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

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Hongos entomopatógenos: ¿Son los aislamientos polisporícicos más patógenos que las cepas monosporíficas?

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 monosporíficas (cultivo en masa de microorganismos que provienen de una sola espora) Las principales razones para el uso de cultivos monosporíficos en lugar de cultivos polisporíficos son: la virulencia atenuada y los posibles “contaminantes” de la misma especie. En este estudio, diferentes aislamientos polisporíficos y sus combinaciones monosporíficas se pusieron a prueba para evaluar su eficacia respecto a larvas de \textit{Tenebrio molitor} (L.) como insecto modelo. Los aislamientos polisporíficos de hongos entomopatógenos fueron obtenidos de muestras de suelo de regiones agrícolas áridas. Se seleccionaron cuatro aislamientos polisporíficos de \textit{Metarhizium} sp. (Metschn.) (CEP413, CEP589, CEP590 y CEP591) para los bioensayos. Se realizaron pruebas de patogenicidad para evaluar la mortalidad en tres tratamientos, Polisporico completo (FP), Polisporico parcial (PP) y Monosporico 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 polisporíficas y monosporíficas para aumentar la eficacia de las aplicaciones es crucial para ayudar a expandir el uso de bio-insecticidas basados en hongos entomopatógenos.
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; Rios-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 x 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; chloranaphenicol 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 10⁷ c/mL from each isolate were mixed in a complete suspension and adjusted to 1 x 10⁸ 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 10⁷ 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 10⁵ c/mL from each monosporic isolate were mixed in a complete suspension and adjusted to 1 x 10⁷ 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 10⁷ 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).

According to the number of sporulated larvae (Fig. 2), at day six the highest proportion (92.4 ± 7.95%) was

![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.](image-url)
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.

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**LITERATURE CITED**


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.
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