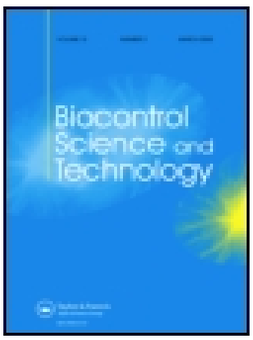

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RESEARCH ARTICLE



# Selection and yield optimisation of a *Beauveria bassiana* isolate for the biological control of leaf cutter ants

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**ABSTRACT**

Leaf cutter ants are one of the most important pests to agriculture and forestry in the Neotropics. One environmentally friendly solution to control them is biological control with the use of conidia of some fungi, such as *Beauveria bassiana*. We tested several isolates of this fungus (obtained from leaf cutter ants) by inoculating ants from seven colonies of *Acromyrmex lundii* with $5 \times 10^6$ conidia $ml^{-1}$ suspensions. All the isolates proved to be good controllers, but isolate 5 (B5) was found to be the best performing option, because it caused the death of 92.3% of the ants in 4–5 days. Isolate 7 (B7) was the second-best option and used for comparative purposes. Further assays were performed with the isolate B5 to optimise yield production. Three types of rice (brown, parboiled and white rice), soybean hull and *yerba mate* (*Ilex paraguariensis* leaves) were tested as substrates, and following that, different conditions of solid-state fermentation (SSF) were changed so as to select the most productive combination. Among treatments, we selected brown rice as substrate and a temperature of 28°C and 75–85% relative humidity in the SSF chamber because this allowed a production of $6.4 \times 10^8$ and $1.2 \times 10^9$ conidia of B5 and B7 per gram of final substrate with a 98% and 97.5% of germination, in 10 days, respectively. The differences in yield obtained under the same conditions for both isolates show that yield is isolate-dependent. A description of the customised design of the SSF chamber used is included.

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**KEYWORDS**
Ant control; entomopathogens; conidia production; solid fermentation

## Introduction

Leaf cutter ants (Hymenoptera: Formicidae) are well known for the damage they cause to vegetation in tropical, subtropical and grassland regions of Latin America. They are represented by two genera: *Atta* and *Acromyrmex*. They cut leaves, flowers and fruits to cultivate a symbiotic fungus (Agaricales: Leucoagaricus) from which they feed the queen and brood of the colony with gongylidia, a nutrient-rich hyphal swelling produced by the fungus (Fisher, Stradling, & Pegler, 1994). Leaf cutter ants are regarded as main herbivores because they can consume more plant biomass than other animals, such as vertebrates in the Neotropics (Hölldobler & Wilson, 1990). *Acromyrmex* spp. are distributed mainly in

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South-America, and some species, such as *A. lundii*, *A. crassispinus* and *A. ambiguus*, are well known as pests in fruit and flowers crops and forestry (Cantarelli, Costa, Pezutti, & Oliveira, 2008; Elizalde, Fernández, Guillade, & Folgarait, 2016; Fiorentino & de Medina, 1991; Foguelman, 2003).

The common method to control these ants is based on synthetic chemical products, primarily sulfluramid and fipronil (Montoya-Lerma, Giraldo-Echeverri, Armbrecht, Farji-Brener, & Calle, 2012). However, due to the negative impact to human health, persistence in the ecosystems and lack of specificity (Guillade & Folgarait, 2014; Hodgson & Rose, 2007; Sidiropoulou et al., 2011), international organisations such as the European Commission (2016) and the Forestry Stewardship Council (FSC 2016) have restricted or banned the use of these pesticides in human food production and for pest control in forestry, respectively.

Mycoinsecticides are gaining increased attention as environmentally friendly insect control agents (Lacey et al., 2015). Up to September 2016, Muñiz-Paredes, Miranda-Hernández, and Loera (2017) recorded 51 fungal bioinsecticides offered online. Although many fungal species infect insects, few have received serious consideration as potential commercial candidates. One of the few is *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Clavicipitaceae), a fungus that has the capacity to infect a large range of insects through conidia (asexual propagules), which attach to the host cuticle, penetrate it and finally cause the death of the insect (Feng, Poprawski, & Khachatourians, 1994).

It is assumed that pest control could be achieved if sufficient conidia inoculum could be produced cheaply enough and applied at sufficiently high rates (Jaronski, 2010), which is an incentive to develop mass production systems to maximise inoculum yield. Most formulations are based on aerial conidia produced by solid-state fermentation (SSF) (Lacey et al., 2015; Mascarin & Jaronski, 2016; Muñiz-Paredes et al., 2017). SSF is an ancient system which allows several types of fungi to produce large quantities of healthy conidia (Bartlett & Jaronski, 1988). SSF has additional advantages compared to the traditional submerged fermentation: mainly, that it is simpler, requires lower capital and energy, uses simpler fermentation media, does not require rigorous control of fermentation parameters, uses less water, produces less wastewater and easily controls bacterial contamination (Pandey, 1994; Soccol et al., 2017). However, some disadvantages should be noticed: SSF also leads to high risk of contamination with fungi and its heterogeneity makes it difficult to standardise batch quality, which are problems that submerged cultures do not present. Nonetheless, conidia cannot always be produced easily in the latter, and blastospores obtained are less resistant to abiotic factors found in nature (Lacey et al., 2015; Muñiz-Paredes et al., 2017), and this represents a disadvantage for formulations that need to be used in the field for biological control purposes. According to Mascarin and Jaronski (2016), some factors such as the cost of the production medium and formulation, production yields, virulence, persistence in the field, tolerance to environmental stresses, host target, delivery system, market size and consistent performance under field conditions allow to decide which is better, conidia or blastospores. Taking into account several of those reasons, this study is focused on conidia production.

Solid substrates have been widely used to produce aerial conidia of entomopathogenic and antagonist fungi (Jaronski & Jackson, 2012; Krishna, 2005; Mascarin & Jaronski, 2016; Wraight, Jacksonz, & De Kock, 2001). Studies on mass production of fungi using SSF have demonstrated that the yield and viability of produced conidia are influenced by several

factors, such as temperature, pH, aeration and substrate components (Jackson, Dunlap, & Jaronski, 2010). Although some of these parameters are difficult to regulate, some SSF bioreactors have been developed in recent years for *B. bassiana* production (Chen, Wang, Ye, & Feng, 2009; Kang, Lee, Yoon, & Kim, 2005; Ye, Ying, Chen, & Feng, 2006), yielding up to $1 \times 10^{10}$ conidia of *B. bassiana* per gram of substrate, at most. However, to the best of our knowledge, there is no SSF bioreactor available for purchase in Latin America. For that reason, we have custom-built a device suited to our needs, such as the possibility to control temperature and relative humidity.

The aims of the present work were: (1) the selection of the best biological control agent from several isolates of *B. bassiana* against *A. lundii* ants, and (2) the optimisation and scale-up of the conidia production of the isolate of *B. bassiana* which presented the highest virulence. To do that, we tested the virulence of seven isolates of *B. bassiana* against seven colonies of *A. lundii*. Then, we assessed several substrates to choose the best to use in the SSF process. Afterwards, scale-up production (from grams to kilograms) was optimised using different treatments with a SSF chamber exclusively designed for this purpose. A second isolate was tested in order to compare conidia production under the best condition of the SSF.

## Materials and methods

### *Fungi used, collection of the ants and infection in laboratory*

Seven isolates of *B. bassiana* (B1 to B7) were isolated from different species of leaf cutter ants in different places from Argentina, and then they were deposited in the Ants Laboratory collection, Quilmes National University, Argentina. Before the isolates were used in any assay, they were stored at −80°C in vials containing 20% glycerol. All were previously identified to the species level morphologically and using the translation elongation factor (TEF-1α) region as marker (Cavallo, 2016).

Ants (300–500) were collected from different colonies located in Entre Ríos (GPS coordinates: −31.66302, −58.02394) and Buenos Aires (GPS coordinates: −34.8772, −58.01562; −35.18138, −57.67092; −34.70873, −58.28146) provinces from Argentina: three colonies from the first state and four from the second. Ants were immediately transported to the laboratory and housed in plastic containers with access to tubes containing water and 20% g $ml^{-1}$ sugar solution *ad libitum*, at room temperature and humidity.

For infection assays, we used at least 30 ants for each treatment, from each of the 7 colonies. Conidia suspensions of *B. bassiana* were obtained from 7-day-old cultures in potato dextrose agar (PDA) (Britania®). We prepared conidia suspensions by harvesting them from the surface culture with 0.01% Tween 80 solution, and we quantified the conidia using a Neubauer haemocytometer. Suspensions at a concentration of $5 \times 10^6$ conidia $ml^{-1}$ were used to immerse ants all together during 10 s. Control ants were immersed in a 0.01% Tween 80 solution. After treatments, ants were placed together in a sterile container (14.5 cm long, 14.5 cm wide and 6 cm high), sealed with aluminium foil and closed with a lid (no openings) with water and 20% sugar solution *ad libitum*. The mortality of the ants was monitored daily for 21 days and dead ants were removed and placed individually in sterile humid chambers. Dead ants were not disinfected in order to preserve those conditions as would be found in a natural situation. They were checked regularly for the

presence of *B. bassiana*, other entomopathogens (considered as such if they appeared from intersegmental membranes and/or the joints of the legs and antennae), or external fungi (non-entomopathogenic), following Goffré and Folgarait (2015), as the way to corroborate the cause of death.

### Evaluation of different types of substrate

We evaluated the following substrates: long-grain white rice, long-grain parboiled white rice, long-grain brown rice, soybean hull and *yerba mate* (dry leaves of *Ilex paraguariensis*, used extensively in Argentina and around countries as infusion). We employed a completely randomised design with six simultaneous replicates for each substrate, and we repeated it three times. We put 2 g of each substrate in a glass Petri dish, and added 2 ml of distilled water. After that, these were autoclaved at 121°C for 15 min.

To measure the moisture content, we placed another six Petri dishes with each substrate at 60°C in a drying oven for 3 days (time required to reach constant weight), calculated the water loss and then the percentage of humidity in the initial weight. For inoculation, conidia were harvested from 20-day-old cultures from the surface of the solid PDA by adding 0.01% (v/v) of Tween 80 (Biopack®), scraping with a sterile spatula and putting it into a 50 ml tube. Conidia suspensions were quantified using a Neubauer haemocytometer, and later diluted to $1 \times 10^6$ conidia $ml^{-1}$ with the Tween 80 solution. We added 100 µl of this conidia suspension to each dish. Petri dishes were individually wrapped, sealed with Parafilm® and put together in a plastic tray, one on top of the other, separated by type of substrate and placed in an acclimatised room at 24°C in darkness for 14 days. Conidia production was evaluated at 10 and 14 days. On the 10th day, we put 1 g of substrate with conidiated fungus in 10 ml of 0.01% Tween 80 solution. The suspension was mixed by vortexing it for 30 s. After that, it was diluted and the number of conidia was quantified as described above. We carried out the same procedure on day 14th.

### Solid fermentation chamber

The device to scale up conidia production was exclusively designed following our specifications, modifying some characteristics of previously published designs (Agamez-Ramos, Zapata-Navarro, Oviedo-Zumaque, & Barrera-Violeth, 2008; Ye et al., 2006). It is a chamber of stainless steel with dimensions of $87 \times 72 \times 64$ cm (long × width × height). It has forced air circulation which maintains temperature and relative humidity uniform inside the chamber. The acclimatised air circulates by convection throughout the chamber, which allows no heat to accumulate, using the motor installed at the rear of the ceramic heat diffuser (9 in Figure 1(a)). There were two accessories for control of humidity. One of them allowed the entrance of moist obtained by a heat resistance inserted in a container with 3 l of sterile distilled water, and the other was full of silica gel in order to retain moisture of circulated air when it was running. Humidity inside the chamber is controlled by the setting of desired conditions with a digital controller (11 in Figure 1(a)), which allows the start of one or another accessory. This system had a precision of ±5%. These outside accessories for the control of moisture are connected to the chamber with flexible hoses, which are inserted into a metal tube to access the chamber (3 and 4 in Figure 1(a)). The hoses can be removed to be sterilised in autoclave

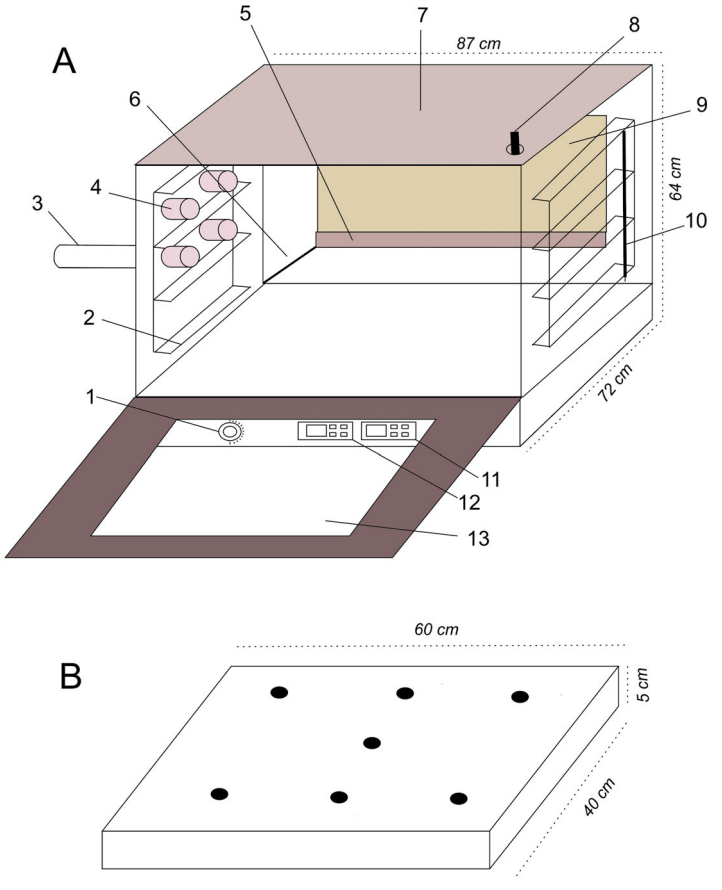


**Figure 1.** (A) Diagram of multi-tray chamber 25 kg bioreactor: (1) knob for sterilisation temperature; (2) metal bracket for mounting the trays; (3) reinforced silicone hose for connection to moisture accessories; (4) metal tube for insertion of hoses and connection with the chamber; (5) sloped trough collecting condensed water from ceramic plate; (6) pipe that transports the water collected from the trough connecting it to outside (which pierces the chamber and ends with a plastic hose inside a bowl with water); (7) stainless steel external structure, with double walled, with glass wool between both walls for temperature maintenance; (8) hygrometer for humidity control, hermetically sealed inside the chamber, mobile (to be removed at the time of sterilisation); (9) ceramic plate for diffusing heat of resistance which is placed in the back, where there are also fans which produce convection for even distribution of temperature; (10) thermometer for temperature control; (11) digital humidity controller; (12) digital temperature controller and (13) reinforced double glass door, with transparent front that allows seeing the interior of the chamber. (B) Dimensions of a tray and pattern for sampling seven samples.

(at 121°C for 15 min). Accessories were cleaned with 10% sodium hypochlorite before each trial. Temperature is also controlled digitally (12 in Figure 1(a)), and an electric resistance was used for heat and a condenser for cooling, with a precision of ±1°C. The sterilisation of the chamber can be done *in situ*, by setting the temperature of the chamber at 220°C with the knob (1 in Figure 1(a)) for 4 h. The reinforced double glass door is transparent (13 in Figure 1(a)), allowing observation during the SSF. The chamber can contain up to four trays, each of 60 cm in length, 40 cm in width and

5 cm in depth. The volumetric capacity per tray is 12,000 $cm^3$ (6.25 kg of brown rice), yielding a total SSF volume of 48,000 $cm^3$ (four trays), equivalent to a total of 25 kg of substrate. This chamber was in a 54 $m^2$ room, which was treated for, at least, 2 h under 40 W germicidal ultraviolet light after a thorough cleaning with 10% sodium hypochlorite.

### *SSF optimisation*

Optimisation of the SSF process included changes in the following variables: volume and type of inoculum; moisture content of the substrate; temperature inside the chamber and additional supplementation (or not) of wheat bran. We carried out six treatments with a combination of those variables (Table 1), designated from A to F. Other variables remained fixed, such as relative humidity inside the chamber (75–80%), type of substrate (brown rice), circulation of the air (on) and darkness during the whole process.

The solid substrate used was brown rice, chosen as the best in the previous evaluation (see section 'Evaluation of different types of substrate' on results). There were two alternative ways to prepare it. In the first one, substrate was cooked with distilled water (one part of substrate to two parts of water) for 30 min in microwave at maximum power (P100), and cooled to ambient temperature (25°C approximately). A moisture content of 60–69% was obtained. In the second one, there was an additional step to the preparation described above: the previously cooked (as above) substrate plus distilled water (two parts of cooked substrate to one part of water) was steamed for 15 min in autoclave at 121°C, which yielded a substrate with higher moisture content (70–79%). The moisture content was measured as the weight of water loss by desiccation at 60°C for 3 days (time needed to constant weight). Each tray contained 6.25 kg of substrate prepared either way. In treatment F, brown rice was supplemented with wheat bran. In this case, 120 g of brown rice + 30 g of wheat bran were cooked with 300 g of water for 30 min in microwaves at maximum power (P100), and cooled to room temperature (25°C approximately).

Each treatment was repeated at least three times (see Table 1). Treatment E was further replicated another three times with isolates B5 and B7 simultaneously. Treatment F was also carried out three times with both isolates.

Inocula with conidia suspensions were prepared harvesting conidia from the surface of the solid PDA by adding 0.01% (v/v) Tween 80 solution, as it was explained before.

**Table 1.** Conditions of each variable selected for the optimisation process of conidia production of *B. bassiana* in brown rice.

| Treatment | Type of inoculum | Volume of inoculum (ml) | Initial conidia concentration (conidia / g) | Moisture content of substrate (%) | Chamber temperature (°C) | Supplementation with wheat bran |
|---|---|---|---|---|---|---|
| A ($N = 3$) | Grown on PDB | 500 | Unknown | 60–69 | 25–26 | No |
| B ($N = 5$) | Conidia suspension | 250 | $4 \times 10^5$ | 60–69 | 25–26 | No |
| C ($N = 4$) | Conidia suspension | 500 | $8 \times 10^5$ | 60–69 | 25–26 | No |
| D ($N = 3$) | Conidia suspension | 500 | $8 \times 10^5$ | 70–79 | 25–26 | No |
| E ($N = 6$) | Conidia suspension | 500 | $8 \times 10^5$ | 70–79 | 27–28 | No |
| F ($N = 3$) | Conidia suspension | 500 | $8 \times 10^5$ | 70–79 | 27–28 | Yes |

Conidia suspensions were quantified using a Neubauer haemocytometer, and later diluted to $1 \times 10^7$ conidia $ml^{-1}$ with the Tween 80 solution. The obtained suspension was immediately used as inoculum in treatments B to E, resulting in an initial conidia concentration between $4 \times 10^5$ and $8 \times 10^5$ conidia $g^{-1}$ when 250 and 500 ml were added on the substrate, respectively (Table 1). For treatment A, we used a biphasic fermentation process: we put 1 µL of $1 \times 10^6$ conidia $ml^{-1}$ suspension in 250 ml of potato dextrose broth (PDB) and let them grow for 48 h at 25°C, room humidity and 16:8 light/dark in an orbital shaker (Thermo Fisher®) at 150 rpm to generate homogeneous suspensions; after that, they were left static for 48 h to produce conidia on the surface. The resulting suspension was used as the seeding inoculum for SSF in treatment A (500 ml per tray).

Inoculum was added to each tray containing cooled solid substrate, followed by thorough mixing, using a sterilised spoon. Subsequently, trays were put into the chamber.

Measurements of temperature and RH inside the chamber during the fermentation process were recorded every 10 min throughout 10 days by a multifunction data logger (V&A® Instruments).

After 10 days of the fermentation process, seven samples were taken from each tray to test conidiation yield for each treatment (Figure 1(b)). We previously tested that the variation in the yield obtained in the different parts of the tray was similar, as well as the average yield obtained with 7–49 samples (unpublished data). Each sample had 1 g of the fungus-colonised substrate, which was suspended in 10 ml distilled water + 0.01% Tween 80, followed by agitation in vortex for 30 s. The samples were successively diluted and quantified for each sample (pseudoreplicate) using a Neubauer chamber, then the number of conidia per gram of solid substrate was calculated. The total average of conidia was expressed as the mean (and standard deviation) of the seven points in each repetition. Therefore, we report the yield as conidia per gram of final substrate; however and to ease the comparison with other publications we added also the estimation of conidia per gram of initial dry substrate. The latter, although an overestimation, was obtained by correcting the final yield with the moisture content measured in the substrate after cooking and before fermentation. For germination evaluation, 20 µL of the previous suspensions were put into Petri dishes containing PDA. After 18–24 h of incubation at room temperature (25°C approximately), the proportion of conidia that had germinated was determined by observation through a microscope at 400× magnification (Nikon Eclipse E200). Germination was considered successful when the size of a germinated tube was equal or bigger than the diameter of conidia. The sample size was 100 conidia per pseudoreplicate, for each of the seven pseudoreplicates.

## Statistical analysis

Survival distributions were compared using the Kaplan–Meier method and the Mantel test was employed to obtain a probabilistic value. We compared the distributions of treatments by colonies and of colonies for each treatment among multiple groups, and then we carried out pairwise comparisons whenever required, adjusting the $P$ value with the Bonferroni correction to maintain $\alpha = 0.05$. Those ants which died 2 days after immersion were not considered in survival analysis, because they probably died for other reasons. The time needed to obtain 50% of the mortality (LT50) was also determined.

Data of conidia yield were subjected to logarithmic transformation [log10 (x)] to improve homogeneity of variances and to be normalised before the data analysis. These two assumptions were verified using the Levene and Shapiro–Wilk tests, respectively. Therefore results were reported as means and standard deviation (STD). Treatments were then compared through analysis of variance (ANOVA) and considered to be statistically different at the 5% significance level. When data did not allow the use of parametric analyses, Kruskal–Wallis nonparametric tests (KW) were performed. In this situation, results were reported as medians with quartiles ($Q_{25\%}$ and $Q_{75\%}$).

All data analyses were conducted using the SYSTAT 13 software (SYSTAT 13©, 2009).

## Results

### *Evaluation of B. bassiana infection in A. lundii ants*

The seven isolates of *B. bassiana* demonstrated to be pathogenic against *A. lundii* workers, i.e. all of them caused a significantly faster mortality in relation to the control ($P < .0018$) (Figure 2). The LT50 for each isolate varied between 4 and 5 days, whereas in the control treatment, it was between 6 and 12 days. Variation observed in the LT50 in control ants was related to differences among colonies, which were also found in survival distributions when they were analysed together ($P < .0024$). Variation among colonies disappeared when ants were treated with conidia of *B. bassiana* isolates. Ants of all the colonies treated with isolates B5 and B7 showed the same pattern of survival curves ($P > .0024$); however, there were statistical differences among colonies when the ants were treated with the other isolates ($P < .0024$).

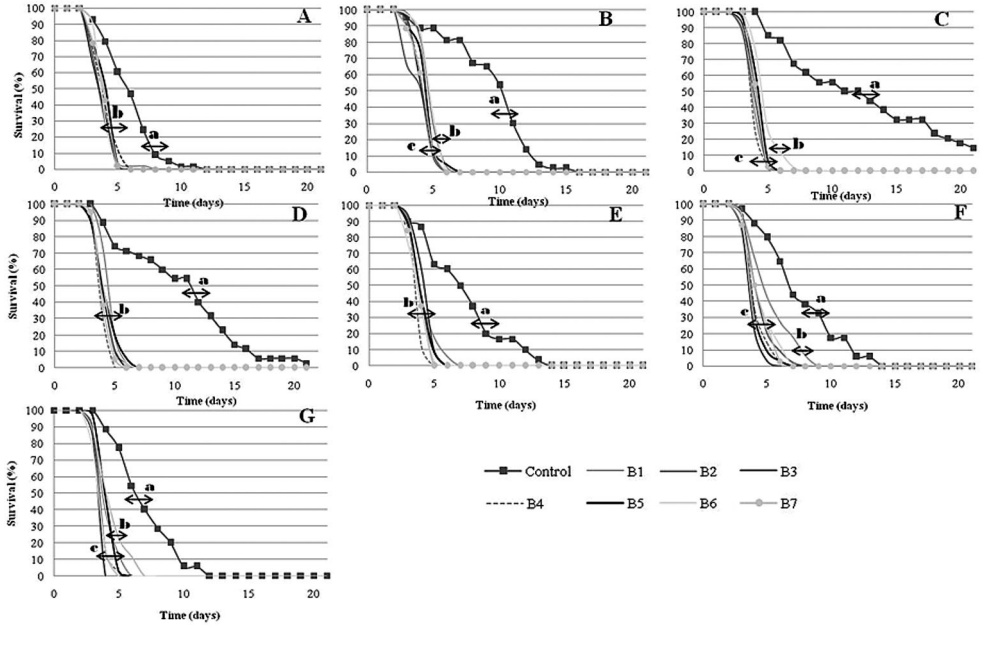


**Figure 2.** Survival time (%) for each colony (A to G) for ants treated with a Tween 80 solution as control, and with the seven isolates of *B. bassiana* (B1 to B7). Survival distributions with the same letter are not significantly different (see text for *P*-values considered).

We analysed each colony separately because we found significant differences among colonies in the control treatments. As we said before, survival time of ants treated with *B. bassiana* was significantly shorter than that recorded in their respective control for each of the colonies (Figure 2). For three out of seven colonies (A, D and E), the survival time of the ants treated with any isolate was statistically shorter than for the control ants ($P < .0018$) and there was no significant differences among survival distribution for all the isolates, so then they showed the same mode of action in these colonies. However, for the other four colonies, the isolates varied in survival distribution among them (Figure 2).

The percentage of dead ants due to *B. bassiana* infection varied between 80% and 93% depending on the isolate. The highest percentages of death caused by *B. bassiana* were obtained for isolates B5 and B7 (92.9% and 87.5%, respectively). We found *B. bassiana* natural infections in ants from the control treatments in five out of seven colonies, with percentages that varied between 2.9% and 11.5%.

The isolate B5 was chosen as the best candidate among the others because this isolate caused the highest percentage of dead ants and the behaviour of survival curves was uniform among colonies, followed by the isolate B7 that shared those same traits but ranked second.

### Evaluation of different types of substrate

The initial moisture contents of the substrates evaluated were 58.8 ± 5.8%, 59.1 ± 6.7%, 59.9 ± 3.3%, 51.9 ± 3.8% and 57.3 ± 4.2% for white rice, parboiled white rice, brown rice, soybean hull and *yerba mate*, respectively.

After 10 days of growth on each substrate, we found that the three types of rice and the soybean hull produced conidia, whereas the *yerba mate* did not, although we saw mycelia growth (Figure 3). In view of these results, *yerba mate* was left aside in the evaluation of yield production, for which just soybean hull and the three types of rice were considered. The yield production was not statistically different (KW = 6.179; $p = .103$; df = 3) among the substrates on the 10th day. On the 14th day, there were still no significant differences among the four substrates (KW = 4.128; $p = .248$; df = 3). The yields seemed to be high on the 14th day, although we were not able to compare non-parametrically both dates due to a possible time-dependent correlation.

The median value of conidia yield for each substrate on the 10th day was increasing as follow: $7.3 \times 10^8$ conidia $g^{-1}$ for soybean hull, $1.9 \times 10^9$ conidia $g^{-1}$ for white rice, $2.0 \times 10^9$ conidia $g^{-1}$ for parboiled rice and $2.2 \times 10^9$ conidia $g^{-1}$ for brown rice. On the 14th day, the median values were $1.4 \times 10^9$ conidia $g^{-1}$ for soybean hull, $3.4 \times 10^9$ conidia $g^{-1}$ for white rice, $3.7 \times 10^9$ conidia $g^{-1}$ for parboiled rice and $3.6 \times 10^9$ conidia $g^{-1}$ for brown rice.

The selection of the best substrate, brown rice, was related to its cost and the highest value of conidia yield on the 10th day as a way to reduce the time of the overall process and the SSF optimisation.

### SSF optimisation

The scale-up in the solid fermentation chamber was successful. Temperature and humidity was easily controlled just by setting the desired parameters, and were fairly stable

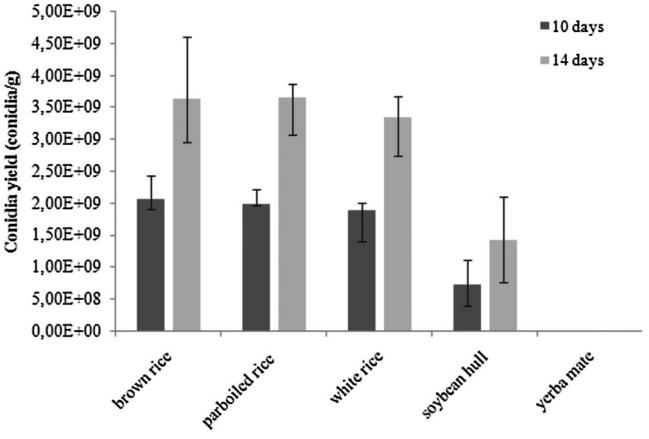


**Figure 3.** Median and quartiles of conidia yield (conidia/g substrate) of *B. bassiana* incubated for 10 and 14 days on five different solid substrates: long-grain brown rice, long-grain parboiled white rice, long-grain white rice, soybean hull and *yerba mate* (dry leaves of *Ilex paraguariensis*). There were no statistical differences in yield production among substrates within each time ($p > .05$).

throughout the process for all the treatments: maximum deviations were 1°C and 2.4% for temperature and relative humidity, respectively (Figure 4). We did not observe self-heating inside the chamber. We had 2 out of 23 cases of contamination, probably related to contaminated inoculum, which we were able to avoid with the subsequent sterilisation cycle. Contaminated replicates were discarded.

Regarding conidia yield using isolate B5, treatment E (conidia suspension as inoculum with $8 \times 10^5$ conidia $g^{-1}$, 70–79% of moisture content of substrate, 27–28°C of temperature and 75–80% of humidity inside the chamber) showed the greatest production, reaching 6.4 (±3.7) $\times 10^8$ conidia $g^{-1}$ ($2.6 \times 10^9$ conidia $g^{-1}$ of initial dry substrate, considering an average humidity of 75%) (Figure 5). However, there were no significant differences among treatments ($F = 1.5$; $p = .239$; $df = 5$). Additionally, treatment E had the lowest variability among replicates (2.5%) in comparison to other treatments (12.7%, 7.2%, 6.3%, 3.8% and 3.2% for treatments A, B, C, D and F respectively). Treatment A was the only one with a PDB inoculum, thus it could be considered biphasic fermentation, and exhibited low yield and the greatest variability among all treatments. The trend observed showed that a lower volume of inoculum (250 ml in B instead of 500 ml in C) seemed to be more efficient in conidia production. Regarding moisture of solid substrate, obtained from different rice cooking procedures, we recorded a better yield in the treatment with higher moisture (treatment D compared to C). Comparison between treatments D and E revealed that the evaluated temperatures in the chamber tended to have a low effect on conidia yield, but 28°C seemed slightly better. Finally, the addition of wheat bran (treatment F) did not increase the conidia yield in comparison to treatment E.

Treatment E was repeated three times with isolate B7 and B5 in the same chamber simultaneously and the conidia yield reached was of 1.2 (±0.6) $\times 10^9$ conidia $g^{-1}$, and 6 (±3.2) $\times 10^8$ conidia $g^{-1}$, respectively. The addition of wheat bran allowed to produce 6.5 (±4.4) $\times 10^8$ conidia $g^{-1}$ for the isolate B5, and 1.2 (±5.8) $\times 10^9$ conidia $g^{-1}$ for the isolate B7, therefore it did not differ the yield in comparison to not adding it (treatment E vs. F).

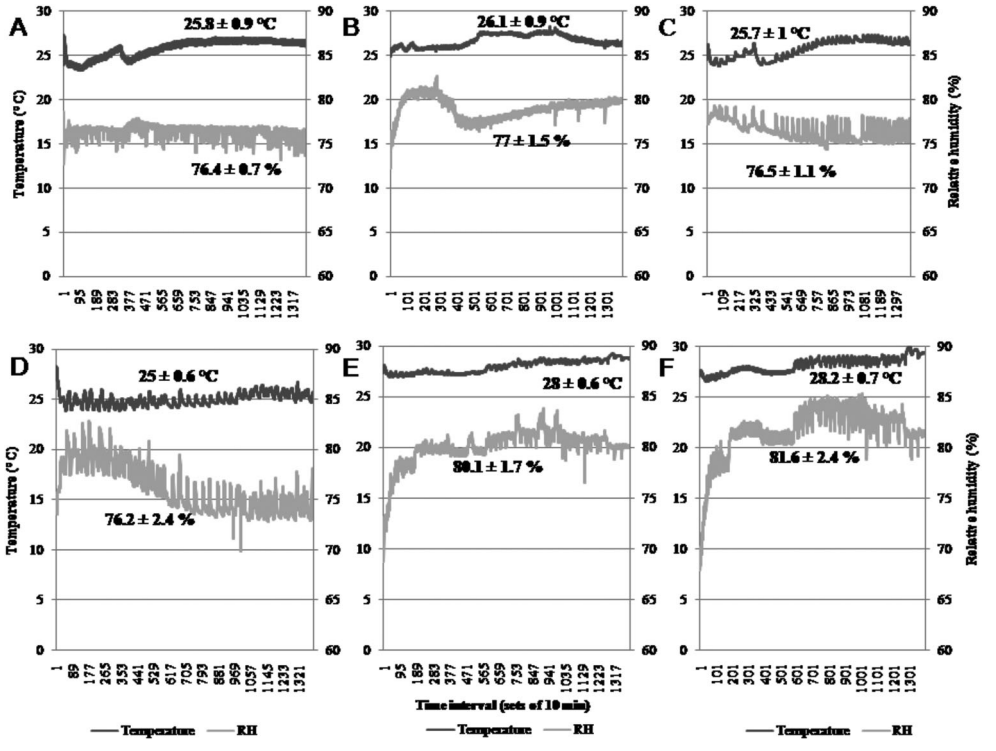


**Figure 4.** Means of temperature and relative humidity inside the chamber for each set of treatments (A to E). The data were collected for 10 days at the interval of every 10 min in each repetition.

Percentages of germination for all treatments exceeded 94%. We found germination means of 94.3 ± 3.4% for treatment A, 96.7 ± 3.5% for B, and 97.2 ± 2.5% for treatments C and D. We found a 98 ± 1.7% and 97.5 ± 3.5% of conidia germinated for isolate B5

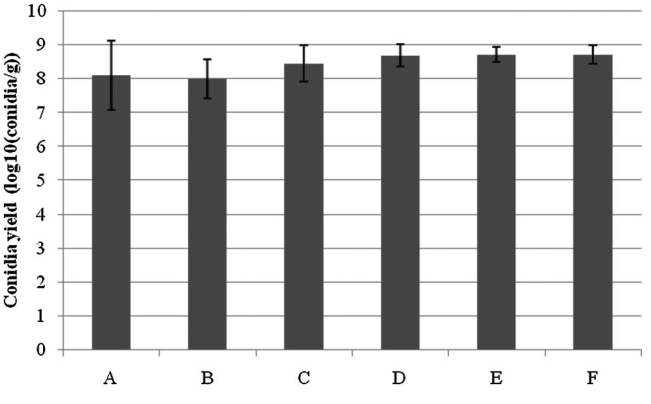


**Figure 5.** Mean and standard deviation of conidia yield (log10 (conidia/g substrate)) of *B. bassiana* incubated for 10 days on brown rice in the solid fermentation chamber, with different conditions (each letter represent one treatment, for details see Table 1). There were no significant differences among treatments ($p > .05$).

and B7, respectively. When wheat bran was added (treatment F), the percentages of germination were 97.6 ± 1.6% and 97.8 ± 1.5% for isolates B5 and B7, respectively. The isolate B5 showed the highest value of germination under treatment E.

## Discussion

The results of this work lead us to propose one out of seven isolates of *B. bassiana* as the best candidate for the control of the leaf cutter ants *A. lundii*, although all of them proved to be good potential control agents. This is the first work with data of *B. bassiana* isolated from *Acromyrmex* ants and their infection in laboratory assays.

The fact that isolates were obtained from *Acromyrmex* ants raises an important safety issue when looking for candidates for biological control purposes because it implies no introduction of foreign strains; furthermore, it will represent just an augmentation of a natural enemy of these ants. The same approach was carried out by Ribeiro et al. (2012) with *Atta bisphaerica* workers, who also isolated potential entomopathogens from the target ants and then tested their virulence. They also got good results with *B. bassiana* as entomopathogen, but to obtain the same LT50 than ours (4 days), they had to inoculate ants with higher conidia concentration ($10^8$ instead of $5 \times 10^6$ conidia $ml^{-1}$). The study of Loureiro and Monteiro (2005) used a conidia concentration similar to ours ($10^6$ conidia $ml^{-1}$) of two isolates obtained from a collection to inoculate *Atta sexdens* ants, and they showed a LT50 of 5.2 and 5.4 days, one day more than in this study. Therefore, the results obtained for *Acromyrmex* ants seemed better than those published for *Atta* species. The reason could be related to the fact that *Acromyrmex* isolates are more virulent than *Atta*'s (and/or collection isolates), or that *Atta* ants have higher capacity to fight against this pathogen.

In virulence assays, isolate B5 was selected as the best of all the isolates tested on account of the highest percentage of infection obtained and similar survival patterns for all colonies. The same reasons apply when it comes to the selection of isolate B7, as the second option. The fact that there was a uniform response in different colonies is a very important issue, as different colonies can have different immunological responses which can affect the efficacy of the biological agent (Goffré & Folgarait, 2015). In addition, it is important to take into account that the infections occurred in non-disinfected ants, i.e. the isolates had to fight and win in the competition with other possible natural entomopathogens present in the ants. Then, the percentages reported here represent a more realistic situation than the results of assays performed with laboratory ants or disinfected ones, which further improves the values found.

After the selection of the isolate B5, the SSF process was evaluated to allow its conidia production. Although conidia yield did not differ among different types of substrate, we selected brown rice due to its higher median value of conidia yield on the 10th day, the possibility to introduce rice as part of a formulation (U.S. Patent No. 9,578,873, 2017) and its lower cost in comparison to the other types of rice (US $1.07 per kg instead of US $1.19 and US $1.56 per kg of white rice and parboiled rice respectively, in Argentina). The conidia yield obtained in brown rice was similar to those obtain previously with one of two tested isolates of *B. bassiana* in Basmati rice in 6 days, but one order of magnitude higher than the another isolate (Taylor, Edgington, Luke, & Moore, 2013). The absence of fungal conidiation in *yerba mate* could be related to the lack of some nutrients,

mainly carbohydrates that are present in lower quantities in this substrate in comparison to the others (12.4 g of carbohydrates in 100 g of *yerba mate* compared to 74.1 g in brown rice, for example). We determined a culture time of 10 days for the following step (scale-up) using brown rice because we tried to reduce the overall time of the process, although the conidia yield obtained at the 10th day continued increasing, so a higher yield can be expected if the process is extended by 4 days.

The scale-up in our custom-built solid fermentation chamber was successful, with easy control of temperature and humidity. Both parameters showed little variability throughout the process, which is crucial given their influence in fungal conidiation (Jackson et al., 2010), so it was of the utmost importance to keep them under control. In addition, despite the inherent difficulty in controlling metabolic heat in SSF processes (Pandey, Soccol, Rodriguez-Leon, & Nigam, 2001), the characteristics of this chamber allowed us to keep this variable under control, given that no temperature increases were observed, as was the case with a similar device presented by Figueroa-Montero et al. (2011). Besides, safety is higher in our chamber than in those designs using open trays, in terms of operation as well as reduced risk of contamination. In addition, in this study we used a higher number of replicates (true repetitions) than SSF studies published with this fungus (Alves & Pereira, 1989; Chen et al., 2009; Kang et al., 2005; Ye et al., 2006), which assures a greater reliability of the optimisation results.

In the optimisation of the isolate B5, we selected the treatment E which allowed us to obtain the highest mean value of conidia yield ($6.4 \times 10^8$ conidia $g^{-1}$ of final substrate). Although there were no statistical differences among treatments, the condition E is also better because there is no need to prepare liquid inoculum previously (as in treatment A) nor to add a supplement substrate, like wheat bran (as in treatment F). Besides, treatment E showed the least variability among replicates and the highest percentage of conidia germination.

However, it could be considered that isolate B5 did not yield as many conidia as reported in other studies (such as $1.1 \times 10^{10}$ conidia $g^{-1}$ of final substrate in Kang et al., 2005, as maximum), although Taylor et al. (2013) reported yields in grams of initial dry substrate lower than ours, using 200 g in its production. Perhaps, changes in other variables could increase the yield, for example, a possible positive effect of light in conidiation (Zhang et al., 2009). As a way to corroborate the optimisation performed with the isolate B5, we also repeated treatments E and F with isolates B5 with B7 growing simultaneously but in different trays. The obtained yield for the isolate B7 was similar to other studies (Chen et al., 2009; Ye et al., 2006), reaching $1.2 \times 10^9$ conidia $g^{-1}$ of final substrate. That result allows us to confirm that, on one hand, different isolates produced different yield under the same conditions (Bena, Talaei, Askary, & Kharazi, 2011) and, on the other hand, the conditions selected for our SSF process also worked well with another isolate. Therefore, it will be interesting to test the conditions selected with other isolates of *B. bassiana* to prove its generality.

Bait formulations for biological control purposes need to be spread in the field and carried by pest organisms as fast as possible, in order to avoid the disintegration of the active ingredient by exposure to climatic conditions and possible infections to non-target organisms (Knowles, 2005). Solid bait formulation is the most popular because it allows, on one hand, to mask the active ingredient and, on the other hand, to add other components that attract ants which collect baits faster (Knowles, 2005). Some promising

approaches have appeared to control leaf cutter ants with fungal conidia as active ingredient, such as Arrieril®, a commercial product derived from the study of Lopez and Orduz (2003). A new patent (U.S. Patent No. 9,578,873, 2017) offers other method to control this pest with solid baits. The formulation includes the selected substrate of this study; thus, this technology becomes more cost-effective, avoiding the need to separate conidia from the substrate.

In conclusion, the different tested isolates of *B. bassiana* showed a good capacity to control *A. lundii* ants, and we proved that the two best isolates could be produced in the SSF chamber shown here, which provides stable conditions of temperature and relative humidity over 10-day periods, yielding a great number of highly viable conidia that could be incorporated with its substrate to solid baits.

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No potential conflict of interest was reported by the authors.

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