PESTICIDE BIOCHEMISTRY AND PHYSIOLOGY 34, 211–217 (1989)

Effect of Triadimefon on Lipids, Sterols, and Membrane Fluidity in Submerged Cultures of *Claviceps purpurea*

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Received September 5, 1988; accepted March 20, 1989

Triadimefon (TDF) (5 mg · liter^{-1}) stimulated the functions of chanoclavine cyclase in submerged cultures of high producing mutant *Claviceps purpurea* 59 with partially blocked oxidation and cyclization of chanoclavine. The culture growth decreased and specific clavine production was raised. The effect of TDF on growth and production was reversed by plant oil (7.2 g · liter^{-1}), but not by ergosterol (10 mg · liter^{-1}). The oil stimulated growth and lowered both specific alkaloid production and chanoclavin cyclase function. Ergosterol showed the opposite effects. TDF reduced the total mycelial lipids to 66%, sterols to 40%, and phospholipids to 72%. In the cultures supplemented with ergosterol, the sterol content increased 16-fold and phospholipids were lowered to 27%; however, the total lipid content remained unchanged. Oil increased all lipid components. The sterol content was indirectly proportional to the PC/PE ratio in all cultures. The membrane fluidity of protoplasts from TDF-treated cultures was higher, in ergosterol-treated cultures lower, and unchanged in the presence of oil. The content of methylated sterols in the TDF-treated cultures was only 2% higher than in controls. The demethylation of sterols in *Claviceps* occurs first in position C-14, then in C-4. The chanoclavine cyclase function probably does not depend on membrane fluidity, but a correlation between higher activity and low phospholipid content was found.

INTRODUCTION

Triadimefon (TDF)[1] is a triazole inhibitor of ergosterol biosynthesis. The main common effect of triazole antifungals is the inhibition of sterol C-14 demethylase. Inhibitors block the reaction by binding the heme iron of cytochrome P450. The accumulation of C-14 methylsterols occurs in most treated fungi. (1, 2). In some fungi, the total sterol content increases (3, 4), which could be explained by a reduction in the feedback inhibition of sterol biosynthesis by ergosterol or one of its metabolites. In other fungi, the total sterol content is reduced (2), which is probably caused by feedback inhibition of HMGCoA reductase by lanosterol, accumulating under the conditions of a demethylase block.

Sterol inhibitors also cause the reduction in phospholipids and the change in the fatty acid content and composition. These alterations are considered as the adaptive-type response to changes in membrane fluidity caused by an altered sterol content (5). The clustering of intramembraneous particles observed in the membranes of *Ustilago avenae*, grown with triadimefon, was probably a reflection of changes in membrane fluidity (6, 7).

This study shows the influence of triadimefon on lipid composition, alkaloid production, and growth of the strain *Claviceps purpurea* 59. As described previously (8), strain 59 is partially blocked in the formation of tetracyclic clavines, thus 70% of the total alkaloids consist of tricyclic clavines. We suppose that the strain is impaired in the function of chanoclavine cyclase which catalyzes the conversion of chanoclavine to agroclavine in two steps. The activity of chanoclavine cyclase is present in the microsomal fraction that im-

[1] Abbreviations used: TDF, triadimefon; DPH, 1,6-diphenyl-1,3,5-hexatriene; PC, phospatidylcholine; PE, phosphatidylethanolamine.

0048-3575/89 $3.00

plies its localization in membranes. The degree of the manifestation of the mutation depends on various environmental factors, especially on those interfering with lipid metabolism and/or membrane properties (9).

MATERIALS AND METHODS

Strain and Cultivation Conditions

C. purpurea 59 was isolated in our laboratory (8), and cultivated first in the inoculation medium TI (sucrose–asparagine) and then in the production medium CS 2 (sucrose–citrate–ammonium) on the rotary shaker as described previously (10). All additives were autoclaved and added prior to the inoculation of the medium CS 2. Triadimefon (Bayleton 25 WP, Bayer) was added as an aqueous suspension, its concentration was expressed as an active ingredient. Ergosterol was added to the medium before sterilization as an ethanolic solution to form microcrystalline suspension. The plant oil consisted mainly of suflower oil; soybean oil gave similar results. Medium CS 2 containing oil was sonicated before inoculation. No solvents were added because they caused undesirable changes in the composition of the clavine mixture. The cultivations were conducted in three to five replications.

Analysis of cultures. Biomass, total alkaloid content, and composition of the clavine mixture were estimated according to published procedures (8, 9). The chanoclavine cyclase activity was characterized by oxidation and the cyclization index, calculated from the proportions of tri- and tetracyclic clavines (9). Lipids were analyzed in 22-day-old mycelium. Lyophilized mycelium (2–6 g) was washed three times with 3 ml of the mixture methanol:chloroform (1:1 v/v) to extract the total lipids (11). The analysis of the total lipid composition was performed by TLC–FID: the chromatogram was developed first in hexane:ether:acetic acid (80:20:1 v/v), then in chloroform: methanol:H_2O (67:35:3 v/v), and analyzed on a Iatroscan TH-10 analyzer (Iatron, Japan) by a flame ionization detector. The sterol fraction was analyzed by gas chromatography–mass spectrometry on a Hewlett–Packard 5995 B instrument (H.-P.) with an Ultra 1 (HP) methylsilicon capillary-fused silica column (25 m × 0.20 mm). The conditions were as described previously (12). The sterol/phospholipid molar ratio was calculated using relative molecular masses 396 and 800, respectively.

Protoplast preparation. All the steps of the protoplast isolation were done in the PM medium (trisodium citrate, 0.01 *M;* $MgCl_2$, 0.01 *M;* $CaCl_2$, 0.01 *M*, pH with citric acid to 4.8) containing various concentrations of sucrose (indicated in parentheses, e.g., PM (0.6 *M*)) (13). Mycelium of a 22-day-old culture was washed three times with distilled water and resuspended 1:5 v/v in the PM (0.8 *M*), containing 3–5% (w/v) lyophilized snail enzyme and 0.2% NovoZym TM 234 (Novo BioLabs, Denmark). The suspension was incubated on a shaker at 24°C for 30 hr, then filtered through a Büchner funnel with filter paper Filtrak No. 388 (VEB Niederschlag, DDR). The sediment of conidia and hyphal fragments was washed with the PM (0.6 *M*). The filtrate containing protoplasts was transferred into centrifugation cuvette, underlayered with PM (0.8 *M*), and overlayered with the PM (0.4 *M*). After centrifugation (2000*g*, 20 min), the protoplast layer in the upper interface was removed with a Pasteur pipette and resuspended in the PM (0.8 *M*).

Anisotropy of Protoplast Membranes

The protoplast suspension, diluted with the PM (0.8 *M*) to OD_{520} 0.20–0.23, was divided into two portions. The fluorescence probe was added to one portion, the other served as a reference. 1,6-Diphenyl-1,3,5-hexatriene (DPH) was dissolved in acetone to avoid the side effects of commonly used tetrahydrofuran. The samples were labeled using DPH according to Shinitzky and Barenholz (14) added 2 hr before the measurement to a final concentration of 10^{-6} *M*. All measurements were carried out

at 22 ± 0.5°C in a SLM 4800 S spectrofluorometer operating the T-format with the light modulation and frequency electronics turned off. The excitation light was polarized vertically by one Glan-Thompson polarizer and the fluorescence emission was observed through two other polarizers. The excitation wavelength was 360 nm and the emission was isolated from Rayleigh and Raman scattering by the use of a Scott KV 420 filter. The steady-state anisotrophy values were calculated according to the equation $r = (I_{\parallel}/I_{\perp} \cdot K - 1)/(I_{\parallel}/I_{\perp} \cdot K + 2)$, where $I_{\parallel}$ and $I_{\perp}$ are fluorescence intensities with parallel and perpendicular polarizer and analyzer, respectively, and K is the correction factor of an instrument.

RESULTS

Alkaloid production in the submerged shaken cultures of *Claviceps* proceeds mainly in the stationary phase of the culture development called idiophase (10). Vegetative growth ceases, lipid reserves accumulate, and morphogenetic changes such as conidiation and differentiation into sclerotia-like cells take place. All of the metabolism of the stationary-phase mycelium is directed toward the alkaloid synthesis (10). Therefore, the effect of triadimefon was studied in this highly specialized *C. purpurea* 59 culture.

Triadimefon, ergosterol, and plant oil were added to the cultures of strain 59 prior to inoculation. The inhibitory action of TDF on total alkaloid production and growth was proportional to its concentration (Fig. 1); however, the specific alkaloid production activity of the mycelium was enhanced by these sublethal doses (Table 1). Ergosterol slightly inhibited growth, but increased specific alkaloid production. Oil stimulated vegetative growth and decreased specific alkaloid production. The suspended oil droplets disappeared after 3–5 days of cultivation.

Untreated cultures consisted of hyphae and single sclerotia-like cells and the conidiation was abundant. Oil stimulated filamentous hyphal growth and reduced the conidiation. Ergosterol increased the proportion of sclerotia-like cells and lowered the level of conidiation, whereas TDF treatment resulted in the formation of irregular swollen large cells often with germ tubes or resembling microcyclic conidiation. The morphological effect of TDF was reversed by oil but not by ergosterol. No pelleting was observed in any culture; the mycelium always grew in suspension.

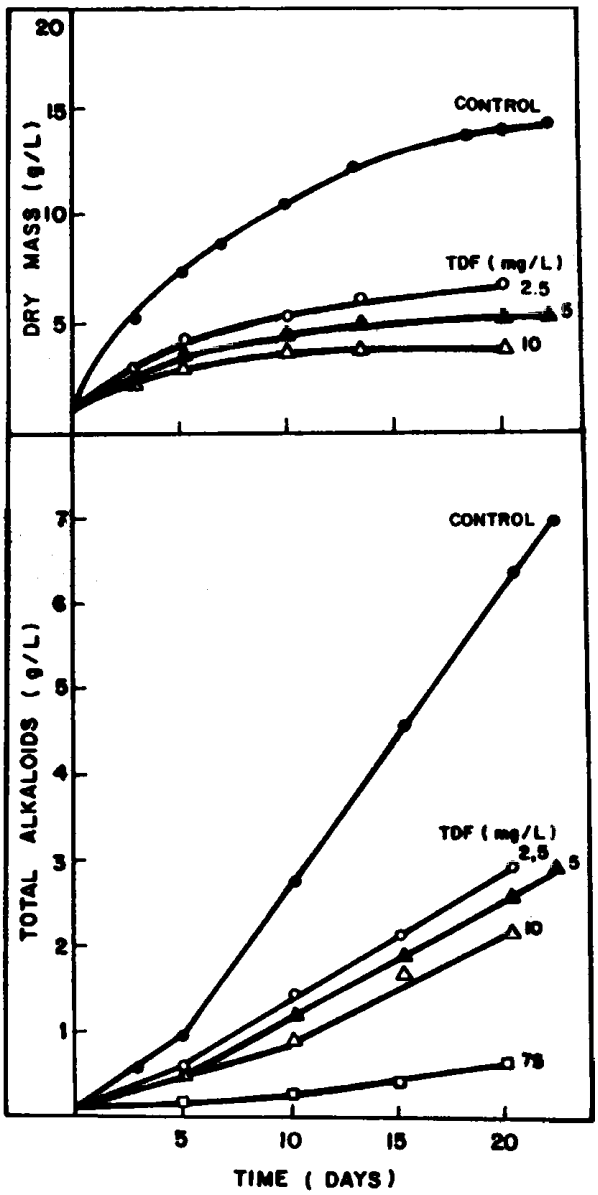

FIG. 1. *Effect of triadimefon on growth and total alkaloid production in submerged cultures of Claviceps purpurea 59.*

TABLE 1
Effect of Triadimefon, Plant Oil, and Ergosterol on Growth, Alkaloid Production and Membrane Properties of C. purpurea 59

Culture	Dose (mg/liter)	Dry mass (g/liter)	Clavine production (mg/g dry mass)	o_i[a]	c_i[b]	Anisotropy
Control	—	14.0	496.1	0.93	1.12	0.1654
Triadimefon	2.5	5.9	590.3	3.27	2.20	ND
	5.0	5.0	622.7	4.76	2.08	0.1326
	10.0	3.5	638.2	3.54	2.09	ND
Oil	7200.0	18.3	384.8	0.79	0.73	0.1642
Ergosterol	10.0	10.9	577.3	2.66	3.49	0.1774
Triadimefon	5.0	17.3	360.5	0.57	1.41	0.1522
Oil	7200.0					
Triadimefon	5.0	4.4	553.5	1.97	1.94	0.1723
Ergosterol	10.0					
Ergosterol	10.0	14.6	434.9	1.00	1.02	ND
Oil	7200.0					

[a] o_i = (100/% chanoclavine)-1.
[b] c_i = (% agroclavine + % chanoclavine)/% chanoclavine-1-aldehyde.

C. purpurea strain 59 produced 70% tricyclic clavines. The intensity of oxidation of chanoclavine to chanoclavine-1-aldehyde is expressed by the oxidation index and the cyclization of aldehyde to agroclavine is characterized by the cyclization index (9). TDF in all concentrations used increased both indices, the same held for ergosterol. However, the synergism of both agents was not complete. The effect of TDF and ergosterol was completely reversed by oil, which is an inhibitor of chanoclavine oxidation and cyclization.

The fluidity of protoplast membranes, which is indirectly proportional to their anisotropy values (r) (Table 1), was increased by TDF. Ergosterol, as expected, decreased the membrane fluidity even in combination with TDF. Oil did not affect the fluidity, but in combination with TDF reversed its impact. The analysis of total lipid revealed the decrease in total lipids, free sterols, triglycerides, and phospholipids in the TDF-treated mycelium (Table 2). The molar ratio of free sterols to phosphatides was also low. On the contrary, the ratio of phosphatidylcholine to phosphatidylethanolamine (PC/PE) was 10 times higher than in the control. All of these effects of TDF were reversed by oil addition. Oil itself increased the lipid and sterol content of the mycelium, decreased the PC/PE ratio, and increased the sterol/phospholipid ratio. Ergosterol elicited sterol accumulation (73.9 mg per flask), well over the amount added to the culture (0.6 mg per flask). The phospholipid content was significantly lower.

TABLE 2
Lipid Composition in Cultures of C. purpurea 59

Culture	Total lipids[a]	Free sterol	Phospholipids	Triglycerides	PC	PE	PC/PE	Sterol/phospholipid (molar ratio)
Control	22.1	0.818	11.67	7.67	9.04	1.35	6.7	0.141
Triadimefon[b]	14.5	0.336	8.50	4.63	8.06	0.13	61.8	0.079
Oil	48.3	2.318	10.85	31.20	8.45	1.84	4.6	0.424
Ergosterol	24.9	13.570	3.34	4.20	2.31	0.69	3.3	8.240
Triadimefon Oil	24.8	1.02	9.50	11.38	7.69	0.67	11.5	0.212

[a] Lipid composition is expressed as the percentage of dry mass.
[b] Dose of TDF was 5 mg/liter, other agents were added as in Table 1.

Sterol analysis (Table 3) showed a 2% increase in the amount of the methylated sterols after TDF treatment. The amount of planar functional sterols (classified according to Ref. (2)) represented 87.8% of the total sterols, whereas 89.7% were found in the control culture. Sterols in cultures with ergosterol and oil were not analyzed because of the possible interference of added compounds. No 4,14-dimethylsterols were found in any of the cultures analyzed. Triadimefon inhibited only demethylation, it interfered neither with desaturation nor with the isomerization of sterols.

DISCUSSION

Submerged 22-day-old cultures of the strain *C. purpurea* contained a considerable amount of lipids in comparison with other fungi (15). Also, the phospholipid content was rather high, which corresponded with the occurrence of numerous membrane structures in older *Claviceps* cells (16). The sterol content was of the same order like that in older cells of plant pathogen *Taphrina deformans* (3), but it was 10 times higher than in other phytopathogens, *Cercospora* and *Cercosporidium* (4).

TDF displayed its effect on total lipid metabolism than specifically on sterol demethylation. The total sterol content was decreased, but the amount of methylsterols increased only by 2%. The accumulation of free fatty acids, observed after triazole treatment in numerous fungi (for review see Ref. (3)), was not observed in *C. purpurea* 59. Free fatty acids represented 2–3% of total lipids in all analyzed cultures.

Sterol analyses of various *Claviceps* species (17) and the analyses of TDF-treated *Claviceps* cultures never revealed 4,14-dimethylsterols (e.g., obtusifoliol). Modifications of lanosterol in most fungi proceed by the sequence C-24 alkylation–C-14 demethylation–C-4 demethylation (1, 2). It seems that in *Claviceps* cells the C-14 demethylation is an absolute prerequisite for the demethylation on C-4 and cannot be overcome even in the presence of the triazole inhibitor. *Claviceps* also belongs to the

TABLE 3
Effect of Triadimefon on Sterol Composition of C. purpurea 59

Sterol	RRT[a]	% of total sterols	
		Control	Triadimefon (5 mg/liter)
8,24-Lanostadienol (lanosterol)	1.52	0.1	2.6
24-Methyl-8,24(28)-lanostadienol	1.75	—	3.4
9(11)-Lanostenol	1.85	—	0.1
4,4-Dimethyl-8,24-ergostadienol	1.83	0.2	0.3
4-Methyl-7,24-ergostadienol	1.87	0.7	0.2
4-Methyl-8,24-cholestadienol	1.38	—	0.1
4-Methyl-8,24(28)-cholestadienol	1.62	4.2	2.6
4-Methyl-8(14)-cholestenol	1.48	0.7	0.3
8,24(28)Ergostadienol (fecosterol)	1.38	7.6	12.4
7-Ergostenol	1.46	1.2	0.4
4-Methyl-7-ergostenol	1.66	0.3	0.3
Ergostanol	1.33	0.3	—
8(14)-Cholestenol	1.13	1.1	0.3
5,7,22,24(28)-Ergostatetraenol	1.34	0.9	0.8
7,22-Ergostadienol	1.28	10.3	9.9
5,8,22-Ergostatrienol	1.19	6.3	6.2
5,24(28)-Ergostadienol	1.27	0.8	1.1
5,22-Ergostadienol (brassicasterol)	1.12	14.1	14.3
5,7,22-Ergostatrienol (ergosterol)	1.26	48.5	42.7

[a] Relative retention time.

group of fungi, which respond to triazole action by decreasing the level of sterols. However, an inhibitory action of methylated sterols on HMGCoA reductase is not probable because the alkaloid synthesis which consumes more isoprenoid units than steroidogenesis is not inhibited by TDF.

The morphological changes caused by TDF in *Claviceps* resembled those reviewed by Weete (1), namely swelling and vacuolization of the cells but generally undisturbed spore germination.

TDF increased membrane fluidity. This effect was observed even in cultures supplemented with ergosterol or oil. The increase of fluidity is accompanied by a decrease in the sterol/phospholipid molar ratio that is reduced by triadimefon to 50% of the corresponding control. Ergosterol did not restore the growth inhibition caused by TDF, but substantially lowered the membrane fluidity both alone and together with TDF. The presence of an ergosterol effect also confirmed that the exogenous sterol entered the *Claviceps* cells.

The increase of sterol biosynthesis after the addition of ergosterol is quite an unexpected result. One possible explanation is the formation of some deregulating substance in ergosterol-supplemented medium during autoclaving. The experiment was also repeated with nonautoclaved ergosterol. The increase of the sterol content was lower but still present (data not shown).

Interestingly, it was found that the triadimefon and ergosterol effect was reverted by plant oil. Oil, as a source of triglycerides and sterols (plant oils usually contain 3–4 g of sterol · liter^{-1}), can replenish the lipid shortage in TDF- and ergosterol-treated cells. Plant sterols from oil do not show the same effect as exogenous ergosterol. Clomiphene lowered the sterol content, and increased the membrane fluidity and cyclase function. The clomiphene effect was also reversible by plant oil (8, 19).

Ragsdale and Sisler (18) achieved a partial reversion of triarimol inhibition of *U. maydis* by the addition of oleic acid and other fatty acids but not by ergosterol. In *Cladosporium cucumerinum* these were ineffective and growth was restored by vitamin A, β-carotene, and even testosterone that, on the other hand, have no effect on *U. maydis*. It seems that triazoles affect several sites in lipid metabolism, but not necessarily the same sites with equal impact in all organisms. Different fungi also counteract the effect of triazoles by different ways.

ACKNOWLEDGMENTS

Thanks are due to Professor J. D. Weete for reading the manuscript and to Novo BioLabs (Denmark) for the generous gift of NovoZym TM 234.

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