storage mechanisms are indicated by the high alkaloid concentrations found in the seed tissues (e.g. embryo) (Table 2). The loline alkaloids stored in the seeds appear not to be degraded during seed germination, but about 20% of total lolines are lost by leaching during seed imbibition. Thus, there is no indication for remobilization of the alkaloid bound nitrogen during germination as in other seed storing nitrogenous defence compounds, e.g. cannavanin in certain legumes [22] or PAs in *Crotalaria* [23], despite the relatively high N/C ratio (2/7) of the lolines. Degradation of loline alkaloids may occur during leaf senescence, since the alkaloids almost completely disappear at the end of the growing season (Figs 3 and 4, and Table 3).

## EXPERIMENTAL

*Plant material.* An *A. uncinatum*-infected *F. pratensis* genotype, field grown at defined plots at the Bundesanstalt für Landwirtschaft (FAL; Braunschweig-Völkenrode) was used throughout the field experiments. All samples were taken from a paddock (14 $m^2$) established in August 1994 from 84% infested seeds (10 kg $hectare^{-1}$). Samples were collected at about 2-week intervals (14 April to 26 October 1995); sampling was always carried out at 9.00–10.00 a.m. Random samples of 50 leaves (samplings 1–3) or 30 stalks (samplings 4–9) or 30 leaves (pseudostems + leaf blades) (samplings 10–14) were collected. Vegetatively growing leaves or stalks were cut just above the ground. Stalks were sectioned at the nodes and the successive internodes dissected further into stem, leaf blade and leaf sheath. The inflorescence (panicle) was sectioned into stem parts and spikelets. Root samples were taken at the beginning, middle and end of the growing season. The samples were combined, weighed (fr. wt), lyophilized, weighed again (dry wt), and ground to pass a 1-mm screen. For the germination experiment, sets of 50 caryopses were soaked in water for 24 hr. Subsequently, one set was analysed quantitatively for loline alkaloids (separated in tissue and soaking water). Soaked caryopses of the other sets were transferred to fresh water and analysed at intervals as indicated. For seed tissue preparation, 50 caryopses were soaked in aerated tap water for 24 hr; the caryopses were then separated into bracts, embryos and remaining tissue (seed coat + endosperm).

*Alkaloid extraction.* Ground plant material (0.2–0.3 g dry wt) was extracted twice with 5 ml 0.5 M $H_2SO_4$ for 30 min. After centrifugation the combined supernatants were made alkaline by adding 0.2 ml $NH_4OH$ (25%) and applied to a Extrelut (Merck) column (1 ml aq. soln/g Extrelut). The lolines were eluted with $CH_2Cl_2$ (10 $ml\, g^{-1}$ Extrelut). After evapn of the eluent the

residue was redissolved in 25–150 $\mu$l MeOH. For GC analysis tropinol (0.5–1 mg ml$^{-1}$) was added as internal standard.

*Capillary GC analysis.* Fused silica column (WCOT, 15 m × 0.25 mm; DB-1, J & W Scientific, CA). Conditions: injector 250°; temp. programme 110–300°, 6° min$^{-1}$; split ratio 1:20; injection vol. 1 $\mu$l; carrier gas He 0.75 bar; detectors, dual FID, PND.

*GC–MS analysis.* Carlo Erba 5160 gas chromatograph equipped with a fused silica column (WCOT, 30 m × 0.32 mm; DB-1). Conditions as specified above, but temp. programme 100–300° and carrier gas He 0.5 bar. The capillary column was directly coupled to a quadrupole mass spectrometer Finnigan MAT 4515. EI-mass spectra were recorded at 50 eV.

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