Revista de la Sociedad Entomológica Argentina
ISSN: 0373-5680
ISSN: 1851-7471
santiago@cepave.edu.ar
Sociedad Entomológica Argentina
Argentina

Entomopathogenic fungi: Are polisporic isolates more pathogenic than monosporic strains?

AGUILERA SAMMARITANO, Juan A.; LECLERQUE, Andreas; DEYMIÉ, María C.; TORRENTE, Karina A.; VAZQUEZ, Fabio; CUTHBERTSON, Andrew G.S.; LÓPEZ LASTRA, Claudia C.; LECHNER, Bernardo

Entomopathogenic fungi: Are polisporic isolates more pathogenic than monosporic strains?

Revista de la Sociedad Entomológica Argentina, vol. 76, no. 3-4, 2017

Sociedad Entomológica Argentina, Argentina

Available in: https://www.redalyc.org/articulo.oa?id=322053335014

El Copyright de cada artículo es retenido por el autor/es del mismo. Los autores ceden a la RSEA la licencia para publicar el artículo y los derechos comerciales para reproducir los volúmenes de la revista para su venta en librerías y particulares. Asimismo, los autores ceden a terceros los derechos para usar el articulo libremente, siempre y cuando se identifique y cite al autor.

Notas

Entomopathogenic fungi: Are polisporic isolates more pathogenic than monosporic strains?

Hongos entomopatógenos: ¿Son los aislamientos polispóricos más patógenos que las cepas monospóricas?

Juan A. AGUILERA SAMMARITANO
aguilerasammaritanoj@unsj.edu.ar
Universidad Nacional de San Juan, Argentina
Andreas LECLERQUE
Geisenheim University, Alemania
María C. DEYMIÉ
Universidad Nacional de San Juan, Argentina
Karina A. TORRENTE
Universidad Nacional de San Juan, Argelia
Fabio VAZQUEZ
Universidad Nacional de San Juan, Argentina
Andrew G.S. CUTHBERTSON
Independent Science Advisor, Reino Unido
Claudia C. LÓPEZ LASTRA
Centro de Estudios Parasitológicos y de Vectores, Argentina
Bernardo LECHNER
Universidad de Buenos Aires, Argentina

Revista de la Sociedad Entomológica Argentina, vol. 76, no. 3-4, 2017

Sociedad Entomológica Argentina, Argentina

Received: 29 March 2017
Accepted: 30 August 2017
Published: 27 December 2017

Redalyc: https://www.redalyc.org/articulo.oa?id=322053335014

Abstract: Currently only monosporic strains from several entomopathogenic fungi have been selected and used for mass production and bioinsecticide manufacturing worldwide. The main reasons for the use of single spore instead of multispore strains are the attenuated virulence and the contaminants of the same species. In this study, different polisporic isolates and their monosporic combinations were tested against *Tenebrio molitor* (L.) larvae as an insect model. Isolates were obtained from arid soils. Four *Metarhizium* sp. (Metschn.) multisporic isolates (CEP413, CEP589, CEP590 and CEP591) were selected for bioassays. Trials were performed to evaluate mortality on three treatments, Full Polisporic (FP), Partial Polisporic (PP) and Pure Monosporic (PM). Cumulative mortality was measured at day 4 post infection. Sporulation percentage was assessed at day 6 post infection. The highest mortality was found at FP treatment (94%), the lowest mortality at day 4 was found at PM-CEP413 (32%). At day 6 the sporulation percentage was higher on FP (94%) and it was different from the rest of the treatments. To elucidate different polisporic and monosporic combinations to improve their effectiveness, may help to expand the use of bioinsecticides based on entomopathogenic fungi.

Keywords: Insect model, Isolate effectiveness, Metarhizium sp, Strain effectiveness, Tenebrio molitor.

Resumen: Actualmente existen varias cepas de hongos entomopatógenos utilizadas para la fabricación de bio-insecticidas comerciales. Sin embargo, la selección de éstas sigue algunas pautas como la obtención y evaluación de cepas monospóricas (cultivo en masa de microorganismos que provienen de una sola espora) Las principales razones para

el uso de cultivos monospóricos en lugar de cultivos polispóricos son: la virulencia atenuada y los posibles "contaminantes" de la misma especie. En este estudio, diferentes aislamientos polispóricos y sus combinaciones monospóricas se pusieron a prueba para evaluar su eficacia respecto a larvas de *Tenebrio molitor* (L.) como insecto modelo. Los aislamientos polispóricos de hongos entomopatógenos fueron obtenidos de muestras de suelo de regiones agrícolas áridas. Se seleccionaron cuatro aislamientos polispóricos de *Metarhizium* sp. (Metschn.) (CEP413, CEP589, CEP590 y CEP591) para los bioensayos. Se realizaron pruebas de patogenicidad para evaluar la mortalidad en tres tratamientos, Polispórico completo (FP), Polispórico parcial (PP) y Monospórico puro (PM). La mortalidad acumulada se midió al día 4 después de la infección. El porcentaje de esporulación se evaluó al día 6 después de la infección. La mayor mortalidad se encontró en el tratamiento PF (94%), la menor mortalidad en el día 4 se encontró en PM-CEP413 (32%). Al día 6 el porcentaje de esporulación fue mayor en FP (94%) y fue diferente del resto de tratamientos. Elucidar diferentes combinaciones polispóricas y monospóricas para aumentar la eficacia de las aplicaciones es crucial para ayudar a expandir el uso de bio-insecticidas basados en hongos entomopatógenos.

Palabras clave: Efectividad de cepas, Efectividad de aislamientos, Insecto modelo, Metarhizium sp, Tenebrio molitor.

INTRODUCTION

An epizootic occurs naturally when an unusual number of insects have been infected at the same time without human intervention (Marcelino, 2007). Epizootic events produced by entomopathogenic fungi (EF) are relatively common, and can be important in the natural regulation of insect populations (Wraight et al., 2007; Skinner et al., 2014). It is well known that some EF species (*i.e. Metarhizium anisopliae* (Metschn.) and *Beauveria bassiana* (Bals.)) have worldwide distributions and are often responsible for wide-scale epizootics (Fuxa, 1987). Although natural epizootics are well documented (Brandenburg & Kennedy, 1982; Ríos-Velasco et al., 2010; Moura-Mascarin et al., 2016), the inherent biological processes involved in the interactions between the fungal pathogen and the insect pest are not completely understood. In their natural state, in which epizootics are produced, some soil EF like *Metarhizium* sp. could act as a multisporic organism rather than a monosporic isolate. Some authors state that "the widespread occurrence of asexual reproduction by asexual propagules (conidia) and of hyphal anastomosing can cause confusion because a mycelium in its natural environment seems to be a single physiological and ecological unit but in reality is a genetic mosaic" (Guarro et al., 1999). It is well known that different strains from the same species usually display great intraspecific variability in respect to their host range, pathogenicity, and morphological and physiological characteristics (Brady, 1979). "Sexual recombination, observed *in planta* and *in vitro*, could be the means by which new genetic variants are generated leading to new biotypes with a selective advantage to colonize new hosts" (Marcelino, 2007). Therefore, to have different strains from the same species may increase and extend the attacking mechanisms like enzyme production, different cuticle penetration rate and differential toxin production.

The aim of this study was to design and test a simple experiment to prove if monosporic *Metarhizium* sp. strains are more, or less, pathogenic

than their parental polisporic isolates, and to know if intermediate combinations have intermediate pathogenicity degree. The hypothesis is that polisporic strains of *Metarhizium* sp. from different regions have different attack mechanisms and therefore have the potential to be more pathogenic than monosporic isolates.

MATERIAL AND METHODS

Soil samples

Soil samples were collected from 4 agricultural fields (sampling points) in San Juan (Argentina). Sample points were located on 4 farms separated by at least 5 km from each other. At each sampling point the soil sample was collected with the use of a garden spade to a depth of between 5 and 15 cm. The soil sample (1.5 - 2 Kg) was formed from five sub-samples (300 - 400 g each). Sub-samples were taken at 20 cm distance from each other. Among samples the spade was sterilized first with 96% ethanol and then washed with sterile distilled water twice. The sub-samples were placed in sterile polypropylene bags (32 × 16 cm) and sealed with a rubber band. In the laboratory the five sub-samples were homogenized, mixed thoroughly, filtered through a 4 mm sieve and used immediately. Between 400 and 420 g of each homogenized soil sample was placed into two clean plastic trays of 900 g capacity. A total of 16 samples of agricultural soil from the 4 farms was obtained.

Fungal bait

Isolates were obtained from soil samples using *Tenebrio molitor* (L.) larvae in a simple fungal bait design (Aguilera Sammaritano et al., 2016) with minor modifications. Twenty *T. molitor* larvae were placed inside each plastic tray (two replicates per sample) and incubated in a Fitotron® controlled environment cabinet at 30 °C and 65% relative humidity for 7 days in the dark (n = 640). Four HOBO data loggers (Onset®) were used to confirm temperature and humidity during the assays. Fresh mycelium from cadavers were scrapped out with a sterile gauge needle and cultivated on complete media (ingredients: dextrose 20 g; peptone 5 g; agar 7.5 g; gentian violet 0.005 g; cycloheximide 0.125 g; chloranphenicol 0.25 g; distilled water 500 mL) (Dobersky & Tribe, 1980) for 10 days at 30 °C in the dark. Morphological determinations of the colony were made under light and stereomicroscopes and the fungal species classified according to Humber (2012). All isolates were stored at the Fungal Culture Collection CEPAVE-EF (La Plata-Argentina).

Bioassays

Four *Metarhizium* sp. isolates CEP413, CEP589, CEP590 and CEP591 were chosen for bioassays because they all sporulated well on the medium

(Saito et al., 2012) and also according to their sporulation speed on *T. molitor* cadavers when on the trays. Three treatments plus one control were performed. For "Full Polisporic" (FP) treatment, 5 x 10^9 c/mL from each isolate were mixed in a complete suspension and adjusted to 1 x 10^7 c/mL. One mL of the FP suspension was sprayed on 10 *T. molitor* larvae and incubated in a Petri dish with moistened filter paper (humidity chamber) for 4 days at 30 °C in the dark. Five replicates were made and the complete treatment was repeated twice (n = 100).

For the "Partial Polisporic" (PP) treatment, 1 mL of an adjusted suspension of 1 x 10^2 c/mL from each polisporic parental were carefully transferred to 5 individual 90 mm Petri dishes with SDAY (dextrose 20 g; peptone 20 g; agar 20 g; yeast extract 10 g; distilled water 1 L) and spread using a sterile Drigalsky spatula. The dishes were incubated for 48 h at 30 °C in the dark. Following this a single germinated conidia was carefully transferred to a sterile Petri dish with SDAY to obtain monosporic strains. The dishes were incubated for 10 days at 30 °C in the dark. Finally, 3 x 10^9 c/mL from each monosporic isolate were mixed in a complete suspension and adjusted to 1 x 10^7 c/mL. One mL of the PP suspension was sprayed onto 10 *T. molitor* larvae which were then incubated in a humidity chamber for 4 days at 30 °C in the dark. Five replicates were made and the complete treatment was repeated twice (n = 100).

The "Pure Monosporic" treatment (PM) was performed with 1 x 10^7 c/mL from each monosporic strain, but in this case, conidia were not mixed. The suspension from each isolate was applied individually to 10 *T. molitor* larvae. Five replicates were made for each strain and the complete treatment was repeated twice (n = 400).

The counting of dead larvae was made on the fourth day of inoculation, and the number of sporulated larvae *per* dish was registered at day six. A sporulated larva was considered a positive count when it was fully covered by green conidia. Five control replicates were made by spraying 1 mL of sterile distilled water onto 10 *T. molitor* larvae (n = 50) and incubated at the same conditions as outlined above. No food was provided throughout the trials to avoid problems with fungal contamination from the diet (Posada &Vega, 2005). The data was analysed using a one-way analysis of variance (Infostat, 2013). In all data sets, normality and variance homogeneity were tested prior to analysis. $p<0.05$ was considered significant.

RESULTS

At the fourth day of inoculation the highest mortality percentage was observed on FP (94 ± 8.4%) and the lowest (32 ± 16.9%) on PM (CEP413) (Fig. 1). Significant differences were found among FP and the rest of the treatments ($p<0.0001$). No statistical differences were found among larvae mortality on PP and PM treatments except for PM (CEP413).

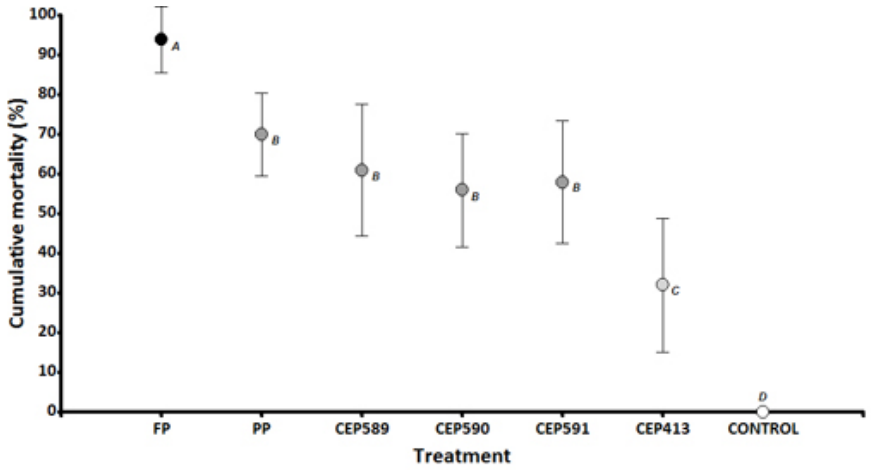

Fig. 1. Cumulative mortality on *Tenebrio molitor* larvae for three bioassays.
Treatments were performed for FP (Full Polisporic) using wild *Metarhizium* sp. isolates, PP (Partial Polisporic) with 4 combined monosporic strains and PM (CEP413, CEP589, CEP590, and CEP591) using monosporic isolates applied individually. The counting of dead larvae was made at the fourth day post infection on 10 *T. molitor* larvae. Different letters indicate significant differences among treatments (LSD test 0.05 significant level). Error bars represent standard deviation for 5 replicates. The complete trial was repeated twice.

According to the number of sporulated larvae (Fig. 2), at day six the highest proportion (92.4 ± 7.95%) was found on FP treatment and the lowest proportion (37.8 ± 31.9%) was found in PM (CEP413). We found significant differences ($p<0.0001$) among FP and the rest of the treatments. However, no difference was found between PP and PM.

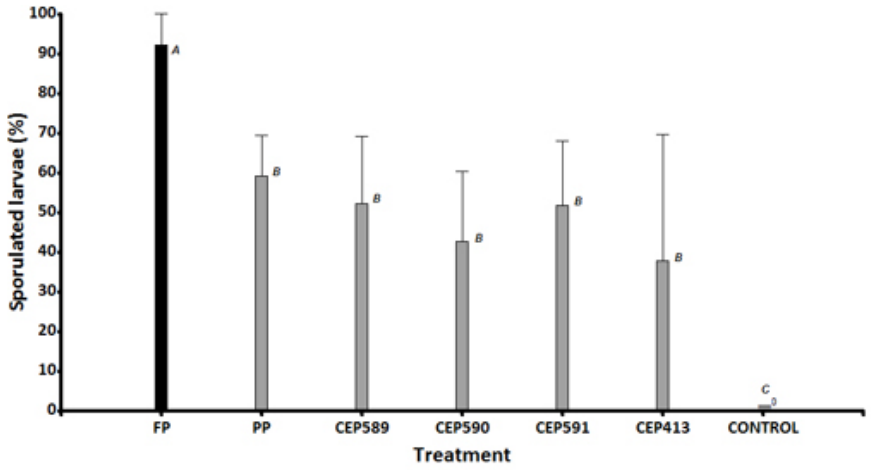

Fig. 2. Proportion (%) of sporulated *Tenebrio molitor* larvae at day six post infection.
Treatments were performed for FP (Full Polisporic) using wild isolates, PP (Partial Polisporic) with 4 combined monosporic isolates and PM (CEP413, CEP589, CEP590, and CEP591) using monosporic isolates applied individually. The counting of dead larvae was made at the fourth day post infection on 10 *T. molitor* larvae. Different letters indicate significant differences among treatments (LSD test 0.05 significant level). Error bars represent standard deviation for 5 replicates. The complete trial was repeated twice.

DISCUSSION

Over the past 20 years a great effort has been directed towards determining the correlation between both the physical and physiological features of some EF and their degree of pathogenicity (Fuxa, 1987;

Jeffs, 1999; Arthurs & Thomas, 2001; Ali-Shtayeh et al., 2003; Jackson et al., 2010; Skinner et al., 2014). In fact, all commercial isolates are strictly purified, classified, tested (germination, sporulation, virulence, pathogenicity, thermal range, etc.) for mass production (Seema et al., 2013; Jaronski, 2014) and commercially distributed in their monosporic (single-spore) state. Until now, the predominant thinking has been that isolates must come from a single spore culture for two main reasons. Firstly, to avoid problems with attenuated virulence and secondly, to eliminate possible contaminants from the same species (Vega et al., 2012). However, Torres de la Cruz et al. (2014) found a wide intra-specific variability among monosporic strains of *M. anisopliae* in terms of their pathogenicity, at least to *Galleria mellonella* larvae.

Unfortunately, little research has been made to elucidate possible multisporic combinations and their effectiveness against their derivative single-spore isolates. In addition, the few studies on this topic reveal contradictory results (Samšiňáková & Kálalová, 1983; Trotter et al., 2004). Therefore, to improve the biocontrol potential of several EF worldwide, it is interesting to know if polisporic isolates may enhance the efficacy of monosporic isolates in controlling insect pest populations. In this study we present original data supporting the hypothesis that multi-spore isolates are more effective than monospore strains. Effectiveness is not only given by the speed of killing a target insect but also for the ability to produce and release infective propagules. In this study, FP isolates were able to kill faster and to sporulate quicker than PP and PM. In our study, PM treatment reached an average of 90% mortality on *T. molitor* larvae at day 7-8 post infection (data not shown); this being 3-4 days after FP. More examples of the advantages of using polisporic isolates instead of their monosporic derivatives on pathogenic fungi can be found in Torres de la Cruz et al. (2014) who found significant differences in pathogenicity trials and physiological characteristics between monosporic and polisporic strains of *M. anisopliae*. In a plant pathogenic fungus, Raabe (1972) also found that the parental isolate of *Armilaria* was always more virulent than their 11 single spore isolates.

Demonstrating the usefulness of polisporic isolates and/or their monosporic combinations according to their pathogenic characterization could help to develop more effective bioinsecticides against crop pests. So far, the presented results are encouraging; however, it is necessary to carry out studies on molecular similitudes or differences between the inoculated polisporic isolates and the emerged isolates from cadavers. Being able to determine if there is a degree of recombination within the host body could also help in choosing the most effective isolates.

Acknowledgements

No potential conflict of interest was reported by the authors. The study was supported by the National Council of Scientific and Technical Research (CONICET) [grant number PIP 11220110101086], and the

Argentinian Education and Sport Ministry under the "University and Cooperatives" research project (Res 2017- N° 777).

LITERATURE CITED

Aguilera Sammaritano, J.A., Lopez-Lastra, C.C., Leclerque, A., Vazquez, F., Toro, M.E., D'Alessandro, C.P., Cuthbertson, A.G.S., & Lechner, B.E. (2016) Control of *Bemisia tabaci* by entomopathogenic fungi isolated from arid soils in Argentina. *Biocontrol Science and Technology*, **26**(12), 1668-1682.

Ali-Shtayeh, M.S., Mara'I Abdel-Basit, B.M., & Jamous, M.R. (2003) Distribution, occurrence and characterization of entomopathogenic fungi in agricultural soil in the Palestinian Area. *Mycopathologia*, **156**, 235-244.

Arthurs, S., & Thomas, M.B. (2001) Effects of temperature and relative humidity on sporulation of *Metarhizium anisopliae* var. *acridum* in mycosed cadavers of *Schistocerca gregaria. Journal of Invertebrate Pathology*, **78**, 59–65.

Brady, B.L. (1979) *Verticillium lecanii* (Zimm.) Viegas. Description of pathogenic fungi and bacteria. *Commonwealth Mycological Institute*. set-61 N° 610.

Brandenburg, R.L., & Kennedy, G.G. (1982) Relationship of *Neozygites floridana* (Entomophthorales: Entomophthoraceae) to two spotted spider mite (Acari: Tetranychidae) populations in field corn. *Journal of Economic Entomology*, **75**, 691-694.

Dobersky, J.W., & Tribe, H.T. (1980) Isolation of entomogenous fungi from EUN BARK and soil with reference to ecology of *Beauveria bassiana* and *Metarhizium anisopliae*. *Transactions of the British Mycological Society*, 74, 95-100.

Fuxa, J.R. (1987) Ecological considerations for the use of entomopathogens in IPM. *Annual Review of Entomology*, **32**, 225-251.

Guarro, J., Gene, J., & Stchigel, A.M. (1999) Developments in Fungal Taxonomy. *Clinical Microbiology Reviews*, **12**(3), 454–500.

Humber, R.A. Identification of entomopathogenic fungi. (2012) *Manual of Techniques in Invertebrate Pathology*. (Ed. Lacey, L.A.), pp. 151-187. Academic Press. London, UK.

Infostat. (2013) Statistical software, professional version. Universidad Nacional de Córdoba. Córdoba, Argentina.

Jackson, M.A., Dunlap, C.A., & Jaronski, S.T. (2010) Ecological considerations in producing and formulating fungal entomopathogens for use in insect biocontrol. *Biocontrol*, **55**, 129–145.

Jaronski, S.T. (2014) Mass production of entomopathogenic fungi: State of the art. *Mass Production of Beneficial Organisms.* Chapter 11. Elsevier Inc.

Jeffs, L.B. (1999) Physical and biochemical properties of entomopathogenic fungal spores. PhD Dissertation. University of Saskatchewan. Saskatoon, Canada.

Marcelino, J.A.P. (2007) Epizootiology and phylogenetics of entomopathogenic fungi associated with *Fiorinia externa* Ferris (Hemiptera: Diaspididae) in the northeastern USA. PhD Dissertation. The University of Vermont.

Moura-Mascarin, G., Guarín-Molina, J.H., Arthurs, S.P., Humber, R.A., de Andrade Moral, R., Demétrio, C.G.B., & Delalibera Jr., I. (2016) Seasonal prevalence of the insect pathogenic fungus *Colletotrichum nymphaeae* in Brazilian citrus groves under different chemical pesticide regimes. *Fungal Ecology*, **22**, 43-51.

Posada, F., & Vega, F. (2005) A new method to evaluate the biocontrol potential of single spore isolates of fungal entomopathogens. *Journal of Insect Science*, **5**(37), 1-10.

Raabe, R.D. (1972) Variation in pathogenicity and virulence in single-spore isolates of *Armillaria mellea. Mycologia*, 64, 1154-1159.

Ríos-Velasco, C., Cerna-Chávez, E., Sánchez-Peña, S., & Gallegos-Morales, G. (2010) Natural epizootic of the entomopathogenic fungus *Nomuraea rileyi* (Farlow) Samson infecting *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Coahuila. México. *The Journal of Research on the Lepidoptera*, **43**, 7-8.

Saito, T., Takatsuka, J., & Shimazu, M. (2012) Characterization of *Paecilomyces cinnamomeus* from the camellia whitefly, *Aleurocanthus camelliae* (Hemiptera: Aleyrodidae), infesting tea in Japan. *Journal of Invertebrate Pathology*, **110**, 14-23.

Samšiňáková, A., & Kálalová, S. (1983) The influence of a single-spore isolate and repeated subculturing on the pathogenicity of conidia of the entomophagous fungus *Beauveria bassiana*. *Journal of Invertebrate Pathology*, **42**, 156-161.

Seema, Y., Neeraj, T., & Krishan, K. (2013) Mass production of entomopathogens *Beauveria bassiana* and *Metarhizium anisopliae* using rice as a substrate by diphasic liquid-solid fermentation technique. *International Journal of Advanced Biological Research*, **3**, 331-335.

Skinner, M., Parker, B.L., & Kim, J.S. (2014) Role of Entomopathogenic Fungi in Integrated Pest Management. *Integrated Pest Management: Current Concepts and Ecological Perspective* (Ed. Abrol, D.P.), pp. 169-191. Academic Press.

Torres de la Cruz, M., Cortez-Madrigal, H., Ortiz-García, C.F., Cappello-García, S., & Pérez de la Cruz, M. (2014) Monosporic strains of *Metarhizium anisopliae* and its pathogenicity to *Galleria mellonella* in Tabasco, México. *Revista Mexicana de Ciencias Pecuarias*, **5**, 171-180.

Trotter, J.R., Darban, D.A., Gowen, S.R., Bishop, A., & Pembroke, B.H. (2004) The isolation of a single spore isolate of *Pasteuria penetrans* and its pathogenicity on *Meloidogyne javanica*. *Nematology*, **6**(4), 463-471

Vega, F.E., Meyling, N.V., Luagnsa-ard, J.J., & Blackwell, M. (2012) Fungal entomopathogens. *Insect Pathology* (ed. Vega F.E., & Kaya, H.K.), Second Edition. Academic Press.

Wraight, S.P., Inglis, G.D. & Goettel, M.S. (2007) Fungi. *Field Manual of Techniques in Invertebrate Pathology*. (ed. Lacey, L.A., & Kaya, H.K.), Chapter IV – pp. 223-248. Springer, Dordrecht, The Netherlands.

Author notes

aguilerasammaritanoj@unsj.edu.ar