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INDUCTION OF PHOTOSPOROGENESIS IN *MACROPHOMINA PHASEOLI* BY AN OCTADECENOIC ACID FROM PEANUTS

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Key Word Index—*Macrophomina phaseoli*; fungus; photosporogenesis; lipids; octadecenoic acid; oleic acid.

Abstract—A qualitative bioassay was developed with *Macrophomina phaseoli* (Maubl.) Ashby to assess photosporogenic activity of different fatty acid fractions. *M. phaseoli* were able to sporulate when treated with the following fractions of the peanut extract: the free fatty acids and the methyl esters of the free fatty acids. The methyl esters of the mono-unsaturated fatty acids containing oleic acid as major component with eicosaenoic acid as contaminant and trace amounts of palmitoleic acid and heptadecenoic acid. By comparing the photosporogenic activity of different monounsaturated fatty acid fractions, it was concluded that oleic acid was the agent which induced photosporogenesis.

INTRODUCTION

THE BIOCHEMICAL mechanism of sporogenesis is still ill understood and it is difficult to find laboratory models for such a study. The fungus *Macrophomina phaseoli* (Maubl) Ashby lends itself to such a study because it sporulates only if treated with an ethyl ether extract of peanuts in the presence of UV light.[1] No sporulation occurs when either the ether extract or UV light is omitted, so that both are necessary to induce sporulation.[2] This study was undertaken to isolate and characterize the compound from the ether extract of peanuts which induces sporulation in the fungus.

RESULTS AND DISCUSSION

Table 1 lists the different purified fractions of the peanut ether extract and the sporogenic response that was obtained with each fraction.

Positive bioresponse was based on the observation of pycnidia, easily detected due to their greater diameter than the black bodies or sclerotia which have much smaller diameters. In order to confirm a positive response, 100 black bodies were taken from a treated segment and crushed between two microscope slides, to liberate the spores from the pycnidium. When hyaline pycnospores were observed under a phase contrast microscope the black body was regarded as a pycnidium.[3] The response was regarded as positive when 95% of the bodies could be classified as pycnidia.

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[1] KNOX-DAVIES, P. S. (1965) *S. Afric. J. Agric. Sci.* **8**, 205.
[2] KNOX-DAVIES, P. S. (1966) *S. Afric. J. Agric. Sci.* **9**, 595.
[3] KNOX-DAVIES, P. S. (1966) *Am. J. Botany* **53**, 220.

The monounsaturated fatty acid methyl ester fraction, obtained by argentation chromatography, was found to be biologically active. GLC analyses showed that this fraction contained the following methyl esters: palmitoleic acid $C_{16:1}$ (0·36%), heptadecenoic acid $C_{17:1}$ (0·08%), oleic acid $C_{18:1}$ (97·38%), eicosaenoic acid $C_{20:1}$ (2·14%). Further purification of this fraction was only possible, on a micro scale, by using GLC. Since it was not possible to use GLC purified fractions for bioassay due to the relatively large amounts needed, other sources of monounsaturated fatty acids were examined where one or more of the fatty acids present in the peanut ether extract were absent to see if the fungus was still able to sporulate. A sample of oleic acid, claimed to be 99+% pure according to GLC and TLC, was therefore employed.

TABLE 1. THE SPOROGENIC ACTIVITY OF THE VARIOUS FRACTIONS OBTAINED FROM AN ETHER EXTRACT OF PEANUTS

Fraction	Sporogenic response	Yield (mg)
Ether extract	+	450
Unsaponifiables	−	11
Free fatty acids	+	359
Methyl esters	+	354
Saturated FAME*	−	72
Monounsaturated FAME	+	94
Poliunsaturated FAME	−	127

* Fatty acid methyl esters.

Analysis of this sample on GLC with maximum attenuation before and after the main peak, showed that it still contained 18 other fatty acids and that oleic acid constituted only 84% of the sample. This sample was then repurified into the monounsaturated fraction which contained the following fatty acids: tetradecenoic acid $C_{14:1}$ (0·15%), palmitoleic acid $C_{16:1}$ (0·50%), heptadecenoic acid $C_{17:1}$ (0·65%), oleic acid $C_{18:1}$ (98·70%). Bioassay performed with this purified fraction indicated that it still contained the sporulation factor, although eicosaenoic acid ($C_{20:1}$), a component of peanut monounsaturates, was absent.

To determine whether the trace contaminants of oleic acid were responsible for sporulation, a highly purified sample of palmitoleic acid (+99% GLC and TLC pure) was employed. This sample contained 26 other fatty acids and when repurified with the aid of argentation chromatography the monounsaturated fraction contained the following fatty acids: palmitoleic acid (98·60%), heptadecenoic acid (0·15%), unknown (0·25%), oleic acid (1·20%).

The lowest effective dose mass of peanut monounsaturates that could induce sporulation was found to be 1 mg per standard segment, of which, in this fraction, palmitoleic acid constituted only 0·37%. It follows then that if palmitoleic acid was the active agent it should be able to induce sporulation at the 5 μg level. Bioassays performed with different levels of palmitoleic acid monounsaturates, i.e. 9, 16, 24, 160 and 240 μg; and 3, 6 and 9 mg were negative in inducing sporulation. Only at the highest concentration, i.e. 9 mg per segment a few pycnidia formed. That can be ascribed to the presence of oleic acid (1·26%) as a trace contaminant. This sample also contained heptadecenoic acid as a contaminant. Due to the overall negative response of the fungus to this fraction at the trace as well as

the higher levels, it can be assumed that palmitoleic and heptadecenoic acid are not active. The only active constituent in the ether extract of peanuts thus appears to be oleic acid.

EXPERIMENTAL

Bioassay. The bioassay was based on the fact that *Macrophomina phaseoli*, when grown on an asparagine-dextrose agar (AGA)[4] medium sporulated only on that part of the medium which contained the sporulation factor. Filter paper segment was used as a carrier for the factor. Due to the nonpolarity of the factor it remained on the segment and did not diffuse into the agar. Equilateral triangular paper segments (area 3·38 cm^2) were cut from Whatman No. 1 filter paper and sterilized in an autoclave. One segment was transferred under sterile conditions with flamed forceps to a prepared AGA plate to serve as a blank. the test segment was prepared by placing it into an empty sterile petri dish and then impregnated with the sample to be tested. The sample, which contained 2·5 mg of the lipid to be tested in 200 μl Et_2O, was applied dropwise to the segment. Each drop was allowed to evaporate before the next applied. This precaution was necessary to prevent hypersaturation which caused loss due to spreading of the sample onto the glass surface. After transferring the impregnated segment into the AGA medium, an inoculum of *M. phaseoli* was placed in the centre of the AGA plate. The test culture was grown for 24 hr in the dark at 30° and then transferred to a box fitted with UV lamp (Philips HPW12) in such a way that its long axis was parallel to and 30 cm from the surface of the culture being irradiated. The Pyrex Petri dishes were kept closed at 30° during the 72 hr irradiation period.

Ether extraction of peanut lipids. Peanuts were crushed in a mortar and pestle and extracted for 18 hr with continuous refluxing and N_2 flushing in a Soxhlet using peroxide-free Et_2O.

Saponification and extraction of unsaponifiable lipids. According to Ast,[5] alkali isomerization of polyunsaturated fatty acids is favoured by long-term elevated temperature conditions. This principle was adopted but the method was adapted to give a more quantitative saponification without interesterification. A volume of 5% (w/v) KOH in 10% aqueous MeOH to give a KOH: lipid ratio of 1:1 was added to the peanut ether extract. After flushing with N_2, saponification was performed at 60° for 30 min in a well stoppered system. The aqueous fatty acid salt phase could not be easily separated from the petrol. phase due to stable interphases. By using MeOH:H_2O (1:1), the foaming action of the fatty acid salts was suppressed and by using a very low b.p. (30–40°) petrol., a quicker dispersion of the interphase was obtained. Extraction was done in a long narrow graduated glass cylinder with the aid of a syringe equipped with a long stainless steel needle. A fraction of the petrol. upper phase was drawn into the syringe. The syringe was lowered until the needle reached the bottom of the cylinder. The petrol. was then ejected with force in order to mix with the lower phase. Completeness of extraction was checked by adding cholesterol to the saponified fraction; disappearance of cholesterol after every following extraction was then tested with the aid of TLC. Three successive extractions were found to be adequate.

Esterification and chromatography of the fatty acids. The fatty acid salt fraction was acidified with 1·0 N HCl and extracted with petrol. The petrol. extract was dried and evaporated to dryness in N_2. Esterification was performed with 12% BF_3 in MeOH according to Metcalfe and Schmitz.[6] Argentation-column chromatography was performed as described by De Vries.[7] The saturated methyl fatty acid esters were eluted with 10% C_6H_6 in petrol. (b.p. 30–40°). Monounsaturates were eluted with 30% C_6H_6 in petrol. and the diunsaturates with 50% C_6H_6 in petrol. Each fraction was analysed by GLC. A Beckman GC4 gas chromatograph fitted with a 1·8 m × 5 mm o.d. stainless steel column packed with chromosorb W (60–80 mesh) coated with 20% DEGS, was employed. The flowrate of the N_2 carrier gas 50 ml per min and the column temp. 198°.

Fatty acid methyl esters were identified by comparison of RR_fs with known standards, and by comparing obtained equivalent chain length values (ECL's) with those published by Hofstetter and Holman.[8] The amount of each fatty acid methyl ester in the mixture was determined by measuring the corresponding peak area (by triangulation) and expressing it as a percentage of the total peak area.

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