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Effect Of Carbon-Nitrogen In The Production Of *Metarhizium anisopliae* Native By Solid Fermentation.

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ABSTRACT

The purpose of this work was to produce conidia of native *Metarhizium anisopliae* in the laboratory by FSS (Fermentation in Solid State), polypropylene sheaths we used as bioreactors. The pre-cooked and sterilized substrates were rice and wheat, supplemented with various sources of carbon-nitrogen (CN), ratio 10: 1. The experimental design was the Categorical Multi-Factor, with 20 treatments and six replicates to determine the concentration variable of conidia/g of bio product; the counting of conidia in the Neubauer chamber. The results we systematized with Statgraphics Plus-Ver.5.1. Mean values of controls were 9,33 $\times 10^8$ conidia/g in rice and wheat 9,67$\times 10^8$ conidia/g. With C-N supplements, the highest mean of *M. anisopliae* in rice was 2,08x10^9 conidia/g (T7) and in wheat were 1,64$\times 10^9$ conidia/g (T17). The lowest mean values were in T10 and T20 with 8x10^8 and 8,4x10^8conidia/g, in the substrate with molasses + amaranth and yeast possibly by antagonistic effect between *Saccharomyces cerevisiae*-entomopathogenic fungi during cultivation. The ANOVA recorded significant statistical differences (*) at ≤ p 0,05 in the C-N, concentration, HEP factors. Interactions AB and BC. We concluded that, the supplementation of C-N on rice and wheat for the mass production of *M. anisopliae* influenced the yield of conidia/g of the bio product.

Keywords: *Metarhizium anisopliae*, carbon-nitrogen sources, production

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INTRODUCTION

The indiscriminate use of insecticides of chemical origin has resulted in the selection of resistant individuals, the resurgence of new pests, human health effects and contamination of ecosystems (Shah & Pell, 2003).The entomopathogen *Metarhizium anisopliae* has received increasing attention in recent decades, since it is used as a biological control agent for insect pest species (Jaronski, 2012), it has a great potential to infect a large group of arthropods (Santi et al., 2010).Their management has been generalized all over the planet as bio pesticides for the control of various insect pests, these entomopathogenic fungi under natural conditions are the pathogens that can frequently control to insect pests that cause deterioration to agricultural crops and constitute the group of greater importance of bio control (Erler & Ozgur, 2015) and cause mortality to insect pests in different agro-ecosystems (Tiago, 2014).EPs, when infecting hosts, produce metabolites, such as cyclic depsipeptides and hydrolytic enzymes that degrade insect, arthropod and / or pest cuticle; In addition, these fungi found in ecological niches with high biodiversity and competition fulfill the role of biological pest controllers.

All bioactive compounds can be used and produced as bioinsecticides or fungicides on cereals through fermentation processes, this opens new areas of agro-biotechnological development, in line with new trends in agricultural research worldwide; (Greenfield et al., 2014). In addition, Entomopathogenic fungi (EPF) are competitive with other control practices, considering their effectiveness, cost of production and safety to the environment (Santi et al., 2010; Gabarty et al., 2014). Among the simple and reliable mass-production technologies of EP, solid-state fermentation (SSF) is mentioned, which 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 potential in the commercial scale production of fungi insecticides (Rousson, 1983).

On the other hand, the lack of appropriate, economical and reliable substrates is an important limitation in the mass production of *M. anisopliae*. Knowledge of nutritional needs using any culture technique is another essential factor for their production. The biomolecules of the substrates are composed of macro elements and these are involved in defense mechanisms between host-pathogen interaction and are responsible for mycelial growth and conidia production (Greenfield et al., 2014). For mass production and commercialization, the supply of simple and cheap solid culture media and substrates is necessary (Raimbault, 1998). In this sense, Miller & Churchill (1986**)** have tabulated many carbon and nitrogen parts and their analysis of ingredients, most of which can be used in SSF, such as soy containing relatively little amount of carbohydrate and can be mixed with wheat or rice that possess a high content of starch for the improvement of spore yield (Maheva et al., 1984). Most microorganisms, including fungi, use carbon compounds as a source of energy, as well as 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 (enzyme activators) and calcium for the synthesis of spore walls (conidia). Vitamins for some microorganisms include inositol, folic acid, vitamin B12 and K (Junco & Rodríguez, 2015). From this arises, the importance of enriching the nutritional solid substrates for mass production of *M. anisopliae*.

Although it is true, some nutritional studies are now known for the production and sporulation of filamentous EPs and the importance of the carbon-nitrogen ratio (C:N) in the culture substrates as one of the most critical parameters for (Shah & Pell, 2003), but very little is known about the effect of the addition of carbon and nitrogen sources on the cereal substrates to increase their production. The development of biological formulations for the control of insect pests; leads the scientific society to seek methods by which effective EPs can be obtained for agroecological systems and to gradually replace chemical pesticide applications (CITAR). In this context, the present investigation evaluated the effect of the C: N ratio on the one for FSS production of *M. anisopliae.*

MATERIAL AND METHODS

The investigation has been development in the period January - July 2015, in the Microbiological Laboratory of the State University of Bolivar, Agricultural Campus Laguacoto II, Guaranda-Ecuador.

Biological specimens

A native isolate of *Metarhizium anisopliae,* isolated from the carcasses of salivazo insects (*Mahanarva andigena.* Jacobi) from sugarcane (*Saccharomyces* sp.) colonized by entomopathogens, was harvested from a sugar cane farm in the province of Pastaza-Ecuador. Strain, which prior to the onset of conidia production was preserved in a sterile 15% glycerol solution at -85°C, subsequently reactivated under conditions essential for providing genetic stability of the strains.

Massive multiplication of *M. anisopliae*

A substrate of wheat (*Triticum vulgare* L.) enriched with nutritive supplements: molasses (source of carbon), amaranth and soybean meal, milk powder and yeast (nitrogen sources), C:N ratio was 10: 1 at two concentrations (2,5 and 5,0 g: 0,25 and 0,50 g, respectively). The methodology developed by Gómez& Mendoza (CINCAE, 2004) with some variation, was measured 100 g of the complex substrates-nutritional supplements in 8 × 12-inch polypropylene bags, the seals sealed sealing three folds and stapling (Aquino et al., 1997). It was sterilized at 121°C, 15 psi, for 30 minutes. The inoculum was the native strain of *M. anisopliae*, obtained by seeding in medium Potato Dextrose Agar + 5% Yeast Extract (PDA-YE) and incubated at 27°C for 10 days (Cañedo & Ames, 2004)**.** The bags were inoculated when their contents were tempered with 1,25 mL suspension of 1×10^{-7} conidia / mL and incubated at 27°C for 15 days (Jaronski, 2012). After incubation, the inoculum was prepared by suspending the conidia in sterile distilled water with 0,1% Tween-80 solution until a serial ten base solution of concentration 1×10^{-3} conidia / mL was obtained (Resquín, 2016). The conidia concentration of the bio product was evaluated (Vélez et al., 1997), through the readings in the chamber of Neubauer (Amalaet al., 2012).

Statistical analyses

A multi-factor categorical design was used with three factors, combining substrates (Factor A), carbon and nitrogen sources (Factor B), two concentrations (Factor C), against an absolute control. Twenty treatments (T) with three replicates were evaluated. Analysis of Variance (ANOVA) at a significance level $p \leq 0,05$ was used to analyze the significance of substrates and carbon-nitrogen sources on the increase of conidia production of EPs and the Multiple Contrast of LSD-Fisher Ranks, to determine whether there is a significance between any pair of means at 95,0% confidence level using the Statgraphics Plus Version 5,1 software package.

RESULTS AND DISCUSSION

In this research, we estimated the effects of three categorical factors, the design was a standard factorial design with a total of 60 executions and consisted of all combinations of factor levels.

Mass production of *M. anisopliae* in wheat

The results of this test revealed that rice was the natural substrate with the best average yield values in conidia / g production relative to wheat. Total production averages in *M. anisopliae* were $1,12 \times 10^9$ / g and $1,18 \times 10^9$ Conidia / g in enriched rice and wheat, respectively.

Comparing the means obtained with the absolute controls in rice ($9,33 \times 10^8$ Conidia/g) and wheat ($9,67 \times 10^8$ Conidia/g) an increase in the production of EP was observed due to the addition of supplements (sources of C and N) to the substrates. The highest mean values in rice were $2,08 \times 10^9$ Conidia / g (T7) and $1,40 \times 10^9$ Conidia / g (T8); in wheat $1,64 \times 10^9$ Conidia/g (T17) and $1,47 \times 10^9$ Conidia/g (T18). The lowest means presented T4 and T10 (range between 8,0-$8,40 \times 10^8$ Conidia/g), in rice with the supplement amaranth flour and yeast at concentration (Molasses 5,0 g + amaranth flour 0,50), this was possibly due to the antagonistic effect between *Saccharomyces cerevisiae* and EPs during cultivation (Figure 1).

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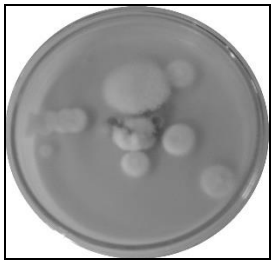

Fig 1: *Metarhizium anisopliae* colonies on agar plate

The mean value of *M. anisopliae* in rice was 1,12 × 10^9 conidia / g (Treatments 1-10), comparing this result with that reported by Nussenbug (2014) (1,18 × 10^9 conidia/g of rice) and by Latifian et al., (2014) (6,32 × 10^6 conidia / g of rice) is observed, in the first case the average value is concordant and in the second it was lower. Comparing *M. anisopliae* means obtained in rice (1,30 × 10^9 / g) and wheat (4,90 × 10^8 / g) by Ibrahim et al, (2015), with those obtained in this test, is observed in rice (1, 12 × 10^9 Conidia/g), the average value close and in wheat was higher (1,18 × 10^9 Conidia/g); Which difference was probably due to the good dispersion of the grains after sterilization, a larger contact surface for the growth and sporulation of the native strains.

Analysis of Variance of the production of conidia of *M. anisopliaein* the laboratory

The ANOVA, summarizing the decomposition of the variability in the production of conidia / g in rice and wheat due to the addition of nutritional supplements, are presented in Table 1.

Table 1: Analysis of Variance of the production of *M. anisopliae* (concentration 1,00×10^9 Conidia/g)

F.V.	SC	gl	CM	F	p-valor
Factor A (rice and barley solid substrates)	5,52067E+16	1	5,52067E+16	2,55	0,1188
Factor B (nutritional supplements amaranth flour, quinoa)	4,33E+18	4	1,08E+18	49,92	<0,0001
Factor C (concentrations)	4,68167E+17	1	4,68167E+17	21,6	<0,0001
Repetitions	5,652E+16	2	2,826E+16	1,3	0,2834
Factor A*Factor B	3,21893E+17	4	8,04733E+16	3,71	0,012
Factor A*Factor C	4,16067E+16	1	4,16067E+16	1,92	0,174
factor B*Factor C	3,712E+17	4	9,28E+16	4,28	0,0059
Factor A*factor B*Factor C..	2,24693E+17	4	5,61733E+16	2,59	0,0519
Error	8,23747E+17	38	2,16775E+16		
Total	6,69E+18	59			

In the ANOVA table, it is observed that 4 p-values are less than 0,05, these factors have a statistically significant effect on conidia / g concentration of *M. anisopliae* for 95,0% confidence; (C and N sources), EP and concentration (Factors B, C, interactions BC and BD) did influence the values of the quantitative variable (production of conidia / g).

Multiple Contrast Ranges of the production of conidia of *M. anisopliae*

Table 2: Multiple Range Contrast of *M. anisopliae* production (concentration 1,00 × 10^9 Conidia / g)

Method: 95,0 percentage LSD				
Substrates	**Number**	**Media LS**	**Sigma LS**	**Homogeneous groups**
1	30	1,18	26880937,4	A
2	30	1,12	26880937,4	B
Contrast	Difference	+/- Limits		
1-2	-0,06566	1,45764		

Different letter, indicates a significant difference

The Multiple Contrast Ranges, was performed by the method of low significant differences of Fisher (LSD) and was determined that means that are significantly different from each other, therefore, there is no statistically significant difference between any pair of means at a 95% confidence level.

CONCLUSIONS

Conidia of native *M. anisopliae* were mass produced under laboratory conditions successfully, applying the FSS method in rice and wheat supplemented with CN sources, using concentration 1 (2,5 g: 0,25 g) in relation C: N ratio of 10: 1. Rice with molasses (source of C), milk powder (source of N) and concentration 1 were the treatments with the highest growth and production of native *M. anisopliae*, followed by molasses-soy flour and molasses-amaranth flour; The yield of conidia / g in yeast rice and wheat, concentration 1 and 2, was lower in all treatments. In ANOVA, 5 p-values were less than 0,05; The factors B, C and AB and BC interactions had statistically significant effects on the production of conidia / g of native *M. anisopliae* at 95.0% confidence level; That is, the different sources of C-N supplemented to rice and wheat did influence the values of the quantitative production of conidia / g bio product. In the multiple comparison of means there was no statistically significant difference between any pair of means at a 95,0% confidence level.

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