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Effect of *Penicillium frequentans* and *Stachybotrys chartarum* on respiratory metabolism of developing eggs of *Ascaris suum* (*Nematoda*)

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Effect of saprotrophic soil fungi *Penicillium frequentans* and *Stachybotrys chartarum* on respiratory metabolism of *Ascaris suum*, during its embryogenesis was determined using histoenzymatic methods. Based on histochemical assessment of the enzyme activity (glycolysis-lactate dehydrogenase-LDH; tricarboxylic acid cycle-succinate dehydrogenase-SDH), changes in the energy metabolism of developing eggs of *A. suum* were detected. Of the fungi species tested – *P. frequentans* caused the most extensive disorders in the processes of cellular oxidation, which were manifested in a decline of SDH activity during gastrulation. Incubation of eggs of *Ascaris* with mycelia of both fungi: *S. chartarum* and *P. frequentans* – in lesser extend affected respiratory metabolism in embryogenesis of this nematode.

Key words: soil fungi, oxidative enzymes, *Ascaris suum*.

INTRODUCTION

Biotic factors along with abiotic ones play a significant role in controlling numbers and dispersal of eggs of *Ascaris* and other geohelminths in soil (Mizgajska 1993). The major groups of soil organisms limiting the quantities of parasite eggs in soil are: fungi, mites, springtails, and bacteria (Lysek 1967, 1986; Mizgajska 1994). Variable-intensity ovistatic and ovicidal

effects on *Ascaris* eggs were observed in relation to different saprotrophic soil fungi (L y s e k 1963, 1967, 1979; L y s e k et al. 1986; K u n e r t 1992).

Antagonistic properties of *P. frequentans* were demonstrated in relation to *Pytium* – a pathogen of sprouts of a beet and charlock (L i u and V a u g h a n 1965). It was also demonstrated that numerous strains of this species inhibited development of *Streptomyces griseus* (cf. M i r c h i u k and B e l a y a 1965) and bacteria Erwinia carotovora (cf. K h a r c h e n k o 1960) and *Staphylococcus aureus* (cf. O r a z o v and S i z o v a 1966). Also, fungistatic and fungicidic properties of this species were stated in relation to *Beauveria bassiana* and *B. brongniartii* (cf. Kal'vish 1972) and *F. sporotrichoides* (cf. S i z o v a and V a s i n 1962). Good confirmations of biological activity of strains of *P. frequentans* are their strong cellulolytic (D o m s c h 1960; K a n e v s k a y a 1966) and proteolytic properties.

S. chartarum is one of common saprotrophs occurring in upper layers of soil. It can also be present in animal-husbandry buildings. Its inhibiting properties were observed in relation to rape, tomato, and linen (D o m s c h 1963). It also demonstrates strong cellulolytic properties (D o m s c h 1960; T r i b e 1960; K a n e v s k a y a 1966). Many strains of this species have pectinolytic (Cochrane 1958), chitinolytic (D o m s c h 1960), and fungistatic properties (D h i n g r a and K h a r e 1973; D o m s c h and G a m s 1968; G h a f f a r and F a t i m a 1970; R a i and S a x e n a 1975).

Ovistatic properties of mycelia of *S. chartarum* and *P. frequentans* in relation to development of *A. suum* eggs were demonstrated in our other paper (K u ź n a - G r y g i e l et al. 2001). They were manifested in retardation of sequential stages of the embryogenesis and occurrence of developmental defects. *P. frequentans* exhibited decisively strongest antagonistic effect among the mould fungi studied.

The aim of the present work was the assessment of the effect of both fungi species (separately and in conjunction) on oxidative metabolism during embryonic development of *Ascaris suum*.

MATERIAL AND METHODS

The specimens of *Ascaris suum* were collected from pigs at the Municipal Slaughterhouse in Szczecin. Fertilised parasite eggs, used in the present study, were dissected from the terminal portion of uteri of nematode females. The eggs were placed in petri dishes in 0.9% NaCl solution (control culture) and in the same NaCl concentration with mycelium fragments of: *Penicillium frequentans* (experimental culture No. 1), *Stachybotrys chartarum* (experimental culture No. 2), and both fungi (experimental culture No. 3).

Saprotrophic mould fungi – *Penicillium frequentans* and *Stachybotrys chartarum* isolated from soil, were cultured on a standard substrate (PDA-Difco medium) in petri dishes at 25°C. Mycelium-substrate circular pieces (40 mm in diameter) were cut out from 21-day cultures of each species and they

were placed in petri dishes along with eggs of *Ascaris suum* in NaCl solution (experimental cultures Nos. 1 and 2). Similarly procedure was applied to the two-species arrangement of mould fungi (culture No. 3). Subsequently the petri dishes were incubated in an incubator at 26°C for 41 days.

In the course of the experiment, eggs from experimental cultures and control culture were monitored daily under a microscope. Based on the observation in control culture, the following developmental stages were defined: 0-day-old eggs, immediately after dissection from the nematode uterus, zygote, early stages of cleavage (2, 4, n-blastomeres), morula, gastrula, L_1 larva, L_2 larva.

Histochemical reactions detecting oxidative enzymes were conducted in nematode eggs immediately after their separation from the uterus (0-day-old) and on days: 2, 8, 11, 14, 17, 22, 25, 28, 31, 35, and 41 of incubation. The procedure involved the following steps: eggs of a defined phase of embryonic development were placed in blocks cut out of a liver, frozen in dry ice, and sectioned in a cryostat at -20°C for 10–μm sections. Unfixed cryostat sections were subjected to reactions detecting NAD-dependent lactate dehydrogenase (LDH) (EC 1.1.1.27) and succinate dehydrogenase (SDH) (EC 1.3.99.1) according to N a c h l a s et al. (P e a r s e 1972). The sections were incubated for 30 min at 37°C and subsequently preserved in Baker solution. Specificity of this enzymatic reaction to substrate was determined based on histochemical reactions carried out in incubation media devoid of substrate.

RESULTS

Based on the microscopic observations of *Ascaris suum* eggs, incubated in 0.9% NaCl solution (control), sequential steps of embryogenesis were determined and studied histochemically. Up to the second day of incubation the majority of eggs were at the stage of zygote. Between days 8 and 11 dominated sequential cleavage stages of blastomeres, although on day 11 the dominant stage was morula. On day 14 of incubation, the majority of eggs reached the stage of gastrula. L_1 stage was observed on day 17 and from day 22 on – L_2 stage.

In experimental culture No. 1 (with *P. frequentans*), the stage of zygote was observed in the eggs of *Ascaris* up to day 22 of incubation. Within 25–31 days of incubation the majority of eggs reached stages ranging from few blastomeres up to morula, while within 35–41 days - the stage of gastrula dominated.

In experimental culture No. 2 (with *S. chartarum*), the stage of zygote was observed until day 14 of incubation. From day 17 on, the majority of eggs featured different cleavage stages up to morula. The stage of gastrula was observed on day 22 of incubation, while larva of stage L_1 – on day 25. From day 28 on, the majority of eggs contained L_2 stage.

In experimental culture No. 3 (with *P. frequentans* and *S. chartarum*) the zygote stage persisted up to day 17 in the majority of eggs. Day 22 revealed

sequential cleavage stages, and day 25 – the stage of gastrula. L_1 stage dominated on day 28, while L_2 larvae were observed on day 31 of culture (Table 1).

Table 1
Effect of *Penicillum frequentans* and *Stachybotrys chartarum* on embryonic development of *Ascaris suum*

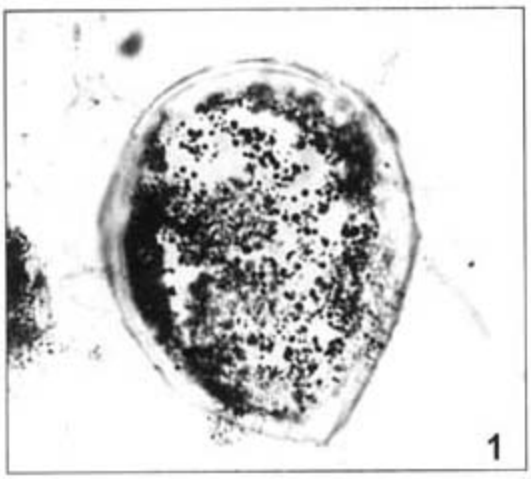
Day of incubation Culture	0	2	8	11	14	17	22	25	28	31	35	41
Control	Z	Z	Bl,M	M	G	L_1	L_2	L_2	L_2	L_2	L_2	L_2
No. 1	Z	Z	Z	Z	Z	Z	Z	Bl,M	Bl,M	Bl,M	G	G
No. 2	Z	Z	Z	Z	Z	Bl,M	G	L_1	L_2	L_2	L_2	L_2
No. 3	Z	Z	Z	Z	Z	Z	Bl,M	G	L_1	L_2	L_2	L_2

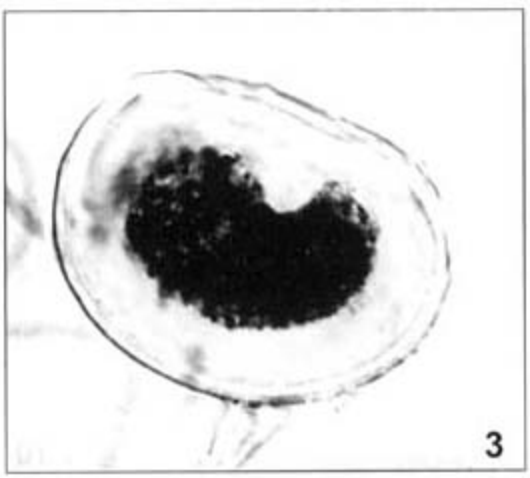
Explanations: Z – zygote; Bl – blastomeres; M – morula; G – gastrula; L_1 – larva L_1; L_2 – larva L_2

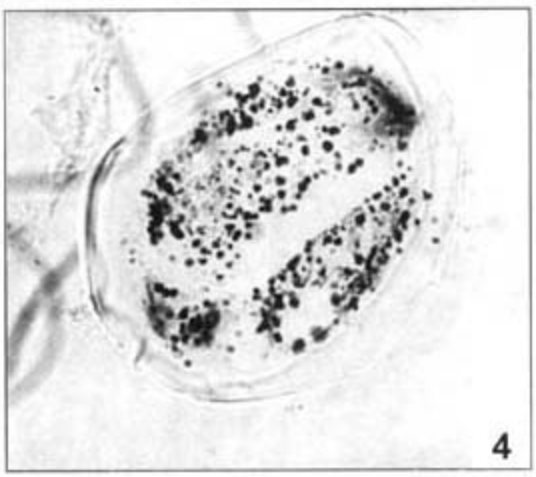
Succinate dehydrogenase (SDH)

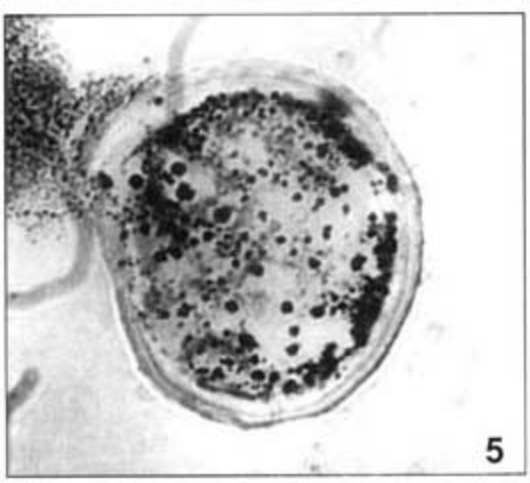
Control culture. In 0-day-old eggs of *Ascaris* – isolated directly from the females uteri – the SDH activity was moderate or strong. The reaction product was visible in a form of numerous, minute formazan granules (Fig. 1). A similar magnitude of SDH reaction was observed in 2-day-old eggs containing zygote and in 8-day-old ones featuring early cleavage stages (Fig. 2). In the stage of morula (approximately day 11 of culture) an increase in the enzyme activity, ranging from a strong to a very strong, was observed. A very strong reaction to SDH in a form of very numerous formazan granules was recorded in *Ascaris* eggs at the stage of gastrula (day 14 of incubation) (Fig. 3). Slight weakening of the reaction to SDH occurred in eggs containing L_1 larvae (day 17 of incubation) where a strong reaction was noted, and in eggs with L_2 (days 22 through 41 of culture) featuring a moderate reaction to SDH (Fig. 4).

Experimental culture No. 1 with *Penicillium frequentans*. On day 2 of *Ascaris* eggs incubation, moderate or strong reactions to SDH were observed in the cytoplasm of zygote, while on day 8 of culture a small decrease to a moderate level was recorded in reaction to SDH. A similarly moderate activity persisted in the cytoplasm of eggs up to day 22 (Fig. 5). On day 25 of culture some 50% of eggs showed initial phases of cleavage, mostly 2 and 4 blastomeres and occasionally the stage of morula. The cytoplasm of the blastomeres exhibited a moderate activity of SDH. Activity increase of SDH up to a moderate or strong level was observed on days 28 and 31 (Fig. 6), when most of eggs reached the stage of morula. In 35- and 41-day-old eggs at the stage of gastrula the reaction to SDH was moderate or strong.









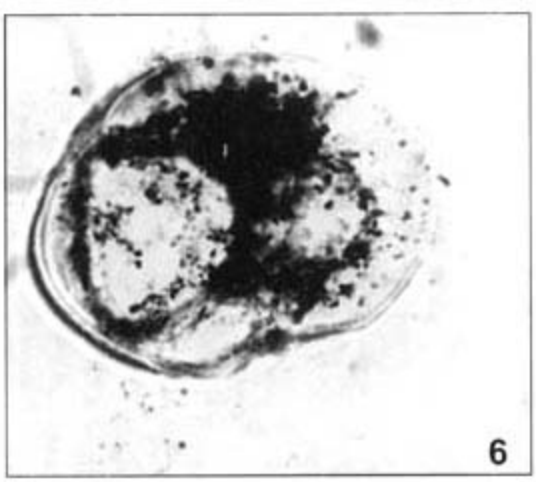


Plate I

Fig. 1. A moderate / strong activity of SDH in 0-day-old egg of *A. suum*, dissected directly from uterus (× 1000)

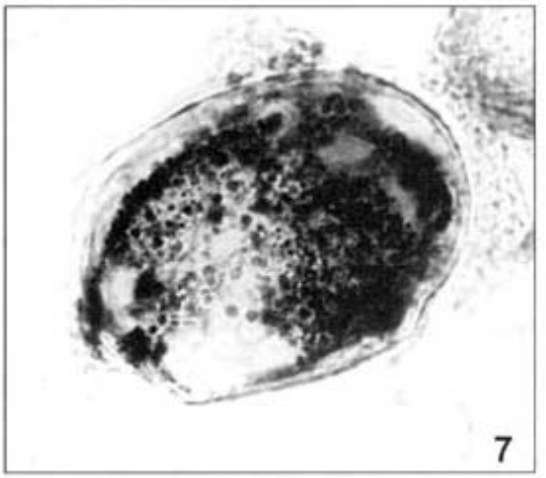
Fig. 2. A moderate / strong activity of SDH in 8-day-old egg of *A. suum* (cleavage stage) in control culture (× 1000)

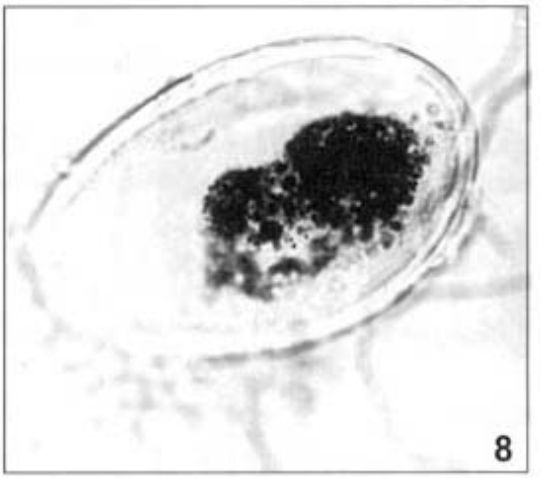
Fig. 3. A very strong activity of SDH in 14-day-old egg of *A. suum* (gastrula) in control culture (× 1000)

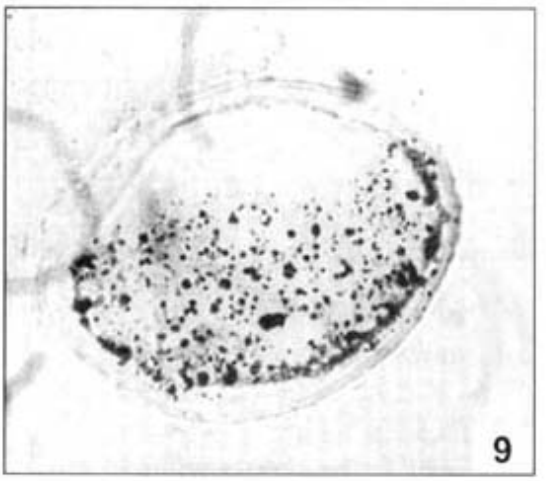
Fig. 4. A moderate activity of SDH in 25-day-old egg of *A. suum* (stage of L_1 larva) in control culture (× 1000)

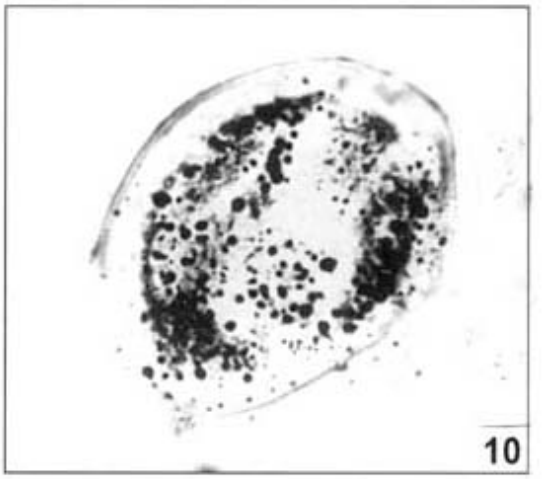
Fig. 5. A moderate activity of SDH in 22-day-old egg of *A. suum* (zygote stage) in experimental culture No. 1, with *P. frequentans* (× 1000)

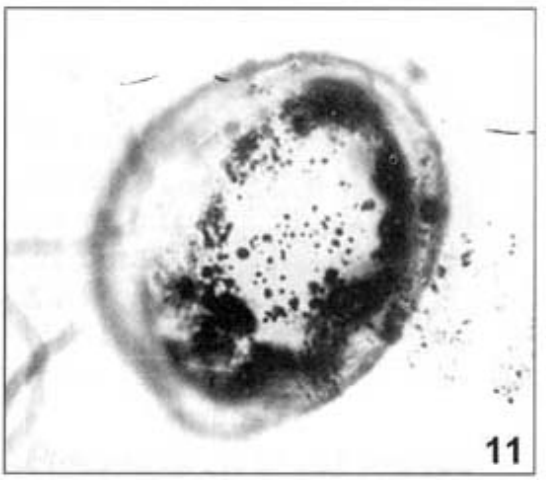
Fig. 6. A moderate / strong activity of SDH in 28-day-old egg of *A. suum* (cleavage stage) in experimental culture No. 1, with *P. frequentans* (× 1000)











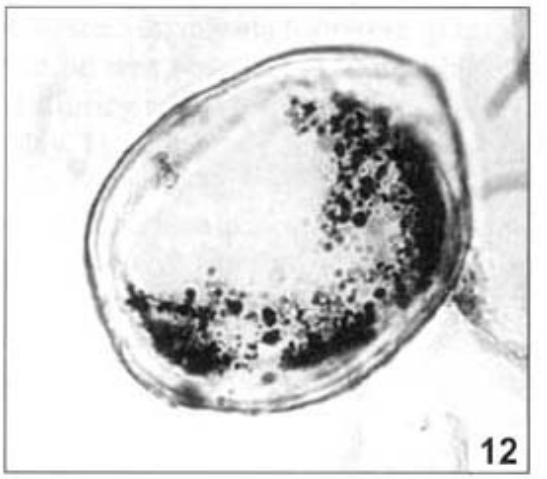


Plate II

Fig. 7. A moderate / strong activity of SDH in 11-day-old egg of *A. suum* (zygote stage) in experimental culture No. 2, with *S. chartarum* (× 1000)

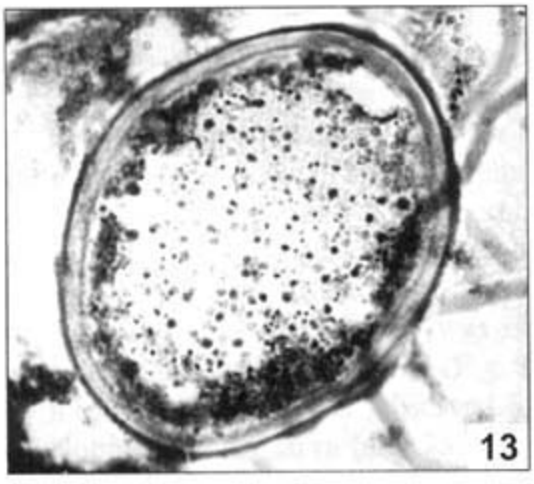
Fig. 8. A strong / very strong activity of SDH in 22-day-old egg of *A. suum* (gastrula stage) in experimental culture No. 2, with *S. chartarum* (× 1000)

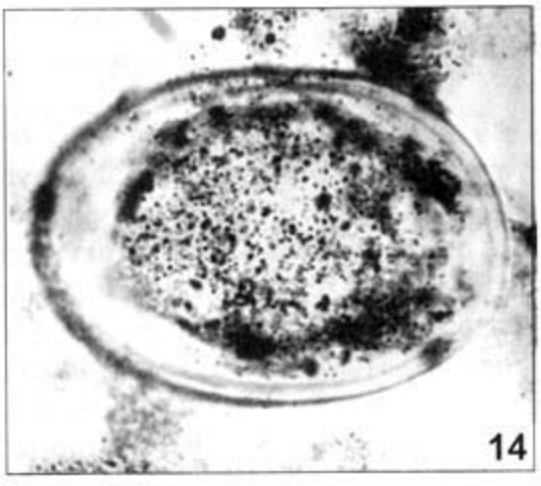
Fig. 9. A moderate activity of SDH in 25-day-old egg of *A. suum* (stage of L_1 larva) in experimental culture No. 2, with *S. chartarum* (× 1000)

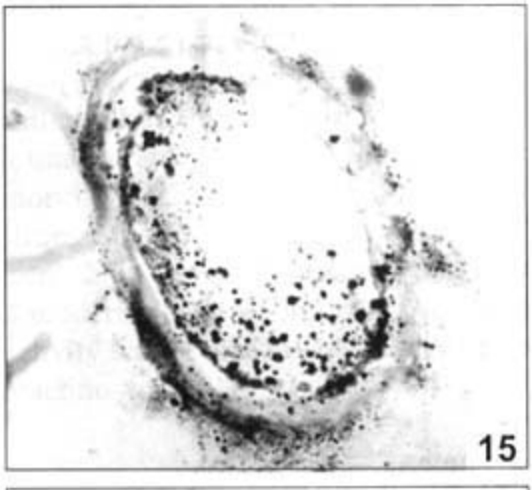
Fig. 10. A moderate activity of SDH in 31-day-old egg of *A. suum* (stage of L_2 larva) in experimental culture No. 2, with *S. chartarum* (× 1000)

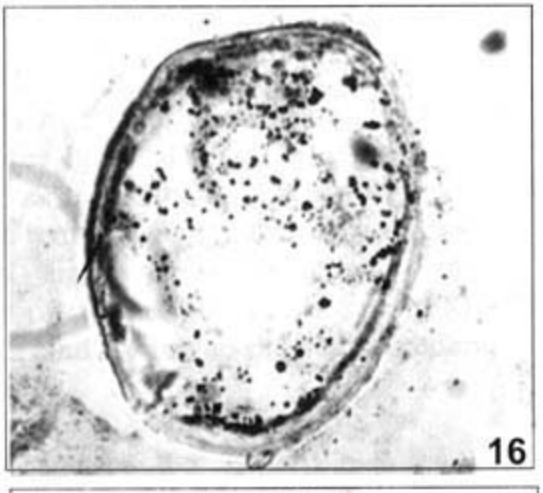
Fig. 11. A moderate activity of SDH in 8-day-old egg of *A. suum* (zygote stage) in experimental culture No. 3, with *P. frequentans* and *S. chartarum* (× 1000)

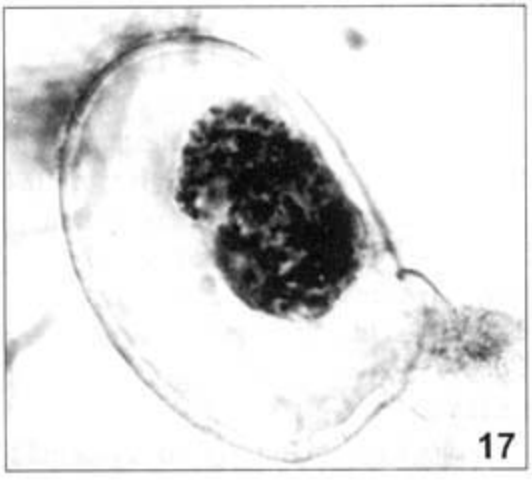
Fig. 12. A moderate activity of SDH in 25-day-old egg of *A. suum* (zygote stage) in experimental culture No. 3, with *P. frequentans* and *S. chartarum* (× 1000)











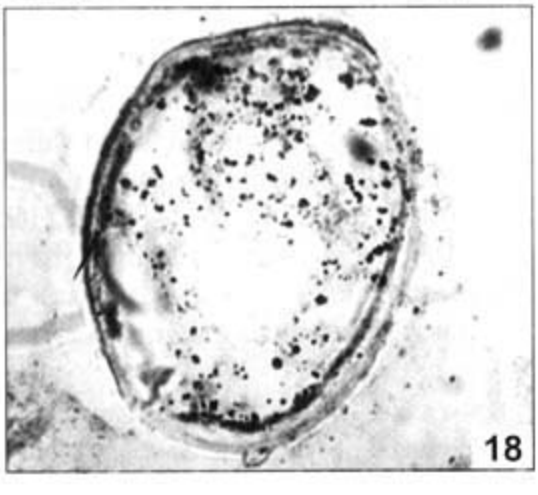


Plate III

Fig. 13. A moderate activity of LDH in 0-day-old egg of *A. suum* dissected directly from uterus (× 1000)

Fig. 14. A moderate / strong activity of LDH in 8-day-old egg of *A. suum* (cleavage stage) in control culture (× 1000)

Fig. 15. A moderate activity of LDH in 22-day-old egg of *A. suum* (stage of L2 larva) in control culture (× 1000)

Fig. 16. A weak activity of LDH in 22-day-old egg of *A. suum* (zygote stage) in experimental culture No. 1, with *P. frequentans* (× 1000)

Fig. 17. A strong activity of LDH in 22-day-old egg of *A. suum* (gastrula stage) in experimental culture No. 2, with *S. chartarum* (× 1000)

Fig. 18. A moderate activity of LDH in 35-day-old egg of *A. suum* (stage of L2 larva) in experimental culture No. 3, with *P. frequentans* and *S. chartarum* (× 1000)

Experimental culture No. 2, with *Stachybotrys chartarum*. Eggs of *Ascaris*, remaining up to day 14 of incubation at the stage of zygote, exhibited a moderate or strong reaction to SDH (Fig. 7). In 17-day-old cleavage eggs (2–4 blastomeres, up to morula) a strong reaction to SDH was recorded. At the stage of gastrula (22-day-old eggs) a strong or very strong reaction of the enzyme was observed in a form of numerous, coarse granules of the reaction product (Fig. 8). A distinct decline of the SDH-reaction to a moderate one was observed in sequential stages of the embryonic development – L_1 larva (day 25 of incubation) (Fig. 9) and L_2 larva (days 28 through 41 of culture) (Fig. 10).

Experimental culture No. 3 with *P. frequentans and S. chartarum*. The cytoplasm of *Ascaris* zygotes up to day 17 of culture showed moderate activity of SDH in a form of unevenly distributed formazan granules, most abundant below the egg shell (Fig. 11). At the stage of morula, reached by most of eggs on day 22 of incubations, a strong or very strong activity of SDH was observed. The remaining eggs, featuring anywhere from 2 to several blastomeres exhibited slightly weaker reaction – from a moderate to a strong one. At the stage of gastrula (day 25) a strong SDH activity was recorded (Fig. 12), whereas L_1 and L_2 larvae showed a moderate reaction to SDH.

Lactate dehydrogenase

Control culture. A weak activity of LDH, in a form of few fine formazan granules was observed in the cytoplasm of 0- and 2-day-old eggs of the nematode. The reaction product was visible mainly below the egg shell (Fig. 13). In early cleavage stages (from day 8 on) the cytoplasm of the blastomeres exhibited weak or moderate activity of LDH (Fig. 14). At the stage of morula a moderate reaction to LDH was observed, while at the stage of gastrula – a strong one. Enzyme activity decrease was stated in subsequent phases of the embryonic development – L_1 and L_2 larvae, showing a moderate reaction to LDH (Fig. 15).

Experimental culture No. 1, with *P. frequentans*. The stage of zygote in eggs of *Ascaris* incubated with *P. frequentans* was observed up to day 22. In this period a weak reaction to LDH – in a from of few fine granules of the reaction product unevenly distributed in the cytoplasm – was observed (Fig. 16). As late as on day 25 of incubation, most of eggs started their cleavage and the reaction to LDH in the blastomere cytoplasm was weak or moderate. Small increase in the of LDH reaction up to a moderate level was observed on days 28 and 31 of incubation, when the majority of eggs still remained at different stages of cleavage (from 2 blastomeres up to morula and blastula). Among 35- and 41-day-old eggs, in addition to the morula stage also gastrula stages were observed, featuring moderate or strong activity of LDH.

Experimental culture No. 2, with *S. chartarum*. Until day 14, the eggs of *Ascaris* at the zygote stage, showed a weak reaction to LDH in a form of few fine granules. On day 17 the cytoplasm of blastomeres in eggs in the cleavage phase exhibited a moderate activity of LDH. At the stage of gastrula (22-day-egg) a strong activity of LDH was stated (Fig. 17). On day 25 of incubation, L_1 larvae showed a decrease in the reaction ranging from a weak to moderate. At the stage of L_2 larva, a moderate LDH activity was observed.

Experimental culture No. 3, with *P. frequentans* and *S. chartarum*. Up to day 17 of incubation, a weak LDH activity was observed in the cytoplasm of *Ascaris* eggs. During the cleavage period, the LDH activity increased up to a moderate activity. At the stage of gastrula (day 25 of incubation) a moderate or strong reaction to LDH was stated, whereas in L_1 larvae (28-day-old eggs) – a weak or moderate one. L_2 larvae exhibited moderate activity of LDH (Fig. 18).

A comparison of activity changes of dehydrogenases studied (SDH and LDH) in the course of embryogenesis of *Ascaris suum* in control and experimental (Nos. 1, 2 and 3) cultures were shown on Figs 19 and 20.

DISCUSSION

The obtained results of histoenzymatic study demonstrated that incubation of eggs of *Ascaris suum* with *Penicillium frequentans* and with *Stachybotrys chartarum* caused changes in the activity of oxidative enzymes during embryogenesis of this nematode. Our study (Kuźna-Grygiel et al. 2001) revealed that the above-mentioned mould fungi caused, along with various morphological disorders, also retardation of the cleavage initiation and prolongation of sequential stages of the embryonic development of *A. suum*.

The study on the energy metabolism, carried out on an experimental model of *Ascaris lumbricoides* indicated that the developing eggs of this nematode represented aerobic metabolism consisting in lipid changes and transformations of triglycerides into carbohydrates (Passey and Fairbairn 1957). Developing eggs of *Ascaris* contained a complete set of glycolytic enzymes and enzymes of tricarboxylic acids (Bloom and Entner 1965; Barret and Beis 1975; Beis and Barrett 1975; Barrett 1976). The major respiratory substrate in early stages of embryogenesis are trehalose and glycogen. Later stages are dominated by lipid metabolism (Barrett 1981) and re-synthesis of glycogen from lipids occurs in the course of glyoxylate cycle and β-oxidation (Ward and Fairbairn 1970; Barrett et al. 1970; Lee and Atkinson 1976).

During embryonic development of *Ascaris* in control culture a small increase in reactions to the oxidative enzymes studied was observed during cleavage as well as a distinct increase of their activity during gastrulation. These facts indicate intensified oxidative metabolism (i.e. oxygen demand and utilization) mainly in the gastrulation period and the early phase of organo-

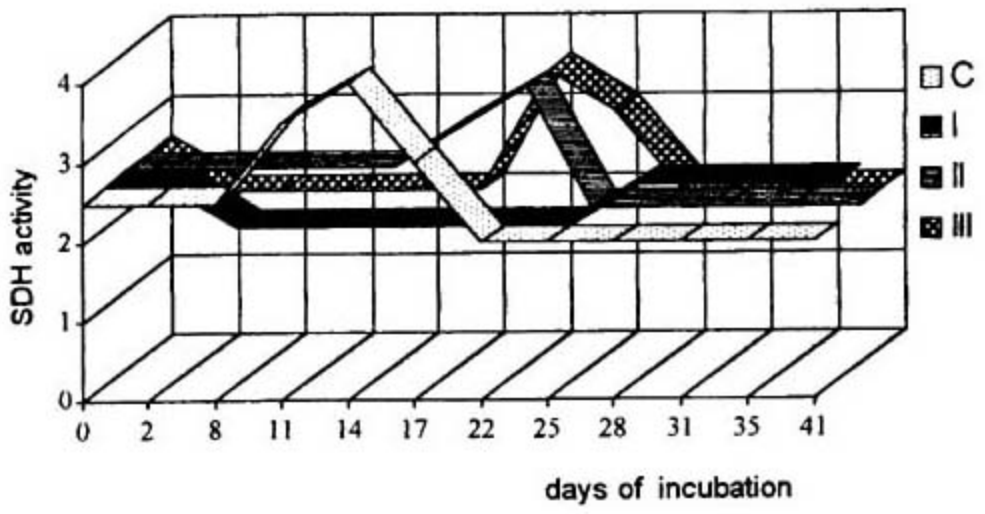


Fig. 19. SDH activity in the course of embryogenesis of *Ascaris suum* (C, control; I, incubation with *P. frequentans*; II, incubation with *S. chartarum*, III, incubation with *P. frequentans* and *S. chartarum*)

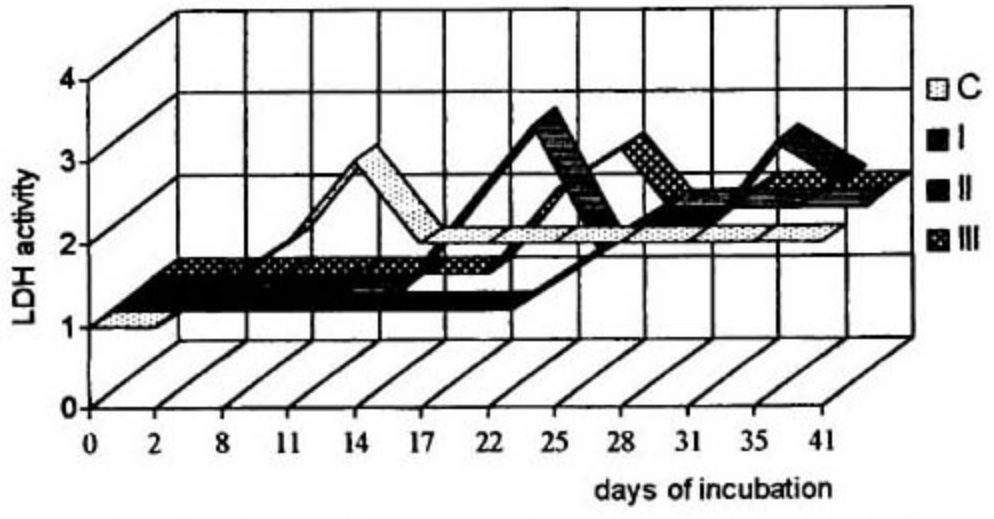


Fig. 20. LDH activity in the course of embryogenesis of *Ascaris suum* (C, control; I, incubation with *P. frequentans*; II, incubation with *S. chartarum*, III, incubation with *P. frequentans* and *S. chartarum*)

genesis, which is undoubtedly associated, among other things, with morphogenetic movements and processes of cellular differentiation occurring at that time. A similar dynamics of changes of oxidative enzymes activity was recorded in embryogenesis of *Toxocara canis* – another parasitic nematode (K o ł o d z i e j c z y k 1998, 1999).

Incubation of *A. suum* eggs with *P. frequentans* caused a small activity decrease of SDH as early as in the zygote stage and in early phases of cleavage, whereas at the stages of morula, blastula, and gastrula in particular, effect of *P. frequentans* on activity of this enzyme was distinctly inhibiting. In the case of

LDH, effect of this fungi species seems to be less significant-the activity of LDH in individual stages of embryonic development of *Ascaris* was similar as in control.

Stachybotrys chartarum similarly as *P. frequentans* delayed cleavage initiation, but in lesser extent it inhibited SHD activity. Only in the phase of gastrulation a small decrease in the activity of this enzyme was observed. Also LDH activity throughout the embryonic development showed small differences in relation to control, because only at the stage of L_1 larva a small activity decrease of LDH was observed.

In the experimental treatment studying the combined effect of *P. frequentans* and *S. chartarum* on energy oxidative metabolism of the nematode eggs, a small decrease of SDH reactions was observed at the stage of zygote (similarly as in experimental culture No. 1) and throughout gastrulation. The observed, gradual increase of activity of this enzyme during cleavage, with a peak in the period of gastrulation, was lower, however, than in control. Also, a small decrease of LDH activity observed in the L_1 stage (similarly as in culture No. 2 with *S. chartarum*) can indicate a certain synergistic action of those two fungi species on the oxidative metabolism of the nematode. The latter metabolism, in this experimental treatment, was expressed in lesser extent because of only half the amount of secondary metabolites. As it has been demonstrated by numerous experiments, the dose of aflatoxin plays a decisive role in its toxicity (S e ń c z u k 1999).

Comparing the activity of the studied oxidoreductive enzymes – lactate dehydrogenase (LDH) and succinate dehydrogenase (SDH) – markers of glycolysis and the Krebs cycle (Figs 19 and 20), it is evident that in the course of *A. suum* embryogenesis, the metabolites of the studied species of mould fungi cause, in particular, a decrease in SDH activity. In addition, the present results give a sound evidence on the inhibiting action of mycelia of *Penicillium frequentans* on the SDH activity in the course of *A. suum* embryogenesis.

The earlier-mentioned antagonistic effects on eggs of *Ascaris* were most probably caused by the secondary metabolites of *P. frequentans* and *S. chartarum* described as mycotoxins (aflatoxins, frequentin, stachybotrytoxin) (P i o n t e k 1999). It has been known that aflatoxin *in vitro* inhibits synthesis of DNA and RNA in nuclei of hepatocytes, which in turn leads to disorders in the synthesis of enzymatic proteins (A l e k s a n d r o w i c z 1970). These facts can explain the retardation of the cleavage and on the other hand the inhibition of SDH activity throughout embryonic development of the large pig roundworm.

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Wpływ *Penicillium frequentans* i *Stachybotrys chartarum* na metabolizm oddechowy rozwijających się jaj *Ascaris suum*

S t r e s z c z e n i e

Przy użyciu metod histoenzymatycznych określono wpływ saprotroficznych grzybów glebowych *Penicillium frequentans* i *Stachybotrys chartarum* na metabolizm oddechowy w czasie embriogenezy pasożytniczego nicienia *Ascaris suum*.

Na podstawie histochemicznej oceny aktywności enzymów glikolizy (dehydrogenaza mleczanowa) (LDH) oraz cyklu kwasów trójkarboksylowych (dehydrogenaza bursztynianowa) (SDH) stwierdzono zmiany w tlenowym metabolizmie energetycznym rozwijających się jaj *A. suum*.

Z testowanych gatunków grzybów – głównie wpływ *P. frequentans* powodował zaburzenia w procesach oddychania komórkowego, które manifestowały się spadkiem aktywności SDH w okresie gastrulacji. Inkubacja jaj *Ascaris* wraz z grzybnią *S. chartarum* oraz razem z *P. frequentans* wpływała w niewielkim stopniu na upośledzenie metabolizmu oddechowego nicienia.