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EFFECT OF TRIDEMORPH AND FENPROPIMORPH ON STEROL COMPOSITION IN FENUGREEK

CLAUDE CERDON,* ALAIN RAHIER,† MARYSE TATON† and YVES SAUVAIRE*

*Laboratoire de Recherche sur les Substances Naturelles Végétales, Université Montpellier II, place Eugène Bataillon, 34095 Montpellier Cédex 5, France; †Institut de Biologie Moléculaire des Plantes, Département d'Enzymologie Cellulaire et Moléculaire, Institut de Botanique, 28 rue Goethe, 67083 Strasbourg Cédex, France

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Key Word Index—*Trigonella foenum-graecum* L.; seedlings; cell suspensions; tridemorph; fenpropimorph; sterol biosynthesis inhibitor; 9β,19-cyclopropyl sterols; Δ^8-sterols; $\Delta^{8,14}$-sterols.

Abstract—The addition of tridemorph (125 μM) during growth of fenugreek seedlings or the addition of fenpropimorph (125 μM) in the culture medium of fenugreek cell suspensions resulted in a large accumulation of 9β,19-cyclopropyl sterols (about 50% of total sterols) concomitant with a decrease in the Δ^5-sterol content (40–50% of total sterols compared with more than 90% in control cultures). However the 9β,19-cyclopropyl sterols accumulated differed depending on the tissue studied—fenugreek roots or cells. In roots of seedlings treated with tridemorph, pollinastanol and 4α,14α-dimethyl-9β,19-cyclo-5α-cholestan-3β-ol were the major cyclopropyl sterols while in treated-cell suspensions with fenpropimorph, 24-methylenepollinastanol and 24-methylpollinastanol were the major sterols. These results indicated that these compounds inhibited the cycloeucalenol-obtusifoliol-isomerase (COI). Moreover, in roots as well as in cell suspensions, these inhibitors led to a weak accumulation of Δ^8-sterols indicating that $\Delta^8 \rightarrow \Delta^7$ isomerase was also affected. Finally, we observed that only fenpropimorph induced an accumulation of $\Delta^{8,14}$-sterols in cell suspensions, showing that Δ^{14}-reductase can also be inhibited by this fungicide.

INTRODUCTION

The morpholines such as tridemorph and fenpropimorph are sterol biosynthesis inhibitors (SBI) used in agriculture for the control of plant pathogenic fungi, particularly the powdery mildews [1]. On the basis of their mode of action in fungal sterol biosynthesis, these systemic fungicides are the second class of SBI after the demethylation inhibitors, DMIs, such as diniconazole, triarimol, diclobutrazol [2–4]. Most investigations on the effects of morpholine fungicides on the growth and morphology have been carried out with tridemorph and fenpropimorph. These fungicides do not suppress the germination of fungal spores to any great extent but are highly effective in inhibiting mycelial growth [5, 6]. The first hint that tridemorph had an inhibitory effect on lipid biosynthesis in general and in sterols in particular was indicated in 1980 by Kato *et al.* [7]. Later, Berg *et al.* [8] showed that in *Saprolegnia ferax*, an oomycete, tridemorph inhibited the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase rather than Δ^{14}-sterol reductase and this was confirmed by Baloch *et al.* [9]. They also showed that fenpropimorph was a potent inhibitor of the Δ^{14}-sterol reductase. It is now apparent that all the morpholines assayed are able to inhibit both enzymes, $\Delta^8 \rightarrow \Delta^7$-isomerase and Δ^{14}-reductase, to a greater or lesser extent [1, 9]. These enzymes, although operating at different stages in the ergosterol biosynthesis pathway, catalyse reactions which proceed through a similar mechanism [10, 11]. The first step in each case consists of the addition of a proton to the sterol substrate, leading to the formation of a carbenium ion transition state intermediate with the positive charge situated at C-14 for the reductase and C-8 for the $\Delta^8 \rightarrow \Delta^7$-isomerase.

Δ^{14}-reductase [12] and $\Delta^8 \rightarrow \Delta^7$-isomerase [13] are processes that occur in the biosynthesis of both fungi and higher plants. Moreover, another enzyme found in plants which is not found in fungi is cycloeucalenol-obtusifoliol isomerase (COI). *In vitro*, this enzyme catalyses the opening of the 9β,19-cyclopropane ring of cycloeucalenol so as to generate the $\Delta^{8(9)}$ double-bound and a β-orientated methyl group at C-10 that are found in the reaction product, obtusifoliol, through a mechanism similar to those used in both the above-mentioned reactions [14, 15]. *In vivo*, the effects of the morpholine fungicides on plant sterol biosynthesis have been the subject of extensive studies of Benveniste and his group [16].

In plants, cholesterol is generally a minor sterol whereas in animals it is the major sterol found [17]. In plants producing saponins (secondary plant metabolites), cholesterol plays a very important role: it is the major precursor of these compounds in species such as fenugreek [18], *Dioscorea* spp. [19–21] and *Avena sativa* [22].

In the present paper, we report the effects of two morpholines, tridemorph or fenpropimorph used at high concentration, (125 μM) on the growth and sterol composition in fenugreek seedlings and in fenugreek cell suspension cultures.

RESULTS AND DISCUSSION

Effect on growth

Tridemorph, a morpholine fungicide, at 125 μM (37.5 mg l^{-1}) induced retardation on fenugreek growth, which was more significant in roots (50%) than in shoots (20%), accompanied by a decrease of dry and fresh wt mg 100^{-1} roots or shoots) compared with the control (Table 1). This inhibition of growth by morpholines (tridemorph or fenpropimorph) was observed by other researchers such as Khalil and Mercer [23], who observed a growth inhibition of about 40% in winter wheat seedlings treated with 250 μM (75 mg l^{-1}) tridemorph, or Mercer *et al.* [24] who showed that tridemorph or fenpropimorph at 125 μM depressed growth of barley seedlings by about 30%. Thus, the morpholine fungicides undoubtedly have an adverse effect on the growth of higher plants but this is only apparent at concentrations much higher than those used to control phytopathogenic fungi. Maize, raised from seeds for 14 days in the presence of 3.36 μM (1 mg l^{-1}) to 67.2 μM (20 mg l^{-1}) tridemorph, grew satisfactorily but the treated plants were only 88% and 44% of the height of the control plants, respectively [25]. Khalil and Mercer [26] showed that the length of wheat shoots or roots decreased by about 10 to 40% when the fenpropimorph concentration increased from 5 μM (1.5 mg l^{-1}) to 250 μM (75 mg l^{-1}).

When fenpropimorph, another morpholine fungicide was added to fenugreek cell suspension, the growth was inhibited by about 10% after 21 days of culture (PCV measurements) and after only one subculture with this inhibitor (Table 2). In bramble suspension cultures, Schmitt *et al.* [27] showed that cells grew satisfactorily in the presence of concentrations of tridemorph in the range 1 to 10 mg l^{-1} (33.6 μM), with only slight inhibition at the highest concentrations.

Table 2. Effect of fenpropimorph (125 μM) on fenugreek suspension cell growth evaluated by packed cell volume measurements

	Treatment	
Days of culture	Control	Fenpropimorph
0	0.05	0.05
21	0.34	0.30
% of growth	100	88

Effect of tridemorph on the sterol content of roots

The sterol composition of the control roots of fenugreek seedlings revealed that Δ^5-sterols were predominant: 4.29 mg g^{-1} dry wt (or 96.9% of total sterols) (Table 3). This fraction contained principally sitosterol (79.1%), campesterol (7.4%), cholesterol (3.7%) and isofucosterol (3.3% of total sterols) (Table 4). These Δ^5-sterols were reduced to 1.58 mg g^{-1} dry wt (or 47.0%) in fenugreek roots treated with 125 μM tridemorph. This decrease was concomitant with the accumulation of 9β,19-cyclopropyl sterols: 1.55 mg g^{-1} dry wt (46.1% of total sterols) while they represented only 1% (or 0.04 mg g^{-1} dry wt) in the control roots.

Mass spectra of sterols that are not presented here have been given previously [28]. We present only mass spectra of the sterols that appeared with tridemorph or fenpropimorph treatment.

In the 4-desmethylsterol fraction of treated roots with tridemorph, three sterols were identified by mass spectroscopy (Table 5) according to the work of Rahier and Benveniste [29]. These sterols were pollinastanol (**34**) (17.7%), 24-methylenepollinastanol (**35**) and 24-methylpollinastanol (**36**) (Fig. 1). Sterol **33** had a RR_t of 1.09 and presented a mass spectrum very similar to that of 14α-methyl-5α-cholest-8-en-3β-ol (**16**) ($RR_t = 1.03$). It appeared that sterol **33** would be the $\Delta^{9('')}$-isomer of **16**, i.e. 14α-methyl-5α-cholest-$9^{('')}$-en-3β-ol. This appeared to be confirmed by the evaluation of RR_t of the three following isomeric sterols: 1.03 for Δ^8-sterol (**16**), 1.09 for $\Delta^{9('')}$-sterol (**33**) and 1.14 for the 9β,19-cyclopropyl sterol (**34**) (Table 4). Moreover, separation factors obtained with these sterols were 1.11 for (RR_t 9β,19-isomer (**34**)/RR_t Δ^8-isomer **16**) and 1.04 for (RR_t 9β,19-isomer (**34**)/RR_t $\Delta^{9('')}$-isomer **33**). These results correspond to those obtained by Goad [30] for the same sterols analysed under acetylated forms and on a OV.1 column.

The analysis of 4α-methyl sterols also showed an accumulation of 9β,19-cyclopropyl sterols in

Table 1. Effect of tridemorph (125 μM) on fenugreek seedling growth

	Roots			Shoots		
	Length (cm)	Dry wt*	Fresh wt*	Length (cm)	Dry wt	Fresh wt
Control	7.72 ± 1.02†	0.29 ± 0.02	6.03 ± 0.52	8.62 ± 0.40	0.63 ± 0.02	14.41 ± 0.91
Tridemorph	3.90 ± 0.21	0.23 ± 0.02	4.34 ± 0.48	7.11 ± 0.14	0.57 ± 0.01	11.13 ± 0.85

*As mg 100^{-1} roots or shoots.

†The data show averages of three experiments ± SD.

Table 3. Types of sterols occurring in control and tridemorph-treated fenugreek roots or fenpropimorph-treated fenugreek cells

	Roots				Cell suspension			
	Control		Tridemorph		Control		Fenpropimorph	
Sterols	mg*	%†	mg	%	mg	%	mg	%
4-Desmethyl sterols	4.32	97.6	2.55	75.8	3.53	98.2	1.68	93.2
4α-Methyl sterols	0.03	0.7	0.69	20.7	0.04	1.2	0.11	6.3
4,4-Dimethyl sterols	0.07	1.7	0.12	3.5	0.02	0.6	0.02	0.5
Δ^5-Sterols	4.29	96.9	1.58	47.0	3.35	93.2	0.69	38.4
Δ^5-C_{10}-SC	3.76	85.1	1.34	30.0	3.12	86.7	0.56	31.0
9β,19-Cyclopropyl sterols	0.04	1.0	1.55	46.1	0.02	0.6	0.89	49.4
9β,19-Cyclopropyl, C_8-SC	0.04	1.0 (100)‡	1.14	33.9 (73.5)‡	0.02	0.6 (100)‡	0.10	5.5 (11.1)‡
9β,19-Cyclopropyl, C_9-SC	—	—	0.4	12.2 (26.5)‡	—	—	0.71	39.7 (80.4)‡
9β,19-Cyclopropyl, C_{10}-SC	—	—	—	—	—	—	0.08	4.2 (8.5)‡
Δ^8-Sterols	0.02	0.4	0.03	1.0	0.02	0.6	0.16	8.8
$\Delta^{8,14}$-Sterols	—	—	—	—	—	—	0.08	4.2
Total sterols (mg g^{-1} dry wt)	4.43		3.36		3.60		1.80	

*As mg g^{-1} dry wt.
†As % of total sterols.
‡As % of 9β,19-cyclopropyl sterols.

tridemorph-treated roots. Those most often found were 4α,14α-dimethyl-9β,19-cyclo-5α-cholestan-3β-ol (**20**)—13.4% of total sterol—and sterol **39**. The characteristic fragments of its mass spectrum (Table 6) led us to suppose that **39** is the 4α,14α-dimethyl-9β,19-cyclo-5α-ergost-24(24^1)-en-3β-ol (cycloeucalenol). Chitwood and Lusby [31], using a similar column, measured RR_t of 1.60, 1.69 and 1.73 for cycloartanyl, cycloeucalenyl and cycloartenyl acetates, respectively. For the same sterols, we obtained 1.45, 1.52 (**39**) and 1.57. Furthermore, cyclopropyl sterol **38** was accumulated weakly (0.7% of total sterols) in treated roots. The analysis of its mass spectrum appears to indicate that it is 4α,14α-dimethyl-9β,19-cyclo-5α-cholest-24-en-3β-ol (**38**). The mass spectrum of sterol **37**, RR_t 1.22, is very similar to that of **19**, RR_t 1.13. It is possible that **37** is the $\Delta^{9(11)}$-isomer of **19**, i.e. the 4α,14α-dimethyl-5α-cholest-9(11)-en-3β-ol. Indeed, separation factors are 1.12 for (RR_t 9β,19-isomer **20**/RR_t Δ^8-isomer **19**) and 1.04 for (RR_t 9β,19-isomer **20**/RR_t $\Delta^{9(11)}$-isomer **37**). The same observation can also be made for sterols **12** (Δ^8-sterol), **13** (Δ^9-sterol) and **14** (9β,19-cyclopropyl sterol).

Similar changes in the sterol composition were obtained in fenugreek roots treated with fenpropimorph (data not shown). The same results, namely a decrease in Δ^5-sterol content and an increase in 9β,19-cyclopropyl sterols have been reported by many workers. Nevertheless, they obtained different sterol compositions from those we have shown. In barley shoots treated with fenpropimorph or tridemorph used at 250 μM, 9β,19-cyclopropyl sterols were 80% of total sterols (against 9% in control) and cycloeucalenol represented 50% of total sterols (against 2% in control) and 24-methylpollinastanol was 20% of the total sterols [24]. In winter wheat shoots treated with tridemorph or fenpropimorph at 250 μM [23] or in maize seedlings [25], similar results were obtained.

Effect of fenpropimorph on the sterol content of cell suspensions

When fenpropimorph was added to fenugreek cell suspensions, a significant decrease of 50% in sterol content was observed (1.8 mg g^{-1} dry wt versus 3.6 mg g^{-1} in control; Table 3).

Moreover, the sterol composition was greatly modified (Tables 3 and 7). These changes were especially present in the 4-desmethylsterol fraction, which nevertheless remained predominant whatever the treatment—93.2% of total sterols (1.68 mg g^{-1} dry wt) in treated suspensions and 98.2% (3.53 mg g^{-1} dry wt) in control cultures. In this class of compound, we observed the appearance of pollinastanol (**34**) (5.2%) which was weak when compared with treated roots (17.7%), 24-methylenepollinastanol (**35**) (15.6%) and 24-methylpollinastanol (**36**) (17.6%). These two sterols were much more important than in roots of fenugreek seedlings treated with tridemorph (3.5% and 4.3%, respectively). Fenpropimorph led to a very important decrease in sitosterol content, the disappearance of isofucosterol and a decrease in stigmasterol and campesterol content. We also noted the increase in cholesterol and 14α-methyl-9β,19-cyclo-5α-stigmast-24(24^1)-en-3β-ol (**28**). Conversely, three sterols, **40**, **41** and **42**, appeared following the treatment with fenpropimorph. These sterols had been identified as a Δ^8-sterol (**41**) and two $\Delta^{8,14}$-sterols (owing to the importance of the molecular peak) (**40**) and (**42**), according to their mass spectra analysis (Table 5) and previous works of Rahier and Benveniste [29] and Schmitt *et al.* [32].

Table 4. Sterol content in roots of fenugreek seedlings treated or untreated with tridemorph (125 μM)

	Control			Tridemorph	
Sterols	RR_t*	mg†	(%)‡	mg	(%)
4-Desmethyl sterols					
Cholesterol (**1**)	1.00	0.16	(3.7)	0.05	(1.5)
14α-Methyl-5α-cholest-8-en-3β-ol (**16**)§	1.03	—	—	0.01	(0.3)
Lathosterol (**2**)	1.09	0.03	(0.7)	—	—
14α-Methyl-5α-cholest-9-en-3β-ol (**33**)§	1.09	—	—	0.10	(3.0)
Pollinastanol (**34**)	1.14	—	—	0.59	(17.7)
Campesterol (**3**)	1.22	0.33	(7.4)	0.13	(4.0)
Stigmasterol (**4**)	1.29	0.12	(2.7)	0.05	(1.5)
24-Methylenepollinastanol (**35**)	1.36	—	—	0.12	(3.5)
24-Methylpollinastanol (**36**)	1.38	—	—	0.14	(4.3)
Sitosterol (**5**)	1.46	3.50	(79.1)	1.27	(37.9)
Isofucosterol (**6**)	1.49	0.15	(3.3)	0.07	(2.1)
Stigmasta-5,24-dien-3β-ol (**7**)	1.56	0.03	(0.7)	—	—
4α-Methyl sterols					
4α,14α-Dimethyl-5α-cholest-8-en-3β-ol (**19**)§	1.13	—	—	0.01	(0.4)
4α,14α-Dimethyl-5α-cholesta-8,24-dien-3β-ol (**8**)§	1.21	0.009	(0.2)	—	—
4α,14α-Dimethyl-5α-cholest-9-en-3β-ol (**37**)§	1.22	—	—	0.06	(1.8)
4α-Methyl-5α-cholest-7-en-3β-ol (**9**)§	1.22	0.004	(0.1)	—	—
4α,14α-Dimethyl-9β,19-cyclo-5α-cholestan-3β-ol (**20**)§	1.27	—	—	0.45	(13.4)
4α,14α-Dimethyl-5α-ergosta-8,24(24^1)-dien-3β-ol (**10**)	1.36	0.004	(0.1)	—	—
4α,14α-Dimethyl-9β,19-cyclo-5α-cholest-24-en-3β-ol (**38**)§	1.36	—	—	0.02	(0.7)
4α,14α-Dimethyl-9β,19-cyclo-5α-ergost-24(24^1)-en-3β-ol (**39**)§	1.52	—	—	0.14	(4.2)
4α,14α-Dimethyl-9β,19-cyclo-5α-ergostan-3β-ol (**29**)§	1.55	—	—	0.006	(0.2)
4α-Methyl-5α-stigmasta-7,24(24^1)-dien-3β-ol (**11**)	1.83	0.01	(0.3)	—	—
4,4-Dimethyl sterols					
4,4,14α-Trimethyl-5α-cholest-8-en-3β-ol (**12**)§	1.31	0.004	(0.1)	0.01	(0.3)
4,4,14α-Trimethyl-5α-cholest-9(11)-en-3β-ol (**13**)§	1.42	0.03	(0.6)	0.04	(1.1)
Cycloartanol (**14**)	1.45	0.02	(0.5)	0.04	(1.2)
Cycloartenol (**15**)	1.57	0.02	(0.5)	0.03	(0.9)
Total sterols (mg g^{-1} dry wt)		4.43 ±0.12		3.36 ±0.07	

*Retention time of steryl acetate relative to cholesteryl acetate.

†As mg g^{-1} dry wt.

‡As % of total sterols.

§Suggested structures from mass spectra alone.

In the 4α-methylsterol class, only one sterol was present, cycloeucalenol (**39**), representing 6.3% in treated cells against 0% in control cultures.

Finally, in the 4,4-dimethylsterol class, we observed a decrease in cycloartenol (**15**) content and the appearance of 24-methylenecycloartanol (**32**).

In treated cells, 9β,19-cyclopropyl sterols were more predominant than Δ^5-sterols—49.4% (0.89 mg g^{-1} dry wt) and 38.4% (0.69 mg g^{-1} dry wt), respectively—while they represented only 0.6% (0.02 mg g^{-1} dry wt in control cells (Table 3). We also noted an increase in Δ^8-sterols principally due to the appearance of stigmasta-8,24(24^1)-dien-3β-ol (**41**) (Fig. 1) representing 8% of total sterols and the presence of $\Delta^{8,14}$-sterols resulting from the appearance of ergosta-8,14-dien-3β-ol (**40**) (Fig. 1) (3.2% of total sterols) and stigmasta-8,14,24(24^1)-trien-3β-ol (**42**) (1% of total sterols).

In bramble cells treated with tridemorph (10 mg l^{-1} = 33 μM), 9β,19-cyclopropyl sterols were also predominant, comprising 82% of total sterols, principally cycloeucalenol (35%), 24-methylenepollinastanol (35%) and 24-methylpollinastanol (7.5%). Conversely, in bramble cells treated with fenpropimorph at 3.36 μM (1 mg l^{-1}), 35% of total sterols are 9β,19-cyclopropyl sterols such as cycloeucalenol (27%) and 24-methylenepollinastanol (4%) [27]. In cell suspension cultures of carrot, tobacco and soybean, treatment with tridemorph always led to an increase of 9β,19-cyclopropyl sterols, from 35 to 60% of total sterols; however, Hosokawa *et al.* [33] obtained other sterol compositions. Cycloeucalenol appeared in the three cultures treated with tridemorph. The presence of 24-methylenepollinastanol was detected in tobacco (39%), in carrot (4%) but not in soybean cultures; 24-methylpollinastanol was present in soybean (45% of total sterols) and in tobacco (4.2%) but not in carrot.

In fenugreek roots, 9β,19-cyclopropyl sterols represented 47% of total sterols compared with 1% in control roots. Furthermore, 73.5% of 9β,19-cyclopropyl sterols

Table 5. Mass spectral analysis of 4-demethyl steryl acetates in fenugreek roots treated with tridemorph or in fenugreek cells treated with fenpropimorph

	4-Desmethylsterols							
	16	**33**	**34**	**35**	**36**	**40**	**41**	**42**
RR_t^*	1.06	1.09	1.14	1.36	1.38	1.24	1.48	1.51
Fragmentation†								
$[M]^+$	442(18)‡	442(13)	442(8)	454(6)	456(5)	440(100)	454(100)	452(100)
$[M - Me]^+$	427(100)	427(100)	427(8)	439(9)	441(7)	425(14)	439(13)	437(42)
$[M - Ac]^+$			382(83)	394(89)	396(70)	380(5)	394(10)	
$[M - Me - Ac]^+$	367(30)	367(90)	367(100)	379(100)	381(100)	365(56)	379(55)	377(12)
$[M - a]^+$								339(70)
$[M - SC]^+$	329(3)	329(8)				313(13)		313(17)
$[M - A]^+$			288(5)	300(8)	302(10)			
$[M - SC - 2H\ (or\ \text{-}H)]^+$							313(13)	312(17)
$[M - a - Ac]^+$				310(10)			296(25)	
$[M - SC - Ac]^+$			269(66)	269(42)	269(98)	253(10)		
$[M - SC - Me - 42]^+$	273(15)	273(42)						
$[M - SC - Ac - Me]^+$	255(6)	255(33)				238(18)		
$[M - SC - Ac - 42]^+$	227(7)	227(25)			227(17)			
$[M - SC - Ac - Me - 42]^+$	213(7)	213(38)						

*Retention time of steryl acetate relative to cholesteryl acetate.

†Ac, acetate; Me, methyl; SC, side chain; a, A, fragments according to ref. [29].

‡Figures in brackets show intensities of ions relative to base peak (100).

were sterols with a C_8 side-chain and only 26.5% of 9β,19-cyclopropyl sterols were sterols with a C_9 side-chain (Table 3). Conversely, in treated cells, 11.1% of 9β,19-cyclopropyl sterols were sterols with a C_8 side-chain, 8.5% were sterols with a C_{10} side-chain and 80.4% were sterols with a C_9 side-chain. This seems to indicate differences in the capacity of roots or cell suspensions to reduce the $\Delta^{24(25)}$ double-bond or to insert a methyl group at C-24. Roots would appear to have an important capacity for reduction whereas cell suspensions appear to possess a greater methylase activity than reductase activity. This phenomenon has been observed with another DMI inhibitor, diniconazole, applied to fenugreek seedlings or cell suspensions [28]. In treated roots and in treated cells, a characteristic feature of the cyclopropyl sterols was the relative percentage of 9β,19-cyclopropyl-C_{10} side-chain sterols. The latter (0% in treated roots and 4.2% of total sterols, or 8% of 9β,19-cyclopropyl sterols, in treated cells) was much lower than the relative percentage of Δ^5-C_{10} side-chain sterols: 85.1% in control roots and 86.4% of total sterols in control cells. This appears to confirm that 24-methylene-9β,19-cyclopropyl sterols would be very poor substrates for the second methyltransferase activity, as suggested by a study using cell-free extract of bramble cells [34].

The high accumulation of 9β,19-cyclopropyl sterols in fenugreek (roots or cell suspensions) treated with the morpholine fungicides (tridemorph or fenpropimorph) indicated that *in vivo*, their primary effect was the inhibition of cycloeucalenol-obtusifoliol isomerase, an enzyme involved in plant sterol biosynthesis. *In vitro*, an enzyme in a cell-free preparation of bramble cells [35] and in microsomes of maize embryos [14] was shown to cleave the cyclopropane ring of cycloeucalenol and isomerize the sterol to obtusifoliol. This enzyme, cycloeucalenol-obtusifoliol isomerase (COI) from maize embryos was shown to be highly specific for cycloeucalenol [15]. In tridemorph-treated fenugreek roots or in fenpropimorph-treated cell suspensions, cyclopropyl sterols accumulated but no obtusifoliol was detected in either treated cultures (Tables 4 and 7). The absence of obtusifoliol and the presence of cycloeucalenol in the treated cultures indicate that tridemorph and fenpropimorph block the conversion of cycloeucalenol to obtusifoliol. Both morpholines appeared to be effective inhibitors of COI in fenugreek.

Some of the 9β,19-cyclopropyl sterols accumulated, pollinastanol (**34**), 24-methylenepollinastanol (**35**) or 24-methylpollinastanol (**36**) are 9β,19-cyclopropyl sterols that are not members of the normal phytosterol biosynthetic pathway and it seems reasonable to assume that they result from the metabolism of the rapidly accumulating cycloeucalenol (**39**) or 4α,14α-dimethyl-9β,19-cyclo-5α-cholestan-3β-ol (**20**) by enzymes whose normal substrates are sterols in the postcycloeucalenol part of the pathway.

In roots treated with tridemorph, Δ^8-sterol content increased weakly (1% in treated roots compared with 0.4% in control) and corresponded with the appearance of **19**, while in fenugreek cells treated with fenpropimorph, 12.2% of total sterols were Δ^8-sterols such as **41** (8% in treated cell against 0% in control cells). The increase in the Δ^8-sterol content of the treated fenugreek indicated that the morpholine fungicides also inhibited the sterol $\Delta^8 \rightarrow \Delta^7$-isomerase as they do in fungi [7, 8]. Similar results have been obtained in soybean cells [33],

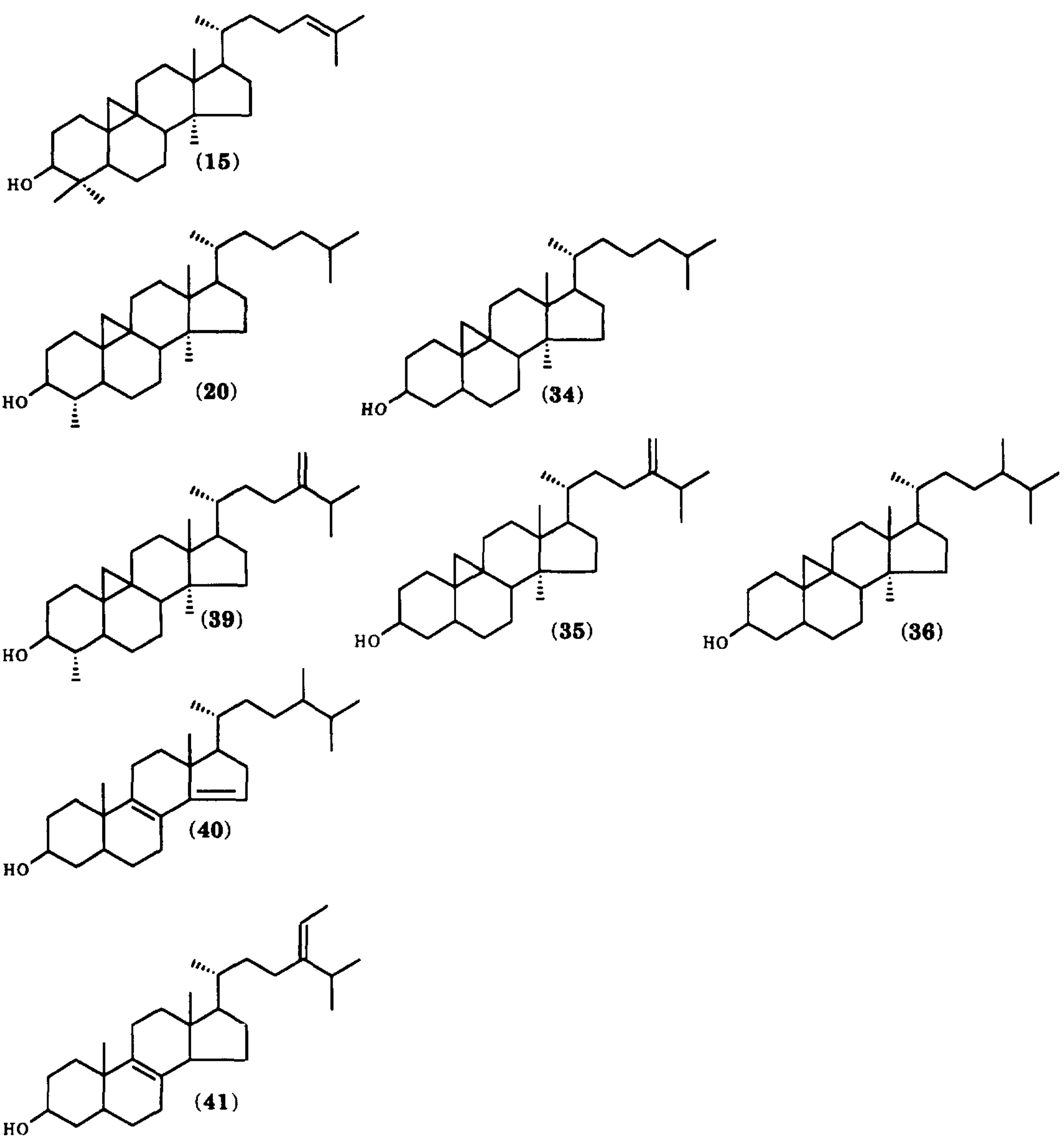

Fig. 1. Some representative structures of sterols detected in roots or in cell suspension cultures of fenugreek.

bramble cells [36] and in seedlings of maize [25] or wheat [26]. It is most likely that the Δ^8-sterols are not accumulated to the same extent as the 9β,19-cyclopropyl sterols because the COI-catalysed step precedes that catalysed by the $\Delta^8 \rightarrow \Delta^7$-isomerase, the former being so strongly inhibited that only small amounts of normal sterol precursors are able to leak past it to reach the subsequent blockage. The weak accumulation of Δ^8-sterols would result from the high concentration of the inhibitor (125 μM = 37.5 mg l^{-1}). Indeed, Schmitt *et al.* [37] have shown that the relative percentage of cyclopropyl sterols increased while that of Δ^8-sterols decreased when the concentration of tridemorph in the bramble cell medium increased. This has also been observed in maize leaves [25]. Furthermore, during *in vitro* assays, fenpropimorph and tridemorph inhibited COI and $\Delta^8 \rightarrow \Delta^7$-isomerase in the same proportions (IC_{50} = 0.4 μM) [12,38].

In cells treated with fenpropimorph, we have observed an accumulation of $\Delta^{8,14}$-sterols, which indicates that fenpropimorph inhibits the Δ^{14}-reductase *in vivo*. These results are consistent with those obtained by Taton *et al.* [12]. They showed that *in vitro*, fenpropimorph was a good inhibitor of $\Delta^{8,14}$-sterol Δ^{14}-reductase (IC_{50} = 0.8 μM) whereas tridemorph was a poor inhibitor of this same enzyme (IC_{50} = 25 μM). They concluded that the weakness of inhibition of the reductase by tridemorph in comparison with fenpropimorph was due to the important role played by the structure of the *N*-substituent of the morpholines.

In bramble cells, Taton *et al.* [36] observed that the replacement of nearly 100% of the Δ^5-sterols normally

Table 6. Mass spectral analysis of 4α-methyl steryl acetates in fenugreek roots treated with tridemorph or in fenugreek cells treated with fenpropimorph

	4α-Methylsterols				
	19	**20**	**37**	**38**	**39**
RR_t*	1.13	1.27	1.22	1.36	1.52
Fragmentation†					
$[M]^+$	456(27)‡	456(4)	456(22)	454(6)	468(12)
$[M - Me]^+$	441(100)		441(100)	394(100)	453(6)
$[M - Ac]^+$	396(5)	396(75)	396(6)		408(100)
$[M - Me - Ac]^+$	381(82)	381(100)	381(84)	379(73)	393(69)
$[M - SC]^+$	343(3)		343(4)		
$[M - a - Ac]^+$				324(7)	
$[M - A]^+$		288(15)		286(7)	300(15)
$[M - SC - 42]^+$	301(5)		301(6)		
$[M - SC - Ac]^+$	283(15)	283(57)		283(13)	283(12)
$[M - SC - Ac - 42]^+$	241(12)	241(10)	241(16)	241(20)	241(12)
$[M - SC - Ac - Me - 42]^+$		226(15)			

*Retention time of steryl acetate relative to cholesteryl acetate.

†Ac, acetate; Me, methyl; SC, side chain; a, A, fragments according to ref. [29].

‡Figures in brackets show intensities of ions relative to base peak (100).

present by 9β,19-cyclopropyl sterols did not affect the growth of the cells. The same result was noted by Grandmougin *et al.* [39]. In fenugreek roots or cells, the replacement of Δ^5-sterols was not total. However, in the case of cells, we believe that most of the Δ^5-sterols present at the end of the treated cultures were those contained in the 'inoculum' and that they represent an important proportion (38.4%) because we made only one subculture. This supposition is consistent with results of Taton *et al.* [36] who observed that after five transfers on medium containing an azadecalin, an inhibitor of the COI, $\Delta^8 \rightarrow \Delta^7$-isomerase and Δ^{14}-reductase, the normal Δ^5-sterols almost completely disappeared (<0.2%). They were replaced by cyclopropyl sterols (95%), supporting the assumption that the cells initially containing Δ^5-sterols were diluted through the successive transfers.

Despite reducing growth, 9β,19-cyclopropyl sterols appear to be well tolerated by fenugreek seedlings, which apparently remain healthy when grown in the dark. The growth of fenugreek cell suspensions treated with fenpropimorph is not significantly changed. This ability to grow even when cyclopropyl sterols almost completely replace the normally occurring Δ^5-sterols has been observed in maize seedlings [25, 39], in bramble cell suspensions, even after several subcultures [36], and in tobacco calli [40]. Grandmougin *et al.* [39] showed that 9β,19-cyclopropyl sterols with a methyl group at C-24 can readily replace Δ^5-sterols as plant membrane components and give an effective packing with the fatty acid chains of phospholipids. More recently, Schuler *et al.* [41] showed that 24-methylpollinastanol, as well as sitosterol, was an efficient sterol in reducing the water permeability of soybean phosphatidylcholine vesicles, owing to the structural features of this sterol molecule. The cyclopropane ring gave flexibility at the level of ring C, affecting the angular orientation of the 14α-methyl group [42] and this would explain why 9β,19-cyclopropyl sterols were relatively good surrogates of Δ^5-sterols.

EXPERIMENTAL

Plant material and growth conditions. Fenugreek (*Trigonella foenum-graecum* L. cv. Gouka) seeds were grown in vermiculite in darkness at a temperature of 22 ± 1°. Tridemorph (2,6-dimethyl-*N*-tridecyl-morpholine, supplied by BASF, with a purity of 99%), was used at 125 μM (37.5 mg l^{-1}) in 0.5% methanolic soln and was applied every 2 days. The control cultures were moistened with a soln containing a mixture of H_2O:MeOH (99.5:0.5). After 10 days of culture, root and shoot lengths were measured and fresh and dry weights were determined.

The fenugreek cell cultures were established in 1990 from callus cultures initiated in 1987 from leaf pieces. Cell suspensions were grown in the following culture medium: macroelements from [43], microelements from [44], sucrose (20 g l^{-1}), glutamine (200 mg l^{-1}), myoinositol (100 mg l^{-1}). Phytohormones were NAA (naphtaleneacetic acid) and BAP (benzylaminopurine) used at 1 mg l^{-1}. Cell cultures were cultivated at 23 ± 1° under fluorescent light (70 μE m^{-2} s^{-1}, 16-hr photoperiod and 120 rpm). Cells were subcultured every 3 weeks. Fenpropimorph (*R*,*S*)-4-[3-(4-tert-butylphenyl)-2-methylpropyl]-2,6-dimethyl-morpholine, (supplied by BASF, with a purity of 95.37%) was added at the start of the culture in methanolic solution. The final concentration of MeOH was 0.5% (v/v) and the final concentration of fenpropimorph was 125 μM (37.5 mg l^{-1}). In the control cultures, MeOH was added at a final concentration of

Table 7. Sterol content in fenugreek cell suspensions treated or not with fenpropimorph (125 μM)

		Control		Fenpropimorph	
Sterols	RR_t^*	mg†	%‡	mg	%
4-Desmethyl sterols					
Cholesterol (**1**)	1.00	0.13	(3.7)	0.11	(6.3)
Lathosterol (**2**)	1.09	0.03	(0.7)	—	—
Pollinastanol (**34**)	1.13	—	—	0.09	(5.2)
Campesterol (**3**)	1.22	0.10	(2.8)	0.02	(1.1)
Ergosta-8,14-dien-3β-ol (**40**)	1.24	—	—	0.06	(3.2)
Stigmasterol (**4**)	1.29	0.86	(23.8)	0.32	(17.9)
24-Methylenepollinastanol (**35**)	1.36	—	—	0.28	(15.6)
24-Methylpollinastanol (**36**)	1.38	—	—	0.32	(17.6)
Unknown (**17**)	1.38	0.06	(1.6)	—	—
Unknown (**18**)	1.40	0.06	(1.6)	—	—
Sitosterol (**5**)	1.46	1.45	(40.2)	0.24	(13.1)
Stigmasta-8,$24(24^1)$-dien-3β-ol (**41**)	1.48	—	—	0.14	(8.0)
Isofucosterol (**6**)	1.49	0.64	(17.9)	—	—
Stigmasta-8,14,$24(24^1)$-trien-3β-ol (**42**)	1.51	—	—	0.02	(1.0)
Stigmasta-5,24-dien-3β-ol (**7**)	1.56	0.06	(1.6)	—	—
Stigmast-7-en-3β-ol (**22**)	1.60	0.05	(1.5)	—	—
Stigmasta-7,$24(24^1)$-dien-3β-ol (**23**)	1.63	0.10	(2.8)	—	—
14α-Methyl-9β,19-cyclo-5α-stigmast-$24(24^1)$-en-3β-ol (**28**)	1.67	—	—	0.07	(4.2)
4α-Methyl sterols					
4α,14α-Dimethyl-5α-cholest-8-en-3β-ol (**19**)§	1.13	0.004	(0.1)	—	—
4α,14α-Dimethyl-5α-cholesta-8,24-dien-3β-ol (**8**)§	1.21	0.004	(0.1)	—	—
4α-Methyl-5α-cholest-7-en-3β-ol (**9**)§	1.21	0.004	(0.1)	—	—
4α,14α-Dimethyl-5α-ergosta-8,$24(24^1)$-dien-3β-ol (**10**)	1.36	0.01	(0.3)	—	—
4α,14α-Dimethyl-9β,19-cyclo-5α-ergost-$24(24^1)$-en-3β-ol (**39**)§	1.52	—	—	0.11	(6.3)
4α-Methyl-5α-stigmasta-7,$24(24^1)$-dien-3β-ol (**11**)§	1.82	0.02	(0.6)	—	—
4,4-Dimethyl sterols					
Cycloartenol (**15**)	1.57	0.02	(0.6)	0.005	(0.3)
24-Methylenecycloartanol (**32**)	1.76	—	—	0.004	(0.2)
Total Sterols (mg g^{-1} dry wt)		3.60 ± 0.3		1.80 ± 0.1	

*Retention time of steryl acetate relative to cholesteryl acetate.

†As mg g^{-1} dry wt.

‡As % of total sterol.

§Suggested structures from mass spectra alone.

0.5% (v/v). Cultures were grown for 21 days, cells were harvested and dry weights were determined. The PCV (packed cell volume) was determined at 0 and 21 days of culture. Aliquots (10 ml of fresh cell suspensions) were centrifugated at 2500 *g* for 10 min. The volume of the cells were compared with the total volume (10 ml). Comparison of results at 0 and 21 days indicates the growth of cells for each treatment.

Analytical procedure. The extraction and the purification of sterols have been described elsewhere [28]. Total sterols were quantified by GC equipped with a flame ionization detector and with a DB1 capillary column (30 m × 0.25 mm i.d. polydimethylsiloxane phase), used at 270° (280° for the injector and detector). Carrier gas (H_2) flow rate was 1.1 ml min^{-1}. Desmethyl sterols, 4α-methyl sterols and 4,4-dimethyl sterols were purified as described elsewhere [28]. The steryl acetates were analysed by GC as above. Chemical structures were determined by GC-MS, ionizing energy 70 eV.

Nomenclature (*IUPAC-IUB* [45]). Cholesterol = cholest-5-en-3β-ol (**1**); lathosterol = 5α-cholest-7-en-3β-ol (**2**); campesterol = campest-5-en-3β-ol (**3**); stigmasterol = stigmasta-5,22-dien-3β-ol (**4**); sitosterol = stigmast-5-en-3β-ol (**5**); isofucosterol = [$24(24^1)Z$]-stigmasta-5,$24(24^1)$-dien-3β-ol (**6**); cycloartanol = 4,4,14α-trimethyl-9β,19-cyclo-5α-cholestan-3β-ol (**14**); cycloartenol = 4,4,14α-trimethyl-9β,19-cyclo-5α-cholest-24-en-3β-ol (**15**); 24-methylenecycloartanol = 4,4,14α-trimethyl-9β,19-cyclo-5α-ergost-$24(24^1)$-en-3β-ol (**32**); pollinastanol = 14α-methyl-9β,19-cyclo-5α-cholestan-3β-ol-(**34**); 24-methylenepollinastanol = 14α,24-dimethyl-9β,19-cyclo-5α-cholest-$24(24^1)$-en-3β-ol (**35**); 24-methylpollinastanol = 14α, 24-dimethyl-9β,19-cyclo-5α-cholestan-3β-ol (**36**).

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