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Effect of oleic acid on vegetative growth of the aphid-pathogenic fungus *Erynia neoaphidis*

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1. SUMMARY

The aphid-pathogenic fungus *Erynia neoaphidis* grew on a semi-defined medium containing 16 $g \cdot l^{-1}$ glucose, 3 $g \cdot l^{-1}$ yeast extract and 5 $g \cdot l^{-1}$ mycological peptone only when the medium was supplemented with low concentrations of certain fatty acids. Of these, oleic acid fulfilled the growth requirement at a concentration of 0.02% (v/v), but higher concentrations were toxic, causing complete loss of viability of cultures at a concentration of 0.2% (v/v) in liquid medium and at 4% (v/v) on solid medium. The reduced viability of the fungus in liquid culture compared to that on equivalent solid medium, and at low inoculum density compared to high inoculum density in liquid medium, is explicable in terms of this toxicity.

2. INTRODUCTION

Erynia neoaphidis is a zygomycete fungus of the order Entomophthorales. It is pathogenic to a wide range of aphids, and is therefore of interest as a potential biological control agent for the control of aphid pests of crop plants. In common with most of the Entomophthorales it has generally been cultivated on complex media containing ill-defined organic components such as egg-yolk and milk [1–3]. This has presented considerable problems in the investigation of the growth physiology of this fungus and for the prospects of its commercial-scale cultivation. Studies by Kerwin [4–6] and Latgé and De Bièvre [7] have shown that several fatty acids can influence the growth and especially the spore germination of closely related species. That work suggested that it might have been the fatty acids contained in milk and egg-yolk that promoted the growth of *E. neoaphidis*. In order to investigate the possibility of replacing these undesirable complex media components with one or a few fatty acids, the influence of such lipids on the vegetative growth of *E. neoaphidis* has been examined. The effect of the mono-unsaturated eighteen-carbon fatty acid, oleic acid, on the vegetative growth of *E. neoaphidis* is reported here.

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3. MATERIALS AND METHODS

3.1. Fungal isolate

Erynia neoaphidis isolate NW115 was collected in July 1981 from the leaf-curling plum aphid, *Brachycaudus helichrysi*, and cultured on Sabouraud dextrose agar supplemented with egg yolk and milk (SEMA) [3]. In April 1983 the isolate was passaged through the pea aphid, *Acyrthosiphon pisum*, and reisolated. The organism has subsequently been stored under liquid nitrogen from which it was retrieved and subcultured on SEMA.

3.2 Media and growth conditions

Semi-defined *Erynia* medium (SDEM) contained, per litre of distilled water: glucose 16 g, yeast extract L21 (Oxoid Ltd) 3 g, mycological peptone L40 (Oxoid Ltd) 5 g, KH_2PO_4 5.97 g and Na_2HPO_4 2.20 g and was autoclaved at 121°C for 15 min. The pH of the medium was 6.0. If oleic acid (Fisons, 0/0200) was to be added it was prepared as a 10% v/v solution in 1.3% (w/v) NaOH, filter-sterilized and added when the temperature of the newly sterilized medium had dropped to 55°C. To neutralize the NaOH, 0.65 ml of 1.0 M KH_2PO_4 per ml of oleic acid solution was added to the bulk of the medium before steam sterilization. Media were solidified by the addition of 1.5% (w/v) agar (Davis Gelatine Ltd) before sterilization.

9 cm diameter Petri dishes containing approximately 15 ml of solid medium were centrally inoculated with a single 7 mm diameter plug cut from the leading edge of a growing colony, and incubated at 20°C in a humidified propagator. Liquid cultures were initially inoculated with 10–20 plugs as above in flasks containing 20 ml of medium and were shaken at 180 r.p.m. Further subcultures from liquid were made by a 1 in 5 dilution of late exponential phase culture into fresh medium.

3.3. Determination of growth

Colony radial growth rates (Kr) [8] were determined by an adaptation of the method of Trinci [9]. Since inoculation with plugs initially results in rather variable growth, the first measurement of colony radius was taken when the colony had been growing for at least 100 h, and the increase in colony radius from that point was plotted against time. Kr was calculated by linear regression as the slope of this graph. At least 5 replicate colonies were measured on at least 5 successive occasions to determine each Kr value.

The dry weight of biomass in liquid cultures was determined by vacuum filtering 10 ml samples through pre-weighed sintered glass filters, porosity 2 (Pyrex), washing twice with an equal volume of distilled water, drying overnight at 105°C and desiccating to constant weight. The growth curves were determined by taking duplicate samples from each of three replicate flasks at each time interval, except for the 0.2% (v/v) oleic acid sample, for which only two replicate flasks were used.

Glucose concentrations in the filtrates from the biomass samples as above were determined using a commercial kit (Boehringer Mannheim GmbH Diagnostica Test-Combination Glucose).

An Olympus BH2 photomicroscope was used for examination and photography of untreated samples from shake flask cultures. Ordinary bright field microscopy was satisfactory for healthy cultures, but Nomarski differential interference contrast optics were used to give better resolution of the contents of damaged or dying mycelium.

4. RESULTS AND DISCUSSION

4.1. Qualitative assessment of growth

As shown by the data in Table 1, the presence of a fatty acid at low concentration was required for growth of *E. neoaphidis* on SDEM. Some growth was observed after the first subculture onto oleic acid free solid medium, and after the first and second subcultures into oleic acid free liquid medium, but this can be attributed to oleic acid or a functionally equivalent derivative being carried over in the inoculum. Palmitoleic acid was found to be suitable as an alternative to oleic acid, but linoleic acid had a particularly toxic effect, causing rapid cessation of colony extension on SDEM which contained it.

It is also evident from the data in Table 1 that the presence of oleic acid in high concentrations was detrimental to growth. While unsaturated fatty

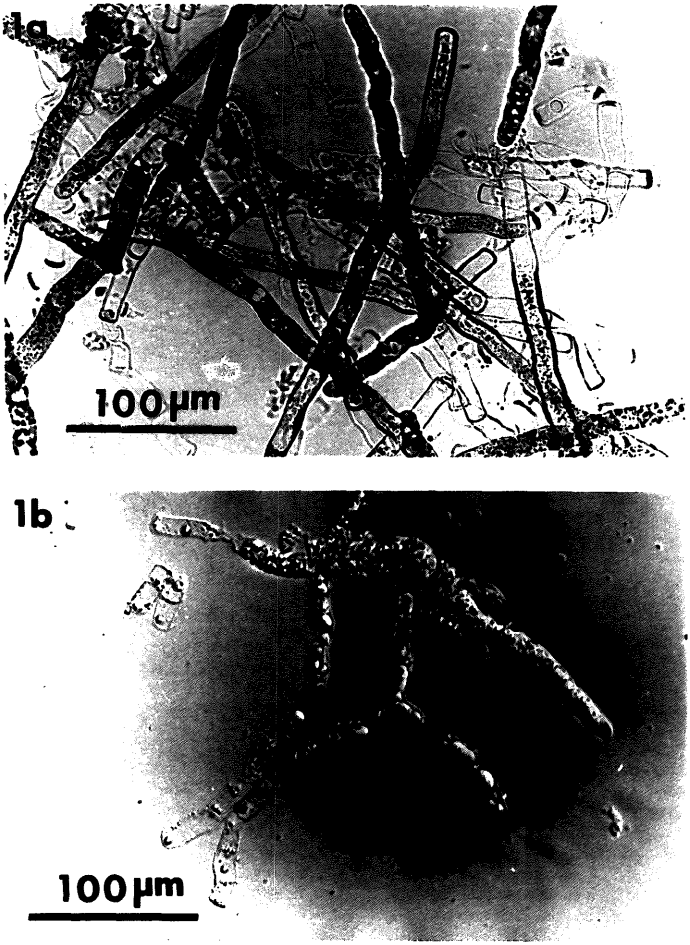

Fig. 1. Mycelium of *Erynia neoaphidis* after 4 days in shake flask culture in SDEM with (a) 0.02% and (b) 0.2% (v/v) oleic acid. Fig. 1a was taken using bright field microscopy, and 1b using Nomarski differential interference contrast optics.

Table 1

Qualitative effect of the concentration of oleic acid on growth of *E. neoaphidis* on solid and in liquid SDEM

Oleic acid concentration (% v/v)	Growth on solid medium	Growth in liquid medium
0.0	− [a]	− [b]
0.01	+	+
0.02	+	+
0.1	+	−
0.2	+	−
2.0	±	ND
4.0	−	ND

+ growth; − no growth; ± very poor growth; ND not determined.

[a] Second subculture onto oleic acid-free medium.

[b] Third subculture into oleic acid-free medium.

acids can be beneficial to micro-organisms for optimization of cell membrane fluidity [6], they have widely been found to have antimicrobial activity due to their incorporation into the cell membrane. For example, palmitic, stearic, oleic and linoleic acids have been shown to cause cell lysis of *Streptococcus faecalis* by destabilizing the membrane [10], and there is evidence that the activity of membrane-bound transport proteins in *Saccharomyces cerevisiae* is influenced by the nature of surrounding fatty acids [11]. The photographs in Fig. 1 show a pronounced difference in appearance between mycelium incubated in medium containing a high concentration of oleic acid (Fig. 1b) compared to normal, healthy growth (Fig. 1a). It is particularly significant that in mycelium grown in the presence of a high concentration of oleic acid, the cell membrane appears to have broken down in some regions and pulled away from the cell wall in others. This supports the hypothesis that a general disruption of the cell membrane is the mechanism of action of the oleic acid in this case, rather than a more specific inhibitory effect.

4.2. *Influence of type of culture and inoculum size*

It can be seen from Table 1 that the toxic effect of oleic acid appeared more pronounced in liquid than in solid culture. Additionally, in liquid medium when the inoculum size was small, for example 0.5 ml inoculum into 24.5 ml fresh SDEM containing 0.02% (v/v) oleic acid, the culture did not grow. However if a larger inoculum of 5 ml into 20 ml was used then growth did occur at the same oleic acid concentration. While the effect of shear forces in liquid culture of material in which the cell membrane is weakened might contribute to this effect, this is probably not the critical difference. When *E. neoaphidis* was grown on solid SDEM, which was opaque due to the presence of oleic acid, a quite sharply defined zone of clearing of 2–3 mm in width appeared around the colony. Fungi of the related genus *Conidiobolus* exhibit extracellular lipolytic activity [12 (as *Entomophthora* spp.), 13,14], and it is not unreasonable to assume that *E. neoaphidis* produces an extracellular enzyme which chemically modifies the oleic acid present in the medium so that its toxic effect is reduced. Thus, when the organism was grown in solid culture it was able to detoxify a zone around the edge of the mycelium before colonizing that area of medium, whereas in shaken liquid culture this could not happen. The inoculum size effect can be explained by the greater capacity to synthesize extracellular enzyme by a large inoculum, which together with carry over of enzyme in the 5 ml of inoculum would enable a much more rapid detoxification of the oleic acid in the medium than with a smaller inoculum.

4.3. *Quantitative assessment of growth*

Kr is not necessarily an accurate measure of the rate of increase in biomass, since it may also be influenced by branching frequency. However, the fact that the values in Table 2 for Kr on SDEM containing a range of oleic acid concentrations from 0.02% (v/v) to 0.1% (v/v) are not significantly different from one another even at the 90% level of confidence strongly suggests that the requirement for oleic acid for growth is adequately met throughout this range.

The concentrations of biomass, and glucose remaining in the medium, in shake flask cultures of *E. neoaphidis* in SDEM over a period of 300 h from inoculation are shown in Fig. 2. When the medium contained 0.02% (v/v) oleic acid the behaviour of the culture can be divided into a growth phase [15] which ended as the supply of glucose

Table 2

Effect of oleic acid concentration on colony radial growth rate of *E. neoaphidis* on SDEM

Oleic acid concentration (% v/v)	Colony radial growth rate (Kr) ($\mu m \cdot h^{-2}$) [a]
0.0	0.0 [b]
0.02	24.89 ± 0.55
0.05	26.38 ± 0.55
0.1	25.03 ± 1.03

[a] ± standard error of the regression coefficient.
[b] Second subculture onto oleic acid-free medium.

was exhausted, followed immediately by a decline phase [15]. The first subculture into oleic acid free medium resulted in a similar pattern of growth, but the dry weight at the end of growth phase was significantly higher at the 95% level of confidence than that obtained in medium containing 0.02% (v/v) oleic acid. This can be explained by assuming that in the first subculture into oleic acid-free medium the requirement for oleic acid was met by material carried over, as outlined above, and that loss of biomass due to cell death and lysis occurring when free oleic acid was a medium component, was either much reduced or eliminated altogether.

By the third subculture into oleic acid-free medium, there was a very low rate of increase in dry weight and a correspondingly low rate of decrease in glucose concentration. It was concluded that the organism was unable to grow because no suitable fatty acid was available. The low rates of change observed were probably due either to assimilation of energy storage compounds or maintenance metabolism, or both.

In SDEM containing 0.2% (v/v) oleic acid there was no increase in biomass, but the concentration of glucose in the medium decreased more quickly than in any other medium over the first few hours of growth, after which it remained constant. This may be because oleic acid in high concentration stimulated an increase in the activity of certain enzymes, an effect which has been demonstrated in *S. cerevisiae* [16]. Subsequent cell death and lysis would prevent any increase in the measured dry weight. Microscopic examination suggested that there was no viable biomasss remaining in cultures containing this concentration of oleic acid after 48 h.

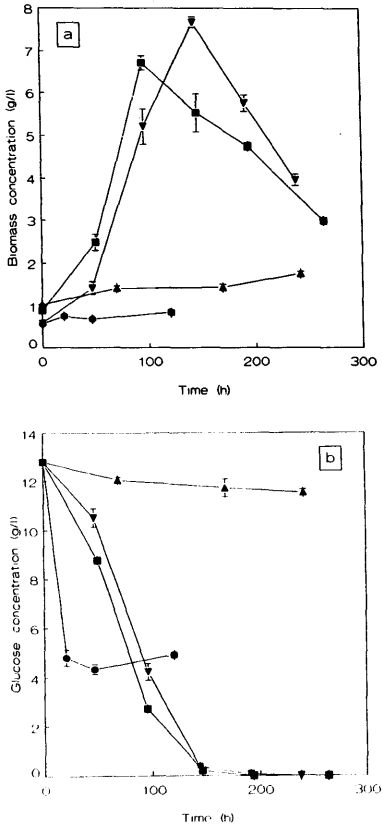

Fig. 2. Growth (a) and glucose uptake (b) of *Erynia neoaphidis* in shake flask culture in SDEM containing 0.02% (■) and 0.2% (●) oleic acid, and in the first (▼) and third (▲) subcultures into liquid SDEM without oleic acid. The error bars indicate the standard errors of the means.

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