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EFFECT OF CARBON-NITROGEN IN THE NATIVE *Beauveria bassiana* PRODUCTION BY SOLID FERMENTATION IN ECUADOR

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ABSTRACT

The purpose of the work was to produce native *Beauveria bassiana* conidia at the laboratory level through Solid State Fermentation (SSF), polypropylene sleeves were used as bioreactors. The pre-cooked and sterilized substrates were: "recycled" rice and wheat, supplemented with various carbon-nitrogen sources (C-N), ratio 10: 1. The experimental design was a Categorical Multi-Factor, with 20 treatments and three repetitions to determine the variable concentration of conidia/g of bioproduct; the count of conidia was in Neubauer chamber. The mean values of controls were 1.15 × 109 Conidia/g in rice and wheat 1.22 × 109 Conidia/g with C-N supplements, the highest means of *B. bassiana* in rice was 1.89 × 109 Conidia/g (T7); in this same order in wheat 1.90 × 109 Conidia/g (T17). The lowest means presented: T10 and T20 (between 1.05 - 1.06 × 109 Conidia / g), in rice and wheat with molasses-yeast, concentration 2 (5.0-0.50 g), possibly due to an antagonistic effect between Saccharomyces cerevisiae-entomopathogenic fungi (EPF) during its cultivation. The ANOVA registered significant statistical differences (*) at ≤ p 0.05 in the factors C-N, and concentration. The Multiple Range Contrast LSD-Fisher, determined that there were no (*) between any pair of means at 95.0% confidence. It is concluded: the supplementation of C-N on rice and wheat for the mass production of *B. Bassiana*, influenced the yield of conidia/g of the bioproduct.

Keywords: *beauveria bassiana*, solid substrates, C and N, mass production.

INTRODUCTION

One of the factors that limits the production of crops are agricultural pests. The indiscriminate use of chemical insecticides has resulted in the selection of resistant individuals, the resurgence of new pests, effects on human health and the contamination of ecosystems. (Gindin *et al*., 2009; Shah and Pell., 2003).

The EPF *Beauveria bassiana* has been the subject of increasing attention, given that, it has been used as an agent for the biological control of insect pests (Jaronski, 2012), this entomopathogen has a great potential to infect a large group of arthropods (Smriti *et al*., 2015). Its management has been generalized throughout the planet as bio pesticides for the control of various insect pests. The *B. bassiana* under natural conditions is the pathogen that can often control pest insects that cause deterioration to agricultural crops and constitute the group of greater importance of biocontrol, causing the mortality of large populations of insect pests in different agro ecosystems (Escamilla et al., 2016, Faria and Wraight, 2007).

The EPF to infect the hosts produce metabolites, such as cyclic depsipeptides and hydrolytic enzymes that degrade the cuticle of the insect, arthropod plague; In addition, these fungi, being found in ecological niches with high biodiversity and competence, fulfill the role of biological controllers. *B. bassiana*, is competitive with other control practices, considering its effectiveness, cost of production and safety to the environment (García, 2015).

Among the simple and reliable EPF mass production technologies, fermentation in solid state (FSS) is mentioned, it has a great advantage in allowing the fungus to sponge naturally (aerial mycelium). For this reason, solid substrates instead of submerged systems have been the methods of choice with Deuteromycetes and need to be further examined for the commercial scale production potential of myco-insecticides (Rousson, 1983).

On the other hand, the lack of appropriate, economical and reliable substrates is an important limitation in the mass production of *B. bassiana*. The knowledge of the nutritional needs using any culture technique is another essential factor for its production. The biomolecules of the substrates are composed of macro elements and these are involved in defense mechanisms between the host-pathogen interaction and are responsible for the growth of the mycelium and the production of conidia (Masoud, 2013). For mass production and commercialization, it is necessary to supply simple and cheap solid culture media and substrates (Raimbault, 1998). In this same sense, Miller and Churchill, (1986), have tabulated a large number of carbon and nitrogen parts and their analysis of ingredients, most of which can be used in FSS, such as soybeans that contain relatively little carbohydrate and can be mixed with wheat or rice (rich in starch) for the improvement of spore yield (Maheva *et al.*, 1984).

Emphasis is made, in the use of the nutritional factors of the substrates for the growth and production of EPF, these nutritional factors must contain all the necessary elements for the biological synthesis. Most microorganisms, including fungi, use carbon compounds as an energy source, in addition to sulfur and phosphorus for the synthesis of cellular elements and nitrogen sources to synthesize enzymes, proteins and nucleic acids. Trace elements such as sodium, chlorine; potassium, zinc, manganese (activate certain enzymes) and calcium for the synthesis of the walls of the spore (conidia). Vitamins for some microorganisms include inositol, folic acid, vitamin B12 and K (Junco and Rodríguez, 2015). Hence the importance of enriching solid substrates with these important nutritional factors for the mass production of *B. bassiana*.

At present, some nutritional studies are known for the production and sporulation of filamentous EPFs and the importance of the carbon-nitrogen (C:N) in the culture substrates as one of the most critical parameters to increase the production of conidia (Shah and Pell, 2003), but very little is known about the effect of the addition of natural carbon and nitrogen sources on cereal substrates to increase their production. The development of biological formulations for the control of insect pests; led researchers to look for methods by which effective EPF can be obtained for agroecological systems and to gradually replace chemical pesticide applications (Cruz, 2014).

In this context, the objective of the present investigation was to evaluate the effect of the C:N ratio on rice and wheat for production by FSS of *B. bassiana*. The obtained results can be used for the biocontrol of the coffee berry borer (*Hypothenemus hampei*) and spittlebug of the sugarcane (*Mahanarva andigena*), with the purpose of contributing to the solution of the problems that affect these crops.

MATERIALS AND METHODS

The investigation was carried out in the period January-July 2015, in the Microbiology Laboratory of the Amazon State University, located in the Central Campus km. 44 via Puyo-Tena, province of Pastaza-Ecuador; its geographic location is 01˚ 14 '4,105 "south latitude and 77˚ 53' 4,27" west longitude, at a height of 584 meters above sea level.

Biological specimens

A native isolate of *Beauveria bassiana* was used from coffee almonds colonized by EPF; They were collected in two coffee farms in the province of Napo and Pastaza-Ecuador. Strains, which before the start of conidia production were conserved in a sterile 15% glycerol solution at a temperature of -85°C, later reactivated to be used, essential conditions to provide genetic stability of the strains.

Massive multiplication of *B. bassiana*

The massive multiplication was made on rice (*Oryza sativa* L.) and wheat (*Triticum vulgare* L.) substrates enriched with nutritional supplements: molasses as a carbon source and amaranth and soybean flours, milk powder and yeast as nitrogen sources, ratio of C: N was 10:1 at two concentrations (2.5 and 5.0 g: 0.25 and 0.50 g, respectively). We applied the methodology developed by Gómez and Mendoza (CINCAE, 2004), with some variation, we measured 100 g of the complex substrates-nutritional supplements in polypropylene covers of 20.32 × 30.48 cm, sealed the covers hermetically making three folds and stapling (Aquino et al., 1997). It was sterilized at 121°C, with pressure of 15 psi, for 30 minutes. The inoculum was aseptically prepared the native *B. bassiana* strains, obtained from the planting in medium Potato Dextrose Agar + 5% Yeast Extract (PDA-YE) and incubated at 27 °C for 10 days (Cañedo and Ames, 2004). The covers were inoculated when their content was tempered with 1.25 mL of suspension of 1×10^7 conidia/ m was incubated at 27ºC in the Memmert incubator for 15 days (Jaronski, 2015). After incubation, the inoculum was prepared by suspending the conidia in sterile distilled water with 0.1% Tween-80 solution, until obtaining a serial solution of base ten of concentration 1×10^{-3} conidia /mL (Resquín, 2016). The concentration of conidia of the bioproduct was evaluated (Vélez *et al*., 1997) through the readings in the Neubauer chamber (Amala and Naseema, 2012).

Statistical design

A categorical Multi-factor design was used, with four factors and 11 levels, combining substrates (Factor A), carbon and nitrogen sources (Factor B), two concentrations (Factor C), and native strains (Factor D), of *B. bassiana* in front of an absolute witness. 40 treatments were evaluated with three repetitions. The Analysis of Variance (ANOVA) at a level of significance $p \leq 0.05$ was used to analyze the significance of the substrates and the carbon-nitrogen sources on the increase of the production of conidia of the HEP and the Multiple Contrast of Ranges LSD- Fisher, to determine if there is significance between a pair of means at 95.0% confidence level, using the statistical software Statgraphics Plus Version 5.1.

RESULTS AND DISCUSSIONS

In this research, the effects of four categorical factors were estimated, the design was a standard factorial with a total of 60 executions.

Mass production *B. bassiana* in rice and wheat

The statistical values of the production of conidia/g of EPF *B. bassiana* obtained in the laboratory, is presented in Table-1.

Table-1. Production statistics *B. bassiana* (1.0×10^9 Conidios/g).

Substrates and sources of C-N	N	Means	Minimum	Maximum
1	20	1.34	1.05	1.89
2	20	1.365	1.06	1.90
Total	40	1.40	1.05	1.90

The total production averages in *B. Bassiana* were 1.34×10^9/g and 1.36×10^9 Conidia/g in enriched rice and wheat, respectively. Comparing with the means obtained with the absolute controls in rice (1.15×10^9 Conidia/g) and wheat (1.22×10^9 Conidia/g) an increase in the production of EPF is observed, due to the addition of nutritional supplements (sources of C and N) to substrates. The highest average values in *B. bassiana* production in rice was 1.89×10^9 Conidia/g (T7); in this same order in wheat 1.90×10^9 Conidia/g (T17). The lowest averages presented the T10 and T20 treatments (range between 1.05×10^9 - 1.06×10^9 Conidia/g), in rice and wheat with the molasses-yeast supplement at concentration 2 (5.0-0.50 g); this was possibly due to the antagonistic effect between *Saccharomyces cerevisiae* and EPF during its cultivation. The value of the means of *B. bassiana* in rice in the absolute control was $1,15\times10^9$ conidia/g (Treatments 1-4), comparing this result with that reported by Nussenbaum, (2014) (1.18×10^9 conidia/g of rice) and by Noboa, (2015) (1.43×10^8 conidia/g of rice) is observed, in the first case, the mean value is concordant and in the second case it was lower; difference that was possibly due to the good dispersion of the grains after sterilization, a greater contact surface for the growth and sporulation of the native strains.

Analysis of variance (ANOVA) of conidia of *B. Bassiana* production

The ANOVA, summarizes the decomposition of the variability in the production of conidia/g in rice and wheat by effect of the addition of nutritional supplements, are presented in the Table-2.

Table-2. ANOVA of *B. bassiana* production (concentration 1.00×10^9 Conidia / g).

Source	Sum of squares	DF	Middle Square	Quotient-F	P-Value
MAIN EFFECTS					
A: Solid substrates	1.84	1	1.84	0.10	0.7513
B: Nutritional supplements	1219.53	4	304.88	16.89	<0.0001
C: Concentration	92.50	1	92.50	5.13	0.0291
INTERACTIONS					
AB	15.89	4	3.97	0.22	0.9256
AC	85.39	1	3.04	0.17	0.6838
BC	85.39	4	21.35	1.18	0.3330
ABC	6.52	4	1.63	0.09	0.9849
RESIDUAL	721.83	40	18.05		
TOTAL (CORRECTED)	2146.55	59			

The F ratios are based on the residual means square error.

In the table of ANOVA, it is observed that 5 p-values are lower than 0.05, these factors have a statistically significant effect on nutritional supplements and conidial concentration of *B. bassiana* and for 95.0% confidence; so, that the different levels of nutritional supplements (Sources of C and N), EPF and concentration (Factors B, C, interactions BC and BD) influenced the values of the quantitative variable (production of conidia).

Multiple Range Contrast of the conidial production of *B. Bassiana*

Table-3. Multiple Range Contrast of *B. bassiana* production.

Method: 95.0 percentage LSD			
Substrates	**Count**	**Mean LS**	**Homogeneous Groups**
1	30	26.93	A
2	30	27.28	A
Contrast	DSM	E.E	
1-2	2.21	18.04	

Different letters show significant statistical difference

The Multiple Range Contrast, was performed by Fisher's method of significant differences (LSD) and was determined that the means that are significantly different from each other, therefore, there is no statistically significant difference between any pair of means at a confidence level 95.0%.

CONCLUSIONS

Wheat with molasses (source of C), milk powder (source of N) and concentration 1, was the treatment with the highest growth and native *B. bassiana* production, followed by molasses-soy flour and molasses-amaranth flour; the yield of conidia/g in rice and wheat with yeast, concentration 1 and 2, was lower in all its treatments. This has shown that testing the mass production of native *B. bassiana* with other cereals as substrates influences the yield of conidia / g of the bioproduct.

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REFERENCES

Amala U., Jiji T and Naseema A. 2012. Mass multiplication of entomopathogenic fungud, *Paecilomyces lilacinus* (Thom) Samson with solid substrates. JBiopest. 5(“): 167.170.

Aquino L.M.N., Vital A.F., Cavalcanti V.L.B and Nascimento M.G. 1997. Culture of *Metarhizium anisopliae* (Metchn.) Sorokin in polypropilene bags. Boletín Técnico CODECAP. 5. pp. 7-11.

Cañedo V. and Ames T. 2004. Manual de laboratorio para el manejo de hongos entomopatógenos. Centro Internacional de la Papa. pp. 1-62.

Cruz I. 2014. Control microbial. UNAN-OEA.

Echeverría F. 2006. Caracterización biológica y molecular de aislamientos del hongo entomopatógeno *Beauveria bassiana* (Bálsamo) Vuillemin. Costa Rica. p. 92.

Escamilla Y., Serrano M.J y Cuapio A. 2016. Agricultural Pest Control with Entomopathogenic Fungi. Revista Científica Tecnológica Miltidisciplinaria del Tecnológico de Estudios Superiores del Oriente del Estado de México.

Faria M and Wraight S.P. 2007. Mycoinsecticids and Mycoacaricides: A comprehensive list worldwide coverage and inernational clasification of formulation types, Bio Control. 43. pp. 237-2007.

García A. 2015. Formulación con Hongos Entomopatógenos para el Control de Plagas. Centro Universitario de Estudios de Investigación de Proyectos. S.A. N° de publicación: WO 2015080545 A1.

Gindin G., Glazer I., Mishoutchenko A. and Samish M. 2009. Entomopathogenic fungi as a potencial control agent against the lesser mealworm, *Alphitobius diaperinus* in broiler houses. BioControl. 54,549-558.

Gómez P & Mendoza J. 2004. (CINCAE) Centro de Investigación de la caña de azúcar del Ecuador. 2004. Guía para la Producción de *Metarhizium anisopliae*. Publicación Técnica. No. 5.

Jaronski S. 2012. Mass Production of Entomopathogenic Hypocreales. En Manual Of Techniques In Invertebrate Pathology. (Second Edition. ed., pág. 255). USA: Lawrence A. Lacey. IP Consulting International Yakima, Washington, USA.

Junco R y Rodríguez C. 2015. Cultivo y crecimiento de microorganismos. pp. 3-5.

Maheva E., Djelveh G., Larroche C. *et al.* 19 84. Sporulation of *Penicillium roqueforti* in solid substrate fermentation. Biotechnol Lett 6: 97. doi:10.1007/BF00127297.

Masoud L. B. 2013. Mass Production Of Entomopathogenic Fungi *Beauveria Bassiana* (Balsamo) By Using Agricultural Products Based On Liquid- Solid Diphasic Method For Date Palm Pest Control. (I. 2.-6.

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Miller. T.L and Churchill. B.W. 1986. Substrates for large-scale fermentations. In: Manual of Industrial Microbiology and Biotechonology, eds (A.L:demain & N.A. Solomon. American Society of Microbiologidts. Washington. D.C. pp. 32, 122.

Noboa G. 2015. Diseño e implementación de un protocolo para mejorar la producción y conservación de *B. bassiana* y *Trichoderma harziunum* como aporte a los productores de café orgánico de la Asociación "Río Intag" cantón Cotacachi.

Nussenbaum A. 2014. Aislamientos de *B. bassiana* y *M. anisopliae* virulentos para el control del picudo del algondonero, Anthonumus grandis (Coleóptera: Curculionidae).

Raimbault M. 1998. General and Microbiologicalaspects of Solid Substrate Fermentation. En International Traiing Course On Solid-StateFermentation. Proceedings. (págs. 1-20.). Curitiba, Brazil.

Resquín G. 2016. Combinación de diferentes estrategias de aplicación de hongos entomopatógenos para el control de *Spodoptera littorales* (Boisduval) (Lepidoptera: Noctuidae) y *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae). Editorial: Servicio de Publicaciones de la Universidad de Córdoba. 2016. p. 114.

Rousson S. R. 1983. Zymotics a Large Scale Fermenter Design and Evaluation, Appl. Biochem. Biotechnol.

Shah P.A and Pell J.K. 2003. Entomopathogenic fungi as biological control agents. Applied Microbiology and Biotechnology. 61, 413-423. 28.

Smriti V., Ravi K and Brajesh S.2015. Mass Production of *Beaveria bassiana* (NCIN N°. 1300) Fungal Spores on Cereal Grains and Agro-Industrial Residues. Reserach Journal of Pharmaceutical, Biological and Chemical Sciences. ISSN: 0975-8585. pp. 58-59.

Vélez P.E., Posada F.J., Marín P., González M.T., Osorio E. and Bustillo A.E. 1997. Técnicas para el control de calidad de formulaciones de hongos entomopatógenos. Boletín Técnico N°. 17. Cenicafé. Colombia. p. 37.