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Effect of carbon sources on the polar lipid fatty acids of *Microsporum gypseum* grown at different temperatures

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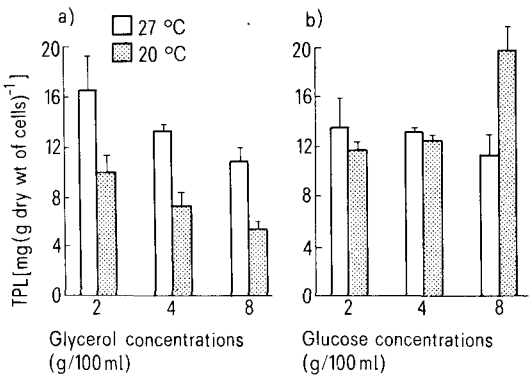
Summary. Total phospholipids and their constituent fatty acids exhibited significant alterations in presence of glucose or glycerol when *M. gypseum* was grown at 20 and 27 °C. Cells grown on a glucose medium showed a higher degree of unsaturation as compared to cells grown on a glycerol containing medium.

Manipulation of membrane lipid components by changing the growth medium composition is a valuable tool for studying the role of phospholipids in microorganisms[3]. Hence, the use of a defined growth medium is essential for bringing about controlled modification in microbial biomembranes[4]. In order to ascertain the role of apolar head groups of phospholipids in biological membranes, extensive studies have been undertaken using either fatty acid auxotrophs[5-7] or microorganisms grown in the presence of cerulenin, an antibiotic that may be used as a tool for introducing controlled alterations in cellular fatty acid composition[8]. However, similar studies on filamentous fungi are scanty[9]. Considerable effort has been made in our laboratory to study the effects of various environmental factors on the phospholipid composition of dermatophytes[10-15], and significant alterations were observed both in polar as well as apolar head groups of phospholipids. In view of the earlier observations on the influence of various carbon sources and temperature on the lipid composition of *Rhizopus arrhizus*[16], we have also compared the effects of glucose and glycerol on the phospholipid fatty acids of *Microsporum gypseum*, grown at different temperatures.

Materials and methods. The source of *Microsporum gypseum* used in this study is the same as has been reported earlier[17]. *M. gypseum* was grown as surface cultures in Sabouraud's media (pH 5.4–5.6) containing 2, 4 or 8% glucose and 1% peptone (Biological grade, Centron, Bombay, India) at 20 or 27 °C. In glycerol media, glucose was replaced with equal concentrations of glycerol. The growth pattern of *M. gypseum* was determined by plotting dry weight of the mycelium against the age of the culture. Various cultures grown at 27 °C were harvested after 15 days, while those grown at 20 °C were harvested after 21 days; at these times the cells were in the log phase of growth. The harvested cells were blotted dry, weighed and a known portion of the fungus kept for dry weight determination while the rest was used for extraction of lipids according to the method of Folch et al.[18]. Phospholipids were quantitated by estimating phospholipid phosphorus after perchloric acid digestion by the method of Bartlett[19] as modified by Marinetti[20]. Phospholipids were separated from total lipids either by acetone precipitation or by silicic acid column chromatography, and methyl esters of phospholipid fatty acids were prepared by transesterification with methanol in the presence of thionylchloride[11]. Fatty acids were identified by comparing their retention times with those of standards. The relative composition of fatty acid was calculated by the triangulation method. Unsaturated/saturated (U/S) fatty acids ratios were calculated by dividing the total unsaturated fatty acids ($C_{16:1}+C_{18:1}+C_{18:2}$) by the total saturated fatty acids ($C_{10:0}+C_{12:0}+C_{14:0}+C_{16:0}+C_{18:0}$).

Results and discussion. Glycerol may be comparable to glucose as the carbon substrate for biosynthesis of *M. gypseum* phospholipids, particularly at higher temperatures and lower concentrations. Total phospholipids showed a significant decrease ($p < 0.05$) with increased glycerol concentration, at both the growth temperatures tested (fig., a). However, no change in total phospholipid content was observed up to 4% glucose, at either temperature (fig., b). With 8% glucose, there was a slight decrease in total phospholipids at 27 °C while a significant increase ($p < 0.001$) was observed at 20 °C. The observed alterations in phospholipid content with these 2 carbon sources may be due to the differences in their utilization for phospholipid biosynthesis by *M. gypseum*. Similar observations have been made with various fungal species, which have been shown to differ in their ability to convert various carbon substrates to lipids[9].

Significant changes in the phospholipid fatty acid composition of *M. gypseum* were observed in the presence of either glycerol or glucose (tables 1 and 2). At 27 °C, there was a decrease in the U/S fatty acid ratio when glycerol concentration was increased from 2% to 4% (table 1). The decrease in unsaturation with 4% glycerol was due mainly to an



Effect of different concentrations of glycerol or glucose on the total phospholipids (TPL) of *M. gypseum* at 27 and 20 °C. Values are mean ± SD of 6 independent determinations.

Table 1. Effect of different concentrations of glycerol on the phospholipid fatty acids of *M. gypseum* grown at different temperatures

Glycerol concentration (%)[a]	Growth temperature	Fatty acid chain length (relative percentage) 10:0	12:0	14:0	15:0	16:0	16:1	18:0	18:1	18:2	U/S fatty acids ratio
2	27 °C	11.0 ± 1.0	4.4 ± 0.9	5.1 ± 1.1	-	46.9 ± 1.7	3.5 ± 0.8	17.7 ± 1.7	9.0 ± 0.9	2.5 ± 0.1	0.18
4		-	4.2 ± 0.0	4.5 ± 0.6	-	64.7 ± 3.1	7.7 ± 1.9	16.7 ± 1.3	-	1.3 ± 0.0	0.10
8		6.4 ± 0.1	7.2 ± 0.9	18.7 ± 0.7	17.6 ± 1.5	19.1 ± 1.6	17.7 ± 1.7	4.8 ± 0.6	3.3 ± 0.9	4.2 ± 0.1	0.34
2	20 °C	12.9 ± 1.5	4.7 ± 1.2	6.3 ± 1.5	-	29.4 ± 0.3	4.7 ± 2.1	6.2 ± 0.2	10.2 ± 0.2	24.1 ± 0.6	0.66
4		3.1 ± 0.7	4.2 ± 0.1	3.6 ± 0.7	-	35.7 ± 2.6	4.9 ± 2.6	9.7 ± 0.2	5.2 ± 0.2	33.7 ± 1.6	0.78
8		t	t	6.5 ± 0.8	6.4 ± 1.3	40.9 ± 2.2	2.8 ± 0.2	8.8 ± 1.2	5.2 ± 1.7	29.9 ± 0.4	0.60

Values are mean ± SD of 3 independent batches analyzed in duplicate. -, Not detected; t, traces (< 0.5%), U/S, total unsaturated/saturated fatty acids; [a]g/100 ml of growth medium.

Table 2. Effect of different concentrations of glucose on the phospholipid fatty acids of *M. gypseum* grown at different temperatures

Glucose concentration (%)[a]	Growth temperature	Fatty acid chain length (relative percentage) 10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	U/S fatty acids ratio
2	27 °C	2.7 ± 0.6	2.1 ± 0.1	4.9 ± 0.8	28.5 ± 2.0	1.9 ± 0.1	14.1 ± 0.6	5.6 ± 1.2	41.0 ± 0.8	0.93
4		3.6 ± 0.1	4.8 ± 0.3	2.9 ± 0.3	33.6 ± 2.0	5.4 ± 0.4	10.0 ± 0.9	6.3 ± 0.8	35.0 ± 5.5	0.85
8		3.5 ± 0.9	1.5 ± 0.6	4.0 ± 1.3	34.6 ± 0.7	1.6 ± 0.5	11.9 ± 2.6	7.2 ± 0.8	36.5 ± 5.2	0.82
2	20 °C	7.8 ± 0.8	11.2 ± 1.0	1.7 ± 0.3	21.0 ± 3.6	4.3 ± 0.3	6.3 ± 0.6	4.0 ± 0.3	45.0 ± 0.6	1.1
4		1.2 ± 0.2	t	0.9 ± 0.1	31.7 ± 3.5	2.9 ± 0.0	6.0 ± 0.7	4.1 ± 0.5	54.4 ± 4.4	1.5
8		-	1.0 ± 0.4	1.9 ± 0.3	20.5 ± 0.0	33.0 ± 1.2	5.1 ± 1.1	1.1 ± 0.1	35.7 ± 1.3	2.4

Values are mean ± SD of 3 independent batches analyzed in duplicate. -, Not detected; t, traces (< 0.5%); U/S, total unsaturated/saturated fatty acids ratio; [a]g/100 ml of growth medium.

increase in palmitic acid content. Moreover, capric and oleic acids, which were present in significant amounts in the presence of 2% glycerol disappeared when the glycerol concentration was increased to 4%. Further, increasing the glycerol concentration to 8% resulted in a 3-fold increase in U/S fatty acid ratio, which was primarily attributed to an increase of about 2.3-fold in the level of palmitoleic acid. Cells grown in a medium containing 8% glycerol showed higher relative amounts of short chain fatty acids ($C_{10:0}$ to $C_{15:0}$). When the fungus was grown at 20 °C in a medium containing 2, 4 or 8% glycerol concentration, it exhibited 3-, 8- and 2-fold increase in the U/S fatty acid ratio, respectively, as compared to the ratios at 27 °C. The increased unsaturation was mainly due to a significant increase in linoleic acid content with a simultaneous decrease in the palmitic acid level. However, in the presence of 8% glycerol there was an increased level of palmitic acid, with a simultaneous disappearance of short chain fatty acids. The degree of unsaturation with 8% glycerol was low as compared to 2 and 4% glycerol grown cells. It appears from these observations that a strong inhibition of the chain elongation system with increasing glycerol concentration (2–8%) at 27 °C takes place, but on lowering the growth temperature, the chain elongating and desaturating enzymes are induced.

As compared to glycerol cultures, glucose grown cells exhibited a lower U/S fatty acid ratio with increasing glucose concentration (2–8%) at 27 °C (table 2). Decreased unsaturation was mainly due to reduction in levels of linoleic acid with a concomitant increase in palmitic acid. In contrast to glycerol grown cells, short-chain fatty acids and palmitoleate did not show significant variation with the increase in glucose concentration at 27 °C. At 20 °C, the U/S ratio of phospholipid fatty acids was significantly higher than that of the lipids from cells grown at 27 °C in all concentrations of glucose. Increased unsaturation at the lower growth temperature was due mainly to a significant increase in linoleic acid levels. With 8% glucose, unsaturation was due mainly to the elevated levels of palmitoleic acid. At 20 °C, the short chain fatty acid content decreased with increasing glucose concentration. Unsaturation, so observed, with decrease in growth temperature was similar to our earlier observations with *M. gypseum*[13], and other fungi[9]. However, certain fungi exhibited a high degree of unsaturation at higher growth temperatures[16,21]. At both the temperatures, a slight inhibition of chain elongation was observed with increasing glucose concentrations as compared to the fatty acids of glycerol grown cells.

This investigation demonstrated that cells grown with various concentrations of glycerol had a lower degree of unsaturation as compared to the glucose grown cells at the usual (27 °C) as well as lower growth (20 °C) temperatures. Variation in the degree of unsaturation with different carbon sources viz. sugars has also been observed earlier in *Penicillium chrysogenum*[22] and *Rhizopus arrhizus*[16]. Thus, the manipulation of fatty acids by the present carbon sources indicates that it may be possible to use this strain of *M. gypseum* as a substitute for fatty acid auxotrophs, which are presently being used to study the role of phospholipid apolar head groups in biological membranes[23].

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Phytoalexins in *Phaseolus vulgaris* and *Glycine max* induced by chemical treatment, microbial contamination and fungal infection

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Summary. Bean seeds, treated with $AgNO_3$ or exposed to the naturally occurring microflora, accumulated phaseollin, phaseollinisoflavan and kievitone in constant relative quantities. In seeds inoculated with *Fusarium oxysporum* f. sp. *phaseoli*, relative quantities of these phytoalexins were changed. In contrast, proportions of glyceollin isomers in soybean hypocotyls were not affected by the phytoalexin-inducing agent.

Attack by nematodes, fungi, bacteria and viruses, and treatment with chemicals, UV-light or temperature shock can induce phytoalexin production in plants. Phytoalexin accumulation seems to be a general response to stress. In plants that synthesize several phytoalexins, the quantitative relationship between these phytoalexins may vary significantly depending on the elicitor[2-5].

The present paper describes a) phytoalexin accumulation in bean seeds after chemical treatment, contamination by the naturally occurring microflora or inoculation with *Fusarium oxysporum* f. sp. *phaseoli*, and b) accumulation of glyceollin isomers in soybeans following chemical treatment or inoculation with *Phytophthora megasperma* f. sp. *glycinea*.

Materials and methods. Seeds of *Phaseolus vulgaris* L. (red kidney) were imbibed in distilled water for 2 h in the dark. Subsequently, the seeds were imbibed in 1 mM $AgNO_3$ for 2 h, rinsed with sterile distilled water and incubated in the dark at 25 °C and 90% relative humidity for 5 days. Seeds imbibed in distilled water for 4 h, but not treated with $AgNO_3$, were either incubated likewise and, thus, allowed to be contaminated by the naturally occurring microflora, or surface sterilized in 2.5% Na-tetraborate for 10 min, rinsed with sterile distilled water, and inoculated with a thick mycelial suspension of *Fusarium oxysporum* f. sp. *phaseoli* Kendrick and Snyder (CMI 141119). *F. oxysporum* was grown in potato broth containing 30 g glucose/l distilled water at 25 °C for 10 days.

Phytoalexins were extracted with ethanol. The solvent was evaporated to dryness at 40 °C under vacuum. The residue was resuspended in ethyl acetate and chromatographed on Merck Silica gel F_{254} TLC-plates with chloroform-methanol (25:1; v/v) as solvent[3]. Phaseollin from beans inoculated with *F. oxysporum* was purified further on Sephadex LH-20 (bed vol. 40 ml, flow rate 0.1 ml/min) eluted with 95% ethanol[6]. Phytoalexins were identified by their R_f-values and absorbance spectra[3], and concentrations were calculated from their absorbance at 280 nm (phaseollin, phaseollinisoflavan) and 293 nm (kievitone), respectively, and their extinction coefficients[7].

Seeds of *Glycine max* (L.) Merr. cv Altona were placed between sheets of moist filter paper for 24 h in the dark. Seed coats were removed. The cotyledons were separated, rinsed with 1 mM $AgNO_3$, and put into Petri dishes with the flat side upward. Prior to incubation in the dark at 25 °C, 90% relative humidity for 48 h, a 50 µl drop of 1 mM $AgNO_3$ was placed on each cotyledon. Soybean seedlings (cv Altona and Maple Arrow) were grown in Vermiculite (previously soaked in water) in the dark at 25 °C, 90% relative humidity for 5 and 10 days, respectively. *Phytophthora megasperma* Drechs. f. sp. *glycinea* Kuan and Erwin *(Pmg)* race 4 (avirulent on cv Altona) was grown on solidified bean broth at 25 °C. Droplets, 10 µl each, of either a suspension of *Pmg* zoospores[8] (10^5/ml) or 1 mM $AgNO_3$ were placed on hypocotyls from the cotyledons to the roots[9]. The seedlings were incubated in the dark at 25 °C, 90% relative humidity for 48 h. Seedling age always refers to the age prior to glyceollin-inducing treatments.

Glyceollin was extracted[10] and chromatographed on Whatman LK6DF TLC-plates with toluene-methanol (95:8; v/v). Glyceollin was located by reference to a co-chromatographed standard, eluted, and the concentration was calculated from its absorbance at 285 nm and its extinction coefficient[11]. Isomers were separated with HPLC, using a