Chemical Control of the Germination of Asexual Spores of *Entomophthora culicis*, a Fungus Parasitic on Dipterans

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Entomophthora culicis, a fungus which parasitizes a variety of small adult dipteran insects, is characterized by the production of asexual spores (conidia) which are forcibly discharged. Of the four developmental possibilities available to conidia, only direct vegetative conidial germination leads to successful infection of a potential insect host. Fatty acids were found to exert profound physiological control over conidium development. In combination with chitin or chitosan, oleic acid ($C_{18:1}$) consistently induced vegetative conidial germination. Palmitoleic acid ($C_{16:1}$), linoleic acid ($C_{18:2}$) and linolenic acid ($C_{18:3}$) were toxic to conidia over a wide range of concentrations. An excess of oleic acid mitigated the toxic effects of linoleic acid. A possible role for lipid hydroperoxides in the mediation of conidial development by fatty acids is discussed.

INTRODUCTION

The genus *Entomophthora* is characterized by its forcibly discharged conidia (asexual spores), the production of zygospores or azygospores, and the entomophagous nature of most of its members. Since epizootics in nature are known to be caused by these organisms, and many species can be grown on defined or semi-defined media on a large scale, *Entomophthora* spp. have been investigated as potential biological control agents for aphids, flies, mosquitoes and other insects (Müller-Kögler, 1959; Roberts & Yendol, 1971; Ignoffo, 1975; Soper *et al.*, 1975).

One fact that has been well documented for all species of *Entomophthora* is the nature of the infective propagule. In all cases investigated, penetration of the insect host's cuticle is initiated by conidia which are forcibly discharged by conidiogenous cells. These adhesive spores have chitinolytic, lipolytic and proteolytic capabilities which enable them to penetrate their host's exoskeleton enzymically as well as mechanically (Gabriel, 1968*a, b*; Garrison *et al.*, 1975).

If conidia land on a suitable substrate, germination results in the formation of vegetative mycelium which continues the invasion of the host or substrate. Conidia which contact substrates not conducive to vegetative germination have several options. The spore can germinate to form a sparsely branched mycelial strand whose growth depends solely on nutrients stored within the conidium; this will, however, ultimately die if it does not contact a medium suitable for growth (Prasertphon, 1963). A second option is the formation and forcible discharge of a smaller secondary conidium which has the same developmental possibilities as the primary conidium. The final possibility for a discharged conidium is death.

The initial metabolic processes of the conidia determine whether an attempt will be made to infect the particular insect to which the spores have adhered. This paper describes the induction of vegetative germination of *Entomophthora culicis* conidia by specific compounds known to be present on the epicuticle of many terrestrial insects. The implications of these results in relation to the host-specific behaviour exhibited by this entomopathogenic fungus are discussed.

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METHODS

Isolation and culture of Entomophthora culicis. Blackflies (*Simulium vittatum*) infected with *E. culicis* were provided by J. A. Shemanchuk of the Lethbridge Research Station, Canada Department of Agriculture, Lethbridge, Alberta, Canada. The infected blackflies were collected along small streams from central Alberta near Athabasca, in an area between 114°20' and 112°0' W and 54°30' and 55°00' N. The fungus was isolated on egg-yolk medium (Müller-Kögler, 1959) by suspending dead infected insects from the lids of Petri dishes, and obtaining vegetative germination of conidia discharged on to the nutrient medium. Evaluation of several dozen media revealed that optimum growth and sporulation of *E. culicis* occurred on quarter-strength Emerson's YpSs (Difco). This medium, termed basal growth medium, was used to provide an ample supply of conidia for the experiments described below.

Chemical separation of egg-yolk fractions. Vegetative germination of *E. culicis* conidia on egg-yolk medium suggested an experiment to determine the type of compounds involved in the regulation of conidium development. Egg-yolk was fractionated into neutral lipid, polar lipid and protein fractions following the procedure of Latgé *et al.* (1978). These fractions were incorporated into the basal growth medium at concentrations of 0.5, 1.0 and 5.0% (w/v) and the activity of conidia discharged on to the surface of the media was then monitored.

It was difficult to incorporate the egg-yolk fractions into the basal growth medium due to the lipophilic nature of most of the egg-yolk components. A suspension of small particles or droplets of each crude fraction was generated by swirling it with basal growth medium at a temperature just above that at which the agar would solidify, then quickly pouring the mixture into Petri plates. Pouring at this temperature resulted in the best dispersion of the crude fractions throughout the agar medium, and ensured a small droplet size. This procedure was followed in subsequent experiments in which other lipophilic compounds were incorporated into aqueous agar media. Sonication of the media did not result in significantly better droplet dispersion.

Conidium development assay. A standard assay for monitoring conidial development was followed for those experiments involving discharge of conidia on to different nutrient media to test the effects of organic compounds on conidial development. Three 100 mm diameter Petri dishes of each kind of augmented medium were prepared, and three 1 cm³ agar blocks of the basal growth medium containing actively discharging colonies of *E. culicis* were placed on the surface of each agar plate. A minimum of 200 conidia discharged on to the various media by each block of inoculum were examined under a dissecting microscope, and their mode of development was recorded. All percentages recorded in these experiments are averages of a minimum of 1800 conidia monitored within 2–4 d of their discharge on to the various agar media.

Agar inoculum blocks were not removed after the discharge of a suitable number of conidia for the assay since the effect of various organic compounds on mycelium growing out on to the medium was also evaluated. This resulted in a combination of recently discharged conidia and conidia that had been in contact with the medium for several days at the time of observation. Only those conidia showing definite development were included in the compilation of percentages. The accuracy and reliability of this scoring technique in which conidia not undergoing noticeable development were ignored was checked and verified by experiments described below.

Effect of fatty acid concentration on control of development. Palmitoleic, oleic and linoleic acids were used in a series of experiments to investigate differential development as a function of fatty acid concentration. Basal growth medium was autoclaved and allowed to cool to just above the temperature of solidification. Each fatty acid was then incorporated at concentrations of 0.5, 0.25, 0.10, 0.01, 0.001 and 0.0001% (w/v). This procedure minimized any physical or chemical changes that the fatty acids, particularly the polyunsaturated fatty acids, might have undergone during autoclaving. Conidia were discharged on to the different media in the standard manner, and the effect on mycelial growth and conidial development was monitored. In other experiments mixtures of oleic and linoleic acids were added in various proportions to give a total fatty acid concentration of 0.1% (w/v).

Determination of a minimal medium for the induction of vegetative germination of conidia. Each of the three major components of basal growth medium, soluble starch, yeast extract, and several salts was incorporated individually into water agar at a concentration corresponding to that at which it was normally present in the basal growth medium, and supplemented with 0.1% (w/v) oleic acid. Various compounds known to be present in yeast extract (which consists of the water soluble components of enzymically lysed yeast cells) were incorporated at concentrations of 0.1% (w/v) in water agar containing 0.1% (w/v) oleic acid. The developmental fate of conidia discharged on to these media was evaluated.

Oleic acid at 0.1% (w/v) was also added to water agar supplemented with different carbon and nitrogen sources reported by Gustafsson (1965) to support growth of *E. culicis*, and conidial development on these media was monitored as usual.

For these minimal media evaluations, the inoculum source consisted of 1 cm³ blocks of agar containing *E. culicis* actively discharging conidia, suspended from the lids of the Petri plates for 24 h. This resulted in a uniform carpet of conidia of similar age whose development could be monitored within 2–4 d. Suspension of the inoculum on the Petri plate lids prevented possible diffusion of compounds from the basal growth medium.

RESULTS

Effect of egg-yolk fractions on conidial development

The four developmental possibilities for germinating conidia of *E. culicis* are depicted in Fig. 1. Of the tens of thousands of conidia monitored following discharge on to the basal growth medium, all developed into secondary conidia. Of the several dozen standard media evaluated for growth of the fungus in the laboratory, only the congealed egg-yolk medium used in the initial isolation of *E. culicis* from blackflies induced vegetative germination of conidia.

Addition of the protein fraction of egg-yolk to the basal growth medium resulted in 100% secondary sporulation of the *E. culicis* conidia discharged on to the agar surface. Vegetative germination of conidia was induced, however, by incorporating either the neutral lipid or polar lipid egg-yolk fractions into the basal growth medium.

Conidia discharged on to the surface of agar supplemented with the sterols cholesterol, fucosterol and desmosterol developed into secondary spores. Addition of myristic ($C_{14:0}$) and stearic ($C_{18:0}$) acids to the basal growth medium induced 4–20% of the spores discharged on to these plates to germinate vegetatively, the remainder developing into secondary spores. These results suggest that fatty acids are probably one of the components of egg-yolk that trigger vegetative germination of *E. culicis* conidia.

Fatty acid induction of vegetative conidial germination

Oleic acid ($C_{18:1}$) induced an average of 93% vegetative germination of conidia when incorporated into the basal growth medium at a concentration of 0.5%. Growth of *E. culicis* was rapid and profuse on this medium. Both linoleic ($C_{18:2}$) and linolenic ($C_{18:3}$) acids, however, were toxic at a concentration of 0.5%, to the point of killing the fungus on the agar inoculum blocks. Palmitoleic acid ($C_{16:1}$) at a concentration of 0.5% stimulated mycelial growth, but depressed sporulation and killed all conidia discharged on to the surface of this medium.

Saturated fatty acids also affected the development of *E. culicis* conidia. Fatty acids with a carbon chain length of 10 or less were toxic at a concentration of 0.5%. Those evaluated included caproic ($C_{6:0}$), heptanoic ($C_{7:0}$), nonanoic ($C_{9:0}$) and capric ($C_{10:0}$) acids. Lauric ($C_{12:0}$) and tridecanoic ($C_{13:0}$) acids were not toxic to the mycelium, but did slow its growth. Conidia discharged on to the latter two media usually developed into secondary spores, but an average of 8% of the conidia were killed.

An average of 14% vegetative germination of conidia occurred on media containing myristic ($C_{14:0}$), palmitic ($C_{16:0}$) and stearic ($C_{18:0}$) acids, the remainder developing into secondary spores; however, saturated fatty acids with more than 18 carbon atoms induced nearly 100% secondary sporulation. Both arachidic ($C_{20:0}$) and behenic ($C_{22:0}$) acids induced less than 0.1% vegetative germination.

A final series of evaluations involved detergents and fatty acid analogues in order to determine the specificity of response of conidia to these types of compounds. Several cationic, anionic and non-ionic detergents were added to the basal growth medium at a concentration of 0.5% and the fate of conidia discharged on to the agar was monitored. Several of the detergents were sulpholipids, and others were used to test the effect of a non-specific wetting action on conidium development. The results of these experiments are summarized in Table 1.

Stearyl and crotyl alcohols and four ketones were also evaluated under the same conditions. Crotyl alcohol and all four long-chain ketones tested (2-tridecanone, 2-pentadecanone, 2-heptadecanone and 3-acetyl-2,6-heptadione) killed conidia discharged on to media into which they were incorporated. All conidia on the stearyl alcohol medium developed into secondary spores.

Effect of fatty acid concentration on control of development

Palmitoleic, oleic and linoleic acids, each of which had different effects on conidial germination and/or mycelial growth, were used in a series of experiments to investigate differential development as a function of fatty acid concentration. As shown in Table 2, the effects of these fatty acids on conidial and mycelial development were concentration-dependent. Interestingly,

Fig. 1. Developmental possibilities for *Entomophthora culicis* conidia. All the bar markers represent 10 μ m.

(a) Vegetative germination of conidia discharged on to the basal growth medium supplemented with 0.1% oleic acid.

(b) Conidia undergoing secondary sporulation following their discharge on to the basal growth medium.

(c) Escape growth (mycelium impastum) exhibited by a conidium discharged on to the lid of a polystyrene Petri dish. Note the absence of cytoplasm in the parent conidium.

(d) Conidia killed following their discharge on to the basal growth medium supplemented with 0.1% linoleic acid. Note the detachment of the spore cell wall from the cell membrane, and the disrupted internal structure.

Table 1. *Effect of detergents on conidial germination of E. culicis*

Detergent type	Detergent*	Type of conidial germination (mean % occurrence)†		
		Vegetative germination	Secondary sporulation	Death
Cationic	Cetyltrimethylammonium bromide	0	0	100
Anionic	Sodium cholate	0	100	0
	Deoxycholic acid	0	0	100
	Sodium dodecyl sulphate	0	0	100
	Sodium dodecyl <i>N</i> -sarcosinate	0	0	100
Non-ionic	Tween 20	3	97	0
	Tween 80	1	99	0

* All detergents were incorporated into the basal growth medium at a concentration of 0.5% (w/v).

† The minimum sample size was 1800 conidia.

Table 2. *Effect of palmitoleic, oleic and linoleic acids on growth and conidial development of E. culicis*

Fatty acid	Concn (%, w/v)	Type of conidial germination (mean % occurrence)*			Effect on mycelial growth†
		Vegetative germination	Secondary sporulation	Death	
Oleic	0.50	94	6	0	++
	0.25	92	8	0	++
	0.10	93	7	0	++
	0.01	81	19	0	+
	0.001	17	83	0	+
	0.0001	3	97	0	+
Palmitoleic	0.50	0	0	100	++
	0.25	0	0	100	++
	0.10	0	0	100	++
	0.01	2	98	0	+
	0.001	2	98	0	+
	0.0001	0	100	0	+
Linoleic	0.50	0	0	100	--
	0.25	0	0	100	--
	0.10	0	0	100	--
	0.01	0	0	100	-
	0.001	12	88	0	+
	0.0001	3	97	0	+

* The minimum sample size was 1800 conidia.

† Mycelial growth relative to that on the basal growth medium: ++, enhanced; +, comparable; -, sparse; --, compound toxic.

palmitoleic acid, although toxic to conidia at concentrations of 0.1% or greater, actually enhanced mycelial growth at these same concentrations. This is in sharp contrast to linoleic and linolenic acids which were toxic to both conidia and mycelium at such concentrations.

Effect of combinations of fatty acids on conidial germination

The very different effects of oleic and linoleic acids on conidial development (induction of vegetative germination by the former and death by the latter) are difficult to explain on a chemical basis. Table 3 summarizes the results of experiments in which oleic and linoleic acids were added in various combinations to the basal growth medium, and their effect on conidium development was monitored. The major point to be made concerning these data is that the

Table 3. *Effect of various concentrations of oleic and linoleic acids on the development of E. culicis*

Oleic acid concn (%, w/v)	Linoleic acid concn (%, w/v)	Type of conidial germination (mean % occurrence)*		
		Vegetative germination	Secondary sporulation	Death
0.1	0	92	8	0
0	0.1	0	0	100
0.05	0.05	0	0	100
0.065	0.035	0	0	100
0.08	0.02	45	0	55
0.09	0.01	89	11	0

* The minimum sample size was 1800 conidia.

effects of a toxic concentration of linoleic acid can be mitigated by adding an excess of oleic acid to the same medium, demonstrating either selective fatty acid uptake or selective metabolism of oleic acid by *E. culicis* conidia.

Determination of a minimal medium for the induction of vegetative germination of conidia

Table 4 summarizes the results of a search for a minimal medium which induces vegetative conidial germination, oleic acid alone being an insufficient stimulus. Chitosan, a polymer of glucosamine, was able to induce vegetative germination most consistently in the presence of oleic acid. Chitin plus oleic acid also induced sporadic vegetative germination. The difficulty of obtaining uniform incorporation of these particulate, water-insoluble compounds into the agar media caused some variation in conidial response. The minimum size of the chitin or chitosan molecule necessary for the induction of vegetative conidial germination is not known, but if small chain length (water-soluble) chitosan or chitin molecules are sufficient, their presence in yeast extract (Rosenberger, 1976) could explain the induction of vegetative germination by this medium. Neither glucosamine nor *N*-acetylglucosamine, the monomeric subunits of chitosan and chitin, respectively, was able to induce a significant percentage of vegetative germination.

DISCUSSION

On the basis of the above series of investigations, it appears that *E. culicis* conidia germinate vegetatively specifically in response to certain fatty acids, a response that is affected by the fatty acid concentration, chain length and degree of unsaturation. Structural differences between oleic, palmitoleic and linoleic acids seem to be insufficient to account for the widely divergent physiological effects of these fatty acids on the conidia of this entomopathogen. Therefore, it is possible that some initial metabolism of these compounds occurs which accounts for the different responses.

Reduction of the unsaturated bonds would not be sufficient to generate these different responses since the fully saturated C₁₈ and C₁₆ fatty acids lead to either vegetative germination or secondary sporulation of conidia.

Another possibility that can be rejected involves the usual fatty acid degradation cycle, β -oxidation, in which C₂ units are sequentially removed from activated fatty acids to form acetyl-CoA and an acyl-CoA with two less carbon atoms (Weete, 1974). The degradation products of this metabolic process involving saturated, monounsaturated or polyunsaturated fatty acids are either identical or very similar (Yamada & Stumpf, 1965; Weete, 1974), and none of the breakdown products are known to be toxic.

The most likely explanation for the varied response of conidia to structurally related fatty acids involves the formation and subsequent metabolism of lipid hydroperoxides. Lipid hydroperoxides are generated in a single chemical step, and their subsequent metabolism would provide the simplest explanation for the observed effects of different fatty acids on *E. culicis*.

Table 4. *Determination of a minimal medium for the induction of vegetative germination of conidia of E. culicis*

Medium*	Type of conidial germination (mean % occurrence)†		
	Vegetative germination	Secondary sporulation	Death
Components of YpSs medium			
Quarter-strength YpSs	0	100	0
Quarter-strength YpSs + oleic acid	93	7	0
Soluble starch	0	100	0
Soluble starch + oleic acid	1	99	0
Agar	0	100	0
Agar + oleic acid	0	84	16
Yeast extract	0	100	0
Yeast extract + oleic acid	91	9	0
Salts in YpSs‡	0	100	0
Salts in YpSs + oleic acid	0	100	0
Chitin and related compounds			
Glucose	0	100	0
Glucose + oleic acid	0	100	0
Glucosamine	0	100	0
Glucosamine + oleic acid	1	99	0
Galactosamine	0	100	0
Galactosamine + oleic acid	0	100	0
N-Acetylglucosamine	0	100	0
N-Acetylglucosamine + oleic acid	2	98	0
N-Acetylgalactosamine	0	100	0
N-Acetylgalactosamine + oleic acid	0	100	0
Chitin	0	100	0
Chitin + oleic acid	14	66	20
Chitosan	0	100	0
Chitosan + oleic acid	23	65	12
Amino acids			
Alanine	0	100	0
Alanine + oleic acid	0	100	0
Asparagine	0	100	0
Asparagine + oleic acid	0	0	100
Glutamine	0	100	0
Glutamine + oleic acid	0	0	100
Casamino acids	0	100	0
Casamino acids + oleic acid	0	100	0
Vitamins§			
Pyridoxine	0	100	0
Pyridoxine + oleic acid	0	0	100
Thiamin	0	100	0
Thiamin + oleic acid	0	100	0
Riboflavin	0	100	0
Riboflavin + oleic acid	0	100	0
Pantothenic acid	0	100	0
Pantothenic acid + oleic acid	0	0	100

* All media included 1.5% agar and were adjusted to pH 7.0 when necessary. Compounds were added at a concentration of 0.1% (w/v) except where specifically stated otherwise. Soluble starch, yeast extract and salts that make up quarter-strength YpSs were added at concentrations comparable to those found in this medium.

† The minimum sample size was 1800 conidia.

‡ Salts in YpSs (Difco) are 1 g K_2HPO_4 and 0.5 g $MgSO_4 \cdot 7H_2O$ per litre distilled water.

§ All vitamins included in this experiment are known to be found in yeast extract (*Difco Manual*).

conidia. It is proposed that, in the case of oleic acid, the synthesis of an oleic acid hydroperoxide derivative would initiate an energetically favourable fatty acid degradative pathway, leading to conditions conducive to vegetative germination of the conidium. The same or a similar enzyme catalyses the formation of hydroperoxide derivatives of palmitoleic, linoleic and linolenic acids, but these compounds are incapable of entering the metabolic pathway which readily accepts the oleic acid derivatives. These toxic compounds accumulate in the conidium, disrupting membranes and initiating autoxidations of enzymes and structural proteins. Figure 1 shows the disruption of membrane integrity induced by spore uptake of linoleic acid, and comparable results were obtained using palmitoleic and linolenic acids. Further support for this hypothesis comes from the fact that oleic acid itself is toxic to conidia when incorporated into certain growth media (Table 4). These results suggest that under some circumstances conidia are unable to metabolize oleic acid hydroperoxides further, leading to death of the conidia.

Fatty acids are common lipid constituents of terrestrial insect cuticles (Thompson, 1973). Development of *E. culicis* conidia adhering to an insect epicuticle will be affected by the type and quantity of fatty acids present on a given epicuticle. It is possible that restriction of this fungus to small adult dipterans (MacLeod *et al.*, 1976) can be partly explained by common chemical characteristics of the cuticles of these insects. This aspect of the interaction of *E. culicis* with host and non-host organisms is currently under investigation.

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