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Note

Analysis of peramine in fungal endophyte-infected grasses by reversed-phase thin-layer chromatography

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The presence of the fungal endophyte *Acremonium lolii* in perennial ryegrass (*Lolium perenne*) has been linked to resistance of infected plants to attack by the Argentine stem weevil (*Listronotus bonariensis*). Production in the infected plants of the alkaloid peramine, a feeding deterrent to adult weevils, is thought to be largely responsible for the resistance[1].

Isolation and identification of peramine was first reported by Rowan and co-workers[1,2], but the method used was too time-consuming and difficult for use in routine analysis. Tapper *et al.*[3] recently reported an improved method for extraction, identification and measurement of peramine. Their procedure involved a two-stage extraction using methanol–chloroform (1:1, v/v); then hexane–water (1:1, v/v) followed by cleanup of the extract on small ion-exchange columns. Peramine was detected on silica gel layers using Ehrlich's reagent or quantified by a high-performance liquid chromatography (HPLC) procedure.

We report here the development of a rapid and sensitive reversed-phase (RP) thin-layer chromatography (TLC) procedure for detection and quantitation of peramine in crude extracts of endophyte-infected grasses.

EXPERIMENTAL

Peramine calibration curve

A solution of peramine bromide (derived from endophyte-infected *L. perenne*) containing 8.5 ng peramine per microliter of 80% aqueous ethanol was used in preparing a calibration curve. Portions of this solution were applied (in duplicate) with a microliter syringe to a Whatman LKC18F reversed-phase plate (10 cm × 16 cm; American Scientific) 2.5 cm from the long edge "A" of the plate. Development of the chromatogram was carried out in two stages. First the plate was immersed with edge A up in 60% aqueous methanol containing 0.1% phosphoric acid adjusted to pH 7.3

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with potassium hydroxide (solvent A) to a depth such that the origin was about 1 cm above the solvent surface. When the solvent reached edge A of the plate the plate was removed and allowed to partially dry. The entire plate was covered with aluminum foil with the exception of two corner areas where the first and last samples of peramine standard had been applied. The exposed areas were sprayed with Van Urk's reagent (1 g *para*-dimethylaminobenzaldehyde in 50 ml concentrated hydrochloric acid plus 50 ml 95% ethanol) and after the color had developed, the foil was removed and a strip of adsorbent approximately 12 mm wide from edge A (approximately 3 mm from edge of spots) was removed. Two strips of adsorbent were also removed at right angles to edge A (first and last lanes which contained the stained peramine spots). The plate was allowed to thoroughly dry and was then developed in 50% methanol containing 0.5 *M* sodium chloride (solvent B) with edge A down until the solvent front had moved approximately 70 to 75 mm. The plate was allowed to partially dry and then sprayed with Van Urk's reagent. After thorough drying, the plate was scanned (could be stored in the freezer overnight) with a Shimadzu High-Speed Thin Layer Chromato Scanner Model CS-920 at 600 nm (reflectance mode). The R_F of peramine was approximately 0.6.

The external standard used in assays of extracts of grasses was purified from endophyte-infected ryegrass according to the methods of Rowan and Gaynor[1].

Extraction

Endophyte-infected grass leaf tissue, either dried at 60°C or freeze-dried, was ground in a Wiley mill to pass through a 40-mesh screen. To each of 3 tubes, 50 mg of dried ground leaves from endophyte-infected perennial ryegrass were added. Aliquots of 2.5 ml aqueous ethanol (80, 90 or 95% ethanol) were added and the tubes were capped and shaken at room temperature for 30 min. The solids were allowed to settle and 2 ml of extract were removed from each tube. To each tube containing the solids were added 2.5 ml 80% ethanol and the tubes were stored at −20°C overnight. The tubes were allowed to warm to room temperature with shaking and the solids were again allowed to settle and supernatant liquid removed. Portions of 0.5 ml of the first and second supernatants from each sample were dried with a stream of nitrogen and the residue was redissolved in 50 μl of 80% ethanol.

"Two-directional" reversed-phase TLC for extracts

Portions of 10 μl of each sample, two peramine standard samples (72 ng/spot), plus peramine in the outside lanes to determine distance for adsorbent removal after development in the first direction, were applied 2.5 cm from edge A of the RP-TLC plate and allowed to dry. Development of the chromatogram was then carried out in two stages as described for the peramine calibration curve. The purpose of the first stage was to remove materials which interfered with peramine measurement. The peramine concentration in samples was quantified by single point calibration using the peramine standards applied to the same plate.

RESULTS AND DISCUSSION

Calibration curve

The area of the stained peramine spots was not a linear function of the amount

of peramine applied to the TLC plate. However, a linear standard curve was obtained if the "linearizer" function of the scanner (position 1) was used. Absorbance measurements on thin-layer materials tend to deviate from the Beer-Lambert Law due to scattering by the adsorbent on the plate. The apparent non-linear relationship between absorbance and concentration is linearized by a microcomputer program in the Model CS-920 scanner[4]. The computer-corrected integration of absorbance (area) shows a linear dependence on weight of peramine in the range of 8.5 ng to 68 ng/spot with a regression equation of $y = 14x$ ($R^2 = 0.9946$; $P = 0.0000$). The detection limit for peramine with the TLC scanner was about 5 ng/spot though smaller quantities could be detected visually.

Extraction of peramine

In the original publication on the isolation of peramine from endophyte-infected perennial ryegrass, Rowan and Gaynor[1] used 95% ethanol for extraction. Some preliminary results of our work (data not shown) suggested that 95% ethanol did not completely extract peramine from freeze-dried ryegrass. Nearly complete peramine extraction (>90%) was possible with either 80% or 90% ethanol, but not with 95% ethanol. For example, when 2.5 ml of 80, 90 or 95% ethanol were used to extract 50 mg samples of infected ryegrass, the recovered alcoholic solvent (2 ml) contained 1201 ng, 1288 ng and 690 ng, respectively. Following a second extraction of each sample residue with 2.5 ml of 80% ethanol, the solvent contained 682 ng peramine when the first extractant was 95% ethanol but only 183 ng and 312 ng if the first extractant was 80% and 90% ethanol, respectively. These results indicate that the first extraction with either 80% or 90% ethanol released essentially all of the peramine in the sample. The second extraction recovered most of the peramine remaining in the void volume of the pelleted material. Using 95% ethanol as the extractant, only about 60% of the peramine was removed with the first solvent extraction. Our routine assay now involves shaking a 150 mg sample with 1.2 ml 80% ethanol for 1 h.

Peramine levels of 8 ppm or greater in tissue of fungal endophyte-infected grass could be measured in crude extracts. If the crude extracts were concentrated 5-fold, 1 ppm peramine on a dry-weight basis could be determined. Isolated peramine was confirmed by NMR and mass spectroscopy by procedures used by Rowan and Gaynor[1].

The use of the first chromatography step in the two-stage TLC procedure is a convenient alternative to preliminary cleanup of the crude extracts prior to TLC. It is especially important for eliminating materials that interfere with resolution and measurement in extracts containing small amounts of peramine. If the first step is not used, the peramine spot tends to become distorted and excessive streaking occurs (especially with highly concentrated extracts) which tends to obscure the peramine after treatment with Van Urk's reagent.

Recovery of peramine standard added to uninfected ryegrass

Purified peramine in 80% ethanol was added to a powdered sample of uninfected ryegrass to give a peramine concentration of 20 μg/g. The mixture was dried, extracted for 1 h at room temperature with 80% ethanol, and then analyzed by our RP-TLC method. Extraction of peramine was essentially complete as recovery values of 94% and 104% were found for two TLC assays on the same extract.

TABLE I

COMPARISON OF PERAMINE LEVELS DETECTED BY RP-HPLC AND RP-TLC METHODS

Grass species and cultivar	*Endophyte*	*Peramine (ppm)*[a]	
		RP-HPLC	*RP-TLC*
Lolium perenne Gator	*Epichloe typhina*[d]	<0.6	n.d.[b]
L. perenne Gator	*E. typhina*[e]	10	16.2
L. perenne Repel	*Acremonium lolii*	29.9	39.8 ± 2.2(3)[c]
Festuca longifolia	*E. typhina*	15.0	20.3 ± 1.7(12)[c]

[a] RP-HPLC analyses carried out at DSIR in New Zealand and RP-TLC analyses carried out at the University of Kentucky.

[b] Not detected.

[c] Mean ± standard error; number in parentheses indicates number of replicate determinations.

[d,e] *E. typhina* from *Festuca rubra commutata* (d), and from *F. longifolia* (e), respectively, were artificially transferred into *Lolium* seedlings by the method of Latch and Christensen[6].

In another test, a powdered sample of infected ryegrass containing 20 μg/g peramine was mixed with powdered non-infected material in a ratio of one part infected ryegrass to nine parts non-infected grass. A value of 2.0 ppm in the mixture was determined by our extraction and RP-TLC quantitation procedure.

Analysis of peramine from endophyte-infected grasses

Analysis of samples that were freeze-dried, ground and then split for analysis by both the RP-HPLC method of Tapper *et al.*[3] and by our RP-TLC method indicated that the two methods were of similar sensitivity but amounts determined with the RP-HPLC method were about 25–30% lower than those for the RP-TLC method (Table I). The reason for these differences is unknown.

The distribution of peramine between roots, stems and blades of some endophyte-infected grasses were checked by the RP-TLC method (Table II). While the

TABLE II

PERAMINE DISTRIBUTION IN ENDOPHYTE-INFECTED TALL FESCUE AND PERENNIAL RYEGRASS

Grass (species)	*Plant part*	*Peramine (ppm), mean ± standard error (n)*
F. arundinacea[a]	Root	n.d.[c]
	Stem	3.4 ± 0.4(3)
	Blade	3.1 ± 0.4(3)
L. perenne[b]	Root	3.7(1)
	Stem	10.2 ± 1.3(3)
	Blade	28.4 ± 2.3(3)

[a] G1-320 tall fescue artificially infected with *Acremonium coenophialum*.

[b] NK 79307 perennial ryegrass (Northrup King) naturally infected with *A. lolii*.

[c] Not detected.

different pattern of peramine distribution between the two endophyte–host combinations may reflect the differences in endophyte distribution within the two different hosts, it also suggests a relatively greater translocation of peramine to the leaf blade in *L. perenne* compared to *F. arundinacea*. The endophyte is not found in roots and lesser amounts of endophyte are found in leaf blade than in stem of both hosts[5].

Using the RP-TLC method, we determined the level of peramine from a number of different endophyte–host combinations. As indicated in Tables I and II, peramine is produced not only in *A. lolii*-infected perennial ryegrass[1], but also in *E. thypina*-infected perennial ryegrass (Table I) and in *A. coenophialum*-infected tall fescue (Table II). The *E. typhina* in infected perennial ryegrass originated from two different species of *Festuca* and were artificially inoculated into *L. perenne* cv. Gator. Rowan *et al.*[2] have shown that peramine is of fungal origin, and as expected, the *E. typhina* from *F. longifolia* produced similar levels in its own host (*F. longifolia*) and in *L. perenne* cv. Gator. On the other hand, the *E. typhina* isolate from *F. rubra*, when transferred to Gator, did not produce appreciable amounts of peramine.

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