Biochem. Physiol. Pflanzen 186, 99–108 (1990)
VEB Gustav Fischer Verlag Jena

Regulation of Lipid and Ergot Alkaloid Biosynthesis in *Claviceps purpurea* by Chlorophenoxy Acids

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Key Term Index: chlorophenoxy acids, membrane fluidity, ergot alkaloids, sterols, fatty acids; *Claviceps purpurea*

Summary

Lipids influence ergot alkaloid biosynthesis in *Claviceps purpurea* mainly as structural elements of membranes. Chlorophenoxy acids (CPA) were applied to *C. purpurea* cultures to modify cellular lipid composition. Mutant strain *C. purpurea* 59 accumulating tricyclic clavines and two parental strains 35 and 129 were used. All the CPA – clofibrate, 2-(4-chlorophenoxy)-acetate and 2-(4-chlorophenoxy)-propionate (CPP) lowered lipid content. Fatty acid (FA) unsaturation index increased indicating a stimulation of FA-desaturation and also FA-elongation. Sterol biosynthesis was impaired mainly in the C(4)- and C(14)-demethylation resulting in a decrease of "membrane functional" (quasiplanar) sterols. Relative content of phosphatidylethanolamine decreased 10fold. Changes in lipids correlated with the decrease of microviscosity of cell membranes (measured as a steady state fluorescence anisotropy of the protoplast membranes with DPH and TMA-DPH as probes). CPP in subtoxic doses stimulated alkaloid production and changed alkaloid composition in the *C. purpurea* 59 strain impaired in chanoclavine cyclase function.

Introduction

The relationship between biosynthesis and composition of lipids and biosynthesis of ergot alkaloids have already been studied by several authors (WAIBLINGER and GRÖGER 1972; DESAI and ŘEHÁČEK 1982; NEUMANN et al. 1979) who assumed that the regulatory interaction of these metabolic pathways occurs at the level of distribution of the acetyl-CoA flow. The hypothesis was based on the fact that the lipid content in mycelium of the fungus *Claviceps* increased almost in parallel with activity of acetyl-CoA-carboxylase and alkaloid production (ŘEHÁČEK and KOZOVÁ 1975).

We assume that lipids influence biosynthesis of alkaloids also via changes in the structure of cell membranes or, occasionally, of lipid inclusions. A major part of alkaloid biosynthesis is

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Abbreviations: CPA, chlorophenoxy acids; CPAc, 2-(4-chlorophenoxy)acetate; CPMP, 2-(4-chlorophenoxy)-2-methylpropionate (clofibric acid); CPP, 2-(4-chlorophenoxy)-propionate; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-(4-trimethylammonium)-phenyl-6-phenyl-1,3,5-hexatriene; FA, fatty acids; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; 2,4-D, 2,4-dichlorophenoxyacetate; U.I., fatty acids unsaturation index; C_i, cyclization index of chanoclavine cyclase; O_i, oxidation index of chanoclavine cyclase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DM, dry mass

localized on endoplasmic reticulum (with the exception of first two enzymes found in cytoplasm (VOŘÍŠEK et al. 1983). The activity of these enzymes is usually associated with the microsomal cell fraction – e.g. reactions mediated by cytochrome P-450 (KIM et al. 1981; KIM et al. 1983). The activity of membrane enzymes is significantly affected by protein-lipid conformation and hence also by the properties of membranes depending on its lipid composition.

To influence lipid biosynthesis of *Claviceps* cultures we used clofibric acid [2-(4-chlorophenoxy)-2-methylpropionate] (CPMP) and its lower homologues DL-2-(4-chlorophenoxy)-propionate (CPP) and 2-(4-chlorophenoxy)-acetate (CPAc). Clofibric acid and its derivates are used as broad-spectrum hypolipaemics in the clinical practice.

CPAc is a derivative of a well-known herbicide 2,4-D. All 3 chlorophenoxy acids also exhibit slight auxin effects.

A mutagenic treatment increasing the production of clavine alkaloids in *Claviceps* strains was associated with a substantial change in the spectrum of cell fatty acids (KŘEN et al. 1985). Therefore, in the present work we aimed at a physiological regulation of lipid composition in cells of *C. purpurea* production cultures with special respect to the production of alkaloids.

The mutant strain, *C. purpurea* 59 (KŘEN et al. 1986) is characterized by a high accumulation of tricyclic clavines (chanoclavine-I and chanoclavine-I-aldehyde) probably due to a damaged function of chanoclavine cyclase. This enzyme is responsible for closing the 4th cycle in the ergoline ring. The phenotype of this mutation can be influenced by certain membrane-affecting agents such as nystatin and clomiphen (PAŽOUTOVÁ et al. 1987). For comparison we also used two ancestor strains *C. purpurea* 129 and 35 having a fully functional chanoclavine cyclase and differing only in the level of alkaloid production.

Material and Methods

Strains and cultivation conditions

Strains *Claviceps purpurea* 129, 129/35 and 59 were obtained after UV mutagenesis (KŘEN et al. 1986, PAŽOUTOVÁ et al. 1987). The strains are deposited in the collection of the Institute of Microbiology, Academy of Sciences (Prague) and were maintained on a medium T2 (agar-solidified), cultivated on media T1 (inoculation medium) and CS 2 (production medium). Media composition and cultivation conditions have been described elsewhere (KŘEN et al. 1984).

2-(4-Chlorophenoxy)-acetic acid, DL-2-(4-chlorophenoxy) propionic acid and 2-(4-chlorphenoxy)-2-methylpropionic acid (Ega-Chemie, Steinheim/Albuch FRG) were added as Na-salt filter-sterilized aqueous solutions at the beginning of cultivation. Ergosterol was added to the medium before sterilization as an ethanolic solution to form a microcrystalline suspension. Sunflower oil was used as plant oil. The oil was added prior to sterilization and during cultivation it formed fine emulsion. Soybean oil yielded similar results.

Analysis of cultures

All analyses were made (if not stated otherwise) in day 22 of production cultivation.

Total alkaloid content was determined by the van Urk reagent (KŘEN et al. 1984), composition of the clavine mixture by TLC (KŘEN et al. 1986a) and biomass was determined gravimetrically.

Total lipids were extracted from lyophilized mycelium as described in (PAŽOUTOVÁ et al. 1988) and weighed. The composition was analyzed by TLC-FID (PAŽOUTOVÁ et al. 1988) in a Iatroscan TH-10 Analyzer (Iatron, Japan). The sterols (as acetyl esters) were analyzed by GC-MS on a Hewlett-Packard 5995B apparatus (Hewlett-Packard, USA) with a capillary fused silica column 25 m × 0,20 Ultra

1 (HP) (methylsilicone) (KŘEN et al. 1986b). Fatty acids composition was determined after alkaline hydrolysis and methyl-esterification by GC-MS under conditions described previously (KŘEN et al. 1985).

The sterol/phospholipid molar ratio was calculated using relative molecular masses 396 and 800, respectively.

Anisotropy of protoplast membranes

The membrane anisotropy was measured as a steady state fluorescence anisotropy of the protoplast suspension with 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethyl-ammonium)-phenyl-6-phenyl-1,3,5-hexatriene (TMA-DPH). The membranes of protoplast prepared as in PAŽOUTOVÁ et al. (1988) were labeled with DPH under conditions described previously (PAŽOUTOVÁ et al. 1988) by adding it as acetone solution to final conc. 10^{-6}M. TMA-DPH was added immediately before the measurement as an acetone solution to final conc. $5 \cdot 10^{-7}$M. All measurements were performed at 22 ± 0.5 °C in a SLM 4800 S (SLM Inc., Urbana, USA) spectrofluorometer operating in the T-format with light modulation and frequency electronics off. All determination were made from 2 independent cultivations from which lipid composition differred in less than 5% and fluidity measurements less than 10%. In results, average values are shown.

Results and Discussion

Alkaloid Production

All chlorophenoxy acids tested decreased biomass production in all *C. purpurea* strains used. The results obtained in *C. purpurea* 129/35 are illustrated in Fig. 1. Alkaloid production was inhibited by most of the CPA used (Fig. 2).

However, the effect of CPP on alkaloid production was quite different. Low concentrations (below 100 m CPP · l^{-1}) increased the alkaloid production (Figs. 2, 3, Table 2). Optimal

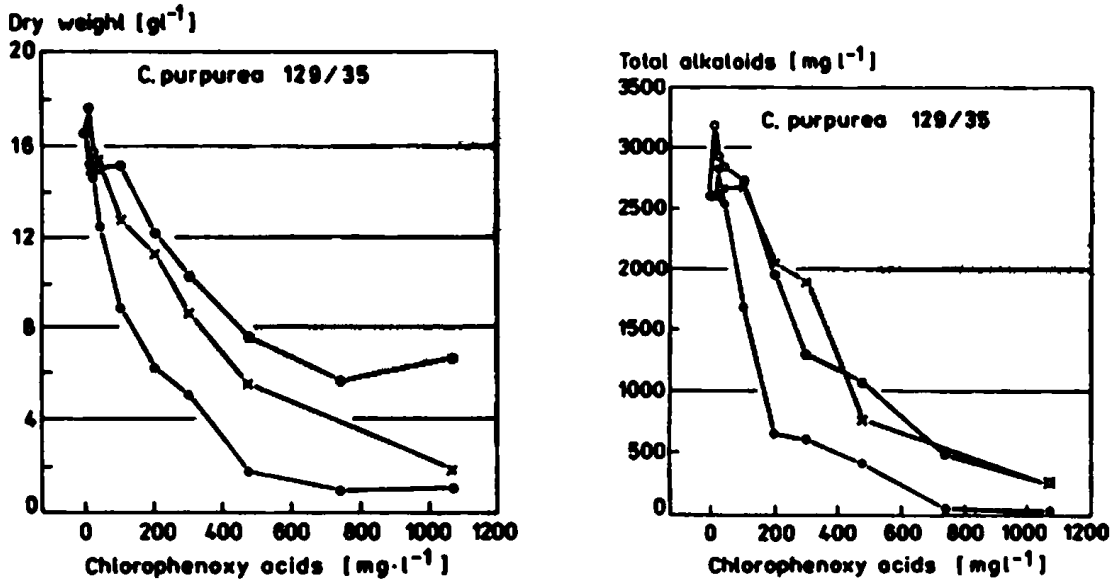

Fig. 1. *Effect of chlorophenoxy acids on biomass production in Claviceps purpurea 129/35.* ● 2-(4-chlorophenoxy)-2-methylpropionate, ○ DL-2-(4-chlorophenoxy)-propionate, × (4-chlorophenoxy)-acetate.

Fig. 2. *Effect of chlorophenoxy acids on ergot alkaloid production in Claviceps purpurea 129/35.* See Fig. 1 for symbols.

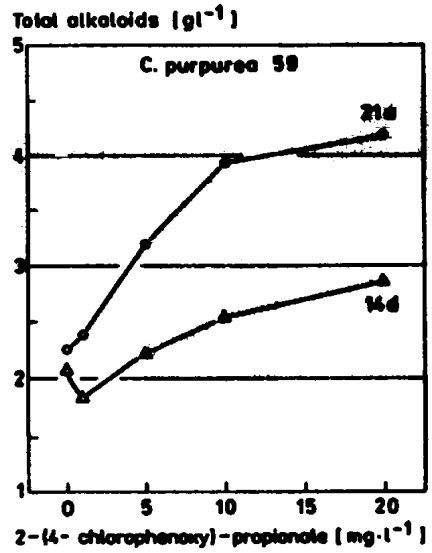

Fig. 3. *Effect of DL-2-(4-chlorophenoxy)-propionate on ergot alkaloid production in Claviceps purpurea 59 after 14 d and 21 d cultivation.*

doses stimulating the production were strain-specific and varied within 10–60 mg · l^{-1}. The same stimulatory effect was observed also in *C. fusiformis* (data not shown).

Chlorophenoxy acid influenced alkaloid biosynthesis more significantly (comparing with other tested strains) in the mutant strain *C. purpurea* 59 (Tables 1, 2). Oxidation of chanoclavine to its aldehyde (O_i) decreased. Cyclization of chanoclavine aldehyde (index C_i) increased proportionally with increasing CPP concentration. The content of chanoclavine-I-aldehyde decreased and that of tetracyclic clavines in the alkaloid mixture increased (Table 2).

Fatty Acids and Phospholipids

The total lipid fraction in the mycelium (22 d) of *C. purpurea* was 22.1 %, which is quite high compared to other fungi (WEETE 1980). CPP and other chlorophenoxy acids (results not shown) decreased substantially the total lipid content.

All chlorophenoxy acids influenced fatty acid composition of the 3 *C. purpurea* strains used in the same manner (Table 3). The content of palmitic acid decreased and that of long-chain FA increased, of C_{18} acids in particular. At the same time the degree of FA saturation changed; the fraction of mono- and di-enoic FA increased, of oleic (not in CPP) and linoleic acid in particular. This situation resulted in an increase of the FA unsaturation index. Chlorophenoxy acids probably stimulated desaturation and elongation of FA. KAWASHIMA et al. (1984) observed similar effects on FA after administration of clofibrate in rats.

CPP, whose effect on lipid composition in strain 59 was studied more in detail, increased a little the percentage of free FA in the mycelium (Table 4). Inhibitors of sterol-C-14-demethylases elicit in fungi similar changes in FA due to adaptive-type response to depletion of membrane active sterols (WEETE 1987; SISLER and RAGSDALE 1977).

However, comparison of total lipid extracts, which include both cytoplasmic acylglycerols and membrane phospholipids, is of limited value particularly as acylglycerols, of which

Table 1. *Effect of 2-(4-chlorophenoxy)-propionate, plant oil and ergosterol on growth, alkaloid production and membrane properties in strain C. purpurea 59* (all data from 22 day of production cultivation). Date are means from 2 parallels.

Culture	Dose (mg · l^{-1})	Dry mass (g · l^{-1})	Total alkaloids (mg · l^{-1})	Specific alkaloids	Anisotropy* of membranes [mg · (g DM)$^{-1}$]	O_i	C_i
Control	–	14.0 ± 0.6	6,850 ± 150	469	0.165 ± 0.004	0.93	1.12
CPP	100	12.4 ± 0.5	6,950 ± 120	533	0.157 ± 0.003	0.68	1.80
Oil	7200	18.3 ± 0.7	7,034 ± 140	385	0.164 ± 0.005	0.79	0.73
Ergosterol	10	10.9 ± 0.4	6,300 ± 90	577	0.177 ± 0.005	2.66	3.49
CPP + Oil	100 / 7200	15.1 ± 1.1	6,500 ± 200	431	N.D.	0.62	0.85
CPP + Ergosterol	100 / 10	9.9 ± 0.5	5,500 ± 170	555	N.D.	1.30	2.85

* Anisotropy of membranes measured with DPH
O_i – oxidation index = ((100 % chanoclavine)-1
C_i – cyclization index = % tetracyclic clavines/% chanoclavine-1-aldehyde (PAŽOUTOVÁ et al. 1987)
N.D. – not determined
DM – dry mass
CPP – 2-(chlorophenoxy)-propionate

Table 2. *Effect of chlorophenoxy acids on ergot alkaloid production in C. purpurea strains 59, 129 and 129/35 (day 22)* – for further data see Table 3.

C. purpurea	Inhibitor		Alkaloids* (Day 22)					O_i	C_i
		conc. (mg · l^{-1})	total (mg · l^{-1})	A %	B %	C %	CA %		
129	control	–	2,877	68	27	4	1	–	–
129	CPP	100	3,473	77	21	4	0	–	–
129/35	control	–	2,851	62	34	4	0	–	–
129/35	CPA	100	2,483	66	27	5	1	–	–
129/35	CPP	100	2,715	68	26	5	1	–	–
129/35	CPB	100	2,639	58	36	3	0	–	–
59	control	–	3,728	18	24	24	33	3.1	1.4
59	CPAc	100	4,311	18	16	50	15	1.0	2.4
59	CPP	20	6,500	10	16	55	21	0.8	1.1
59	CPP	100	5,100	9	14	50	19	1.4	2.3
59	CPP	500	2,966	23	37	33	7	1.9	12.9
59	CPB	100	3,232	13	35	38	13	1.4	3.0

*A – agroclavine, E – elymoclavine, C – chanoclavine, CA – chanoclavine aldehyde
O_i – oxidation index of chanoclavine cyclase
C_i – cyclization index of chanoclavine cyclase

Table 3. *Changes in composition of main fatty acids in mycelium of Claviceps purpurea strains 129, 129/35 and 59 (expressed as % of total FA), in FA unsaturation index and protoplast membrane fluidity after the treatment with chlorophenoxy acids (day 22).*

C. purpurea	Substance		Fatty acids+					U.I.	Membrane anisotropy (TMA-DPH)
		conc. ($mg \cdot l^{-1}$)	16:0	18:2	18:1	18:0	OH-18:1		
129	control	–	27.7	45.1	14.3	7.5	0.2	0.985	0.243
129	CPP	100	17.5	55.2	12.6	8.8	0.4	1.277	N.D.
129/35	control	–	32.2	41.3	14.3	7.9	0.2	0.985	0.250
129/35	CPAc	100	28.6	41.9	15.7	8.1	0.4	1.008	0.197
129/35	CPP	100	30.0	51.2	13.5	7.9	0.4	1.171	0.183
129/35	CPMP	100	20.3	51.1	16.7	6.1	0.4	1.200	0.222
59	control	–	26.3	35.8	28.0	9.7	0.3	1.006	0.200
59	CPAc	100	16.9	36.1	30.8	10.7	0.4	1.039	0.181
59	CPP	20	22.9	44.8	20.0	7.1	0.4	1.105	N.D.
59	CPP	100	22.9	46.0	18.2	8.1	0.5	1.112	0.166
59	CPP	500	18.0	54.6	14.5	7.4	0.5	1.247	N.D.
59	CPMP	100	16.0	40.5	32.6	6.4	0.6	1.147	N.D.

+ In addition to the acids presented, FA 16:1, anteiso-17:0, 20:0, 22:0 and 24:0 were detected at concentrations lower than 1 % and their changes were insignificant. The FA 18:2 was identified as linoleic acid. OH-18:1 Ricinooleic acid. U.I. – Fatty acid unsaturation index (EVANS et al. 1985), N.D. – Not determined.

Table 4. *Lipid composition in cultures of Claviceps purpurea 59.* Influence of DL-2-(4-chlorophenoxy)-propionate

		Total lipids in dry biomass (%)	Triglycerides (% of DM)	Groups of lipids+ (% of total lipids)											other minor lipid
				HC	SE	FS	WE	TG	FFA	PA	PE	PI	PS	PC	
Dose ($mg \cdot l^{-1}$)															
control	0	22.1	7.7	0.7	2.4	3.7	0.4	34.7	2.1	0.7	6.1	2.4	2.7	40.9	3.3
CPP	100	9.8	3.2	1.1	1.1	2.1	0.6	32.1	2.7	1.1	0.7	2.3	1.4	52.7	2.1
CPP + Oil	100 7,200	26.1	13.4	0.8	1.9	3.3	0.4	51.4	4.2	1.6	2.6	1.4	2.1	23.6	6.7
Oil	7,200	48.3	31.2	0.8	3.2	4.8	0.9	64.8	0.8	0.3	3.8	0.7	0.0	17.5	2.4

+ Abbreviations: HC, hydrocarbons; SE, sterolesters; FS, free sterols; WE, wax esters; TG, triglycerides; FFA, free fatty acids; PA, phosphatidic acid; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine

triglyceride is the most abundant fraction, may comprise over 90 % of total fungal lipid content (WEETE 1980). It is therefore reasonable to assume that analysis of total phospholipids alone may prove to be a reliable index describing changes in membrane components.

After the addition of CPP substantial changes in phospholipid composition in *C. purpurea* 59 could be detected (Table 4).

Table 5. *Composition of sterol fraction of C. purpurea 59* (% of total sterols). Influence of DL-2-(4-Chlorophenoxy)-propionate (100 mg · l^{-1})

No.	Sterols	Control	CPP
1	8,24-lanostadien-3β-ol	0.1	1.9
2	9(11)-lanosten-3β-ol	0.0	1.8
3	24-methyl-8-lanosten-3β-ol	0.0	0.1
4	4,4-dimethyl-8,24-cholestadien-3β-ol	0.2	1.7
5	4-methyl-7,24-ergostadien-3β-ol	0.7	0.6
6	4-methyl-8,24-cholestadien-3β-ol	0.0	0.3
7	4-methyl-8,24 (28)-ergostadien-3β-ol	4.2	2.8
8	4-methyl-(14)-cholesten-3β-ol	0.7	0.4
9	8,24 (28)-ergostadien-3β-ol	7.6	15.1
10	7-ergosten-3β-ol	1.2	3.9
11	4-methyl-7-ergosten-3β-ol	0.3	4.3
12	ergosten-3β-ol	0.3	0.2
13	8 (14)-cholesten-3β-ol	1.1	0.7
14	5,7,22,24 (28)-ergostatetraen-3β-ol	0.9	1.0
15	7,22-ergostadien-3β-ol	10.3	2.7
16	5,8,22-ergostatrien-3β-ol	6.3	2.2
17	5,24 (28)-ergostadien-3β-ol	0.8	15.3
18	5,22-ergostadien-3β-ol	14.1	6.4
19	5,7,22-ergostatrien-3β-ol	48.5	32.2
	other minor sterols	2.7	6.4
	14-methylsterols	0.1	3.8
	4,4-dimethylsterols	0.3	5.5
	4-methylsterols	5.9	8.4
	membrane functional "quasiplanar" sterols	89.7	78.8
	24-methylsterols	95.2	86.8

Total phospholipid content as related to dry mass decreased to about 50 % control, which is associated with the overall decrease of lipids in the mycelium. Representation of PE decreased almost 10 times, so that the PC/PE index rose from 6.7 to 75.3. This finding indicates that pronounced changes in the quality of membranes occurred.

The increase of the PC/PE index and the decrease of the total lipid content could be reversed by the addition of oil to the cultivation medium (Table 4).

Sterol Biosynthesis

CPP brought about a decrease of total sterols (free sterols + sterylesters) of 25 % of the control and a decrease of their relative proportion in the lipid fraction (Table 4). The content of free functional sterols dropped from 0.73 % of DM (control) to 0.15 % (100 mg CPP · l^{-1}).

The effect of CPP on the cellular sterol composition resembled that of sterol demethylation inhibitors, namely the increase of C(4) and C(14)-methyl-sterols (Table 5) associated with a decrease in the content of membrane "quasiplanar" demethylsterols from 89.7 down to 78.8 % total sterols. A typical inhibitor of sterol demethylation, triadimefone, decreased the content of "functional" sterols to about 87.8 % only (PAŽOUTOVÁ et al. 1989).

Also C(24)-methylation is inhibited by CPP (Table 5). In *C. purpurea* 59 C(24)-methylation probably proceeds only after C(14)-demethylation. In fungi the order of these reactions is not fixed (WEETE 1987).

In spite of the fact that the representation of "non-functional" sterols increased after the CPP treatment, the total sterol content decreased. After the treatment with sterol inhibitors most fungi produce roughly two times more sterols than the non-treated cells (WEETE et al. 1983).

The fungus *Claviceps purpurea* as a species belonging to the subdivision *Ascomycotina* is characterized by the occurrence of brassicasterol in cell membranes. Its biosynthetic pathway is thus specific for representatives of this subdivision to a certain extent (WEETE et al. 1985). The effect of CPP on sterol biosynthesis was particularly pronounced in biosynthesis of brassicasterol, due primarily to the inhibition of isomerisation of Δ24 to Δ22 in 5,24(28)-ergostadienol and (4)-demethylation of 4-methyl-7-ergosterol. As a result, the precursors of brassicasterol 5,24(28)-ergostadienol and 4-methyl-7-ergosterol accumulate (about 20 times and 14 times more than in control, respectively) (Table 5).

Growth inhibition caused by sterol-inhibitors can be only partially reversed by ergosterol added to the cultivation medium or cannot be reversed at all (SHERALD et al. 1973; SANCHOLLE et al. 1984; LEROUX et al. 1976). However, a variety of unsaturated lipophilic substances such as Tween 20 and 40, oleic acid, tocopherol, β-carotene, farnesol, trilinolein and several others can alleviate the growth inhibitory effect to triazoles (SHERALD et al. 1973; LEROUX et al. 1976) as well as morpholines (KERKENAAR et al. 1979). Therefore, in the culture of *C. purpurea* 59 we also studied the effect of plant (sunflower) oil and ergosterol.

The oil stimulated growth of the strain 59 but decreased specific alkaloid production (related to DM) to 78 % of the control. The oxidation and cyclization function of chanoclavine cyclase also decreased (Table 1).

The total content of lipids and sterols in the mycelium increased by more than two times due to incorporation of fats from the added oil, the PC/PE ratio decreased and the sterol/phospholipid ratio was restored nearly to the control value (Table 4).

Fluidity of protoplast membranes slightly increased after the addition of the oil (Table 1).

Effect of CPP (100 mg · l^{-1}) on lipid composition in the mycelium was partially compensated by addition of the plant oil (Table 1; 4). However, the total alkaloid production and specific alkaloid production decreased. The oxidation function of chanoclavine cyclase decreased as a result of a combined effect of both compounds but the cyclization function was of intermediary value close to that of the control (Table 1).

Plasma Membrane Fluidity

Anisotropy of protoplast membranes was followed by means of two methods. The first method utilized as a probe TMA-DPH entering only surface structures of the cell membrane due to the polar moiety of the molecule (trimethylammonium) (KUHRY et al. 1983), the second method used DPH which is distributed among other membrane and lipid structures, so that the measurement reflects rather the average anisotropy of most cell membranes (BOROCHOV et al. 1979). The absolute values of anisotropy measure with TMA-DPH and DPH are not comparable.

From all the chlorophenoxy acids tested the highest decrease of anisotropy is caused by CPP. Measurements using the DPH probe showed that anisotropy also decreases in deeper

cell membrane structures. The decrease of anisotropy (microviscosity) is in agreement with a decreased molar ratio sterol/phospholipid (55 % of the control) and with an increased FA unsaturation index and decreased content of free functional (quasiplanar) sterols (SHINITZKY and BARENHOLZ 1974), influencing strongly the behaviour of the lipidic bilayer as plastifiers. On the contrary, the addition of ergosterol to the cultivation medium increased anisotropy of the membranes (Table 1).

Changes in the quality of cell membranes can influence the alkaloid synthesis by protein – lipid interaction and also their transport out of the cells. Alkaloids are probably exported either by a non-specific diffusion preferring more lipophilic clavines (e.g. agroclavine). Another proposed mechanism is exocytosis, during which alkaloids containing vesicles fuse with the cytoplasmic membrane which is followed by subsequent exocytosis (PERIOT et al. 1987).

The overall effect of chlorophenoxy acids on ergot alkaloids production in *C. purpurea* is apparently pleiotropic. The increased membrane fluidity after the application of CPP may lead to a partial restoration of chanoclavine cyclase function in *C. purpurea* 59. However, cytochrome P-450 can be partially induced by this compound resulting in a stimulation of the alkaloid biosynthesis. It should be also mentioned here that rigidization of membranes caused by ergosterol in this strain does not induce an opposite effect.

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Received May 2, 1989; *revised form accepted* September 15, 1989

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