PERGAMON

Phytochemistry 62 (2003) 147–153

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Sterol composition of mycelia of the plant pathogenic ascomycete *Leptosphaeria maculans*

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Received 25 July 2002; received in revised form 18 September 2002

Abstract

Analysis of sterols in mycelia of the ascomycete, *Leptosphaeria maculans* by gas chromatography–mass spectrometry revealed that ergosterol comprised 95% of the total sterols, with eight other sterols comprising the remaining 5%. Six of these latter sterols were putative precursors of ergosterol and their presence suggested a pathway for ergosterol biosynthesis in this fungus. Ergosterol biosynthesis in fungi is inhibited by the triazole antifungal agent flutriafol. When *L. maculans* was grown in the presence of flutriafol, ergosterol content decreased while two 14α-methylated sterols, 24-methylene dihydrolanosterol and obtusifoliol, accumulated.

Keywords: *Leptosphaeria maculans*; Ascomycota; Plant pathogen; Gas chromatography–mass spectrometry; Sterols; Triazole fungicide; Flutriafol

1. Introduction

The Dothideomycete *Leptosphaeria maculans* (Desm.) Ces. Et de Not. [anamorph: *Phoma lingum* (Tode ex Fr.) Desm.] causes blackleg disease of oilseed *Brassica* spp. such as *B. napus* (canola), and results in severe yield losses worldwide (Howlett et al., 2001). Triazole fungicides are used to control *L. maculans*, and inhibit the ergosterol biosynthetic pathway in fungi such as *Neurospora crassa* (Connerton et al., 1991) and *Pyrenophora teres* (Loeffler and Hayes, 1992).

Ergosterol is the predominant sterol of most fungi, where its primary function is maintenance of membrane structure and function (Weete, 1989). In filamentous fungi, ergosterol biosynthesis begins with the cyclization of squalene to produce lanosterol. Subsequent reactions include: methylation at C-24, demethylations at C-14 and C-4, double bond transformations in the nucleus ($\Delta^8 \rightarrow \Delta^7 \rightarrow \Delta^{5,7}$), saturation of the C-24(28) double bond and formation of a C-22(23) double bond in the side chain (Mercer, 1984; Kato, 1986). Reactions usually occur in this order although variations are possible, particularly in the sequence of the final three double bond transformations (Kato, 1986; Mercer, 1984).

Triazole fungicides inhibit 14α-demethylase which catalyses the C-14 demethylation of 24-methylene dihydrolanosterol in filamentous fungi. The fungicide binds to the active site of 14α-demethylase and interacts with the sixth coordinate position of the haem prosthetic group of this cytochrome P-450 enzyme. This position is normally occupied by activated oxygen, which is required for hydroxylation of the 14α-methyl group on the developing sterol molecule (Sanglard et al., 1998). Inhibition of 14α-demethylase in fungi results in the accumulation of C-14 methyl sterols and a depletion of ergosterol (Kelly et al., 1989). In other Dothideomycetes such as *Pyrenophora teres* (Loeffler and Hayes, 1992) and *Septoria tritici* (Joseph-Horne et al., 1996) the enzyme C-4 demethylase then converts the accumulated C-14 methyl sterols to obtusifoliol and 14α-methyl fecosterol. In *Aspergillus fumigatus* and *Saccharomyces cerevisiae* (Venkateswarlu and Kelly, 1996; Kelly et al., 1995), further metabolism by the ergosterol biosynthetic enzyme C-5,6 desaturase results in production of the toxic sterol 14α-methyl-3,6-diol.

Despite the agricultural importance of *L. maculans* and the extensive use of triazole fungicides to control blackleg disease, there is no knowledge of the ergosterol biosynthetic pathway in this pathogen. In this paper we describe the major sterols in *L. maculans*, and use this information to deduce the pathway of ergosterol biosynthesis and the site of action of flutriafol.

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0031-9422/03/$ - see front matter
PII: S0031-9422(02)00505-8

Table 1
Sterol composition and ergosterol content in mycelia of *Leptosphaeria maculans* isolate M1 grown in the absence or presence of flutriafol at the EC_{50} concentration[a]

Sterol	Flutriafol	
	−	+
	(mol%)	
24-Methylene dihydrolanosterol (**1**)	Tr	3
4,4-Dimethyl fecosterol (**2**)	Tr	Tr
4-Methyl fecosterol (**3**)	1	1
Fecosterol (**4**)	1	1
Episterol (**5**)	2	1
Ergosta-7,22-dienol (**6**)	Tr[b]	Tr
Ergosterol (3β-hydroxy) (**7**)	95	91
Ergosta-5,7,9(11),22-tetraenol (**8**)	Tr	Tr
Epiergosterol (3α-hydroxy) (**9**)	1	1
Obtusifoliol (**10**)[c]	—[c]	2
Ergosterol (μg mg^{-1} dry wt mycelia)	16.9[d]	11.9[d]

[a] EC_{50} is 1.5 + 0.24 μM.
[b] Tr, trace.
[c] Not detected.
[d] ±1.4.

2. Results and discussion

2.1. Sterol composition of L. maculans and deduced ergosterol biosynthetic pathway

The sterol composition of *L. maculans* mycelia grown in 10% Campbells V8 juice was determined by gas chromatography–mass spectrometry (GC–MS). Ergosterol comprised 95% of the total sterol content at a concentration of 16.9 μg mg^{-1} dry wt mycelia (Table 1). These values were much higher than those reported for other Dothideomycetes; for example, in *P. teres* and *Septoria tritici* the ergosterol content was 58% and between 47–60% of total sterols, respectively (Loeffler and Hayes, 1992; Joseph-Horne et al., 1996). In contrast, in *Venturia inaequalis* the ergosterol content was 3% of total sterols (Shirane et al., 1996). The concentration of ergosterol was only reported for *P. teres* and this was 2.8 μg mg^{-1} dry wt mycelia.

In addition to ergosterol, eight other sterols which were identified using a mass spectral database or by comparison with published ion fragmentation data, were present in mycelia of *L. maculans* (Table 1): 24-methylene dihydrolanosterol (**1**), 4,4-dimethyl fecosterol (**2**), 4-methyl fecosterol (**3**), fecosterol (**4**), episterol (**5**), ergosta-7,22-dienol (**6**), ergosta-5,7,9(11),22-tetraenol (**8**) and epiergosterol (**9**). The relative retention times and ion fragmentation patterns for these sterols are presented in Table 2.

The former six sterols are typical intermediates of the ergosterol biosynthetic pathway of filamentous fungi. Their presence suggests a scheme for ergosterol biosynthesis in *L. maculans* (Fig. 1). The presence of 24-methylene dihydrolanosterol (**1**), a 4,4,14-trimethyl sterol which is unsaturated at C-24, indicates that alkylation at C-24 to form a $\Delta^{24(28)}$ methylene precedes all C-4 and C-14 demethylations. Sequential demethylations at C-14 and C-4 are represented by 4,4-dimethyl fecosterol (**2**), 4-methyl fecosterol (**3**) and fecosterol (**4**),

Table 2
Ion species in the mass spectral data of sterols (as TMSi ethers) isolated from mycelia of control and flutriafol-treated *Leptosphaeria maculans*. (Intensities as a percent of the base peak are in parentheses)

Sterol number	1	2	3	4	5	6	7	8	9	10
RR_t[a] (min)	4.58	5.11	4.04	2.69	3.21	2.44	2.16	1.54	1.69	3.71
Fragmentation										
$[M]^+$	512 (35)	498 (60)	484 (100)	470 (63)	470 (12)	470 (53)	468 (29)	466 (8)	468 (29)	498 (54)
$[M]^+ - 15$ ($-CH_3$)	497 (32)	483 (13)	469 (43)	455 (63)	455 (24)	455 (40)	453 (3)	451 (3)	453 (6)	483 (92)
$[M]^+ - 90$ (–TMSiOH)	–	408 (22)	394 (29)	380 (23)	–	380 (12)	378 (15)	376 (38)	378 (6)	–
$[M]^+ - 105$ ($-CH_3$–TMSiOH)	407 (100)	393 (54)	379 (30)	365 (40)	365 (12)	–	363 (100)	361 (8)	363 (100)	393 (100)
$[M]^+ - 131$ (–TMSiO=CH–CH_2–CH_3[b])	–	–	353 (8)	–	–	–	337 (64)	–	337 (69)	–
$[M]^+ - 129$ ($-C_{20}$–C_{28})	383 (2)	–	–	343 (100)	–	–	–	–	–	–
$[M]^+ - 127$ ($-C_{20}$–C_{28}–2H)	–	–	357 (13)	–	343 (100)	343 (97)	–	–	–	–
$[M]^+ - 215$ ($-C_{20}$–C_{28}–TMSiOH)	297 (2)	–	269 (21)	355 (18)	255 (19)	255 (100)	253 (19)	251 (100)	253 (21)	–
$[M]^+ - 217$ ($-C_{20}$–C_{28}–TMSiOH–2H)	–	–	–	253 (40)	253 (14)	253 (35)	251 (6)	249 (17)	251 (7)	–
$[M]^+ - 241$ ($-C_{20}$–C_{28}–TMSiOH–C_2H_2)	–	–	243 (16)	229 (32)	–	229 (40)	227 (5)	225 (7)	227 (5)	–
$[M]^+ - 242$ [$-(C_{20}$–C_{28} + H)–TMSiOH–C_2H_2]	–	257 (26)	–	–	–	–	226 (5)	224 (4)	226 (3)	–
$[M]^+ - 243$ [$-(C_{20}$–C_{28} + 2H)–TMSiOH–C_2H_2]	–	–	241 (38)	227 (40)	227 (14)	–	225 (5)	223 (10)	225 (4)	–
$[M]^+ - 257$ ($-C_{20}$–C_{28}–TMSiOH–42[c])	255 (13)	255 (22)	227 (58)	213 (58)	213 (19)	213 (35)	201 (18)	209 (15)	211 (16)	241 (26)
Additional ions	241 (22)	153 (100)	213 (40)							309 (24)
			337 (18)							227 (40)
			297 (11)							201 (24)

Sterols are identified as **1**, 24-methylene dihydrolanosterol; **2**, 4,4-dimethyl fecosterol; **3**, 4-methyl fecosterol; **4**, fecosterol; **5**, episterol; **6**, ergosta-7,22-dienol; **7**, ergosterol; **8**, ergosta-5,7,9(11),22-tetraenol; **9**, epiergosterol; **10**, obtusifoliol.
[a] RR_t, retention time relative to epicoprostanol.
[b] CH–CH_2–CH_3, from ring A of nucleus?
[c] 42, loss of C_3H_6 from C_{15} to C_{17}.

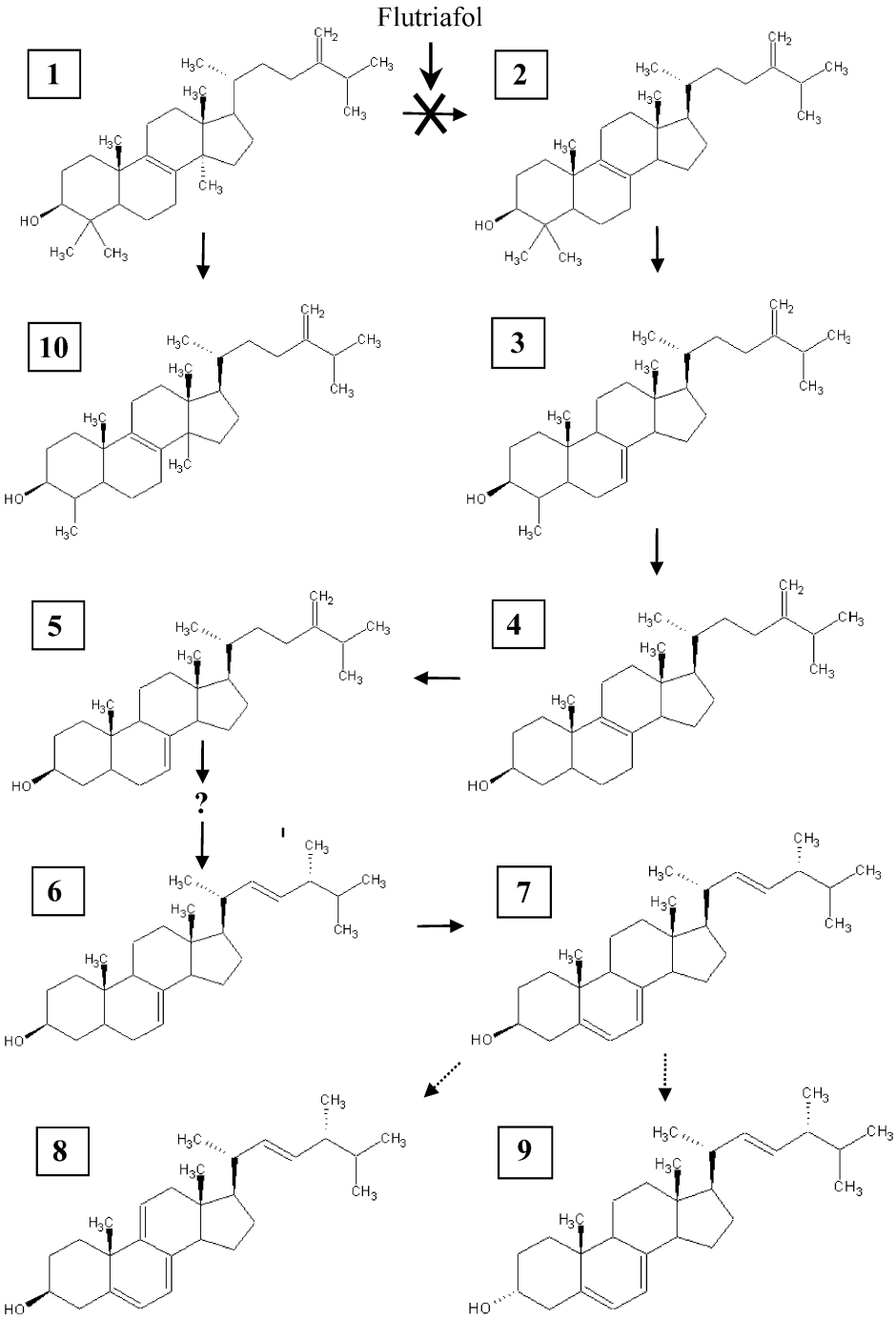

Fig. 1. A proposed model for ergosterol biosynthesis in *Leptosphaeria maculans*. Sterols from vegetative mycelia of *L. maculans* were analysed by GC–MS and a pathway for ergosterol biosynthesis was deduced using the model for filamentous fungi described by Mercer (1984) and Kato (1986). Sterol code: **1**, 24-methylene dihydrolanosterol; **2**, 4,4-dimethylfecosterol; **3**, 4-methylfecosterol; **4**, fecosterol; **5**, episterol; **6**, ergosta-7,22-dienol; **7**, ergosterol; **8**, ergosta-5,7,9(11),22-tetraenol; **9**, epiergosterol; **10**, obtusifoliol. **?** represents a probable intermediate which was not detected. ·····▶ signifies possible downstream metabolic processing. Flutriafol (indicated by **X**) caused a decrease in ergosterol and an accumulation of 24-methylene dihydrolanosterol and obtusifoliol.

while $\Delta^8 \rightarrow \Delta^7$ isomerisation is represented by the presence of fecosterol and episterol (**5**). The presence of ergosta-7,22-dienol (**6**) suggests that C-24(28) saturation and C-22(23) desaturation precede the $\Delta^7 \rightarrow \Delta^{5,7}$ transformation in *L. maculans*. All ergosterol precursors detected in *L. maculans* (sterols **1**–**6**) have also been found in *P. teres* (Loeffler and Hayes, 1992) and other filamentous fungi such as *Eutypa lata* (Sordariomycetes) (Chapuis et al., 1996) and *Botrytis cinerea* (Leotiomycetes) (Loeffler and Hayes, 1990).

Typical pathway intermediates that were not detected in *L. maculans* included lanosterol and an intermediate

for the conversion of episterol to ergosta-7,22-dienol. Diversity in sterol composition and ergosterol content among fungi is partly a reflection of variation among species and isolates. It is also due to the media composition, growth temperature, growth stage examined and to the sterol extraction method used (Losel, 1988; Elliott, 1977). However, the media composition did not necessarily influence sterol content of *L. maculans,* since mycelia grown in liquid Czapek-Dox media containing 0.1% yeast extract had an identical sterol composition to that grown in 10% Campbell's V8 juice (data not shown).

Additional sterols detected in mycelia of *L. maculans* were ergosta-5,7,9(11),22-tetraenol (**8**), and epiergosterol (**9**). Ergosta-5,7,9(11),22-tetraenol has been found in *B. cinerea, P. teres* (Loeffler and Hayes, 1990) and *E. lata* (Chapuis et al., 1996) and was hypothesized to be a dehydrogenated metabolite of ergosterol used for storage or downstream metabolic processes (Atherton et al., 1972). Epiergosterol was identified in *L. maculans* according to its identical properties to this molecule in *S. tritici* where it was predicted to be an ergosterol isomer that differs at C-3 (3α hydroxyl instead of 3β) (Joseph-Horne et al., 1996). In *L. maculans*, the formation of epiergosterol appears to occur after that of ergosterol, since isomers of other ergosterol precursors were not detected. This is the only known report of epiergosterol in any organism other than *S. tritici.*

2.2. Effects of flutriafol on sterol composition and ergosterol content of L. maculans

The EC_{50} concentration for flutriafol was determined to be 1.5 μM (±0.24) for isolate M1 and 3 μM for isolate 60.1. Growth of both isolates was completely inhibited at flutriafol concentrations of 10 μM and higher (Fig. 2). The well-characterised isolate, M1 (Cozijnsen et al., 2000), was subsequently grown under two conditions to examine the effects of flutriafol on sterol composition. In the first, flutriafol was included in the growth media at the EC_{50} concentration, and cultures were harvested after 7 days. In the second, flutriafol was added at a final concentration of 15 μM (10 times the EC_{50} concentration) to cultures that had been grown for six days and mycelial samples were removed at 0, 4 and 24 h following this addition. The sterol content of mycelia from both treatments was analysed by GC–MS.

Following growth in both conditions, the ergosterol (**7**) and episterol (**5**) content of mycelia decreased, while the C-14 methyl sterols 24-methylene dihydrolanosterol (**1**) and obtusifoliol (**10**) content increased. The relative retention time and ion fragmentation pattern for obtusifoliol are in Table 2. After growth in the presence of flutriafol for 7 days at the EC_{50} concentration, the ergosterol composition of mycelia decreased by 4% to comprise 91% of the total sterol content, and decreased

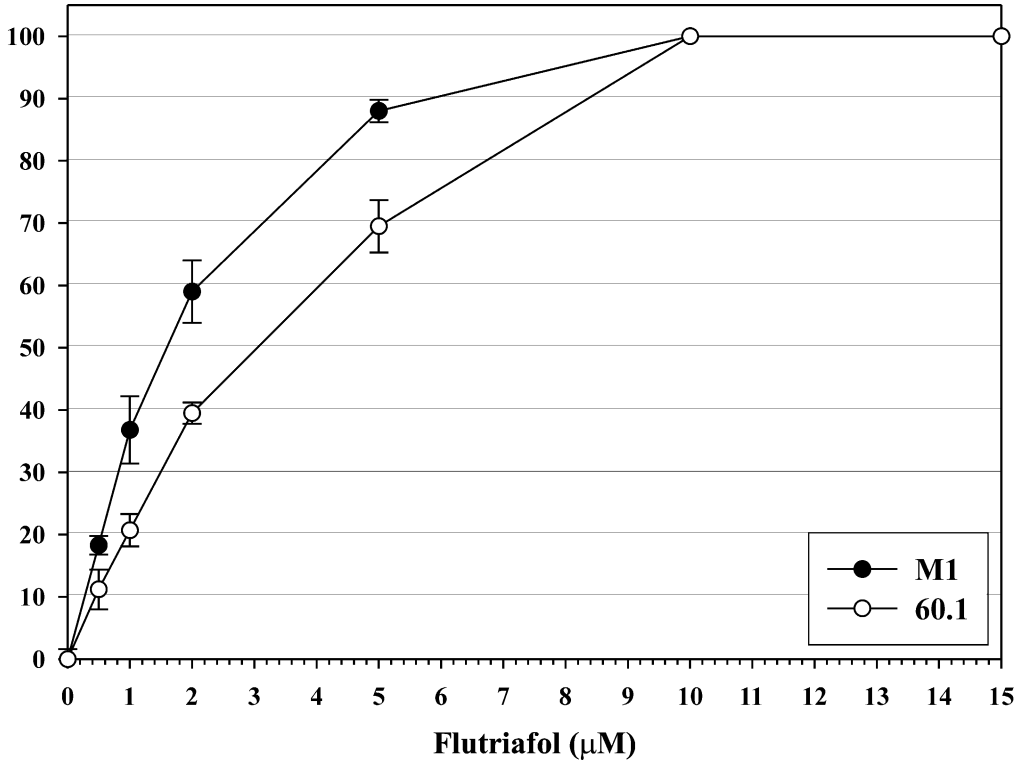

Fig. 2. Effect of flutriafol concentration on radial mycelial growth of *Leptosphaeria maculans* isolates M1 and 60.1. Agar discs containing each isolate were grown for 8 days at 22 °C on solid media amended with flutriafol at 6 different concentrations.

Table 3
Sterol composition and ergosterol content in mycelia of *Leptosphaeria maculans* isolate M1 following treatment with flutriafol at ten times the EC_{50} concentration[a]

Sterol	Hours after addition of flutriafol			
	0	4	24	24 (CONT [b])
	(mol%)			
24-Methylene dihydrolanosterol (**1**)	Tr	3	11	Tr
4,4-Dimethyl fecosterol (**2**)	Tr	Tr	Tr	Tr
4-Methyl fecosterol (**3**)	1	1	Tr	–
Fecosterol (**4**)	1	1	1	1
Episterol (**5**)	3	2	1	3
Ergosta-7,22-dienol (**6**)	Tr[c]	Tr	Tr	Tr
Ergosterol (3β-hydroxy) (**7**)	94	92	85	93
Ergosta-5,7,9(11),22-tetraenol (**8**)	Tr	Tr	Tr	Tr
Epiergosterol (3α-hydroxy) (**9**)	1	1	1	1
Obtusifoliol (**10**)	–	Tr	1	–
Ergosterol (μg mg^{-1} dry wt mycelia)	22.6[d]	18.2[d]	16.0[d]	22.2[d]

[a] Ten times the EC_{50} concentration = 15 μM.
[b] CONT, untreated control.
[c] Tr, trace
[d] ±1.6.

from 16.9 to 11.9 μg mg^{-1} dry wt mycelia (Table 1). During this time 24-methylene dihydrolanosterol (**1**) and obtusifoliol (**10**) accumulated to comprise 3 and 2%, respectively, of the total sterol content.

When *L. maculans* was exposed to flutriafol at 10 times the EC_{50} concentration for varying periods, ergosterol composition decreased from 94 to 85% 24 h after addition of this compound (Table 3). The amount of ergosterol decreased during this time from 22.6 to 16.0 μg mg^{-1} dry wt mycelia. Four hours after the addition of flutriafol, 24-methylene dihydrolanosterol accumulated to 3%, and then further increased to comprise 11% of the total sterol content at 24 h. Obtusifoliol was not detectable until 24 h after addition of flutriafol, when it comprised 1% of the total sterol content. The sterol composition of an untreated control was identical to that of cultures prior to addition of flutriafol (Table 3).

The finding that flutriafol caused a decrease in ergosterol content and an accumulation of two C-14 methyl sterols in *L. maculans* is consistent with the reported mode of action of this fungicide, i.e. inhibition of 14α-demethylation. The absence of 14α-methylfecosterol and 14α-methyl-3,6-diol from flutriafol-treated mycelia suggested that enzymes in the ergosterol biosynthetic pathway of *L. maculans* are sensitive to the retention of methyl groups at C-4 or C-14 on 24-methylene dihydrolanosterol and obtusifoliol. As has been proposed for other fungi by Vanden Bossche (1989), the altered sterol composition following triazole treatment of *L. maculans* is likely to hinder the formation of normal membranes, thus explaining the inhibition of growth observed in radial growth inhibition assays (Fig. 2) and *in planta* (Ballinger et al., 1988). It remains to be elucidated whether triazole-mediated growth inhibition of fungi is primarily attributable to ergosterol depletion or to the accumulation of abnormal sterols (Buchenauer, 1987; Quail et al., 1993; Venkateswarlu and Kelly, 1996; Loeffler et al., 2000). Therefore, further experimentation is required to determine the predominant sterol effector of flutriafol-mediated growth inhibition in *L. maculans*.

This is the first report of the sterol composition and deduced pathway for ergosterol biosynthesis in this economically important plant pathogenic fungus. Such information has potential application in the analysis of *L. maculans* isolates that are resistant to fungicidal inhibitors of ergosterol biosynthesis. Furthermore, ergosterol may be used as a chemical indicator to quantify *L. maculans* biomass in infected host plants and seed batches.

3. Experimental

3.1. General experimental procedures

Flutriafol was obtained from Syngenta, USA (as Impact in-furrow®). Ergosterol and epicoprostanol were from Sigma, USA. The derivatising reagent, BSTFA (+1%TMCS) [*N,O*-bis (trimethylsilyl) trifluoroacetimide + trimethylchlorosilane], was purchased from Pierce, USA. GC–MS was performed using a Hewlett-Packard HP 6890 gas chromatograph equipped with an autosampler and Hewlett-Packard 5973 mass selective detector. The GC column was WCOT

(CP-SIL5: 30 m by 0.25 mm internal diameter with a film thickness of 0.25 μm) and was obtained from Chrompak, Varian, Australia. A Wiley 275 Mass Spectral database was used to analyse mass spectral data.

3.2. Fungal culturing

A well-characterized isolate of *L. maculans*, M1 (Cozijnsen et al., 2000), was grown on 10% Campbell's V8 juice agar under constant white light fluorescent light at 22 °C. Pycnidiospores (asexual spores) were harvested from sporulating cultures, inoculated into 50 ml of 10% V8 juice at a concentration of 2×10^6 spores per ml and grown for 7 days at 22 °C in the dark without shaking. Mycelia were harvested by filtration through Miracloth (Calbiochem), rinsed with double distilled water, frozen and then freeze-dried.

Radial growth inhibition assays were used to determine the flutriafol EC_{50} concentration (effective concentration that inhibited growth by 50%) for this isolate and another, 60.1. A sterile hollow metal tube was used to cut agar discs (3.5 mm diameter) from the perimeters of cultures growing actively on 10% Campbell's V8 juice. These were placed mycelia-side down on 10% V8 juice agar plates amended with concentrations of flutriafol ranging from 0.5 to 15 μM. Six agar plugs were placed on each plate. The cultures were incubated in the dark at 22C and colony diameter was measured to the nearest 0.5 mm after 8 days. The assay was repeated three times for isolate M1 and once for isolate 60.1.

Two different treatments were used to examine the effect of flutriafol on sterol composition of mycelia from isolate M1. In the first treatment, pycnidiospores were inoculated into 10% V8 juice (in triplicate) as described above, then flutriafol was immediately added at the EC_{50} concentration. The cultures were grown for 7 days at 22 °C in the dark without shaking and the sterol content of mycelia was compared with that from three cultures that were not treated with flutriafol. In the second treatment, flutriafol was added at 10 times the EC_{50} concentration (15 μM) to three cultures that had been grown for 6 days. Mycelial samples were then removed 0, 4 and 24 h following this addition and the sterol content was compared with a culture that was not treated with flutriafol.

3.3. Sterol isolation and analysis by gas chromatography—mass spectrometry

Sterols were isolated from mycelia and analysed by GC–MS. Freeze dried mycelia (5 mg) from cultures grown in triplicate were ground in a mortar and pestle, the internal standard epicoprostanol (5 μg) was added and the mixture was saponified for 1 h at 70 °C with 5 ml ethanolic potassium hydroxide [10% KOH (w/v) in 60% aq. ethanol (v/v)], under nitrogen gas. Non-saponifiable lipids (which included sterols) were extracted with 5 ml hexane, evaporated to dryness using a nitrogen stream then silylated with BSTFA (+1% TMCS). Derivatised samples were evaporated to dryness, resuspended in hexane and then analysed by GC–MS. Two separate sterol extracts were made from each mycelial sample: one was resuspended in 100 μl of hexane (for spectral identification of minor peaks) the other was resuspended in 400 μl of hexane (for ergosterol quantification). Injections (1 μl) were made in split (10:1) mode and helium, at a flow rate of 1 ml/min was used as the carrier gas. The oven temp. was programmed to increase from 260 to 300 °C at a rate of 2 °C per min, which according to Quail et al. (1993), are suitable conditions for the detection of all fungal sterols produced during treatment with a triazole fungicide. The injector temp. was maintained at 240 °C and the interface temp. between the gas chromatograph and the mass spectrometer was held at 260 °C. Data were acquired in full scan mode (total ion chromatogram) to detect ions from m/z 40–550 using electron impact with an ionisation energy of 70 eV. Sterol components were identified by mass spectral fragmentation and retention time comparison with an authentic standard (for ergosterol), or by mass spectral matching with published data or the commercial library (other sterols). Except where indicated, all sterols had the 3β-hydroxy-5α-configuration. The following trivial names are used in the text: 4,4-dimethyl fecosterol, 4,4-methylergosta-8,24(28)-dienol; epiergosterol, 3α-ergosta-5,7,22-trienol; episterol, ergosta-7,24(28)-dienol; ergosterol, ergosta-5,7,22-trienol; fecosterol, ergosta-8,24(28)-dienol; lanosterol, 4,4,14α-trimethylcholesta-8,24-dienol; 4α-methylergosta-8,24(28)-dienol; 14-methyl fecosterol, 14α-methylergosta-8,24(28)-dienol; 24-methylene dihydrolanosterol, 4,4,14α-trimethyl-ergosta-8,24(28)-dienol; obtusifoliol, 4α,14α-dimethyl ergosta-8,24(28)-dienol. Ergosterol was quantified in mycelial extracts using the pre-determined response factor of 0.76 + 0.02 (mean of six replicates), with respect to the epicoprostanol internal standard. The response factor was linear in the ergosterol concentration range present in mycelia. To estimate the amount of ergosterol lost during the sterol extraction process, 6 or 12.5 μg of ergosterol standard was added to 5 mg dry wt of mycelia (in duplicate). The amount of ergosterol recovered in sterol extracts of these samples was 27% (2% error). Accordingly, all values for ergosterol concentration were multiplied by a factor of 3.7.

Acknowledgements

K.M.G. receives a student scholarship from the Victorian Department of Natural Resources and Environment. Research in B.J.H. and A.B. laboratories is funded by the Australian Grains Research and Development

Corporation and the Australian Research Council. The authors are grateful for expert advice provided by Zofia Felton and Drs Filomena Pettolino and Graeme Currie, School of Botany, the University of Melbourne.

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