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In Vitro Methodology to Reproduce the Conditions of the Gastrointestinal Tract of Ruminants: A Useful Tool to Assess Spores' Viability from Nematophagous Fungi for the Biological Control of Parasitic Nematodes

Martha Orozco-Aceves[1], José A. Calvo-Araya[2], Ana E. Jiménez-Rocha[3], Oscar Acuña-Navarro[4], Víctor Álvarez-Calderón[5]

1. Corresponding author. Universidad Nacional de Costa Rica, Escuela de Ciencias Agrarias, Maestría en Agricultura Alternativa con mención en Agricultura Ecológica. Heredia, Costa Rica. Email: marthaorozcoaceves@gmail.com
2. Universidad Autónoma Chapingo, Departamento de Parasitología Agrícola. Texcoco, Edo. de Méx. Email: josealonsocalvoaraya@gmail.com
3. Universidad Nacional de Costa Rica, Escuela de Medicina Veterinaria, Laboratorio de Parasitología. Heredia, Costa Rica. Email: ana.jimenez.rocha@una.cr
4. Universidad de Costa Rica, Centro de Investigaciones Agronómicas, Laboratorio de Bioquímica de Procesos Orgánicos. San José, Costa Rica. Email: oscar.acuna@ucr.ac.cr
5. Ministerio de Agricultura y Ganadería, Servicio Nacional de Salud Animal, Programa de la garrapata. Heredia, Costa Rica. Email: viacal@racsa.co.cr

Abstract **– To be considered as a promising biological control agent of parasitic nematodes of ruminants, a nematophagous fungus must be an efficient predator of nematodes. Additionally, the fungus must be able to survive the conditions of the gastrointestinal tract of the animals when orally administered. The viability of fungal material after its passage through the gastrointestinal tract is traditionally assessed using living animals, which can be expensive and non-practical. Therefore, an *in vitro* test that can produce similar results as those obtained from an *in vivo* test is desirable. The main aim of this work was to determine the utility of Tilly & Terry's methodology (1963; a methodology originally designed to test forage digestibility), which reproduces the conditions prevalent in the gastrointestinal tract. During the experiment, conidia or chlamydospores produced by 11 strains of nematophagous fungi native to Costa Rica were subjected to Tilly & Terry's methodology, afterwards the viability of spores was tested. Out of the 11 strains, only two *Candelabrella musiformis* strains that were chlamydospore-forming fungi remained viable after the test. Finally, we discuss diverse aspects regarding the use of Tilly & Terry's methodology for the assessment of fungal material viability, for example, type and number of spores, incubation times, and aliquot volume used in the viability test.**

Keywords **- Arthrobotrys Oligospora, Biological Control, Candelabrella Musiformis, Parasitic Nematodes.**

I. Introduction

Nematophagous fungi used as biological control agents of parasitic nematodes in animals must be efficient predators. Additionally, when orally administered the fungal structures (i.e., mycelium, conidia, chlamydospores) must be capable to survive the passage through the gastrointestinal tract of the animals (GTA), remain viable, be able to germinate, grow, trap and destroy infective larvae in faeces [1]. Frequently, living animals are used to assess the viability of fungal structures after their passage through the GTA [2]-[6], however, this method can be costly. For example, to test different treatments with suitable number of replicates a high number of animals are needed, animals have to stay enclosed to avoid re-infections, which imply suitable facilities are required, as well as enough food and labour. Finally, depending on the aims of the research, animals are sacrificed to obtain results, which translates into animal losses. For these reasons, a reliable and low-cost *in vitro* methodology to assess the viability of fungal structures is desirable.

Despite its utility, attempts to develop an *in vitro* methodology to simulate the conditions of the GTA to assess the viability of nematophagous fungi material are scarce. [7], [8]. In this research we used the original Tilly & Terry's methodology to simulate the conditions of the GTA to assess the viability of spores of nematophagous fungi strains that are native to Costa Rica. The overall aim of the study was to determine the feasibility of the original Tilly & Terry's methodology, specifically we were interested in technical aspects to consider, modify or adjust when utilizing such methodology.

II. Materials and Methods

A. *Nematophagous Fungi*

Eleven strains of predatory nematophagous fungi that are native to Costa Rica were used during the experiment. The strains were isolated from productive systems located in five counties of Costa Rica: Alajuela, Cartago, Heredia, Limón and San José, and specifically from dung, pasture soil, or organic fertilizers elaborated with dung (Table I). Of the 11 strains, five were identified as *Candelabrella musiformis*, and six strains were identified as *Arthrobotrys oligospora*, both are trap-forming fungus. For the experiment, fungal strains were cultivated in water-agar (WA), incubated at room temperature (23-26 °C) and artificial light for 12 weeks.

Table I. Strains of nematophagous fungi used during the test and their origin.

Strain	Species	Substrate	Origin
BBP2	*A. oligospora*	Organic fertilizer made with cattle dung	Poasito, Alajuela
C4	*A. oligospora*	Goat faeces	Coronado, San José
DAP	*A. oligospora*	Pasture soil from an organic dairy farm	Moravia, San José
DEPG	*C. musiformis*	Pasture soil from an organic free range system	África, Limón
G1	*A. oligospora*	Cattle faeces	Guápiles, Limón
G3	*C. musiformis*	Cattle faeces	Guápiles, Limón
MO1	*A. oligospora*	Forage plantation fertilized with organic fertilizer	Santa Lucía, Heredia
PFC	*C. musiformis*	Pasture soil from a diversified farm in transition to organic	Coronado, San José
PFSL1	*C. musiformis*	Pasture soil from dairy farm	Santa Lucía, Heredia
UC1a	*A. oligospora*	Cattle faeces	Turrialba, Cartago
UC1b	*C. musiformis*	Cattle faeces	Turrialba, Cartago

B. *Suspensions of Spores*

After 12 weeks of incubation, *C. musiformis* strains produced chlamydospores that are resistance spores, whereas *A. oligospora* strains produced conidia that are dispersion spores. Chlamydospores and conidia were removed from culture media by scrapping the surface with scalpel, submerging the scalpel into distilled water and shaking gently to release the spores. Numbers of chlamydospores and conidia were estimated with haemocytometer. Spore concentration was adjusted to 1.5×10^5 chlamydospores ml^{-1} or 1.5×10^6 conidia ml^{-1}. Differences in spore number in suspensions responded to differences in spore production, chlamydospores were always produced in lower numbers as compared with conidia. In addition, given that conidia are dispersion spores and not resistance spores, we tried to increase the survival probability of conidia by increasing their number in suspensions.

C. *Artificial Digestive Process: Tilly & Terry's Methodology (1963)*

Plastic tubes with rubber lids were added with 10 ml of McDougal saliva, 2.5 ml of goat rumen fluid and 2 ml of spore suspension (i.e., chlamydospores or conidia). A CO_2 current was introduced into the tubes for 15 s to displace air, then the tubes were sealed and incubated at 39 °C. The tubes were shaken three times a day per two days. After 48 h, tubes were added with HCl_{conc} to adjust the pH to 1.3 - 1.5, then 0.5 ml pepsin (5%) was added. Incubation at 39 °C continued for 48 h more shaking the tubes three times a day [9]. A control was included to detect the presence of contaminant nematophagous fungi (i.e., from rumen fluid). This control was processed as described above but instead of suspension of spores, distilled water was added.

D. *Viability Test of Spores*

At the end of the artificial digestive process, viability of spores was determined. We verified if spores were able to survive, germinate, grow and trap nematodes. To do this, 50 µl of the digestion mixture were extracted and placed on petri dishes (9 cm diameter) containing WA supplemented with chlortetracycline (0.02%), this procedure was repeated three times per strain. After 24 h, 500 µl of a suspension containing at least 100 free-living nematodes was added to each petri dish to stimulate fungal predatory activity. Petri dishes were incubated in darkness at 28 °C for two weeks and observed at day five, nine and 14 in search of fungal structures, conidiophores, mycelium, traps, and trapped nematodes. Presence or absence of fungal structures was reported.

III. Results and Discussion

The methodology used in this work was useful but some aspects must be considered if used to determine survival of nematophagous fungi spores, for example, the type and number of spores, incubation times, and sample volume used for the viability test.

During the study only two out of five *C. musiformis* strains; DEPG and PFC that produced chlamydospores survived the artificial digestive process, and showed positive results during the viability test at day 14. This indicated that some spores survived the conditions of Tilly & Terry's methodology, were able to germinate, grow and trap nematodes. In case of DEPG strain, the three replicates contained viable spores, whereas for the PFC strain only one replicate contained viable spores. In contrast, none of the *A. oligospora* strains that produced conidia survived the conditions of the methodology. Controls were negative to the presence of contaminant nematophagous fungi.

The result suggests that there are more probabilities of obtaining a positive result during the viability test if chlamydospores are used, a finding that has been evidenced by other works [2], [3], [10]. This result is expected due to structural characteristics of conidia and chlamydospores. Conidia have simple and thin walls that are easily degraded by digestive processes. Chlamydospores have thick walls that are resistant to heat, toxic chemicals and desiccation [11].

The number of spores used for the viability test is an aspect that must be standardized, since there is any reference value in previous reports [7], [8]. In *in vitro* tests where faeces that contained eggs of parasitic nematodes were directly inoculated with 250 – 100,000 conidia g^{-1} a substantial reduction of parasitic larvae was demonstrated [12]-[15]. However, to expel through faeces the number of spores indicated above, an animal must ingest millions of spores, since thousands of spores lose viability after passing through the GTA. For this reason, during *in vivo* tests higher number of spores are administered to animals (i.e., 10^6 spores kg^{-1} animal weight or single doses

containing 1.5×10^5 – 1.1×10^7 spores; [2], [4], [16]. In the present study 3×10^5 chlamydospores or 3×10^6 conidia in 2 ml were used, these concentrations fall within the range reported for *in vivo* tests, despite this, conidia did not survive the conditions of the artificial GTA.

Strains of *A. oligospora* (i.e., C4, G1, G3, UC1a, UC1b), all conidia-forming strains, were isolated from faeces directly collected from animal's rectum, which implies that some conidia were capable to survive the adverse conditions of the GTA [17]. Therefore, the probability of these strains to survive the conditions of the *in vitro* test is theoretically high. However, any of these strains survived the conditions of Tilly & Terry's methodology. This suggests that the conditions of the methodology were unfavourable for spore survival. Specifically, we suggest that two incubation periods of 48 h each produced detrimental effects on spores and do not reflect what occurs in living animals. Studies using sheep indicated that residence times of spores in rumen and abomasum are much shorter. Conidia were recovered from samples collected from a cannulae located in the pyloric region of the abomasum within an hour after oral administration of spores. The greatest flow concentration passed this site from four to 12 h after administration [8], which is substantially less than times established by Tilly & Terry's methodology. For this reason, in order to use Tilly & Terry's methodology to assess spore viability of nematophagous fungi, it is necessary to adjust incubation times to reflect what occurs in living animals.

Another aspect to be considered when utilizing Tilly & Terry's methodology is the volume of the digestion mix that is sampled and then plated during the viability test of spores. In this study three aliquots of 50 µl from a total of 15.5 ml were used. Since aliquots volume is small, the probability of taking viable spores might be low. Therefore, it is recommended to standardize the volume of aliquots taken from the digestion mix in order to increase the probability of sampling viable spores. Another option is to standardize the number of aliquots/ replicates taken from the digestion mix to augment the possibility of detecting viable spores.

Finally, in order to optimize the use of Tilly & Terry's methodology, we suggest the inclusion in the digestion mix of a standardized amount of plant material to make the conditions of the test more similar to those present during the digestion process that occurs *in vivo*. It is likely that food material physically protects the spores from the aggressive conditions of the GTA, raising the probabilities of spore survival. The presence of food material might have protected the conidia that were isolated from faeces of living animals (i.e., strains C4, G1, G3, UC1a, UC1b). In Tilly & Terry's methodology the spores are exposed to the direct action of the chemicals causing their degradation and viability lost. In practice, oral administration of fungal material can be accompanied of food, such as commercial food, forages, barley grains, soy bran, and others [3], [4], [12], [18]-[20].

In conclusion, Tilly & Terry's methodology can be used to assess the viability of nematophagous fungi spores but it is necessary to standardize and adjust several aspects in order to obtain reliable and realistic results. Once adjusted, the methodology can be useful in applied or industrial research, since it has the advantages of being low-cost, simple, and fast with respect to *in vivo* tests. This methodology could be used as a screening method to select promising strains, to simultaneously assess numerous treatments; for example, individual and combinations of strains, as preliminary test to asses protecting agents such as sodium alginate, to test new formulations, or as preliminary tests of more complex devices such as intraruminal devices of controlled spores' release.

REFERENCES

[1] M. Larsen, "Biological control of helminthes" 1999. Int. J. Parasitol. 29, 139-146

[2] P. Mendoza de Gives, C.J. Flores, R.D. Herrera, P.V. Vázquez, H.E. Liébano, F.G.E. Ontiveros, "Biological control of *Haemonchus contortus* infective larvae in ovine faeces by administering an oral suspensión of *Duddingtonia flagrans* chlamydospores to sheep". 1998. Rev. Latinoam. Microbiol. 72, 343–347

[3] M.E. Fontenot, J.E. Miller, M.T. Peña, M. Larsen, A. Gillespie, "Efficiency of feeding *Duddingtonia flagrans* chlamydospores to grazing ewes on reducing availability of parasitic nematode larvae on pasture" 2003. Vet. Parasitol. 118, 203–213

[4] E.B.N. Graminha, A.J. Costa, G.P. Oliveira, A.C. Monteiro, S.B.S. Palmeira, "Biological control of sheep parasite nematodes by nematode-trapping fungi: *in vitro* activity and after passage though the digestive tract" 2005. World J. Microbi. Biot. 21, 717-722

[5] J.M. Araujo, J.V. Araújo, F.R. Braga, R.O. Carvalho, "*In vitro* predatory activity of nematophagous fungi and after passing though digestive tract of equine on infective larvae of *Strongyloides westeri"* 2010. Parasitol. Res. 107, 103-108

[6] A.O. Tavela, J.V. Araújo, B.F. Ribeiro, W.F. Silveira, H.V. Dornelas e Silva, J.M. Carreta, B.L. Alcântara, J.M. Araujo, B.L. dos Anjos, C.G. Ribeiro, P.A. Teixeira, "Coadministration of sodium alginate pellets containing the fungi *Duddingtonia flagrans* and *Monacrosporium thaumasium* on cyathostomin infective larvae after passing though the digestive tract of horses" 2013. Res. Vet. Sci. 94, 568-572

[7] M. Larsen, J. Wolstrup, S.A. Henriksen, C. Dackman, J. Grønvold, P. Nansen, "*In vitro* stress selection of nematophagous fungi for biocontrol of parasitic nematodes in ruminants" 1991. J. Helminthol. 65, 193-200

[8] P.J. Waller, M. Larsen, M. Faedo, S.R. Hennessy, "The potential of nematophagous fungi to control the free-living stages of nematode parasites of sheep: *in vitro* and *in vivo* studies" 1994. Vet. Parasitol. 51, 289-299

[9] J.M. Tilly, R.A. Terry, "A two-stage technique for the *in vitro* digestion of forage crops" 1963. J. Br. Grassl. Soc. 18,104-111.

[10] N. Soto-Barrientos, J. de Oliveira, R. Vega-Obando, D. Montero-Caballero, B. Vargas, J. Hernández-Gamboa, C. Orozco-Solano, "*In-vitro* predatory activity of nematophagous fungi from Costa Rica with potential use for controlling sheep and goat parasitic nematodes" 2011. Rev. Biol. Trop. 59, 37-52

[11] A. Rubner, "Revision of predacious hyphomycetes in the *Dactylella-Monacrosporium* complex" 1996. Centraalbureau Voor Schimmelcultures Baarn and Delft. Stud. Mycol. 39

[12] J. Grønvold, J. Wolstrup, P. Nansen, S.A. Henriksen, "Nematode-trapping fungi against parasitic cattle nematodes" 1993a. Parasitol. Today 9, 137-140

[13] P. Mendoza de Gives, V.M. Vázquez-Prats, "Reduction of *Haemonchus contortus* infective larvae by the nematophagous fungi in sheep faecal cultures" 1994. Vet. Parasitol. 55, 197-203

[14] P. Chandrawathani, J. Omar, P.J. Waller, "The control of the free-living stages of *Strongyloides papillosus* by the nematophagous fungus, *Arthrobotrys oligospora"* 1998. Vet. Parasitol. 76, 321-325

[15] A.S. Maciel, J.V. Aráujo, A.K. Campos, E.A. Lopes, L.G. Freitas, "Predation of *Ancylostoma* spp. dog infective larvae by nematophagous fungi in different conidial concentrations" 2009.Vet. Parasitol. 161, 239-247
[16] R.O. Carvalho, F.R. Braga, J.V. Araújo, "Viability and nematophagous activity of the freeze-dried fungus *Arthrobotrys robusta* against *Ancylostoma* spp. infective larvae in dogs" 2011. Vet. Parasitol. 176, 236-239
[17] J. Grønvold, J. Wolstrup, P. Nansen, S.A. Henriksen, M. Larsen, J. Bresciani, "Biological control of nematode parasites in cattle with nematode-trapping fungi: a survey of Danish studies" 1993b. Vet. Parasitol. 48, 311-325
[18] M. Larsen, P. Nansen, S.A. Henriksen, J. Wolstrup, J. Grønvold, A. Zorn, E. Wedø, "Predacious activity of the nematode-trapping fungus *Duddingtonia flagrans* against cyathostome larvae in faeces after passage though the digestive tract of horses" 1995. Vet. Parasitol. 60, 315-32
[19] B.F. Ribeiro, J.V. Aráujo, A.R. Silva, R.O. Carvalho, J.M. Araujo, S.R. Ferreira, G.R. Carvalho, "Viability of the nematophagous fungus *Pochonia chlamydosporia* after passage through the digestive tract of horses" 2010. Vet. Parasitol. 168. 264-268
[20] S.R. Ferreira, J.V. Araújo, B.F. Ribeiro, M.J. Araujo, F.F. Mara, "*In vitro* predatory activity of nematophagous fungi *Duddingtonia flagrans* on infective larvae of *Oesophagostomum* spp. after passing though digestive tract of pigs" 2011. Trop. Anim. Health Prod. 43, 1589-1593

AUTHOR'S PROFILE

Martha Orozco-Aceves was born in Mexico City on 17th January 1978. She is microbiologist, degree granted in 2001 by the Escuela Nacional de Ciencias Biológicas (National School of Biological Sciences) of the Instituto Politécnico Nacional (Polytechnical National Institute) in México City. She obtained a Master's degree in Ecological Agriculture in 2005 by the Universidad Nacional de Costa Rica (National University of Costa Rica) in Costa Rica, and a PhD in Soil Ecology applied to restoration of ecosystems in 2015 by The University of Western Australia.
She has worked as Professor and Researcher in Mexico, Costa Rica and Australia in diverse areas such as Agroecology, biological control of pests and diseases, soil ecology, clean alternatives to pesticides' use, and restoration of ecosystems. Currently, she is researcher at the Instituto Regional de Estudios en Sustancias Tóxicas (Central American Institute for Studies in Toxic Substances) in Costa Rica. Some of her relevant publications are:
Orozco-Aceves, M., Standish, R.J., Tibbett, M. 2015. Soil conditioning and plant-soil feedbacks in a modified forest ecosystem are soil-context dependent. Plant and Soil 390:183-194.
Orozco-Aceves, M., Standish, R.J., Tibbett, M. 2015. Long-term conditioning of soil by plantation eucalypts and pines does not affect growth of the native jarrah tree. Forest Ecology and Management 338:92-99
Orozco A. Martha, Jiménez R. Ana, Acuña N. Oscar, Álvarez C. Víctor. 2015. Crecimiento de hongos nematófagos en fuentes de carbono diversas (Determination of the growth of nematophagous fungi on diverse carbon sources). Agronomía Costarricense 39:143-151
Dr. Orozco-Aceves obtained an honorific mention *summa cum laude* by the Universidad Nacional de Costa Rica for her thesis with ttitle: "Aislamiento y caracterización de hongos nematófagos como potenciales controladores biológicos de nemátodos gastrointestinales de rumiantes" (Isolation and characterization of nematophagous fungi for the biological control of gastrointestinal nematodes of ruminants). She also obtained recognition diplomas in 2004 and 2005 by the Universidad Nacional de Costa Rica for being postgraduate model student during her Masters studies.

José Alonso Calvo-Araya was born in Alajuela, Costa Rica on 27th March 1985. He gained his Bachelor of Science in Agronomy in 2008, at the National University of Costa Rica, he obtained another degree in Ecological Agriculture at the National University of Costa Rica in 2011, and his M.Sc degree in Plant Protection at the Chapingo Autonomous University in Mexico. He has worked as a researcher in Costa Rica in the field of Entomology. From 2010 to 2012 he worked as a Research assistant in Entomology at The University of Western Australia. After that he received a scholarship from the Inter-American Institute for Cooperation on Agriculture (IICA) to study a Master Program in Plant Protection in Mexico. From January 2016 he is Professor of plant diseases and pest diagnostic at the National University of Costa Rica.
Some of his relevant publications are:
Calvo, J.; Herrera, A. 2015. Rove diversity (Coleoptera: Staphylinidae) in six coffee agroecosystems of valley central of Costa Rica. ECORFAN Journal. December 2015 Vol.1 No.1 27-36
Calvo-Araya, J.A. 2014. Control biológico del moho gris en el cultivo de la mora. Editorial Académica Española. ISBN: 978-3-659-02396-5. 92 pp.
Calvo-Araya, J.A; Rivera-Coto, G; Orozco-Cayasso, S; Orozco-Rodríguez, R. 2012. Aislamiento y evaluación in vitro de antagonistas de Botrytis cinerea en mora. Agronomía Mesoamericana 23(2):225-231

M.Sc. Oscar Acuña Navarro was born in San José, Costa Rica, on June 22, 1957. Bachelor of Science, Liceo J.J. Vargas Calvo, Costa Rica, 1974. Bachelor of Agricultural Engineering, University of Costa Rica, San José, Costa Rica, 1980. Bachelor of Agricultural Engineering with emphasis on Crop Science, University of Costa Rica, San José, Costa Rica, 1983. M.Sc. Agricultural and Natural Resources Soil Field, University of Costa Rica, San José, Costa Rica, 1993.
He works as an Associate Researcher at CIA (Agricultural Research Center), UCR (University of Costa Rica), since 1982 and is Head of the Biochemistry Laboratory since 2003 at CIA. Professor of the courses: Soil Microbiology and Organic Farming & Production at the Agronomy Faculty, UCR. Also is the Institutional Head of the Organic Agriculture Program (OAP), UCR.
He was the Vice Principal of the School of Crop Science, Agronomy Faculty, UCR, for two periods; Vice Principal (CIA), for one period and the Head of Soil Microbiology Laboratory (CIA), from 1987-2001.
Work experience: preparation and evaluation of organic substrates and their biological activity on crop production; the use of clean technology to improve crop productivity and recovery of quality and soil health.
2015 -2016 Emphasizes on biotransformation of agricultural and industrial waste to obtain nutraceuticals and bio fertilizers. Biodiversity and ecology of soil organisms.
M.Sc. Acuña has been a Member of the Committee on Recognition (1994 to date) and representative at the Collegiate Representative Assembly of the School of Agronomy (1998 to date), at the UCR. Member of the Programme for Research and Technology Transfer in Organic Agriculture of the Government of Costa Rica. Costa Rican Association of Soil Science. College of Agricultural Engineers. Costa Rican Association of Phytopathology. Latin American Association of Rhizobiology.
Publications:
Acuña, O. 2011. Manejo adecuado de los rastrojos de piña. Piña de Costa Rica, Ed. 7 pp. 68-69
María Araya, Ana Tapia, Rafael Mata, Edgardo Serrano, Oscar Acuña. 2014. Efecto de la aplicación de compost y nematicida sobre la dinámica de las poblaciones de microorganismos, nematodos fitoparásitosdel suelo y la salud del sistema radical en el cultivo del banano. Agronomía Costarricense 38(2)93-105
Rafael A. Segura,, Edgardo Serrano, Luis Pocasangre, Oscar Acuña, Floria Bertsch, Jetse J. Stoorvogel, Jorge A. Sandoval 2015. Chemical and microbiological interactions between soils and roots in commercial banana plantations (Musa AAA, cv. Cavendish) Scientia Horticulturae 197 (2015) 66–71

M.Sc. Víctor Álvarez Calderón was born in San José, Costa Rica on 22nd of June 1955. He is Veterinary and gained his degree in 1982 at the Agricultural Academy of Ukraine, and his M.Sc degree in Tropical diseases at the National University of Costa Rica in 1994. He has worked as Researcher for 22 years in the Ministry of Agriculture in Costa Rica. Research lines: ecto and endoparasites of animals.