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Istituto di Nematologia Agraria, C.N.R. - 70126 Bari, Italy

RESPIRATION AND TERMINAL OXIDASES OF *XIPHINEMA INDEX*

by
S. Molinari and C. Miacola

Summary. Active specimens of *Xiphinema index* showed a respiratory activity markedly influenced by the pH of the assay mixture. The optimum for nematode respiration was pH 7. Sodium azide severely inhibited the oxygen uptake of the nematodes but it did not kill them. Homogenates of nematodes actively oxidized succinate and ascorbate. Sodium azide showed a partial inhibition on both these oxidations. Ascorbate oxidation, after the addition of sodium azide, was sensitive to inhibition by the hydroxamic acid *m*-CLAM and the enzyme SOD. Conversely, duroquinol oxidation was inhibited by the sole *m*-CLAM whilst SOD showed very little effect. LDH activity was found in *X. index* homogenates to be 3-fold higher than in *Meloidogyne hapla* and *Heterodera schachtii* homogenates.

Reports in the literature on the respiration of plant parasitic nematodes are scarce (Rhode, 1971). Because of the difficulty of detecting oxygen uptake by small juvenile nematodes, investigations on their respiratory activity have been rare (Reversat, 1977). In general, it is well known that cells of soil-inhabiting nematodes possess an efficient aerobic metabolism which has characteristics similar to those of mammalian cells and which is well adapted to overcome periods of low oxygen regimes (Atkinson, 1980). Moreover, properties which are specific to plant cell respiration such as the presence of hydroxamic acid-sensitive terminal oxidases and a certain resistance of respiration to cyanide inhibition have been reported in *Aphelenchus avenae* (Mendis and Evans, 1984a; Navas *et al.*, 1992). This study, for the first time, describes the respiratory activity of plant ectoparasitic nematodes such as *Xiphinema* spp., and, with a comparative approach, it also focuses on the biochemical mechanisms by which nematodes adapt the oxidative metabolism to their own type of parasitism and life environment.

Materials and methods

Xiphinema index Thorne *et* Allen were obtained from a culture maintained on fig in a glasshouse. Nematodes were extracted by the Cobb wet sieving technique. Juveniles (J_2) of *Meloidogyne hapla* Chitw. were obtained by incubating at 27 °C egg masses from a culture maintained on tomato. Juveniles (J_2) of *Heterodera schachtii* Schmidt were obtained by hatching cysts from roots of sugarbeet in 0.6 mM sodium metavanadate at 20 °C.

Samples of *X. index* generally consisted of 40-60 hand-picked nematodes in 0.5 ml of 0.1 M potassium phosphate buffered at pH 5, 6, 7 and 8. Suspensions of *M. hapla* and *H. schachtii* were filtered through 1 µm pore-sized filters (Whatman, cellulose nitrate). Filters were washed with 0.5 ml of 0.1 M potassium phosphate, pH 7.0, in order to obtain highly concentrated nematode suspensions.

For respiration assays of live *X. index* 0.5 ml of nematode suspensions in 0.1 M potassium phosphate buffers at various pH were trans-

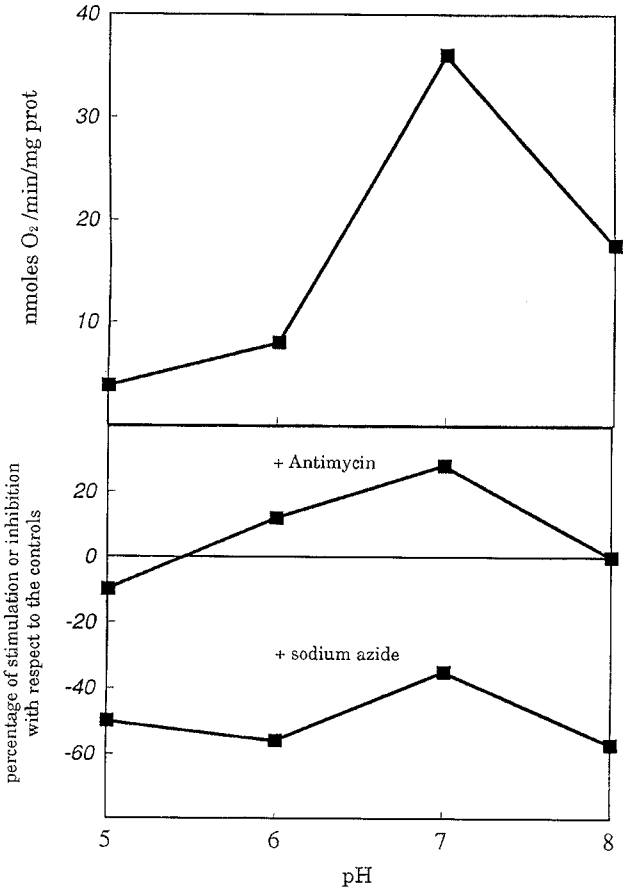

Fig. 1 - Oxygen uptake of active specimens of *Xiphinema index* at different pH. Assay medium was 0.1 M potassium phosphate (0.5 ml) at pH 5, 6, 7, 8. Values are expressed as nmoles O_2 per min per mg of total protein measured after the homogenization of each sample. Antimycin (5 µg) and sodium azide (10 mM) were added to test the effect on respiration.

ferred to a Clark type oxygen electrode chamber (Rank Bros., Cambrige, UK), and oxygen uptake monitored at 30 °C. When a constant rate of respiration was established, respiratory inhibitors such as antimycin (10 µg/ml) and sodium azide (10 mM) were added. After the respiration assay nematodes were recovered, rinsed in distilled water, filtered through a 5 µm pore-sized filter (Whatman, cellulose nitrate) and suspended in a minimum volume of a grinding medium consisting of 0.3 M sucrose, 10 mM $MgCl_2$, 1 mM EDTA and 10 mM TES, pH 7.4. The suspension of nematodes was then homogenated in a chilled 2 ml glass mortar. Rates of respiration of active juveniles are expressed as nmoles min^{-1} mg^{-1} of total proteins detected in these homogenates. When measurements required the integrity of cell membranes, freshly collected nematodes were gently comminuted in a glass mortar and pestle and the homogenate immediately used for the assays. In other experiments homogenization of the nematodes was done with a pestle connected to a rotor, the speed of pestle rotation and the time for homogenization determined when rough particles were no more present in the homogenate by observing it under a light microscope. The homogenates were clarified by means of centrifugation at 5000 rpm for 3 min in a bench centrifuge. Some of the samples were stored overnight at 4 °C before the analysis while others were frozen to test the effect of freezing and thawing on the enzyme activities tested. These clarified samples were defined as crude fractions.

Respiration assays of fresh homogenates were carried out in the grinding buffer described above. Respiration was maintained by adding substrates oxidable by mitochondria such as succinate (10 mM) or ascorbate (10 mM). When succinate was used as substrate, 10 µg/ml rotenone and 0.5 µM of the uncoupler of mitochondrial oxidative phosphorilation CCCP (Carbonyl Cyanide *m*-Chloro Phenylhydrazone) were added to the assay mixture to inhibit the production of oxalacetate (a strong inhibitor of succinate dehydrogenase) and to stimulate the oxygen uptake, respectively. TMPD (0.1 mM) (N,N,N,N-Tetramethyl-*p*-phenylendiamine) was previously added in ascorbate oxidation to promote the reduction of mitochondrial cytochrome oxidase.

Oxygen uptake due to ascorbate and duroquinol oxidase activities of crude fractions was monitored in 0.5 ml grinding medium by add-

ing 10 mM ascorbate and 0.1 mM duroquinol, respectively. Sodium azide (10 mM), SOD (10 µg/ml) (superoxide dismutase) and *m*-CLAM (1 mM) (*m*-chlorobenzhydroxamic acid) were tested as inhibitors of these activities.

Lactate dehydrogenase (LDH) activity was detected in a 557 Perkin-Elmer double-beam spectrophotometer. The final assay mixture (570 µml) consisted of 10 mM lactate, 2 mM NAD^+ and approximately 50 µg protein in 0.1 M sodium phosphate buffer, pH 7.0. Wavelength was set at 340 nm ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$).

The protein content of each sample was determined by the Lowry procedure (Lowry *et al.*, 1951) with bovine serum albumin as the standard.

Experiments were carried out with homogenates obtained from 15 different lots of nematodes. Protein content of homogenates was in the range of 0.1-0.4 mg/ml.

Results

Respiratory activity of active specimens of *X. index* was much higher at pH 7 and 8 compared with that at pH 5 or 6 (Fig. 1). Generally, antimycin, which is an inhibitor of mitochondri-

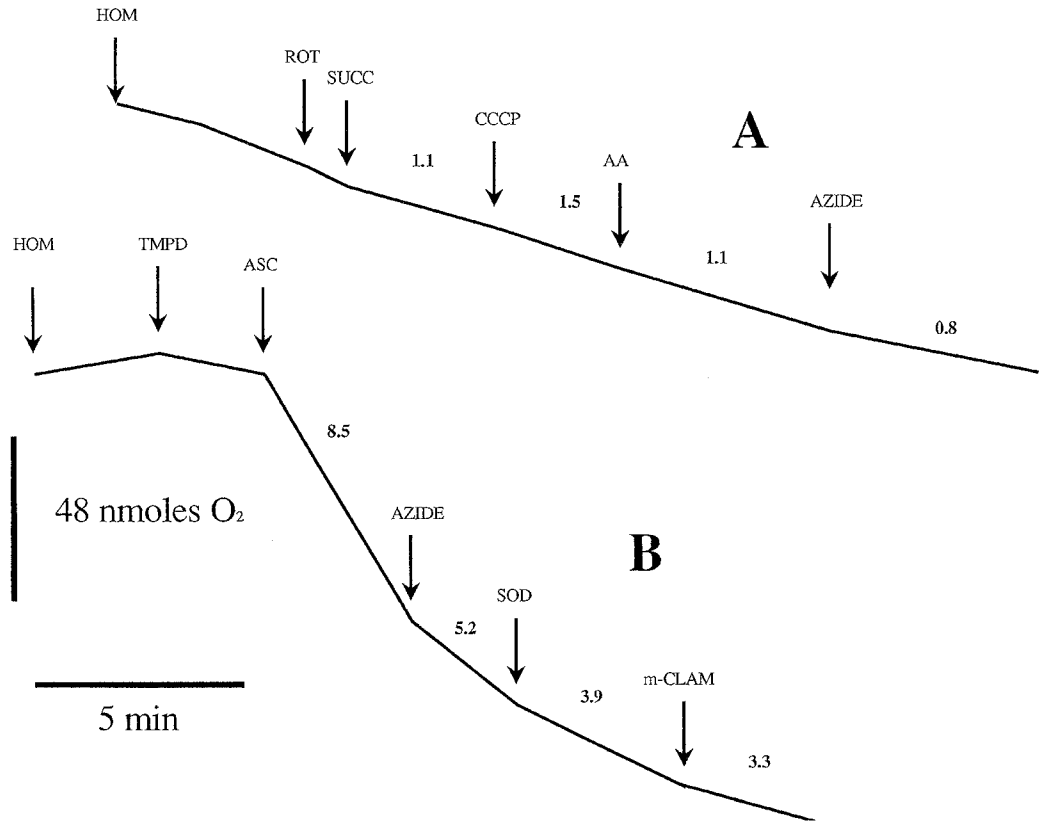

Fig. 2 - Oxygen uptake of fresh homogenates of *X. index* oxidizing 10 mM succinate (A) and 10 mM ascorbate (B). Values are expressed as nmoles O_2 per min per ml of assay medium. Homogenates contained about 50 µg prot. Concentrations of the chemicals added are as follows: (A),10 µg/ml rotenone (ROT), 0.5 µM CCCP, 10 µg/ml antimycin (AA), 10 mM sodium azide (AZIDE); (B), 0.2 mM TMPD, 10 mM sodium azide, 10 µg/ml superoxide dismutase (SOD), 1 mM *m*-CLAM.

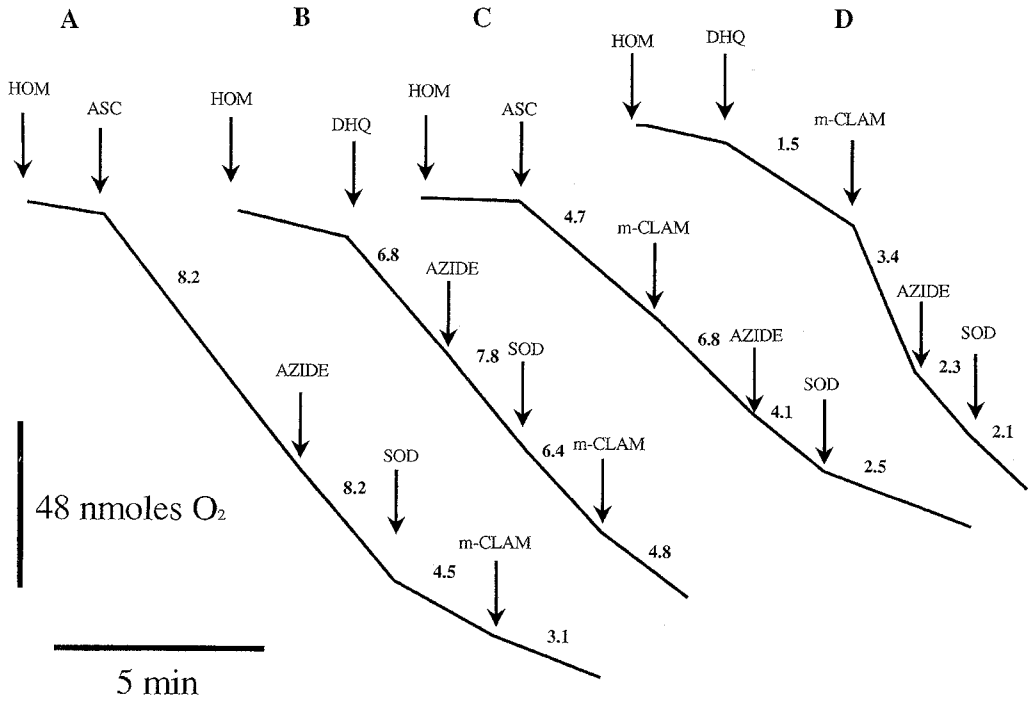

Fig. 3 - Oxygen uptake of crude fractions of *X. index* stored overnight at 4 °C (A, B) or frozen and thawed (C, D). Duroquinol (O.1 mM) oxidation is shown in B and D. Concentrations of the other chemicals added are as described in fig. 2.

al electron transport chain, had no or a slightly stimulating effect on respiration of living nematodes. Sodium azide, which is an inhibitor of mitochondrial cytochrome oxidase, strongly inhibited the respiration of the juveniles at any of the pH values assayed.

Gentle homogenization of freshly collected *X. index* juveniles was followed by immediate monitoring of the oxygen uptake of the homogenate in order to save membrane integrity, thus allowing the detection of respiration in which intact mitochondria were involved (Fig. 2). Samples obtained by this procedure were able to oxidize succinate. The occurrence of this oxidation, which is specifically mitochondrial, as well as its stimulation when the uncoupler of the mitochondrial oxidative phosphorilation CCCP was added, confirmed the involvement of mitochondria in the oxygen uptake observed. Antimycin and sodium azide actually inhibited the oxygen uptake by about 40% and 30%, respectively (Fig. 2A). Taking into account that 0.5-0.6 nmoles O_2 per min per ml is the aspecific oxygen consumption of the assay system, the association of the inhibitors of mitochondrial respiration, antimycin and sodium azide, blocked 70-80% of the uncoupled succinate oxidation by these nematode homogenates. Ascorbate oxidation in the presence of TMPD was almost halved by the addition of sodium azide (Fig. 2B). SOD, which is a very active scavenger of superoxide radicals ($O_2^{\cdot}$), and *m*-CLAM, which is a strong inhibitor of the alternative oxidase found in plant mitochondria (Molinari, 1991) and can also act as a radical scavenger and a peroxidase inhibitor, further decreased the oxygen uptake left after the addition of sodium azide.

Additional samples of *X. index* juveniles were ground at a very high speed of pestle ro-

tation and then centrifuged. Some of these crude fractions were left overnight at 4 °C whilst some others were frozen and then thawed before measurements were made. Ascorbate and duroquinol oxidase activities of these fractions were monitored in a polarographic assay (Fig. 3). Frozen samples (Fig. 3C-D) had an oxygen uptake much lower than samples left overnight in the refrigerator (Fig. 3A-B) with both ascorbate and duroquinol as substrates. Sodium azide had no inhibitory effect on ascorbate oxidase activity in this assay (Fig. 3A); SOD had a similar inhibitory effect whilst *m*-CLAM doubled its ability to inhibit ascorbate oxidation with respect to data shown in Fig. 2B. Fig. 3B shows that duroquinol oxidation was not susceptible to inhibition by sodium azide and scarcely susceptible to inhibition by SOD; conversely, *m*-CLAM was a good inhibitor of the activity in the presence of both sodium azide and SOD. Surprisingly, *m*-CLAM, when added before sodium azide, markedly stimulated the oxygen uptake with both the substrates used (Fig. 3C-D). In this case, the oxygen uptake of both ascorbate and duroquinol oxidation was inhibited by sodium azide which did not show the same property when *m*-CLAM was not previously present in the assay medium (Fig. 3A-3B). Generally, SOD was an inhibitor of ascorbate oxidation whilst it did not show the same effect on duroquinol oxidation.

Homogenates of *X. index*, *M. hapla* and *H.*

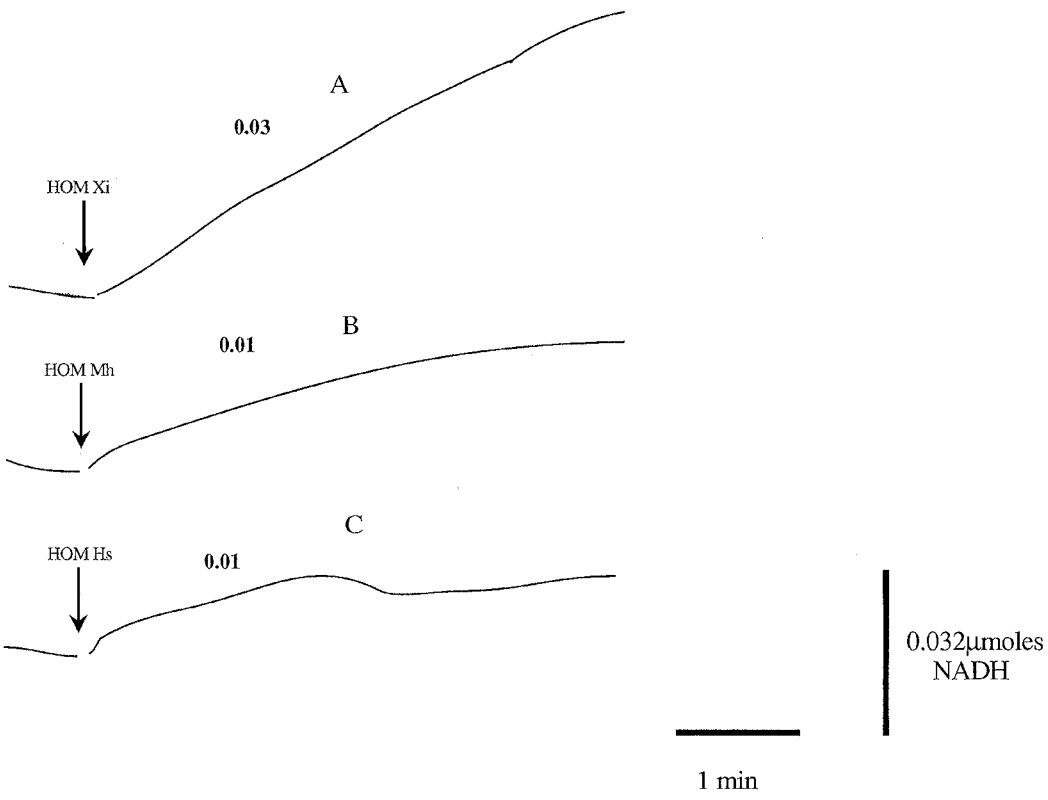

Fig. 4 - Spectroscopic measurements of lactate dehydrogenase activity in homogenates of *X. index* (Xi), *Meloidogyne hapla* (Mh) and *Heterodera schachtii* (Hs). Reaction mixture consisted of 10 mM lactate, 2 mM NAD^+, approx. 50 µg prot in 0.1 M sodium phosphate buffer, pH 7.0. Values are expressed as µmoles of NADH produced per min per mg prot. Wavelength was set at 340 nm (ε = 6.22 mM^{-1} cm^{-1}).

schachtii were able to oxidize lactate, producing NADH from NAD^+ (Fig. 4). Specific lactate dehydrogenase activity was found to be 3-fold higher in *X. index* than in *M. hapla* and *H. schachtiiI.*

Discussion

This paper presents preliminary data on the characteristics and properties of the respiratory activity of the ectoparasitic nematode *X. index in vitro.* Calculation of the oxygen uptake of active *X. index* juveniles based on total protein content of the relative homogenates was considered more accurate and appropriate than calculation based on fresh or dry weight of the nematodes. Respiratory activity of *X. index* seems to be repressed by acidic media; the increase of one unit of pH, from 6 to 7, resuted in an increase of oxygen uptake of approximately 5-fold. Oxygen uptake of second-stage juveniles of *Heterodera oryzae* increased by only 12% when the pH of the respiration buffer was increased from 5 to 8 (Reversat, 1977). Antimycin did not inhibit respiration of living nematodes which may simply result from the difficulty of a large molecule, such as antimycin, entering the thick cuticle of the nematode. Antimycin also had little effect also on the respiration of several populations of *Aphelenchus avenae* (Navas *et al.*, 1992). Conversely, sodium azide severely affected the respiration of the juvenile *X. index.* When recovered after treatment, nematodes appeared motionless but after rinsing with fresh water their movements were restored. This indicates that the aerobic metabolism supporting muscle contraction was totally suppressed by sodium azide although a putative alternative aerobic pathway, responsible of the residual oxygen uptake observed, and/or an anaerobic metabolism must be active to furnish the minimal energy necessary for surviving.

Two types of sample preparation were undertaken in the homogenization procedure: the first one was intended to preserve the integrity of mitochondria by immediately analyzing the fresh homogenates; the second did not preserve mitochondrial integrity since terminal oxidase activities were detected in the resulting crude fractions.

Respiration of fresh homogenates, when succinate was provided as the specific substrate, was suppressed by 70-80% of the oxygen uptake in the presence of antimycin and sodium azide. When ascorbate and TMPD were used as substrate, sodium azide suppressed only about 40% of the oxygen uptake; an additional 20% was suppressed by SOD and *m*-CLAM. A similar partial inhibition by azide on ascorbate/TMPD oxidase activity was obtained by Mendis and Evans (1984b) with *Apelenchus avenae.*

m-CLAM was found to be a good inhibitor of ascorbate and duroquinol oxidations by *X. index* homogenates, thus suggesting the presence of hydroxamic acid-sensitive terminal oxidases in this plant parasitic nematode. Such a presence has been already proposed in the free living nematode *Aphelenchus avenae* (Mendis and Evans, 1984a). Furthermore, generation of superoxide radicals is evident from the inhibition by SOD of ascorbate oxidation in mitochondrial and non mitochondrial-type samples. On the contrary, duroquinol oxidation did not show this same characteristic. The presence of SOD in homogenates of *X. index* has been already detected (Molinari, personal communication). As evidence is emerging on the capability of oxygen radical generation by plant parasitic nematodes, the issue of an enzymatic system required to cope with the oxidative stress caused by this production must be considered. It should be noted that the degree of activity of this system would also determine, in the nematode, the opportunity of defence against the oxygen radicals produced by the attacked plants. Experiments will be done to ascertain the presence of a O_2^{-0} generating NAD(P)H oxidase similar to that found in membrane fractions of tomato roots (Molinari, 1994).

Rates of ascorbate and duroquinol oxidation were lowered by freezing and thawing crude fractions of nematode homogenates thus suggesting a possible involvement of membrane bound enzymes in these oxidations. For instance, when tomato root microsomes were exposed to this treatment, $O_2^{\ominus}$ generating NAD(P)H oxidase activity was completely inhibited (Molinari, 1994).

It is generally recognized that plant parasitic nematodes are aerobic organisms but they are able to survive periods of partial or complete anaerobiosis. Energy production in anaerobic conditions is related to the reoxidation of NADH by lactate dehydrogenase (LDH) in the cytoplasm of cells. There are only old and negative reports on the production and excretion of lactate by plant parasitic nematodes (Myers and Krusberg, 1965). On the other hand, LDH has been detected in soil-inhabiting nematodes such as *Aphelenchus avenae* and *Caenorhabdtis* spp. (Cooper and Van Gundy, 1971). Data presented in this paper reveal the presence of such activity in *X. index* which has been found to be much higher than that of juveniles of endoparasitic nematodes such as *M. hapla* and *H. schachtii*. This finding might be related to the different type of parasitism of *X. index*: ectoparasites spend their life span in the soil, which is an environment with a highly fluctuating oxygen content, compared with the more constant oxygen content of root tissues where endoparasites live and develop. Thus it would appear likely that *X. index* frequently undergoes periods of anaerobiosis and develops a more active anaerobic metabolism in which LDH certainly plays a key role.

Literature cited

ATKINSON H. J., 1980. Respiration in Nematodes. pp. 101-142. *In Nematodes as Biological Models* (B. M. Zuckerman ed.), Academic Press, New York.

COOPER A. F. and VAN GUNDY S. D., 1971. Ethanol production and utilization by *Aphelenchus avenae* and *Caenorhabdtis* sp. *J. Nematol., 3*: 205-214.

LOWRY O. H., ROSEBROUGH N. J., FARR A. L. and RANDALL R. J., 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem., 193*: 265-275.

MENDIS A. H. W. and EVANS A. A. F., 1984a. Substrates respired by mitochondrial fractions of two isolates of the nematode *Aphelenchus avenae* and the effects of electron transport inhibitors. *Comp. Biochem. Physiol., B (Comp. Biochem.I),* 78: 373-378.

MENDIS A. H. W. and EVANS A. A. F., 1984b. First evidence for the occurrence of cytochrome-*o* in a free living nematode. *Comp. Biochem. Physiol., B (Comp. Biochem.), 78*: 729-735.

MOLINARI S., 1991. Role of alternative pathway respiration in tomato roots attacked by *Meloidogyne incognita*. *Ann. Appl. Biol., 119*: 373-379.

MOLINARI S., 1994. Regulation of the superoxide anion generation in tomato roots susceptible and resistant to nematodes. *Nematol. medit., 22*: 69-74.

MYERS R. F. and KRUSBERG L., 1965. Organic substances discharged by plant-parasitic nematodes. *Phytopathology, 55*: 429-437.

NAVAS A., MOLINARI S., NOMBELA G. and MUNDO-OCAMPO M., 1992. Relationship between population structure and respiration activity in *Aphelenchus avenae*. *Nematol. medit., 20*: 105-112.

REVERSAT G., 1977. Influence of some external factors on the rate of oxygen uptake by second stage juveniles of *Heterodera oryzae*. *Nematologica, 23*: 369-381.

RHODE R. A., 1971. Respiration. pp. 235-246. *In Plant Parasitic Nematodes. Vol. II Cytogenetics, Host-Parasite Interactions, and Physiology* (P. M. Zuckerman, W. F. Mai, R. A. Rhode eds), New York, Academic Press.

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