Cessation of Polyunsaturated Fatty Acid Formation in Four Selected Filamentous Fungi When Grown on Plant Oils

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ABSTRACT: Four fungi, *Conidiobolus nanodes, Entomophthora exitalis, Mortierella isabellina,* and *Mucor circinelloides,* were grown on various oils (triolein, sesame, safflower, linseed, and oil from *M. isabellina*) and produced lipids in which the fatty acids were predominantly the same as those of the original starting substrate. Only in the first two cases was there evidence of a small amount of chain elongation and of fatty acid desaturation taking place. The extent of this was only about 10% of that seen in glucose-grown cells. The apparent repression of the fatty acid desaturases and elongases was not reversed by growing cells on glucose and oils as mixed substrates—the fatty acid profiles were the same as when the fungi had grown in oils alone. Neither was the cessation of polyunsaturated fatty acid synthesis due to the presence of nonoil components (NOC) in the oil. Only the NOC from sesame oil affected one single conversion, that of 20:3n-3 to 20:4n-6. We conclude that fatty acid desaturase and elongase systems are repressed either partially or completely in a filamentous fungi grown on triacylglycerol oils.

JAOCS 73, 431–435 (1996).

KEY WORDS: *Conidiobolus, Entomophthora,* fungi, *Mortierella, Mucor,* oil conversion, polyunsaturated fatty acids.

The current interest in the nutritional role of polyunsaturated fatty acids (PUFA) has stimulated research into their production by a number of fungal and algal sources (1,2). Numerous groups have reported the range of fatty acids that are produced by various microorganisms when growing on glucose or other carbohydrates. However, when microorganisms are grown on oils from plant or animal sources, there is generally little change in the fatty acid profile of the added oil; the organism appears to cease lipid synthesis, as well as the desaturation and elongation of the presented fatty acids (3). Thus, growing microorganisms on the precursors of PUFA does not enhance PUFA formation but leads to cessation of PUFA production. There are, however, some exceptions: Shinmen *et al.* (4) found that of 18 strains of *Mortierella* only three (all strains of *M. alpina*) produced as much arachidonic acid (ARA) when grown on olive oil as they did when grown on glucose. The best strain, *M. alpina* 1S-4, showed a 30% increase in ARA production with olive oil in place of glucose. All other 15 strains showed apparent repression of ARA formation. Shimizu *et al.* (5) similarly described another strain of *M. alpina,* 20-17, that could convert α-linolenic acid (18:3n-3), presented as linseed oil, directly into 20:5n-3; the presence of linseed oil did not affect formation of ARA, which continued to be produced. From this and later work (6,7), it would seem that some species of *Mortierella,* especially selected strains of *M. alpina* or *M. elongata,* are able to grow on various oils and, by various elongation and desaturation reactions, continue to produce longer-chainlength fatty acids with higher degrees of unsaturation. It has also been reported (8) that *Conidiobolus* spp. are able to effect conversion of various oils to dihomo-γ-linolenic acid (20:3n-6) from both 18:2n-6 and γ-18:3n-6, but not from α-18:3n-3.

Some caution in the interpretation of reported claims for desaturation/elongation is required because small amounts of some PUFA could be selectively incorporated into cell lipids, whereas other fatty acids in the substrate oils were being used for β-oxidation degradation to provide the necessary energy and intermediates for cell metabolism. Also, when oils are presented to fungi along with other sources of carbon, such as glucose or in components such as yeast extract or corn steep liquor, it is not always certain if the oil is being assimilated—continued formation of PUFA in such cells might be due to *de novo* synthesis and exclusion of the oil (or fatty acids) from being taken up into the cell. Therefore, apparent PUFA formation from preformed oils always needs to be rigorously checked.

The conversion of preformed fatty acids to various PUFA is of current interest, but the exact mechanism of how this may be accomplished, or even how the process may be self-inhibited by the microorganism when presented with an exogenous oil, is unclear. We have therefore examined the process in four filamentous fungi. The fungi were chosen because of the range and type of fatty acids they produce; two *(Conidiobolus nanodes* and *Entomophthora exitalis)* produce fatty acids beyond C_{18} in chainlength (9), and the other two *(M. isabellina* and *Mucor circinelloides)* were chosen because of their utility in the production of γ-linolenic acid (18:3n-6)

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(2,3,10,11). The oils upon which the fungi were grown (triolein, sesame, safflower, linseed, and an oil from *Mortierella* sp.) were also chosen for their range and type of fatty acids.

EXPERIMENTAL PROCEDURES

Fungi and growth. Conidiobolus nanodes (IMI 92299), *E. exitalis* (NRRL 3742), *M. isabellina* (CBS 224.35), and *M. circinelloides* (CBS 108.16) were grown for 72 h at 30°C in vortex-aerated, 1-L bottles with semi-defined medium at pH 5.5 as previously described (9). With the two former fungi, sodium glutamate at 3 g/L was used as source of N in place of ammonium tartrate (3.3 g/L). Glucose was at 30 g/L, and oils were also at 30 g/L. When mixed substrates were used, each component was at 15 g/L.

Cell dry weights of cultures were determined directly, usually by filtration of 10–20 mL of culture through a preweighed glass-filter washing with 3 × 25 mL deionized water and drying at 90°C to a constant weight. Fungi grown on oils were first washed extensively with distilled water, then with Tween 80 (50 mg/L), followed by ethanol and, on occasion, with a rapid wash with chloroform. Cells were finally washed with distilled water before proceeding further.

Lipid analysis. Lipid content of cells, fractionation of lipids, and fatty acid analyses were as previously described (9).

Preparation of nonoil components (NOC) from oils. We followed the procedure of Shimizu *et al.* (12): Oil (25 g) was dissolved in acetone (150 mL) and held at −70°C overnight, after which the precipitate was removed by filtration. The filtrate was held in a rotary evaporator to remove all solvent, and the final residue, the NOC, was taken up in a minimum quantity of acetone. This was stored at 4°C.

Oil substrates. These were obtained from Sigma Chemical Co. (St. Louis, MO), except for the oil from *Mortierella,* which was produced by extraction of approximately 0.5 kg of *M. isabellina* grown in 5-L fermenters on medium as given previously.

RESULTS AND DISCUSSION

Utilization of oils. All four filamentous fungi grew well on most of the oils, though neither *M. circinelloides* nor *M. isabellina* grew on either linseed oil or mortierella oil. The reason for these failures was not pursued. In comparison with glucose-grown cells (Table 1), all fungi produced more lipid and, although some of this may have been original substrate oil strongly absorbed to the surface layers, this is unlikely as all cells were thoroughly washed with detergent and, on occasion, rapidly with chloroform without changing the apparent lipid contents.

The fatty acid profiles of the lipid from glucose-grown cells (Table 2) established the range of fatty acids that these cells could synthesize *de novo*: ranging from 9% myristic acid (14:0) in *E. exitalis* to 20% ARA (20:4) in *C. nanodes* and small amounts of eicosapentaenoic acid (EPA) (20:5) and docosahexaenoic acid (DHA) (22:6) in both of these fungi. Following growth of the fungi on the individual oils, the fatty acid profiles of the lipids were predominantly like that of the original substrate (Table 3). There were, however, some exceptions, both *C. nanodes* and *E. exatilis* grown on triolein now synthesized 20:1 (at 14 and 7%, respectively) with little 20:4 or 22:6. Only with Mortierella oil did these two fungi produce 20:4 and 22:6 in proportions similar to those seen in glucose-grown cells. With both *M. circinelloides* and *M. isabellina,* there was no evidence that any change had occurred to the fatty acyl residues of the substrate oil. What changes were noted could be explained by selective uptake of hydrolyzed fatty acids arising by action of extracellular lipases on the oils. Although these two fungi are not able to elongate fatty acids beyond C_{18}, they do possess a Δ6 desaturase that produces γ-linolenic acid (18:3n-6), but only small amounts of this acid were formed, even with safflower oil, which contains the highest proportion of 18:2n-6 and is the immediate precursor of γ-18:3. Thus, the conclusion appears inevitable that these four fungi have a much decreased capacity for PUFA synthesis when grown on pre-formed oils.

Although it is unlikely, it is nevertheless difficult to eliminate direct uptake of oil into the fungal cell as a cause of the similarity between the fatty acyl profiles of substrate and cell lipids. If this were so, it would imply that the triacylglycerol fraction of the cell, which is the major lipid type that is present, then obscures the minor lipid components, which may show evidence for genuine changes occurring in the substrate fatty acids. Therefore, we examined the fatty acyl groups of the phospholipid and sphingo- plus glycolipid fractions from *E. exitalis,* being typical of the four fungi, grown on glucose

TABLE 1
Growth Yield and Lipid Accumulation in Four Filamentous Fungi Grown on Glucose and on Various Oils

Fungus	Substrate	Cell yield (g dry wt/L)	Lipid (% w/w dry wt)
Conidiobolus nanodes	Glucose	7.3	26
	Triolein	10.8	44
	Sesame oil	9.1	43
	Safflower oil	3.9	36
	Linseed oil	12.3	42
	Mortierella oil	11.1	40
Entomophthora exitalis	Glucose	6.3	25
	Triolein	6.8	35
	Sesame oil	8.8	36
	Safflower oil	7.1	25
	Linseed oil	5.3	41
	Mortierella oil	6.1	48
Mortierella isabellina	Glucose	8.2	20
	Triolein	8.4	43
	Sesame oil	6.9	46
	Safflower oil	9.7	46
Mucor circinelloides	Glucose	7.1	30
	Triolein	4.8	38
	Sesame oil	4.1	42
	Safflower oil	6.4	35

TABLE 2
Fatty Acyl Profiles of Total Lipid Extracted from Four Filamentous Fungi Grown on Glucose

	Relative % (w/w) fatty acyl groups														
Fungus	14:0	16:0	16:1	18:0	18:1	18:2	α-18:3	γ-18:3	20:0	20:1	20:2	20:3	20:4	20:5	22:6
Conidiobolus nanodes	1.5	17.2	3.5	6.6	35.8	2.6	1.4	2.1	1.6	trace[a]	3.9	1.5	19.7	0.5	2.5
Entomophthora exitalis	9.0	16.6	12.4	2.4	32.0	5.4	trace	0.8	—	0.5	trace	trace	10.2	2.9	1.8
Mortierella isabellina	0.6	23.9	2.4	10.1	43.4	13.8	—	3.9	0.5	trace	trace	—	0.6	—	trace
Mucor circinelloides	2.7	23.4	13.1	4.3	31.5	15.3	—	8.3	—	—	—	—	—	—	—

[a]<0.05%.

and on safflower oil. The results (Table 4) showed that some elongation and desaturation was occurring, as evidenced principally by the occurrence of 20:4 at 10% and γ-18:3 at 2.5% of the total fatty acids in the phospholipid fraction, neither of which are detectable in the original substrate (see Table 3). There was little evidence of such fatty acids being transferred from the phospholipids, which is their site of synthesis, into the sphingo- plus glycolipids. However, the high proportion of palmitic acid (16:0) and oleic acid (18:1) in both these fractions (the phospholipid and the sphingo- plus glycolipids) means that these acids were probably being selectively incorporated into them from these same fatty acids that were present in the original substrate, even though in lower proportion.

There is, therefore, some evidence to suggest that desaturation and elongation reactions are not completely repressed in *E. exitalis*. By inference, the same will hold for *C. nanodes*, but not necessarily for other two molds (*viz.* results in Table 3). The extent of repression of PUFA synthesis can clearly vary from microorganism to microorganism.

Growth of fungi on mixed carbon sources. As growth of fungi on the various oils as sole carbon sources led to apparent repression, either partial or complete, of the desaturation and elongation processes, we attempted to show that this was not due to absence of necessary co-factors for these enzymes: i.e., acetyl-CoA for elongation or NADPH for elongation and desaturation, by growing the cells on both glucose and oils as mixed substrates. The presence of glucose might also prevent repression of the key enzymes. Certainly glucose metabolism would generate the necessary co-factors for the desaturases and the elongases if they were not repressed. If the strategy failed, then it could be inferred that the enzymes themselves were being repressed.

The growth yields of the three fungi (*M. circinelloides* was not examined in this respect) that were grown on glucose plus oils were all better than on glucose alone (Table 5), but were generally less than on oils alone (see Table 1). Lipid contents of the fungi grown on the mixed substrate were usually intermediate between the values obtained when grown on glucose

TABLE 3
Fatty Acyl Profiles of Total Lipid Extracted from Four Filamentous Fungi Grown on Various Oils Compared to the Profile of the Original Oil

	Relative % (w/w) fatty acyl groups														
Fungus	14:0	16:0	16:1	18:0	18:1	18:2	α-18:3	γ-18:3	20:0	20:1	20:2	20:3	20:4	20:5	22:6
Triolein	2.5	5.6	6.4	1.8	68.1	11.1	1.2	1.7	0.5	0.5	0.6	—	—	—	—
Conidiobolus nanodes	1.0	8.9	3.6	1.1	50.6	6.0	trace[a]	1.0	trace	14.0	2.1	1.4	2.4	trace	1.4
Entomophthora exitalis	2.1	5.1	0.9	1.1	42.2	31.3	1.3	1.9	—	7.5	4.0	trace	0.6	trace	trace
Mortierella isabellina	2.1	5.8	6.2	1.6	68.2	10.9	0.9	0.5	1.4	trace	trace	trace	trace	—	—
Mucor circinelloides	2.4	5.2	5.8	0.6	60.3	15.1	—	7.8	—	—	—	—	—	—	—
Sesame oil	trace	9.1	trace	4.9	41.5	42.8	trace	0.7	trace	trace	—	—	—	—	—
C. nanodes	trace	12.8	0.6	2.1	16.3	47.2	2.0	0.6	trace	1.9	4.7	1.1	5.8	1.1	2.2
E. exitalis	1.1	9.4	trace	trace	45.7	37.8	0.8	0.3	—	0.6	0.5	trace	1.1	1.5	0.7
M. isabellina	trace	9.9	trace	6.0	39.6	42.3	—	0.9	0.7	trace	—	trace	trace	—	—
M. circinelloides	trace	16.2	1.1	0.7	25.4	49.0	—	2.6	—	—	—	—	—	—	—
Safflower oil	2.3	4.2	trace	0.7	13.3	76.8	0.8	—	trace	trace	trace	—	—	—	—
C. nanodes	0.5	11.7	0.7	2.1	29.7	26.6	2.1	trace	trace	6.5	7.2	2.3	5.7	1.4	2.6
E. exitalis	2.8	14.6	0.5	trace	1.4	72.6	0.8	trace	—	0.5	0.7	trace	2.2	1.0	2.8
M. isabellina	trace	8.1	trace	3.5	18.5	65.1	1.8	1.4	0.5	trace	—	trace	trace	—	—
M. circinelloides	trace	16.2	4.9	0.7	14.8	61.9	—	1.1	—	—	—	—	—	—	—
Linseed oil	trace	9.0	0.1	3.3	20.8	14.6	51.5	trace	—	—	—	—	—	—	—
C. nanodes	trace	7.9	trace	3.0	14.7	10.6	38.9	3.7	3.7	1.7	trace	0.9	10.9	3.0	0.7
E. exitalis	1.3	7.6	0.7	2.6	27.1	14.0	40.8	1.2	—	0.8	trace	0.5	1.5	0.5	1.8
Mortierella oil	0.5	22.8	0.9	4.1	38.0	20.3	0.7	9.8	—	—	—	1.1	1.8	—	—
C. nanodes	0.5	17.5	0.6	3.6	29.5	14.2	0.5	7.4	0.5	2.3	1.8	trace	18.8	trace	2.4
E. exitalis	0.8	13.8	0.9	2.7	39.0	14.0	0.6	6.2	—	trace	2.1	2.3	13.7	trace	2.7

[a]<0.05%.

TABLE 4
Fatty Acyl Profiles of Sphingo- Plus Glycolipid (S + G) and Phospholipid (P) Fractions from *Entomophthora exitalis* Grown on Either Glucose or Safflower Oil

Fatty acid	Glucose-grown		Safflower oil-grown	
	S + G	P	S + G	P
14:0	9.9	9.6	1.2	4.6
16:0	15.1	24.0	10.7	22.9
16:1	4.3	11.2	trace	1.7
18:0	4.9	1.8	2.9	1.2
18:1	6.6	13.4	17.4	18.1
18:2	9.7	9.9	59.9	36.8
α-18:3	trace[a]	trace	trace	trace
γ-18:3	2.9	3.3	trace	2.5
20:0	3.5	—	0.8	trace
20:1	—	—	0.6	trace
20:2	0.6	trace	0.5	trace
20:3	0.6	0.7	trace	trace
20:4	4.8	17.5	1.8	9.6
20:5	4.2	0.1	1.1	trace
22:5	1.2	1.2	1.0	trace
22:6	—	3.0	1.0	trace

[a]<0.5%.

and when grown on oil alone. However, the fatty acid profiles were almost identical to ones obtained when the fungi were grown on the oils alone (see Table 3) and, because of this similarity, are not given here separately because the essential details are already given in Table 3. Thus, we can conclude that growth on the fungi on oils is probably causing enzyme repression and is not due to any failure of the cells to generate essential metabolites or co-factors because no advantage accrues by providing an ancillary source (glucose) of metabolites to the oil-growing cultures.

Growth of fungi on glucose in the presence of NOC. The possibility that the failure of the fungi to desaturate or elongate the substrate fatty acids could have been due to the presence of some NOC in the oil acting as an inhibitor was also explored. Shimizu *et al.* (12) showed that sesame oil, when fed to the ARA-producing fungus *M. alpina,* led to increased formation of dihomo-γ-linolenic acid 20:3(8,11,14) at the expense of 20:4(5,8,11,14) formation. This was subsequently attributed to the presence of sesamin in the oil that was acting as a specific inhibitor of the Δ5 desaturase in the fungus (13). We consequently tested the NOC of triolein, sesame oil, safflower oil, and linseed oil, obtained by cold acetone precipitation, on *E. exitalis* as a model for the other three molds. Each NOC was added at the time of inoculation at the equivalent of 30 g oil/L.

There was no demonstrable effect of the NOC on growth or lipid accumulation (Table 6), nor on the fatty acyl profile, except with the NOC from sesame oil, which produced, as found by Shimizu *et al.* (12,13), an increase in formation of 20:3 at the expense of 20:4 (Table 6). No other desaturation appeared to have been affected by this or any other NOC. Thus, the absence of changes to the substrate fatty acids by growing fungi on the oils is not attributable, except in a small way with the NOC from sesame oil, to anything other than the oils themselves.

The effect of the NOC from sesame oil on the 20:3 to 20:4 conversion was presumably due to sesamin, as found by Shimizu *et al.* (13). Examination of the neutral, sphingo- plus glycolipid, and phospholipid fractions indicated that the principal fraction affected was the phospholipid: the ratio of 20:4/20:3 changed from 25:1 in the phospholipids of glucose-grown cells to 2.6:1 in those from cells exposed to sesame NOC at 0.08%. This would be in keeping with phospholipids being the site of the desaturase reactions (14,15).

We therefore conclude that PUFA biosynthesis in fungi growing on triacylglycerol oils is strongly repressed in *M. cirinelloides* and *M. isabellina.* With *E. exitalis* and *C. nanodes,* both of which can produce PUFA up to 22:6, there is partial repression of elongation reactions so that some of the long PUFA can still be formed from the pre-formed fatty acids. The amounts produced are substantially less than are found in glucose-grown cells. The desaturases for producing C_{18} PUFA, i.e., the Δ12 and Δ6 desaturases for the respective conversions of 18:1 to 18:2 and then to 18:3, appear to be strongly repressed in all four fungi. Even with safflower oil, with almost 80% of its total fatty acids being linoleic acid (18:2), there was little conversion to γ-18:3 by the Δ6 desaturase in any fungus.

As both desaturation and elongation of fatty acids are carried out by complexes of enzymes and must include enzymes for provision of NADPH needed in the desaturation, and acetyl-CoA and NADPH for elongation, only by a detailed study of the desaturases and elongases will it be possible to say whether all enzymes or just one component in each is repressed. It would only take one enzyme of the desaturase or elongase complex to be repressed for the whole pathway to become inoperative. Reversal of such repression or repressions in some way—either by producing nonrepressible (i.e., constitutive) mutants or by genetic manipulation—would open up opportunities for carrying out biotransformations of low-

TABLE 5
Growth and Lipid Accumulation in Three Fungi Grown on Glucose and Oils as Mixed Substrates

Fungus	Oil[a]	Biomass (mg/mL)	Lipid content (%, w/w)
Entomophthora exitalis	None[b]	6.4	26
	Triolein	7.3	26
	Sesame	7.4	40
	Safflower oil	6.9	30
Conidiobolus nanodes	None[b]	5.9	23
	Triolein	6.6	32
	Sesame oil	6.5	29
	Safflower oil	6.2	26
Mortierella isabellina	None[b]	6.3	28
	Triolein	9.2	33
	Sesame oil	8.1	34
	Safflower oil	8.4	31

[a]Added at 15 g/L with glucose at 15 g/L. [b]Glucose at 30 g/L

TABLE 6
Effect of Nonoil Components (NOC) from Four Oils on Growth (biomass), Lipid Accumulation, and Fatty Acyl Profile of the Total Lipid of *Entomophthora exitalis* Grown on Glucose

Source of NOC	NOC[a] (% w/w)	Biomass (g/L)	Lipid (% w/w)	Relative % (w/w) of fatty acyl groups[b]											
				14:0	16:0	16:1	18:0	18:1	18:2	γ-18:3	20:0	20:3	20:4	20:5	22:6
Control	—	4.7	13	9.2	18.2	17.4	2.8	23.3	7.2	1.1	0.9	1.3	8.3	1.7	2.7
Triolein	3.9	4.8	14	7.7	17.0	15.2	2.7	25.0	9.2	1.5	1.0	0.8	9.9	1.3	2.5
Sesame	1.3	4.8	13	7.4	13.9	15.8	2.4	30.6	6.7	1.8	0.5	2.9	6.6	0.9	1.5
Safflower	1.4	5.2	13	8.4	18.1	15.3	2.7	20.4	12.3	1.1	1.0	1.2	8.7	1.5	2.6
Linseed	2.1	5.0	10	7.4	17.9	12.9	2.8	19.7	12.0	1.0	1.0	1.2	7.6	1.9	2.7

[a]NOC: values given are the contents in the four oils, each was added at the equivalent of 30 g oil/L medium.
[b]All samples also contained traces (<1%) of 12:0, 14:1, 15:0, α-18:3, 20:1, 20:2, and 22:5.

cost PUFA from plant sources into higher value-added commodities. Such work is now continuing in these laboratories.

ACKNOWLEDGMENTS

AK was supported by a Co-Operative Board in Science and Engineering from the Science & Engineering Research Council in collaboration with J & E Sturge (now Rhône-Poulenc Chemicals, Woodley, Cheshire, United Kingdom). We thank Philip Milsom and Dr. Neville Fish for their interest in, and support of, this research.

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[Received August 8, 1995; accepted January 11, 1996]