Journal of Chemical Ecology, Vol. 31, No. 10, October 2005 (©2005)
DOI: 10.1007/s10886-005-7107-y

ROQUEFORTINE/OXALINE BIOSYNTHESIS PATHWAY METABOLITES IN *Penicillium* SER. *Corymbifera*: *IN PLANTA* PRODUCTION AND IMPLICATIONS FOR COMPETITIVE FITNESS

D. P. OVERY,* K. F. NIELSEN, and J. SMEDSGAARD

Center for Microbial Biotechnology, Technical University of Denmark, BioCentrum-DTU, Building 221, DK-2800 Kgs. Lyngby, Denmark

(Received March 5, 2005; accepted May 18, 2005)

Abstract—Three strains of each of the seven taxa comprising the *Penicillium* series *Corymbifera* were surveyed by direct injection mass spectrometry (MS) and liquid chromatography–MS for the production of terrestric acid and roquefortine/oxaline biosynthesis pathway metabolites when cultured upon macerated tissue agars prepared from *Allium cepa*, *Zingiber officinale*, and *Tulipa gesneriana*, and on the defined medium Czapek yeast autolysate agar (CYA). A novel solid-phase extraction methodology was applied for the rapid purification of roquefortine metabolites from a complex matrix. *Penicillium hordei* and *P. venetum* produced roquefortine D and C, whereas *P. hirsutum* produced roquefortine D and C and glandicolines A and B. *P. albocoremium*, *P. allii*, and *P. radicicola* carried the pathway through to meleagrin, producing roquefortine D and C, glandicolines A and B, and meleagrin. *P. tulipae* produced all previously mentioned metabolites yet carried the pathway through to an end product recognized as *epi*-neoxaline, prompting the proposal of a roquefortine/*epi*-neoxaline biogenesis pathway. Terrestric acid production was stimulated by all *Corymbifera* strains on plant-derived media compared to CYA controls. *In planta*, production of terrestric acid, roquefortine C, glandicolines A and B, meleagrin, *epi*-neoxaline, and several other species-related secondary metabolites were confirmed from *A. cepa* bulbs infected with *Corymbifera* strains. The deposition of roquefortine/oxaline pathway metabolites as an extracellular nitrogen reserve for uptake and metabolism into growing mycelia and the synergistic role of terrestric

* To whom correspondence should be addressed. E-mail: do@biocentrum.dtu.dk

acid and other *Corymbifera* secondary metabolites in enhancing the competitive fitness of *Corymbifera* species *in planta* are proposed.

Key Words—*Penicillium* ser. *Corymbifera*, roquefortine C, glandicoline A, glandicoline B, meleagrin, *epi*-neoxaline, terrestric acid, *Allium cepa.*

INTRODUCTION

Taxa of the *Penicillium* ser. *Corymbifera* are pathogenic agents causing blue mold storage rot upon a variety of flower and vegetable bulbs (Saaltink, 1971; Prince et al., 1988; Vincent and Pitt, 1989; Bertolini and Tian, 1996; Filtenborg et al., 1996), especially *Allium cepa* (onion) (Overy et al., 2005a). Seven species are recognized within the ser. *Corymbifera*: *P. albocoremium* (Frisvad) Frisvad, *P. allii* Vincent and Pitt, *P. hirsutum* Dierckx, *P. hordei* Stolk, *P. radicicola* Overy and Frisvad, *P. tulipae* Overy and Frisvad, and *P. venetum* (Frisvad) Frisvad. Extracellular enzyme profiling of the series demonstrated that all of the *Corymbifera* (with the exception of *P. hordei*) produce high levels of cellulase and hemicellulases compared to other *Penicillia*, but do not excrete extracellular proteases (Overy et al., 2005b). The *Corymbifera* extracellular enzymatic profile corresponds to the composition of *A. cepa* cells (high levels of cellulose, hemicellulose, and pectin with only a 1% protein content per cell dry weight; Mankarios et al., 1980; Séné et al., 1994), indicating that *A. cepa* bulbs are an ideal ecological niche for these specialized fungal invaders. An exception to this is *P. hordei*, which produces moderate levels of proteases (Overy et al., 2005b), reflecting this species' known association with cereal grains.

The *Corymbifera* species are reported to produce the alkaloids roquefortine C and sometimes meleagrin (Frisvad and Filtenborg, 1989; Overy and Frisvad, 2003); however, the extent to which each of the taxa produce intermediates of the roquefortine/oxaline (r/o) biosynthesis pathway (Figure 1) has not been evaluated for the series. Roquefortine C is a diketopiperazine, synthesized from tryptophan, histidine, and dimethylallyl pyrophosphate (Ohmomo et al., 1979). Both dehydrohistidyl–tryptophanyl–diketopiperazine and 3,12-dihydroroquefortine (roquefortine D) are known precursors to roquefortine C (Kozlovsky et al., 1996). C^{14} labeling experiments by Reshetilova et al. (1995) have demonstrated that roquefortine C is a precursor to glandicolines A and B (Kozlovsky et al., 1994), meleagrin (Kawai et al., 1984), and oxaline (Nagel et al., 1976), a series of biosynthetically related and structurally similar metabolites comprising the r/o biosynthesis pathway. A few reports of antibiotic activity (Kopp and Rehm, 1979) and neurotropic properties (Scott et al., 1976) of roquefortine C have prompted multiple studies into roquefortine production (Boichenko et al., 2002), uptake (Reshetilova et al., 1994; Kulakovskaya et al., 1997), and metabolism (Reshetilova and Kozlovsky, 1990).

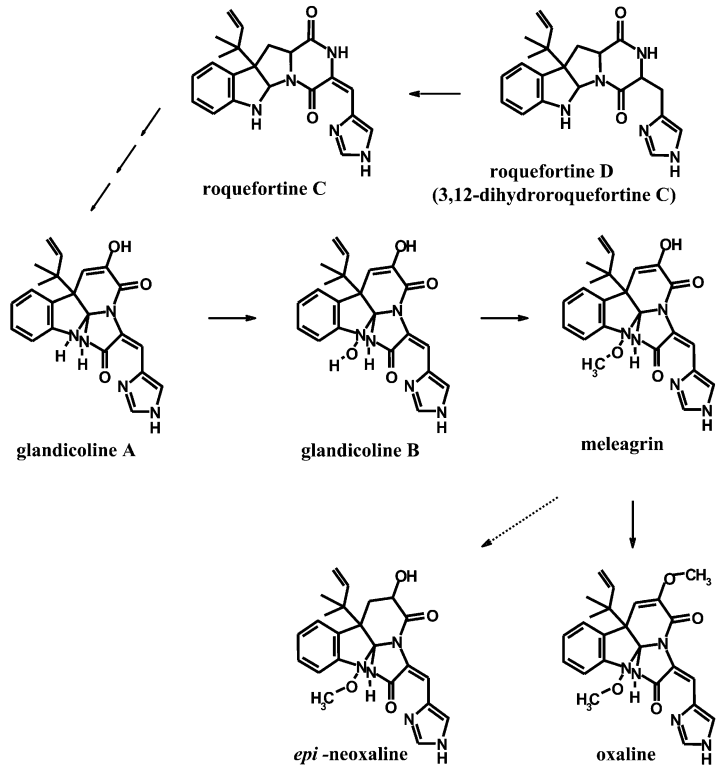

FIG. 1. Roquefortine/oxaline biosynthesis pathway as proposed by Reshetilova et al. (1995) and the proposed roquefortine/*epi*-neoxaline biogenesis pathway proposed for *Penicillium tulipae*.

The use of alkaloids as an exclusive, transportable nitrogen source has been previously proposed in plants (Wink, 2003), and we hypothesize that this strategy is also employed by fungi. Roquefortine C biosynthesis and excretion from fungal mycelia has been determined to be dependent upon the quantity of exogenous roquefortine C present in the surrounding media (Kulakovskaya et al., 1997). Additionally, ^{14}C-roquefortine C experiments demonstrated that roquefortine C was not only taken up by growing mycelia, but was also incorporated into protein and mycelial residues (Reshetilova et al., 1986, 1994; Reshetilova and Kozlovsky, 1990; Kulakovskaya et al., 1997). From *in vitro* experimentation on strains of *P. crustosum*, it was determined that transport of roquefortine C across the cellular membrane occurs via both energy-dependent and energy-independent processes (Reshetilova et al., 1994; Kulakovskaya

et al., 1997); thus, Kulakovskaya et al. (1997) proposed that roquefortine C may function as an extracellular nitrogen source for the producing organism.

Recent research efforts culturing ser. *Corymbifera* species upon host tissue-derived media have led to the stimulation and structure elucidation of a series of novel naphthalene lactones (Overy and Blunt, 2004). Preliminary investigation into the r/o biosynthesis pathway metabolites indicated that discrepancies in production might exist with the reported literature (Frisvad and Filtenborg, 1989). Media-dependent production of terrestric acid, a low molecular weight metabolite produced by five out of seven of the *Corymbifera* species, has also been observed (unpublished results). We conducted experiments testing the degree to which stimulation of the r/o biosynthesis pathway metabolites and terrestric acid occurs when *Corymbifera* strains are grown on plant-based media. To facilitate analysis of r/o metabolites from complex matrices, a preferential solid-phase extraction (SPE) methodology was applied. Subsequent *in planta* studies using the common host *A. cepa* (yellow onion) were carried out (1) to see if production of terrestric acid, the r/o pathway metabolites, and additional secondary metabolites, associated with the *Corymbifera* strains occurred, and (2) to suggest an ecological, synergistic function for these metabolites *in planta*.

METHODS AND MATERIALS

Fungal Isolates. The following isolates were revived from IBT culture collection (BioCentrum-DTU, Kgs. Lyngby, Denmark) and streak plated on Czapek yeast autolysate agar (CYA) to ensure purity: *P. albocoremium* (Frisvad) Frisvad (IBT 21502, IBT 22521, IBT 21071), *P. allii* Vincent and Pitt (IBT 3772, IBT 4112, IBT 21503), *P. hirsutum* Dierckx (IBT 12398, IBT 13033, 19340), *P. hordei* Stolk (IBT 4154, IBT 21532, IBT 23024), *P. radicicola* Overy and Frisvad (IBT 10693, IBT 10696, IBT 22526), *P. tulipae* Overy and Frisvad (IBT 3458, IBT 10676, IBT 10681), and *P. venetum* (Frisvad) Frisvad (IBT 22111, IBT 23039, IBT 23040). Spore suspensions of each isolate were then prepared by removing a loopful of conidia from axenic cultures into a semisolid medium (for media recipes, see Frisvad and Samson, 2004) and vortexed.

Preparation of Macerated Plant Tissue Agar. Plant tissue-based agars were prepared according to Overy and Blunt (2004). Bulbs of *A. cepa* (red and yellow onion) and *Tulipa gesneriana* (tulip) and tubers of *Zingiber officinale* (ginger) were inspected for symptoms of infection, and healthy tissue was selected and individually macerated in a blender. The macerate was transferred into weighed 2-l screw cap bottles, and deionized water was added to yield a

40% slurry (w/v) to which agar was added (2%; w/v). The mixture was autoclaved at 121°C for 20 min and then poured into 9-mm Petri dishes (15 ml/dish).

Strain Cultivation and Extraction. The selected ser. *Corymbifera* strains were three-point inoculated onto CYA and the various plant extract agar plates and incubated in darkness at 25°C for 14 d. Following this, colony extraction was divided into two parts. For metabolomic profiling by direct infusion electrospray mass spectrometry (Di-ESI-MS), three plugs were removed from each culture and individually extracted according to Smedsgaard (1997) [using a one-step extraction with ethyl acetate (EtOAc)]. For solid-phase extraction (SPE), an entire colony from one selected strain from each species (see Table 1) was removed in strips from the plate and inserted into 14-ml glass vials to which 8 ml of EtOAc were added. The vials were sonicated for 1 hr and the extraction solvent was subsequently decanted into clean 14-ml glass vials and evaporated *in vacuo* using a Christ rotational vacuum concentrator (RVC). The dry residues were dissolved in 2 ml of EtOAc, filtered through 0.45-μm PTFE filters to remove conidia and particulates, and evaporated to dryness under a stream of nitrogen in weighed glass vials. Selected extracts were subsequently dissolved in a volume of EtOAc to give the same concentration of raw extract, and a 1-ml subsample of the extract was dried down to be used for solid-phase extraction (equivalent to approx. 1 g of extract).

In Planta *Infection Trials.* Infection trials of *A. cepa* (yellow onion) bulbs and *Z. officinale* tubers were carried out using the selected ser. *Corymbifera* strains according to Overy et al. (2005a,b). Tubers and bulbs were purchased in

TABLE 1. SUMMARY OF SER. *corymbifera* SECONDARY METABOLITES PRODUCED *In Planta* IN INFECTED *A. cepa* BULBS[a]

Species	*In planta* secondary metabolite production
Penicillium albocoremium	Andrastin A, b. acid A, cyclopenin, gland A&B, meleagrin, roq C, viridicatin
Penicillium allii	Andrastin A, cyclopenin, fulvic acid, gland A&B, meleagrin, roq C, viridicatin
Penicillium hirsutum	Compactin, cyclopenin, gland A&B, roq C, terrestric acid, viridicatin
Penicillium hordei	Roq C (1/3), terrestric acid (1/3)
Penicillium radicicola	Andrastin A, cyclopenin, gland A&B, meleagrin, roq C, terrestric acid, viridicatin
Penicillium tulipae	Andrastin A, *epi*-neoxaline, gland A&B, meleagrin, roq C, terrestric acid
Penicillium venetum	Andrastin A, cyclopenin, roq C, terrestric acid, viridicatin

[a]Secondary metabolites were confirmed by LC-DAD-HR-MS analysis from EtOAc tissue extracts of three different species strains individually inoculated into separate *A. cepa* bulbs.
b. acid A = Barceloneic acid A; gland A&B = glandicolines A and B; roq C = roquefortine C.

late spring and inspected for signs of damage or infection (bulbs had their outer skin removed). Tubers or bulbs exhibiting signs of damage or infection (this included physical damage, sunken lesions, brown spots, or visible rot) were discarded. Surface sterilization was done in three steps in a laminar flow bench: submerging in 96% ethanol for 1 min, then in a 3% hypochlorite solution for 1 min, and resubmerging in 96% ethanol for 1 min. Once dry, bulbs were inoculated with a spore suspension in the center of the basal root plate and tubers through the epidermis with a flame sterilized needle, and placed into perforated plastic bags, and incubated in the dark at 20°C. After 3 wk, explants approximately 2 cm^3, consisting of infected tissue displaying visible sporulation (including exposed conidiophores and hyphae), were removed to 14-ml screw cap vials to which 8 ml of EtOAc were added. Control samples were similarly prepared using visually uninfected tubers and bulbs. The vials were shaken on a rotary shaker for 2 hr, the extraction solvent subsequently decanted into clean 14-ml screw cap vials, and evaporated to dryness *in vacuo* prior to SPE.

To confirm the identity of the fungal cultures excised and extracted for analysis, tissue explants were removed from the infected tissues where sporulation was visible and placed onto Petri dishes containing CYA media (+chloramphenicol, 100 ppm). Petri dishes were then incubated in the dark at 25°C and monitored for 3–7 d. Colonies emerging from the tissue explants were transferred to streak plates from which purified cultures were three-point inoculated onto CYA and incubated in the dark at 25°C for 7 d for identification based upon colony characteristics and micromorphology.

Solid-Phase Extraction. Solid-phase extraction columns were packed with approximately 300 mg of Silica 60 (0.015–0.040 mm, Merck, Darmstadt, Germany) pressed between two disks of 3-mm Vyon sheet (porous high-density polyethylene, FilterServe, West Midlands, UK) in 2-ml disposable syringes. The columns were placed onto a vacuum manifold, wetted with 2 ml of MeOH, and conditioned with 2 ml toluene/EtOAc/formic acid (TEF, 5:4:1, v/v/v). Sample extracts were redissolved in 200 µl TEF and loaded onto the column. The loaded column was then washed with 3.5 ml of TEF (fraction 1) and then with 1.5 ml EtOAc/MeOH (1:1, v/v; fraction 2). The fractions were dried *in vacuo* and resuspended in MeOH for liquid chromatography–ultraviolet–mass spectrometry (LC-UV-MS) evaluation.

Time Scale Study. P. tulipae IBT 3458 was three-point inoculated onto 18 CYA plates and incubated in the dark at 25°C. Three plates were grown to 5, 6, 7, 8, 9, and 10 d. One colony was removed from each dish with a scalpel, further cut into 2- to 3-mm rectangles, transferred to a 16-ml vial, and shaken twice with 10 ml EtOAc for 1 hr. The EtOAc phases from the double extraction were combined, evaporated *in vacuo*, and redissolved in 1000 µl MeOH for LC-UV-MS analysis. From the culture plates, ten plugs (6 mm ID) were taken from

the agar outside the colonies, transferred into a 4-ml vial, and shaken twice with 3 ml EtOAc for 1 hr. The combined EtOAc phases were evaporated *in vacuo* and redissolved in 500 μl MeOH for LC-UV-MS analysis.

LC-UV-MS Analysis. Extracts fractionated by solid-phase extraction were filtered through a 0.45-μm PFTE syringe filter and analyzed by reversed-phase chromatography combined with photodiode array detection (DAD) and high-resolution mass spectrometry on an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to a LCT (Waters-Micromass, Manchester, UK) orthogonal *Time of Flight* mass spectrometer operated in the positive electrospray ionization (ESI^+) mode as described in detail by Nielsen and Smedsgaard (2003). Samples were separated on a Phenomenex Luna C_{18} (II) column (2×50 mm, with 3-μm particles) using a flow of 0.3 ml/min. A linear acetonitrile–water (AcN:H_2O) gradient system (starting at 15% AcN and increasing to 100% AcN over 20 min and holding for 5 min) was used to elute compounds from the column. The water (MilliQ) contained 10 mM ammonium formate and 20 mM formic acid (both analytical grade) and AcN (gradient grade) containing 20 mM formic acid.

Peaks, from UV and/or ESI^+ MS, excluding those found in negative controls, were matched against the Mycology reference standard database (~550 compounds; Nielsen and Smedsgaard, 2003). Metabolites not available as reference standards were dereplicated (tentatively identified) by UV spectra combined with high-resolution ESI^+ spectra as illustrated in Nielsen and Smedsgaard (2003), and matched against literature data of *Corymbifera* species and other *Penicillia* as well as Antibase 2003 (ca. 30,000 microbial secondary metabolites, Wiley & Sons, Hoboken, NJ, USA). ESI^+ spectra confirmed by deconvolution, v.s., by plotting ion traces of ions found in the background subtracted spectra of the peak of interest. Target analyses of selected metabolites, andrastin A, barceloneic acid A, cyclopenin, compactin, *epi*-neoxaline, fulvic acid, glandicolines A and B, meleagrin, roquefortine C, terrestric acid, viridicatin, and viridicatol, were performed by plotting their $[M+H]^+$ ions ± 0.01 Da.

Di-ESI-MS Fingerprinting Analysis. Plug extracts developed from each of the three selected strains of the ser. *Corymbifera* species grown on the various experimental media were additionally fingerprinted by using direct infusion positive ES-MS on a QTOF (Waters-Micromass) orthogonal quadrupolequad-rupole *Time of Flight* mass spectrometer. A constant flow of 15 μl methanol/min was delivered from an Agilent 1100 (Agilent Technologies), and 1 μl of sample was injected into the carrier stream. Just prior to the source, 5 μl/min of water with 2% formic acid were added, giving a combined flow of 20 μl/min entering the ESI source. Spectra were collected from 100 to 1000 Da/e, at a rate of 1 spectrum/sec. The scans collected during the elution time (about 15 scans) were summarized into one scan for further processing. As roquefortine C is produced

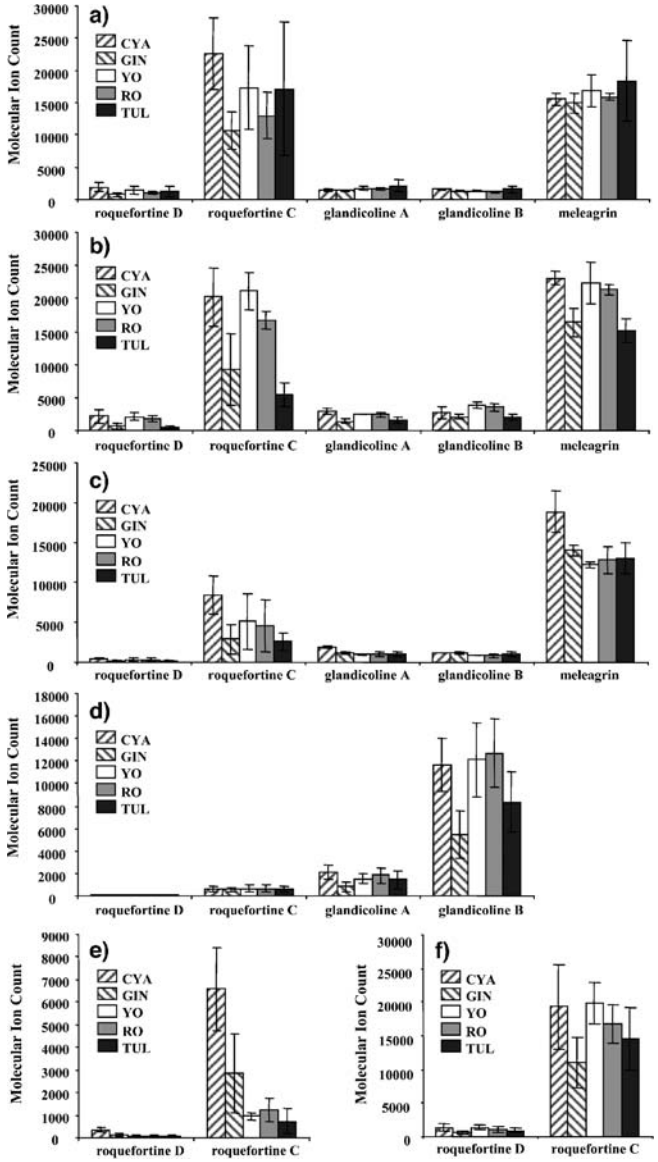

FIG. 2. Average molecular ion count calculated for roquefortine/oxaline biosynthesis pathway metabolites as produced by *Corymbifera* strains grown on Czapek yeast autolysate (CYA), ginger (GIN), yellow onion (YO), red onion (RO), and tulip (TUL) agars. (a) *Penicillium albocoremium*, (b) *Penicillium allii*, (c) *Penicillium radicicola*, (d) *Penicillium hirsutum*, (e) *Penicillium hordei*, (f) *Penicillium venetum*.

by all species and under all conditions, protonated roquefortine C (390.1930 Da/e) was used for internal mass calibration. In case of high roquefortine C intensity, the 13-C isotope was used instead. The ions from protonated terrestric acid and protonated metabolites from the roquefortine/oxaline biosynthesis pathway from each strain were averaged to give a representative species measure.

RESULTS

P. albocoremium, *P. allii*, and *P. radicicola* proceeded through the r/o pathway to meleagrin as an end product, producing roquefortine D and C, glandicolines A and B, and meleagrin. *P. hirsutum* produced roquefortine D and C and glandicolines A and B with glandicoline B accumulating as the end product. Both *P. hordei* and *P. venetum* produced roquefortine D and C, with roquefortine C accumulating as an end product. Although *P. tulipae* produced roquefortine D and C, glandicolines A and B, and meleagrin, it differed from other species in the production of *epi*-neoxaline. The time scale study of *P. tulipae* strain IBT 3485 (Figure 3b) showed that the internal mycelial concentration of meleagrin was stable after 7 d, whereas *epi*-neoxaline accumulation continued to increase gradually as colony diameter increased. Only *epi*-neoxaline could be detected as diffusing into the agar adjacent to the colonies after 8 d (3–4 ng/cm^2 detected), corresponding to approx. 20 times less than the internal mycelial concentration. Interestingly, meleagrin did not diffuse into the media.

Regardless of the media type applied for growth, r/o pathway metabolite expression for all of the *Corymbifera* species was carried through to the respective end product (Figures 2a–f, 3a). Some variation in the production of the various pathway metabolites was present between the three strains surveyed for each species as demonstrated by the error bar length for several of the media. It is important to note that the colony fitness of the species strains upon the various media were approximately equivalent as judged by the amount of sporulation as well as colony diameter (although colonies developing on TUL, YO, and RO agars did tend to produce more biomass). Colonies developing on ginger agar were an exception as they had a smaller diameter compared to the others, yet were otherwise phenotypically comparable to the CYA controls.

In general, when the margin of error was taken into consideration, the average r/o pathway metabolite expression observed for the *Corymbifera* strains grown on the various media remained relatively constant as compared to expression on CYA. For *P. albocoremium*, a decreased average production of the intermediate roquefortine C was observed on GIN and RO media; however, production of the remaining intermediates and the pathway end product meleagrin was comparable to production on CYA (Figure 2a). For *P. allii*, a

decreased production of all r/o pathway intermediates and the end product meleagrin was observed from GIN and TUL agar extracts compared to CYA, RO, and YO agars (Figure 2b). An average production of all r/o pathway metabolites was observed for *P. radicicola* strains grown on the various plant-derived media compared to production on CYA (Figure 2c). Of all the plant media used for *P. hirsutum* strains, GIN agar resulted in a decreased production of glandicoline B compared to CYA, RO, and YO agars (Figure 2d). Strains of *P. hordei* showed a definite decrease in roquefortine D, even more so in roquefortine C, on all of plant-based media (Figure 2e). However, *P. venetum* strains had relatively consistent production of these compounds on all plant-based media (although slightly lower on ginger agar; Figure 2f). For *P. tulipae* strains, an overall reduced average production of *epi*-neoxaline was observed when strains were cultured on GIN, RO, and YO media compared to CYA; in contrast, an increased expression of meleagrin production was observed for TUL compared to CYA, GIN, and YO media (Figure 3a).

Stimulation of terrestric acid production was evident when molecular ion counts were compared between culture extracts from plant-derived media and CYA (Figure 4). Terrestric acid stimulation was most pronounced for *P. venetum* grown on YO, RO, and TUL agars. For *P. hirsutum*, terrestric acid production was also stimulated on YO, RO, and TUL agars. In the case of *P. radicicola* and *P. tulipae* strains, terrestric acid stimulation was pronounced on TUL agar; however, stimulation did occur on YO and RO agars. Terrestric acid stimulation for *P. hordei* strains was noted from YO and RO agars. For all of the species, only minor terrestric acid stimulation occurred on GIN agar compared to the other plant-derived media. The greatest production stimulation occurred from *P. venetum* strains. Terrestric acid production for *P. albocoremium* and *P. allii* strains did not occur on CYA or the other plant extract agars.

With the exception of *P. hordei*, all of the ser. *Corymbifera* series species produced extensive rot (root disk covered in sporulating mycelium) in yellow onion bulbs after 3-wk incubation. Internal and external tissue necrosis was most extensive in *P. albocoremium*, *P. allii*, *P. hirsutum*, and *P. venetum* samples (with *P. hirsutum* and *P. venetum* being most extensive) and characterized by total necrosis of the root disk and meristematic region, tissue discoloration and rot of the outerleaf, first leaves, and leaf initials accompanied by pronounced sporulation of the bulb cavity (Figure 5a, d). Although the external symptoms of the *P. tulipae* and *P. radicicola* infection were similar in appearance, the internal decay caused by these strains was limited to necrosis and sporulation of the root disk, and meristematic tissue with the discoloration of first leaf and leaf initials (Figure 5b, e). For the *P. hordei* isolates, only minor sporulation was apparent, extending to cover one third of the root disk surface and internal discoloration limited to the root disk region (Figure 5c, f). In addition to the aforementioned compounds, the *Corymbifera* species we tested

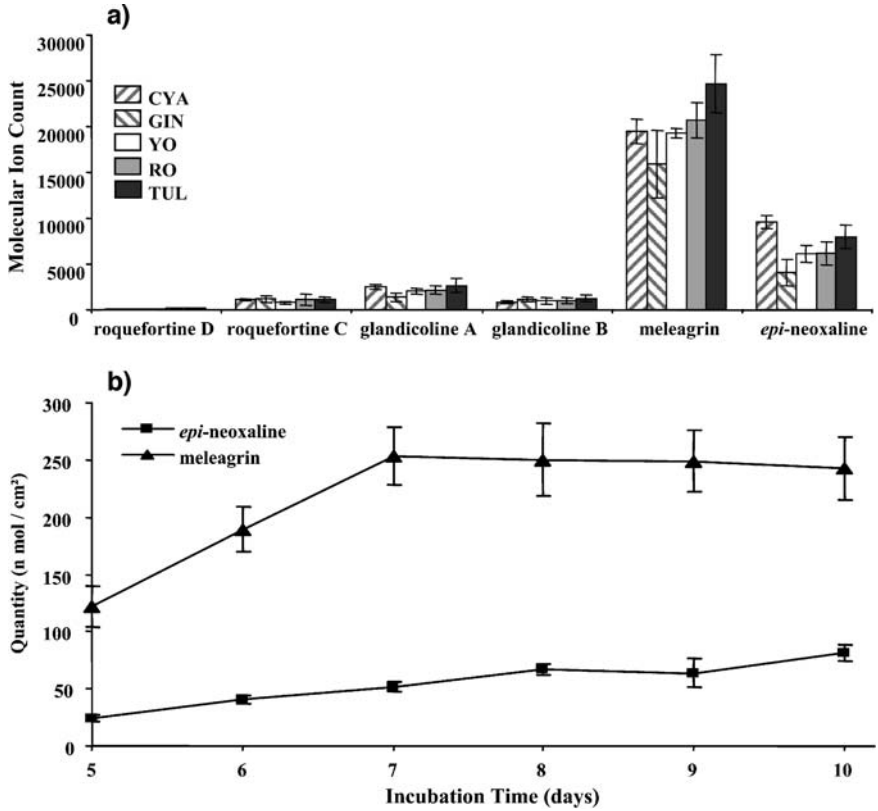

FIG. 3. Summary of roquefortine/*epi*-neoxaline biogenesis pathway metabolites produced by strains of *P. tulipae*. (a) Average molecular ion count calculated for *P. tulipae* strains grown on Czapek yeast autolysate (CYA), ginger (GIN), yellow onion (YO), red onion (RO), and tulip (TUL) agars. (b) Production of meleagrin and *epi*-neoxaline by *P. tulipae* strain IBT 3458 grown on CYA from days 5 to 10.

produced a variety of secondary metabolites (Table 1). None of the *Corymbifera* species we tested produced a rot on *Z. officinale* tubers.

DISCUSSION

Most fungi can absorb and utilize inorganic nitrogen (nitrates and ammonium) as well as organic nitrogen-containing compounds (proteins and amino acids) as a source of nitrogen. This is essential for the biosynthesis of complex molecules (i.e., amino acids, proteins, and nucleic acids); however, larger

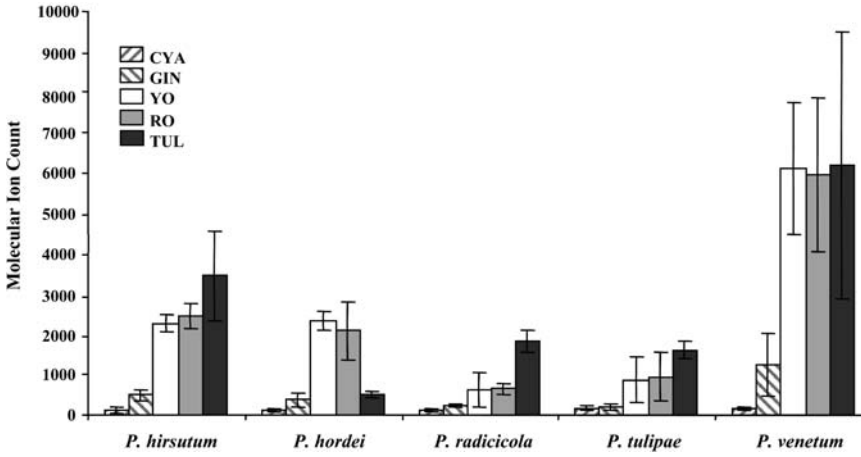

FIG. 4. Average molecular ion count of terrestric acid produced by *Corymbifera* strains grown on Czapek yeast autolysate (CYA), ginger (GIN), yellow onion (YO), red onion (RO), and tulip (TUL) agars.

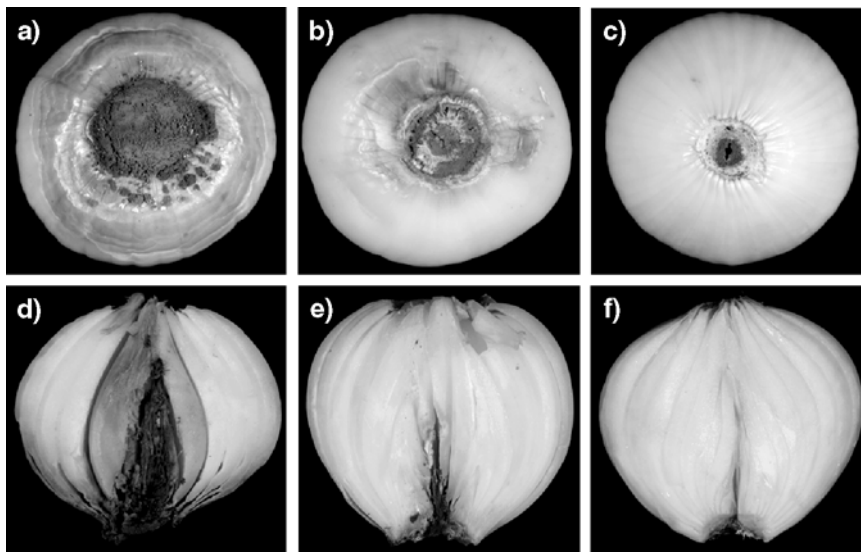

FIG. 5. Pathological symptoms displayed by *Allium cepa* bulbs infected with *Corymbifera* strains following 3-wk incubation in the dark at 20°C. (a–c) Root disk region of *A. cepa* bulbs inoculated with (a) *P. hirsutum*, (b) *P. radicicola*, and (c) *P. hordei*. (d–f) Bisection of *A. cepa* bulbs inoculated with (d) *P. hirsutum*, (e) *P. radicicola*, and (f) *P. hordei*.

extracellular polymers such as proteins must first be broken down by proteases into smaller units prior to transport. With the exception of *P. hordei*, all of the ser. *Corymbifera* species do not produce exogenous proteases and are, therefore, unable to breakdown extracellular proteins into transportable amino acids (Overy et al., 2005b). Perhaps in compensation, roquefortine C (and subsequent products of the r/o pathway) may serve as exogenous nitrogen sources. The fact that these compounds can enter and exit mycelia via both energy-independent and succinate energy-dependent mechanisms (Kulakovskaya et al., 1997) may make them particularly advantageous. In this case, nitrogen would not have to be actively pumped into mycelia. Following colonization and growth, r/o biosynthesis pathway intermediates and products accumulated in mycelia and bulb tissue infected by *P. albocoremium*, *P. allii*, *P. hirsutum*, *P. radicicola*, and *P. venetum* strains. Once accumulated in colonized tissue, these alkaloids could subsequently be taken up into growing hyphae and/or germinating conidia, metabolized into primary metabolites, and incorporated into proteins (Reshetilova et al., 1986; Reshetilova and Kozlovsky, 1990) to facilitate colonial expansion. Moreover, the antimicrobial properties reported for roquefortine C may help prevent the utilization of accumulated reserves of this amino acid resource by bacterial secondary infectors.

All of the ser. *Corymbifera* species produce and excrete organic acids: *P. hirsutum*, *P. hordei*, *P. radicicola*, *P. tulipae*, and *P. venetum* produce terrestric acid, whereas *P. albocoremium* produces barceloneic acids A and B, and *P. allii* produces fulvic acid (Overy and Frisvad, 2003, Frisvad and Samson, 2004); all of which are confirmed to be produced *in planta* in infected *A. cepa* bulbs. While r/o metabolites are deposited during colony development, accumulation of the *Corymbifera* organic acids *in planta* would lower the pH of the plant cellular environment. The pH optimum for roquefortine C excretion is 6–7 and uptake is 4.5 for *P. crustosum* strains (Kulakovskaya et al., 1997). Acidification of the infected region during colony development would therefore facilitate the uptake and subsequent metabolism of deposited r/o pathway alkaloids into germinating conidia and developing hyphae. Regional acidification would have an additional bactericidal effect, preventing the growth and competition of host-associated bacteria.

Several additional fungal secondary metabolites were produced *in planta* by *Corymbifera* strains. These compounds may act synergistically with terrestric acid and the r/o metabolites to improve the pathogenic ability and competitive fitness of the infecting strains. In addition to terrestric acid and roquefortine C, both cyclopenin and viridicatin have antibacterial properties (Bracken et al., 1954; Taniguchi and Satomura, 1970). Viridicatin also possesses phytotoxic properties, as is reported to inhibit root and shoot development in rice seedlings (Taniguchi and Satomura, 1970). Viridicatin may also act synergistically with the protein farnesyl transferase (PFTase)

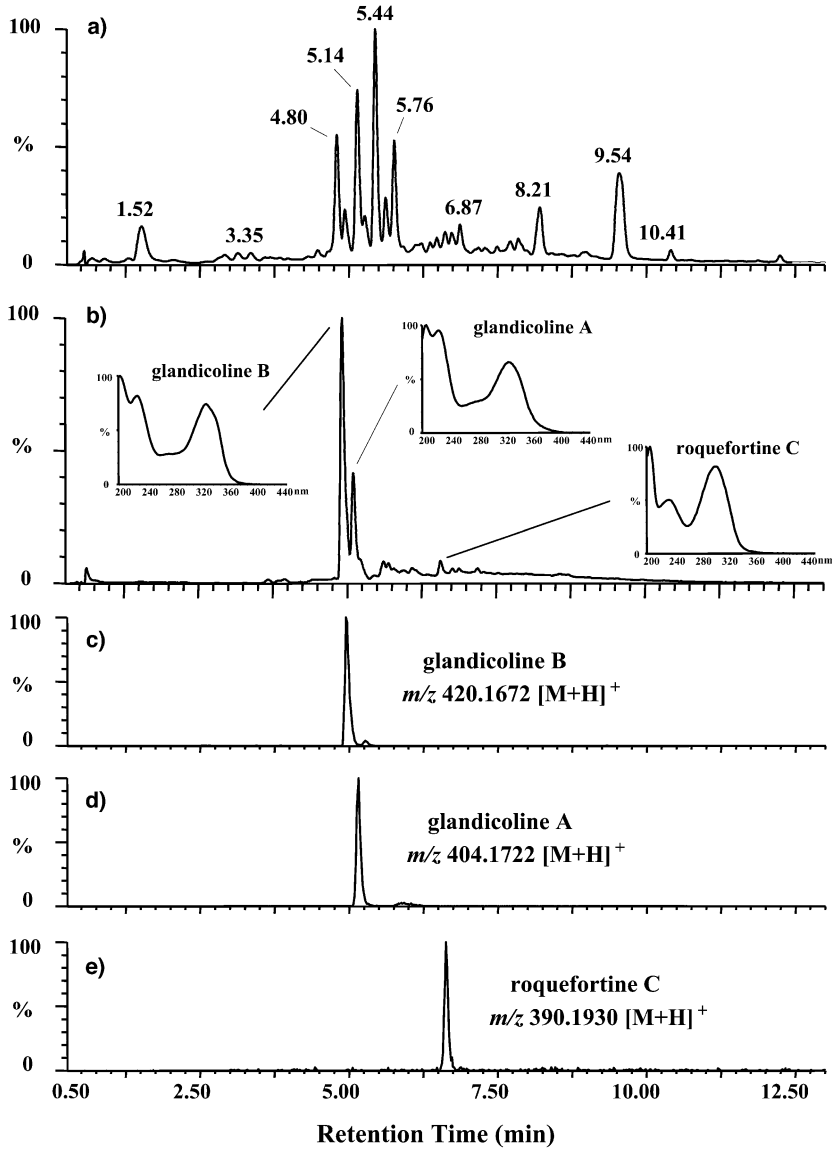

FIG. 6. LC-UV-MS traces demonstrating the preferential separation of r/o pathway metabolites from a *P. hirsutum*-infected *A. cepa* tissue extract: (a) crude extract, UV trace 300 nm (*y*-axis = relative absorbance); (b) following SPE, UV trace 300 nm (*y*-axis = relative absorbance); (c–e) accurate mass confirmation of $[M + H]^+$ ion using 100 ppm search window (*y*-axis = relative ion count).

inhibitors barceloneic acid A (Jayasuriya et al., 1995) and andrastin A (Omura et al., 1996) to weaken plant cell response to fungal infection (Overy et al., 2005c). PFTase inhibitors disrupt cellular signaling and division in developing plant cells (Morehead et al., 1995; Qian et al., 1996) resulting in decreased growth rates and stunted plants (Running et al., 2004). Compactin, produced *in planta* by *P. hirsutum* strains, is a well-known hydroxymethylglutaryl (HMG)–CoA reductase inhibitor, an enzyme responsible for the production of mevalonic acid, the biochemical building block involved in terpene biosynthesis. Although compactin has not been examined for phytotoxicity, lovastatin (a structurally similar compound) does inhibit cellular development in cultured carrot cells (Chen et al., 1987). Both compactin and lovastatin have reported antifungal properties (Brown et al., 1976; Huang et al., 1999), attributed to the inhibition of ergosterol biosynthesis in other fungi (Huang et al., 1999).

Terrestric acid production is stimulated by plant-based media. This compound is formed from the condensation of a C_4-TCA cycle intermediate with the methylene group of a fatty acid (Turner, 1971). Production of this fungal metabolite is, therefore, directly dependent upon the catabolism of glucose or other simple sugars to pyruvate during glycolysis. Ser. *Corymbifera* species produce an increased quantity of extracellular cellulases and hemicellulases as compared to other *Penicillia* (Overy et al., 2005b). This characteristic extracellular enzyme expression is ideal for the liberation and mycelial uptake of simple sugars through the enzymatic degradation of plant cell wall structural polymers (that are present in high concentrations in *A. cepa* cells; Mankarios et al., 1980). We conclude, therefore, that increased catabolic rates in the *Corymbifera* strains we tested were responsible for stimulating the production of TCA intermediates and subsequently terrestric acid production following an increased rate of respiration.

The SPE technique employed in this survey was successful in the preferential separation of the targeted r/o pathway metabolites from the various complex matrices (Figure 6), allowing the profiling of the r/o biosynthesis pathway in each of the ser. *Corymbifera* species. The r/o pathway profiles produced for five out of the seven species were in accordance with literature reports, with *P. hirsutum* and *P. tulipae* as exceptions (Frisvad and Filtenborg, 1989; Overy and Frisvad, 2003). *P. hirsutum* has been reported in the past to produce meleagrin based on TLC and HPLC-DAD analysis (Frisvad and Filtenborg, 1989); however, the results here based on preferential purification by SPE followed by LC-UV-MS analysis clearly demonstrates that glandicoline B is the r/o pathway end product for this species rather than meleagrin. Results from SPE profiling for *P. tulipae* allowed for the structural confirmation of *epi*-neoxaline (unpublished data), a metabolite previously reported as being similar to neoxaline and produced by *P. tulipae* (Overy and Frisvad, 2003). As *epi*-neoxaline rather than meleagrin was excreted by the *P. tulipae* strain IBT 3458 into the surrounding media, and because of the

progression of structural differences between glandicoline B, meleagrin, and *epi*-neoxaline, we propose that *epi*-neoxaline is the end product in the roquefortine/*epi*-neoxaline biogenesis pathway for *P. tulipae* (Figure 1).

In general, when cultured *in vitro*, terrestric acid production was stimulated, whereas the overall production of r/o metabolites was comparable to the CYA controls for most of the *Corymbifera* grown on most of the plant tissue agars. However, all of the ser. *Corymbifera* species demonstrated only minor stimulation of terrestric acid and a decreased overall production of r/o metabolites when cultivated on GIN media. Colony size on GIN media was reduced in comparison to CYA controls; however, sporulation and general colony morphology were comparable. None of these species have been reported to produce a rot on ginger and failed to grow when inoculated. An inability to grow on this substrate *in planta* may have resulted in decreased overall production of r/o metabolites when cultured *in vitro* on GIN media. This trend was also apparent for *P. hordei* when cultured *in vitro* and *in planta* on *A. cepa* (yellow onion), and for *P. allii* cultured on TUL agar. *P. hordei* did not cause rot in infected *A. cepa* bulbs, and *P. allii* is nonpathogenic on tulip bulbs (Overy et al., 2005a). With the exception of *P. hordei*, all of the ser. *Corymbifera* species are capable of producing rot on *A. cepa* (yellow onion), accompanied by the *in planta* production of terrestric acid and associated r/o pathway metabolites. The rot produced by *P. radicicola* and *P. tulipae* strains was less severe compared to the remainder of the infectious *Corymbifera*, which corresponds to a reduced production of r/o pathway end products when strains were cultured *in vitro* on YO agar. We propose that *in vitro* activities of fungi on plant-based media are predictive of *in planta* metabolite expression and pathogenic activity.

Acknowledgements—Research activities were funded by the Danish Research Council under the project "Functional biodiversity in *Penicillium* and *Aspergillus*" (grant No. 9901295). The authors would like to acknowledge Hanne Jacobsen for MS technical support, Xuiping Liu for laboratory assistance with the processing of the SPE samples, and Richard Phipps and Kristian Karlshøj for bulb inoculation.

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