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EFFECT OF DINICONAZOLE ON STEROL COMPOSITION OF ROOTS AND CELL SUSPENSION CULTURES OF 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*; Leguminosae; seedlings; cell suspensions; diniconazole; sterol biosynthesis inhibitor; 14α-methyl Δ^8-sterols.

Abstract—Diniconazole, a triazole-type fungicide had no plant growth regulating activity on fenugreek seedlings but decreased the growth of fenugreek cell suspensions by about 20%. Exposure of the fenugreek seedlings or cells to diniconazole caused inhibition of sterol biosynthesis at the 14α-demethylation step leading to accumulation of 14α-methyl Δ^8-sterols concomitant with a decrease in the sitosterol and stigmasterol content. 14α-Methyl-Δ^8-sterols represented 67.8% of total sterols in treated cell suspensions and 28% in treated roots compared with 0.5 and 0.4%, respectively, in untreated tissues. An accumulation of 14α-methyl Δ^8-sterols with a C_8-side chain (92% of the 14α-methyl Δ^8-sterols) such as 14α-methyl-5α-cholest-8-en-3β-ol and 4α,14α-dimethyl-5α-cholest-8-en-3β-ol was found in treated roots. In contrast, in fenugreek cell suspensions, 52.8% of the accumulated 14α-methyl Δ^8-sterols had a C_9-side chain, such as in 14α-methyl-5α-ergosta-8,24(24^1)-dien-3β-ol, 14α-methyl-5α-ergost-8-en-3β-ol and 4α,14α-dimethyl-5α-ergosta-8,24(24^1)-dien-3β-ol, and 43.8% had a C_{10}-side chain, such as in 14α-methyl-5α-stigmasta-8,24(24^1)-dien-3β-ol and 4α,14α-dimethyl-5α-stigmasta-8,24(24^1)-dien-3β-ol. Results point to the early reduction–methylation bifurcation at the Δ^{24} double bond of sterol precursors which are converted with few interconnecting routes to the end-product sterols, i.e. cholesterol or phytosterols.

INTRODUCTION

In higher plants, sterol biosynthesis has been widely investigated in different species by means of inhibitors of different enzymatic steps, either used in *in vitro* microsomal preparations or *in vivo* [1–3]. Cytochrome P-450-dependent obtusifoliol 14α-methyl demethylase is a key step in plant sterol biosynthesis. It has been shown that removal of the 14α-methyl group might occur only after removal of the 4β-methyl group and cleavage of the 9β,19-cyclopropane ring [4] that is at the level of obtusifoliol which is metabolized to the corresponding 4α-methyl-5α-ergosta-8,14,24(24^1)-trien-3β-ol. The demethylation requires NADPH and oxygen and is photoreversibly inhibited by carbon monoxide [5]. Inhibitors of the 14α-demethylase step constitute an important class of agrochemical fungicides, widely used for the control of fungal diseases [6–8], or of plant growth regulators (PGR) [9–11]. They belong to a large family of compounds containing a pyrimidine, pyridine, imidazole, triazole or norbornenodiazetine group. Due to subtle structural changes, a given molecule may have fungicidal or (PGR) activity, or, more often, both [2, 11, 13]. Moreover, in a few cases, these compounds have been shown to inhibit directly *ent*-kaurene hydroxylase from fungi [9, 14] or higher plants [9, 15, 16]. This key enzyme involved in gibberellin biosynthesis is also a cytochrome P-450-dependent monooxygenase [16].

The demethylation inhibitors (DMIs) are believed to operate through the binding of a heterocyclic nitrogen atom in the inhibitor to the ferric protohaeme iron of the enzyme [6]. The resulting inhibition leads to a depletion of terminal Δ^5-sterols and an accumulation of 14α-methyl Δ^8-sterols in plants [17–20] as well as in fungi [21–23].

Diniconazole is a triazole-type fungicide with a broad antifungal spectrum. This compound shows excellent efficacy against various diseases on cereal, fruits and other fields crops by both preventive and curative applications [24]. It is a specific inhibitor of the 14α-demethylation step of ergosterol biosynthesis in fungi [25]. Diniconazole possesses two enantiomers due to one asymmetrical carbon atom. The (*R*)-(−)-isomer is more fungitoxic than the (*S*)-(+)-isomer against fungal species belonging to the Ascomycotina, Basidiomycotina and Deuteromycotyna [26]. On the other hand, the (*S*)-(+)-isomer shows a much stronger plant growth regulating activity against some species of plant than the (*R*)-(−)-isomer [26].

This paper describes the effect of the (*R*)-(−)-isomer of diniconazole on growth and sterol biosynthesis in fenu-

greek seedlings and cell suspensions. The interest in this plant is due to the fact that it produces saponins, secondary plant metabolites that possess a variety of important biological activities [27]. Moreover, a particular sterol, cholesterol, is the major precursor to these compounds [28, 29].

RESULTS AND DISCUSSION

Effect of diniconazole on growth

Treatment of fenugreek seeds with diniconazole at 125 μM slightly affected the growth of seedlings (Table 1). Nevertheless, we observed that the length of treated roots was greater than the length of control roots, whereas fr. wt (g 100^{-1} roots) was slightly greater in control roots pared with treated roots. Dry wt (g 100^{-1} roots) was unaltered by the inhibitor. With shoots, diniconazole induced no important change of length, fr. wt or dry wt. Other DMIs such as triarimol, triadimefon or diclobutrazol (2*R*,3*S*) have been shown to reduce growth of winter wheat by 20 to 40%, depending on the parameter measured [19]. Our results could be due to the fact that we used (*R*)-(−)-diniconazole. Triazole derivatives constitute a group of chemicals that have been developed for use either as fungicides or plant growth regulators. In varying degrees, they exhibit both properties and the intensity of their biological activity is dependent on the isomeric form used. The (*S*)-(+)-enantiomer of triapenthenol causes growth inhibition by inhibiting gibberellin biosynthesis whereas the (*R*)-(−)-enantiomer inhibits plant growth only at higher concentrations [30]. Takano *et al.* [26] showed that, with cucumber seedlings, the (*S*)-(+)-isomer of diniconazole exhibited a stronger plant growth regulating activity than the (*R*)-(−)-isomer.

When diniconazole was added to cell suspension cultures, we observed that, after 21 days, the growth of cells, evaluated by PCV measurements, had decreased by about 20% compared with the control cell growth (Table 2).

Effect of diniconazole on sterol profile of roots

Control roots had a sterol composition similar to that of fenugreek leaves [31], from which cell suspensions were derived. No significant change of total sterol content between control and treated roots was observed. Total sterols represented about 4.5 mg g^{-1} dry wt (Table 3).

In control roots, Δ^5-sterols were predominant: 96.9% of the total sterols (Table 4). The main sterols were sitosterol (79.1% or 3.56 mg g^{-1} dry wt), campesterol (7.4% or 0.33 mg g^{-1} dry wt), cholesterol (3.7% or 0.17 mg g^{-1} dry wt), isofucosterol (3.3% or 0.15 mg g^{-1} dry wt) and stigmasterol (2.7% or 0.12 mg g^{-1} dry wt). In the 4-demethyl sterols class, representing 97.6% of total sterols (or 4.39 mg g^{-1} dry wt), other sterols have been identified as their acetate derivatives by capillary gas chromatography coupled with mass spectroscopy analysis (Table 5) (i.e. by coincidental retention time and an identical electron impact spectrum to that of authentic standards or comparison with literature data [32–35]). Particularly, in control roots, lathosterol (**2**) (Δ^7-sterol) has been identified by its typical fragmentation of a Δ^7-sterol without other unsaturation, thus presenting a strong molecular ion and an intense peak at *m/z* 255 $[M - SC - Ac]^+$ (Table 5) [33]. The sterol (**7**) has been identified as stigmasta-5,24-dien-3β-ol. This sterol had a slightly longer RR_t than that of isofucosterol (**6**) and a very similar mass spectrum with a strong peak at *m/z* 296 $[M - a - Ac]^+$, 'a' corresponding to the allylic cleavage of the 22–23 bond due to a McLafferty-type rearrangement [33]. In accordance with the location of the double bond at C-5, the molecular ion is very weak, in contrast to Δ^7-sterols in which it is strong. Identification of sterol **7** as stigmasta-5,24-dien-3β-ol is in accordance with data from Chitwood and Lusby [34], who identified this sterol previously and found that its RR_t was very close to that of isofucosterol.

In treated roots, amounts of sitosterol (47.9% or 2.11 mg g^{-1} dry wt) and stigmasterol (0.6% or 0.03 mg g^{-1} dry wt) decreased whereas amounts of campesterol (11.3% or 0.50 mg g^{-1} dry wt) increased and Δ^5-sterols represented only 70.1% of total sterols against 96.9% in control roots (Table 4). Moreover, we observed the presence of a new sterol (**16**) with a molecular mass of 442. The mass spectrum of **16** possessed strong peaks at *m/z* 427 $[M - Me]^+$ and *m/z* 367 $[M - Me - Ac]^+$, characteristic of 14α-methyl sterols [33]. Thus sterol **16** was probably 14α-methyl-5α-cholest-8-en-3β-ol. 14α-Methyl-5α-ergost-8-en-3β-ol (**24**) was identified by comparison of its mass spectrum with that of the same compound found in bramble cell suspensions treated with fenarimol, another inhibitor of the plant sterol 14α-demethylase [17].

Low amounts of 4α-methyl sterols were detectable (0.7% of total sterols) in control roots. The presence of obtusifoliol (**10**) was indicated by comparison of its mass

Table 1. Effect of diniconazole on fenugreek seedling growth

	Roots			Shoots		
	Length	Dry wt	Fr. wt	Length	Dry wt	Fr. wt
Control	8.86 ± 0.05†	0.31 ± 0.02	5.92 ± 0.30	8.85 ± 1.40	0.59 ± 0.02	14.30 ± 0.40
Diniconazole*	9.57 ± 0.80	0.26 ± 0.04	5.10 ± 0.20	8.55 ± 1.20	0.57 ± 0.02	13.42 ± 0.40

*Cultures treated with diniconazole at 125 μM.
†The data show the averages of three experiments ± SD.

Table 2. Effect of diniconazole on fenugreek cell suspensions growth evaluated by PCV measurements

	Treatment	
Days of culture	Control	Diniconazole*
0	0.05	0.05
21	0.34	0.28
% of growth	100	82

*Cultures treated with diniconazole at 125 μM.

spectrum with that previously described (as it acetate derivative) [4, 17]. 4α-Methyl-5α-stigmasta-7,24(24^1)-dien-3β-ol (**11**) was identified by comparison of its mass spectrum with that published by Chitwood and Lusby [34]. Sterol **19**, detected only in treated roots, had a strong $[M - Me]^+$ fragment (*m/z* 441) indicating that it was probably a 14α-methyl sterol (Table 6). A similar fragment was observed for sterol **8**, which had 2 units less than sterol **19**, but gave identical fragments after cleavage of the side chain. The presence of a double bond in the side chain of **8** between C-24 and C-25 was indicated by the fragment at *m/z* 369 $[M - a - Me]^+$. Thus, **19** was probably 4α,14α-dimethyl-5α-cholest-8-en-3β-ol and **8** was 4α,14α-dimethyl-5α-cholest-8,24-dien-3β-ol. Sterol **9** had a strong molecular ion at *m/z* 442 $[M]^+$, in accordance with Δ^7-unsaturation. This was confirmed by the presence of a fragment at *m/z* 243 $[M - SC - Ac - C_2H_2]^+$ with a relative intensity of 17% (this fragment is much weaker in Δ^5-sterols). Sterol **9** was thus identified as 4α-methyl-5α-cholest-7-en-3β-ol (or lophenol).

In diniconazole-treated roots, an increase in 4α-methyl sterols was observed (7.2% of total sterols against 0.7% in control) with the appearance of 4α,14α-dimethyl-5α-cholest-8-en-3β-ol (**19**), 4α,14α-dimethyl-5α-ergost-8-en-3β-ol (**25**) in small amounts (0.1% of total sterols, identified according to Schmitt and Benveniste [17], and 4α,14α-dimethyl-9β,19-cyclo-5α-cholestan-3β-ol (**20**), identified on the basis of its mass spectrum. The presence of a fragment at *m/z* 288 $[M - A]^+$ indicated a 9β,19-cyclopropyl ring, the fragment 'A' resulting from the fragmentation of the B-ring [33]. Study of the other fragments indicated the presence of a C_8-side chain without unsaturation and of a 14α-methyl group based on the presence of the fragment at *m/z* 241 $[M - SC - Ac - 42]^+$ [33].

The control and the treated roots contained 4,4-dimethyl sterols, including cycloartenol (**15**) [32], cycloartanol (**14**) [34] and sterols **12** and **13** (Table 7). The last

Table 3. Sterol content in roots of fenugreek treated or not with diniconazole (125 μM)

		[Control]		[Diniconazole]	
Sterols	RR_t*	mg†	(%)‡	mg	(%)
4-Demethyl sterols:					
Cholesterol (**1**)	1.00	0.17	(3.7)	0.23	(5.3)
14α-Methyl-5α-cholest-8-en-3β-ol (**16**)§	1.03	—	—	0.86	(19.5)
Lathosterol (**2**)	1.09	0.03	(0.7)	0.03	(0.6)
Campesterol (**3**)	1.22	0.33	(7.4)	0.50	(11.3)
14α-Methyl-5α-ergost-8-en-3β-ol (**24**)	1.25	—	—	0.08	(1.8)
Stigmasterol (**4**)	1.29	0.12	(2.7)	0.03	(0.6)
Sitosterol (**5**)	1.46	3.56	(79.1)	2.11	(47.9)
Isofucosterol (**6**)	1.49	0.15	(3.3)	0.19	(4.3)
Stigmasta-5,24-dien-3β-ol (**7**)	1.56	0.03	(0.7)	0.03	(0.7)
4α-Methyl sterols:					
4α,14α-Dimethyl-5α-cholest-8-en-3β-ol (**19**)§	1.13	—	—	0.25	(5.8)
4α,14α-Dimethyl-5α-cholesta-8,24-dien-3β-ol (**8**)§	1.21	0.009	(0.2)	0.01	(0.3)
4α-Methyl-5α-cholest-7-en-3β-ol (**9**)§	1.22	0.005	(0.1)	0.009	(0.2)
4α-14α-Dimethyl-9β,19-cyclo-5α-cholestan-3β-ol (**20**)§	1.27	—	—	0.004	(0.1)
4α,14α-Dimethyl-5α-ergosta-8,24(24)^1-dien-3β-ol (**10**)	1.36	0.005	(0.1)	0.02	(0.4)
4α,14α-Dimethyl-5α-ergost-8-en-3β-ol (**25**)	1.38	—	—	0.004	(0.1)
4α-Methyl-5α-stigmasta-7,24(24^1)-dien-3β-ol (**11**)	1.82	0.01	(0.3)	0.01	(0.3)
4,4-Dimethyl sterols:					
4,4,14α-Trimethyl-5α-cholest-8-en-3β-ol (**12**)§	1.31	0.005	(0.1)	0.004	(0.1)
4,4,14α-Trimethyl-5α-cholest-9(11)-en-3β-ol (**13**)§	1.42	0.03	(0.6)	0.02	(0.4)
Cycloartanol (**14**)	1.45	0.02	(0.5)	0.004	(0.1)
Cycloartenol (**15**)	1.57	0.02	(0.5)	0.009	(0.2)
Total sterols (mg g^{-1} dry wt)		4.5 ± 0.1		4.4 ± 0.1	

*Retention time of steryl acetate relative to cholesteryl acetate (DB1 capillary column.)
†As mg g^{-1} dry wt.
‡As % of total sterol.
§ Suggested structure from mass spectra alone.

Table 4. Distribution of sterol structural groups in control and diniconazole-treated fenugreek roots and cell suspensions

Sterols	Roots				Cell suspensions			
	[Control]		[Diniconazole]		[Control]		[Diniconazole]	
	(mg)*	(%)†	(mg)	(%)	(mg)	(%)	(mg)	(%)
4-Demethyl sterols	4.39	97.6	4.04	92	3.53	98.2	1.25	73.7
4α-Methyl sterols	0.03	0.7	0.32	7.2	0.04	1.2	0.44	25.7
4,4-Dimethyl sterols	0.08	1.7	0.03	0.8	0.02	0.6	0.01	0.6
Δ^5-Sterols	4.36	96.9	3.08	70.1	3.35	93.2	0.47	27.7
Δ^5-C_{10}-SC	3.86	85.8(88.5)‡	2.35	53.5(76.4)‡	3.12	86.7(94.7)‡	0.38	22.8(82.3)‡
$\Delta^{5,24(24^1)}$-sterols	0.15	3.3(3.5)‡	0.19	4.3(6.1)‡	0.64	17.9(19.6)‡	0.04	2.8(10.5)‡
14α-Methyl Δ^8-sterols	0.02	0.4	1.23	28.0	0.02	0.5	1.15	67.8
14α-Methyl $\Delta^{8,24(24^1)}$	0.005	0.1(25.0)§	0.02	0.4(1.4)§	0.01	0.3(75.0)§	0.86	50.6(74.6)§
14α-Methyl Δ^8, C_8-SC	0.01	0.3(75.0)§	1.13	25.7(92.0)§	0.007	0.2(40.0)§	0.04	2.3(3.4)§
14α-Methyl Δ^8, C_9-SC	0.005	0.1(25.0)§	0.10	2.3(8.2)§	0.01	0.3(60.0)§	0.61	35.8(52.8)§
14α-Methyl Δ^8, C_{10}-SC	—	—	—	—	—	—	0.50	29.7(43.8)§
14α-Methyl $\Delta^{8,24(24^1)}$-C_{10}-SC	—	—	—	—	—	—	0.50	29.7(43.8)§
TS. (mg g^{-1} dry wt)	4.5 ± 0.1		4.4 ± 0.1		3.6 ± 0.3		1.7 ± 0.02	

*As mg g^{-1} dry wt.
†As % of total sterols.
‡As % total Δ^5-sterols.
§As % of 14α-methyl Δ^8-sterols.

two had very similar mass spectra. We noted the presence of a peak at m/z 255 $[M - SC - Ac - 42]^+$, detected in mass spectra of 4,4-dimethyl sterols [33]. In both mass spectra, the fragment $[M - A]^+$ (m/z 288) was not present, indicating the absence of a 9β,19-cyclopropyl ring. Sterol **12** was thus probably Δ^8-lanostenol and sterol **13** was Δ^9-lanostenol, since Δ^8-isomers are less retained in GC than the corresponding Δ^9-isomers [35].

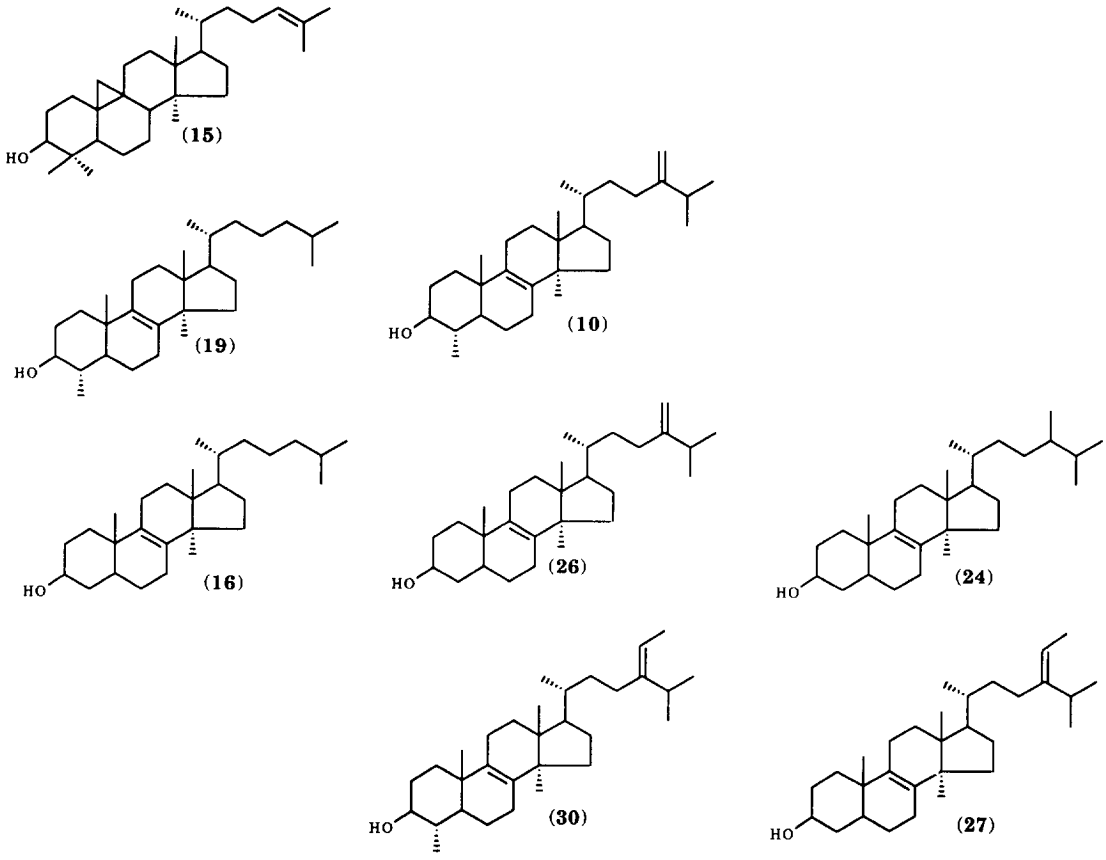

Table 5. Mass spectral analysis of 4-demethyl steryl acetates of fenugreek roots or cell suspensions treated or not with diniconazole

	4-Demethyl sterols															
Sterols	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**17**	**18**	**16**	**22**	**23**	**24**	**26**	**27**	**28**
RR_t^*	1.00	1.09	1.22	1.29	1.46	1.49	1.56	1.38	1.40	1.06	1.60	1.63	1.25	1.23	1.52	1.67
Fragmentation†																
$[M]^+$		428 (100)					454 (1)			442 (18)	456 (100)	454 (2)	456 (20)	454 (38)	468 (30)	468 (10)
$[M - Me]^+$		413 (22)					439 (1)			427 (100)	441 (35)	439 (2)	441 (100)	439 (100)	453 (100)	453 (14)
$[M - Ac]^+$	368 (100)‡	368 (10)	382 (100)	394 (100)	396 (100)	394 (15)	394 (13)	394 (100)	394 (100)		396 (20)	395 (16)				408 (10)
$[M - Me - Ac]^+$	353 (20)	353 (10)	367 (28)	379 (8)	381 (16)		379 (3)	379 (5)	379 (16)	367 (30)	381 (23)		381 (30)	379 (33)	393 (20)	393 (10)
$[M - a]^+$												356 (23)				
$[M - Me - a]^+$														355 (12)	355 (25)	355 (100)
$[M - SC]^+$		315 (13)								329 (3)	315 (15)					327 (16)
$[M - A]^+$																314 (10)
$[M - SC - 2H]^+$												313 (100)				
$[M - a - Ac]^+$						296 (100)	296 (100)									
$[M - Me - Ac - a]^+$							281 (23)							295 (17)	295 (20)	
$[M - SC - 42]^+$		273 (26)									273 (16)		287 (12)	287 (17)	285 (8)	
$[M - SC - Ac]^+$	255 (10)	255 (85)	255 (28)	255 (95)	255 (10)	255 (5)		255 (52)	255 (27)		255 (75)				267 (20)	267 (40)
$[M - SC - Ac - 2H]^+$				253 (25)		253 (10)	253 (16)	253 (90)	253 (50)			253 (16)				
$[M - SC - Ac - C_2H_2]^+$		229 (22)									229 (23)					
$[M - SC - Me - 42]^+$										273 (15)						
$[M - SC - Ac - Me]^+$										255 (6)				255 (17)		
$[M - SC - Ac - 42]^+$	213 (10)	213 (36)	213 (28)	213 (24)	213 (10)	213 (27)	213 (10)	213 (16)	213 (10)	227 (7)	213 (33)	213 (8)	227 (8)	227 (18)		
$[M - SC - Ac - Me - 42]^+$										213 (7)			213 (8)	213 (30)		

*Retention time of steryl acetate relative to cholesteryl acetate. (DB1 capillary column.)

†Ac, acetate; Me, methyl; SC, side chain.

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

In roots treated with diniconazole, the percentage of 4,4-dimethyl sterols decreased (0.8% of total sterols versus 1.7% in control roots) because of the reduction in cycloartanol (**14**) and cycloartenol (**15**) content.

In treated roots, 28.0% of total sterols were 14α-methyl Δ^8-sterols against 0.4% in control roots (Table 4). This increase of 14α-methyl Δ^8-sterols took place at the expense of Δ^5-sterols, since diniconazole induced no change in total sterol content. This indicates that *R*-(−)-diniconazole inhibits 14α-demethylase *in vivo* as has been shown by many investigators using other azole DMIs [17–20]. A number of triazole fungicides and plant growth retardants have been shown *in vitro* to inhibit 14α-demethylase contained in microsomes of maize seedlings and the IC_{50} for the different triazoles were demonstrated to be in the range 0.05 to 10 μM [18]. Among these 14α-methyl Δ^8-sterols present in treated roots, 92% had a C_8-side chain and 8.2% had a C_9-side chain (Table 4) such as 14α-methyl-5α-cholest-8-en-3β-ol (**16**) and 4α,14α-dimethyl-5α-cholest-8-en-3β-ol (**19**). The percentage of obtusifoliol (**10**) and 14α-methyl Δ^8-sterols with a C_9-side chain increased slightly in treated fenugreek roots (2.3% against 0.1% in control roots) whereas they were often present in much greater amounts after treatment of plants by a DMI:17.7% of total sterols against 0.2% in control in bramble cells treated with fenarimol [17], 6% in wheat shoots treated with triadimefon or triarimol [19] or 45% in maize seedlings treated with the triazole Lab 170250F [18]. The relatively weak accumulation of 14α-methyl Δ^8-sterols with a C_9-side chain (2.3% of total sterols or 8.2% of 14α-methyl Δ^8-sterols, Table 4) and the strong accumulation of 14α-methyl Δ^8-sterols with a C_8-side chain (25.7% of total sterols, or 92% of 14α-methyl Δ^8-sterols, Table 4) indicate that, in fenugreek roots, reduction of the $\Delta^{24(25)}$ double bond of cycloartenol or of biosynthetic intermediates is important compared with its methylation. 14α-Methyl Δ^8-sterols with a C_{10}-side chain were not detected. This indicates that the presence of the 14α-methyl group inhibits the second side chain methylation, as shown previously [36].

Effect of diniconazole on sterol composition in cell suspensions

In contrast to the results obtained with roots, in cell suspensions, diniconazole treatment led to a decrease of about 50% of total sterol content when compared with that of the control cells (Table 8).

Study of the sterol composition in control cell suspensions (Table 8) showed differences in the sterol composition compared to that observed in control roots (Table 3). In cell suspensions, stigmasterol represented 23.8% of total sterols whereas in roots, it represented only 2.7%. This increase seems to have taken place at the expense of sitosterol (40.2% in cell culture against 79.1% in roots) and could be the result of the passage of leaf pieces in *in vitro* culture, which constituted a kind of stress. These data were in accordance with those of others in which different stresses led to an accumulation of stigmasterol in different plants [37–39].

There was a great difference in the amount of isofucosterol between cell culture (17.9%) and roots (3.3%). A similar result was obtained by Fernandes-Ferreira *et al.* [40] in cell suspensions of *Euphorbia characias* L. subsp *characias*. Thus, it is possible that the reduction of the 24(24^1) double bond of isofucosterol is a limiting step in the biosynthesis of sitosterol in *in vitro* culture. The enzymes or enzyme systems involved in this step, might be lacking or operating at low activity.

Stigmast-7-en-3β-ol (**22**) and stigmasta-7,24(24^1)-dien-3β-ol (**23**) were present in cell suspension, but they were not detected in leaves or roots. Sterol **22** was also detected in fenugreek seeds and **23** was observed in seeds of other species of *Trigonella* [31].

In control cells, 4-demethyl sterols represented 98.2% of total sterols (or 3.53 mg g^{-1} dry wt) and Δ^5-sterols represented 93.2% of total sterols (or 3.35 mg g^{-1} dry wt) (Table 4). We also noted the presence of two sterols, **17** and **18**, which remained unidentified after mass spectral analysis (Table 5). Both had RR_fs close to that of stigmasterol (Table 5). Moreover, their mass spectra were very similar showing a very low intensity for the molecular ion, one of the characteristics of Δ^5-sterols, and a strong peak at *m/z* 394 $[M - Ac]^+$. These sterols might be side chain isomers of stigmasterol (**4**). The mass spectrum of sterol **22** showed that it was probably stigmast-7-en-3β-ol, since it contained an intense molecular ion, characteristic of Δ^7-sterols with a saturated side chain. The presence of the fragments $[M - SC - Ac]^{+1}$ (*m/z* 255) and $[M - SC - Ac - C_2H_2]^+$ (*m/z* 229) confirmed this structure. Sterol **23**, had a base peak at *m/z* 313 $[M - SC - 2H]^+$, characteristic of a Δ^7-sterol with an unsaturated side chain [33]. Other fragmentations allowed **23** to be identified as stigmasta-7,24(24^1)-dien-3β-ol (Δ^7-avenasterol).

In treated cell suspensions, the proportion of 4-demethyl sterols decreased (73.7% of total sterols or 1.25 mg g^{-1} dry wt) as well as that of Δ^5-sterols (27.7% against 93.2% of total sterols in control cells, Table 4). The decrease of stigmasterol content in treated cells especially (5.2% against 23.8% in control cells), shown also by other workers [17, 41–43], could result from the inhibition of the Δ^{22}-desaturase. This enzyme which is involved in the conversion of sitosterol to stigmasterol has been shown to be cytochrome P-450-dependent in yeast [44]. This reaction has not yet been characterized in plant tissues although a similar mechanism could be assumed [18, 41, 45]. Three 4-demethyl sterols, **26**, **27** and **28**, were found in treated cell suspensions, in addition to those present in control cell or those present in treated roots. According to the data of Schmitt and Benveniste [17] and to their mass spectra, we deduced that **26** was 14α-methyl-5α-ergosta-8,24(24^1)-dien-3β-ol and **27** was 14α-methyl-15α-stigmasta-8,24(24^1)-dien-3β-ol. Sterol **28** was probably 14α-methyl-9β,19-cyclo-5α-stigmast-24(24^1)-en-3β-ol based on the presence of the fragment $[M - A]^+$ (*m/z* 314).

In treated cells, 4α-methyl sterols content increased 10-fold (0.44 against 0.04 mg g^{-1} dry wt) compared with

Table 6. Mass spectral analysis of 4α-methyl steryl acetates of fenugreek roots or cell suspensions treated or not with diniconazole

	4α-methyl sterols									
Sterols	**8**	**9**	**10**	**11**	**19**	**20**	**25**	**29**	**30**	**31**
RR_t^*	1.21	1.21	1.36	1.82	1.13	1.27	1.38	1.52	1.67	1.84
Fragmentation†										
$[M]^+$	454 (30)‡	442 (100)	468 (44)	468 (2)	456 (27)	456 (4)	470 (25)	470 (10)	482 (40)	482 (2)
$[M - Me]^+$	439 (100)	427 (17)	453 (100)		441 (100)		455 (100)	455 (77)	467 (100)	467 (12)
$[M - Ac]^+$		382 (12)			396 (5)	396 (75)	410 (3)	410 (4)		
$[M - Me - Ac]^+$	379 (50)	367 (17)	393 (45)	393 (2)	381 (82)	381 (100)	395 (50)	395 (100)	407 (35)	407 (10)
$[M - a]^+$				370 (34)						384 (66)
$[M - Me - a]^+$			369 (3)						369 (28)	369 (100)
$[M - SC]^+$		329 (9)			343 (3)					341 (13)
$[M - A]^+$						288 (15)		302 (12)		314 (3)
$[M - SC - 2H]^+$				327 (100)						
$[M^+ - Me - Ac - a]^+$			309 (20)						309 (34)	
$[M - SC - 42]^+$			301 (11)		301 (5)		301 (8)			282 (4)
$[M - SC - Ac]^+$	283 (10)	269 (60)			283 (15)	283 (57)		282 (22)		
$[M - SC - Ac - 2H]^+$				267 (13)						
$[M - SC - Ac - C_2H_2]^+$		243 (17)								
$[M - SC - Ac - Me]^+$									269 25)	
$[M - SC - Ac - 42]^+$	241 (16)	227 (40)	241 (22)	227 (11)	241 (12)	241 (10)	241 (8)		241 (20)	
$[M - SC - Ac - Me - 42]^+$						226 (15)				

*Retention time of steryl acetate relative to cholesteryl acetate. (DB1 capillary column.)

†Ac, acetate; Me, methyl; SC, side chain.

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

Table 7. Mass spectral analysis of 4,4-dimethyl steryl acetates in fenugreek roots or cell suspensions treated or not with diniconazole

	4,4-Dimethyl sterols				
Sterols	**12**	**13**	**14**	**15**	**32**
RR_t^*	1.31	1.42	1.45	1.57	1.76
Fragmentation†					
$[M]^+$	470 (16)‡	470 (18)	470 (11)	468 (20)	482 (13)
$[M-Me]^+$	455 (60)	455 (64)	455 (14)	453 (18)	467 (6)
$[M - Ac]^+$			410 (94)	408 (100)	422 (100)
$[M - Me - Ac]^+$	395 (100)	395 (100)	395 (100)	393 (90)	407 (66)
$[M - SC]^+$			356 (23)		354 (10)
$[M - a - Ac]^+$				339 (40)	
$[M - A]^+$			288 (70)	286 (50)	300 (30)
$[M - SC - 42]^+$		315 (4)			
$[M - SC - Ac]^+$			297 (57)		297 (18)
$[M - SC - Ac\text{-}C_2H_2]^+$	273 (8)	273 (12)			
$[M - SC - Ac - Me]^+$					282 (15)
$[M - SC - Ac - 42]^+$	255 (4)	255 (10)	255 (30)		255 (10)
$[M - SC - Ac - Me - 42]^+$	241 (10)	241 (10)			

*Retention time of steryl acetate relative to cholesteryl acetate. (DB1 capillary column.)
†Ac, acetate; Me, methyl; and SC, side chain.
‡Figures in brackets show intensities of ions relative to base peak (100).

the control (Table 4). This increase in 4α-methyl sterols class was principally due to an increase of obtusifoliol (**10**) content and the accumulation of 4α,14α-dimethyl-5α-stigmasta-8,24(24[1])-dien-3β-ol (**30**). Treated cells contained 4α-methyl sterols **29**, **30** and **31**, that were not present in roots or untreated cells. Sterol **30** was identified as 4α,14α-dimethyl-5α-stigmasta-8,24(24[1])-dien-3β-ol according to Schmitt and Benveniste [17]. Sterols **29** and **31** were 9β,19-cyclopropyl sterols because of the presence of the characteristic fragment $[M - A]^+$. Sterol **29** was 4α,14α-dimethyl-9β,19-cyclo-5α-ergostan-3β-ol and sterol **31** was 4α,14α-dimethyl-9β,19-cyclo-5α-stigmast-24(24[1])-en-3β-ol.

In control cell suspensions, only one 4,4-dimethyl sterol, cycloartenol (**15**), was detected. It was also present in treated cell suspensions together with 24-methylenecycloartanol (**32**), identified by its mass spectrum and according to the works of Combaut [32], Rahier and Benveniste [33] or Chitwood and Lusby [34].

In treated cell suspensions as well as in treated roots, more than 90% of 14α-methyl sterols were Δ^8-sterols, which indicates that the presence of the 14α-methyl group hinders the isomerization of the Δ^8-double bond [46]. In treated cells, 67.8% of sterols were 14α-methyl Δ^8-sterols against 28.0% in treated roots. This difference possibly explains the growth inhibition of about 20% observed in treated cell suspensions. This growth inhibition might be due to the fact that the replacement of Δ^5-sterols by 14α-methyl Δ^8-sterols perturbs membrane structure [47] and physiological functions normally observed in fungi [2, 48] and in plants [17, 18]. Among 14α-methyl Δ^8-sterols, we observed that the distribution was different from that noted in treated roots. Indeed, in treated cells, 52.8% of 14α-methyl Δ^8-sterols had a C_9-side chain, 43.8% had a C_{10}-side chain and 3.4% had a C_8-side chain, against, respectively, 8.2%, 0.0% and 92.0% in roots (Table 4). These results indicate differences in sterol biosynthetic fluxes arising from the methylation of cycloartenol or the reduction of the $\Delta^{24(25)}$ double bond. In cell suspensions, the reductase activity is apparently less important than the methylase activity, whereas the opposite pertains to roots. This difference between the distribution of the biosynthetic fluxes (methylation or reduction of the $\Delta^{24(25)}$ double bond of cycloartenol or biosynthetic intermediates leading to phytosterols or cholesterol, respectively) according to the type of culture (cell suspensions or roots), could explain the weak steroidal saponin production observed in control cell suspensions (0.14 mg g^{-1} dry wt) compared with the control root content (8.8 mg g^{-1} dry wt) [49]. A special feature in fenugreek is the presence of steroid saponins which consist of several aglycones (sapogenins) with sugar residues at C-3 and C-26. Cholesterol, a sterol with a C_8-side chain, has been shown to be the precursor to fenugreek saponins [28] or oat saponins [29] in a sequence of reactions in which hydroxylation at C-26 is thought to be the first step [29]. Results obtained with fenugreek are in accordance with the proposals that cholesterol is the precursor to saponins, which are produced in higher amounts in the roots than in cell suspension cultures.

In treated cells the presence of sterols **31**, **28**, **30** and **27**, 14α-methyl sterols with a C_{10}-side chain, seems to indicate that the methylation on C-24[1] of cycloeucalenol (not detected) or obtusifoliol (**10**) can take place under certain

Table 8. Sterol content in cell suspension of fenugreek treated or not with diniconazole (125 μM)

Sterols	RR_t*	[Control] mg†	[Control] (%)‡	[Diniconazole] mg	[Diniconazole] (%)
4-Demethyl sterols:					
Cholesterol (**1**)	1.00	0.13	(3.7)	0.08	(4.9)
14α-Methyl-5α-cholest-8-en-3β-ol (**16**)§	1.03	—	—	0.04	(2.3)
Lathosterol (**2**)	1.09	0.03	(0.7)	0.003	(0.2)
Campesterol (**3**)	1.22	0.1	(2.8)	—	—
14α-Methyl-5α-ergosta-8,24(24^1-dien-3β-ol (**26**)	1.23	—	—	0.22	(13.1)
14α-Methyl-5α-ergost-8-en-3β-ol (**24**)	1.25	—	—	0.23	(13.3)
Stigmasterol (**4**)	1.29	0.86	(23.8)	0.09	(5.2)
Unknown (**17**)	1.38	0.06	(1.6)	—	—
Unknown (**18**)	1.40	0.06	(1.6)	—	—
Sitosterol (**5**)	1.46	1.45	(40.2)	0.25	(14.8)
Isofucosterol (**6**)	1.49	0.64	(17.9)	0.05	(2.8)
14α-Methyl-5α-stigmasta-8,24(24^1)-dien-3β-ol (**27**)	1.52	—	—	0.27	(16.1)
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)	0.005	(0.3)
Stigmasta-7,24(24^1)-dien-3β-ol (**23**)	1.63	0.1	(2.8)	0.003	(0.2)
14α-Methyl-9β,19-cyclo-5α-stigmast-24(24^1)-en-3β-ol (**28**)	1.67	—	—	0.008	(0.5)
4α-Methyl sterols:					
4α,14α-Dimethyl-5α-cholest-8-en-3β-ol (**19**)§	1.13	0.004	(0.1)	—	—
4α,14α-Dimethyl-5α-cholest-8,24-dien-3β-ol (**8**)§	1.21	0.004	(0.1)	—	—
4α-Methyl-5α-cholest-7-en-3β-ol (**9**)§	1.22	0.004	(0.1)	—	—
4α-14α-Dimethyl-5α-ergosta-8,24(24^1)-dien-3β-ol (**10**)	1.36	0.01	(0.3)	0.13	(7.8)
4α,14α-Dimethyl-5α-ergost-8-en-3β-ol (**25**)	1.38	—	—	0.03	(1.6)
4α,14α-Dimethyl-9β,19-cyclo-5α-ergostan-3β-ol (**29**)§	1.52	—	—	0.03	(1.6)
4α,14α-Dimethyl-5α-stigmasta-8,24(24^1)-dien-3β-ol (**30**)	1.67	—	—	0.23	(13.6)
4α-Methyl-5α-stigmasta-7,24(24^1)-dien-3β-ol (**11**)	1.82	0.02	(0.6)	—	—
4α,14α-Dimethyl-9β,19-cyclo-5α-stigmast-24(24^1)-en-3β-ol (**31**)§	1.84	—	—	0.02	(1.1)
4,4-Dimethyl sterols:					
Cycloartenol (**15**)	1.57	0.02	(0.6)	0.005	(0.3)
24-Methylenecycloartanol (**32**)	1.76	—	—	0.005	(0.3)
Total sterols (mg g^{-1} dry wt)		3.6 ± 0.3		1.7 ± 0.02	

*Retention time of steryl acetate relative to cholesteryl acetate. (DB1 capillary column.)
†As mg g^{-1} dry wt.
‡As % of total sterols.
§Suggested structure from mass spectra alone.

conditions. Fonteneau *et al.* [36] could not demonstrate this methylation in microsomes of bramble cells incubated in the presence of *S*-adenosyl methionine and cycloeucalenol or obtusifoliol. Sterols **31**, **28**, **30**, **27**, **26** and **10** possess the 24(24^1) double bond, suggesting that diniconazole might have an indirect effect on the reduction of this double bond of $\Delta^{8,24(24')}$-sterols (Table 4). These effects have been observed in bramble cells treated with fenarimol [17]. However, in roots, this phenomenon was not detected.

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°. Diniconazole = [(*R*)-1-(2,4-dichlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)-1-penten-3-ol] (supplied by Rhône-Poulenc with a purity of 100%), was used at 125 μM (= 40.7 mg l^{-1}) in 0.5% methanolic soln and was applied every 2 days. The control plants were moistened with a soln containing a mixt. of H_2O–MeOH (99.5:0.5). After 10 days, root and shoot lengths were measured and fresh and dry wts 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 Murashige and Skoog [50], microelements from Nitsch *et al.* [51], sucrose (20 g l^{-1}), glutamine (200 mg l^{-1}), myoinositol (100 mg l^{-1}). Phytohormones were NAA (naphthaleneacetic acid) and BAP (benzylaminopurine) used at 1 mg l^{-1}. Cell cultures were cultivated at 23 ± 1° under fluorescent light (70 μE m^{-2} sec^{-1}, 16 hr photoperiod and 120 rpm) and cells were subcultured every 3 weeks. Diniconazole was added at the beginning of the culture in methanolic soln.

The final concn of MeOH was 0.5% (v/v) and the final concn of diniconazole was 125 μM. In the control cultures, MeOH was added at a final concn of 0.5% (v/v). Cultures were grown for 21 days, cells were harvested and dry wt was determined. The PCV (packed cell volume) was determined at 0 and 21 days of culture. Aliquots (10 ml of fresh cell suspensions) were centrifuged at 2500 *g* for 10 min. The vol. of cells was compared with the total vol. (10 ml). Comparisons of results between 0 and 21 days indicate the growth of cells according to the treatment.

Extraction, purification and determination of sterols. Freeze-dried material (200 mg) was ground to a powder in a mortar and extracted with *n*-hexane (× 3) then (× 3) with *i*-PrOH–H_2O (7:3). Aliquots of the 2 extracts were combined, betulinol added as an int. standard, and total sterols isolated according to the method of Grunwald [52]. 0.18N H_2SO_4 in 95% EtOH (10 ml) was added to the dried extract and refluxed for 12 hr to cleave steryl glycosides. 10% KOH in 95% EtOH (5 ml) was added and refluxed for 30 min to hydrolyse the esterified sterols. This gave total sterols. They were extracted (× 3) with Et_2O and enough H_2O (15 ml) to obtain 2 layers. The 3 frs were combined, washed (× 2) with H_2O and dried under vacuum. After purification on silica gel plates using $CHCl_3$–Et_2O (9:1) as a developing solvent and betulinol, cholesterol and lanosterol as standards, total sterols were acetylated with a mixt. of pyridine–Ac_2O (1:2) and 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). The carrier gas (H_2) flow rate was 1.1 ml min^{-1}. Cholesterol was used as a standard for RR_t determination.

Demethyl sterols, 4α-methyl sterols and 4,4-dimethyl sterols were purified by TLC. Total sterols (not acetylated) were chromatographed on silica gel plates using CH_2Cl_2 as a developing solvent (2 runs). After sepn, each of the 3 classes (after spraying with 0.1% berberine in 95% EtOH) was eluted and acetylated. The steryl acetates were analysed by GC as above. Chemical structures were determined by GC-MS: gas chromatography, under the same conditions as those already quoted, coupled with mass spectroscopy using a mass spectrometer at an ionizing potential of 70 eV [33].

Nomenclature. Cholesterol = cholest-5-en-3β-ol (**1**); lathosterol = cholest-7-en-3β-ol (**2**); campesterol = campest-5-en-3β-ol (**3**); stigmasterol = (22*E*)-stigmasta-5,22-dien-3β-ol (**4**); sitosterol = stigmast-5-en-3β-ol (**5**); isofucosterol = (24 × *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**).

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