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DETECTION AND MEASUREMENT OF THE ALKALOID PERAMINE IN ENDOPHYTE-INFECTED GRASSES

B. A. TAPPER* and D. D. ROWAN
Biotechnology Division, Department of Scientific and Industrial Research, Private Bag, Palmerston North (New Zealand)
and
G. C. M. LATCH
Plant Diseases Division, Department of Scientific and Industrial Research, Private Bag, Palmerston North (New Zealand)
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SUMMARY

Two methods are described for the detection and measurement of peramine, an alkaloid from endophyte-infected grasses with insect feeding deterrent activity. Both procedures involve ion-exchange work up of extracts followed by either thin-layer chromatography with detection by Ehrlich's reagent or quantitation by reversed-phase high-performance liquid chromatography with UV detection at 280 nm.

INTRODUCTION

Perennial ryegrass (*Lolium perenne*) infected with the endophytic fungus *Acremonium lolii* has field resistance to infestation by the Argentine stem weevil (*Listronotus bonariensis*)[1,2]. A major factor in this resistance is the production of a chemical deterrent to feeding of adult weevils. The chemical basis of the feeding deterrence has been examined by solvent extraction and fractionation procedures in conjunction with a bioassay test for activity[3]. The predominant activity was attributed to a single compound, peramine[4], the structure of which is shown in Fig. 1. Recent studies[5] have demonstrated the presence of peramine in some other endophyte-grass combinations.

Endophyte-infected perennial ryegrass also contains the neurotoxin lolitrem B and related compounds which may cause the "ryegrass staggers" disorder in grazing animals[6].

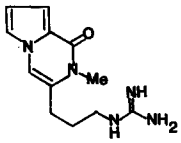

Fig. 1. Structure of peramine. Me = methyl.

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A method for measuring peramine in small samples of ryegrass herbage was required for studies on variation in peramine content of a range of endophyte-grass combinations. A target for accuracy of ± 10% was considered sufficient provided the method was relatively simple and convenient. The peramine isolation method of Rowan and Gaynor[3] involved solvent partitioning, followed by high-performance liquid chromatography (HPLC) using sodium *n*-heptanesulfonate as a reversed-phase modifier in a gradient elution system. This was considered to be unsuitable for routine analytical measurements.

We report herein on convenient methods for the detection and measurement of peramine, based on knowledge of its chemical structure and properties. Ion-exchange methods of purification were chosen because of the relatively strong basic character of the guanidino group of the peramine molecule. A two-phase extraction process permits a parallel determination of lolitrem B by minor modifications of the method of Gallagher *et al.*[7].

EXPERIMENTAL

Plant material

For method development plants of perennial ryegrass either infected with, or free from, *A. lolii* were grown in pots containing composted bark in a greenhouse (12–24°C) and harvested when 20–30 cm tall. Herbage was cut from plants at the crown and in some cases was divided into top and basal portions with a further cut 8–10 cm from the crown. Herbage from Italian ryegrass (*L. multiflorum*), free from endophyte and peramine, was used in tests measuring the recovery through extraction and work up of added peramine. All herbage samples were freeze dried, fine ground in a coffee mill and stored at 4°C.

Standard peramine

Crystalline peramine sulphate and peramine bromide were obtained from ryegrass seed[8] and solutions in 20% (v/v) aq. propan-2-ol were calibrated based on an extinction coefficient for peramine at 290 nm of 8300 l mol^{-1} cm^{-1}.

Extraction of plant material

Weighed samples, typically 100 mg, of dry ground herbage were extracted in two stages into a two-phase solvent system. The first stage of the extraction was with 3 ml of methanol–chloroform (1:1, v/v) for 30 min at about 18°C in polyethylene-capped glass vials with mixing by continuous gentle inversion. For the second stage 3 ml of hexane and 3 ml of water were added for a further 30 min of mixing before centrifugation to separate the two phases and sediment the plant residues. The two solvent phases were each of about 4.5 ml in volume.

TLC detection of peramine

A 3-ml volume of the aqueous lower phase extract was aspirated through small tandem ion-exchange columns of (i) 0.5 ml bed of BioRad AG2 × 8 (200–400 mesh) (hydroxide form) approximately 9 × 8 mm, followed by (ii) an Analytichem Bond Elut CBA column, 100 mg absorbent, in the free acid form. The columns were washed with 3 ml of 80% (v/v) methanol, separated, and the peramine eluted from the Bond

Elut CBA with 0.5 ml of 80% aq. methanol containing 5% (v/v) formic acid. The eluted sample was concentrated to dryness under a stream of nitrogen and redissolved in 50 or 100 μl of 80% aq. methanol.

Small volumes (5–20 μl) of the concentrated extracts were spotted on to Merck silica gel 60 aluminum-foil-supported layers and chromatograms developed with a solvent of chloroform–methanol–acetic acid–water (20:10:1:1) until the solvent front advanced 6–10 cm. The chromatograms were dried before dipping very briefly in an Ehrlich's reagent consisting of nine parts of 1% (w/v) *p*-dimethylaminobenzaldehyde dissolved in acetone and one part of aqueous 6 *M* hydrochloric acid mixed immediately before use. Peramine, with an R_F of 0.43, developed as a purple-blue spot on standing or with gentle warming for a few minutes. Alternative spray versions of Ehrlich's reagent were also used successfully.

HPLC measurement of peramine

A 1-ml portion of the lower aqueous phase of the extract was passed through a single Analytichem Bond Elut CBA column, 100 mg absorbent, in the ammonium ion form. The sample was washed through with 1 ml of water and the bound peramine eluted with 0.5 ml of 5% (v/v) aqueous formic acid. Gentle centrifugation was used at each step. The net weight of the solution and an estimate of its density was used to calculate the eluted volume of peramine containing solution.

The efficiency of the extraction of peramine from herbage and of the subsequent work up by ion exchange was tested by adding peramine standard to dry herbage samples, removing the solvent under vacuum, and then extracting the sample for 30 min for each stage.

Peramine was measured by reversed-phase HPLC using a Waters Assoc. Liquid Chromatograph fitted with a Waters Radial-Pak Resolve C_{18} column, 5 μm particle size, 100 × 8 mm. Detection was with a fixed-wavelength UV detector at 280 nm, close to a peak of peramine UV absorption. The isocratic mobile phase was 33% aq. acetonitrile containing guanidinium formate and excess formic acid to give a solution of approximately pH 3.7. This buffer was prepared by dissolving guanidinium carbonate (1.44 g/l) in water, adding reagent grade (98–100%) formic acid (1.6 ml/l), chromatography grade acetonitrile (330 ml/l), and making up to volume before degassing. The flow-rate was 1 ml/min.

The quantity of peramine in 50-μl injection samples was estimated by comparisons of peak heights with those from standard solutions of peramine bromide. Peramine eluted at about 7 min after injection and was often followed by some other unidentified UV absorbing substances. Each HPLC analysis could be completed in approximately 12 min.

The efficiency of extraction of peramine from herbage and subsequent work up by ion exchange was tested by adding small standard amounts of peramine to dry herbage samples and then extracting with the two-phase solvent system.

RESULTS AND DISCUSSION

Thin-layer chromatographic detection of peramine

The tandem ion-exchange step offered convenient purification and concentration of peramine from extracts into a solution with relatively low salt content. The

procedure for detecting peramine by thin-layer chromatography (TLC) is a refinement of the methods used by Rowan and Gaynor[3]. The addition of small amounts of water and acetic acid to the TLC solvent improved reproducibility of the peramine R_F at 0.43, while the tandem ion-exchange work up was effective in removing other compounds in the extracts which react with Ehrlich's reagent. The detection limit was approximately 10 ng of peramine per spot. The intensity of colour reaction may be used to indicate a broadly high or low concentration of peramine in the samples.

Measurement of peramine

The two-phase extraction of peramine from freeze dried herbage samples was adopted after preliminary tests indicated that, as a single cycle of extraction, it was more efficient and convenient than extraction with single phases of aqueous ethanol, aqueous methanol, or chloroform–methanol followed by separate partitioning steps. When using 3 ml of chloroform–methanol (1:1, v/v) and subsequently adding 3 ml each of hexane and water the final extract partitioned into very nearly equal volumes of lipophilic top phase and aqueous bottom phase. By repartitioning portions of the top phase it was shown that negligible amounts of peramine were in the top phase of the extracts. Two notable advantages were (i) the simple removal of lipids and pigments from the peramine extract and (ii) the use of the lipophilic top phase for measurement of lolitrem B by a modification of the method of Gallagher *et al.*[6].

A single step extraction under mild conditions cannot be expected to be complete, therefore reproducible extraction efficiency must be sought and quantified by standard addition methods.

Variation of extraction times (Table I) for each of the two stages of the extraction demonstrated that doubling the times from the standard 30 min did not significantly enhance extraction while a shorter 10-min first stage was less efficient. The two 30-min extraction periods were adopted as convenient and near optimum times.

The overall recovery measured by standard additions was 79% with standard deviation of 2–4%. The degree of extraction of the added peramine was shown to be similar to that of naturally occurring peramine by reextracting the plant residue for 30 min with 50% aq. ethanol and showing that the residual peramine was extracted in proportion regardless of whether or not peramine had been added. The range of

TABLE I

EFFECT OF VARIATION OF EXTRACTION TIMES RELATIVE TO THE STANDARD PROCEDURE OF 30 min FOR EACH STAGE OF EXTRACTION

1st stage of extraction (min)	*2nd stage of extraction (min)*	*Relative extraction efficiency (%)*	*S.D. (%)*
10	30	93.7	1.3*
30	30	100	1.8*
60	30	101.1	2.6*
30	10	98.0	1.6*
30	60	98.8	2.3**

* $n = 4$.
** $n = 3$.

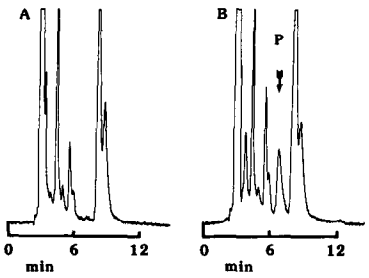

Fig. 2. Typical chromatograms of extracts from ryegrass. (A) endophyte-free herbage, (B) endophyte-containing herbage with peramine, P.

response of the overall extraction and HPLC analysis was demonstrated with standard additions up to the equivalent of 75 ppm of peramine as the free base in dry plant material. The response was shown to be linear by regression analysis through the origin with a standard error of 4.7%. There was no indication of lack of linearity at concentrations higher than the equivalent of 75 ppm. The lower limit for routine detection was approximately 1 ppm.

The isocratic HPLC solvent of 33% (v/v) aq. acetonitrile, 8 m*M* guanidinium formate with excess formic acid was adopted after trials with solvents containing methanol, propan-2-ol and various other salts at various concentrations. Guanidinium salts reduced the tailing of the peramine peak in comparison with sodium, ammonium, or trimethylammonium acetates. Acetonitrile was preferred for the longer retention of peramine on the column relative to other compounds from the extracts with UV absorption. Typical elution patterns are shown in Fig. 2. In as far as no peak was observed at the normal peramine elution time for samples from endophyte-free grasses there was no interference in the HPLC assay. The typical time for each peramine measurement of 12 min compares favorably with the 27 min elution time for peramine in the gradient system of Rowan and Gaynor[3].

Peramine measurements have been made on a range of grasses. No peramine was detected in endophyte-free plants and up to 47 mg peramine/kg dry weight of whole plant herbage was found in plants of perennial ryegrass infected with *A. lolii*. Where plants were divided into top and basal portions the latter contained relatively higher concentrations of peramine (Table II). The age of plants and growth condi-

TABLE II

TYPICAL RESULTS OF PERAMINE MEASUREMENT OF ENDOPHYTE-INFECTED *L. Perenne* HERBAGE

Cultivar (clone)	*Part of plant*	*Peramine (ppm)*
Grasslands Nui (1)	Basal	25
Grasslands Nui (1)	Upper	16
Grasslands Nui (3)	Basal	15
Romanian selection	Basal	9
Romanian selection	Upper	5
Regal (5)	Basal	19
Regal (7)	Basal	32

tions affected peramine concentration. These biological factors and their effect on peramine production will be reported elsewhere. The natural variation from a variety of biological and environmental factors was considerably greater than the variation in results attributable to the analysis method.

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