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Short communication

An isolation procedure for arachidonic acid producing *Mortierella* species

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Abstract

Malt extract agar and an incubation temperature of 5 °C were used to selectively isolate representatives of the genus *Mortierella* from soil. Fungi in a soil sample from mountain grassland able to grow under these conditions, amounted to a total of 2640 colony forming units per gram soil. *Circa* 94% of the total fungal isolates represented *Mortierella* subgenus *Mortierella*. The rest of the colony-forming units consisted of *Mucor* isolates (6.0%) and higher fungi (1.5%). All the *Mortierella* isolates produced arachidonic acid.

Introduction

Oils containing dietetically important polyunsaturated fatty acids (PUFAs) such as arachidonic acid [20:4(ω6)] are considered to be of high value (Ratledge 1992). A current commercial source of oils containing 20:4(ω6) is porcine liver, but an alternative source which has been extensively studied is fungi belonging to the genus *Mortierella* (Streekstra 1997). Attempts have been made to improve 20:4(ω6) production in *Mortierella* species by changing the culture conditions (Bajpai et al. 1991). In addition, various fungal strains have been screened in order to find the best producer of this fatty acid (Bajpai et al. 1991; Kendrick & Ratledge 1992).

Interestingly, Eroshin et al. (1996) found that 20:4(ω6) producing *Mortierella* strains are sensitive to aspirin in the growth medium. After screening 87 *Mortierella* strains they proposed a screening procedure, utilising aspirin, to distinguish 20:4(ω6) producing *Mortierella* strains from other members of this genus that do not produce this fatty acid.

Although the incidence of 20:4(ω6) production by representatives of *Mortierella* has been well documented (Amano et al. 1992), the ecological reason why only representatives of *Mortierella* and not other mucoralean fungi produce this PUFA (Amano et al. 1992), still needs to be elucidated. However, it was found that fungi produce more PUFAs at lower growth temperatures (Manocha & Campbell 1978). More PUFAs in the membranes increase the membrane fluidity and presumably is an adaptive response to a cold environment (Walker & Woodbine 1979; Lomascolo et al. 1994). Interestingly, Carreiro & Koske (1992) found that an isolation temperature of 0 °C instead of 25 °C, mainly selects for representatives of *Mortierella* subgenus *Mortierella*. Representatives of this subgenus have been found by other authors to produce 20:4(ω6) (Amano et al. 1992).

With the above as background, it became the aim of this study, to develop and test a low temperature isolation procedure for 20:4(ω6) producing members of the genus *Mortierella.*

Materials and methods

Isolation of Mortierella

Malt extract agar (MEA) as isolation medium and an incubation temperature of 5 °C, were evaluated for the ability to isolate *Mortierella* species from soil. The sampling site comprising an area of 10 m^2, is situated in Alti Mountain Grassland (Low & Rebelo 1996) within an humid summer rainfall region in Kwazulu-Natal, South Africa. The mean annual rainfall is 1340.8 mm, while the mean annual temperature is *circa* 10 °C. The soil temperature at the time of sampling (mid-winter) was 5 °C , while the soil moisture content was 28.79 ± 0.18% (w/w) determined by drying soil in an electric oven at 105 °C for 12 h (Eicker 1970). The soil had an hydrogen ion concentration of pH 4.96 determined according to the method of Spotts & Cervantes (1986). The organic matter content of the dried soil was 39.55 ± 0.40% (w/w) determined by ignition (Eicker 1970).

At the sampling site, the surface litter was first scraped away to reduce contamination from this habitat. A soil sample of 919 g, comprising nine subsamples, was taken at random over the area of the site, each to a depth of 10 cm. The subsamples were mixed in the laboratory to produce the sample which was then further processed.

The soil plate technique of Warcup (1950) utilising MEA was then used to isolate the fungi from the sample. Soil plates were prepared by transferring 0.005 g of soil from the sample to each of five sterile Petri dishes. Eight millilitres of cooled molten MEA was then added to each Petri dish. The cultures were incubated at 5 °C in the dark and observed for growth. After 8 days of incubation, growth from each of the developing colonies were transferred to fresh MEA and further purified. Isolates were identified on MEA or potato-carrot agar according to the keys and descriptions given by Zychae & Siepmann (1969), Gams (1976, 1977), Schipper (1978), Domsch et al. (1980) and Sutton (1980). In order to confirm the identity of certain isolates, their characteristics were compared to those of CBS cultures grown under identical conditions.

Culture conditions for fatty acid analyses

A pre-inoculum was prepared for each *Mortierella* isolate by preparing plate cultures grown for one week at 20 °C on MEA. A square (9 mm^2) containing growth was then cut from the culture and used as inoculum. Each isolate was inoculated in triplicate onto malt extract gelatine (MEG) contained in Petri dishes (diameter 80 mm).

The MEG consisted of 2% (w/w) malt extract (Difco) and 10% (w/w) gelatine (Biolab). The triplicate cultures of each isolate were incubated at 20 °C, because it is known that, at this temperature 20:4(ω6) accumulation is stimulated in *Mortierella* (Bajpai & Bajpai 1993). At lower temperatures (e.g. 5 °C), a temperature-sensitive Δ17 desaturase is activated which catalyses the formation of eicosapentaenoic acid [20:5(ω3)] from 20:4(ω6).

As soon as the colony diameter reached 80 mm the fungal growth was harvested. This was accomplished by washing the MEG from the colony on preweighed filter paper (Whatman no. 1.) using 250 ml of distilled water at 30 °C. The biomass was then freeze dried.

Fatty acid analyses

The lipids were extracted from the freeze-dried biomass using chloroform/methanol (2:1, w/w) (Kendrick & Ratledge 1992). The extracted lipids were dried under nitrogen gas and then methylated by the addition of trimethyl sulphonium hydroxide (TMSH) (Butte 1983). The methylated fatty acids were analysed with a Varian 3300 gas chromatograph and a Supelcowax 10 glass capillary column (0.75 mm × 30 m) with nitrogen (5.00 ml/min) as carrier gas (Kock 1988). Peaks were identified by reference to authentic standards and the percentage 20:4(ω6) in the lipid was calculated relative to the other long-chain fatty acids present. These fatty acids included palmitic acid (16:0), palmitoleic acid [16:1(ω7)], stearic acid (18:0), oleic acid [18:1(ω9)], linoleic acid [18:2(ω6)], and γ-linolenic acid [18:3(ω6)].

Results and discussion

Isolation of Mortierella

The isolates obtained when MEA as isolation medium and an incubation temperature of 5 °C were used to select *Mortierella* species from soil, are listed in Table 1. Fungi from the soil sample able to grow amounted to 2640 colony-forming units per gram soil (CFU/g), as determined using the soil plate technique of Warcup (1950). *Mortierella* isolates comprised 92.4% of the total number of fungi obtained, while 6% com-

Table 1. The fungal isolates obtained from the soil sample

Species	CFU/g	Isolate no.	Percentage 20:4(ω6)
Mortierella alpina			
Peyronel	560	4j	62.0
		5d	57.0
		5i	38.4
		4k	35.3
		5c	23.1
		2k	15.5
		5h	14.0
		10c	13.3
		2h	4.8
		4f	3.9
		2c	3.1
		4h	2.3
		9a	1.9
		4r	1.7
Mortierella angusta			
(Linnem.) Linnem.	40	10g	23.8
Mortierella antarctica			
Linnem.	240	4a	62.4
		4q	44.6
		4g	22.6
		10L	8.0
		9g	2.3
		9o	2.3
Mortierella basiparvispora			
W. Gams & Ginsbergs	200	5a	28.3
		10d	13.8
		9i	12.6
		4p	8.4
		9j	2.9
Mortierella camargensis			
W. Gams & R. Moreau	40	2i	0.6
Mortierella clonocystis			
W. Gams	40	10e	29.6
Mortierella elongata			
Linnem.	40	5j	5.3
Mortierella epicladia			
W. Gams & van Emden	160	2L	1.8
		10i	1.6
		2g	1.6
		9f	1.0
Mortierella gamsii Milko	120	4d	20.0
		4e	7.5
		4s	7.2
Mortierella globalpina			
W. Gams & Veenbaas-Rijks	40	9d	0.7
Mortierella horticola			
Linnem.	80	10f	13.6
		4L	4.0
Mortierella minutissima			
Tiegh.	40	4o	7.6
Mortierella parazychae			
W. Gams	40	5b	10.6
Mortierella parvispora			
Linnem.	40	9c	1.8
Mortierella sarnyensis	80	9e	13.5
Milko			
		4n	8.1
Mortierella selenospora	40	2d	2.0
W. Gams			
Mortierella verticillata			
Linnem.	40	2b	1.9
Mortierella zychae			
Linnem.	40	9q	14.1
Mortierella sp*	400	9n	8.8
Mortierella sp*		10j	23.4
Mortierella sp*		9k	18.6
Mortierella sp*		2e	18.5
Mortierella sp*		2a	12.4
Mortierella sp*		10m	11.6
Mortierella sp*		10a	9.6
Mortierella sp*		5g	7.0
Mortierella sp*		9m	4.5
Mortierella sp*		5e	0.8
Mortierella sp**	160	5f	40.6
Mortierella sp**		2j	4.8
Mortierella sp**		9h	0.3
Mortierella sp**		10h	0.2
Mucor sp.	160		ND
Pestalotiopsis sp.	40		ND

The percentage 20:4(ω) in the lipid was calculated relative to the other long-chain fatty acids pesent. These fatty acids included palmitic acid (16:0), palmitoleic acid [16:1(ω7), stearic acid (18:0) oleic acid [18:1(ω9)], linoleic acid [18:2(ω6)] and gamma-linoleic acid [18:3(ω6)]. Values are the means of three repetitions. The standard deviations of the values are less than 10%. ND= not determined.

*Identifiable as *Mortierella* due to colony odour and habit, however no distinguishing characteristics were noted.

**Identifiable as *Mortierella* due to colony odour and habit and the presence of smooth chlamydospores, however no distinguishing characteristics were noted.

prised of *Mucor* isolates and 1.5% were higher fungi representing the genus *Pestalotiopsis*.

Interestingly, when soil plates were prepared from the same soil sample, using MEA and an incubation temperature of 20 °C, the total number of fungi obtained was 5500 CFU/g (Results not shown). In this case *Mortierella* subgenus *Mortierella* isolates comprised only 64% of the total number of fungi obtained, *Mortierella* subgenus *Micromucor* comprised 2%, while *Mucor* isolates comprised 15% and 20% were higher fungi. These higher fungi comprised of *Trichoderma* and *Penicillium* isolates.

At an isolation temperature of 5 °C, the most abundant identifiable *Mortierella* species in the soil sample was *Mortierella alpina* (560 CFU/g) followed by *Mortierella antarctica* (240 CFU/g) and *Mortierella basiparvispora* (200 CFU/g). In addition, rep-

resentatives of fifteen other species of *Mortierella* subgenus *Mortierella*, were also isolated from the soil sample (Table 1).

As in the case of *Thamnidium* (Hesseltine & Anderson 1956; Benny & Benjamin 1975), all the *Mortierella* isolates isolated at 5 °C were psychrotolerant and hence able to grow at 5 °C as well as at 20 °C and 25 °C. This characteristic distinguishes these fungi from true psychrophilic fungi, which includes members of *Chaetocladium* and *Helicostylum*, that grow poorly at temperatures of 25 °C and above (Brooks & Hansford 1923; Hesseltine & Anderson 1957; Benny 1995).

All the *Mortierella* isolates isolated at 5 °C were able to produce 20:4(ω6) at 20 °C (Table 1). The highest percentages 20:4(ω6) in the lipids, relative to the other long chain fatty acids present, were obtained with *Mortierella antarctica* 4a (62.4%) and *Mortierella alpina* 4j (62.0%). The overall lowest percentage 20:4(ω6) was obtained with *Mortierella* species 10h, the percentage 20:4(ω6) obtained was 0.2%. It is also noteworthy that the percentage 20:4(ω6) differed significantly within species, for example within *Mortierella alpina* it ranged from 1.7%, obtained with isolate no. 4r, to 62%, obtained with isolate no. 4j.

Conclusions

The results showed that certain 20:4(ω6) producing *Mortierella* species can selectively be obtained from soil, using MEA as isolation medium and 5 °C as incubation temperature. The growth and 20:4(ω6) production of the *Mortierella* subgenus *Mortierella* strains obtained in this study are currently being compared to growth and 20:4(ω6) production of *Mortierella* strains isolated at 20 °C.

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