

## Mycotoxins and Other Secondary Metabolites Produced in Vitro by *Penicillium paneum* Frisvad and *Penicillium roqueforti* Thom Isolated from Baled Grass Silage in Ireland

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Secondary metabolites produced by *Penicillium paneum* and *Penicillium roqueforti* from baled grass silage were analyzed. A total of 157 isolates were investigated, comprising 78 *P. paneum* and 79 *P. roqueforti* isolates randomly selected from more than 900 colonies cultured from bales. The findings mostly agreed with the literature, although some metabolites were not consistently produced by either fungus. Roquefortine C, marcfortine A, and andrastin A were consistently produced, whereas PR toxin and patulin were not. Five silage samples were screened for fungal metabolites, with two visually moldy samples containing up to 20 mg/kg of roquefortine C, mycophenolic acid, and andrastin A along with minor quantities (0.1–5 mg/kg) of roquefortines A, B, and D, festuclavine, marcfortine A, and agroclavine. Three visually nonmoldy samples contained low amounts of mycophenolic acid and andrastin A. The ability of both molds to produce a diverse range of secondary metabolites in vitro and in silage should be a concern to livestock producers.

**KEYWORDS:** *Penicillium paneum*; *Penicillium roqueforti*; baled grass silage; secondary metabolites; patulin; roquefortine C; mycophenolic acid; PR toxin; andrastin A

### INTRODUCTION

Grass harvested using the baled silage system accounts for approximately 30% of silage fed annually to livestock in Ireland (1). Recent surveys to establish the incidence of fungal growth on baled grass silage in Ireland have shown that up to 90% of bales examined had visible fungal growth present (2–4). Fungal growth on silage reduces its nutritional value (5) and may result in the growth of other secondary allergenic, toxicogenic, and pathogenic microorganisms (6, 7). Fungi of the genera *Aspergillus*, *Penicillium*, and *Monascus* are frequent contaminants of silage (2, 8, 9), with *Penicillium roqueforti* (*sensu lato*) being one of the most common spoilage molds (2, 9–11).

*P. roqueforti* (*sensu lato*; subgenus *Penicillium*) is now considered to consist of three different species, *P. roqueforti* Thom, *P. paneum* Frisvad, and *P. carneum* Frisvad, on the basis of ribosomal and  $\beta$ -tubulin DNA sequence comparisons, random amplified polymorphic DNA (RAPD) profiles, and secondary metabolite profiles (11–13). *Penicillium* species isolated from

baled grass silage in Ireland were almost exclusively represented by *P. roqueforti* and *P. paneum*, and in one study they represented 52 and 4.5%, respectively, of all fungal isolates detected (2). To date, there have been only a few reports of *P. paneum* occurring on silage (14, 15) and on feed grain stored under low-oxygen conditions (16), originally misidentified as *P. roqueforti* (11). *P. carneum* is associated with meat products such as sausages, as well as cheese, bread, and barley (17) and perhaps silage (18). *P. roqueforti* is common on substrates with high levels of organic acids, high concentrations of carbon dioxide, and low levels of oxygen (18); accordingly, silage provides a very favorable substratum for growth of this mold.

The secondary metabolite profiles of *P. roqueforti*, *P. paneum*, and *P. carneum* differ. All three produce roquefortine C and andrastin A, whereas *P. roqueforti* and *P. carneum* produce mycophenolic acid and both *P. carneum* and *P. paneum* produce patulin. *P. paneum* produces marcfortines and perhaps botryodiplodin, whereas *P. carneum* produces penitrems and *P. roqueforti* PR toxin (Figure 1) (19). The mechanisms by which these mycotoxins could adversely affect the health of livestock are not fully understood. Patulin is known to be antibacterial, cytotoxic, and perhaps even carcinogenic (19, 20). PR toxin inhibits protein and RNA synthesis (21, 22), is mutagenic in the *Salmonella typhimurium* assay (23), and is

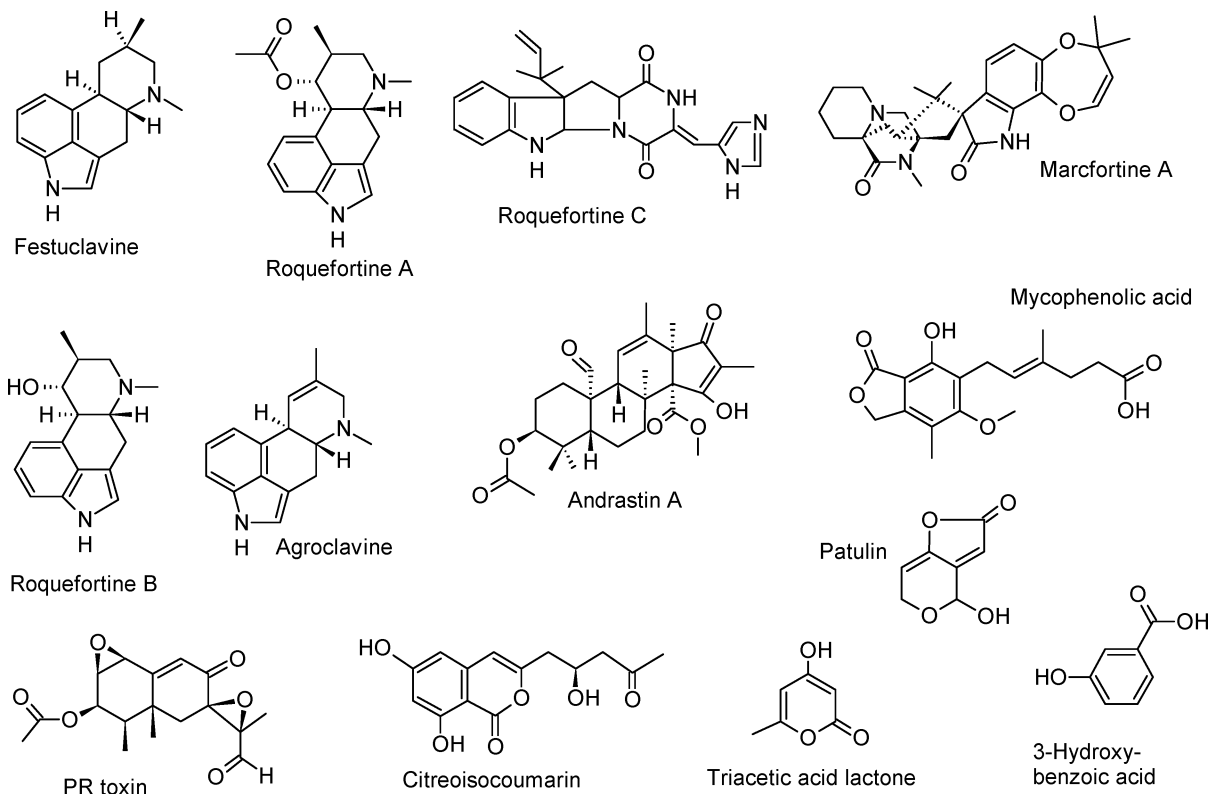
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**Figure 1.** Structures of selected described metabolites from *P. roqueforti* and *P. paneum* in this and other studies.

carcinogenic in rats (24). Mycophenolic acid is antibacterial and immunosuppressive in higher animals (25). In a feeding experiment with sheep, no roquefortine toxicity was revealed (26); however, others have shown it to be antibacterial (27), and in a more recent study, it (and mycophenolic acid) has been shown to be highly toxic when inhaled by mice (28). The toxicological properties of andrastin A, previously “metabolite A” (12), are unknown, but it is present in all blue cheeses (29) and has been considered for use as an anticancer agent (30).

Acute toxic syndromes and even fatal poisoning of unknown etiology have been observed in livestock that have consumed molded silage (31–33). With improved methods for the detection of mycotoxins in feed, it is becoming apparent that mycotoxins are regularly formed in silage (10, 34). Roquefortine C and mycophenolic acid are the two most frequently detected mycotoxins produced by the *P. roqueforti* group in silage (10, 35), whereas PR toxin and patulin have been detected only occasionally owing to their unstable nature in this substratum (9, 36).

Previous studies have screened *Penicillia* from a wide variety of substrata for secondary metabolite production, but no study to date has explored the consistency of secondary metabolite production by a large number of *Penicillium* isolates from grass silage. There is also a gap in our knowledge as to the types of secondary fungal metabolites commonly present in mold-contaminated silage. This study aimed to characterize the secondary metabolite profiles of *P. roqueforti* and *P. paneum* isolated from baled grass silage in Ireland, to induce *P. paneum* isolates to produce patulin by supplementing the growth medium with trace metals, for example, manganese, and to assess the ability of these *P. paneum* isolates to produce patulin after storage for 30 weeks. Another objective was to screen visually moldy and visually nonmoldy grass silage samples for secondary fungal metabolites.

## MATERIALS AND METHODS

**Sample Collection and Isolate Selection.** The incidence of fungal growth on baled grass silage ( $n = 464$  bales) on Irish farms ( $n = 235$  farms) was recorded in three separate studies undertaken in March 2003 (2), from November 2003 to March 2004 (3), and in February 2004 (4). A total of 2277 visible fungal colonies were enumerated on these bales, and 1190 fungal colonies were sampled and later identified following an established protocol (2). Of this total, 830 were identified as *P. roqueforti* and 78 as *P. paneum* by their macro- and micromorphology features, using appropriate identification keys (18, 37). Approximately 10% of the *P. roqueforti* isolates were chosen with the random function in Microsoft Office Excel, and these selected isolates ( $n = 79$ ) were screened for their secondary metabolites. All 78 *P. paneum* isolates were screened for secondary metabolite production. The isolates selected, of both species, were sourced from 102 bales on 77 farms. Isolates were maintained throughout the study on malt extract agar (MEA) plates (Oxoid, Basingstoke, U.K.) at 2–4 °C in darkness and were subcultured no more than six times prior to secondary metabolite analyses.

In a separate study, silage samples from three bales sourced from different farms (one bale per farm) were collected. A visually moldy and a visually nonmoldy sample were collected from two of the bales (i.e., two samples per bale), and a fifth sample was taken from a third bale free of all visible mold growth on its surface. A representative silage grab sample (100 g) was collected in each case. The visible mold on the surface of two of these bales was recognized as *Penicillium* spp., and a small sample of mold was collected from each bale for species identification. Silage samples were stored at ca. 4 °C while in transit and then at –18 °C until required for secondary metabolite analysis. Five grab samples of silage were taken from nonmoldy parts of each of these bales and assayed for dry matter (DM) concentration by drying (85 °C for 16 h) in a forced-air oven.

**Growth Media and Conditions.** *Screening Penicillium Isolates for Secondary Metabolites.* *Penicillium* isolates were three-point-inoculated onto yeast extract sucrose (YES) agar (18) and Czapek yeast autolysate agar (CYA) (17) and incubated for 7 days at 25 °C in darkness. The yeast extract in both media was purchased from Difco (Becton Dickinson and Co., Franklin Lakes, NJ). To induce patulin production

in *P. paneum*, isolates were three-point-inoculated onto potato dextrose agar (PDA) (Oxoid) and incubated for 7 days at 25 °C in darkness.

**Inducing *P. paneum* Isolates To Produce Patulin.** PDA medium supplied by Difco (Becton Dickinson and Co.) was supplemented with 0.003%  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  (38), 0.001%  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , and 0.0005%  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ . Forty-two *P. paneum* isolates in which patulin was not detected when grown on CYA, YES, and Oxoid PDA were inoculated onto the medium as outlined above, incubated for 7 days at 25 °C in darkness, and rescreened for their ability to produce patulin.

**Assessing the Ability of *P. paneum* Isolates To Produce Patulin after a Prolonged Storage Period.** Eleven isolates identified as producers of patulin were rescreened for their ability to produce patulin after 30 weeks of storage on MEA at 2–4 °C. These isolates were inoculated onto PDA (Oxoid) as outlined above and incubated for 7 days at 25 °C in darkness before being analyzed for patulin production.

**Secondary Metabolite Extraction from Cultures.** Extraction of secondary metabolites was based on a standard method for cultures grown on solid medium (39). Five agar plugs were removed from a *Penicillium* colony on CYA and likewise from a YES plate and additionally from PDA for *P. paneum* isolates. Plugs were cut with a stainless steel corer (diameter = 6 mm) under aseptic conditions and collectively transferred to a 4-mL glass vial (10–15 agar plugs per vial for each isolate). To each vial was added 1.8 mL of solvent (ethyl acetate/dichloromethane/methanol, 3:2:1, v/v/v, + 1% formic acid) (39), and the mixture was left overnight. All solvents were of HPLC grade, and formic acid was of analytical grade. The liquid fraction was decanted into clean 4-mL glass vials and evaporated in vacuo in a vacuum centrifuge at 1 mbar for ~35 min or by passing pure nitrogen over the solvent using a drying block set to 35 °C. The samples were then redissolved in 500  $\mu\text{L}$  of methanol (HPLC grade) and kept for 1–2 h with intermittent shaking, before filtering through a 0.45- $\mu\text{m}$  Teflon syringe filter (SRI, Eatontown, NJ) into a 2-mL vial before HPLC analysis.

**Secondary Metabolite Extraction from Silage Samples.** One gram samples were transferred to 16-mL extraction vials and extracted using 10 mL of ethyl acetate for ~3 h in a shaker at ~200 rpm and subsequently filtered through a Whatman (Brentford, U.K.) PS1 phase separation filter. The ethyl acetate was then evaporated in vacuo, reconstituted in 3  $\times$  1.0 mL of water containing 0.5% formic acid, and loaded onto a 60 mg Strata-X module (Phenomenex, Torrance, CA). The solid-phase extraction (SPE) cartridge had previously been conditioned with 2 mL of methanol and 2 mL of water containing 0.5% formic acid. After sample loading, the cartridge was washed with 2 mL of water containing 0.5% formic acid and eluted with 80% acetonitrile/water containing 0.2% formic acid, and the eluate was evaporated in vacuo. The sample was redissolved in 2  $\times$  100  $\mu\text{L}$  of methanol and filtered through a 0.45- $\mu\text{m}$  PFTE syringe filter (Chromacol, Herts, U.K.) into a 2-mL vial. Fortified samples were prepared (in duplicate) by the addition of 100  $\mu\text{L}$  of ethyl acetate mixture of penicillic acid, mycophenolic acid, roquefortine C, and patulin in levels of 60, 15, and 2  $\mu\text{g/g}$ .

**HPLC Analysis.** Culture extracts were analyzed on an Agilent (Torrance, CA) 1100 liquid chromatographic system equipped with a photodiode array detector (DAD), a fluorescence detector, and a 100  $\times$  2 mm i.d., 3  $\mu\text{m}$ , Luna C<sub>18</sub> II column (Phenomenex) fitted with a Phenomenex SecurityGuard C<sub>18</sub> precolumn. Two different water/acetonitrile systems were used at a flow rate of 0.4 mL/min. The first (standard system) started at 15%  $\text{CH}_3\text{CN}$ , which was increased to 100% over 20 min and then held at 100% for 5 min. The second system started at 5% acetonitrile for 3 min before increasing to 100% over 17 min and then holding at 100% for 5 min. Sample volumes of 1–5  $\mu\text{L}$  were injected onto the column.

**HPLC-MS Analysis.** Silage samples as well as representative culture extracts were also analyzed by liquid chromatography–electrospray high-resolution mass spectrometry, using a Micromass LCT orthogonal time of flight mass spectrometer (Micromass, Manchester, U.K.) equipped with a Z-spray electrospray source and a LockSpray probe. The LC system was the same as described above except that the column was 50 mm long and a fluorescence detector was not used. Samples were analyzed in both negative and positive electrospray modes with gradient systems started at 5 and/or 15% acetonitrile (11, 40).

**Analysis of HPLC and HPLC-MS Data.** Peaks, excluding those found in noninfected silage samples or from blank agar plates, were matched against an internal reference standard database (~630 compounds) (41), and occasionally some of the following metabolites were included in the same sequence as the samples: cyclopiiazonic acid; cyclopaldic acid; gentisyl alcohol; mycophenolic acid; orsellinic acid; patulin; penicillic acid; penitrem A; PR toxin; roquefortines A–D; agroclavine I; festuclavine; secoclavine; citreoisocoumarin; marfortines A and B; 6-methylsalicylic acid; salicylic acid; 2,5-dihydroxybenzoic acid; 2,4-dihydroxybenzoic acid; andrastins A and E; linolenic acid; 3-hydroxybenzoic acid; bis(dethio)bis(methylthio)gliotoxin; gliotoxin; and 4-methylsalicylic acid.

## RESULTS AND DISCUSSION

***P. roqueforti* and *P. paneum* Secondary Metabolites in Culture.** The range of secondary metabolites detected in both *P. roqueforti* and *P. paneum* in this study (Tables 1 and 2, respectively) was broadly in agreement with the findings of previous studies (11, 12, 19), but not all metabolites were consistently produced (Table 3). Figures 2 and 3 show HPLC-UV chromatograms of typical *P. roqueforti* and *P. paneum* extracts, respectively, with the predominant peaks identified. Approximately 90% of *P. roqueforti* isolates were consistent producers of roquefortine C and andrastin A, but varied greatly in their ability to produce roquefortine A, citreoisocoumarin, andrastin C, PR toxin, eremofortin C, and an unidentified metabolite. This unidentified metabolite is probably an analogue of scytalone or hydroxymellein and had a molecular composition of  $\text{C}_{10}\text{H}_{10}\text{O}_4$  (from ESI<sup>-</sup> LC-MS, mass deviation <5 ppm). UV absorptions were 216 nm (100%), 260 nm (62%), and 334 nm (21%). Patulin, marfortine, gentisic acid, and botryodiploidin production by *P. roqueforti* were not detected. The majority of *P. paneum* isolates were consistent producers of andrastin A, citreoisocoumarin, marfortines, and roquefortine C but were not consistent in their ability to produce roquefortine A, andrastin C, gentisic acid, and patulin (Table 3). Lack of consistency in metabolite production was also observed in isolates collected from the same silage bale (e.g., isolates WH121E–WH121M in Table 2). Nonproduction of patulin by *P. paneum* has been reported previously (11); however, in this study, the *P. paneum* isolates that did not produce patulin produced 6-methylsalicylic acid, which is a patulin precursor. It is not known if these isolates are unable to synthesize patulin or in certain circumstances shunt precursors toward the synthesis of other metabolites. Roquefortine C and andrastin A were the two most consistently produced metabolites by both species, and in the case of roquefortine C, *P. roqueforti* typically produced 5–20-fold more than *P. paneum*. The ratio of mycophenolic acid and PR toxin production was found to vary greatly among *P. roqueforti* isolates; the mean ratio was ca. 50:1 (range from <0.01:1 to 424:1), with more mycophenolic acid produced than PR toxin in most cases (Table 1). *P. paneum* did not produce detectable amounts of mycophenolic acid, PR toxin, botryodiploidin, metabolite T, and eremofortine. A report on botryodiploidin production by *P. paneum* (42), identified originally as *P. roqueforti* (11), could not be confirmed in this study, but perhaps with different cultural conditions it may have been possible to induce its production.

**Factors Affecting Patulin Production. Trace Metal Enriched Difco PDA.** Production of secondary metabolites by *Penicillium* species is generally favored on CYA and YES media (43), but there are exceptions. *P. paneum*, for example, will produce patulin only when grown on PDA and occasionally on YES (11). There is also anecdotal evidence to suggest that Difco PDA is better for patulin production than Oxoid PDA. When

**Table 1.** Secondary Metabolites Produced in Culture by *P. roqueforti* ( $n = 79$  Isolates) Isolated from Baled Grass Silage in Ireland

isolate <sup>a,c</sup>	IBT no. <sup>d</sup>	RA <sup>f</sup>	RC	CIC	AA	AX	SC	EC	MPA	PR	MPA/PR ratio <sup>e</sup>	isolate <sup>a,c</sup>	IBT no. <sup>d</sup>	RA <sup>f</sup>	RC	CIC	AA	AX	SC	EC	MPA	PR	MPA/PR ratio <sup>e</sup>
CN100E	25410	+	+		+			+	+	+	2.70	MH02D	25374	+	+	+	+		+	+	+	+	2.23
CN102A	25416	+	+		+		+	+	+		na	MH02F <sup>b</sup>	25371	+	+		+	+	+	+	+	+	0.02
CN104C	25386	+	+	+	+		+	+	+	+	45.18	MH03C <sup>b</sup>	25334	+	+	+	tr	+	+	+	+	+	1.25
CN105C <sub>1</sub>	25370		+		+		+	+	+	+	6.80	MH03G	25367	+	+		+		+				na
CN105C <sub>2</sub>	25408	+	+	+	+			+	+	+	31.40	MH04F <sup>b</sup>	25337	+	+	+	+	+		+	+	+	na
G203B <sub>1</sub> <sup>b</sup>			+	+	+	+			+	+	0.97	MH07B	25470		+		+		+	+	+	+	99.21
G203B <sub>2</sub> <sup>b</sup>			+	+	+				+	+	0.72	MH08F	25378		+	+	+	+	+	+	+	+	85.63
KE10D <sub>2</sub>	25376	+	+	+	+	+	+	+	+	+	6.14	MH09E			+		+	+		+	+	+	3.40
KE10G	25373	+	+	+	+	+	+	+	+		na	MH10A	25335	+	+			+	+	+	+	+	26.53
KE11B	25377	+	+					+	+	+	24.53	MH12D	25395		+	+	+	+		+	+	+	7.18
KE11G <sup>b</sup>	25372	+	+	+	+		+		+		na	MH14C <sup>b</sup>	25336	+	+		+	+		+	+	+	0.45
KE17E	25369	+	+	+	+	+	+	+	+		na	OY100G	25418	+	+	+	+	+	+	+	+	+	na
KE18C	25375	+	+	+	+	+	+	+	+	+	28.51	OY101B	25417	+	+			+	+	+	+	+	7.07
KE18F	25384	+	+	+	+	+	+	+	+		na	OY107C	25404	+	+		+	+	+	+	+	+	38.69
KE18G <sup>b</sup>	25389	+	+		+		+	+	+	+	<0.01	OY108A	25385	+	+		+	+	+	+	+	+	45.55
KE19E	25400	+	+	+	+	+	+	+	+	+	16.33	OY109C	25382	+	+		+	+	+	+	+	+	1.33
KE19I <sup>b</sup>			+	+	+				+	+	117.83	OY111H	25381	+	+		+	+	+	+	+	+	na
KE25D	25390	+		+	+		+		+		na	OY111K	25473	+	+		+		+				na
KE28G <sup>b</sup>	25338	+	+		+	+			+		na	OY113F <sup>b</sup>				+	+	+		+	+	+	1.25
KE28I	25387		+					+	+	+	137.73	OY128T <sub>1</sub> <sup>b</sup>			+	+	+			+	+	0.01	
KE29E	25368	+	+	+	+		+	+	+	+	107.75	RN209B <sup>b</sup>			+	+	+		+	+	+	+	21.53
KE29H	25471	+	+	+	+		+	+	+	+	178.80	TN101C	25419		+		+	+		+			na
KE29I	25396	+	+	+	+		+	+	+	+	6.29	TN102A	25472		+		+		+		+	+	28.40
KK09A	25399	+	+	+	+		+	+	+	+	6.31	TN103C	25429	+	+		+	+		+	+	+	106.90
KK09B <sup>b</sup>	25388	+	+		+	+	+	+	+	+	47.28	TN106H	25398	+	+			+	+		+	+	na
LD100A	25421	+	+	+	+		+	+	+	+	33.55	TN106I	25426	+	+		+		+	+	+	+	116.30
LD100E	25422		+	+	+	+	+	+	+	+	9.93	TN107A	25403	+	+		+	+	+	+	+	+	17.95
LD101B	25424		+	+	+	+	+	+	+	+	37.50	TN107I	25405		+		+	+	+	+	+	+	53.83
LD101F	25420		+	+	+	+	+	+	+	+	70.71	TS12B	25379	+	+		+	+	+	+	+	+	na
LD102B	25412	+	+	+	+			+	+	+	97.55	TS14C	25383	+			+		+	+	+	+	61.95
LD103D	25411	+	+		+			+	+	+	50.50	WH100A	25414		+		+			+	+	+	6.31
LD104G	25409	+	+	+	+			+	+	+	200.00	WH100F	25413	+	+	+	+	+		+	+	+	424.70
LD104I <sub>2</sub>	25401	+	+		+	+	+	+	+	+	22.07	WH101E		+	+		+	+		+	+	+	1.98
LD105B	25406		+	+	+		+	+	+	+	209.33	WH102E	25407	+	+		+	+	+	+	+	+	98.73
LD105D	25402	+	+					+	+	+	44.59	WH103B <sub>2</sub>	25397	+	+				+	+	+	+	23.45
LS01G	25391	+	+	+		+	+	+	+		na	WH103B <sub>1</sub>	25427	+	+		+	+	+	+	+	+	na
LS04A	25392	+	+	+	+	+	+	+	+	+	10.69	WH103E	25333	+	+	+		+	+	+	+	+	na
LS05A <sup>b</sup>	25393		+	+	+		+	+			na	WX210A <sup>b</sup>			+		+	+	+	+	+	+	0.01
LS06H	25380	+	+	+				+	+	+	15.53												
LS08A	25394	+	+	+	+		+	+	+	+	1.88												
LS08C <sup>b</sup>			+	+	+			+	+	+	0.47												

<sup>a</sup> Isolates were grown on YES and CYA media for 7 days at 25 °C, and secondary metabolites were extracted from both media and analyzed using LC-UV. <sup>b</sup> The presence of metabolites was confirmed using LC-MS; +, denotes the presence of a metabolite; tr, trace. <sup>c</sup> Isolates denoted by the same two-letter code followed by an identical two- or three-digit number were collected from different points on the same bale of silage. <sup>d</sup> IBT, culture collection at the Centre for Microbial Biotechnology, Lyngby, Denmark. <sup>e</sup> The peak area of mycophenolic acid was compared to PR toxin and the ratio calculated; na, not applicable. <sup>f</sup> Abbreviations: RA, roquefortine A (isofumigaclavine A); RC, roquefortine C; CIC, citreoisocoumarin; AA, andrastin A; AC, andrastin C; SC, scyatalone or hydroxymellein derivative (see text); EC, eremofortin C; MPA, mycophenolic acid and analogues; PR, PR toxin.

50% (39/78) of the *P. paneum* isolates screened in this study failed to produce detectable amounts of patulin on Oxoid PDA, they were re-inoculated onto Difco PDA supplemented with trace metals (i.e., manganese, zinc, and copper). For metabolite analyses, zinc and copper have routinely been included in YES and CYA media, as in this study, and the addition of manganese has been shown to be important for patulin production in a number of different *Penicillium* spp. (38). However, only 4/39 isolates were induced to produce patulin on the modified medium. The addition of manganese to PDA is known to enhance patulin production by increasing the conversion of 6-methylsalicylic acid to patulin in a number of *Penicillium* spp. (38, 44). In this study, the addition of manganese resulted in a 5–20-fold increase in the peak area of 3-hydroxybenzoic acid (a patulin precursor) and citreoisocoumarin, indicating that the addition of manganese may redirect synthesis toward citreoisocoumarin from 3-hydroxybenzoic acid rather than patulin.

**Storage.** To investigate if storage can have an effect on patulin production, 11 *P. paneum* isolates that were known to be patulin

producers were rescreened for their production after a further 30 weeks of storage at 2–4 °C. Following storage, no detectable levels of patulin could be found in 4 isolates; levels were reduced on average by 77% in 3 isolates and were raised, on average, by 68% in the remaining 4 of the 11 isolates tested.

In relation to storage, a further anomaly was the inconsistency in patulin production in two different batches of *P. paneum* isolates. One batch consisting of 19 isolates was collected in March 2003, and 12 of the 19 (63%) isolates produced patulin. In contrast, in a second batch of isolates collected from November 2003 to March 2004, only 27 of 59 (45%) isolates were patulin producers. This is of interest because the first batch was in storage for approximately 72 weeks prior to analysis and was subcultured more frequently, whereas the second batch was in storage for approximately 33 weeks prior to analysis and subcultured less frequently. Others (45) have reported that patulin production decreases with time for *Penicillium expansum* when preserved by subculturing and maintenance at 4 °C. The same authors found that patulin production and detection were

**Table 2.** Secondary Metabolites Produced in Culture by *P. paneum* ( $n = 78$  Isolates) Isolated from Baled Grass Silage in Ireland

IBT										IBT									
isolate <sup>a,b</sup>	no. <sup>c</sup>	RA <sup>e</sup>	RC	CIC	AA	AC	MA	PA	GA	isolate <sup>a,b</sup>	no. <sup>c</sup>	RA <sup>e</sup>	RC	CIC	AA	AC	MA	PA	GA
C203A			+	+	+		+	+	+	OY112H			tr	+	+	+	+	+	
CN100J			+	+	+		+			OY113A			tr	+	+	+	+	+	<sup>d</sup>
CN100K	25415		tr	+	+	+	+			OY116D			+	+	+		+		
CN103C			tr	+	+		+		<sup>d</sup>	OY118B			tr	+	+		+	+	
CN204A		+	+	+	+		+	+		OY118K			tr	+	+		tr	+	
DL205A			+	+	+		+			OY122C			tr	+	+		+	+	
DL227A		+	+	+	+		+	+		OY123A			+	+	+		+		
DL251J			+	+	+		+			OY127E		+	+	+	+		+		<sup>d</sup>
DL253A			+	+	+		+	+	+	OY127H		+	+	+	+	+	+		
G216B		+	tr	+	+		+			OY127I		+	+	+	+		+	+	
G218A		+	+	+	+		+	+		OY128H		+	+	+	+		+		
G223A		+	+	+	+		+	+		OY128O		+	+	+	+		+		
KE26B		+	+	+	+	+	+	+		OY129B		+	+	+	+		+	+	
KE26C		+	+	+	+	+	+			RN203A		+	+	+	+		+	+	
KE27D			+	+	+	+	+	+		SO202A		+	+	+	+		+		
KE27E		+	+	+	+		+	+		TN103A		+	+	+	+		+		
KE28B		+	+	+	+	+	+	+		TN104C			tr	+	+		+		
KE28C		+	+	+	+		+	+		TS13G		+	+	+	+	+	+		
KE28D		+	tr	+	+		+	+		TS20G		+	+	+	+		+		
KE29F			tr	+	+		+	+		WD203A			tr	+	+		+	+	
KE29G			+	+	+		+			WD208A		+	tr	+	+		+		
KK04A	25331		+	+	+	+	+	+	+	WD214A			+	+	+		+		
KK04B	25332	+	+	+	+		+	+	+	WD217A			+	+	+		+		
KK06B			+	+	+		+			WH101C	25423		+	+	+	+	+		
KK08B		+	+	+	+		+			WH105D		+	+	+	+		+	+	
KK214B			+	+	+		+			WH109D			tr	+	+		+		
KY222B		+	+	+	+		+			WH109F		+	+	+	+		+	+	+
LD1041I	25330	+	+	+	+	+	+	+		WH113D		+	tr	+	+		+	+	
LD106C		+	+	+	+	+	+		+	WH115B		+	+	+	+		+	+	
LK207A		+	+	+	+		+			WH116H		+	+	+	+		+		<sup>d</sup>
LK210B			+	+	+	+				WH120S			+	+	+	+	+		
LK225B			+	+	+	+	+	+		WH121E			+	+	+	+	+		
LS06C		+	tr	+	+		+	+		WH121G		+	tr	+	+		+	+	
LS06E2		+	+	+	+		+	+		WH121H			+	+	+		+		
LS08E2		+	+	+	+		+	+		WH121I		+	+	+	+		+		
MH08G		+	+	+	+		+			WH121J		+	+	+	+		+		
MN204B		+	+	+	+		+	+		WH121K		+	tr	+	+		+		
MO204A		+	+	+	+	+	+	+		WH121L		+	+	+	+	+	+		
OY112C		+	+	+	+		+			WH121M			+	+	+		+		

<sup>a</sup> Isolates were grown on YES, CYA, and PDA media for 7 days at 25 °C. Secondary metabolites were extracted from the media and analyzed using LC-UV and LC-MS.

<sup>b</sup> Isolates denoted by the same two-letter code followed by an identical two- or three-digit number were collected from different points on the same bale of silage. <sup>c</sup> IBT, culture collection at Centre for Microbial Biotechnology, Lyngby, Denmark. <sup>d</sup> Patulin production detected only when grown separately on Difco PDA supplemented with manganese and other trace metals. <sup>e</sup> Abbreviations: RA, roquefortine A (isofumigaclavine A); RC, roquefortine C; CIC, citreoisocoumarin; AA, andrastin A; AC, andrastin C; MA, marcfortine A; PA, patulin; GA, gentisic acid; +, denotes the presence of a metabolite; tr, trace.

**Table 3.** Consistency of Secondary Metabolite Production in Culture by *P. roqueforti* and *P. paneum* Isolates from Baled Grass Silage in Ireland

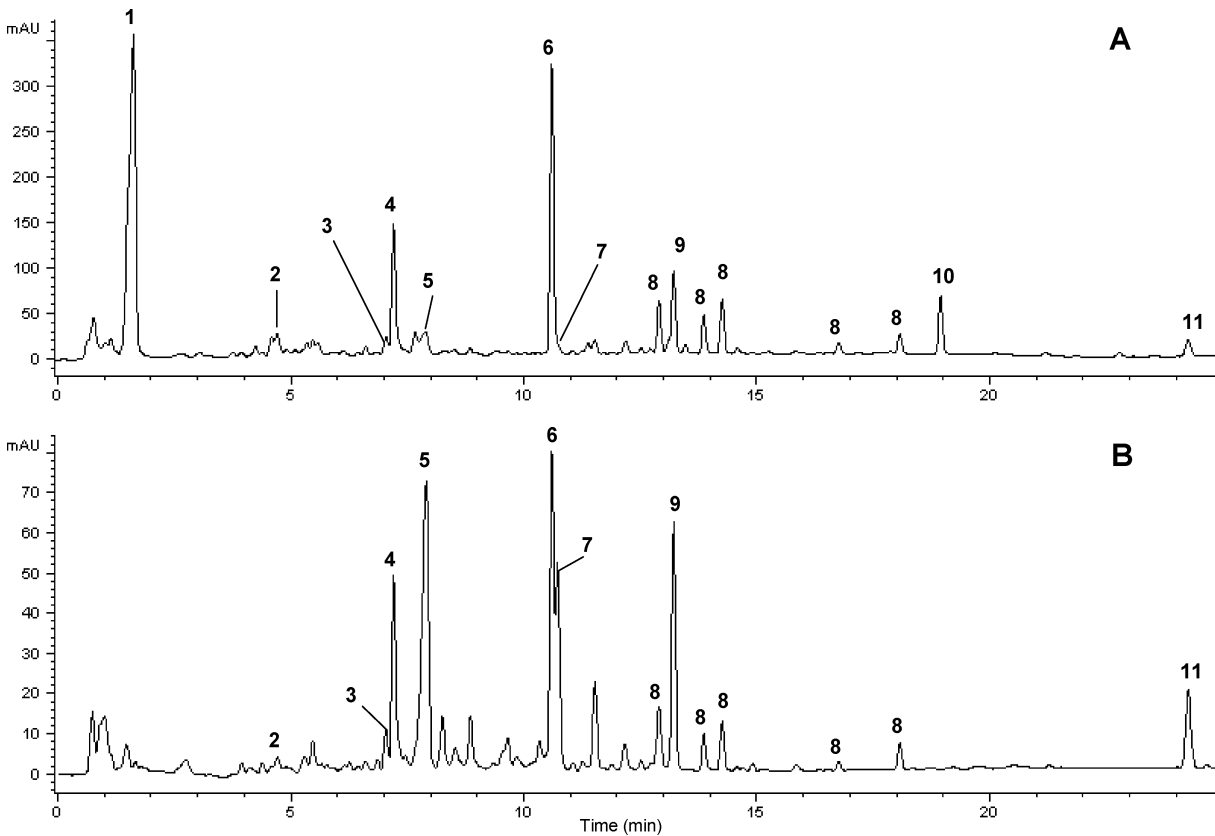
secondary metabolite	no. of isolates <sup>a</sup> (% of total isolates)	
	<i>P. roqueforti</i> ( $n = 79$ )	<i>P. paneum</i> ( $n = 78$ )
mycophenolic acid and analogues	67 (84.8)	ND
roquefortine A	55 (69.6)	42 (53.8)
roquefortine C	76 (96.2)	76 (97.4)
marcfortines	ND	76 (97.4)
citreoisocoumarin	33 (41.7)	78 (100)
andrastin A	72 (91.1)	78 (100)
andrastin C	30 (38.0)	17 (21.8)
metabolites T	45 (57.0)	ND
PR toxin	61 (77.2)	ND
eremofortin C	63 (79.7)	ND
patulin	ND	39 (50)
gentisic acid	ND	6 (7.7)

<sup>a</sup> ND, not detected.

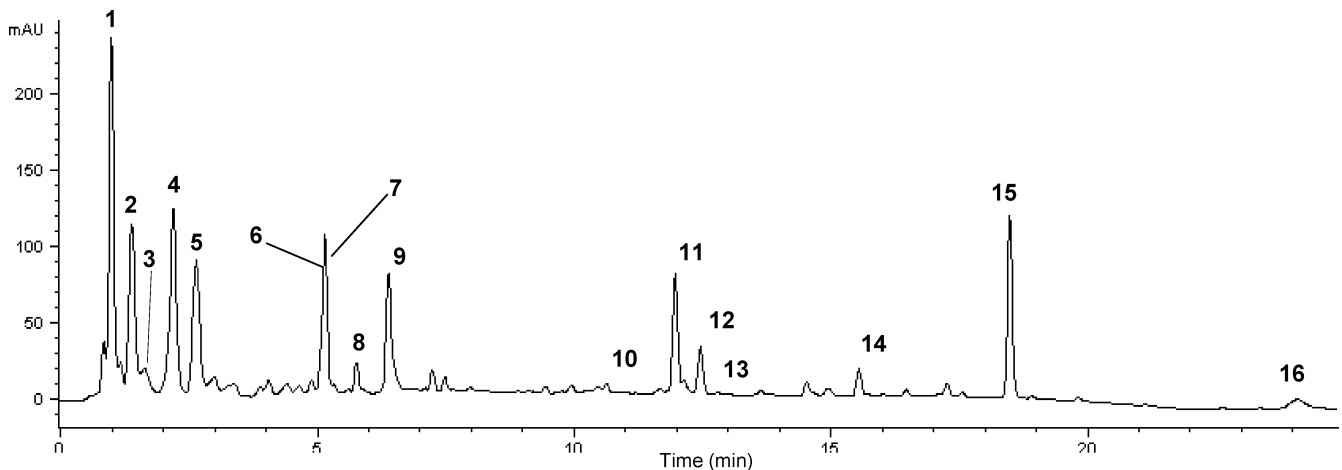
more consistent in *P. expansum* preserved using silica gel or the freeze-drying methods, compared to subculturing and maintenance at 4 °C.

***P. roqueforti* and *P. paneum* Secondary Metabolites in Silage.** The chromatograms in **Figure 4** clearly confirm the presence of andrastin A, mycophenolic acid, roquefortines A and C, marcfortine A, and festuclavine in mold-contaminated silage. The recoveries ( $\pm$  standard deviation, three levels analyzed in duplicate) of metabolites were determined to be  $80 \pm 15\%$  for roquefortine C,  $70 \pm 10\%$  for mycophenolic acid,  $40 \pm 20\%$  for patulin, and  $60 \pm 20\%$  for penicillic acid. Patulin was detected by UV at 276 nm and ESI<sup>-</sup> as the  $[M - H]^-$  ion and penicillic acid as the  $[M - H]^-$  ion with an approximate limit of detection of 0.1–0.5 mg/g of silage. As sufficient quantities of the marcfortines and roquefortines A, B, D, and OH-C (detected by ESI<sup>+</sup>) were not available for a fortification experiment, the quantities of these were estimated from calibration curves using pure substances and also assuming the same recovery as for roquefortine C and a detection limit of 40–200  $\mu\text{g}/\text{kg}$  of silage estimated from the lowest amount of the fortified sample (2 mg/kg). Mycophenolic acid and andrastin A were detected in both polarities with a negative LC-ESI-MS being most sensitive, giving a detection limit of 100–200  $\mu\text{g}/\text{kg}$  of silage (based on the recovery level of mycophenolic acid).

Visually moldy silage samples contained up to 20 mg/kg (74



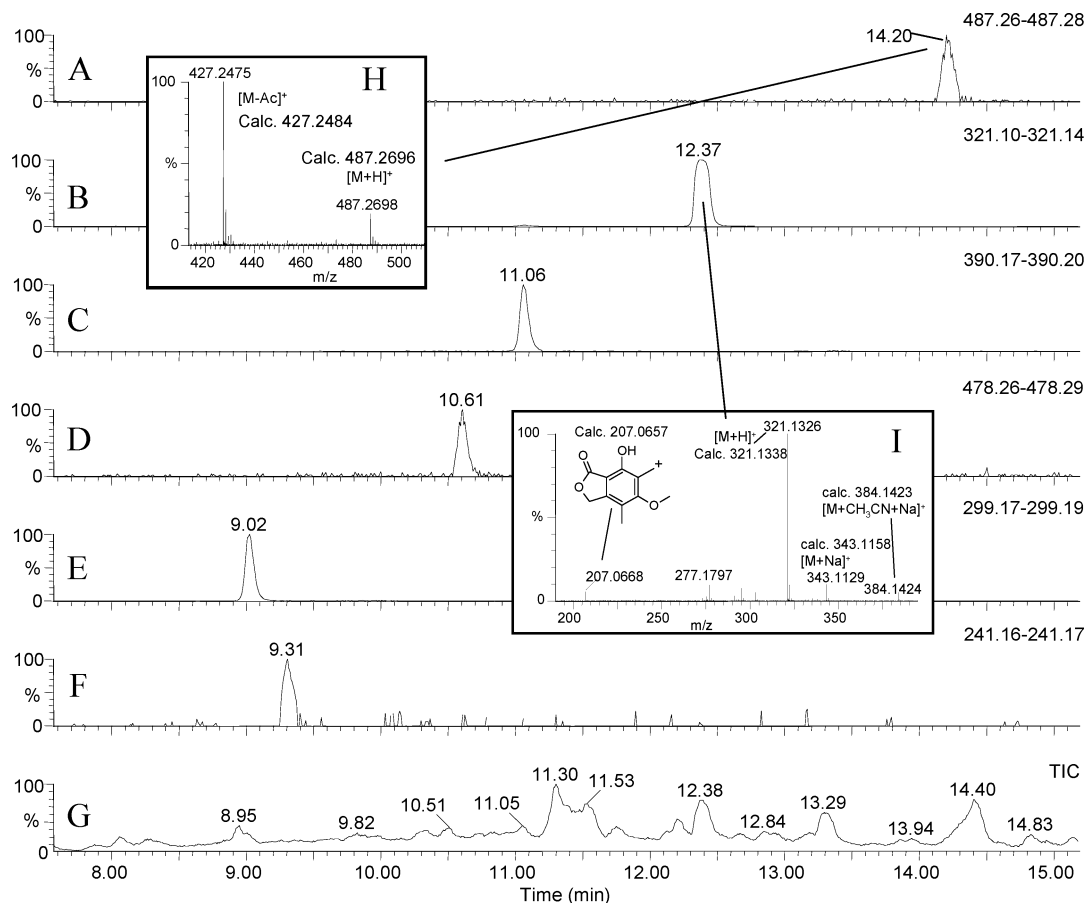
**Figure 2.** HPLC-UV chromatograms at (A) 210 nm and (B) 254 nm of extract of *P. roqueforti* KK09A grown on yeast extract sucrose agar and Czapek yeast autolysate agar at 25 °C for 7 days. The selected metabolites annotated are (1) an unidentified metabolite, (2) roquefortine A, (3) citreoisocoumarin, (4) roquefortine C, (5) eremofortin C, (6) mycophenolic acid, (7) PR toxin, (8) mycophenolic analogue, (9) andrastin A, (10) linoleic acid, and (11) ergosterol.



**Figure 3.** HPLC-UV chromatograms at 210 nm of extract of *P. paneum* KE28B grown on yeast extract sucrose agar, Czapek yeast autolysate agar, and potato dextrose agar (Oxoid) at 25 °C for 7 days. The selected metabolites annotated are (1) an unidentified metabolite, (2) patulin, (3) triacetic acid lactone, (4) 2,5-dihydroxybenzoic acid, (5) 3-hydroxybenzoic acid, (6) citreoisocoumarin, (7) marcfortine A, (8) roquefortine C, (9) 6-methylsalicylic acid, (10) andrastin B, (11) andrastin A, (12) an unidentified metabolite (MW 304), (13) andrastin D, (14) andrastin C, (15) linoleic acid, and (16) ergosterol.

mg/kg of DM) each of roquefortine C, mycophenolic acid, and andrastin A along with minor quantities (0.1–5 mg/kg) of the roquefortines A, B, and D, festuclavine, marcfortine A, and agroclavine (**Table 4**). Visually nonmoldy silage samples contained low amounts (<0.1–5 mg/kg) of mycophenolic acid and andrastin A, confirming the observations of both Auerbach et al. and O'Brien et al. (10, 46) that *P. roqueforti* may be found in apparently clean silages. Roquefortine C levels in both visually nonmoldy and moldy wilted grass silage in northern Germany ranged from 0.1 to 0.3 mg/kg of DM and from 0.2 to

1.5 mg/kg of DM, respectively (10), and this compares to none detected in visually nonmoldy samples and between 11.1 and 73.5 mg/kg of DM (5–20 mg/kg) in moldy silage samples in this study (**Table 4**). However, in the German study, moldy whole-crop maize silage samples contained up to 36 mg/kg of DM of roquefortine C (10). Armbruster (47) detected roquefortine C in 3 of 24 grass silage samples in concentrations ranging from <0.1 to 0.58 mg/kg, but, similar to the findings of Auerbach et al. (10), higher concentrations of up to 28 mg/kg were found in whole-crop maize silage. Mycophenolic acid



**Figure 4.** HPLC-MS chromatograms of naturally contaminated moldy silage, showing the extracted ion chromatograms of the  $[M + H]^+$  ion of (A) andrastin A, (B) mycophenolic acid (same as PR toxin), (C) roquefortine C, (D) marcfortine A, (E) roquefortine A, and (F) festucalvine. (G) Total ion chromatogram (TIC) and the  $ESI^+$  spectra of (H) andrastin A and (I) mycophenolic acid.

**Table 4.** Secondary Metabolites Detected in Fresh Samples of Grass Silage from Bales in Ireland

sample	FC <sup>d</sup>	AC	RA	RB	OHRC	RC	RD	MA	CIC	MPA	AA
1 <sup>a</sup>	++	++	+++	++	++	++++	++	++	tr	++++	++++
2 <sup>a</sup>	++	++	+++	++	ND	++++	++	++	tr	++++	++++
3 <sup>b</sup>	ND	ND	ND	ND	ND	ND	ND	ND	ND	tr	++
4 <sup>b</sup>	ND	ND	ND	ND	ND	ND	ND	+	ND	+++	++
5 <sup>c</sup>	ND	ND	ND	ND	ND	ND	ND	ND	ND	tr	++

<sup>a</sup> Visually moldy silage (fungus identified as *P. roqueforti*). <sup>b</sup> Visually nonmoldy silage adjacent to mold colonies on the same bale. <sup>c</sup> Visually nonmoldy silage from a bale free of all visible mold growth on its surface. Bale 1, samples 1 and 3 [dry matter (DM) = 27.2%]; bale 2, samples 2 and 4 (DM = 44.8%); bale 3, sample 5 (DM = 51.9%); +++++, 5–20 mg/kg range; +++, 1–5 mg/kg range; ++, 0.1–1 mg/kg range; +, <0.1 mg/kg. Metabolites were detected by LC-ESI<sup>+</sup>-MS, except CIC, which was detected by only LC-ESI<sup>-</sup>-MS. MPA and AA were detected in both polarities with LC-ESI<sup>-</sup>-MS being the most sensitive. ND, not detected, which equals <40–200  $\mu$ g/kg of all metabolites except CIC; tr, trace; peak detected ( $s/n < 10$ ). <sup>d</sup> Abbreviations: FC, festucalvine; AC, agroclavine; RA, roquefortine A, RB, roquefortine B; RC, roquefortine C; OHRC, 16-hydroxyroquefortine C; RD, roquefortine D; MA, marcfortine A; CIC, citreoisocoumarin; MPA, mycophenolic acid; AA, andrastin A.

levels in grass silage in Bavaria, Germany, ranged from 0.02 to 35 mg/kg (35); it was detected in 30% of samples, but as this concentration was not corrected for DM content, it is difficult to make comparison to values recorded in this study. Müller and Amend (36) studied the accumulation of mycotoxins in maize silage inoculated with different strains of *P. roqueforti* (*sensu lato*) over 160 days of aerobic storage and detected levels

of mycophenolic acid, patulin, penicillic acid, and PR toxin to levels of 4, 15, 3, and 2 mg/kg of silage, respectively.

Notwithstanding that the *Penicillium*-like colonies growing on bales were identified as *P. roqueforti*, the presence of marcfortine A with mycophenolic acid in three of the silage samples suggests that *P. paneum* and *P. roqueforti* were present together because *P. paneum* is the only known producer of marcfortine. Nielsen et al. (11) have suggested that the occurrence of *P. paneum* in silage is probably underestimated due to its misidentification as *P. roqueforti* and also possibly due to its comparatively lower levels of sporulation. An alternative explanation that another fungus in silage is also capable of producing marcfortines seems to be unlikely.

The following metabolites were not detected in any of the silage samples: verrucologen, fumitremorgin B, bis(dethio)-bis(methylthio)gliotoxin, or gliotoxin (*Aspergillus fumigatus* metabolites); citrinin (*Monascus* metabolite); patulin or marcfortine B (*P. paneum* metabolites); penicillic acid or penitrem A (*P. carneum* metabolites). The presence of patulin in feed for livestock would be a concern because it can alter metabolism of nutrients by ruminal microbes (48).

This is the first report of the toxigenic potential of *P. roqueforti* and *P. paneum* growing on grass silage in Ireland. In a Canadian study (15) of mold spoilage of animal feeds, *P. paneum* was associated with ill-thrift of dairy cows, whereas *P. roqueforti* was linked to toxicoses in the animals. Owing to the large number of secondary metabolites produced by these two species in this and other studies, both in culture and in silage, there is a need to carry out multimycotoxin analysis on this feedstuff on a larger scale. A combination of mycotoxins

and compounds not themselves being toxic rather than any one mycotoxin may be a more likely cause of livestock health problems, and previous studies have not adequately investigated synergistic effects.

In conclusion, this study established the secondary metabolites produced *in vitro* by *P. roqueforti* and *P. paneum* isolated from baled grass silage in Ireland. Two factors that may affect patulin production by *P. paneum*, that is, trace metal supplements and duration of isolate storage prior to secondary metabolite screening, require further research. Secondary metabolites produced by *P. roqueforti* and also presumably *P. paneum* were detected in visually moldy silage contaminated with *P. roqueforti* and in silage samples that did not appear to be moldy.

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