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Mass production studies in solid substrates with the entomopathogenic fungus, *Purpureocillium lilacinum*

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ABSTRACT

The objective of this study was to evaluate several substrates to determine the optimal condition of entomopathogenic mass production with the purpose of developing a mycoinsecticide to be used for an integrated pest management. Conidial quality and yield for two isolates of *Purpureocillium lilacinum* were determined. For quality parameters assessment, germination and purity percentage and viability of conidia were determined. For the production parameters, the conidial yield was calculated. The main results obtained show the type of substrate that had the greatest effect on the viability, germination and yield of conidia of *P. lilacinum*. The substrate that presented the highest production of conidia per gram was rice, showing 2.1×10^9 for isolate JQ926212 and 1.6×10^9 for JQ926223. In the particulate substrates, the percentage purity was about 99%, while in powder substrate, the percentage purity was low with values ranging from 23 to 75%. The highest percentage of moisture content was about 40% for rice and wheat, while for maize meal and vermiculite the percentage was close to 20%. In conclusion, *P. lilacinum* can be mass multiplied in rice grains and vermiculite.

INTRODUCTION

This study began when the authors started looking for an alternative method of preventing the damage caused by insect pest in stored maize grains. The damage, caused by insect pest in stored grains, act as reservoirs of disease and distribution of microorganisms (White, 1995), like toxigenic *Aspergillus flavus* (Nesci et al., 2011). The interaction of these toxigenic fungi with substrate and abiotic predisposing factors may promote a moldy substrate (Barra et al., 2013b) and the accumulation of the mycotoxin carcinogenic aflatoxin B_1 (IARC, 1993), in stored grains.

The development of resistance to chemical insecticides and concerns about the harmful effects of chemicals on the environment, human and animal safety are the main causes for the development of alternative strategies for integrated pest management. One of these strategies in research and development is biological control. After isolation of the potential biocontrol agents from the same ecosystem in which they must be effective (Lacey et al., 1996) and pathogenicity against the target pests is confirmed, a suitable quantity of good quality inoculum of the biocontrol agent is the next stage of the biocontrol program (Jenkins et al., 1998).

Previously, the entomopathogenic fungus *Purpureocillium lilacinum* JQ926212 and JQ926223 was isolate from the soil, which showed pathogenicity against most important insect pests in stored maize (Barra et al., 2013a). *P. lilacinum* (Thom) Samson is a soil saprophyte and has shown promise for use as a biocontrol agent (Khan et al., 2006). Therefore, there is a need for further progress in this line of research, continuing the study of

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the mode of application of this prevention strategy in stored maize.

The propagules of the entomopathogenic fungi responsible for the dispersion and infection under natural conditions are aerial conidia (Wraight et al., 2001). Applications by spray designed to deposit fungal spores more or less uniformly over a field or a crop is not necessarily the most efficient mode of application of the entomopathogenic fungal inoculum on stored grains. A granular formulation contained in a trap, attracting insects to feed on it or enter to be inoculated with spores of the entomopathogen and then released to spread the pathogen to the rest of the population, a process known as autodissemination (Wraight and Carruthers, 1999), can be considerably more effective. Because the intention is not to spray the grains with the entomopathogenic fungus to make contact with the insect pests, the infectious units must survive until the insect inadvertently contacts the fungal inoculum or is attracted to a trap. In this situation, granular formulations can provide the necessary protection to ensure the survival of the entomopathogen (Pereira and Roberts, 1991; Wraight and Ramos, 2002).

The development of a simple and reliable production system follows basic multiplication procedures using a two stage system in which fungal inoculum formed by mycelium or hyphal bodies in liquid culture is produced, either in shake flasks or fermenters. After that it is transferred to a solid substrate for production of conidia (Jaronski and Jackson, 2012).

Fungal enzymes that hydrolyze proteins and chitin, which represent the main components of the insect cuticle, are considered vital for the infection process (Fang et al., 2009). It is known that enzymatic activity is directly related to fungal growth, which shows that mycelial growth is crucial for an adequate production of different extracellular enzymes (Carlsen et al., 1996). To determine the optimum physiological acclimatization conditions of the entomopathogenic fungus, it is important to find the best substrate to achieve the highest growth. It is also important to compare whether these optimal conditions agree with the increased production of enzymes which could improve the pathogenicity and virulence of the entomopathogenic fungus (Barra et al., 2015).

There is a wide range of solid substrates available for use in the production of fungi in a biological control process. To achieve maximum sporulation, a good surface/volume ratio is essential. An ideal substrate should not only contain particles of the correct dimensions, but also maintain its structural integrity during preparation for the production process (Lopez-Perez et al., 2015).

According to Jenkins et al. (1998), the most commonly selected substrate for the production of entomopa-thogenic fungi conidia have been rice grains. Other authors have supported this situation (Tarocco et al., 2005; Ye et al., 2006; Posada-Florez, 2008), probably related to a combination of factors including nutritional balance, cost, worldwide accessibility, including physical characteristics that influence culture conditions such as grain size and shape, hydration properties and structural integrity even after colonization by fungi.

It is therefore essential to determine the best substrate for spore production and their viability. The success of microbial control of insect pests depends not only on the isolation, characterization and pathogenicity, but also on the successful mass production of the microbial agents in the laboratory. For a successful integrated pest manage-ment program, the agents like the entomopathogenic fungi should be according to easy and cheap mass multiplication. It is important to determine if the studied fungus is able to develop on a variety of cheap and easily available grains.

The objective of this study was to evaluate several solid substrates to determine the optimal condition for conidial production.

Conidial quality and yield for two isolates of *P. lilacinum*, previously selected by its pathogenicity against the most important insect pest vectors of aflatoxigenic fungi in stored maize were determined.

MATERIALS AND METHODS

Fungal isolates

P. lilacinum isolates JQ926212 and JQ926223 were used in these experiments. These entomopathogenic isolates were recovered from soil samples collected from the University of Río Cuarto Experimental Field Station in Río Cuarto, Córdoba, Argentina, and were identified and deposited in GenBank (Barra et al., 2013a). These isolates are held in the Microbial Ecology Laboratory Collection, Microbiology and Immunology Department of the National University of Río Cuarto, Córdoba, Argentina. Both isolates showed pathogenicity against *Tribolium confusum*, *Rhyzopertha dominica* and *Sitophilus zeamais*, three insect pest vectors of aflatoxigenic fungi in stored maize (Barra et al., 2013a). Moreover, both *P. lilacinum* isolates are compatible with food grade antioxidants, enhancing their insecticidal and/or fungicidal effect (Barra et al., 2013b).

Previously the pathogenicity of both isolates was increased by passage through the insect host according methodology of Fargues and Robert (1983). The fungi grew on synthetic hydrocarbon-enriched media for stimulate the pathogenicity against the insects. Then it was cultivated under optimal conditions to achieve the physiological improvement as described previously in Barra et al. (2015).

Evaluation of conidial production in several solid substrates

The particulate rice, wheat and vermiculite substrates and powder substrates maize meal and oat bran were used. Fifty grams of each substrate were placed in 20 × 40 cm polypropylene bags and 26.45, 23.41, 16.14, 26.52 and 13.38 mL of distilled water were added in order to reach 45% relative humidity (RH) in rice, wheat, maize meal, oat bran and vermiculite respectively. In order to allow the space and oxygen for fungal growth, bags were half-filled with the substrate. The bags were sealed and autoclaved for 20 min at 121°C and then inoculated. An aliquot of 1 mL of 1 × 10^7 spores mL^{-1} suspension (physiological improved previously) was used to inoculate each bag in a laminar flow chamber. They were closed and vigorously manually shaken for uniform distribution of the propagules in the substrate. After inoculation, the bags were incubated at 25±1°C with 12:12 h light:dark cycle for 7 days. Three replicates were done for each substrate.

To determine the production parameter, the conidial yield was calculated. From each substrate contained in bags, 1 g of the fungus-colonized substrate was taken and homogenized with 9 mL of 0.01% tween 80 in water. Conidia were separated from each substrate by filtering the resulting mixture through a double layer of muslin. Serial dilutions were performed and the number of conidia g^{-1} of each solid substrate was quantified using a Neubauer chamber (Espinel et al., 2008).

Quality parameters determinations

For quality parameters assessment, germination and purity percentage and viability of conidia were determined.

Conidial germination was examined according the method adapted from Smith and Edgington (2011). Ten grams of the fungus-colonized substrate was suspended in 90 mL 0.1% peptone water solution. Serial dilutions from each sample were performed and aliquots of 1 mL from 10^{-3} dilution were centrifuged at 1.3226 *g* for 10 min. 5 µL droplet of the supernatant was inoculated onto Petri dishes containing 0.1% water agar medium. After 24 h incubation at 25±1°C with 12:12 h light:dark cycle one drop of lactophenol cotton blue and then a coverslip were placed on the plates. Germination was observed at 400 x magnification in an optical microscope. For each plate four microscopic fields, each containing a minimum of 100 conidia was evaluated. For this assay, spores were considered to have germinated when the germ tube was longer than the diameter of the spore, and a test was considered positive when at least 10% of spores had germinate. Each assay was repeated three times.

The purity percentage was evaluated on the same times and under the same conditions as for the conidial viability assay. Ten grams of the fungus-colonized substrate was suspended in 90 mL 0.1% peptone water solution. Serial dilutions from each sample were performed and aliquots of 0.1 mL from 10^{-4} dilution for purity assay and from 10^{-6} to 10^{-10} for viability assay was spread on Sabouraud Dextrose Agar (SDA) (trypticase 5 g l^{-1}, peptone beef 5 g l^{-1}, glucose 20 g l^{-1}, agar–agar 15 g l^{-1}, water 1.000 ml) or PDA (dextrose 10 g l^{-1}, potato 200 g l^{-1}, agar-agar 15 g l^{-1}) with the addition of chloramphenicol 0.5% (w/v) respectively. The plates were incubated at 25±1°C with12:12 h light:dark cycle for 7 days for purity and 15 days for viability determinations. Moreover, for each evaluates substrate 10 g of uncolonized substrate was used as control treatment. The counting of fungal propagules was recorded as CFU g^{-1} for each substrate. Each assay was repeated three times. The conidial viability was expressed as $\log_{10}$ g^{-1} of substrate. The purity percentage (% P) was calculated as:

% P = (CFU of the evaluated fungus / total CFU) × 100.

Substrates parameters

The fungus-colonized solid substrates were taken from the bags and drying using a stove at 80-90°C until constant weight to evaluate the moisture of the substrate at the end of the incubation period. The following formula was then applied:

100 - (Dry weight remaining substrate *100 / initial weight of the remaining substrate).

Statistical analyses

The data were subjected to ANOVA using InfoStat program (Di Rienzo et al., 2008). Means of quality, productivity and substrates parameters were compared using Tukey test ($p < 0.05$).

RESULTS AND DISCUSSION

Statistical analyses on conidial viability and conidial germination for *P. lilacinum* mass production on several solid substrates were determined. The most significant effect was that of substrate (F=809.60). Conidia of both isolates germinate within 20–24 h on rice and vermiculite substrates, and at 7 days of incubation presented values of conidial germination higher than 90% (Figure 1a and b). In these conditions we observed good conidial viability ranged from 8.5 to 12 log according to the different substrates assayed (Figure 1c and d). The values of

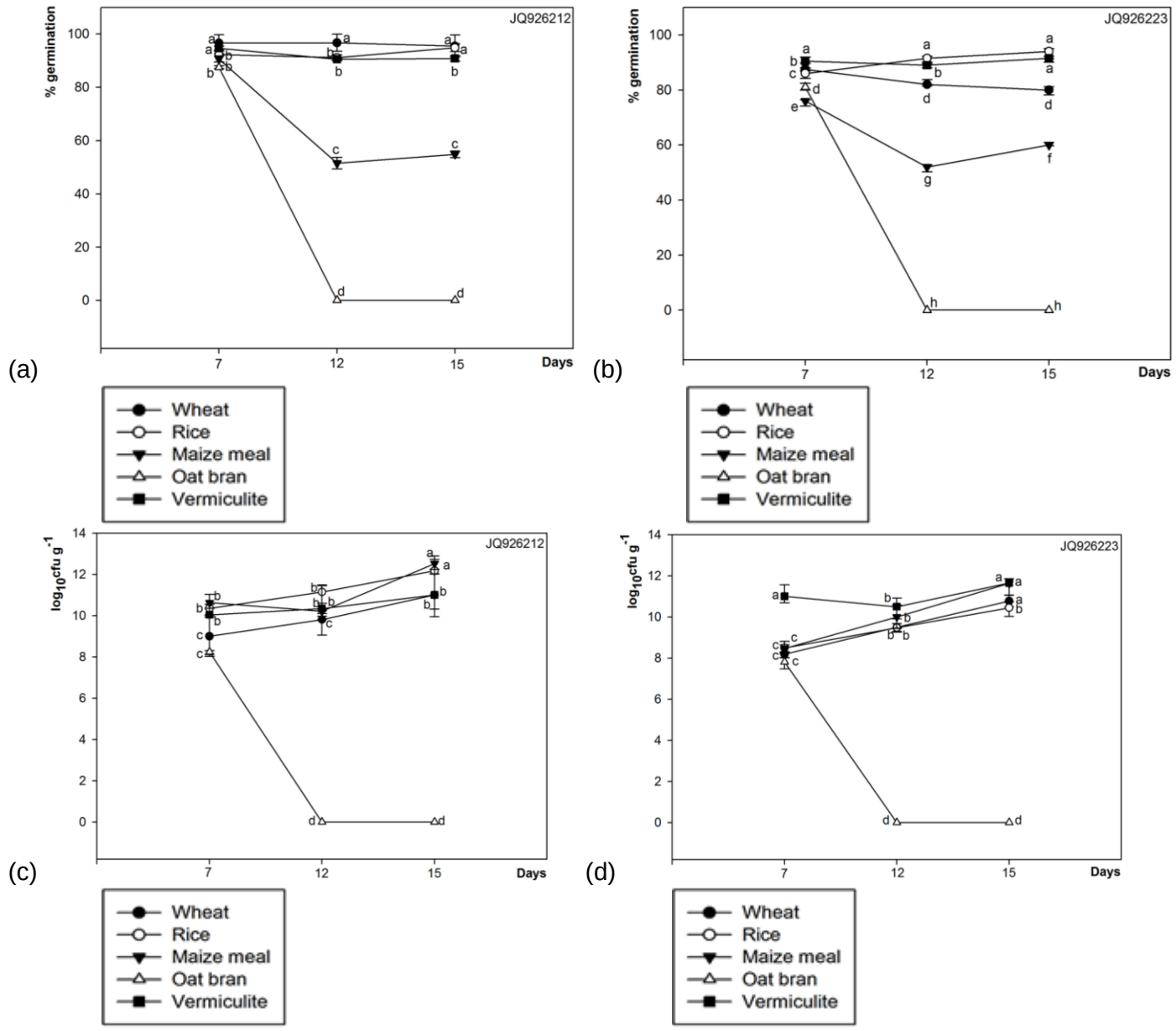

Figure 1. a and b, Effect on conidial germination percentage; **c and d,** conidial viability of two isolates of *P. lilacinum* using several solid substrates and different incubation times. Data with different letters for the same incubation and different substrate are significantly different based on Tukey test ($p<0.05$).

conidial germination percentage and viability decrease significantly ($p<0.05$) with the nutritive powder substrates. Maize meal and oat bran showed high percentage of contamination at the end of incubation period. The germination percentage measured through a protocol based on a short incubation period is a more consistent predictor of the potency of a conidial preparation than measures based on viability alone (Faria et al., 2015). Despite that, spore viability is widely relied upon as a measure of the quality of fungal inocula used in biological control research and applications, being a critical variable of quality control (Jenkins and Grzywacz, 2000; Goettel et al. 2000).

Moreover, the conidial production of both entomopathogenic fungal isolates on solid substrates was evaluated. Statistical analysis of the yield of conidia on the solid substrates of each isolates and their interactions were performed. The only effect was that of substrate ($F=12.09$). No significant differences between isolates ($F=0.39$, $P>0.53$) were observed. The substrate

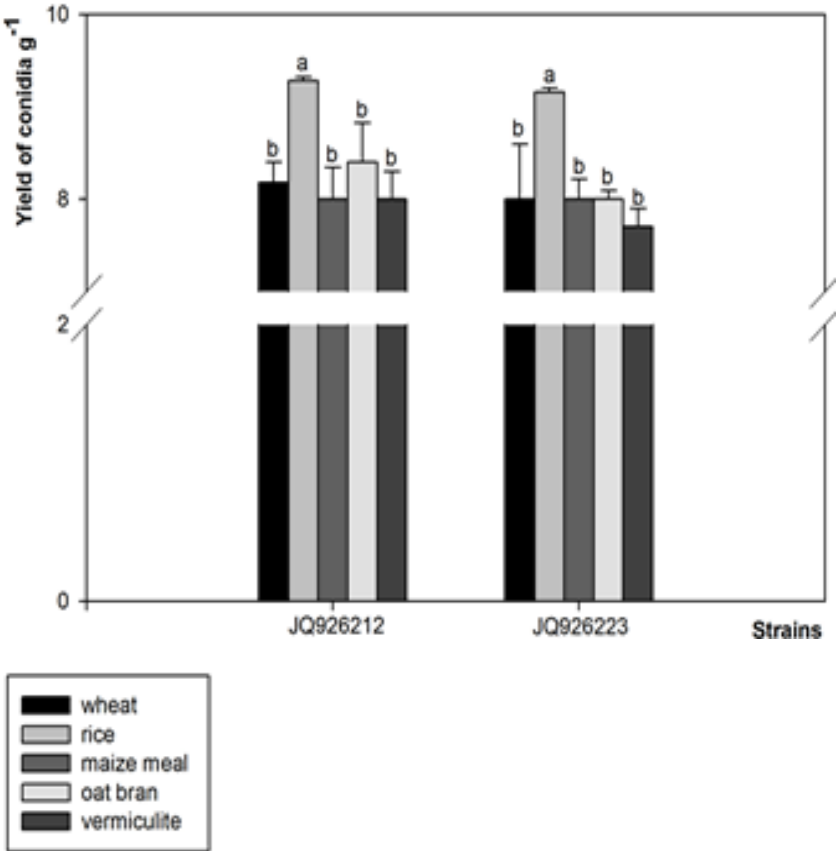

Figure 2. Conidial production of two isolates of *P. lilacinum* on different solid substrates. Data with different letters for the same incubation time and different substrate for each isolate are significantly different based on Tukey test ($p<0.05$).

that presented the highest production of conidia per gram was rice, showing 2.1×10^9 for isolate JQ926212 and 1.6×10^9 for JQ926223. In agreement with authors of other studies *P. lilacinum* has good development on a wide variety of substrates. Presoaked sorghum grains supported maximum multiplication of *Paecilomyces lilacinus* (Gogoi and Neog, 2003; Hasan, 2004; Verma et al., 2004) and other substrates like pounded rice (Khan and Goswami, 1999) and sugarcane by-products (Somasekar et al., 1998). Besides, Mar and Lumyong (2012) proved that *P. lilacinus* strains were able to maintain a high germination percentage (of 80%) after 60 days of incubation in rice, wheat, maize and sorghum. Similar observations were recorded by Amala (2012) who showed the maximum viability of *P. lilacinus* (8 log) on rice bran at 7 days of incubation. Similarly, but with other entomopathogenic genera Kruger (2014) obtained 3.72×10^9 conidia per gram of *Metarhizium anisopliae* in white rice.

Rice is the most chosen substrate for fungal conidia production. However, nutritive substrates permit far less control over the nutritional environment. This variation can affect fungal production (Jenkins et al., 1998). Therefore, an alternative is the use of synthetic materials such as amberlite or polyurethane or natural materials such as sugarcane bagasse as inert solid support (Pandey et al., 2000). In this study, high conidia yield with the inert substrate vermiculite was obtained, with counts of the order of 10^7 conidia per gram at 7 days of incubation (Figure 2). We agree that although during the mass production of entomopathogenic fungi there are important parameters that must be studied as: yield, versatility of the substrate, concentration of conidia in the final product, purity and application rate (Patil, 2011), the efficiency tests must take into account mainly the sporulation capacity of the final product (Wraight et al., 2001), that is, the percentage of germination and viability.

The projection of this work aims to conclude with the development of a trap with a unidirectional path. This trap will allow the passage of insects inside it to contaminate their bodies with a sufficient amount of conidia and then disseminate them in the insect population through indirect contamination. Therefore, small amounts of material are required for this development, unlike the production of

Table 1. Percentage of purity and moisture content of different substrates for the production of both strains at the end of the assay.

Substrate	Parameter			
	Purity percentage		Moisture content	
	JQ926212	JQ926223	JQ926212	JQ9026223
Wheat	98.00a	98.00a	39.35a	31.45a
Rice	99.80a	99.60a	34.95a	43.05a
Maize meal	75.00b	62.00b	21.10ab	25.05ab
Oat bran	23.00c	32.00c	nd	Nd
Vermiculite	100.0a	100.0a	15.70b	21.15b

large quantities of formulations that are needed in an application at field level (Mendonça, 1992; Lomer et al., 1993).

On the other hand, water availability in solid substrate fermentation system is a crucial factor, which must be critically evaluated. Water activity (a_W) of the substrate has determinant influence on microbial activity (El Damir, 2006). The appropriate moisture level in the substrate is essential for optimal mass production of entomopathogenic fungi (Young et al., 2002). Besides this, individual substrate particles should remain separate after hydration and sterilization. Substrate particles which clump together when water is added reduce the surface area to volume ratio, limiting the space on which sporulation can occur (Lopez-Perez et al., 2015). In our study, the moisture content at the end of the experiment remained the same as at the beginning of the assay when particulate nutrient substrates were used, mainly rice (45%). However, in the powdered and non-nutritive substrates the humidity showed a reduction of 50% (Table 1). Because the high percentage of moisture necessary to achieve a good production process, it is difficult to reach the end of the experience with the evaluated substrates in good conditions. This could explain the differences in germination for some isolates. For that reason, the particulate substrates showed higher percentages of purity than powder substrates. According to quality control standards of entomopathogenic fungi, the purity must be greater than 90% (Vélez et al., 1997; Bastidas et al., 2009). In this study, results using particulate substrates agree with these standards. Vermiculite showed the highest percentage of purity (100%) and with rice the purity was >99%, while in powder substrates purity percentage was low with values between 23 to 75% (data showed in Table 1).

Despite of the elevated moisture present in the rice at the end of the incubation period, the substrate was maintained unalterable and water was available for *P. lilacinum* development. This is evidenced by the absence of contaminant development. According to the results shown by Pham et al. (2010), rice with a moisture level of 40% had the highest conidial yield of *B. bassiana* (3.17×10^9 conidia g^{-1}). Their result demonstrates that conidia yield strongly depend on the moisture content of rice. In another report, moisture levels from 35 to 80% were utilized in solid fermentation, depending on the fungal strain and substrate (Raimbault, 1998).

The type of substrate and the nutritional and physical environment of the mass production system are important factors because they could greatly affect the number, type, stability, durability, pathogenicity and virulence of fungal propagules.

In conclusion the strains of *P. lilacinum* evaluated can be mass multiplied in rice grains and vermiculite. Others parameters of formulation with both substrates, to improve the applicability and effectiveness are in progress.

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REFERENCES

Amala U. (2012). Mass multiplication of entomopathogenic fungus, *Paecilomyces lilacinus* with solid substrate. J. Biopest. 5:168-170.

Barra P., Etcheverry M. & Nesci A. (2015). Improvement of the insecticidal capacity of two *Purpureocillium lilacinum* strains against *Tribolium confusum*. Insects. 6:206-223.

Barra P., Nesci A. & Etcheverry M. (2013b). *In vitro* compatibility of natural and food grade fungicide and insecticide substances with *Purpureocillium lilacinum* and their effect against *Aspergillus flavus*. J Stored Prod. Res. 54:67-73.

Barra P., Rosso L., Nesci A. & Etcheverry M. (2013a). Isolation and identification of entomopathogenic fungi and their evaluation against *Tribolium confusum*, *Sitophilus zeamais*, and *Rhyzopertha dominica* in stored maize. J. Pest Sci. 86:217-226.

Bastidas A., Velásquez S. E., Marín P., Benavides P., Bustillo A. E. & Orozco F. J. (2009). Evaluación de preformulados de *Beauveria bassiana* (bálsamo) Vuillemin, para el control de la broca del café. Agronomia. 17: 44-61.

Carlsen M., Spohr A. B., Nielsen J. & Villadsen J. (1996). Morphology and physiology of an α amylase producing strain of *Aspergillus oryzae* during batch cultivations. Biotechnol Bioeng. 49:266-276.

Di Rienzo J. A., Casanoves F., Balzarini M. G., Gonzalez L., Tablada M. & Robledo C. W. (2008). InfoStat. GrupoInfoStat, FCA: Universidad Nacional de Córdoba, Córdoba, Argentina.

El Damir M. (2006). Effect of growing media and water volume on conidial production of *Beauveria bassiana* and *Metarhizium anisopliae*. J Biol. Sci. 6:269-274.

Espinel C., Torres L., Grijalba E., Villamizar L. & Cotes A. M. (2008). Preformulados para el control de la moscablanca *Bemisia tabaci* (Hemiptera: Aleyrodidae) en condiciones de laboratorio. Rev colom Entomol. 34:351-360.

Fang W. G., Feng J., Fan Y. H., Zhang Y. J., Bidochka M. J., Leger R. J. S. & Pei Y. (2009). Expressing a fusion protein with protease and chitinase activities increases the virulence of the insect pathogen *Beauveria bassiana*. J. Invertebr. Pathol. 102:155-159.

Fargues J. F. & Robert P. H. (1983). Effects of passage through scarabeid host on virulence and host specificity of two strains of the entomopathogenic Hyphomycete *Metarhizium anisopliae*. Can. J. Microbiol. 29:576-583.

Faria M., Lopes R. B., Souza D. A. & Wraight S. P. (2015). Conidial vigor vs. viability as predictors of virulence of entomopathogenic fungi. J. Invertebr. Pathol. 125:68-72.

Goettel M. S., Inglis G. D. & Wraight S. P. (2000). Overview of pathogen groups: fungi. In: Lacey L. A., Kaya H. K. (eds). Field manual of techniques in invertebrate pathology. Kluwer Academic Publishers, Dordrecht, The Netherlands. Pp. 255-282.

Gogoi B. B. & Neog P. P. (2003). Vegetable and pulse crops – Host for culturing bacterial antagonists of root knot nematode. Ann. Plant Protect. Sci. 11:403-404.

Hasan N. (2004). Evaluation of native strains of *Paecilomyces lilacinus* against *Meloidogyne incognita* in Cowpea followed by Lucerne. Ann. Plant Protect. Sci. 12:121-124.

Jaronski S. T. & Jackson M. A. (2012). Mass production of entomopasthogenic Hypocreales. In: Lacey LA, (ed) Manual of techniques in invertebrate pathology. 2nd Edition. Academic Press, London, UK. Pp. 255-284.

International Agency for Research on Cancer, IARC (1993). Monograph on the evaluation of carcinogenic risk to humans. IARC, Lyon, France.

Jenkins N. E. & Grzywacz D. (2000). Quality control of fungal and viral biocontrol agents - assurance of product performance. Biocontrol Sci. Technol. 10:753-777.

Jenkins N. E., Heviefo G., Langewald J., Cherry A. J. & Lomer C. J. (1998). Development of mass production technology for aerial conidia for use as mycopesticides. Biocontrol News and Information. 19:21-31.

Khan A., Williams K. L. & Nevalainen H. K. M. (2006). Control of plant parasitic nematodes by *Paecilomyces lilacinus* and *Monacrosporium lysipagum* in pot trials. Biocontrol. 51:643-658.

Khan M. R. & Goswami B. K. (1999). Selection of suitable media for mass culture of *Paecilomyces lilacinus* isolates. Agric. J. India. 43:203-206.

Kruger R. D. (2014). Control microbiano de la chinchedeltallo de arroz, *Tibracalimbativentris* Stal. 1860 (Hemiptera: Pentatomidae) con hongos entomopatógenos. [PhD thesis]. Universidad Nacional de Buenos Aires.

Lacey L. A., Fransen J. J. & Carruthers R. (1996). Global distribution of naturally occurring fungi of *Bemisia*, their biologies and use as biological control agents. In: Gerling D, Mayer R (eds) Taxonomy, biology, damage, control and management, Intercept, Andover. Pp. 401-433.

Lomer C. J., Bateman R. P., Godonou I., Kpindou D., Shah P. A., Paraiso A. & Prior C. (1993). Field infection of *Zenocerus variegatus* following application of an oil based formulation of *Metarhizium flavoviridae* conidia. Biocontrol Sci. Technol. 3:337-346.

Lopez-Perez M., Rodriguez-Gomez D. & Loera O. (2015). Production of conidia of *Beauveria bassiana* in solid-state culture: current status and future perspectives. Critical Reviews Biotechnol. 35(3):334-341.

Mar T. T. & Lumyong S. (2012). Conidial production of entomopathogenic fungi in solid state fermentation. KKU Res J. 17:762-768.

Mendonca A. F. (1992). Mass production, application and formulation of *Metarhizium anisopliae* for control of sugarcane frog hopper, *Mahanarva posticata* in Brazil. In: Lomer C. J., Prior C. (eds). Biological control of locust and grasshoppers. CAB International, Wallingford, pp 239-244.

Nesci A., Barra P. & Etcheverry M. (2011). Integrated management of insect vectors of *Aspergillus* in stored maize using synthetic antioxidants and natural phytochemicals. J. Stored Prod. Res. 47:231-237.

Pandey A., Soccol C. R. & Mitchell D. (2000). New developments in solid state fermentation: I-bioprocesses and products. Process Biochem. 35:1153-1169.

Patil S. (2011) Development of entomopathogenic fungi based biopesticide technology. LAP LAMBERT Academic Publishing.

Pereira R. M. & Roberts D. W. (1991) Alginate and cornstarch mycelial formulations of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae*. J. Econ. Entomol. 84:1657-1661.

Pham T. A., Kim J. J. & Kim K. (2010). Optimization of solid-state fermentation for improved conidia production of *Beauveria bassiana* as a Mycoinsecticide. Mycobiology. 38:137-143.

Posada-Florez F. J. (2008). Production of Beauveria bassiana fungal spores on rice to control the coffee berry borer, *Hypothenemus hampei*, in Colombia. J. Insect Sci. 26:1-13.

Raimbault M. (1998). General and microbiological aspects of solid substrate fermentation. EJB Electron. J. Biotechnol. 1:1-20.

Smith C. & Edgington S. (2011). Germination at different wáter activities of similarly aged *Metarhizium* conidia harvested from ageing cultures. J. Stored Prod. Res. 47:157-160.

Somasekar N., Mehta U. K. & Hari K. (1998). Evaluation of sugarcane byproducts for mass multiplication of nematode antogonistic fungi. In: Nematology challenges and opportunities in 21st Century. Coimbatore, India: Proceedings of the Third International Symposium of Afro–Asian Society of Nematologists. Pp. 199-202.

Tarocco F., Lecuona R. E., Couto A. S. & Arcas J. A. (2005). Optimization of erythritol and glycerol accumulation in conidia of *Beauveria bassiana* by solid-state fermentation, using response surface methodology. Appl. Microbiol. Biotechnol. 68:481-488.

Vélez P. E., Posada F. J., Marín P., Bustillo A. E., González M. T. & Osorio E. (1997). Técnicas para el control de calidad de formulaciones de hongos entomopatógenos. Boletin Técnico. 17:37. Cenicafé, Colombia.

Verma A. C., Singh H. K. & Khan M. N. (2004). Evaluation of substrates for mass multiplication of *Paecilomyces lilacinus*. Ann. Plant Protect. Sci. 12:459-460.

White N. D. G. (1995). Insects, mites and insecticides in stored-grain ecosystems. In: Jayas P., White N. D. G, Muir W. E. (eds). Stored grain ecosystems. Marcel Dekker, New York, pp 123–167.

Wraight S. P., Carruthers R. I. (1999). Production, delivery and use of my coinsecticides for control of insect pests of field crops. In: Hall FR, Menn JJ (eds) Methods in Biotechnology, Vol 5, Biopesticides: use and delivery. Humana Press, Totowa, New Jersey, pp 233–269.

Wraight S. P., Ramos M. E. (2002). Application parameters affecting field efficacy of *Beauveria bassiana* foliar treatments against Colorado potato beetle *Leptinotarsa decemlineata*. Biol. Control 23:164–178.

Wraight S. P., Jackson M. A. & de Kock S. L. (2001). Production, stabilization and formulation of fungal biocontrol agents. In: Butt T. M., Jackson C., Magan N. (eds). Fungi as biocontrol agents. Progress, problems and potential. CABI Publishing, Walling Ford, UK, pp.253-287.

Ye S. D., Ying S. H., Chen C. & Feng M. G. (2006). New solid-state fermentation chamber for bulk production of aerial conidia of fungal biocontrol agents on rice. Biotechnol. Lett. 28:799-804.

Young S. E., Kwang Hee S., Dong Ha K., Ki Duk J., Cheol J., Yong Man Y. & Yong P. H. (2002). Cultivation optimization of insect-pathogenic fungi *Paecilomyces lilacinus* HY-4 to soil-pest *Adoretus tenuimaculatus*. Kor. J. Entomol. 32:133-139.