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Influence of excretions of chosen *Penicillium* species on the population of *Globodera rostochiensis*

MAZURKIEWICZ-ZAPAŁOWICZ KINGA[1], JANOWICZ KRYSTYNA[2], and KUŹNA-GRYGIEL WANDA[3]

[1] Department of Zoology, Agricultural University
Dr Judyma 20, PL-71-466 Szczecin, Poland
[2] Department of Applied Entomology, Agricultural University
Słowackiego 17, PL-71-434 Szczecin, Poland
[3] Department of Medical Biology and Parasitology, PAM
Al. Powstańców Wielkopolskich 72, PL-71-110 Szczecin, Poland

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It was stated that the contact of *Penicillium frequentans* and *P. verrucosum* var. *cyclopium* with *Globodera rostochiensis* in the medium stimulated reduction in the number of cysts of nematodes. Among 16 *P. verrucosum* var. *cyclopium* metabolites, the strongest destruction of eggs and larvae in *G. rostochiensis* cysts was observed in the case the $R_f = 0.31$ and $R_f = 0.75$ substances. The above metabolites caused that irregular lipid-like granules formed in the intestine tracts of the *G. rostochiensis* larvae. Most probably, the pathological presence of lipides in the intestine resulted from the distemper of synthesis of lipolythic enzymes in the nematodes under the effect of the *P. verrucosum* var. *cyclopium* extracts.

Key words: soil fungi, entomopathogenic fungi, *Globodera rostochiensis.*

INTRODUCTION

Fungi metabolites have been successfully applied in medicine, industry, and agriculture. The revolutionary discoveries made by Fleming and concerning the properties of penicillin have triggered intensive investigations on fungi, which yielded new and surprising information on the properties of fungi. In the scope of plant protection biotechnology, the research focuses on identification and extraction of substances (Evans et al. 1969) that have the antibiotic effect on some bacterial and fungous phytopatogenes (Truszkowska et al. 1986; Wilson et al. 1986; Szewczuk et al. 1990; Feldlaufer 1993; Kita et al. 1999).

Several-year-long research confirms also the biological effect of fungi excretions on the reduction of the population of potato nematode – *Globodera rostochiensis* (J a n o w i c z et al. 1994). This way, they can diminish meaningfully the crop losses of cultivated plants – host for *Globodera rostochiensis*. Particularly, saprophytic fungi, e.g.: *Penicillium frequentans* (Westling) and *Penicillium verrucosum* var. *cyclopium* (Westling) Samson, Stolk et Hadlok, deserve a closer insight (M a z u r k i e w i c z-Z a p a ł o w i c z unpubl.). They appear commonly in the soil and their antagonistic properties are underestimated. Particularly *P. verrucosum* var. *cyclopium* could be applied as a factor diminishing the development of nematodes, as it has antibiotic properties against *Mycobacterium tuberculosis* (C u n n i n g h a m and F r e e m a n 1953).

Hence, the present research was focused on isolation and further investigation of the active substances produced by *P. verrucosum* var. *cyclopium*, which can repress development of potato nematodes. The aim of the research was also to analyse how the active substances affect the growth stages of the nematodes.

MATERIAL AND METHODES

C u l t u r e s of *Penicillium frequentans* and *Penicillium verrucosum* var. *cyclopium*. First, strains of *P. frequentans* and of *P. verrucosum* var. *cyclopium* were isolated from the cysts of *G. rostochiensis*. Then, one-sporous cultures of the above fungi were grown for 21 days in the standard PDA difco medium, in Petri dishes with diameter of 100 mm. The temperature equalled 22–25°C. The culture was used in pot investigations and in biochemical research in order to extract the metabolites.

P o t e x p e r i m e n t s. In order to determine the effect of *P. frequentans* and *P. verrucosum* var. *cyclopium* on the density of cysts of *G. rostochiensis*, pot experiments were carried out with potato cv. Aster as the host plant. Experiments were carried out in the following combinations:

G. rostochiensis – control
G. rostochiensis + *Penicillium frequentans*
G. rostochiensis + *Penicillium verrucosum* var. *cyclopium*

The initial concentration of *G. rostochiensis* was 10 cysts in 100 cm^3 of soil whereas the mycelium area of each inoculate of *P. frequentans* and *P. verrucosum* var. *cyclopium* reached ca 180 mm^2 per pot. The nematodes and fungi were introduced into the soil at the moment when the bulbs were planted. Each combination of the experiment was repeated four times. After the potato vegetation ceased, the density of *G. rostochiensis* cysts was estimated. The Buhra method was applied (W i l s k i 1967). Average size of the cysts was also estimated by means of microscope measurements of 40 cysts from each repeat.

The results were statistically elaborated by means of the one-factor variance analysis. Semi-intervals of confidence were calculated from the Newman-Keuls test, at the significance level equal to 0.05.

Extraction of *Penicillium verrucosum* var. *cyclopium* metabolites. Mycelium of *P. verrucosum* var. *cyclopium*, grown in 60 Petri dishes, was used to extract the metabolites. The cultures and their media were rubbed with anhydrous sodium sulfate (Na_2SO_4). Then, the mass was homogenized for 5 min. and by 3000 rotations per min. Chloroform ($CHCl_3$) was applied as dissolvent. The homogenizate was then placed in centrifugal tubes and centrifuged 10 min. by 4000 rotations per min. Next, the chloroform extracts were filtrated and collected in tubes with microsection. The remaining, jelly-like substance was treated similarly.

The chloroform extracts obtained in both processes were then put together and condensed in a rotary evaporator at the temperature of 40°C and under diminished pressure.

The dry residue was placed in tubes with microsection by means of chloroform and treated with preparatory chromatography (PLC).

Preparatory plates were made from a mixture of Merck silicagels G and HF_{254} with the addition of starch. The layer of adsorbent was equal to 1 mm.

The extract prepared in this way was placed on conditioned plates in the system of chloroform and aceton in the proportion of 9 to 1, respectively. The above method made it possible to separate 16 substances from *P. verrucosum* var. *cyclopium*. Then, they were collected, together with the gels, and applied in further experiments concerning their direct effect on nematodes. The gel collected from the plate with no extract of *P. verrucosum* var. *cyclopium* was the control.

Research on the influence of *P. verrucosum* var. *cyclopium* metabolites on health state of eggs and larvae of *G. rostochiensis* and on the size of nematode cysts. Biological activity of each of the 16 fractions was tested on *G. rostochiensis* cysts. Each of the fractions was mixed with soil, to which *G. rostochiensis* cysts had been previously introduced in the amount of 90 cysts per 100 cm of soil. The cysts remained in the contact with the excretions of particular fractions for four weeks. Then, the health-state of the eggs and larvae of *G. rostochiensis* was analysed. The cysts from the medium without any of the above mentioned fractions were the control. The health-state of the 500 randomly chosen eggs and larvae was analysed. Proportional amount of morphologically and anatomically changed eggs and larvae was determined in each of repeat.

Analysis of anatomical and morfological changes of eggs and larvae of *G. rostochiensis*. Eggs and larvae of *G. rostochiensis* were isolated from the medium and preserved in dyed and non-dyed samples. Larvae set free from the egg thecae, eggs with larvae, and cysts were observed under the light-microscope.

The samples were dyed by oil red for lipids according to the Lille method, and by black Sudan (Zawistowski 1983).

RESULTS AND DISCUSSION

After the pot experiments had been carried out, the high reduction of *G. rostochiensis* was revealed in the medium in which the nematodes were in contact with the fungi. In the case of *P. frequentans*, the reduction was confirmed statistically (Table 1).

Table 1

The influence of *Penicillium verrucosum* var. *cyclopium* and *Penicillium frequentans* on the number of *Globodera rostochiensis* cysts

Combination	Average number of cysts in 100 ml of soil after the vegetation period
Penicillium verrucosum var. *cyclopium* + *Globodera rostochiensis*	101.85 b
Penicillium frequentans + *Globodera rostochiensis*	61.50 a
Globodera rostochiensis	146.20 b

It was also proved that the contact of *G. rostochiensis* with *P. frequentans* diminished relevantly the size, of cysts in comparison to the remaining combinations (Table 2).

Table 2

The influence of *Penicillium verrucosum* var. *cyclopium* and *Penicillium frequentans* on the size of *Globodera rostochiensis* cysts

Combination	Size of cysts ($\bar{x}$ from 40 cyst) [μm]
Globodera rostochiensis	537.4 b
Penicillium verrucosum var. *cyclopium* + *Globodera rostochiensis*	546.0 b
Penicillium frequentans + *Globodera rostochiensis*	499.2 a

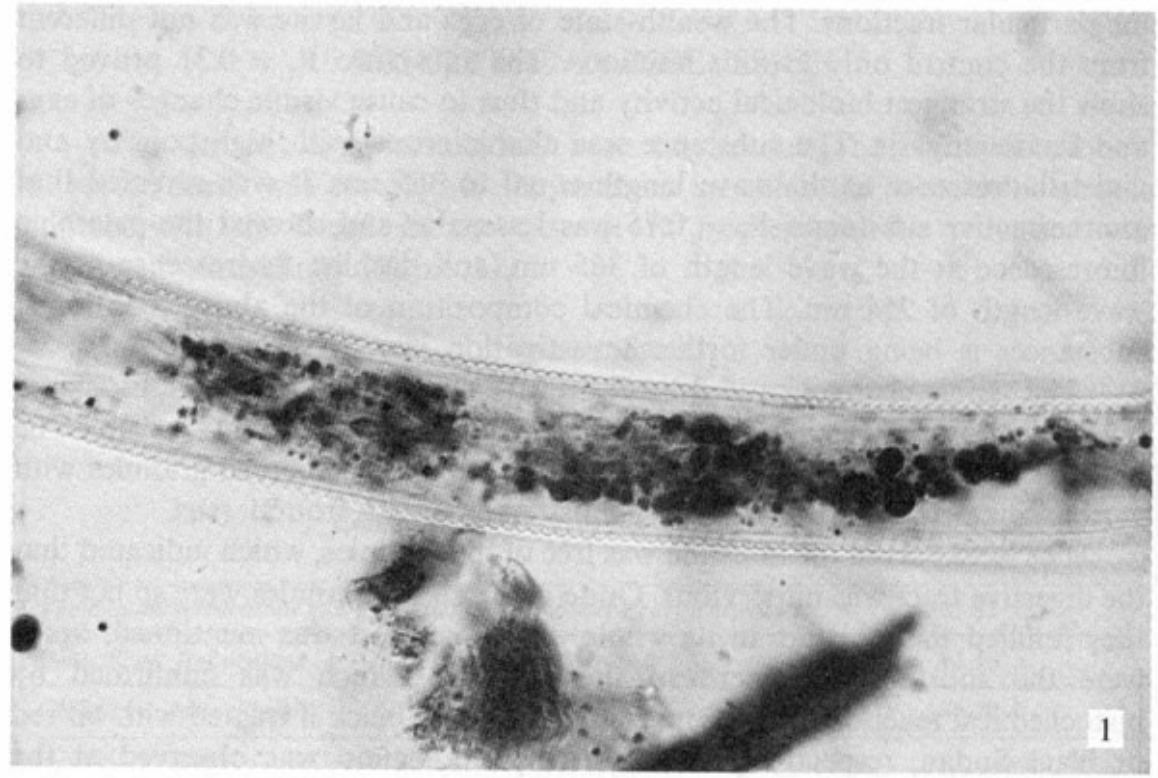
Eight chromatographic fractions were extracted from *P. frequentans*. They had a slight influence on the morphology of *G. rostochiensis* larvae of type L_2. Hence, the stated meaningful restriction on the development of *G. rostochiensis* in the presence of *P. frequentans* results most probably from the advantageous influence of the fungus on the well-being of the plant, which stimulates, in turn, the plant's resistance to the nematode.

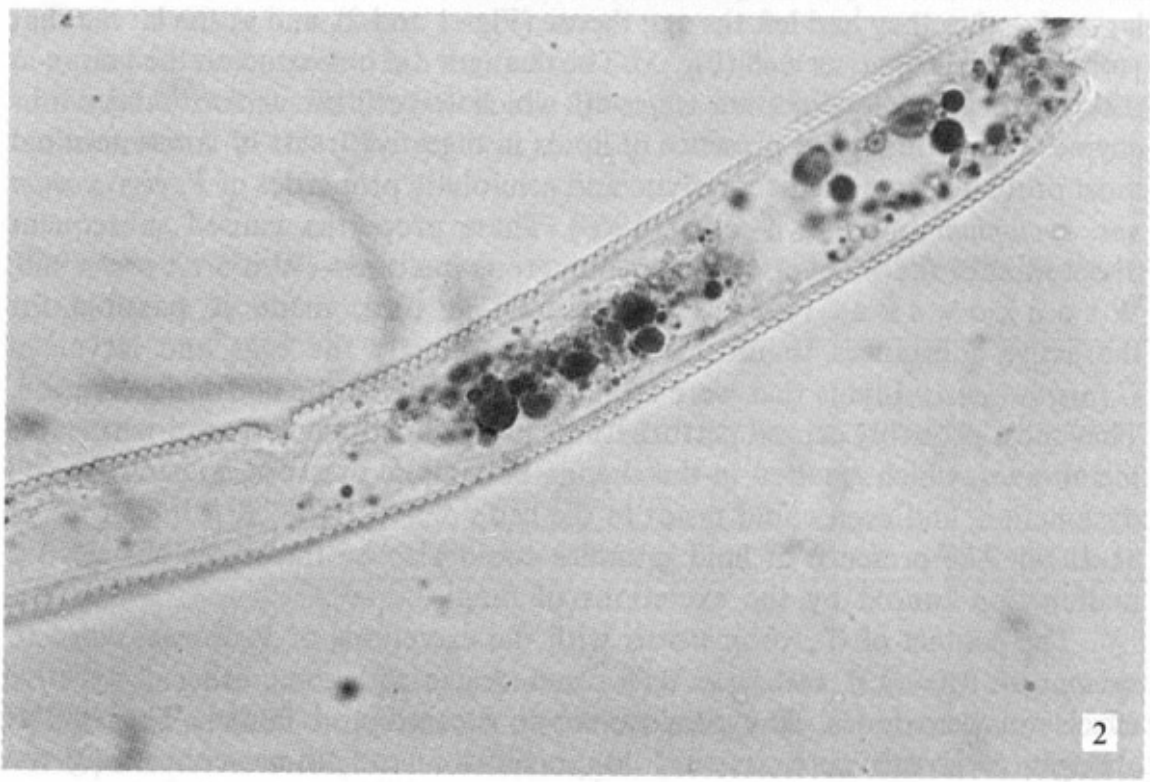
On the other hand, *P. verrucosum* var. *cyclopium* proved to be chemically more active. In as many as 12 fractions, eggs and larvae with anatomical and morphological changes amounted 15–80% of the whole number, differently for particular fractions. The wealth-state of eggs and larvae was not different from the control only in four fractions. The substance $R_f = 0.31$ proved to show the strongest biological activity and thus to cause visible changes of eggs and larvae in cysts. The substance was characterized with high polarity and violet fluorescence at the wave length equal to 365 nm. It was revealed that another active substance: $R_f = 0.75$ was less polar and showed the pale-blue fluorescence at the wave length of 365 nm, and dashing fluorescence at the wave length of 254 nm. The chemical composition of the above mentioned substances is being under further investigation.

The visible changes caused by $R_f = 0.31$ and $R_f = 0.75$ metabolites were revealed most often in digestive tracts of the investigated larvae. The destructive effect resulted in the formation of irregular, spherical granules with various diameters in the intestine, most often in its frontal part.

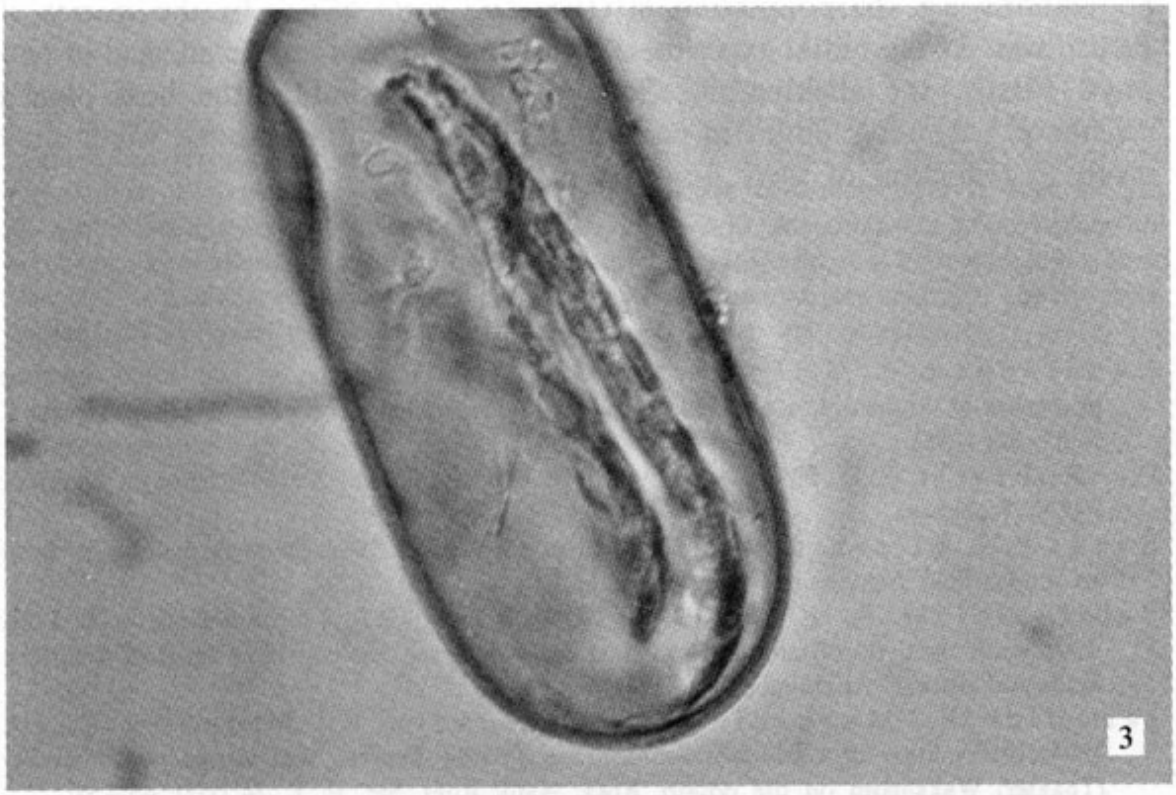
The hind part of the intestine was free of the granules, which indicated that the digestive tract was impervious. Quite often, single granules were so big that they fulfilled the intestine in its whole diameter. As it was mentioned, lipids were the substantial element of the granules, which was confirmed by histochemical reaction. The granules turned red or black if treated with oil red or black-Sudan, respectively. The above phenomenon was observed at the larvae L_2 after they had left the egg thecae (Figs 1 and 2), and at the larvae that yet remained in eggs, as well (Fig. 3). The changes did non concern the larvae in the control (Fig. 4), digestive tracts of which were filled with homogeneous chyme. The pathological presence of lipids in digestive tracts of larvae resulted most probably from the pectinolytic and celulolytic properties of *P. verrucosum* var. *cyclopium* (M i l a n et al. 1969). These properties caused subsequent dissolution of the walls of cysts and numerous fractures (W h a r t o n 1980; W r o n k o w s k a 1993). The fractures, in turn, made it possible for the active substances to intrude and affect directly the eggs and larvae of *G. rostochiensis*. Lipids that were found in cysts proved the above observation. They most probably caused perturbations in the functioning of semi-permeable membranes, which resulted in the change of osmotic pressure, causing the cell dysfunction, and even could result in the cell's death (D e R o b e r t i s et al. 1974). The presence of lipid granules could also be the result of digestive malfunction caused by the excretions of fungi.

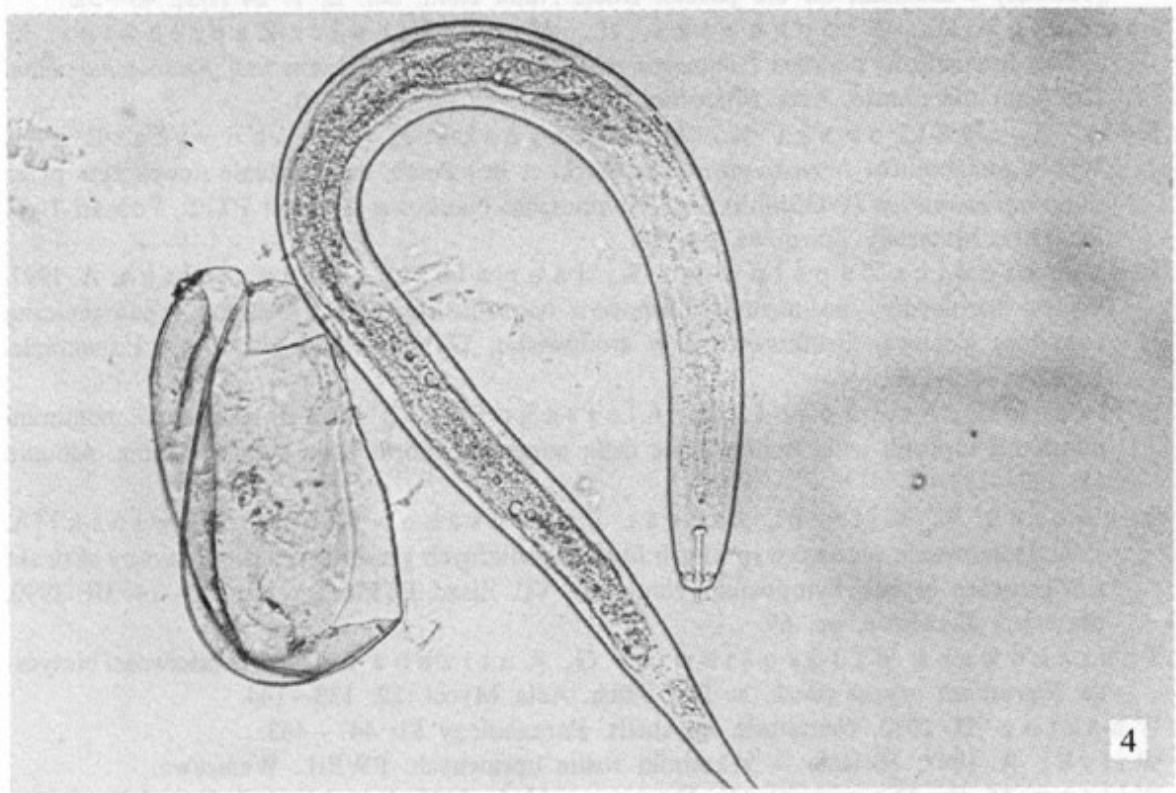
The contact of *G. rostochiensis* with the excretions of *P. verrucosum* var. *cyclopium*: $R_f = 0.31$ and $R_f = 0.75$ resulted also with some other destructive effects on nematodes. The skin of larvae creased and folded. The similar changes of larvae were revealed in previous investigations concerning the contact of *G. rostochiensis* with other fungi, e.g. *Rhizoctonia solani* (J a n o w i c z et al. 1994a). Herbicides affected nematodes in a similar way (M a z u r k i e w i c z-Z a p a ł o w i c z et al. 1997).





Figs 1, 2. Fig. 1. The larvae L_2 of *Globodera rostochiensis* under the influence of the *Penicillium verrucosum* var. *cyclopium* excretion $R_f = 0.31$. Fig. 2. The frontal part of the intestine filled with numerous spherical granules of lipids. (Phot. W. Kuźna-Grygiel), × 1000





Figs 3, 4. Fig. 3. Larvae of *Globodera rostochiensis* still remaining in the egg, under the influece of the *Penicillium verrucosum* var. *cyclopium* excretion $R_f = 0.31$. (Phot. W. Kuźna-Grygiel), × 1000; Fig. 4. Larvae L_2 of *Globodera rostochiensis* in the control – without spherical granules of lipids. (Phot. W. Kuźna-Grygiel), × 1000

The present investigations confirmed the broad possibilities of application of microorganizms that coexist in biocoenosis with nematodes to reduce their populations, e.g. the population of *Globodera rostochiensis* by the species of *Penicillium*. As the initial experiments show, nematodes can be affected in two ways. Firstly, the allelopathic effect of fungi can induce the host plant's resistance. Secondly, fungi can act antagonistically on nematodes, which may result in reduction of their population.

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Wpływ wydzielin wybranych gatunków *Penicillium* na populację nicieni *Globodera rostochiensis*

Streszczenie

Stwierdzono, że kontakt *Penicillium frequentans* i *Penicillium verrucosum* var. *cyclopium* z *Globodera rostochiensis* w podłożu, wpływa na redukcję cyst tego nicienia. Spośród 16 metabolitów *P. verrucosum* var. *cyclopium*, najistotniejszą destrukcję jaj i larw w cystach *G. rostochiensis*, wykazały substancje $R_f = 0.31$ i $R_f = 0.75$. Metabolity te doprowadziły do wytrącania się w jelicie larw nieregularnych ziarnistości o budowie lipidów. Patologiczna obecność tłuszczów w jelicie larw, wynika prawdopodobnie z zaburzenia syntezy enzymów lipolitycznych nicieni, pod wpływem testowanych ekstraktów *P. verrucosum* var. *cyclopium*.