v. 2, n. 12, 2022

Journal of Agricultural Sciences Research

PRODUCTION OF BLASTOPORES AND COLONIES FORMING UNITS OF *BEAUVERIA BASSIANA* IN THE PRESENCE OF SOURCES AND DOSE OF BIOCHAR

Lucas Rietjens
Graduating in Agronomy - Federal University of Mato Grosso, Cuiabá - MT
https://orcid.org/0000-0001-5913-1943

Ana Lucia da Silva
Doctoral Student in Tropical Agriculture - Federal University of Mato Grosso, Cuiabá - MT
https://orcid.org/0000-0002-0375-2035

Daniela Tiago da Silva Campos
Profª Drª - Federal University of Mato Grosso, Cuiabá - MT
https://orcid.org/0000-0001-5066-9317

Abstract: The submerged cultivation of entomopathogenic fungi is a growing technique that aims to increase productivity and reduce time and costs in the production process of these pathogens. Biochar provides nutrients and habitat for microorganisms to colonize, grow and reproduce. The aim of this study was to evaluate the production of blastospores and colony forming units of *Beauveria bassiana* IBCB 66 in liquid medium enriched with types and doses of biochar. Three types of biochars were tested in potato dextrose (BD) medium: cotton boll, swine manure and eucalyptus wood, and four doses: 0.5; 1.5; 3.0 and 5.0 g L^{-1} + 1 control (without biochar). The production of blastospores and colony forming units (CFUs) were evaluated at 4 days after incubation. There was interaction between the types and doses of biochar for the variables analyzed. Eucalyptus wood biochar at a dose of 1.5 g L^{-1} was superior in the production of blastospores (15.5 %) and CFU (28.24 %), respectively, when compared to the control. The dose of 5.0 g L^{-1}, regardless of the source of biochar, reduced the production of blastospores, however, it was verified that there was an increase in the germination of blastospores, that is, greater production of CFU when the highest dose was used, with the exception of cotton boll biochar. The production of blastospores and colony-forming units of *B. bassiana* IBCB 66 varies according to the type and dose of biochar used in the culture medium.
Keywords: Submerged cultivation, pyrogenic carbon, entomopathogenic fungus.

INTRODUCTION

The use of biological inputs based on entomopathogenic fungi has been highlighted against the use of chemical pesticides in the control of agricultural insect pest populations. The fungus *Beauveria bassiana* has proven entomopathogenicity, high growth rate, resisting the physicochemical barriers of the tegument, which is its penetration route, and the host's hemolymph (Dong et al., 2017) producing a diversity of antimicrobial peptides that they act on immune suppression, followed by the dismantling of internal tissues and the reduction of nutrients causing the death of the host insect (Mascarin et al., 2016).

For fungi to be effectively used as efficient control agents, there must be a high potential for inoculum in the area. For this, they need to be produced in large quantities, maintaining their morphological and virulence characteristics at an affordable cost (Zorzetti et al., 2014).

B. bassiana is a cosmopolitan microorganism, capable of growing in both solid and liquid media. The production by fermentation in liquid culture media, despite being less used, allows better control of the physical and nutritional conditions required by the microorganism (Jackson et al., 1996; Jackson, 1997) in addition to allowing, in a short time, the production of high concentrations of vegetative cells, known as blastospores, developed under liquid fermentation and exhibit high virulence capacity compared to aerial blastospores produced in solid culture medium (Mascarin et al., 2015).

The development of production techniques for entomopathogenic fungi seeking to increase the productivity of these pathogens and reduce process costs is increasing due to their use in the biological control of insects. Sources of nutrients, carbon and nitrogen, especially the C/N ratio, are essential for the proper development of the cultured microorganism, especially submerged production.

Biochar is characterized as a material fundamentally different from its biomass of origin, with the formation of structures rich in carbon and elements such as phosphorus (P), calcium (Ca) or magnesium (Mg) and

sometimes even nitrogen (N) (Lehmann and Joseph, 2015). The porosity of biochar, its high internal surface area and its ability to adsorb soluble organic matter, gases and inorganic nutrients provide a habitat for microorganisms to colonize, grow and reproduce (Verheijen et al., 2010).

These characteristics of biochar are associated with the need to develop alternative culture media that offer, in addition to the balance of the C/N ratio, other sources of macro and micronutrients. The investigation of the addition of biochar in culture media and its effects on microbial growth is an important knowledge gap to be investigated. Thus, this study aimed to evaluate the production of blastospores and colony forming units of *Beauveria bassiana* IBCB 66 in liquid medium containing types and doses of biochar.

METHODOLOGY

The experiment was carried out in a completely randomized design, under a 3 x 4 + 1 factorial scheme, the first factor being: three types of biochars (cotton boll, swine manure and eucalyptus wood) and the second factor: four doses of biochar (0.5, 1.5, 3.0 and 5.0 g L^{-1}) + 1 control (without biochar), with the production count of blastospores and colony forming units (CFUs) evaluated at 4 days after inoculation.

The biochars were produced commercially (SPPT Pesquisas Tecnológicas Ltda, Mogi Mirim - São Paulo/Brazil), at 600 °C. Details regarding the physical and chemical properties of each of the biochars can be verified in Sousa (2018). The composition of the biochar is shown in Table 1, where there is the composition of N, P_2O_5, K_2O and Ca, in addition to the elemental composition (%) of hydrogen, oxygen, carbon, nitrogen and the carbon/nitrogen ratio of cotton boll biochars (BC), swine manure (BS) and eucalyptus wood (BE) evaluated in this study.

The fungus strain was provided by the Biological Institute of São Paulo - SP and stored in the Soil Microbiology Laboratory of the Faculty of Agronomy and Animal Science (FAAZ) of the Federal University of Mato Grosso - UFMT, Campus Cuiabá, MT, SISGEN process number AB0E8A9.

REACTIVATION OF THE ISOLATE AND OBTAINING THE PRE-INOCULUM

Initially, the fungus was cultivated in Petri dishes containing potato dextrose agar (PDA) culture medium, incubated in B.O.D. (Biochemical Oxygen Demand) at 26 ± 2 ºC with a photoperiod of 12 hours for a period of 15 days. After sporulation, 5 fungal discs were aseptically removed and placed in an Erlenmeyer flask (250 mL) containing 50 mL of sterile potato and dextrose medium (liquid BD) and kept under stirring at 150 rpm at 26 ± 2 °C for 4 days. Subsequently, a 1 mL aliquot of the pre-inoculum was collected and serially diluted in distilled water + 80 tween at 0.01% (Wollum, 1982). Then, the concentration of blastospores was estimated in a Neubauer chamber and the fungal suspension was standardized to 1 x 10^6 blastospores/mL.

CULTURE MEDIUM WITH BIOCHAR

The biochars were ground to < 0.5 mm and the doses corresponding to the treatments were added to 50 mL of the medium (BD), then the pH of the medium was adjusted to 5.8 and the medium was transferred to a 250 mL Erlenmeyer flask. and sterilized for 20 minutes at 121°C under 1 atm pressure. After cooling, each Erlenmeyer flask was inoculated with 1mL of the pre-inoculum suspension of *B. bassiana*, closed with aluminum foil and placed in a refrigerated orbital incubator with agitation of 150 rpm and temperature of 26 ± 2 ºC, without light for a period of four days.

After the incubation period, an aliquot of 1

mL was removed from the medium and serially diluted for later counting of blastospores and CFUs production.

The blastospore count was performed with the aid of a Neubauer chamber under an optical microscope at 40X. For each treatment, the spores were counted in duplicate and the two fields of the chamber, counting in each field, five subcompartments, obtaining the average of the results obtained for the two fields. The counting results were expressed in mL, taking into account the dilution used.

The counting of CFUs was performed directly in Petri dishes containing PDA medium. 0.1 mL of the suspension (1×10^3 blastospores mL^{-1}) was inoculated onto the surface of the medium and incubated at 26 ± 2 ºC. After 72 hours of incubation, the number of colonies formed was quantified. Three replications were performed per treatment.

The means of the variables were evaluated by means of "ANOVA" analysis of variance and Tukey's test (p< 5%) when significant, followed by unfolding of the interactions of factors and contrasts with the reference treatment (control).

RESULTS AND DISCUSSION

There was a significant interaction between the types and doses of biochar for the production of blastospores and for colony-forming units of *B. bassiana* after four days of incubation.

Cotton boll biochar promoted greater production of blastospores when used at a dose of 3.0 g L^{-1}. For swine manure biochar, the highest production of blastospores was obtained at a dose of 1.5 g L^{-1}, while for eucalyptus wood biochar, doses of 1.5 and 3.0 g L^{-1} promoted higher means in the production of blastospores of *B. bassiana* (Table 2).

Still in Table 2, it is noted that the production of blastospores did not differ significantly when using doses of 0.5 and 3.0 g L^{-1}, regardless of the biochar used. At doses of 1.5 and 5.0 g L^{-1}, higher blastospores production were observed when swine manure and eucalyptus wood biochars were used.

When comparing each treatment with the control, without the addition of biochar, it was verified that only the treatment with eucalyptus wood biochar at a dose of 1.5 g L^{-1} was significantly higher in the production of blastospores (15.5 %) in relation to witness. The dose of 3.0 g L^{-1} for the three types of biochar (8.30, 8.14 and 8.87), respectively, as well as the dose of 1.5 g L^{-1} of swine manure biochar (9.46), had a similar effect to the control (8.64). It is noteworthy that regardless of the biochar used, the dose of 5.0 g L^{-1} had a negative effect on the production of blastospores, being lower than that of the control (Table 2).

The CFUs were not influenced by the doses of cotton boll biochar and did not differ statistically from the control. The swine manure biochar presented the best average for the production of CFU when used at a dose of 5.0 g L^{-1}, while for the eucalyptus wood biochar, the best average was verified at the dose of 1.5 g L^{-1} (Table 3).

Regarding the doses studied, no differences were observed when the dose of 0.5 g L^{-1} was used, regardless of the type of biochar used. When applied at a dose of 1.5 g L^{-1}, there was a higher production of CFU for biochar from eucalyptus wood, while at a dose of 3.0 g L^{-1}, the highest production of CFU was for biochar from cotton boll. At the dose of 5.0 g L^{-1}, the best CFU production was obtained when using swine manure biochar (Table 3).

The biochar from swine manure at a dose of 5.0 g L^{-1} and from eucalyptus wood at a dose of 1.5 g L^{-1} differed significantly from the control, presenting higher averages for the production of CFU with an increase of 31.75 and 28.24%, respectively, in relation to the treatment without the addition of biochar.

Biochar	N	P_2O_5	K_2O	Ca	H	O	C	N	C/N
	g Kg^{-1}				%				
BC	9.2	127.3	58.9	14.7	3.21	18.54	55.35	1.84	30.12
BS	10.1	105	9.1	125	1.26	7.34	31.72	1.91	16.58
BE	1.2	5.0	2.2	19.5	3.63	17.01	73.9	0.70	106.01

Table 1. Macronutrient composition (N, P_2O_5, K_2O and Ca) and elemental composition of H, O, C and N in cotton wool (BC), swine manure (BS) and eucalyptus (BE) biochars pyrolysed at 600°C.

Source: Adapted from Souza, 2018.

Biochar	Doses				Mean
	0.5	1.5	3.0	5.0	
	Blastospores/mL (x10^5)				
BC	7.86 aAB	6.71* bB	8.30 aA	5.31* bC	7.04
BS	7.18* aAB	9.46 aA	8.14 aB	6.58* aC	7.84
BE	7.60* aB	9.98* aA	8.87 aA	5.90* abC	8.09
Mean	7.55	8.72	8.44	5.93	
Control: 8.64					
CV (%): 13.84					

Data transformed into $\sqrt{(x + 0.5)}$.

* Means differ compared to the control by Dunnett's test at 5% significance level (bilateral).

Means followed by the same letter, uppercase in the row and lowercase in the column, do not differ statistically from each other by the Tukey Test (p>0.05).

Table 2. Production of total blastospores of *Beauveria bassiana* IBCB 66 in doses of cotton boll (BC), swine manure (BS) and eucalyptus (BE) biochar after 4 days of incubation.

Source: Prepared by the authors.

Biochar	Doses				Mean
	0.5	1.5	3.0	5.0	
	UFC/mL (x10^3)				
BC	8.72 aA	9.21 bA	11.15 aA	11.12 bA	10.05
BS	9.31 aB	11.4 abAB	6.27* bC	13.9* aA	10.22
BE	10.39 aB	13.53* aA	4.66* bC	6.18* cC	8.69
Mean	9.47	11.38	7.36	10.40	
Control: 10.55					
CV (%): 11.58					

Data transformed into $\sqrt{(x + 0.5)}$.

* Means differ compared to the control by Dunnett's test at 5% significance level (bilateral).

Means followed by the same letter, uppercase in the row and lowercase in the column, do not differ statistically from each other by the Tukey Test (p>0.05).

Table 3. Production of colony forming units of *Beauveria bassiana* IBCB 66 in biochar doses of: cotton boll (BC), swine manure (BS) and eucalyptus (BE) after 4 days of incubation.

Source: Prepared by the authors.

The production of blastospores and colony-forming units did not follow a production pattern as a function of the types and doses of biochar, except for the production of blastospores in swine manure and eucalyptus biochars, which followed an increasing order up to a dose of 1, 5 g L^{-1} and decreased with increasing biochar dose from 3.0 g L^{-1}. The variability in the blastospore and CFU production curve as a function of the types and doses of biochar can be seen in the scatter plot of the results (Figure 1).

The germination of blastospores/spores is a crucial factor for the formation of CFU. This fact may be related to the results observed in this study, in which the dose of 5.0 g L^{-1}, despite higher average values for the production of colony-forming units, presented a lower amount of blastospores, with the exception of boll biochar. of cotton that no difference was observed for CFU between the doses studied.

Another factor that may have influenced the growth of the fungus is the composition of nutrients and compounds that each biochar has. Cotton boll biochar has higher levels of K_2O and significant amounts of N and P_2O_5, in turn, swine manure biochar has higher rates of N, P_2O_5, Ca, Mg, Cu, Zn and Na, as characterized by Souza (2018).

Eucalyptus biochar, despite having lower nutrient content and high C/N, produced a greater amount of blastospores at doses of 1.5 and 5.0 g L^{-1}, while at doses of 0.5 and 3.0 g L^{-1} did not differ from cotton boll and swine manure biochars. It is believed that the amount of nutrients, mainly N, Ca, Mg, Cu, Zn and Na contained in the eucalyptus biochar was responsible for favoring the production and germination of blastospores. Being an acceptable nutritional condition for spore reproduction and mycelial growth.

Some nutrients such as C, N and ions are essential for the growth of microorganisms. The C/N ratio is one of the main factors that affect microbial growth and the ability to produce assimilable nitrogenous forms. The results observed in this study can be justified by the high carbon and low nitrogen content, whose C/N ratio is high and which were not adjusted, with the C/N of cotton boll, swine manure and eucalyptus biochars of 30.12:1, 16.58:1 and 106.01:1, respectively, as shown in Table 1, a fact that may have induced a depletion of N, due to the great demand by microorganisms, causing the temporary immobilization of this nutrient implying longer time for decomposition/assimilation of nutrients and higher energy expenditure to seek the balance of C/N.

The results of this study allow us to corroborate the influence of biochar on submerged cultures of fungi. Based on these results, further studies may be guided, in an attempt to obtain the concentrations and types of biochars suitable for promoting the growth and sporulation of entomopathogenic fungi.

CONCLUSION

The production of blastospores and colony forming units of *B. bassiana* IBCB 66 exhibits variation in the type and dose of biochar used in the culture medium.

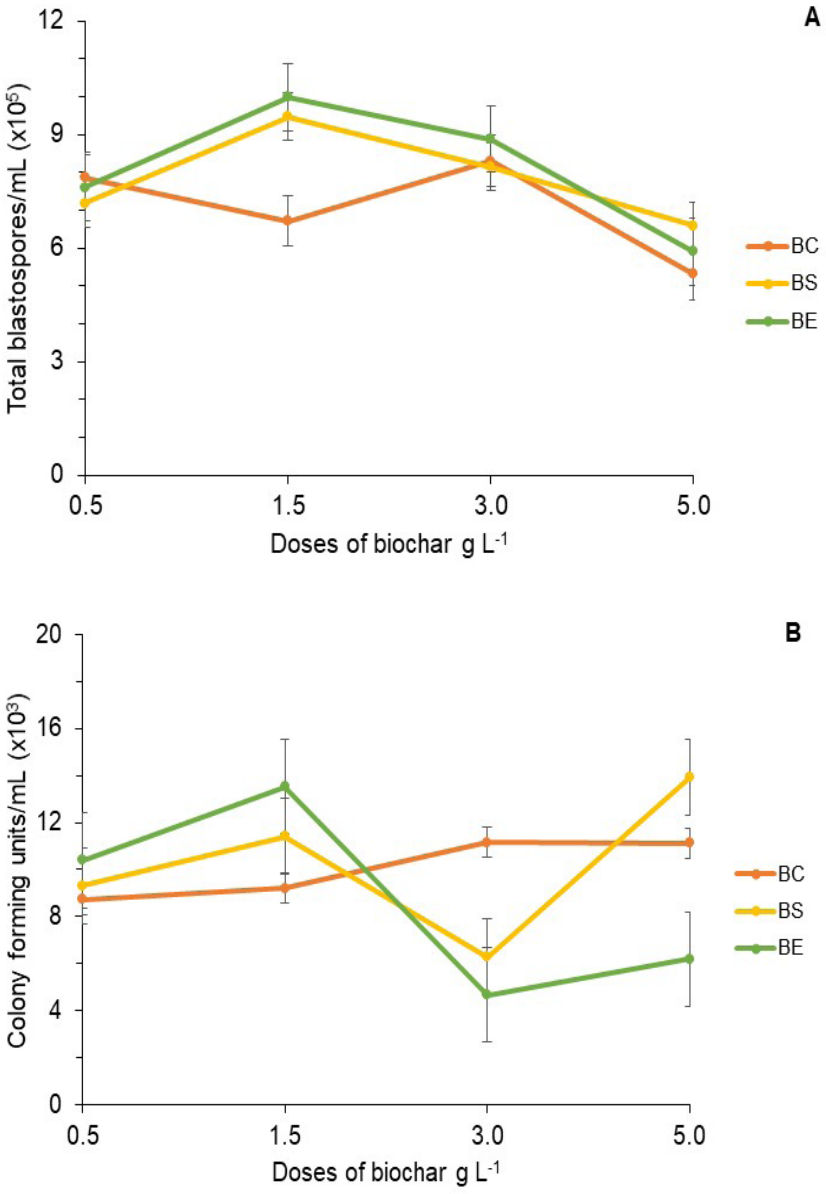

Figure 1. Mean curve for the production of blastospores (A) and colony forming units (B) of *Beauveria bassiana* IBCB 66 in biochar doses of: cotton boll (BC), swine manure (BS) and eucalyptus (BE). Bars represent standard error.

Source: Prepared by the authors.

REFERENCES

DONG, W.; DING, J.; GAO, Y.; PENG, Y.; FENG, F. Transcriptomic insights into the alternative splicing-mediated adaptation of the entomopathogenic fungus *Beauveria bassiana* to host niches: autophagy-related gene 8 as an example, **Environmental Microbiology,** v. 19, n.10, p. 4126-4139, 2017.

JACKSON, M. A.; SCHISLER, D. A.; SLININGER, P. J.; BOYETTE, C. D.; SILMAN, R. W.; BOTHAST, R. J. Fermentation strategiesfor improving the fitness of a bioherbicide. **Weed Technology**, v. 10, p. 645-650,1996.

JACKSON, M. A.; Mcguire, M. R; Lacey, L. A.; Wraight, S. P. Liquid culture production of desiccation tolerant blastospores of the bioinsecticidal fungus *Paecilomyces fumosoroseus*. **Mycological Research,** v. 101, n. 1, p. 35-41, 1997.

LEHMANN, J.; JOSEPH, S. **Biochar for Environmental Management: Science, Technology and Implementation.** Routledge, London, 2015. 994 p.

MASCARIN, G. M; JARONSKI, S. T. A produção e usos de *Beauveria bassiana* como inseticida microbiano. **Revista Mundial de Microbiologia e Biotecnologia,** v. 32, n. 11, pág. 1-26, 2016.

MASCARIN, G.M. Jackson, M. A.; Kobori, N. N.; Behle, R. W.; Delalibera Junior, I. Fermentação de cultura líquida para produção rápida de blastósporos tolerantes à dessecação de cepas *Beauveria bassiana* e *Isaria fumosorosea*. **Journal of Invertebrate Pathology,** v. 127, p. 11-20, 2015.

SOUSA, H. M. **Caracterização e efeitos de biocarvões para uso agronômico em solos arenoso.** Universidade Federal de Mato Grosso. Tese de Doutorado. Faculdade de Agronomia e Zootecnia. Programa de Pós-Graduação em Agricultura Tropical. 2018.

VERHEIJEN, F., JEFFERY, S., BASTOS, A. C., VAN DER VELDE, M., & DIAFAS, I. **Biochar application to soils. A critical scientific review of effects on soil properties, processes, and functions.** EUR, 24099, 162. 2010.

WOLLUM, A.G. **Cultural methods for soil microorganisms**. In: PAGE, A.L.; MILLER, R.H.; KEENEY, D.R. (Ed.). Methods of soil analysis. Madison: Soil Science Society of America, p.781- 802, 1982.

ZORZETTI, J.; SANTORO, P. H.; CONSTANSKI, K. C.; OLIVEIRA, JANEIRO NEVES, P. M. Sensibilidade de conídios de *Beauveria bassiana* a fatores abióticos após sucessivos cultivos in vitro**. Semina: Ciências Agrárias,** vol. 35, núm. 4, pp. 1773-1783. 2014.