COLONIZATION OF ASCARIS LUMBRICOIDES EGGS BY THE FUNGUS VERTICILLIUM CHLAMYDOSPORIUM GODDARD

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Abstract. The process of colonization of Ascaris lumbricoides eggs by the fungus Verticillium chlamydosporium was studied by scanning electron microscopy. The preparations were made by fractionation of egg suspension exposed to the fungus for four days and frozen in liquid nitrogen according to Štěrba and Miláček (1980). Ovicidal fungus forms an abundant ramifying mycelial network in the area between the eggs. However, egg-shells are penetrated only by some hyphae without any penetration organs produced (simple hyphal penetration). In a liquid medium, after penetration, hyphae inside the eggs rapidly grow among inner structures of egg-shells and on the surface of developing larvae. In the next phase, hyphae colonize the developing larva. The eggs attacked by this fungus remain morphologically unchanged for a long time except the sites of penetration. Verticillium chlamydosporium is a fungus with unique ovicidal properties. It colonizes the eggs of Ascaris lumbricoides at all stages of embryo development and also attacks larvae inside the eggs.

Ovicidal fungi represent an important biological phenomenon providing a possible application of these fungi for biological control of parasitic and especially phytopathogenic nematodes (Tribe 1980). They are saprophytic fungi so that they are not dependent on the presence of nematode eggs in the soil. This typical feature allows them to survive for a long time in the intact soil, i.e. in the soil without parasitic nematodes and their eggs. Consequently, potential value of these fungi for biological control of parasitic nematodes seems to be considerable. Therefore, much more attention has been paid to the study of ovicidal fungi. Nevertheless, the papers specialized in the process of destruction of the eggs of parasitic nematodes, causal organisms of human and/or animal helminthoses by means of ovicidal fungi appear sporadically. Most studies focus on colonization of cysts containing eggs of phytopathogenic nematodes (Tribe 1977, 1980, Stirling and Mankau 1978 a, b, Stirling et al. 1979, Kerry et al. 1980, 1984, Dunn et al. 1982, Kerry 1982, Dunn 1983, Jatała 1986). The authors conclude that only a small number of fungi are specialized for parasitism on nematode eggs. Significant ovicidal properties have been reported particularly for the following three species of fungi: Verticillium chlamydosporium Goddard, Paecilomyces lilacinus (Thom) Samson, and Dactylella oviparasitica Stirling et Mankau.

One of us has been intensively dealing with isolation of ovicidal fungi from various geographical localizations and soil types. So far, 33 fungal species with ovicidal activity from soil samples collected in Czechoslovakia, Cuba, Turkey, Afghanistan, Pakistan, Mexico and USSR have been isolated (Lýsek 1963, 1966, 1967, 1975, 1979, Lýsek et al. 1982, 1986). The ovicidal fungi isolated by us are discussed in detail elsewhere (Fassatiová and Lýsek 1982). Ovicidal properties of these fungi differ in the way of destruction of nematode eggs, the mass of eggs that can be attacked during a given period, and the rate of destruction of eggs (Lýsek 1982).

Only some authors have studied the course of ovicidal process (Stirling and Mankau 1978, 1979, Tribe 1980, Dunn et al. 1982, Lýsek 1982, Jatała 1986, Lýsek 1982).
and Krajčí 1987). The course and conditions of this process play an important role in the whole ovicidy and have been studied mainly morphologically under light microscope, TEM and SEM. The present paper reports on the process of ovicidy of the fungus *Verticillium chlamydosporium* studied on fracture preparations under SEM.

**MATERIALS AND METHODS**

Ovicidal process was studied using the fungus *Verticillium chlamydosporium* Goddard, strain No. 9 from the collection of ovicidal fungi kept at the Faculty of Medicine, Palacký University in Olomouc. This strain is kept also at the American Type Culture Collection, Rockville, Maryland, USA as No. 42184, and at the collection of micromycetes of the Institute of Soil Biology, Czechoslovak Academy of Sciences in České Budějovice. The experiments were carried out with the eggs of *Ascaris lumbricoides* Linné, 1758 obtained from female round worms removed from slaughter pigs. The eggs were washed with sterile distilled water and a very dense egg suspension was placed in the form of elongated piles on sterile Petri dishes. The mycelium of the fungus was inoculated on the top of the piles. *Verticillium chlamydosporium* had overgrown egg piles densely in the course of four days. The fungus and the eggs formed a compact mass which was fixed with paraformaldehyde. That suspension was frozen in liquid nitrogen and fractured by a sharp tool, frozen under the same conditions (Štěrba and Mládeček 1986). The sample to which a sharp tool was applied gave cutting surfaces and fracture surfaces projecting space among the eggs, egg contents and their internal structures. The preparations were transferred into 100 % acetone by means of rising alcohol series. Dehydrated samples were further processed in a special pressure chamber (FOLARON) enabling a precise thermal and pressure regulation. After reaching 6 MP acetone was replaced by liquid carbon dioxide according to Anderson (1951). Saturated preparations were heated to 36 °C which is associated with increased pressure, conversion of liquid into gas and simultaneously with a complete dehydration of the subject (critical point CO₂ method). Then the preparations were stuck on aluminium discs with upside fracture surface, coated with gold under vacuum and observed in a SEM TESLA BS 300.

**RESULTS**

The preparations comprised both surface areas and inner areas of the eggs of *Ascaris lumbricoides* at the site where they were fractured. These areas were filled with a dense network of mycelium of *Verticillium chlamydosporium* overgrowing the eggs. Egg fracture surfaces showed inner structures of the eggs and their inner surfaces (Plts. I—IV).

The eggs of *Ascaris lumbricoides* used in the experiment were without external uterine shells which are usually released during preparation. The external surface of the eggs visible in Figures consists of chitin-protein layer of egg-shells. Heinetic value of fractured preparations depends on the area where egg suspension was fractured. When fracture surface goes through the space among the eggs (Pl. I, Fig. 1), egg colonization can be seen from external surface projection. The fungus grew rapidly in egg suspension and formed a dense network among the eggs. The majority of hyphae do not contact or even attack the eggs. Other hyphae grow on egg surface forming a network. Only some hyphae contact by their tips on the external surface of the eggs and penetrate their egg-shells, namely these hyphae which contact the egg-shell in the most perpendicular position as described previously (Lýsek 1977, 1982). In the present experiment, a specific penetration organ termed by some authors appressorium was never observed. The fungus penetrated the eggs by direct, simple penetration of individual hyphae.

Some fractures allowed study of the process of colonization of nematode egg content. After penetration the fungi continue to grow. Egg-shells remain intact for a long time, except the places of penetration. Within the egg, the fungus begins rapidly

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ramify and gradually destroys the whole content, including the developing embryo. A rapid ramification of the fungus occurs first in the inner space of the egg between the ascaroside layer of egg-shells and the surface of the embryo. The fungus thus can surround the developing nematode embryo by a dense hyphal network (Pl. II, Fig. 1). In the next phase the fungus attacks the developing embryo (Pl. II, Fig. 2, Pl. III, Fig. 1).

As soon as the interior of the egg, including the embryo, is destroyed and no nutrient substrate is present, the fungus consumes the residues of egg-shells and the final phase of ovicidal process, deliberation phase, develops. The fungus penetrates back to the external space among the eggs and individual hyphae can colonize again other eggs (Pl. III, Fig. 2).

The fungus *Verticillium chlamydosporium* is able to attack the eggs of *Ascaris lumbricoides* in any phase of embryonal development. It our previous experiments the fungus attacked even mobile larvae (Lýsek 1982, Lýsek et al. 1987, 1989). The course of liquidation of the larva by the fungus is shown on Pl. IV. Fig. 1 illustrates an early phase of colonization of an egg containing a larva when the fungus has penetrated egg-shells. Fig. 2 gives an advanced stage of destruction of a larva.

**DISCUSSION**

The mechanism of penetration of ovicidal fungus through resistant egg-shells inside the egg has not yet been elucidated. Some authors believe it is mainly a mechanical process where enzyme activity of the fungus may be involved as an accessory factor (Stirling et al. 1979). These conclusions are based on the fact that fungi are able to produce a penetration organ (apressorium) at the site of contact with egg surface. It is presumed that the appressorium is able to press strongly to surface structure of egg-shells, perforate them and thus penetrate into egg. However, recent studies suggest that this organ is not prerequisite to penetration of the fungus into the egg (Lýsek 1978, 1982, Dunn et al. 1982, Lýsek et al. 1987). The enzymatic activity of the fungus plays an important role in the process of attacking the eggs and penetration through their egg-shells. In one of our previous papers we described two ways by which the fungus *Verticillium chlamydosporium* penetrates the eggs of *Ascaris lumbricoides*: either by simple hyphal penetration or by means of a specific organ that we termed penetration organ. Thus mechanical pressure is neither the only nor the main factor of ovicidal process. In case of a simple hyphal penetration, a single hypha is not able to produce such a strong pressure to perforate mechanically a resistant chitin-protein layer of the eggs of *Ascaris lumbricoides*. No adhesive structures enabling the hypha to develop a necessary pressure were found in our TEM studies (Lýsek and Krajčí 1987). The possibility can not be excluded, however, that the penetrating fungus may develop a certain pressure during penetration through egg-shells as demonstrated by Jatala (1986). In his electron optical pictures there are visible small invaginations of egg-shells at the site of penetration of *Paecilomyces lilacinus* into the eggs of *Tylenchus semipenetrans*. The author suggests that the origin of these invaginations may be correlated with two simultaneous processes: activities of chitinase and efficiency of even though slight pressure developed by the penetrating hypha. In our above mentioned study no invaginations of this type were observed at the point of penetration. The area of the contact between the fungus and the egg surface was smooth in the first and the second phase of ovicidal process, i.e. in the phase of contact and in the phase of adhesión. In the third phase of ovicidal process the fungus *Verticillium chlamydosporium* produced a conic haustorium in the vicinity.
of which there was a distinct halo of different electron density in TEM pictures. This made us suppose that the penetration of the haustorium through egg-shells is mainly of enzymatic character. Different electron density in the vicinity of the haustorium indicates that chitin-protein complex is decomposed there.

Dunn (1983) studied the fungus Paecilomyces nostocoides displaying ovicidal properties which penetrated the eggs of Heterodera zeae and formed at the spot of contact with egg-shell a penetration organ of hockey stick shape. The identical formation was observed in our experiments with Verticillium chlamydosporium during penetration into the eggs of Ascaris lumbricoides (Lýsek 1978, Lýsek and Krajčí 1987).

The question still remains open if ovicidal fungi are able to colonize nematode eggs at all stages of development of nematode embryo or if ovicidal fungi prefer the eggs at certain developmental stages. Jatala (1986) reports that the fungus Paecilomyces lilacinus colonizes the eggs of the nematode Globodera pallida much more easily at early developmental stages, namely before gastrulation. At higher developmental stages, especially when a larva is developed inside the egg, ovicidal activity, i.e. ability to colonize the eggs, is reduced in most ovicidal fungi. Kerry (1982) also concludes that the eggs before the development of the second larval stage are highly sensitive to ovicidal fungi. Another study (Irving and Kerry 1986) of the fungus Verticillium chlamydosporium proved that the fungus had been able to colonize the eggs of phytopathogenic nematodes at all stages of embryonal development. Ovicidal activity differed in various strains of the same fungal species.

All our experiments were carried out with the fungus Verticillium chlamydosporium, strain No. 9, Collection of ovicidal fungi, Medical Faculty, Olomouc. The present paper confirms again that Verticillium chlamydosporium is able to attack even those eggs of Ascaris lumbricoides which contained a developed mobile larva of the second stage. This finding corresponds to all conclusions published previously (Lýsek 1982, Lýsek and Krajčí 1987).

The SEM study supports the data published by us and of other authors that ovicidal fungi form a relatively dense mycelial network around the eggs of nematodes. Ovicidal fungus does not attack the eggs of Ascaris lumbricoides by the first hypha which comes into contact with egg surface, but this possibility may exist as it is suggested by the experiments of Irving and Kerry (1986). These authors report that Verticillium chlamydosporium attacked easily and more frequently the eggs of Heterodera avenae when the eggs were still in mucoid cysts and even in female bodies. The mucus surrounding the eggs was probably a good nutrient substrate increasing the intensity of mycelial growth and subsequently led to egg colonization. As is illustrated in Pl. I, Fig. 1, most of hyphae of the experimental fungus first multiply among the eggs where residues of cellular detritus occur due to egg dislodgement from female uteri. This detritus may serve as a suitable nutrient source allowing a rapid growth of the fungus which is then able to attack intensively nematode eggs.

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Fig. 1. Fracture surface of cross section of suspension of *Ascaris lumbricoides* eggs, 400×. a — a dense network of ovidial fungus *Verticillium chlamydosporium* among the eggs; b — fracture surfaces across the eggs. Arrows indicate probable spots of a simple hyphal penetration without penetration organ. Fig. 2. Cross-section of the egg immediately after penetration of the hypha inside the egg (arrow). 1 600×. a — intact parts of egg-shells; b — still intact, no gastrulated embryo; c — artificial fibers produced by coagulation of serous fluid inside the egg.
Fig. 1. Cross section of the egg without its embryo. 1600x. a — mycelial network closely under the internal surface of ascaroside layer of egg shells; b — intact parts of egg-shells; c — mycelial network in spaces among the eggs.

Fig. 2. Cross section of the egg in the initial phase of colonization by the fungus. 1600x. a — intact sections of egg-shells; b — embryo inside the egg in the initial phase of colonization by hyphae of the ovicidal fungus; c — spot of penetration into the egg. Hyphal residue is visible.
Fig. 1. Cross section of the egg in advanced stage of destruction by the fungus. 1600×. Fig. 2. Final phase of egg destruction by ovicidal fungus. Residues of egg-shells only outlined. 1600×.
Fig. 1. The initial phase of colonization of *Ascaris lumbricoides* egg containing a developed larva. 1600×. a — developed larva; b — hypha penetrated the egg and grows in the space among inner surface of egg-shells and mobile larva. Fig. 2. The advanced phase of egg colonization. 1600×. a — overgrown and destroyed egg-shells; b — the embryo at advanced stage of development.