PHYTONEMATODE PATHOLOGY: ULTRASTRUCTURAL STUDIES. I. PARASITISM OF MELOIDOGYNE ARENARIA EGGS BY VERTICILLIUM CHLAMYDOSPORIUM

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ABSTRACT


An isolate of Verticillium chlamydosporium Goddard, from females of Meloidogyne arenaria (Neal) Chitwood, was evaluated *in vitro* for its ability to parasitize eggs of the nematode. It prevented egg hatching and colonized eggs by hyphal penetration. Both the egg shell and larval cuticle were disrupted, and hyphae readily proliferated endogenously within the eggs and larvae. Hyphae within the egg were able to reemerge through the shell. Some ultrastructural disorganization of the cutin and lipid layers of the egg shell and the basal layer of the larval cuticle was evident. Invaded larvae soon became totally necrotic and disintegrated internally.

Additional key words: root-knot nematodes, biological control, population dynamics, fungal ecology, soil fungi.

RESUMEN


Se evaluó la capacidad de una cepa de Verticillium chlamydosporium Goddard, obtenida de una hembra de Meloidogyne arenaria (Neal) Chitwood, para invadir *in vitro* huevos del mismo nematodo. El hongo impidió la eclosión de los huevos colonizándolos al penetrar hifas dentro de los mismos. También, el hongo causó desorganización extensa tanto en la estructura de la cascara de los huevos como en la de la cutícula de las larvas, observándose proliferación endógena y abundante de las hifas dentro de los huevos y de las larvas. Las hifas dentro de los huevos pudieron desarrollarse y reemergir a través de las cascaras. El ataque del hongo causó rupturas en la capa de lípidos y la de quitina de la cascara de los huevos así como en la capa basal de la cutícula de las larvas. Las larvas invadidas por el hongo mostraron rápidamente necrosis y desintegración interna.

Palabras claves adicionales: nematodos noduladores, combate biológico, dinámica poblacional, ecología de los hongos, hongos del suelo, manejo de plagas.
INTRODUCTION

The hyphomycetous, soil-borne fungus *Verticillium chlamydosporium* Goddard has been implicated on a number of occasions in the pathology of both cyst and root-knot phytonematodes. It is known to occur in a variety of soils and is probably cosmopolitan in its distribution. An account of its known geographical occurrence, host substrate relationships, and nomenclatural history has been published (16).

The association between *V. chlamydosporium* and nematodes was first documented by Willcox and Tribe (20) who encountered it in cysts of *Heterodera schachtii* Schmidt collected in a field near Ely, Cambridge, England. The majority of eggs in naturally occurring cysts invaded by this fungus were found to be dead, and the fungus was isolated from both eggs and hatched larvae. On PDA medium and in shallow soil layers in Petri dishes, the ability of the fungus to enter apparently healthy cysts and eggs was demonstrated. Overall, however, only a limited degree of infection was obtained, particularly in white cysts developing on roots of sugar beet host plants in pot culture. The somewhat negative results were interpreted as evidence that *V. chlamydosporium* was only weakly parasitic upon eggs contained in mature cysts.

In an extended survey involving additional soils in different localities in England, Dursnell and Tribe (3) found *V. chlamydosporium* to be the most common fungal parasite of *H. schachtii* eggs, occurring in five of seven samples examined. Preliminary pathogenicity tests, in which conidia of *V. chlamydosporium* were added to *H. schachtii* cysts developing in potting soils, indicated positive though low degrees of re-infection. This was thought to reflect inadequate inoculum levels rather than inherent low infective capability. Infected eggs were said to be characteristically yellow-brown with slightly shrunken content permeated by hyphae and sometimes bearing a convoluted wall.

Kerry (11) commonly found *V. chlamydosporium* in females and new cysts of *Heterodera avenae* Woll. where continuous cereal cultivation was practiced in England. The fungus was described as being capable of killing all eggs within young cysts. The fungus was isolated from eggs and from infected second stage larvae, suggesting invasion at a relatively early stage in embryonic development. Females infected early became aberrant, failing to grow and producing small cysts containing but few eggs. No firm conclusions were drawn concerning the role of *V. chlamydosporium* in regulating cyst nematode populations.

Tribe (18) found chlamydospores of *V. chlamydosporium* in slides prepared from cysts of *H. avenae* reported by Graham and Stone (10) to be diseased and containing granular or shrivelled eggs. The fungus was categorized as a major egg pathogen and some of its characteristics were
described. It was said that the contents of invaded eggs were converted into a hyphal mass but chlamydospore production was rare. Young white females on roots were said not to be attacked, an observation somewhat contradictory to that of Kerry (11). In pot experiments, Kerry and Crump (13) added eggs to soils known to contain *V. chlamydosporium*. Recovery of cysts from host plants after a period of time showed the fungus to be capable of attacking eggs of *Globodera rostochiensis* Mulvey and Stone and six species of *Heterodera* Schmidt, namely *H. goettingiana* Liebscher, *H. trifolii* (Goffart) Oostenbrink, *H. carotae* Jones, and *H. cruciferae* Franklin in addition to *H. avenae* and *H. schachtii*. It was reported that all stages of egg development can be attacked.

In a survey of the extent of disease in populations of *H. avenae* and *H. schachtii*, Tribe (19) showed *V. chlamydosporium* to be implicated in the pathology of cysts and eggs of these nematodes in Belgium, Czechoslovakia, Italy, the Netherlands, Poland, and Sweden, as well as in England. It was again considered to be the principal egg pathogen, occurring in about 25% of all diseased cysts examined. Stirling (17) recorded the presence of *V. chlamydosporium* in brown cysts of *H. avenae* in Australia.

Kerry (12) and Kerry et al. (14,15) showed *V. chlamydosporium* to have an adverse effect on *H. avenae* multiplication in fields and to play a significant role in controlling cyst nematode populations in Europe. Again, the fungus was reported to attack young females as well as eggs. Under continuous or intensive cereal cultivation, *H. avenae* populations had been found to decline (5). This is now thought to be associated with fungal parasitism (12). Tribe (18) noted high frequency of *V. chlamydosporium* in German *H. schachtii* cysts where beet monoculture had been practiced. Crop monoculture and its attendant phytonematode populations apparently allows build up of fungal inoculum sufficient to exercise significant and detectable control. Where crop plants are grown in monoculture, an enhanced suppressive effect can occur after a period of time (4). Not only may pathogenic fungal biomass build up, but selection pressures may favor a particular fungus species such as *V. chlamydosporium*. There is some evidence that *V. chlamydosporium* is not normally a highly successful competitor in soils. Godoy et al. (9) failed to recover it with high frequency from soil to which it had been added.

*Verticillium chlamydosporium* has been found to be a parasite of *Meloidogyne arenaria* females and eggs in Alabama peanut monoculture field soils (9,16). In greenhouse studies the fungus was shown to exercise a considerable suppressive effect on the nematode. Gintis et al. (7) have
recently encountered it, in low frequency, in cysts of *Heterodera glycines* Ichinohe from Alabama soybean field soils. Its highly distinctive chlamydospores were occasionally present in cysts but the fungus often failed to grow out and form colonies when these were placed onto PDA plates. This was taken to indicate that the fungus has specialized nutritional requirements or, more likely, is sometimes unable to overcome fungistatic effects, leading to dormancy.

Although the consistent association of *V. chlamydosporium* with reproductive stages of both cyst and root-knot phytonematodes is now well documented, and its capacity to penetrate females and eggs and to increase its biomass within these structures is known, the precise mode of action of its parasitism remains to be elucidated. Likewise, relatively little is known of its physiology. Two possible types of activity, operating separately or in combination, are thought to be involved in parasitism of cysts and eggs by fungi (6,7). It seems likely that diffusible fungal toxins can deleteriously affect eggs and even lead to premature death of larvae. Exoenzymes, which might affect the permeability of egg shells, could operate in tandem with mycotoxins to predispose eggs to potential invasion. One or more of these factors might render females and hatched larvae vulnerable to infection.

Among the most important aspects of cyst and root-knot nematode pathology is the condition of egg shells and larval cuticles following exposure to fungal metabolites. Of particular interest is the permeability of egg shells as related to the presence or absence of a lipid layer, the condition of that later, and any changes in its lipoprotein membranes. In order to determine if ultrastructural changes are brought about in mature eggs and larvae as a result of invasion by *V. chlamydosporium*, the study reported herein was undertaken.

MATERIALS AND METHODS

An isolate of *V. chlamydosporium* obtained from females of *Meloidogyne arenaria* on roots of ‘Rutgers’ tomato (*Lycopersicon esculentum* Mill.) planted in infested sandy loam field soil from Headland, Alabama (16) was maintained in axenic culture on 2% colloidal chitin agar (8). The soil had been in peanut (*Arachis hypogaea* L.) monoculture for eight years. Eggs of *M. arenaria* were extracted from galled roots of other ‘Rutgers’ tomato plants grown in the same soil. Infected roots bearing galls were cut into pieces 0.5-1.0 cm long and washed in running tap water for 24 hr. The root segments were blended with 150 ml of 10% (v/v) Clorox® [5.25% (w/w) NaOCl] solution in a Virtis® 45 homogenizer at medium setting. The homogenate was passed successively through four nested, stainless steel sieves (eight-cm diam) with openings of 250, 150,
75, and 30μ (500 mesh), respectively. Eggs retained in the 30μ sieve were further axenized by three serial rinses in sterile demineralized water. The egg suspension was stored in a refrigerator until processed further.

A small volume of egg suspension was placed in a sterile watch glass. Using a stereomicroscope at 40X magnification, healthy, mature eggs containing viable second stage larvae (as evidenced by their movement within the egg shells) were located. Forty such eggs were carefully removed one or two at a time with a micropipette, and transferred to four thinly-poured water agar plates (ten eggs to each plate). Two of the plates were inoculated in the vicinity of the eggs with mycelial fragments of *V. chlamydosporium*. All four plates were incubated in the dark at 25C for ten days and then examined microscopically. Parasitized eggs, where there was evident hyphal contact and penetration, were removed in small excised agar cubes. These were then prepared for scanning and transmission electron micrography. Specimens were fixed for 24 hr at 4C in 2% glutaraldehyde (pH 7), followed by rinsing in cacodylate buffer solution (pH 7) and post-fixation treatment for 3 hr in 1% osmium tetroxide. Following a second rinsing in buffer solution, the specimens were dehydrated through a graded ethanol series to 100%.

For scanning electron micrography, specimens in 100% ethanol were infiltrated in two steps with amyl acetate and dried in a Denton® DCP-1 critical point drying apparatus followed by coating with gold and palladium using a Denton® DV-502 vacuum evaporator. Observations were made using an AMR 1000 scanning electron microscope.

For transmission electron micrography, specimens were passed through a graded series of ethanol-L R White R (hard) embedding medium into 100% embedding medium. Infiltration was conducted over a period of three days, with the specimens transferred to fresh embedding medium daily. Embedded specimens were encased in gelatin capsules and the medium polymerized by incubation for 24 hr at 60C. Sections were cut using a Sorvall® MT-2 “Porter Blum” ultra microtome and stained in 1% uranyl acetate for 2 hr. The sections were examined with a Philips 300 D701 transmission electron microscope.

Similarly treated and sectioned unparasitized eggs from uninoculated water agar plates served as control. These eggs were ruptured with the aid of fine needles during fixation to facilitate subsequent infiltration of the embedding medium.

**RESULTS**

Within the 10 day incubation period, mostly healthy larvae hatched from control treatment eggs. On inoculated plates larvae hatched from a few eggs where there were no proximal fungal hyphae. Where hyphae
were present in the vicinity of eggs or in actual contact with the egg shell, no hatching took place. After 10 days 70% of the eggs on inoculated plates were heavily parasitized and the majority bore fungal hyphae endogenously, obliterating the larvae. Fungal mycelium radiated profusely from such eggs. Upon hyphal contact with eggs, growth continued to form a branched, mycelial network closely prostrate over the smooth shell surface (Figs. 1A, 1B). A spiral, primary hyphal growth pattern closely adhering to the surface was frequently evident. With progressive hyphal proliferation, lateral branches penetrated the host.

Satisfactory fixation, impregnation with the embedding medium, and staining were influenced by egg parasitization. The various chemicals penetrated into the parasitized eggs easily, whereas there was little or no penetration of healthy eggs. An alteration in the permeability of the shell of a parasitized egg was evidenced by the uptake of osmium tetroxide and subsequent darkening of the egg contents and larvae. The larvae within healthy, uninvaded eggs showed no sign of being affected by the fixative or stain after 24 hr exposure to 2% glutaraldehyde and 3 hr to 1% osmium tetroxide. The larvae appeared to be just as active after the fixation procedure as before. Following mechanical rupturing of the egg shell, many larvae could not be infiltrated with the embedding medium nor stained. Presumably, intact, unaffected cuticles were impermeable.

Sections of eggs from uninoculated agar plates (Figs. 2A, 2B) showed undisrupted shells enclosing healthy larvae. The egg shell (Fig. 4A) consisted of three layers: a narrow outer vitelline layer, a middle chitinaceous layer, and an inner lipid layer. The inner limits of the latter layer were not clearly discernible and it varied considerably in thickness, being much wider polarly. The chitin layer was more or less evenly thickened throughout and appeared somewhat more condensed adjacent to the lipid layer. There was a clear line of demarcation between the two layers. The cuticle of the healthy second stage larvae (Figs. 6A, 6B) was also made up of three main layers: a cortical layer with discrete external and internal zones, a median layer, and a basal layer. The median layer varied in appearance between sections and was not always easily discernible. Globular, electron-dense bodies were apparent in some instances (Fig. 6A). Sometimes a continuous, electron dense band was present (Fig. 6B). The basal layer contained electron dense striations lying perpendicular to the larval surface and embedded in less electron-dense material. Where globular bodies were evident in the median layer, the basal layer striae were barely visible, whereas in the presence of a continuous band of electron dense material above, the striae were prominent. Subtending the basal layer a membrane-bound hypodermis is present, bordered internally by a basal lamella (Fig. 6A). Below this lies somatic musculature.
Fig. 1. A & B) SEM of *Meloidogyne arenaria* eggs showing prostrate, encircling fungal hyphae and lateral penetrating elements (PH), (x1000), C) Section of infected egg: EH = emerging hypha, ES = egg shell, FH = fungal hyphae, L = larva (x9000).
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Fig. 2. A & B) Sections of healthy, uninfected eggs: ES = egg shell, MC = molted older cuticle, LC = larval cuticle (x17,000).
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Sections of infected eggs showed hyphae abundantly present in both the egg venters and enclosed larvae (Fig. 1C). The presence of fungal hyphae exogenously in close proximity to the egg shell surface and endogenously within the egg venter resulted in visible structural changes in the shell. Where a hypha is recumbent and closely appressed to the egg shell surface (Fig. 3B) the vitelline layer is seen to be, in part, disorganized or even broken down. This relatively narrow electron-dense layer (Fig. 3A) is normally discrete and continuous. Following the advent of a fungal hypha in proximity to the egg surface, it becomes somewhat diffuse, splitting irregularly into two ill-defined electron-dense bands. It eventually becomes discontinuous, bearing easily discernible gaps peripheral to which more or less globose aggregations of material occur (Fig. 3B). Where a hypha lies close to the egg shell endogenously (Fig. 3C) electron-dense material is evident in the region of contact but not elsewhere. Changes in the chitin and lipid layers of the egg shell also occur in diseased eggs heavily invaded by hyphae. A comparison between the shell of a healthy egg (Fig. 4A) and that in a diseased condition (Fig. 4B) shows the normally well-defined margin of the chitin layer to be not easily distinguishable. There appears to be some dissolution of the chitin layer where it is condensed at its junction with the lipid layer. Likewise, the indefinite lipid layer appeared to be largely dissolved.

Verticillium chlamydosporium hyphae readily penetrate egg shells of M. arenaria both from without and from within. Once the fungus enters, hyphal branching and proliferation occurred. This was followed by hyphal penetration of the larval cuticle. Ultimately, the entire contents of the shell became occupied by mycelial biomass (Figs. 5A, 5B). At this stage of pathogenesis, several hyphae may emerge, further disrupting the egg shell layer in the process (Fig. 4C).

As a fungal hypha entered a larva (Fig. 4D) the cuticle was broken. Larvae occupied by hyphae were necrotic, and internal deliquescence had occurred resulting in the central part becoming largely vacuolated (Fig. 3C). No trace of somatic musculature could be detected in the diseased larvae. The remaining body contents assumed a granular appearance in a broad, irregular, peripheral band (Fig. 6C). The cuticle of such larvae had become heavily folded and individual cuticular layers and the hypodermis were no longer discernible.

DISCUSSION

Our results indicate that V. chlamydosporium is capable of effectively parasitizing Meloidogyne arenaria eggs and leads to the ultimate demise of the larvae. A number of significant ultrastructural changes are seen following egg contact by a hypha. No larval hatching takes place when
Fig. 3. A) Section of healthy cuticle: VL = vitelline layer, CL = chitin layer, B) section of cuticle with proximal fungal hypha: FH = fungal hypha, HW = hyphal wall, VL = vitelline layer, CL = chitin layer (white arrows indicate gaps in vitelline layer). (both x100,000), C) section of cuticle with proximal endogenous fungal hypha: VL = vitelline layer, CL = chitin layer, FH = fungal hypha, EDI = electron-dense inclusions (x46,000).
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Fig. 4. A) Section of healthy egg cuticle, B) section diseased egg cuticle: VL = vitelline layer, CL = chitin layer, LL = lipid layer (both x50,000), C) section through emerging hypha: EH = emerging hypha, DVL = disrupted vitelline layer, DCL = disrupted chitin layer, D) hypha penetrating larva: PH = penetrating hypha, DC = disrupted cuticle (both x40,000).
Fig. 4. A) Section of healthy egg cuticle, B) section diseased egg cuticle: VL = vitelline layer, CL = chitin layer, LL = lipid layer (both x50,000), C) section through emerging hypha: EH = emerging hypha, DVL = disrupted vitelline layer, DCL = disrupted chitin layer, D) hypha penetrating larva: PH = penetrating hypha, DC = disrupted cuticle (both x40,000).
Fig. 5. A & B) Sections through infected eggs and larvae: V = vacuoles, L = larvae, ES = egg shells, FHc = exogenous fungal hyphae, FHb = intra-egg fungal hyphae, FHc = intra-larval fungal hyphae (A x9000, B x20,000).
Fig. 5. A & B) Sections through infected eggs and larvae: V = vacuoles, L = larvae, ES = egg shells, FHa = exogenous fungal hypha, FHb = intra-egg fungal hyphae, FHc = intra-larval fungal hyphae (A x9000, B x20,000).
Fig 6. A & B) Sections through healthy larvae: ECL = external cortical layer, ICL = internal cortical layer, ML = median layer, BL = basal layer, H = hypodermis, SM = somatic musculature, C) section through diseased larvae: ECL = external cortical layer, FH = fungal hyphae (all x50,000).
Fig 6. A & B) Sections through healthy larvae: ECL = external cortical layer, ICL = internal cortical layer, ML = median layer, BL = basal layer, H = hypodermis, SM = somatic musculature, C) section through diseased larvae: ECL = external cortical layer, FH = fungal hyphae (all x50,000).
the fungus is near eggs, suggesting an exogenous effect, possibly involving the presence of a translucatable, physiologically disorganizing factor such as a diffusible toxic metabolite. The partial disintegration of the vitelline layer in the presence of proximal fungal hyphae may involve exoenzymes. This disruption not only predisposes the egg to fungal infection by physical weakening of the shell, but increases permeability, thus facilitating inward passage of fungal metabolites, both toxic and enzymatic. The detected differences in stain and fixative penetration in our study between healthy and infected eggs provides evidence for changes in permeability brought about by the presence of the fungus. The total, progressive exopathic effect is sufficient to abort the reproductive process.

Once a fungal hypha enters an egg, presumably involving localized enzymatic dissolution of the chitin layer, mycelial proliferation ensues resulting in probable biosynthesis of destructive metabolites endogenously. The end result of this is further breakdown of the chitin and lipid egg shell layers. Permeability of the egg shells of at least some nematodes is said to be directly related to the presence or absence of a lipid layer which is thought to consist of lipoprotein (1). Bird and McClure (2) showed that an egg of *Meloidogyne javanica* (Treub) Chitwood is permeable to osmium tetroxide when this layer is broken down prior to hatching. The presence of electron-dense inclusions where an endogenous hypha assumes contact with the inner shell surface indicates other biochemical disturbances. Exogenous and endogenous changes render the egg shell ineffective as a resistant, protective entity surrounding the larva.

Following endogenous mycelial proliferation, supported in part nutritionally by the lysis of egg shell material, hyphae are able to penetrate the larval cuticle with apparent ease. As a result it is clear that all the component parts of the larvae are deleteriously affected. Some of the changes in the cuticle appear to be closely related to those known to occur during larval invasion of host tissue. The basal layer of the egg shell of *M. javanica*, which is distinctly striated in an infective larva (1), becomes progressively granular in appearance up to one week after the onset of parasitism. A similar change, together with muscular atrophy, occurs prematurely in *V. chlamydosporium* infected eggs. The endopathic activity of the fungus leads to total degeneration of the egg contents.

The capacity of *V. chlamydosporium* to parasitize nematode eggs is now established but its potential as an effective biological control agent remains unresolved and will remain so until more is known about pathogen physiology. There is some evidence that it cannot compete successfully in soils in the absence of a selective, promotional substrate. If soil additives, such as chitin, can provide a selective advantage, it seems possible that *V. chlamydosporium* populations might be enhanced
sufficiently to give an inoculum level high enough to exercise a controlling influence. The addition of *V. chlamydosporium* together with chitin may prove to be a practical biocontrol method for root-knot and other nematodes.

**LITERATURE CITED**


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