Agronomy Research **16**(5), 1921–1930, 2018
https://doi.org/10.15159/AR.18.197

Evaluation of rice by-products, incubation time, and photoperiod for solid state mass multiplication of the biocontrol agents *Beauveria bassiana* and *Metarhizium anisopliae*

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Abstract. The success of biological control of insect pests depends not only on the isolation, characterization, and pathogenicity, but also on the success of the mass production of the microbial agents. The biological control strategy using entomopathogenic fungi like *B. bassiana* and *M. anisopliae* can only be useful if practical and economic methods of mass multiplication are available. Rice by-products like broken rice grains, rice hulls and their combination was evaluated for solid state multiplication of *B. bassiana* and *M. anisopliae*. The influence of photoperiod and incubation time in the production of conidia was also evaluated. This study showed that, broken rice was the most productive substrate for conidial production of both fungal genera, with a yield of 4.62×10^7 and 2.22×10^6 conidia g^{-1} respectively. Also, under the evaluated solid state multiplication conditions, the best conidia production was achieved with a photoperiod of 24 h of light for *B. bassiana* (with 4.43×10^7 conidia g^{-1}) and *M. anisopliae* (with 1.35×10^6 conidia g^{-1}). The results here demonstrated that these two fungal species could viably be multiplied with good yields of conidia on agro-industrial by-products using solid-state culture and regulating some culture conditions.

Key words: entomopathogenic fungi, solid substrates, light, incubation time, propagule, production.

INTRODUCTION

The scenario of pests' treatment in developed agricultures has been changed to an integrated management especially after the development of resistance in pests, the resurgence of pest outbreaks, and different environmental issues with pesticides. The integrated management includes the use of cultural, biological, biotechnical, mechanical and physical methods, and more innovative microbial pesticides (Blanco-Metzler, 2004). In this scenario, entomopathogenic fungi are frequently employed as biocontrol agents reducing insect pest populations in different agro-ecosystems (Bradley et al., 1992; Inglis et al., 2001).

The entomopathogenic fungi have unique mechanisms of invasion, persistence, and propagation that characterize them as excellent agents of biological control against

different insect pests (Charnley, 1997; Shah & Pell, 2003; Santos et al., 2007; Hajek & Delalibera, 2010). Entomopathogenic fungi that are being studied most for the biological control of insect pests are *Metarhizium anisopliae*, *Beauveria bassiana*, *Lecanicillium lecanii*, among others (Lecuona, 1996; Wraight et al., 2000; Butt et al., 2001; Faria & Wraight, 2007).

The success of biological control of insect pests depends not only on the isolation, characterization, and pathogenicity but also on the successful mass production of the microbial agents (Sahayaraj & Namasivayam, 2008).

Similar, for the development and use of a biological pesticide based on fungi, large amounts of inoculum of the biocontrol agent are required for field application (Ibrahim et al., 2002; Babu et al., 2008; Pham et al., 2009; Gao, 2011). Hyphae (biomass) and conidia of fungi are the main infective fungal structures used in biocontrol strategies (James, 2001; Jaronski, 2014; Mascarin & Jaronski, 2016; Jaronski & Mascarin, 2017).

The biological control strategy using entomopathogenic fungi could only be useful if practical and economic methods of mass multiplication are available (Kleespies & Zimmermann, 1992; Pham et al., 2009). However, only a limited number of methods of mass production for some fungi are being studied, developed and updated. Commercially, the most used method for mass production of biocontrol fungi is the fermentation in standard media (Thakre et al., 2011). The fermentation in solid substrates like low-cost agriculture by-products is a prominent method, especially in emerging countries (Prakash et al., 2008; Jaronski, 2014). Currently, solid substrate fermentation of fungi with agriculture by-products and conditions like incubation time and photoperiod remains mainly studied independently.

The multiplication in solid substrates has generated great interest due to advantages of economic and ecological importance that it offers in comparison with the liquid culture, among which we can mention: the use of solid support for microorganisms, low demand of water, simulation of the natural environment, lower sterility requirements, easy aeration using small batches, high productivity, among other features (Chahal, 1985; Deschamps & Huet, 1985; Acuña et al., 1995; Polizeli et al., 2005; Rodríguez & Sanromán, 2005; Ruiz-Leza et al., 2007; Prakash et al., 2008). In addition, this type of multiplication offers the possibility of using substrates that are abundant and cheap as waste and by-products of food or forest industries (Hölker & Höfer, 2004).

In the evaluation of solid substrates for the mass production of fungi, several authors have studied a variety of plant materials like rice grains, broken rice, rice bran, rice husk, barley, cassava chips, sugarcane bagasse, wheat, wheat bran, among others, with different results (Dorta et al., 1996; Taylor et al., 2013; Jaronski, 2014). Also, mass production of entomopathogenic fungi is dependent on different factors, such as the isolates selected, inoculum density and diverse environmental conditions like photoperiod and incubation time (Taylor et al., 2013).

The present study was undertaken to evaluate combinations of different rice milling by-products for mass production of three different strains of *B. bassiana* and two strains of *M. anisopliae*. It was also evaluated the effect of light and incubation time in the conidial production of strains of those entomopathogenic fungi.

MATERIALS AND METHODS

Three strains of *B. bassiana* sensu lato (accession numbers LBM216, LBM211, and LBM192) and two strains of *M. anisopliae* sensu lato (LBM218, and LBM217) were used in the evaluation of solid mass production of entomopathogenic fungi. These fungal strains are deposited in the culture collection of the Universidad Nacional de Misiones.

Three different treatments with locally available substrates were evaluated in the solid state multiplication of entomopathogenic fungi in small scale evaluations. The evaluated treatments comprised 15 x 30 cm polypropylene bags containing either 100 g of broken rice grains, 100 g of rice hulls or a combination of 50 g of broken rice grains and 50 g of rice hulls. Each bag opening was arranged with cotton plugs for better inoculation, aeration, and sampling under aseptic conditions.

After soaking the substrate with 30 mL of distilled water, the bags were autoclaved at a 15-psi pressure at 121 °C for 30 min (Prakash et al., 2008; Pham et al., 2010; Jaronski, 2014). After cooling, the clumps of the substrates were broken and 1 mL of a conidial solution with a concentration of 10^7 conidia mL^{-1} was added. Each bag was inoculated with a single strain of entomopathogenic fungi. This procedure was carried out under aseptic conditions. Each bag (treatment) was thoroughly agitated for proper distribution of the conidia. Three replicates were maintained for each treatment.

The polypropylene bags were incubated at 28 ± 1 °C and high humidity level (> 80%) for 28 days after inoculation with the entomopathogenic fungi. The samples were taken every seven days for determination of the number of conidia produced.

Also, the influence of light (photoperiod) in the production of conidia was evaluated and three types of photoperiods were considered: 24 h of light, 12 h of light followed by 12 h of dark, and 24 h of dark. The supplementary light was provided by a white light tube at 20 cm (6500 K, 18 w) and the light/dark periods were regulated by a Zurich XTIM03205 digital timer.

To determine the conidia produced by each treatment, the conidia were harvested by suspending under aseptic conditions one gram of each substrate in 10 mL of sterile distilled water containing 0.1% Tween 80 (v v^{-1}) as surfactant agent (Gandarilla et al., 2013; Ibrahim et al., 2015). The number of conidia produced was determined microscopically from each replicate with a Neubauer hemocytometer at 400 x magnification (Alves & Faria, 2010).

The analysis of variance (ANOVA) was carried out using the Statgraphics Centurion XV program (Statpoint). In addition to the tests of overall significance with ANOVA, the Tukey's HSD test was used to check significant differences between the variables with a confidence level of 95%. All figures were generated using the Statgraphics Centurion XV program (Statpoint) by analizing the data of two factor at time (interaction plots).

RESULTS AND DISCUSSION

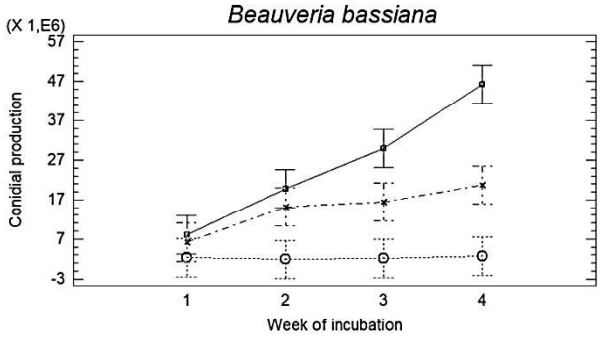
Various fermentation containers such as conical flasks, Petri's plates, tubes, trays, and plastic bags can be used for the mass production of entomopathogenic fungi (Wraight et al., 2001; Jaronski, 2014). One of the advantages of solid multiplication using plastic bags is the possibility of breaking the substrate clumps formed and in some cases the use of light for optimal sporulation (Jaronski, 2014).

In our study, mass production potential of *B. bassiana* and *M. anisopliae* were assessed (Fig. 1). Conidial production among different strains of the same species of entomopathogenic fungi (*B. bassiana* or *M. anisopliae*) showed only small, statistically insignificant differences ($F = 2.14$, $df = 2$, $p = 0.12$; and, $F = 2.75$, $df = 1$, $p = 0.1$; respectively).

Figure 1. Small-scale plastic bag-based mass production of *B. bassiana* and *M. anisopliae*.

However, strains of *Beauveria* produced higher amounts of conidia per gram of substrate than strains of *Metharizium*. The results indicated that the sporulation of these fungi differed significantly among different substrates ($F = 133.8$, $df = 2$, $p = 0$, for *Beauveria*; and $F = 141.6$, $df = 2$, $p = 0$, for *Metarhizium*). Highest sporulation was recorded after four weeks of incubation on broken rice for both fungi, with a mean value of 4.62×10^7 ($\pm 0.2 \times 10^7$) conidia g^{-1} for *B. bassiana* and 2.22×10^6 ($\pm 0.09 \times 10^6$) conidia g^{-1} for *M. anisopliae* (Fig. 2).

a)



b)

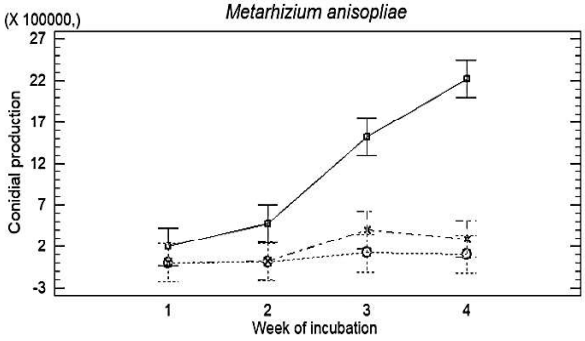


Figure 2. Solid multiplication of the entomopathogenic fungi *B. bassiana* (a) and *M. anisopliae* (b) on different solid substrates. Treatments: —□— 100% Broken rice, – ✕ – 50% Broken rice: 50% Rice hulls. ··○·· 100% Rice hulls.

The evaluated treatments (substrates combinations) differed significantly with respect to sporulation for both entomopathogenic fungal genera. In all treatments broken rice obtained the highest amounts of sporulation for *B. bassiana* and *M. anisopliae* strains. Always the lowest sporulation was recorded on rice hulls (both for *B. bassiana* and for *M. anisopliae* strains) followed by an intermediate sporulation of the combination of 50% of broken rice and 50% of rice hulls treatments.

Values comparable to the present study were reported by Sahayaraj & Namasivayam (2008) with a production close to 1.1 x 10^7 conidia g^{-1} of substrate, and in their work they proposed rice grains as the most suitable substrate for the mass multiplication of *B. bassiana*. Latifian et al. (2013) evaluated the solid state multiplication of *B. bassiana* on different plant materials, including sugarcane, corn, barley, rice, millet, and sorghum. They found that a selected strain of *B. bassiana* (IRAN441c) recorded a maximum of conidia production of 6.24 x 10^4 conidia g^{-1} on rice.

Nonetheless, better spore production has been reviewed and commented by Bradley et al. (1992) and Bradley et al. (2002) on different substrates, e.g., barley, where selected *Beauveria* strains produced in the order of 2.6 x 10^{10} conidia g^{-1} on culture reactors.

Babu et al. (2008) reported that conidial production of the fungus *M. anisopliae* on rice (amended with yeast extract) was significantly greater than on other solid plant substrates, with a mean value of 1.1 x 10^9 conidia g^{-1} of substrate. When multiplying *M. anisopliae* on rice in conical flasks Latifian et al. (2014) recorded a maximum of conidial production of 2.8 x 10^6 conidia g^{-1}. Loera et al. (2016) using rice grains as the only substrate for the production of conidia with a selected strain of *M. anisopliae* in plastic bags managed to obtain about 1 x 10^9 conidia g^{-1} of substrate.

Some authors maintain that the structure of the substrate is as important as the availability of nutrients and that an ideal substrate should provide a large surface area to favor aeration and formation of conidia (Lomer & Lomer, 2008; Machado et al., 2010; Mascarin et al., 2010). Rice hull is a by-product of the rice industry, which has more surface area per gram than the rice grain. However, in the present work any of the rice hulls combinations as a solid mass multiplication substrate produced fewer conidia per gram of substrate than the rice grain for the fungal strains evaluated. This could be due to the fact that rice hulls have few nutrients or little availability of the same for the fungal strains. So, even if rice hull is a by-product of rice milling cheaper than the broken rice, the proportion of nutrient in broken rice is higher, making the last a better option for mass multiplication of biocontrol fungi.

Also, different published protocols of mass multiplication use additives such as *Torula* yeast extract or sugarcane molasses to bypass the need of nutrients of some agricultural substrates and increase the production of conidia (Prakash et al., 2008; Sene et al., 2010; Jaronski, 2014, Mishra et al., 2016). Thus the use of additives could be one possible option to optimize the production the conidia of these entomopathogenic fungal strains in further studies.

We also observed that the incorporation of light has a significant positive effect in the production of conidia by *B. bassiana* ($F = 159$, $df = 2$, $p = 0$) and *M. anisopliae* ($F = 29.1$, $df = 2$, $p = 0$) (Fig. 3). Also, 24 h of light incubation showed higher production of conidia than the treatments with a photoperiod of 12 h of light followed by 12 h of dark, and 24 h of dark.

With respect to the influence of the photoperiod, Kuźniar (2011) and Zhang et al. (2009) observed that exposure to light increased the growth and sporulation of

B. bassiana. Similarly, Onofre et al. (2001) proposed that continuous illumination gave them 2.5 to 5-fold more conidia production of *M. flavoviride*. Oliveira et al. (2017) found that *M. robertsii* grown under blue light produce more conidia than the fungus grown in the dark. Also, they found that white light induced the production of conidia in *Metarhizium* that germinated faster and were more virulent to insects, which is a key factor when the aim is to produce high amounts of fungal propagules (Ibrahim et al., 2002).

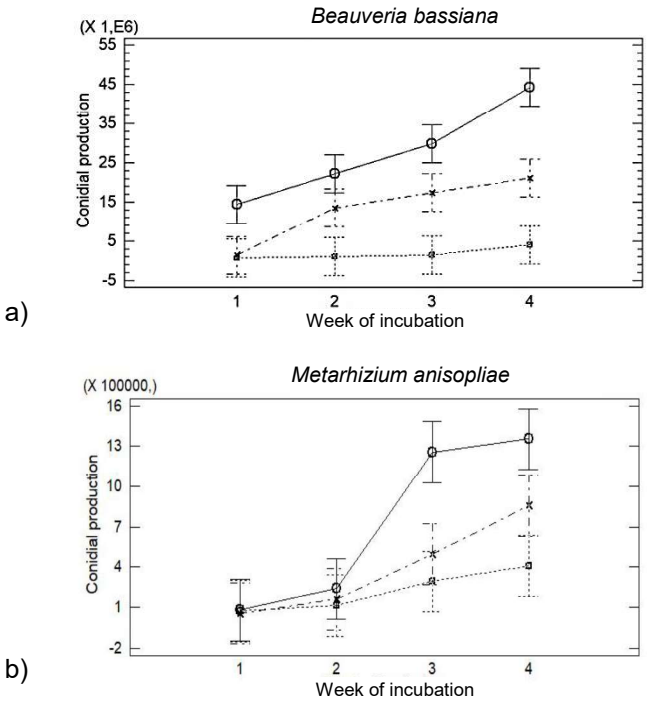


Figure 3. Conidial production by the entomopathogenic fungi *B. bassiana* (a) and *M. anisopliae* (b) with different photoperiods. Treatments: —□— 24 h of light. – ✕ – 12 h of light / 12 h of dark. ···○··· 24 h of dark.

However, Bradley et al. (1992) suggested high conidial production of various strains of *B. bassiana* in a completely dark fermentation environment; or Rangel et al. (2011) who evaluated the growth and sporulation of a strain of *M. robertsii*, and observed that the sporulation of the fungus was equivalent under conditions of continuous light or darkness. Therefore, the requirement of a parameter such as light may be a requirement of each fungal strain rather than a general rule.

Similar to the results above, in the simultaneous evaluation of the influence of the factors solid substrates and photoperiod on mass production of the entomopathogenic fungi *B. bassiana* and *M. anisopliae* the best combination was broken rice and 24 h of light ($F = 39$, $df = 4$, $p = 0$; and, $F = 9.09$, $df = 4$, $p = 0$; respectively) (Fig. 4).

Small and medium-scale conidia production varies according to different key parameters like substrate used, pH, temperature, moisture, light, aeration (structure of the substrate), different additives, among others, and optimal conditions must be evaluated for each entomopathogenic fungal species, and even each particular strain (Mascarin et al., 2010; Mar & Lumyong, 2012; Taylor et al., 2013; Muñíz-Paredes et al.,

2017). Further studies with our fungal strains could be deepened in the assessment of mass production on different rice structures or conformations like the grain size or parboiled rice.

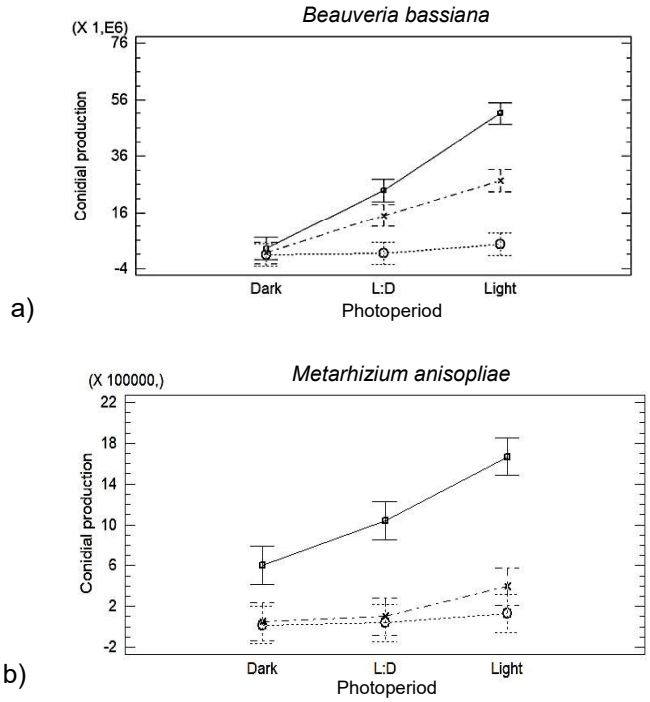


Figure 4. Influence of solid substrates and photoperiod evaluated simultaneously on mass production of the entomopathogenic fungi *B. bassiana* (a) and *M. anisopliae* (b). Treatments: —□— 100% Broken rice. – ✕ – 50% Broken rice: 50% Rice hulls. ··○·· 100% Rice hulls.

CONCLUSIONS

The data of this study showed that broken rice substrate and incubation with 24 h of light were better conditions for mass production of aerial conidia of different strains of *B. bassiana* and *M. anisopliae*. The substrates and parameters evaluated in this study will be a promising strategy even for medium-scale production of conidia for mycoinsecticides with low costs.

For all the above, the results of the present work confirm that each fungal strain has optimal conditions for mass multiplication. In addition the results obtained provide information for a better understanding of key nutritional requirements and culture conditions that can improve the mass production of *Beauveria* and *Metarhizium*. This information can be useful even to small-scale farmers with basic infrastructure to culture these biocontrol fungi easily.

ACKNOWLEDGEMENTS. The authors are sincerely thankful to the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) Argentina for postdoctoral fellowships of Castrillo and Bich.

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