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Research Article

Biphasic production of the fungus *Beauveria bassiana* (Bals.-Criv.) Vuill. in an attract-and-infect device for control of the yerba mate ampoule *Gyropsylla spegazziniana* (Lizer & Trelles, 1919) (Hemiptera: Aphalaridae)

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Abstract. An 'attract-and-infect' device for controlling adult yerba mate ampoule *Gyropsylla spegazziniana* (Lizer & Trelles, 1919) (Hemiptera: Aphalaridae) was designed and tested in the lab. The device was constructed from plastic sheets containing small, recessed wells. The wells were filled with liquid culture medium and inoculated with conidia of *Beauveria bassiana* (Bals.-Criv.) Vuill.. After incubation, the devices were hung in cages containing a yerba mate seedling and adults of *G. spegazziniana*. For comparison, yellow cardboard cards impregnated with conidia were used. There were 28 times more conidia in plastic device than in the yellow card. Conidia had a germination level above 90% and infected approximately 56% of the insects. Further tests in the field are planned to evaluate its effectiveness for managing populations of *G. spegazziniana* in yerba mate.

Keywords: biological control, entomopathogenic fungi, conidia production, *Ilex paraguariensis.*

Yerba mate is a popular tea in South America which is made from the leaves of the same-named plant (*Ilex paraguariensis* A. St-Hil. (Aquifoliaceae). The trees are commercially grown for tea and pharmaceutical extracts in Brazil, Argentina, and Paraguay. The yerba mate ampoule, *Gyropsylla spegazziniana* (Lizer & Trelles, 1719) (Hemiptera: Aphalaridae), is one of the most important pest insects of yerba mate in all production regions. Adult females oviposit near new shoots, and after hatching, the feeding activity of the immatures causes the formation of galls (or ampoules) on the shoots. The immatures complete their development in the gall, which protects them from predators and adverse weather. Gall formation results in leaf drop and lateral shoot development and may lead up to 35% reduction in harvestable leaves (Penteado et al. 1995; Leite & Zanol 2001; Borges & Lazzari 2008; Formentini et al. 2015).

There are no insecticides registered in Brazil for use against *G. spegazziniana* in commercial yerba mate plantations (Agrofit 2023). However, there is natural occurrence of epizootics due to the fungus *Zoophthora radicans* (Bref.) A. Batko (Entomophthorales) in *G. spegazziniana* populations in Brazil and Argentina (Sosa-Gómes et al. 1994; Alves et al. 2009). Additionally, the fungus *Beauveria bassiana* (Bals.-Criv.) Vuill. (Hypocreales: Cordycipitaceae) was found to be pathogenic to *G. spegazziniana* in laboratory bioassays (Alves et al. 2013). An isolate of *B. bassiana*, Unioeste 44, was shown to be highly pathogenic to *G. spegazziniana* and showed promise as a bioinsecticial agent against this pest (Formentini et al. 2015). Although adjuvants and carriers make it possible to make spore formulations to apply to *G. spegazziniana*, numerous applications were found to be necessary to achieve an adequate level of control (Loeblein et al. 2022).

So-called 'attract-and-infect' devices (or 'autodisseminators') have been developed as a means of introducing and disseminating entomopathogenic fungi into populations of pest insects. The devices use strong visual and/or scent attractants to lure pest insects to them. The device is coated with entomopathogen spores and, after alighting on it, the insects self-inoculate (Patt et al. 2015). After leaving the device, they can directly transfer spores to conspecifics in the near term, and later via horizontal transmission. Attract-and-infect devices have been shown to be an efficient method for infecting individuals and establishing epizootics within pest populations (Lopes et al. 2014; Chow et al. 2018; Hassemer et al. 2020; Srei et al. 2020).

We have been developing attract and infect devices, using Unioeste 44 conidia as the biocontrol agent, to control *G. spegazziniana* in yerba mate plantations. In a preliminary field test, we showed that *G. spegazziniana* individuals visited a prototype attract-and-infect device and became self-inoculated with conidia (unpublished data). Manufacturing the attract-and-infect devices requires developing a source of conidia. Typically, the fungus is produced in a solid medium, such as parboiled rice loaded into polyethylene bags. After sporulation, the conidia are collected, dried, and then applied to the surface of the devices. This method is quite frequently used for the commercial mass production of entomopathogenic fungi (Faria & Wraight 2007; Li et al. 2010; Almeida 2020). However, the process is labor intensive, requiring many steps to obtain the conidia, and rearing the fungus may take 20 to 30 additional days under sterile conditions to reduce the risk of contaminations (Roberts & St. Leger 2004; Jackson et al. 2010). Production of conidia in static liquid medium is also possible, and the process can be automated to rapidly produce a large amount of fungus. But it poses difficulties, such as a high risk of contamination to large volumes of liquid medium.

It is also possible to produce conidia on the surface of a liquid medium. Lopes et al. (2019) developed a device to produce aerial conidia using a biphasic system: a liquid-phase fermentation followed by solid-phase fermentation on a plastic support resembling a

honeycomb with the liquid culture media placed in wells.

An attract-and-infect device was manufactured with a liquid culture of *B. bassiana*, which allowed the development and production of conidia (Srei et al. 2020). It is also possible to use a loofah sponge, or similar material, as an inert support for fungal cultures in attract-and-infect devices (Nascimento et al. 2020). In the present study, we developed and tested a novel attract and infect device to control *G. spegazziniana* with conidia from the Unioeste 44 isolate of *B. bassiana*. Our attract-and-infect device featured a biphasic fungal medium system to produce conidia *in situ*. The goal of the research is to provide proof of concept of this novel device as a potential means of controlling *G. spegazziniana* in yerba mate plantations.

Material and Methods

Fungus. The fungus *B. bassiana* isolate Unioeste 44 (*B. bassiana sensu lato*) (GenBank sequence OK004060), was used as the infection agent. The isolate was previously selected as the most virulent for *G. spegazziniana* (Formentini et al. 2015). Conidia were obtained from a culture medium (ME) (10 days, 26 ± 1 °C, 14:10 light:dark photoperiod) (Alves et al. 1998). The conidia were collected by scraping the medium surface and then transferred to a flask with a 0.05% Tween 80 solution in distilled water.

Insects. The insects were obtained from a commercial yerba mate plantation. In the laboratory, galls with last-instar nymphs (Leite & Zanol 2001) were maintained in plastic containers with a screened lid and bottom covered by paper towel and maintained at 26 ± 1 °C; 12:12 light:dark photoperiod; and R.H. of 60 ± 10% until the emergence of adults. Adults which emerged from the galls were used in the bioassays (Loeblein et al. 2022).

Preparation of attract-and-infect devices. Liquid medium for blastospore production (41.7 g yeast extract, 5.02 g sucrose, 1.0g KCl, 0.36 g KH_2PO_4, 0.6 g $MgSO_4$ and 1 L distilled water) was prepared and 150 mL aliquots of the medium were added to 250 mL Erlenmeyer flasks (Lopes et al. 2019). After sterilizing and cooling, the flasks were inoculated with 1 mL of conidial suspension (approximately 1×10^9 conidia/mL). The flasks were incubated in an orbital shaker (25 ± 1 °C, 200 rpm) for 2 days.

The inert support for the blastospore production medium on the attract-and-infect device was made from a piece (2.5 × 2.5 cm) cut from a transparent food grade plastic sheet (Pact Group, Richmond, Australia, https://pactgroup.com.au/). The support contained 12 triangular wells (approximately 0.43 cm^2; 0.5 mm depth) for holding the fungal medium. The support was painted bright yellow, since this color is highly attractive to *G. spegazziniana* (unpublished data). The devices were surface sterilized by successively submersion in 5% sodium hypochlorite, 70% alcohol, and distilled water for 1 minute. After drying for 5 minutes in a laminar flow chamber under UV lights, each well was filled (180 µL) with the 2-day-old inoculated liquid medium. This volume was previously determined as sufficient for completely filling the wells without overflowing (Fig. 1A). The devices were packaged in sterilized Petri dishes containing 2% agar-agar and maintained in an incubator for 4 days (26 ± 1 °C, 14:10 light:dark photoperiod) which was a sufficient time for fungal sporulation (Fig. 1B). This approach will be referred to as the biphasic device, as it involves a two-stage process. The first stage involves the liquid phase production, yielding blastospores and biomass. Subsequently, the process transitions into a static liquid phase, with the goal to obtain aerial conidia. A total of 5 devices were prepared, each one with one replication. The entire experiment was replicated twice.

A bright yellow cardboard card dusted with conidia, which functioned effectively as an attract-and-infect device (unpublished data), was used as a control, and will be referred as the card-shaped device. The cards (2.5 cm of side) were painted with a thin layer of emulsified wax SPLAT® (ISCATech), which helped the conidia to adhere to the device (Patt et al. 2015). The aerial conidia were applied to the card + SPLAT devices with a sieve and the conidia excess was removed by slightly shaking the cards afterwards.

Evaluation of conidial yield and activity. The amount of conidia produced in the wells of the biphasic devices was determined at the end of the incubation period by placing the devices in glass tubes, submerging them in a 0.05% Tween 80 solution, and then vortexing them for 2 min to remove the conidia. The same procedure was performed to obtain the conidia produced by the card-shaped control devices. Conidia concentrations were measured in a Neubauer chamber observed at ×400 magnification with a light microscope.

The initial viability of conidia from both devices was also evaluated. After dilutions, 130 µL of conidial suspensions were applied to a potato dextrose agar (PDA) culture medium surface, in a Rodac® dish (Replicate Organism Detection and Counting). After drying in a laminar flow, the dishes were incubated for 10 days at a temperature of 26 ± 1 °C and a 14:10 light:dark photoperiod (Oliveira et al. 2015). Both germinated and non-germinated conidia were counted using a light microscope at ×400 magnification. Conidia were considered germinated when the size of the germination tube was twice as long as the diameter of the conidia. Each device was considered a replication, and three dishes were prepared for each replica.

The bioinsecticidal activity of the conidia was evaluated in both types of devices using cage tests. An *I. paraguariensis* seedling and 15 adult *G. spegazziniana* were placed inside a cage (30 cm high × 12 cm diameter) with transparent plastic panels equipped with side openings (6 cm height × 5 cm length), a screened top for ventilation (Loeblein et al. 2019). A single attract-and-infect device of either type was attached to the upper section of the test cage (Fig. 1C). In this test, the control treatment was the same as above except that no device was added to the cage. Mortality of *G. spegazziniana* was evaluated daily for 15 days by visually inspecting each cage. Insect cadavers were removed, surface sterilized by submersion in a solution of 2% sodium hypochlorite, 70% alcohol, and distilled water, and then maintained in the humid chamber for 5 days. Mortality by fungus was confirmed by observing the insect cadavers for mycosis under a stereomicroscope. Four cages were prepared for each test device and for the control, and each one was considered a replication. The entire experiment was replicated twice, at two different times.

Statistical analysis. All data were evaluated regarding homogeneity and homoscedasticity (Shapiro-Wilk and Barttlet) ($p < 0.05$). The quantification of conidia present in the devices and confirmed mortality were analyzed using the Student *t*-test. Total mortality data were analyzed using the One-Way ANOVA test followed by Tukey HSD ($p < 0.05$) (Software Statistica® 7.0, StatSoft 2014).

Results and Discussion

There was a significant difference in the amount of conidia produced by each type of attract-and-infect device ($F=0.027$; $p < 0.01$). A total of 254×10^8 conidia, with an estimated concentration of (4×10^9 conidia/cm^2), was produced by the biphasic device while the mean total number of conida produced on the card-shaped control device was 9.0×10^8, with an estimated concentration of 1.4×10^8 conidia/cm^2. Thus, the concentration of conidia obtained from the biphasic device was approximately 28 times higher than that obtained from the card-shaped control device. Conidial germination in both types of devices, observed at the beginning of the experiment, was higher than 90% (Tab. 1).

Both devices infected similar proportions of insects, causing 55.5 and 55.9% total mortality in cages with the biphasic and card-shaped devices, respectively. The proportions of infected insects differed between cages with attract-and-infect devices and the control cages, which lacked devices ($F = 20.159$; $P < 0.01$). Mycosed insect cadavers were present in both treatments, yet the number of cadavers showing confirmed mortality i.e., mycosis, was significantly higher in the card-shaped device (30.5%) than in the well device (16%) ($F = 6.11$; $P < 0.05$). There was no confirmed mortality in the control group (Tab. 2).

The difference in confirmed mortality of insects between the two devices may be due to competition for resources inside the insects. In tests with the biphasic device, the insects were exposed to a very large number of conidia. Their subsequent germination and proliferation

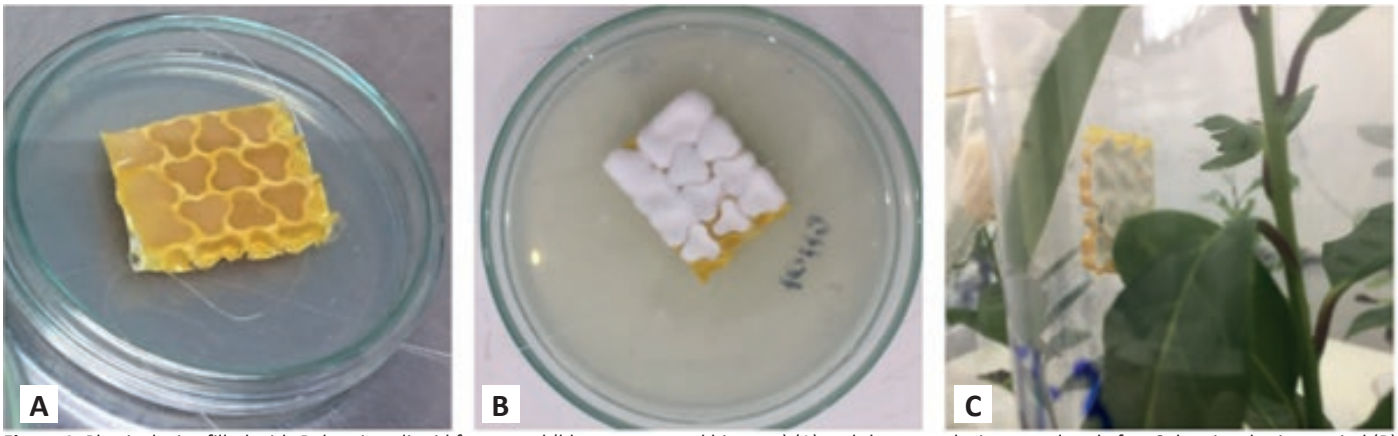

Figure 1. Plastic device filled with *B. bassiana* liquid fermented (blastospores and biomass) (A) and the same device sporulated after 6 days incubation period (B); Biphasic device hanged on PVC cages containing a yerba-mate seedling and insects (C).

inside individual hosts resulted in the depletion of nutrients (Hajek & St. Leger 1994) leaving none for reproduction (Shimazu 1994). Insects that visited the card-shaped device were inoculated with fewer conidia, so resource competition would not be expected to be so high that it results in nutrient depletion in the cadaver. Thus, it is possible that some cadavers exposed to the biphasic devices did not mycose and the pathogen could not complete its infection cycle. This phenomenon was observed previously with *G. spegazziniana* (Formentini et al. 2015). In addition to the low confirmed mortality, cadavers from the biphasic device had a reduced body volume, without any sign of conidiogenesis, as was observed with *Diaphorina citri* (Kuwayama, 1908) (Hemiptera: Liviidae) (Padulla & Alves 2009).

Table 1. Conidia of *B. bassiana* strain Unioeste 44 produced by the biphasic and card-shaped attract-and-infect devices.

Type of device	Mean (± SE) number of conidia/device (× 10^8)	Estimated concentration of conidia/cm² (× 10^9)
Biphasic	254 ± 1.42 a	4.0
Card-shaped	9.01 ± 0.15 b	0.14

Means followed by different letters differ from each other using the Student *t*-test ($P < 0.05$).

Table 2. Mortality (mean ± SE) of *G. spegazziniana* adults after 15 days of exposure to attract-and-infect devices containing conidia of *B. bassiana* Unioeste 44 (25 ± 2 °C; RH = 65 ± 5%; 14:10 light:dark photoperiod).

Type of device	Mortality (%)	
	Total	Confirmed
Card-shaped	55.9 ± 10.83 a	30.5 ± 6.40 a
Biphasic	55.5 ± 2.20 a	16.0 ± 1.70 b
Control (No device)	19.1 ± 4.58b	0.0 ± 0.00 c

Means followed by different letter in each column differ statistically from each other (Tukey HSD, $P < 0.05$).

The concentration of conidia obtained from the biphasic device can be considered quite high, since the production was approximately 40 times higher than the obtained in a Petri dish with the same isolate in culture medium by Formentini et al. (2015). Using the same biphasic device, a concentration of 2.2 × 10^8 conidia/cm² of *B. bassiana* isolate J21 was obtained in Sabouraud-dextrose-yeast extract culture medium (Lopes et al. 2019). These variations in the conidia yield are expected due to the variation in production system, availability of nutrients in the medium, and characteristics of the isolates used. Moreover, both germination and fungal activity did not change with the variation in production system (Lopes et al. 2019).

The results showed that high concentrations of conidia could be produced in liquid medium on the plastic support of the biphasic attract-and-infect device. It indicates that the biphasic approach is a potentially viable and a promising means of producing conidia *in situ* in attract-and-infect devices designed to control *G. spegazziniana*. As noted earlier, the yellow cards impregnated with fungal conidia were effective as attract-and-infect devices against *G. spegazziniana* adults in the field (unpublished data). However, conidia for the card device, using the conventional method with parboiled rice in plastic bags, takes 20-30 days. An important advantage of the biphasic device is that viable conidia are produced relatively quickly (6-8 days), enabling the devices to be rapidly completed and deployed. Studies under field conditions are necessary to prove the attract-and-infect capacity of the biphasic device for *G. spegazziniana* control. However, card-shaped devices achieve a higher confirmed mortality rate, indicating that in the field, there may be a greater occurrence of cadavers using this approach and could contribute to the enhanced dissemination of the fungus in the environment.

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Authors' Contributions

IF: planning, execution, tabulation, data curation and analysis, writing and review; LFA: planning, supervision, financing, writing and review; JSL: planning; ATBG: planning and statistical treatment; JMP: planning, writing and reviewing.

Conflict of Interest Statement

No potential conflict of interest was reported by the authors.

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