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BASIDIOMYCETE-BASED METHOD FOR BIOCONTROL OF PHYTOPATHOGENIC NEMATODES

Tiberius BALAEŞ[1*], Leonard BOROŞ[2], Tatiana Eugenia ŞESAN[3], Cătălin TĂNASE[4]

Abstract: Phytopathogenic nematodes represent one of the most important groups of pathogens in crops. The use of chemical to control the nematodes attack in crops is decreasing every year due to the concern of the toxicity and side effects of such compounds. In the course for finding alternatives to the use of chemicals, biological control of nematodes is gaining much attention. Some saprotrophic fungi are able to feed on invertebrates, thus becoming efficient agents of control. In this study, three species of basidiomycetes were analyzed for their potential to be used as control agents of phytopathogenic nematodes. Through on *in vitro* investigation of these potential, one strain – *Gymnopilus junonius* was further selected for a pot test against *Meloidogyne incognita*, a very important phytopathogenic species of nematodes. The fungal treatment strongly decreased the *M. incognita* population on the tested pots, proving the potential of *G. junonius* strain to be used in biocontrol.

Keywords: biocontrol, phytopathogenic nematodes, *Meloidogyne incognita*, nematophagous fungi, *Gymnopilus junonius*

Introduction

The nematodes are primarily aquatic organisms, or organisms that develop in humid soils, in many types of habitats. Most of the nematodes have microscopic dimensions (0.3–3.0 mm). The soil is very rich in nematodes, representing approximatively 26% of all the invertebrates genera [WHARTON, 1986]. The root knot nematodes from the genus *Meloidogyne* is one of the most worldwide-spread group of plant parasites that affect most of the crops [PERRY & MOENS, 2009; BOROŞ & al. 2015], being obligate-parasites. The use of nematicidal compounds have significantly decrease the *Meloidogyne* populations sizes, but due to the strong toxicity and side-effects of these chemical, many of nematicidal compounds have been banned for commercializing [RAVICHANDRA, 2010]. For this reason, new types of treatment for controlling the phytopathogenic nematodes are required, and the biological ones are the most promising.

Lignicolous saprotrophic fungi degrade dead wood, but can also use other substrates as an alternative source of nutrients. In this respect, many species of lignicolous basidiomycetes capture and consume invertebrates for an increase uptake of nitrogen and phosphorous [DIX & WEBSTER, 1995].

[1] "Alexandru Ioan Cuza" University of Iaşi, "Anastasie Fatu" Botanical Garden, Dumbrava Rosie Street, No. 7-9, Iaşi – Romania.
[2] Regional Laboratory of Nematology, Phytosanitary Agency, 47 Lânii Street, 500465, Braşov – Romania.
[3] University of Bucharest, Faculty of Biology, 36-46 M. Kogalniceanu Boulevard, Bucharest – Romania.
[4] "Alexandru Ioan Cuza" University of Iaşi, Department of Biology, Bd. Carol I, No. 20 A, Iaşi – Romania.
[*] Corresponding author. E-mail: tiberius.balaes@uaic.ro

Although most of the studies on biocontrol of nematodes involved imperfect nematophagous specialized fungi [YANG & al. 2007], the effectiveness of these species is limited due to their reduced development in agricultural soils. In this respect, the basidiomycete species appear to be better adapted to agricultural environment, many of these species having the ability of colonizing nematodes [KUMAR & KAVIYARASAN, 2012; LI & al. 2007; TRUONG & al. 2007; TZEAN & LIOU, 1993]. The involved mechanisms are either biological, based on specialized structures used for actively capturing nematodes [LUO & al. 2004; KARASIŃSKI, 2013; TRUONG & al. 2007] or biochemical ones, with the secretion of nematicidal compounds [DONG & ZHANG, 2006; LIU & al. 2008].

When nutrients are available in large quantities, the carnivory of fungal species involved do not occur. However, in the agricultural soils, the characteristic substrata for lignicolous fungi are missing, but the mycelium develops in vegetative form, using different organic compounds as sources of nutrients. In a previous study concerning the potential of basidiomycetes in biocontrol of nematodes (*in press, corrected proof*) we have tested 68 strains of basidiomycetes for selection of strains with high potential of capturing / killing nematodes. Three saprotrophic species of fungi – *Gymnopilus junonius*, *Fomitopsis pinicola* and *Daedalea quercina* proved a strong efficiency in colonizing nematodes cadavers when the medium was low in nutrients.

In the present study, we have analyzed three species o lignicolous basidiomycetes for their potential as biological control agents. The most promising strains, *G. junonius* was selected for further investigations against phytopathogenic nematodes – *Meloidogyne incognita.* The obtained data suggest a biochemical mechanism involved, as *Gymnopilus junonius* produces gymnopillins, gymnoprenol and related products [KUSANO & al. 1986] with various biological effects, such as antibacterial [AL-FATIMI & al. 2013], antimycotic [VAHIDI & al. 2006] and cytotoxicity against different types of animal cells [KAYANO & al. 2004; TOMASI & al. 2004].

Our results proved a good potential of *G. junonius* strain for the production of nematicidal compounds, opening new perspectives in biocontrol of phytopathogenic nematodes.

Materials and methods

Fungal strains and nematodes

The tested fungal strains: *Daedalea quercina*, *Fomitopsis pinicola* and *Gymnopilus junonius*, previously isolated from fruit bodies [BALAEŞ & TĂNASE, 2012] belong to the Culture Collection of Fungal Research Laboratory (RECOSOL), Faculty of Biology, Alexandru Ioan Cuza University of Iasi. The nematode species – *Steinernema feltiae* was purchased as a commercial available product, Entonem® (Koppert B.V, The Netherlands). The product consists in a powder with juveniles at the same stage of development. A suspension of approximatively 10,000 individuals per milliliter was prepared in distilled sterile water and used for inoculation. The phytopathogenic nematodes – *Meloidogyne incognita*, was obtained from the collection of Regional Laboratory of Nematology – Brasov, Romania [BOROŞ & al. 2015]. The juvenile nematodes (second stage, J_2) were extracted from tomatoes roots of 12 weeks old and treated with 1% streptomycin sulfate for 3 minutes.

Media and cultures

All the media have been prepared using reagents of analytical grade. An organic medium was used for preparation the inoculum used in all experiment (L^{-1}): glucose 8 g, malt extract 20 g, yeast extract 2 g, peptone 2 g, agar 15 g. Except when stated, no other minerals have been added to the media composition. Sterilization of media was done using a 75 liters upright model autoclave (Raypas, Barcelona, Spain), at 120 ºC. The pH was adjusted at value 5.5, with hydrochloric acid 0.1 M or potassium hydroxide 0.1 M, using an electronic pH/ion-meter (model INOLAB, WTW, Weilheim, Germany).

Influence of carbon and nitrogen sources. For testing the influence of the carbon and nitrogen sources over the colonization of nematodes by the fungal mycelium, eight types / concentrations of carbon sources (C1-C8) and seven for nitrogen sources (N1-N7) have been used (Tab. 1) in solidified media (15 g agar L^{-1}).

Tab. 1. Composition of nutrient media used for screening of C and N sources

Media	C (g L^{-1})	N (g L^{-1})	Mycelium development (cm radius)		
			D. quercina	*F. pinicola*	*G. junonius*
C1	sucrose (2)	peptone (0.5)	1.7	1.8	2.2
C2	maltose (2)	peptone (0.5)	4.0	4.1	3.2
C3	sorbitol (2)	peptone (0.5)	1.6	3.0	1.6
C4	starch (2)	peptone (0.5)	2.4	4.5	3.1
C5	glucose (1)	peptone (0.5)	3.0	4.5	2.4
C6	glucose (3)	peptone (0.5)	4.5	4.1	4.1
C7	glucose (5)	peptone (0.5)	4.5*	4.2	4.5
C8	glucose (7)	peptone (0.5)	4.5*	4.2	4.5
N1	glucose (2)	peptone (0.5)	4.0	4.1	4.0
N2	glucose (2)	peptone (1)	4.1	4.2	4.1
N3	glucose (2)	peptone (2)	2.3	4.0	3.1
N4	glucose (2)	peptone (4)	2.2	3.1	2.4
N5	glucose (2)	ammonium sulphate (0.5)	4.1	3.7	4.0
N6	glucose (2)	urea (0.5)	3.5	4.0	2.4
N7	glucose (2)	sodium nitrate (0.5)	4.0	3.7	4.5

*the mycelium reached the edge of plate prior to analysis, and develop a very thick net

Influence of nutritive salts. The influence of salts, has been tested both on solid (15 g agar L^{-1}) and liquid media, containing 2 g of glucose and 0.5 g of peptone L^{-1} for solid media (S1-S3) and for first variant of liquid medium (S4) and respectively 4 g of glucose and 1 g of peptone L^{-1} for second variant of liquid media (S5). Five variants of media have been used (L^{-1}): **S1** 2 g KH_2PO_4 + 1 $MgSO_4 \cdot 7H_2O$; **S2** 1 g KH_2PO_4 + 0.5 $MgSO_4 \cdot 7H_2O$; **S3** 0.5 g KH_2PO_4 + 0.1 $MgSO_4 \cdot 7H_2O$; **S4** 1 g KH_2PO_4 + 0.5 $MgSO_4 \cdot 7H_2O$ + 0.1 $FeSO_4 \cdot 7H_20$ + 0.1 $NaNO_3$; **S5** 1 g KH_2PO_4 + 0.5 $MgSO_4 \cdot 7H_2O$ + 0.1 $FeSO_4 \cdot 7H_20$ + 0.1 $NaNO_3$. The test tubes with liquid media were shaken at 150 rpm.

Influence of metals. The influence of metals was tested only using *G. junonius*, on liquid media (L^{-1}: 2 g glucose, 0.5 g peptone, 1 g KH_2PO_4, 0.5 $MgSO_4 \cdot 7H_2O$) supplemented with sulfates of heavy metals: Cu^{2+}, Mn^{2+}, Fe^{2+} and Zn^{2+}, in concentration of 1 mM and 2 mM respectively. The cultivation was performed in tubes of 10 mL, with 2 mL working

volume, and the addition of nematodes have been done after 7 days of incubating fungi. The control consisted in fungi-free medium with nematodes.

Influence of inoculum quantity. To assess the optimum quantity of inoculum, for the colonization / nematicidal effect to occur, and for establishing economical variants of media for fungal cultivation, three types of media have been used (L^{-1}): A: 2 g glucose + 0.5 g peptone; B: 2 g sucrose + 0.5 malt extract; C: 10 g sucrose + 5 g malt extract. The sucrose and malt extract used, were cheap supplements acquired from commerce, as food sugar for human consumption and malt extract for beverages. Nematodes were added as a suspension of 0.1 mL, containing 1000 individuals. In order to keep the pH at an approximately constant value, a lactate buffer of 0.05 M, 5.5 pH was used. Quantities of inoculum from 0.1 to 1 mL were used.

Each sample / combination on Petri dishes were made in three replicate, while on test tubes five replicates were used for each. The plates / test tubes have been incubated in the dark at 25 ºC, for 7 days before suspensions with nematodes being added.

The inoculation of Petri dishes have been achieved using agar plugs of 11 mm diameter, taken from actively growing colonies (2 weeks old cultures, grown on the inoculum medium) and placed in the center of each Petri dishes. 0.1 mL of nematodes suspension (1000 nematodes) has been pulverized after 7 days of incubation, on the surface of each Petri dishes, then being analyzed for 15 days further. Controls, consisting of Petri dishes inoculated with nematodes but with no fungi, were used for each type of media. From the three fungal species tested, *G. junonius* was selected as the most efficient species, and used further in experiments. For test tubes, inoculation was performed using mycelium grown on liquid medium (the inoculum medium described above, without agar) for two weeks and homogenized thereafter using a Heidolph Homogenizer, at 9000 rpm.

Analytical methods and measurements

The effect of fungal mycelium over the viability of nematodes was assessed after 7 days of co-incubation (after nematodes addition to experimental variants). Visual observation made both under the stereomicroscope (stereomicroscope SZM2 Optika, at a magnification of 20-45x) and at microscope using glass slides (phase contrast microscope NIKON, at a magnification of 200-1000x) were made, and the viability of nematodes was tested by mechanically stimulating the bodies with a very thin needle. The degree of nematodes colonization by fungal mycelium was assessed visually, after staining. Pictures have been taken using a photo camera at all stages.

Pots experiment

A total number of 10 pots filled with a mixture of 6:3:1 clay-sand-peat, pH 7.2, sterilized through autoclaving at 120 °C, 20 minutes. Seeds of *Lycopersicum esculentum* "ClaussF1" variety were placed in the pots, and after 14 days from the germination, the substrate was inoculated with root-knot nematodes from *Meloidogyne incognita* species, 80 individuals (second stage juveniles, J_2) per pot. From these pots, five were co-inoculated with tested fungi (*G. junonius* cultivated on liquid medium for inoculum, as described above) and five remained un-inoculated and used as control. After 20 days of cultivation of nematodes-inoculated tomatoes, the nematodes extraction from soil was performed using the Cobb's method, through sieving and decantation and afterwards using the Baermann modified method [Southey, 1985]. The nematodes were collected in aqueous suspension

during no more than three days, numbered on counting dish, using a binocular stereomicroscope (Leica MZ95). The features of roots and the morphological aspect of juveniles were also analyzed.

Data interpretation and calculations

The rate of fungal colonization of nematodes bodies and viability of nematodes was evaluated under the microscope by randomly choosing seven squares of one cm^2 for each Petri dishes and counting the nematodes in designated area, or by taking 0.1 mL of liquid for samples in liquid media. For each Petri dishes, 200-250 nematodes were observed and analyzed. The data presented in current paper represents the mean value for all the replicates of each sample (after normalizing and eliminating from calculation the values with a high standard deviation). The finally obtained value for each set was considered the percentage of mortality / colonization.

Results and discussion

Many lignicolous basidiomycete species kill and colonize invertebrates when nutrients availability is very low. Among these basidiomycete species, some have particular adaptations for actively capturing insect larvae and worms, especially nematodes. Synthesis of compounds with nematicidal activity is another mechanisms involved in capturing nematodes for using their cadavers as sources of nutrients. As it can be observed in the Fig. 1 (A-E) and Fig. 2 (A-E), the nematodes cadavers are being gradually colonized by fungal hyphae until the total degradation of cadavers occur.

Influence of carbon and nitrogen sources. In order to optimize the process and to develop an easy and cheap method for using these fungi against phytopathogenic nematodes, we have tested different sources of carbon and nitrogen (Tab. 1) in preparing the media. In the same time, the quantity of glucose and peptone (the two nutrients widely used in our previous study that gave good rates of both mycelium development and nematicidal effect) ranged from 1 to 7 g L^{-1} for glucose, and from 0.5 to 4 g of peptone L^{-1}.

The mycelium development varied widely. Among the sugars, maltose had the strongest stimulating effect for the three fungal species. Increased concentration of glucose lead to a very fast development of mycelium. The preferences for the nitrogen sources was different from a species to another. While peptone and sodium nitrate stimulated the mycelium development for all the fungi, urea gave positive results only for *F. pinicola* and ammonium sulfate stimulated the development for *D. quercina* and *G. junonius*. Increasing concentrations of peptone from 0.5 to 1 g L^{-1} stimulated the mycelium development and extension of it on the medium surface, but increasing the concentration above this value lead to a very slow extension of mycelium, forming a thick and dense net around the inoculation point.

These observations are very important for elaborating a biocontrol strategy for field trials, as the concentration and type of nitrogen sources are affecting positively or negatively the process. Uptaking inorganic compounds of nitrogen, such as ammonia or nitrate, is an advantage as many of the used fertilizers in agriculture contain such compounds.

The used nutrients have had a different effect over the nematicidal activity and fungal colonization of nematodes. Overall, *G. junonius* presented the strongest nematicidal

effect and the highest colonization rate (Tab. 2). All the carbon sources used in quantity of 2 g L^{-1} were satisfying the fungal requirements. Although the results were different for the three fungi, on the maltose media, strong nematicidal activity was observed for all the fungi. *G. junonius* killed all the nematodes also on the medium with starch. Regarding the nitrogen sources, both ammonium sulfate and sodium nitrate gave good results concerning the nematicidal activity of fungi. For *G. junonius* was effective as well.

Concerning the colonization rate, the results were comparable, the same nutrients stimulating the process. However, the increasing concentration of peptone strongly decreased the colonization rate. As the fungi meets their nutritional requirements in the medium, no longer colonize nematodes.

Tab. 2. The effect of various C and N sources on colonisation and nematodes mortality (after 15 days of co-incubation with fungi)

Media	*D. quercina*		*F. pinicola*		*G. junonius*		Control
	colonization %	nematodes mortality%	colonization %	nematodes mortality%	colonization %	nematodes mortality%	nematodes mortality%
C1	0	68	0	55	11	92	17
C2	14	59	13	71	26	100	16
C3	0	19	12	71	0	89	17
C4	15	38	0	28	31	100	15
C5	8	12	11	49	14	93	16
C6	26	59	27	56	61	100	15
C7	31	61	36	62	56	100	17
C8	30	60	34	61	31	100	16
N1	17	49	0	49	78	100	17
N2	15	47	12	42	49	88	16
N3	11	40	10	36	37	67	16
N4	0	36	0	22	0	25	14
N5	16	62	54	62	17	100	17
N6	8	25	0	21	0	79	18
N7	15	64	16	63	16	100	17

Influence of nutritive salts. Ions such as Mg^{2+}, K^{+} and PO_4^{3-} are important for both plant and fungal development. Although less important, SO_4^{2-} is also required in sufficient concentration for fungi to grow. In agricultural practices, magnesium, potassium and phosphorus are usually added as fertilizers if the soil do not contain such minerals in adequate concentrations. In this study, we have tested the influence of these minerals, added as salts in nutritive media. For liquid media, other two salts – iron sulfate and sodium nitrate have been added in small concentrations.

It can be observed from the results that, except the case of *F. pinicola*, increasing concentration of minerals led to significantly decreasing the colonization rate (Tab. 3), while the nematicidal activity remained constant. This phenomenon suggests that fungi are colonizing nematodes cadavers not only for sugars and nitrogen compounds, but also for essential minerals.

Tab. 3. Mortality of nematodes on solid (S1-S3) and liquid (S4-S5) media supplemented with nutritive salts (after 15 days of co-incubation with fungi)

Media	*D. quercina*		*F. pinicola*		*G. junonius*		Control
	colonization%	nematodes mortality%	colonization%	nematodes mortality%	colonization%	nematodes mortality%	nematodes mortality%
S1	25	58	12	62	43	100	11
S2	51	59	0	37	54	100	10
S3	53	59	0	37	55	100	10
S4	5	14	8	39	39	100	8
S5	6	20	10	50	33	90	7

For liquid media (with previously mentioned minerals in concentration equal to the richest solid medium) *D. quercina* and *F. pinicola* presented the highest activity on the medium with higher concentration of glucose and peptone, while *G. junonius* did the opposite. As already stated, for the fungal metabolisms to be switched in the direction of using invertebrates as sources of nutrients, it is very important that in the substratum these fungi develop, there are enough nutrients for mycelium to grow, but in concentration small enough to represent a limitative factor.

Influence of heavy metals ions. In agriculture, there are frequently used pesticides that contain ions of heavy metals, particular fungicides. The cupric fungicides were historically used. Considering that for biocontrol strategy fungi will be applied as a liquid inoculum to soil, it is very important that these fungi resist to the concentration of heavy metals as high as in the agricultural soils. In this respect, the strain that presented the highest activity, *G. junonius*, was further tested for its resistance to heavy metals. As it can be observed in the Tab. 4, addition of copper and zinc manifested o strong inhibitory effect over the mycelium development and nematicidal activity as well. Iron and manganese ions had a stimulating effect and gave positive results, especially on the media with higher concentration of these ions (2 mM). Other researchers [CAMPOS & al. 2009] have proved that *G. junonius* is a metal accumulator, meaning that it resists to high levels of metals. The results suggest that when applying fungi as biocontrol agents, the use of chemical compounds containing copper or zinc should be limited.

Tab. 4. Mortality of nematodes on media supplemented with heavy metals (after 15 days of co-incubation with fungi)

Metals	plates inoculated with *Gymnopilus junonius*		Control
	fungal development	nematodes mortality%	nematodes mortality%
1mM Cu^{2+}	weak	20	16
1mM Mn^{2+}	strong	55	9
1mM Fe^{2+}	strong	85	18
1mM Zn^{2+}	weak	12	7
2mM Cu^{2+}	no development	25	25
2mM Mn^{2+}	strong	95	17
2mM Fe^{2+}	strong	100	30
2mM Zn^{2+}	weak	13	11

Pots experiment. In order to test the nematicidal activity of fungi in laboratory simulated agricultural condition, plants of tomatoes infected with phytopathogenic nematodes were treated with liquid inoculum of *G. junonius.* We have used five pots for control and five for fungi-inoculated plants, with 80 individual of nematodes in each pot (Tab. 5). After incubation time, the average number of individuals were recovered on pots inoculated with fungi was approximatively three time lower that the number recovered from control pots, showing a significant nematicidal activity of *G. junonius*'s mycelium. The treated (with fungi) and un-treated plants did not look different (there was any significant evidence of the nematodes attack), due to the small incubation period (20 days). For severe symptoms to occur in attacked plant an entire generation of nematodes (60 days) should develop. The nematodes obtained from control pots, through extraction, at the end of the experiment were in the pre-adult stage (J3 and J4), meaning their life cycle was normal, and not affected by the culture condition, but individuals on treated pots were affected by the presence of fungi. The strong decrease of nematodes' number on the treated pots is an evidence of *G. junonius* efficiency in controlling nematodes.

The possible involved mechanisms might be the production of nematicidal compounds, as different authors [KAYANO & al. 2004; KUSANO & al. 1986] observed the production of gymnopilins, gymnoprenols and other related compounds, with different physiological action on animals, such as cytotoxicity against human tumor cell lines [KIM & al. 2012] or mobilization of Ca^{2+} from nervous cells [MIYAZAKI & al. 2012]. In the same time, other species from family Strophariaceae, such as *Stropharia rugosoannulata*, are nematophagous [LUO & al. 2006] and there is a possibility of these mechanisms to occur in the case of *G. junonius* as well.

Tab. 5. The effect of *G. junonius* inoculum addition on the tomatoes inoculated with *Meloidogyne incognita* (after 15 days of fungal inoculum addition)

Type of experiment	Pot	Initial number of juveniles (J2)	Final number of juveniles (J2)	Examination of nematodes	Symptoms on tomatoes
with *J. junonius*	1	80	16	The cuticle thinned, abnormal positions, faint stylet, granulations on digestive tube	Small zoocecidia in formation
	2	80	18		
	3	80	29		
	4	80	21		
	5	80	19		
	average	80	20.6		
without fungi	1	80	68	Strong movements for advancing, prominent stylet, retractile, the hyaline part of the tail prominent	Small zoocecidia in formation
	2	80	71		
	3	80	55		
	4	80	63		
	5	80	59		
	average	80	63.2		

Tab. 6. The mortality of nematodes in dependence of *G. junonius* inoculums quantity (after 1 day of co-incubation)

Inoculum quantity (mL)*	Type of medium		
	medium A	medium B	medium C
	nematodes mortality%		
0.1	53	22	30
0.2	75	27	48
0.4	92	39	51
0.6	100	52	100
0.8	100	100	100
1.0	100	100	100
control	20	18	17

*out of a total mixture of 2 mL (inoculum, fresh medium and 100 µL nematodes suspension)

Influence of inoculum's quantity. After one day of incubation, a quantity of inoculum as high as 0.6 mL/2 mL (30%) was enough for killing all the nematodes. Considering the short period for incubation, the involved mechanism should be of biochemical type, as no colonization occur. Concerning the media used in this experiment, the standard medium was the most effective, but also the cheap variants gave positive results, especially on the C medium, rich in nutrients, where mycelium developed very strong. There are necessary additional studies for testing the chemical compounds with nematicidal activity, purifying and researching for understanding the conditions in which these compounds present the nematicidal activity.

Conclusions

The tested fungi are colonizing nematodes when nutrients are available in low quantity. This property gives the possibility of using fungi in biological control strategies.

G. junonius presented the highest nematicidal activity and was effective in simulated conditions of plants attacked by nematodes.

The chemical compounds used as fertilizers in agriculture have a positive effect over the process if they are not used in very large quantities.

The tolerance to the high concentrations of iron and manganese ions of *G. junonius* is an advantage and recommends the use of this species in biocontrol of nematodes. However, the concomitant usage of fungicides or compounds containing copper or zinc should be avoided.

The proved biochemical mechanism involved in the process is very important and can lead to isolation and purification of the active compound for using it in biocontrol. In this respect, additional studies are required for completely understanding the mechanisms.

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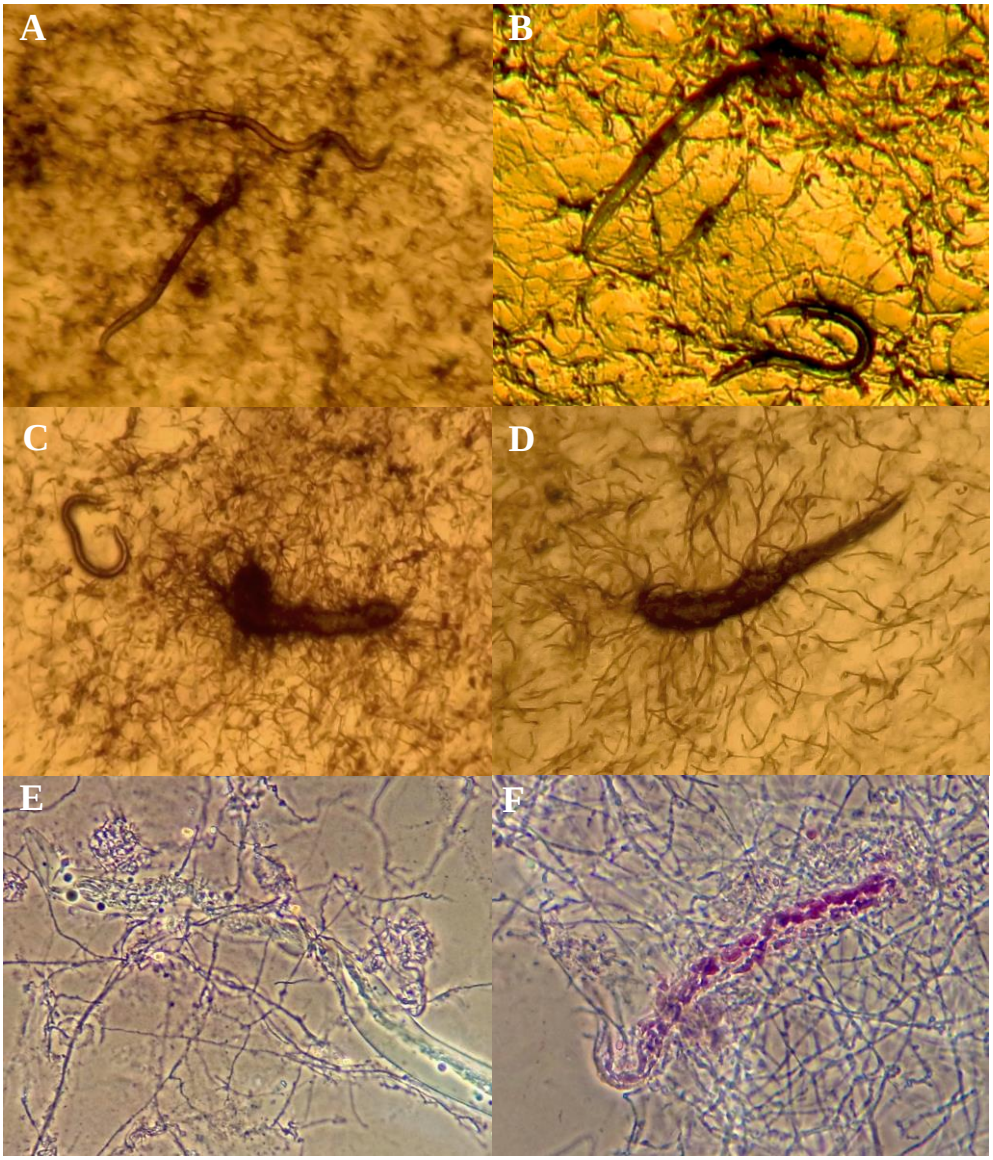

Fig. 1. Colonisation of nematode's bodies: A and B – initial stages of infection, stereomicroscope image, 25x; C and D – the end of colonisation and degradation, stereomicroscope image, 25x; E and F – different stages of colonisation, phase contrast microscope image, after Acid Fucsine staining, Microscope 400x.

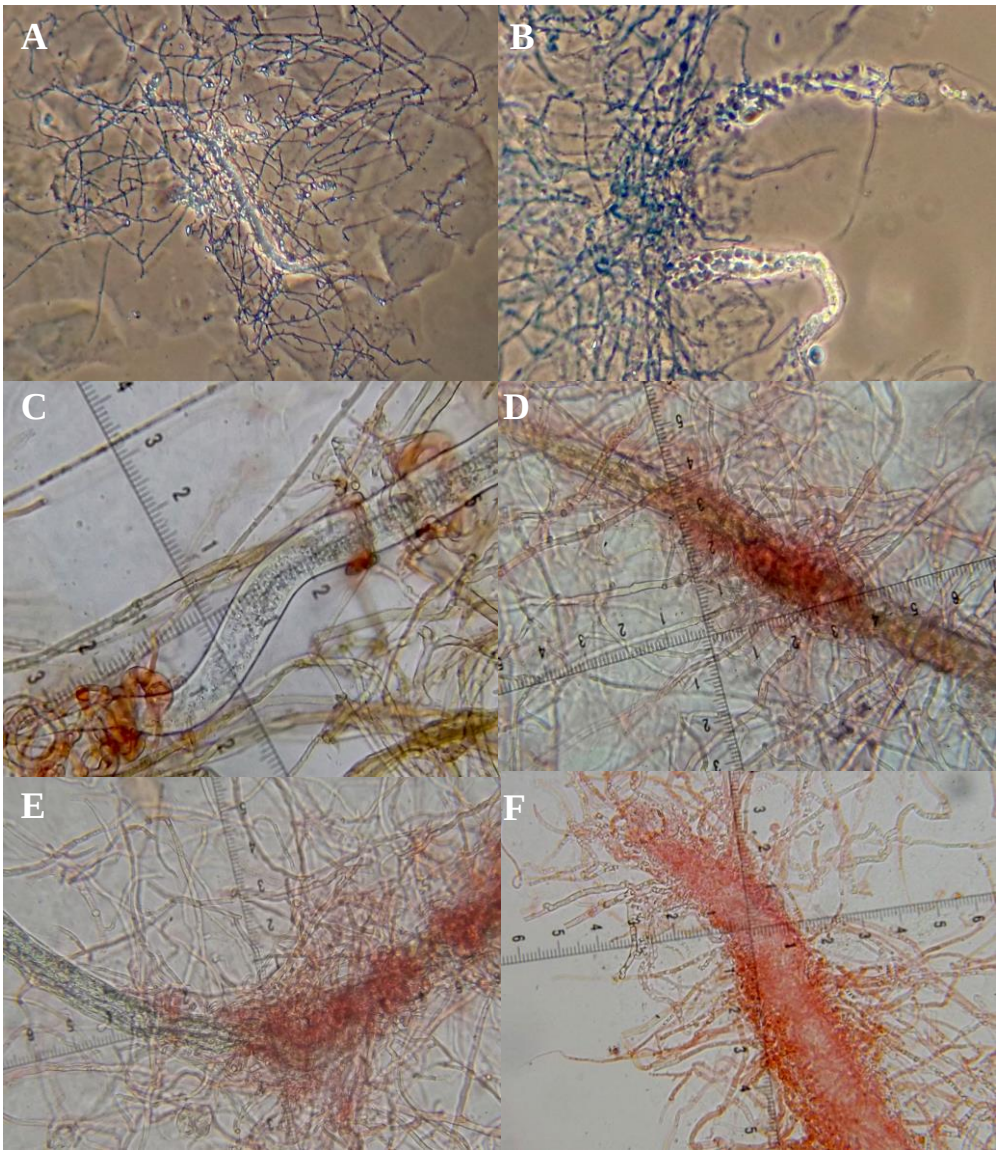

Fig. 2. Fungal degradation of nematode's bodies: A and B – phase contrast microscope image, after Methyl Blue staining, 400x; C – F – upright normal microscope image, Congo red staining, Microscope 400x.

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