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Effects of Tetcyclacis on Growth and on Sterol and Sapogenin Content in Fenugreek

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Abstract. Tetcyclacis, a norbornanodiazetine plant growth retardant, used at 10 mg · L^{-1} (36 μM), caused greater growth inhibition in the shoots of fenugreek (*Trigonella foenum-graecum* L.) seedlings (60%) than in the roots (30%), compared with control. This greater retardation was reversed by a supplement of gibberellin (200 mg · L^{-1}). The total sterol composition of control and treated seedlings was analyzed and quantified. In the roots especially, treatment of seedlings with tetcyclacis resulted in a modification of the sterol profile, leading to an accumulation of 14α-methyl sterols, presumably as a consequence of the inhibition of cytochrome P-450-dependent obtusifoliol 14α-demethylase. In addition, tetcyclacis caused a significant increase in the cholesterol content of the roots: 38.1% of total sterols against 3.7% in the control roots. However, tetcyclacis was shown to be an ineffective inhibitor of the *S*-adenosyl-L-methionine (Adomet):cycloartenol-C24-methyltransferase (EC 2.1.1.41) in fenugreek microsomes indicating that cholesterol accumulation does not result from the inhibition of the sterol side chain-alkylating enzyme. Moreover, this accumulation was shown to be concomitant with a significant decrease of the sapogenin content in the treated roots. This last result is discussed with respect to the current proposed pathway by which cholesterol is metabolized to saponins.

The norbornanodiazetine compound tetcyclacis (BAS 106. W, LAB 102 883) (Fig. 1) has been shown to function as a potent plant growth retardant by reducing the shoot gibberellin (GA) content as a result of its inhibition of ent-kaurene hydroxylase (Graebe 1987, Rademacher 1992). This key enzyme involved in gibberellin (GA) biosynthesis is a cytochrome P-450-dependent monooxygenase (Graebe 1987). Evidence has been obtained that tetcyclacis, as well as other azole and pyrimidine derivatives widely used as PGRs or fungicides, bind strongly to the protoheme iron of cytochrome P-450 because of their heterocyclic sp^2 nitrogen atom (Vanden Bossche et al. 1987). Depending on their structures, these compounds interfere with a number of distinct biosynthetic pathways involving P-450-dependent reactions such as those for the GAs, phytosterols, ABA, etc. (Grossmann 1990, Rademacher et al. 1987). Moreover, several agricultural fungicides and PGRs have been shown also to inhibit the cytochrome P-450-dependent obtusifoliol 14α-methyl demethylase (Taton et al. 1988), a key enzyme in phytosterol synthesis, which was recently shown to be the target of 1-*N*-substituted imidazole carboxymethylester herbicides (Salmon et al. 1992).

In plants, cholesterol (Fig. 1) is generally a minor sterol, whereas in animals it is usually the only sterol (Nes and Mac Kean 1977). However, in plants that produce saponins, cholesterol plays a very important role: it is the major precursor of these compounds in fenugreek (Hardman and Fazli 1972), *Dioscorea* (Bennett and Heftmann 1965, Joly et al. 1969, Stohs et al. 1969), and *Avena sativa* (Eichenberger 1982). Saponins are secondary plant metabolites that possess a variety of important biologic activities. For example, they act on metabolism and on the cardiovascular system and have antimicro-

Abbreviations: GA, gibberellin; PGR, plant growth regulator; GA_3, gibberellin acid; FW, fresh weight(s); DW, dry weight(s); Adomet-CMT, *S*-adenosylmethionine-cycloartenol-C24-methyltransferase; GC, gas chromatography; TLC, thin layer chromatography; MS, mass spectroscopy; FS, free sterol(s); SE, steryl ester(s); SG, steryl glycoside(s); ASG, acylated steryl glycosides; SAM, *S*-adenosylmethionine.

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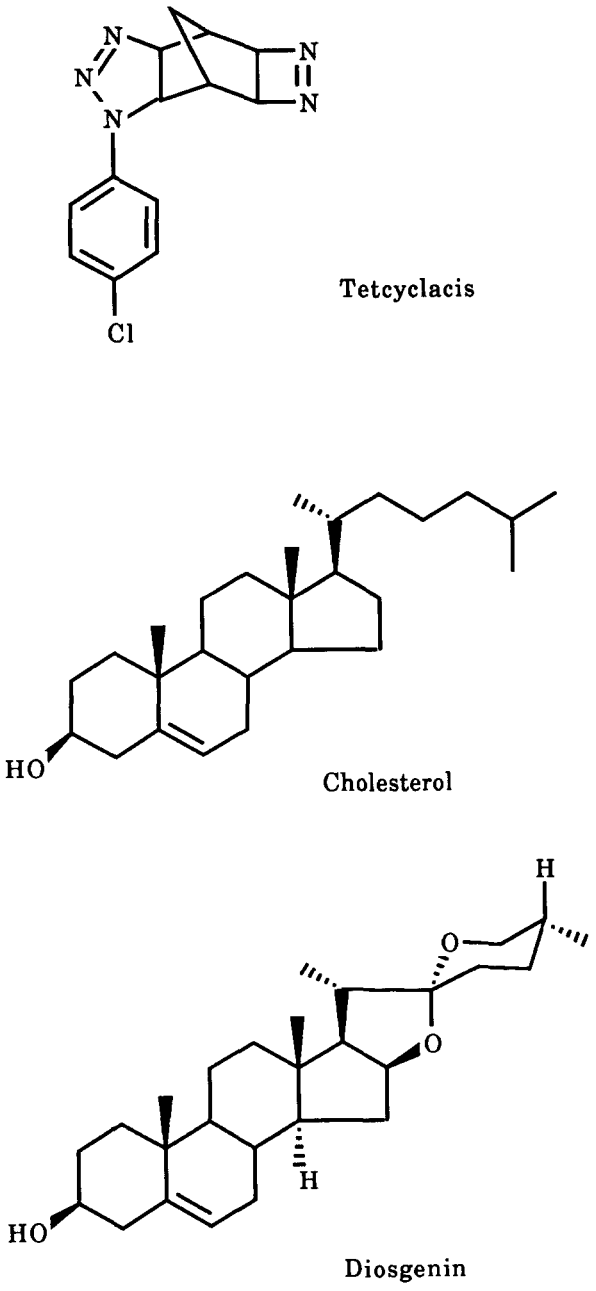

Fig. 1. Chemical structures of tetcyclacis, cholesterol, and diosgenin.

bial (Mahato et al. 1982) and hypocholesterolemic activity (Sauvaire et al. 1991, Stark and Madar 1993). After hydrolysis, saponins are converted to sapogenins, such as diosgenin (Fig. 1), a valuable substance often used in the pharmaceutical industry for the synthesis of active compounds such as sex hormones or cortisone (Lemin and Djerassi 1954, Marker 1940, Nomine 1980). Therefore, manipulation of saponin contents in plants is of interest because of their numerous biologic activities and because they are a source for the production of important pharmaceutical compounds.

The present paper describes the effects of tetcyclacis on the growth, and sterol and sapogenin composition of fenugreek seedlings.

Materials and Methods

Plants and Growth Conditions

Fenugreek (*Trigonella foenum-graecum* L. cv Gouka) seeds were grown in vermiculite in darkness at a temperature of 22 ± 1°C. Tetcyclacis, 5-(4-chlorophenyl)-3,4,5,9,10-pentaazatetracyclo [5.4.$10^{2,6}$.$0^{8,11}$]-dodeca-3,9-diene (supplied by BASF, Aktiengesellschaft, Limburgerhof, Germany), was used at 10 mg · L^{-1} (36 μM) in 0.5% methanolic solution and was applied every 2 days. This concentration was chosen to give results comparable to those of Burden et al. (1987) on the sterol composition of oat and to those obtained in our laboratory in studies on the effects of different concentrations of tetcyclacis on growth and sterol and sapogenin content in fenugreek seedlings (Cerdon 1993). The control cultures were moistened in a solution containing a mixture of water/methanol (99.5:0.5, v/v). In experiments with added GA, tetcyclacis was applied (or not) as above, together with GA_3 at 200 mg · L^{-1} (gibberellic acid, Sigma G-7645). After 10 days of culture, root and shoot lengths were measured and fresh weights (FW) and dry weights (DW) were determined.

Fenugreek cell cultures (for Adomet-CMT assay only) were established in 1990 from callus cultures initiated in 1987 from leaf pieces. Cell suspensions were grown in the following culture media: macroelements from Murashige and Skoog (1962), microelements from Nitsch (Nitsch et al. 1968), sucrose (20 g · L^{-1}), glutamine (200 mg · L^{-1}), myoinositol (100 mg · L^{-1}). Phytohormones were naphthaleneacetic acid and benzylaminopurine, both used at 1 mg · L^{-1}. Cell cultures were cultivated at 23 ± 1°C under fluorescent light (70 μE · m^{-2} · s^{-1}, 16 h photoperiod and 120 rpm), and cells were subcultured every 3 weeks.

Extraction, Purification, and Determination of Sterols

Freeze-dried tissue (200 mg) was ground to a powder in a mortar and extracted with *n*-hexane (three times) and then three times with 2-propanol/water (70:30, v/v). Aliquots of the two extracts were combined. 250 μg of betulinol (Sigma) dissolved in ethyl acetate was added as an internal standard and total sterols isolated according to the method of Grunwald (1970). Sulfuric acid (0.18 N) in 95% ethanol (10 ml) was added to the dried extract and refluxed for 12 h to cleave steryl glycosides. Ten percent KOH in 95% ethanol (5 ml) was added and the mixture refluxed for 30 min to hydrolyze the esterified sterols. This gave total sterols. They were extracted three times with diethyl ether and enough water (15 ml) to obtain two layers. The three fractions were combined, washed twice with water, and taken to dryness under vacuum. After purification on silica gel plates (Macherey Nagel SIL G-25) using chloroform/diethyl ether (90:10, v/v) as developing solvent and betulinol, cholesterol, and lanosterol as standards, total sterols were acetylated with a mixture of pyridine/acetic anhydride (1:2, v/v) and quantified by GC on a DI 700

Table 1. Effects of tetcyclacis or of tetcyclacis + GA_3 on seedling growth and shoot:root ratio of fenugreek.

	Control[a]	GA_3[b]	Tetcyclacis[c]	Tetcyclacis + GA_3[d]
Root length	8.03 ± 0.44[e]	8.32 ± 0.10	5.71 ± 0.20	8.34 ± 0.10
Shoot length	9.06 ± 0.44	12.00 ± 0.40	3.77 ± 0.10	8.30 ± 0.10
Shoot:root ratio	1.1	1.4	0.7	1.0
FW ($g \cdot 100^{-1}$ roots)	5.15 ± 0.22	5.36 ± 0.28	4.94 ± 0.15	5.05 ± 0.20
FW ($g \cdot 100^{-1}$ shoots)	12.30 ± 0.30	13.08 ± 0.32	4.38 ± 0.12	11.62 ± 0.31
Shoot:root ratio	2.4	2.4	0.9	2.3
DW ($g \cdot 100^{-1}$ roots)	0.27 ± 0.02	0.24 ± 0.02	0.38 ± 0.04	0.30 ± 0.01
DW ($g \cdot 100^{-1}$ shoots)	0.57 ± 0.02	0.60 ± 0.01	0.45 ± 0.01	0.50 ± 0.02
Shoot:root ratio	2.1	2.5	1.2	1.7

[a] Cultures were moistened with water/methanol (99.5:0.5, v/v).
[b] GA_3 was used at 200 mg $\cdot L^{-1}$.
[c] Tetcyclacis was used at 10 mg $\cdot L^{-1}$ (36 μM).
[d] Tetcyclacis was used at 10 mg $\cdot L^{-1}$ (36 μM) and GA_3 at 200 mg $\cdot L^{-1}$.
[e] The data are mean values for three experiments ± SD.

(Delsi) equipped with a flame ionization detector and with a DB1 capillary column (30 m × 0.25 mm inner diameter polydimethylsiloxane phase, J & W), used at 270°C (280°C for the injector and detector). 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 dichloromethane as a developing solvent (two runs). After separation, each of the three classes (after spraying with 0.1% berberine in 95% ethanol) was eluted and acetylated. The steryl acetates were analyzed by GC as above. Chemical structures were determined by GC-MS: gas chromatography (Hewlett Packard HP 5890 series II) coupled with mass spectroscopy using a HP 5971 series at an ionizing energy of 70 eV.

The different sterol derivatives were determined as follows. The free sterols (FS) and steryl esters (SE) were present essentially in the hexane extract, and the steryl glycosides (SG) and acylated steryl glycosides (ASG) (analyzed together) were mainly in the 2-propanol extract. For FS and SE, the hexane extract, mixed with 100 μg of betulinol, was concentrated and chromatographed on silica gel plates using hexane/ethyl acetate (60:40, v/v) as a developing solvent. After spraying with berberine, FS were eluted and acetylated. SE plus 100 μg of betulinol were saponified, extracted, and acetylated. Each sterolic form was analyzed by GC.

Extraction, Purification, and Determination of Sapogenins

Total saponins contained in the 2-propanol acid extract were hydrolyzed according to the method of Sauvaire and Baccou (1978a, 1978b) slightly modified by Drapeau et al. (1986). Sulfuric acid (2 N) in 70% 2-propanol (15 ml) was added to the dried extract and refluxed for 12 h to cleave sapogenin-oses bonds. After adding betulinol as an internal standard, sapogenins were extracted with diethyl ether, acetylated, and analyzed by GC equipped with a DB1 capillary column as for the sterols.

Adomet-CMT

Adomet-CMT activity was determined according to the method of Rahier et al. (1984). Fenugreek cells (50 g) were drained and homogenized in a medium containing 0.1 M Tris-HCl, 1 mM EDTA, 5 mM glutathione, 10 mM $MgCl_2$, and 0.5 mM sucrose, pH 7.4. The homogenate was centrifuged at 7000 ×g for 10 min, and the supernatant was centrifuged again at 100,000 ×g for 90 min. The pellet (microsomal fraction) was resuspended in a medium containing 0.1 M potassium phosphate, 1 mM glutathione, 1 mM EDTA, and 4 mM $MgCl_2$, pH 7.5. Microsomes were incubated for 2 h at 30°C in the presence of 50 μM (50 μCi $\cdot$ μmol^{-1}) [^{3}H]SAM (*S*-adenosyl-L-methionine) and 50 μM cycloartenol ± 50 μM tetcyclacis. The reaction was stopped by KOH and ethanol. The neutral lipids were extracted with hexane and purified by TLC as described above on silica gel using dichloromethane as solvent. The 4,4-dimethyl sterol fraction was scraped off and the associated radioactivity determined in a liquid scintillation spectrometer.

Results

Growth Retardation and Its Reversibility

Although tetcyclacis (10 mg $\cdot L^{-1}$) had no effect on fenugreek seed germination, it inhibited root length by 30% and shoot length by 60%, compared with control (Table 1). This was accompanied by a significant decrease (65%) in shoot FW and in shoot DW (20%), whereas root DW increased (40%). The decrease in shoot growth resulted in a concomitant reduction in the shoot:root ratio (by length and by weight). When GA_3 was applied, stimulation of shoot length was the major effect, and when GA_3 and tetcyclacis were applied simultaneously shoot and root lengths were similar to those of the control (Table 1).

Sterol Composition

The mass spectra from GC-MS analysis of sterols were compared with those obtained by other work-

Table 2. Total sterol composition of fenugreek roots.

Sterols	RRT[a]	Control[b]		Tet[c]		Tet + GA[d]	
		mg[e]	(%)[f]	mg	(%)	mg	(%)
4-Demethyl sterols							
Cholesterol	1.00	0.16	(3.7)	2.31	(38.1)	2.45	(37.1)
14α-Methyl-5α-cholest-8-en-3β-ol	1.02			0.17	(2.9)	0.18	(2.8)
Lathosterol	1.09	0.03	(0.7)	0.05	(0.8)	0.05	(0.7)
Campesterol	1.22	0.33	(7.4)	0.32	(5.3)	0.38	(5.8)
Stigmasterol	1.24	0.12	(2.7)	0.006	(0.1)	0.01	(0.2)
Unknown 1	1.37			0.04	(0.6)	0.05	(0.7)
Unknown 2	1.39			0.04	(0.7)	0.05	(0.7)
Sitosterol	1.45	3.56	(79.1)	2.18	(36.3)	2.48	(37.6)
Isofucosterol	1.48	0.14	(3.3)	0.53	(8.9)	0.57	(8.7)
Stigmasta-5,24-dien-3β-ol	1.55	0.03	(0.7)	0.06	(1.0)	0.08	(1.2)
4α-Methyl sterols							
4α,14α-Dimethyl-5α-cholest-8-en-3β-ol	1.13			0.05	(0.8)	0.05	(0.7)
4α,14α-Dimethyl-5α-cholesta-8,24-dien-3β-ol	1.21	0.004	(0.1)	0.02	(0.3)	0.01	(0.2)
4α-Methyl-5α-cholest-7-en-3β-ol	1.22	0.009	(0.2)	0.04	(0.6)	0.03	(0.4)
4α,14α-Dimethyl-9β,19-cyclo-5α-cholestan-3β-ol	1.26			0.006	(0.1)	0.006	(0.1)
4α,14α-Dimethyl-5α-ergosta-8,24(24[1])-dien-3β-ol	1.35	0.004	(0.1)	0.02	(0.3)	0.01	(0.2)
4α-Methyl-5α-ergosta-7,24(24[1])-dien-3β-ol	1.46			0.006	(0.1)	0.006	(0.1)
4α-Methyl-5α-stigmasta-7,24(24[1])-dien-3β-ol	1.82	0.01	(0.3)	0.05	(0.9)	0.05	(0.7)
4,4-Dimethyl sterols							
4,4,14α-Trimethyl-5α-cholest-8-en-3β-ol	1.30	0.004	(0.1)	0.006	(0.1)	0.006	(0.1)
4,4,14α-Trimethyl-5α-cholest-9(11)-en-3β-ol	1.41	0.03	(0.6)	0.02	(0.4)	0.03	(0.5)
Cycloartenol + β-amyrine	1.44	0.02	(0.5)	0.07	(1.2)	0.06	(1.0)
Cycloartenol	1.56	0.02	(0.5)	0.03	(0.5)	0.03	(0.5)
Δ^5-Sterols		4.36	(96.9)	5.46	(91.0)	6.07	(92.0)
14α-methyl sterols		0.09	(1.9)	0.41	(6.8)	0.41	(6.2)
All sterols (mg · g^{-1} DW)		4.5 ± 0.2[g]		6.0 ± 0.1		6.6 ± 0.4	

[a] Relative retention time of steryl acetates compared with cholesterol acetate obtained on GC with a DB1 capillary column.
[b] Control roots.
[c] Roots treated with 36 μM tetcyclacis.
[d] Roots treated with tetcyclacis + GA_3 (200 mg · L^{-1}).
[e] Sterols in mg · g^{-1} DW.
[f] Percent of total sterols.
[g] The data are mean values of three experiments ± SD.

ers (Chitwood and Lusby 1991, Combaut 1986, Goad 1991, Rahier and Benveniste 1989). In tetcyclacis-treated roots, sterol analysis revealed important qualitative and quantitative changes in the total sterol content, which increased from 4.5 mg · g^{-1} DW in control to 6.0 mg · g^{-1} DW in treated plants (Table 2). However, there was little or no effect of tetcyclacis on the sterol content of shoots (data not shown). Tetcyclacis treatment resulted in a dramatic increase in the cholesterol content, from 0.16 mg · g^{-1} DW and 3.7% of total sterols in the control to 2.31 mg · g^{-1} DW, 38.1% of the total sterols, or 42% of the Δ^5-sterols (Table 2), in the treated roots. Moreover, this increase in cholesterol was not entirely realized at the expense of C24-alkylated sterols and accounted, in part, for the quantitative increase in total sterol content following tetcyclacis treatment.

Accordingly, sitosterol content decreased from 3.56 mg · g^{-1} DW (79.1%) in control roots to 2.18 mg · g^{-1} DW (36.3%) in tetcyclacis-treated roots, along with a large decrease in stigmasterol content, which resulted in a decline in the stigmasterol:sitosterol ratio from 0.03 in control to 0.003 in treated roots. There was also a small increase in Δ^7-sterols (2.4% in treated roots compared with 1.2% in controls), which was the result of an increase in 4α-methyl-5α-stigmasta-7,24(24[1])-dien-3β-ol content and the appearance of 4α-methyl-5α-ergosta-7,24(24[1])-dien-3β-ol.

In the roots of tetcyclacis-treated plants, along with the increase in cholesterol, we found a small accumulation of 14α-methyl sterols, comprising mainly 14α-methyl-5α-cholest-8-en-3β-ol and 4α,14α-dimethyl-5α-cholest-8-en-3β-ol. The 14α-methyl sterols represented 6.8% of total sterols in the roots of treated plants compared with 1.9% in the controls. This indicated that tetcyclacis inhib-

Table 3. Sterol derivatives and free sterol composition of 5 major sterols in fenugreek roots.

	Steryl esters				Free sterols				SG + ASG[a]			
	Control[b]		Tet[c]		Control		Tet		Control		Tet	
	mg	(%)	mg	(%)	mg	(%)	mg	(%)	mg	(%)	mg	(%)
Cholesterol	0.07	(5.1)	1.42	(56.5)	0.04	(2.1)	0.68	(35.7)	0.04	(4.7)	0.71	(47.0)
Campesterol	0.12	(8.7)	0.11	(4.6)	0.17	(8.6)	0.12	(6.3)	0.07	(6.6)	0.07	(4.7)
Stigmasterol					0.06	(3.1)	0.01	(0.8)	0.02	(2.2)		
Sitosterol	1.20	(83.4)	0.95	(37.6)	1.70	(84.0)	1.01	(52.9)	0.86	(84.8)	0.68	(45.6)
Isofucosterol	0.04	(2.8)	0.03	(1.3)	0.04	(2.2)	0.08	(4.3)	0.01	(1.7)	0.04	(2.7)
TS[d] (mg · g^{-1} DW)	1.44		2.53		2.03		1.92		1.02		1.51	
Ratio			Control						Tetcyclacis			
FS:(SE + SG + ASG)			0.82						0.48			

[a] Steryl glycosides + acylated steryl glycosides.
[b] Control roots.
[c] Roots treated with tetcyclacis at 10 mg · L^{-1}.
[d] Total sterols. The data are mean values of three experiments.

ited slightly the cytochrome P-450-dependent 14α-methyl sterol demethylase in fenugreek.

The sterol composition of roots treated simultaneously with tetcyclacis and GA_3 was the same as that of roots treated only with tetcyclacis, suggesting that the reduction in root length (Table 1) was independent of the change in sterol composition.

Furthermore, in tetcyclacis-treated roots, analysis of the sterol derivatives revealed that although treated tissues contained a greater amount of sterols, particularly more Δ^5-sterols than control tissues, the FS content remained similar (Table 3), while the amounts of SE and SG plus ASG increased (1.44 to 2.53 mg · g^{-1} DW and 1.02 to 1.51 mg · g^{-1} DW, respectively). This led to a decrease in the FS:(SE + SG + ASG) ratio of about 40% in treated roots compared with control roots (Table 3).

Adomet-CMT activity in isolated microsomes from cell suspension cultures of fenugreek was unaffected by tetcyclacis (Table 4). This indicated that the accumulation of cholesterol was not the result of inhibition of C24 methylation by tetcyclacis.

Sapogenin Content

In fenugreek roots, nine different sapogenins were found. However, with our analytic method, we were unable to separate tigogenin and yamogenin. Nevertheless, sapogenins were divided into monohydroxylated (smilagenin, sarsasapogenin, diosgenin, tigogenin, yamogenin, and neotigogenin) and dihydroxylated forms (yuccagenin, gitogenin, and neogitogenin). Sapogenin composition was altered by tetcyclacis treatment. Decreases were found in the total sapogenin content from 8.8 mg · g^{-1} DW in controls to 5.3 mg · g^{-1} DW in treated roots and in the relative proportions of the monohydroxylated sapogenins (monoOH Sg), principally because of a decrease in diosgenin content. On the other hand, the relative proportion of dihydroxylated sapogenins (diOH Sg) increased compared with control (Table 5).

In the roots of plants given simultaneous applications of GA_3 and tetcyclacis, the sapogenin composition was similar to that in plants treated with tetcyclacis alone.

Discussion

Tetcyclacis is a plant growth retardant that inhibits GA biosynthesis and thereby reduces shoot length, an effect clearly demonstrated in fenugreek plants, in which the tetcyclacis-treated (10 mg · L^{-1}) shoots were appreciably shorter than controls (Table 1). Similar effects of tetcyclacis on shoot growth have been reported by other groups (Burden et al. 1987, Rademacher et al. 1984, Vavrina et al. 1986, Yang and Naylor 1988). In contrast, tetcyclacis treatment has been reported to stimulate root growth through an increase in length and thickening of the main root (Rademacher et al. 1984). However, in our experiments with tetcyclacis, used at 10 mg · L^{-1}, we did not observe such effects. There was a significant shortening of the roots; the decrease in the shoot:root ratio was due to the reduction in shoot growth rather than an increase in root growth (Table 1).

In fenugreek, the growth retarding effects of tetcyclacis were reversed by addition of GA_3 (Table 1), suggesting that they may be mediated by a re-

Table 4. Determination of the Adomet-CMT activity in microsomes from cell suspensions cultures of fenugreek.

Treatment	Adomet-CMT activity (pmol · h^{-1} · mg^{-1} protein)
Control[a]	12
Tetcyclacis[b]	12

[a] Microsomes were incubated without tetcyclacis.
[b] Microsomes were incubated with tetcyclacis (50 μM).

duction in the endogenous GA content of the plant. Similar results have been observed in various species (Rademacher 1992). Accordingly, specific inhibition of GA biosynthesis in a cell-free system of pumpkin endosperm by 10^{-7} M tetcyclacis has been shown (Graebe 1982). Moreover, Zeevaart (1985) showed that *Agrostemma githago,* retarded in growth with tetcyclacis, contained lower concentrations of endogenous GAs than the corresponding control plants. In addition, a model has been proposed in which low concentrations of tetcyclacis ($<10^{-6}$ M) inhibit GA synthesis in plants leading to reduced cell elongation, whereas concentrations ($>10^{-6}$ M) appear to have an effect on sterol synthesis and related cell division (Nitsche et al. 1985). Furthermore, when GA_3 was added alone in culture, the growth was stimulated compared with the control. This stimulation was comparable to that observed in tetcyclacis + GA_3 cultures, indicating that it is independent of tetcyclacis (Table 1).

In fenugreek roots treated with tetcyclacis (10 mg · L^{-1}), we observed important changes in the sterol composition compared with the control (Table 2). First, a decrease in the relative content of Δ^5-sterols (91% of total compared with 96.9% in controls) was observed concomitant with a small, but significant, increase in 14α-methyl sterols (6.4% in tetcyclacis-treated roots compared with 1.9% in controls), providing evidence for very low inhibition of 14α-demethylase by tetcyclacis. This confirms the data of Taton et al. (1988), who showed that in vitro, tetcyclacis has a low affinity for obtusifoliol-14α-methyl demethylase (IC_{50} = 9 μM) compared with the triazole fungicide LAB 170 250F (IC_{50} = 0.05 μM).

Second, a decrease in stigmasterol was found in tetcyclacis-treated roots which could result from the inhibition of the Δ^{22}-desaturase. Indeed, this enzyme, which is involved in the conversion of sitosterol to stigmasterol, has been shown to be cytochrome P-450 dependent in yeast (Hata et al. 1981). However, this reaction has not been yet characterized in plant tissues, although a similar mechanism has been proposed (Grossmann 1992, Haughan et al. 1989, Taton et al. 1988).

Third, in fenugreek roots, a major effect of tetcyclacis treatment was the large increase in cholesterol concomitant with an important increase in total sterol content. Accumulation of cholesterol has already been observed in *A. sativa* after treatment with tetcyclacis, but it was in the shoots (Burden et al. 1987). Although, in fenugreek, this increase in cholesterol was found in all the sterol forms, FS, SE, SG, and ASG, it was proportionally more important in the sterol conjugates (SE, SG, and ASG)

Table 5. Steroidal sapogenin composition in fenugreek roots.

	Control[a]		Tet[b]		Tet + GA[c]	
Sapogenins	mg	(%)	mg	(%)	mg	(%)
Smilagenin	0.04	(0.5)	0.02	(0.4)	0.02	(0.3)
Sarsasapogenin	0.03	(0.3)	0.02	(0.3)	0.01	(0.2)
Diosgenin	2.34	(26.6)	1.15	(21.8)	1.31	(19.9)
Tigogenin + yamogenin	1.39	(15.9)	0.75	(14.3)	0.99	(15.1)
Neotigogenin	0.17	(2.0)	0.11	(2.2)	0.14	(2.2)
Yuccagenin	1.42	(16.2)	1.08	(20.5)	1.43	(21.7)
Gitogenin	2.39	(27.2)	1.55	(29.3)	2.08	(31.5)
Neogitogenin	0.99	(11.3)	0.59	(11.2)	0.6	(9.1)
Mono-OH Sg[d]	3.99	(45.3)	2.07	(39.0)	2.49	(37.7)
Di-OH Sg[e]	4.81	(54.7)	3.23	(61.0)	4.11	(62.3)
TSg[f] (mg · g^{-1} DW)	8.8 ± 0.6		5.3 ± 0.4		6.6 ± 0.5	

[a] Control roots.
[b] Roots treated with tetcyclacis at 10 mg · L^{-1}.
[c] Roots treated with tetcyclacis at 10 mg · L^{-1} and GA_3 at 200 mg · L^{-1}.
[d] Monohydroxylated sapogenins (smilagenin + sarsasapogenin + diosgenin + tigoyamogenin + neotigogenin).
[e] Dihydroxylated sapogenins (yuccagenin + gitogenin + neogitogenin).
[f] Total sapogenin. The data are mean values of three experiments ± SD.

(Tables 2 and 3). The results suggest that the excess of sterol and particularly of cholesterol would be esterified or glycosylated in the treated fenugreek tissues. This esterification could reflect a regulation of the pool of FS in this plant to maintain the optimal functioning of the cell. Such regulation of the FS pool mediated by a balance between sterol-acyl transferase and sterol ester hydrolase activities has already been shown in yeast (Taylor and Parks 1981) and more recently in higher plant cells (Maillot-Vernier et al. 1991).

The accumulation of cholesterol was in part concomitant with a decrease in the sitosterol content, suggesting a possible effect of tetcyclacis on Adomet-CMT. However, we could not observe any direct inhibition of Adomet-CMT by tetcyclacis in fenugreek microsomes (Table 4). Moreover, we observed that in the presence of tetcyclacis, total sapogenin content in the roots decreased to 40% of control. This observation supports the proposal that this decrease in sapogenin content might result from an inhibition of the early steps in the metabolism of cholesterol to saponins (Bennett and Heftmann 1965, Eichenberger 1982, Hardman and Fazli 1972). An attractive possibility would be that tetcyclacis inhibits the first committed step of this pathway, that is, cholesterol 26-hydroxylase, which has been shown to be a cytochrome P-450-dependent monooxygenase in animals (Wikvall 1993). Therefore, it is possible that in fenugreek, tetcyclacis inhibits oxidation of cholesterol to 26-hydroxycholesterol, which, in turn, leads to the synthesis of saponins. Further work will be necessary to test this hypothesis.

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