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Endophytic Development of the Entomopathogenic Fungus *Beauveria bassiana* Reduced the Development of Galls and Adult Emergence of *Leptocybe invasa* in Susceptible *Eucalyptus*

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Abstract: *Eucalyptus* cultivation in arid regions is limited by the losses caused by the galling wasp, *Leptocybe invasa* Fisher and La Salle, 2004 (Hymenoptera: Eulophidae); it oviposits below the epidermis of the central veins, mainly on seedlings in nurseries and/or young plants in the field, causing galls where the insects develop. Galls modify the normal circulation of sap in vascular tissues, ultimately affecting the development of infested plants and resulting in slower growth. *Eucalyptus* genotypes tolerant to water stress are susceptible to *L. invasa*, and this pest has no effective control methods. Here, we aimed to (i) determine the initial infestation dynamics of *L. invasa* in *Eucalyptus* plants inoculated with *Beauveria bassiana* and (ii) identify any change in the development of galls. *B. bassiana*-inoculated seedlings were infested with *L. invasa*. Susceptible plants were unaffected, and gall development was negatively affected at multiple stages. A fungal solution containing *B. bassiana* reduced gall development and the emergence of *L. invasa* adults in eucalyptus plants at considerable rates. The total number of adult emergence holes was 89.74% fewer for inoculated plants when compared to uninoculated plants. These results can help develop *B. bassiana* products to control *L. invasa* in susceptible eucalyptus plants for improved, sustainable forestry.

Keywords: gall wasp; biological control; fungus; entomopathogen

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1. Introduction

The extensive and indiscriminate use of pesticides has caused serious imbalances in the ecosystem, severely affecting pest control through natural processes and resulting in the resurgence of secondary pests and the elimination of natural enemies. In addition, concerns for human health and the environment have motivated the development of more sustainable and safer strategies for controlling pests. An example of using more sustainable strategies is the biological control of insect pests by entomopathogenic [1,2]. This method reduces crop damage and is key to integrated pest management programs. The advantages that make its use feasible include the positive interaction between the ecosystem and beneficial organisms, lower environmental chemical residues, and greater biodiversity in

ecosystems managed by humans [3]. In addition to their use as biological insecticides, there is increasing evidence that some species of entomopathogenic fungi can colonise the tissues of certain plants. Although only a selected group of these have been reported as naturally occurring endophytes, many successful attempts have artificially introduced them into plants using different techniques [4]. This natural or artificial colonisation can be beneficial for the plant, as studies show increases in plant growth and reductions in pest infestation in crops of economic importance [5,6].

In managing pests in commercial crops, the entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuills (Ascomycota: Hypocreales) is a viable alternative for controlling biological pests [7–9]. Actions to stimulate parasitoid populations have already been developed, and promising results have been obtained for controlling forest pests [6]. However, little information is available on the dynamics of ecophysiological defence responses in *Eucalyptus* L'Hér plants colonised by entomopathogenic fungi in response to insect pest herbivory and/or parasitism.

The forestry sector plays a strategic role in supplying raw materials to transformation industries. The genera *Eucalyptus* and *Corymbia* are prominent among the species that form planted forests. They originate from the Australian continent and include large native forest massifs. Phenotypic plasticity, rapid growth, high productivity, and industrial yield form the basis for the expansion of crops in tropical and subtropical regions worldwide [10]. Plantations of forest species with reduced genetic diversity express vulnerability to pests. The eucalyptus gall wasp, *Leptocybe invasa* Fisher and La Salle, 2004 (Hymenoptera: Eulophidae), is one of the main pests of eucalyptus crops. *Leptocybe invasa*, in its juvenile stages, induces hyperplasia (galls) in the tender tissues of young leaves, petioles, internodes, and branches of *Eucalyptus* L'Hér, *Corymbia* KD Hill, and LAS Johnson plants. In addition, they induce physiological disturbances that are initiated shortly after oviposition. Substantial productivity losses of seedlings have been attributed to the pest, with estimates of losses in regions with infestation-intense conditions [11]. The formation of galls and feeding of larvae in the internal chambers of the gall may reduce the ability of *Eucalyptus* plants, at different levels of susceptibility, to redistribute photoassimilated material [12] which consequently impairs the growth and development of seedlings and young plants13. Currently, there is no efficient control method for *L. invasa* in *Eucalyptus* plantations, not even chemical control, which has been inappropriately used in past situations [13].

Thus, studies on sustainable pest management strategies are needed. In this context, biological control with entomopathogenic fungi could be used as a sustainable pest control method because it is friendlier to non-target species, the environment, and human health than synthetic insecticides. Based on that, here we investigated (i) the dynamics of initial infestation of *L. invasa* in eucalyptus plants inoculated with the entomopathogenic fungus *B. bassiana* and (ii) the effect of this inoculation on the development of *L. invasa* galls as well as adult emergence in susceptible *Eucalyptus* plants.

2. Materials and Methods

2.1. Experimental Site

The study was carried out in the forest nursery area of the Laboratory of Applied and Functional Ecology at the Federal University of Tocantins (UFT) Brazil, Gurupi *Campus*, at 11°44′36.86″ South latitude and 49°2′57.18″ West longitude; humid tropical with dry winter Aw [14].

2.2. Plant Source

Rooted cuttings were produced from a hybrid clone VS058, obtained from crossing *Eucalyptus tereticornis* × *Eucalyptus camaldulensis*, which is highly susceptible to *L. invasa*, initially from the S&D Florestal nursery, Martinho Campos-MG, Brazil. When they reached the age of 120 days (d), they were transplanted from the tubes into 3.8 L pots containing commercial Bioplant® (Bioplant holding LTDA, Nova Ponte, Brazil) substrate based on pine bark, carbonised rice husk, vermiculite, macronutrients, and micronutrients. After

transplanting, the seedlings were acclimatised in a greenhouse for 30 days in the sun at the UFT/Campus de Gurupi, Experimental Station. Throughout the experiment, the plants were exposed to local meteorological conditions: an average temperature of 27.5 °C (Tmax 35 °C and Tmin 23 °C) and relative humidity (R.U. max 90% and R.U. min 35%). The plants were maintained under daily irrigation with a blade adjusted to replace the volume referring to the crop's evapotranspiration (ETc) during the experimental period from December 2020 to July 2021.

2.3. Fungi Culture

The fungus *B. bassiana* (Beauveril strain PL 63) was obtained from the mycological collection of the Laboratory of Symbiosis, Insect Microorganisms at UFT/Campus de Gurupi. The collected plates were subcultured on new plates containing PDA (Potato, Dextrose, Agar) medium, supplemented with amoxicillin 500 μg mL^{-1}, and maintained in BOD (biological oxygen demand) for 15 days at 25 °C ± 2 °C and 12 hours of photophase. After this period, the plates were opened in a flow chamber under aseptic conditions, and the spores were removed from the colonies. For this purpose, 10 mL of autoclaved distilled water with 0.02% (v/v) Tween 80 was added to the plate, followed by delicate scraping of the spores with a sterilised spatula. The spores were transferred to a sterile beaker containing 100 mL of sterilised distilled water. The solution was then shaken in an orbital-shaking incubator at 150 rpm for 10 min at room temperature. The suspension was then filtered through a double layer of fabric (sterilised gauze) to retain the mycelial fragments and remnants of the culture medium, which were then poured into an autoclaved beaker. This solution was placed in a Neubauer chamber for spore counting using an optical microscope and automatic counting (Counter model Milky Way, Taichung, Taiwan). The concentration of the solution was adjusted to 1×10^8 conidia mL^{-1}, with autoclaved distilled water containing Tween 80 at 0.02% (v/v). Inoculation was performed immediately after solution preparation. An autoclaved solution of distilled water containing 0.02% (v/v) Tween 80 was used for the control plants.

2.4. Insect Source

The individuals of *L. invasa* used in the present study were obtained from an organza-lined cage with dimensions of 2.8 × 5.2 × 3.0 m in height, length, and width, respectively, with infested plants destined to create the galling insects. Twigs that presented galls in stages close to emergence were cut from the infested eucalyptus plants. These branches were taken to the laboratory and placed in a beaker with distilled water inside a bench cage lined with organza. One hundred percent *Apis mellifera* honey (undiluted) was offered at room temperature (25 °C ± 2.0; 12 hour photophase) to maintain the survival conditions of the newly emerged adults from the galls [15]. After 24 h, the emerged individuals were collected using a manual entomological aspirator and placed in centrifuge microtubes [12].

2.5. Plant Inoculation

Plants were inoculated with *B. bassiana* through the adaxial epidermis of the fourth, fifth, and sixth fully expanded leaves from the apices of each of the 8 plants, and 8 plants were uninoculated, totaling 16 plants. These leaves were slightly injured with a polyurethane sponge with dimensions of 4.00 × 3.75 × 2.5 cm in length, width, and height, respectively. Eight plants were sprayed with a standardised *B. bassiana* conidial solution as previously described. The remaining eight plants were sprayed with distilled water and autoclaved with 0.02% (v/v) Tween 80. The sprays were carried out mainly on injured leaves, and nearby leaves consequently experienced spray drift. Six branches from the upper third of each plant were covered with transparent plastic bags and stored for a set period.

2.6. *Beauveria bassiana* Colonisation in Eucalyptus Plants

Beauveria bassiana colonisation was confirmed by collecting three leaves from the upper third of four plants in the inoculated group (*B. bassiana*) and four plants in the uninoculated group (control). Three circular fragments (0.25 cm^2) were removed from each leaf, which were then superficially sterilised in 1% NaCl (5 min), 70% ethyl alcohol (3 min), and sterile water and incubated in a Petri dish containing PDA culture medium supplemented with Chloramphenicol (100 µg mL^{-1}). They were kept in B.O.D. chambers for 16 days at 25 °C ± 2 °C and a photophase of 12 h. At the end of this period, the plates were evaluated for the presence (+) and absence (−) of *B. bassiana* colonies (Figure S1) in the Environmental Microbiology and Biotechnology Laboratory—LAMBIO, in UFT—Campus Palmas.

2.7. Infestation with *Leptocybe Invasa*

At 45 d after *B. bassiana* inoculation, all *Eucalyptus* plants (inoculated and non-inoculated) were infested with *L. invasa*. Two newly emerged females were collected from pruned apices and placed in beakers with distilled water in the laboratory's rearing chamber (organza chamber) at 25 °C ± 2.0. We followed the methodology described by Sarmento et al. [12], who showed that using two females per plant to infest the apices wrapped in an organza bag was sufficient to obtain the oviposition required for the studies [6,12]. The microtubes were then opened so the wasps could initiate infestation and lay eggs on the apex (leaves, petioles, or internodes). Given that Sarmento et al. [12] observed that many individuals were still alive and active after 24 h of oviposition, extending the period to 48 h was necessary to obtain more oviposition. Thus, after 48 h, the wasps that were still alive were eliminated, and the bags were removed.

From that moment onwards, the collection of data on infestation began, and the number of oviposited plants was quantified by totaling the plants that showed signs of oviposition on the petiole, central veins, and even on the internodes of the apexes exposed to infestation. The number of oviposited leaf primordia per plant was quantified in the apexes exposed to infestation. Verification of temporal variation in the duration of developmental stages: E1: brown ring, E2: suberised scar, E3: greenish gall, E4: reddish gall, and E5: adult emergence hole, as described by [12], was performed daily from the first to the one hundred and twenty-sixth day after infestation (DAI). The number of galls per plant was obtained between 28 and 42 DAI with *L. invasa*. The width and average length of the galls were obtained with the aid of a calliper (digital in carbon fibre with an accuracy of ±0.20 mm) at 14, 28, 35, 42, 52, and 66 (DAI), and for the length, it was only possible to start the collection at 28 DAI. For this purpose, the apexes, leaves, and internodes that formed the galls were marked with synthetic tape to allow the collection of measurements over the days of development. The number of emergence holes per plant was obtained daily from the first emergence between 45 and 126 DAI [12]. The experimental arrangement was randomised into two rows organised by treatment, with eight replicates per treatment. The treatments consisted of seedlings inoculated with *B. bassiana* and subsequently infested with *L. invasa* and non-inoculated seedlings infested with *L. invasa* (control), totaling 16 plants.

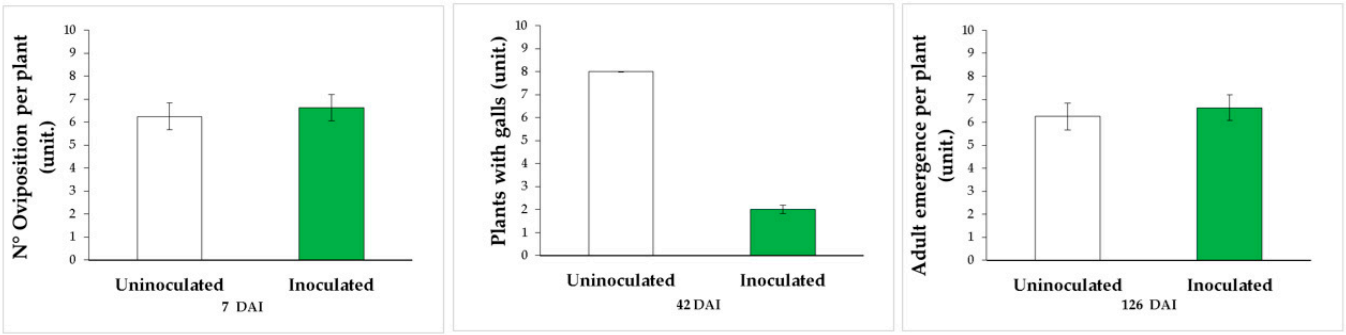
2.8. Statistical Analysis

The data obtained were subjected to the Shapiro–Wilk test, and when normal, they were subjected to analysis of variance, and the averages were subjected to the Student's *t*-test ($p < 0.05$). An analysis of variance and a standard error calculation were performed for the infestation data. Non-parametric analysis was performed using the Kruskal–Wallis test, and the medians were compared using the Mann–Whitney test for the group of variables in which the assumption of normality was not met, both performed using JAMOVI 2.3 software, Sydney, Australia (2022).

3. Results

By analysing the dynamics of parasitism by *L. invasa* in a hybrid *Eucalyptus* clone, it was possible to verify that, seven days after infestation, there were no significant differences

between treatments and all eight plants of each treatment were oviposited (GL = 14; t = −0.46; $p = 0.0653$). The same occurred for the intensity of infestation, and no statistically significant difference was observed for the number of ovipositions per plant (6.25 ± 0.59 and 6.63 ± 0.56) between plants uninoculated and inoculated with *B. bassiana*, respectively (Figure 1a). At 42 days after infestation, the plants developed galls, but the group that had been previously inoculated with *B. bassiana* had 75% fewer gall-producing plants (2.0 ± 0.56) than the uninoculated plants in the control group (8.0 ± 0.00) (Figure 1b). The same occurred for the number of adult emergence holes per plant (4.87 ± 2.09) in the group of plants uninoculated with *B. bassiana*, which had at least one adult emerging hole per plant. In contrast, in the inoculated plants, there were fewer adult emergence holes (0.50 ± 0.16), with less than one adult emergence per plant (Figure 1c). When analysing the total number of emergence holes per group of plants, it was found that at 126 days after infestation, there were 89.74% fewer adult emergence holes for plants inoculated with *B. bassiana* fungus when compared to the total obtained in the control plants (Figure 1c).



(**a**) (**b**) (**c**)

Figure 1. (**a**) Mean number of oviposition per plant 7 days after infestation (DAI) with *L. invasa*; (**b**) total plants with galls at 42 DAI; and (**c**) mean adult emergence per plant at 126 DAI.

Temporal data referring to the duration of each stage of gall development did not meet the normality assumption of the Shapiro–Wilk test at 95% significance. Except for the E1 stage in the group of plants inoculated with *B. bassiana* ($p = 0.092$) and E2 and E4 in Eucalyptus plants from the control group ($p = 0.094$ and 0.097, respectively) (Table 1). Thus, all results obtained for the temporal variation in the developmental stage of the galls were subjected to the Kruskal–Wallis test at a 95% significance level.

For the brownish ring (E1) and suberised scar (E2) stages with χ^2 critical = 3.841, no statistically significant difference was observed ($\chi^2 = 0.28$; df = 1; $p = 0.597$) or ($\chi^2 = 3.57$; df = 1; $p = 0.059$) between the plants inoculated and uninoculated with *B. bassiana*, respectively. At greenish gall (E3), there was a statistically significant difference in the temporal response between the inoculated and uninoculated treatments with *B. bassiana* (Table 1). Positive asymmetry was observed in plants inoculated with *B. bassiana*, as the values were concentrated above the median of 6.00 days. The opposite seemed to occur for temporal responses at the same stage: a brownish ring (E1). However, even with less variability in uninoculated plants, the asymmetry was negative when more values were concentrated below the median of 6.50 days.

At the suberized scar stage (E2), it was possible to verify the maintenance of the trend of greater variability in the data for the group of plants inoculated with *B. bassiana*, as the interquartile interval was 44.59 days, which was numerically higher than that obtained for plants in the control group at 23.90 days. However, asymmetry was not observed in *B. bassiana* plants. Negative asymmetry occurred when the concentration was below the median of 88.44 days. Uninoculated plants showed less data dispersion, with an interquartile interval of 23.90 days and slightly positive asymmetry, with data concentrations above the median of 58.31 days.

Table 1. Normality test and multivariate analysis for temporal responses of galls development induced by *Leptocybe invasa* (days) in *Eucalyptus* plants previously inoculated with the entomopathogenic fungus *B. bassiana* and in the uninoculated plants (control) 126 days after infestation of susceptible *Eucalyptus* seedlings.

Gall Stage	Inoculation	Mean ± SE	Quartile		Shapiro–Wilk			Kruskal–Wallis		
			1 st	3rd	Interval	W	P	χ^2	df	*p*
Brow Ring (E1)	Yes	6.06 ± 0.55	4.63	7.50	2.87	0.849	0.092	0.280	1	0.597
	No	5.89 ± 0.44	5.38	6.69	1.31	0.814	0.04			
Suberized Scar (E2)	Yes	81.31 ± 7.99	57.78	102.37	44.59	0.822	0.049	3.570	1	0.059
	No	57.48 ± 8.13	45.94	69.84	23.90	0.977	0.944			
Greenish Gall (E3)	Yes	5.77 ± 3.95	0.00	4.21	4.21	0.607	<0.001	5.000	1	0.024
	No	17.08 ± 4.72	6.09	20.59	14.50	0.843	0.080			
Reddish Gall (E4)	Yes	1.06 ± 0.72	0.00	0.81	0.81	0.608	<0.001	9.116	1	0.003
	No	12.08 ± 3.72	9.00	15.96	6.96	0.851	0.097			
Emergency Hole (E5)	Yes	0.30 ± 0.25	0.00	0.09	0.09	0.514	<0.001	11.907	1	≤0.001
	No	6.91 ± 1.60	4.38	9.62	5.24	0.806	0.033			

The gall development stage described as E3 (greenish gall) revealed a reversal of the previously observed trend, considering (df = 1, $p < 0.05$, and χ^2 critical = 3.841). Thus, for the E3 stage, there was a statistically significant difference ($\chi^2 = 5.0$; df = 1; $p = 0.024$) between plants inoculated with *B. bassiana* and uninoculated plants for this stage of development of *L. invasa* gall on *Eucalyptus* plants (Table 1). Plants inoculated with *B. bassiana* were temporally restricted, so the median value was 0.00 days, less than the median of 15.19 days for uninoculated plants. Thus, the inoculated plants also showed less variability, with an interquartile range of 4.21 days, 29.03% lower than that observed in the control treatment group with 14.50 days of interquartile range.

For the reddish gall stage (E4), there was greater data variability for the control treatment; therefore, the interquartile range at 6.96 days was 88.32% higher than that observed in the group of inoculated plants, which presented an interquartile range of 0.81 days. However, when analysing the responses related to data asymmetry, it was observed that the inoculated plants exhibited positive asymmetry and a concentration of values above the median of 0.00 days (Table 1). As for the uninoculated plants, the development of galls remained in evolution and consequently favoured temporal verification, with a median of 9.00 days for E4 (Table 1).

The developmental stage, described as the gall with adult emergence hole (E5), showed a statistically significant difference between treatments ($\chi^2 = 11.96$; df = 1; $p = 0.001$) (Table 1). The first quartile and median of plants inoculated with *B. bassiana* were 0.00 days. Consequently, it was lower than the median of uninoculated plants in the control group, which obtained a value of 4.38 days for this stage of gall development of *L. invasa* in susceptible *Eucalyptus* plants. The variability in the E5 stage was greater in the control treatment (uninoculated plants), as the interquartile interval of 5.24 days was more significant than that observed in the group of plants inoculated with *B. bassiana* at 0.09 days. Asymmetry was positive in control plants without inoculation with *B. bassiana*. The extent to which the greatest concentration of data was observed was above the median of 4.38 days, as shown in the box plot.

The gall development stages of *Leptocybe invasa* in uninoculated and inoculated plants with the *Beauveria bassiana* fungus are shown Figure 2.

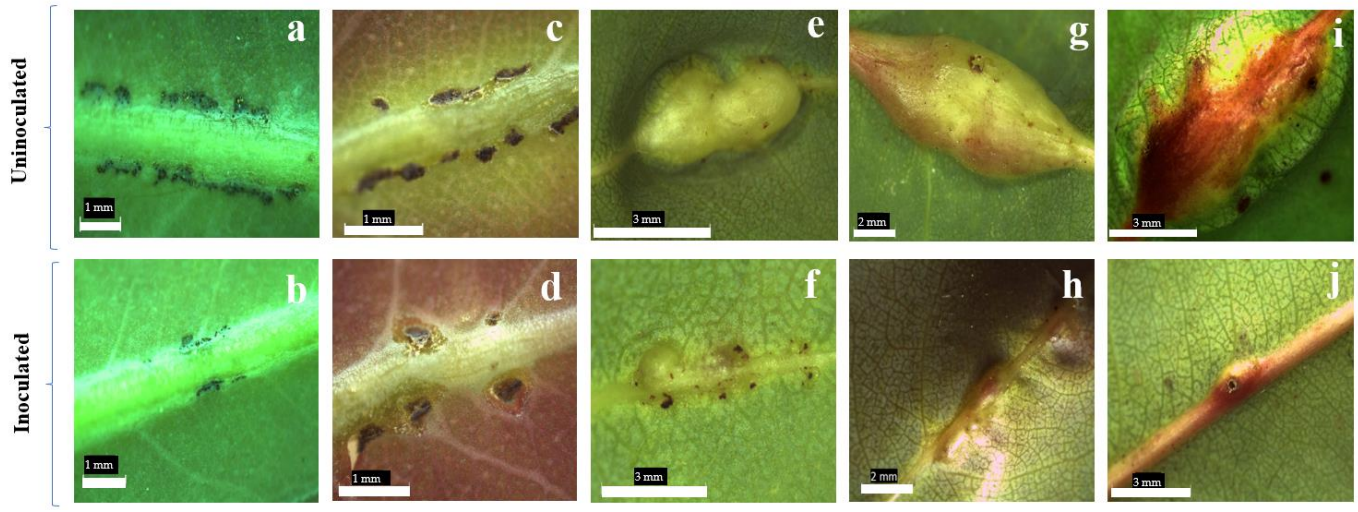


Figure 2. *Leptocybe invasa* Fisher & La Salle (2004) wasp gall development stages (**a**,**c**,**e**,**g**,**i**) for uninoculated plants and (**b**,**d**,**f**,**h**,**j**) for inoculated plants with the fungus *Beauveria bassiana*. (**a**,**b**) Brownish ring; (**c**,**d**) suberized scar; (**e**,**f**) greenish gall; (**g**,**h**) reddish gall; and (**i**,**j**) adult emergency hole for the midrib of the abaxial surface of hybrid clone leaves (*E. tereticornis* × *E. camaldulensis*) susceptible to infestation by *L. invasa* analysed from 4 to 126 days after infestation.

There was a statistically significant difference in the number of galls per plant between treatments, uninoculated and inoculated ($p = 0.03$ and $p < 0.001$), respectively, according to the Kruskal–Wallis test ($p \leq 0.05$). In uninoculated plants, the median value was 2.50 galls per plant (Figure 3). The observed value for inoculated plants was less than one gall per plant, with a median of 0.00 (Figure 3). Uninoculated plants showed greater variability and negative asymmetry (Figure 3). However, this was not observed in the inoculated plants, which showed less variability and positive asymmetry. The third quartile value for the inoculated treatment was 0.750 galls per plant, lower than that observed in the uninoculated treatment; the first and third quartiles averaged 1.0 and 3.5 galls per plant, respectively (Figure 3). This suggests a trend towards possible defence responses and a lower presence of galls in plants inoculated with *B. bassiana*.

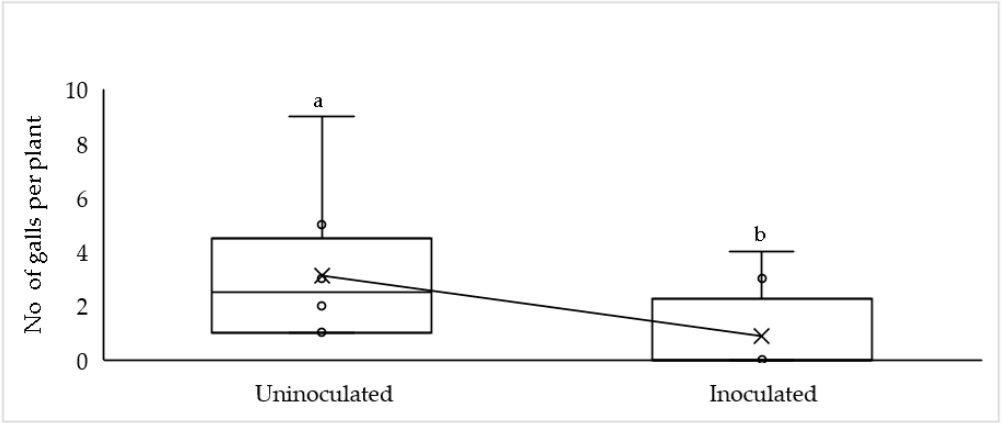


Figure 3. The number of *Leptocybe invasa* galls per plant in a hybrid Eucalyptus clone inoculated with *Beauveria bassiana* and in uninoculated plants (control). Horizontal lines cutting through the plot box represent the position of the median; points external to the diagram represent outliers. Raw data: Uninoculated 8 plants with 1, 3, 2, 1, 3, 1, 5, and 9 galls; inoculated 8 plants with 0, 3, 0, 4, 0, 0, 0, and 0 galls. Plot boxes followed by the same lowercase letter do not differ statistically from each other according to the Mann–Whitney test ($p < 0.05$).

At 14 DAI, the average gall width of *L. invasa* between uninoculated and inoculated plants was measured, and the t-statistic value was 4.29 with 30 degrees of freedom. The control treatment presented an average gall width of 0.525 ± 0.046 mm, and plants inoculated with *B. bassiana* had an average of 0.313 ± 0.018 mm, which represented a 40.38% reduction in gall width compared to the uninoculated group of plants (Figure 4). At 21 DAI, there was no significant difference in the mean gall width (mm) (t = 1.13; df = 30; $p = 0.268$) (Figure 4). At 35 DAI, when analysing gall width, non-normality was verified ($p = 0.03$ and $p < 0.001$) in both treatments. However, there was a significant difference in the Kruskal–Wallis test at $p < 0.05$ ($\chi^2 = 7.16$; df = 1; $p = 0.006$) (Figure 4). Plants inoculated with *B. bassiana* showed a 22.22% reduction (0.70 mm) in median gall width. This value was lower than the median for the control group (uninoculated plants), which was 0.9 mm according to the Mann–Whitney U test (U = 56.5; $p = 0.006$) (Figure 4). At 42 DAI, the same occurred. There was a statistically significant difference in the Kruskal–Wallis test ($p \leq 0.05$) ($\chi^2 = 9.10$; df = 1; $p = 0.003$). Uninoculated plants had a median of 1.00 mm and an interquartile range of 0.30 mm (Figure 4), superior to the inoculated group that had a median of 0.80 mm according to the Mann–Whitney test (U = 49.0; $p = 0.003$). Data dispersion for the *B. bassiana* treatment was reduced considering the values of the first and third quartiles (0.60 and 0.82 mm, respectively) (Figure 4). At 52 DAI, the median and first and third-quartile values were 1.00, 0.950, and 3.075 mm, respectively. Relative to the gall width values in uninoculated plants, there was a high dispersion of this external morphological response, with interquartile ranges of 2.13 mm (Figure 4). The same was not observed in the inoculated plants; low data dispersion and an interquartile range of 0.200 mm were observed when analysing variability. The median and first and third quartile values (0.800, 0.700, and 0.900 mm, respectively) were significantly lower than those of the uninoculated group (Figure 4).

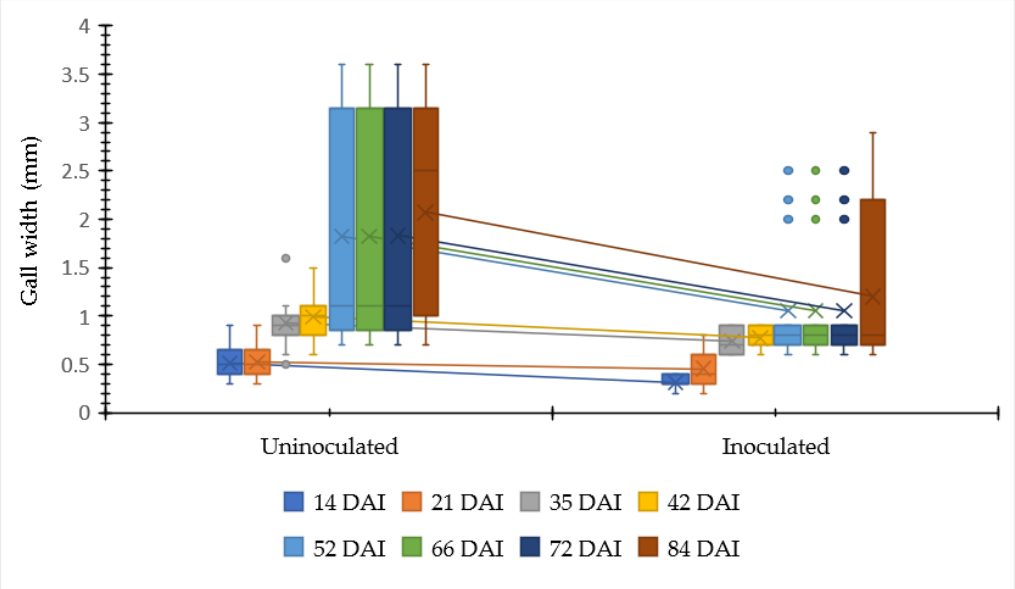


Figure 4. Differential responses for gall width (mm) of *Leptocybe invasa* in *Eucalyptus* hybrid plants inoculated with *Beauveria bassiana* and uninoculated plants (control).

The observed responses to gall development (length) during the evaluation period reinforced the tendency to maintain lower gall development in the plants inoculated with *B. bassiana* compared with the uninoculated control group (Figure 5).

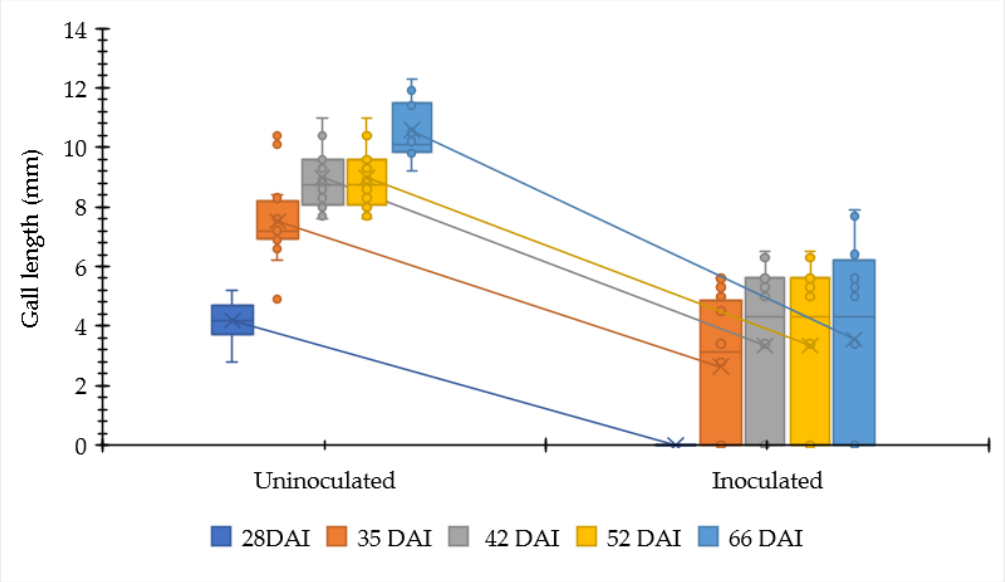


Figure 5. Differential responses for gall length (mm) of *Leptocybe invasa* in *Eucalyptus* hybrid plants inoculated with *Beauveria bassiana* and uninoculated plants (control).

The total number of adult *L. invasa* emergence holes per plant did not meet the normality assumption of the Shapiro–Wilk test. However, there was a statistically significant difference between the treatments (χ^2 = 7.73; df = 1; p = 0.005). Therefore, there was a significant reduction of 46.24% in the total number of emergence holes for the plants inoculated with *B. bassiana* compared to that observed in the group of control plants that were uninoculated, according to the Mann–Whitney test (U = 6.50; p = 0.006) (Figure 6).

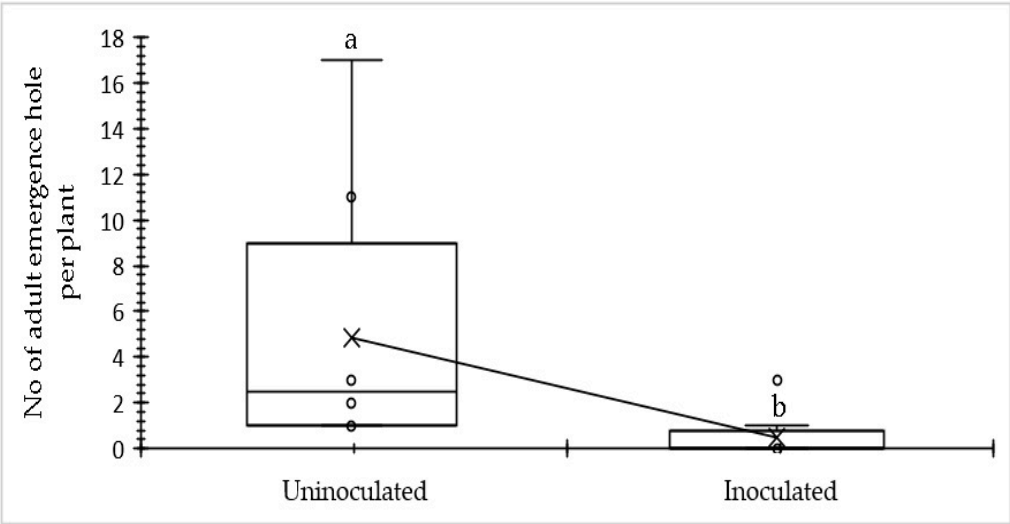


Figure 6. The number of adult emergence holes per plant for both plant groups. Box plots followed by the same lowercase letter do not differ statistically according to the Mann–Whitney test ($p \leq 0.05$).

4. Discussion

Here, we show that the initial infestation dynamics of *Eucalyptus* plants susceptible to the galling insect *L. invasa* were unaffected by plants previously inoculated with the entomopathogenic fungus *B. bassiana*. Furthermore, the development of galls in plants susceptible to *L. invasa* inoculated with *B. bassiana* was reduced at the E3, E4, and E5 gall development stages.

Temporal variation in the E1 and E2 stages of gall development after *L. invasa* infestation was not directly affected by the inoculation process with the fungus *B. bassiana*. Biotic or abiotic signalling pathways generally activate constitutive defences in plants. Calazans et al. [16] verified the microbiome dynamics in *Eucalyptus* plants under infestation by *L. invasa* on the African continent and variations associated with different infestation levels by *L. invasa*. Among the genetically identified endophytes in the *Eucalyptus* microbiome, a significant part was historically related to phytopathogenicity [16]. Therefore, further studies are needed to elucidate the possible initial antagonisms of *B. bassiana* and the pre-existing microbiome in host plants to understand the levels of inactivation of the benefits already evidenced in the routes of metabolism of defences in plants challenged by biotic and abiotic damage [7,12,17].

The fungus *B. bassiana,* under endophytic interactions, secretes enzymes and secondary metabolites essential for plant defence, such as proteases, lipases, and chitinases [8]. When studying the dynamics of defence activation in eucalyptus plants from two groups (susceptible and tolerant), Pinto et al. [17] found divergences in the spatial dynamics of cell death in tissues surrounding the oviposition point of *L. invasa*; this suggests the activation of the metabolic pathway of constitutive defences for tolerant plants through programmed tissue death. Such responses can significantly influence the duration between stages. Under natural conditions of infestation and susceptibility, the suberized scar phase lasts 7–15 days after infestation [17].

The endophytic colonisation of *Beauveria bassiana* in various plants, such as tomatoes [18], beans [19], corn [20], and wheat [21], among others, can bring benefits to the hosts, such as increased resistance to pathogens and environmental stresses. Understanding the mechanisms of interaction between the fungus and host plants can lead to the development of more efficient biological control and disease management strategies [22,23].

The developmental stages of *L. invasa,* E3, E4, and E5, showed significantly different responses between treatments. The fungus *B. bassiana* can significantly contribute to the signalling of plant defence metabolism by activating primary or secondary metabolism [22–24].

The dynamics of initial infestation by *L. invasa* did not significantly influence *Eucalyptus* plants inoculated with *B. bassiana* because of the total number of plants being oviposited by *L. invasa*. Few studies have been conducted on the effectiveness of the perception and selectivity of target plants attributed to the olfactory system of *L. invasa* and the synthesis of volatile compounds in plants colonised by *B. bassiana*. However, evidence points to the influence of endophytic fungi, their potential to activate secondary metabolism for jasmonic acid synthesis, and the accumulation of defence volatiles in plants [25]. Nonetheless, the opposite has also been observed for the modulation of the attraction of the insect pest *Ostrinia furnacalis* (Lepidoptera: Crambidae) in corn plants inoculated with *B. bassiana*. There is an increase in attractive volatiles and reduced levels of defensive volatiles, influencing oviposition behavior. However, consuming tissues colonised by *B. bassiana* results in lower survival of larvae, pupae, and adults of *O. furnacalis* [26].

The reproductive success of *L. invasa* was negatively affected in plants inoculated with *B. bassiana*. Our results suggest that inoculation through the leaves and a fungal solution containing viable conidia of *B. bassiana* induced a 75% reduction in the appearance of galls at 42 DAI. It also resulted in 85% less emergence of *L. invasa* adults at 126 DAI than in the control treatment (uninoculated). Unlike other fungi, such as *Metharizium anisopliae*, which predominate in the rhizosphere [27], *B. bassiana* actively colonises the tissues of the aerial parts of the plant.

Zhu et al. [26] studied the life cycle of *O. furnacalis* in maize plants colonised by *B. bassiana* and found a reduction in adults by the end of the evaluation period. They also stated that this could be attributed to low levels of nitrogenous compounds in plants inoculated with *B. bassiana* [26].

Gall width was negatively affected and associated with previous inoculation with *B. bassiana* [6]. The length of the galls was also affected, and there was a significant reduction in the dimensions of these parameters in the galls of plants inoculated with *B. bassiana*. The

induced and constitutive resistance in *E. camaldulensis* resulted in a decline in the size of galls, which was attributed to the defence responses of plants tolerant to infestation by *L. invasa* [28]. In contrast to the uninoculated plants, there was a significant reduction in adult emergence holes in plants inoculated with *B. bassiana*. Colonisation of *Sorghum bicolor* plants by the fungus *B. bassiana* resulted in 70–100% larval mortality for the stem borer *Sesamia nonagrioides* (Lepidoptera: Noctuidae) [13]. Applying a fungal solution containing *B. bassiana* reduced the development of galls by 75% at 42 DAI and the emergence of *L. invasa* adults in *Eucalyptus* plants by more than 85% at 126 DAI.

5. Conclusions

The results presented here will help to develop products and registers of *B. bassiana* to control *L. invasa* in *Eucalyptus*. The possibility of using entomopathogenic fungi to endophytically colonise *Eucalyptus* plants opens a vast field of exploration for these interactions. It brings the possibility of taking advantage of the ability of these microorganisms to act as potential pest control agents, allowing for more sustainable forestry production through a reduction in the use of pesticides. Further research is needed to better understand the mechanisms underlying this interaction and to explore the long-term effects on both the pest and the host plants. This study contributes valuable insights into developing sustainable pest management strategies in forestry and agriculture, emphasising the importance of biological control methods in reducing the impact of invasive pests on plant ecosystems.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su152316411/s1, Figure S1. Mean colony count of *B. bassiana* obtained per plant on Petri dishes, originating from leaves of Eucalyptus seedlings, inoculated and uninoculated.

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References

1. Mahar, A.N.; Jan, N.D.; Mahar, G.M.; Mahar, A.Q. Control of insects with entomopathogenic bacterium *Xenorhabdus nematophila* and its toxic secretions. *Int. J. Agric. Biol.* **2008**, *10*, 52–56.
2. Fanning, P.D.; Grieshop, M.J.; Isaacs, R. Efficacy of biopesticides on spotted wing drosophila. *Drosophila suzukii* Matsumura in fall red raspberries. *J. Appl. Entomol.* **2018**, *142*, 26–32. [CrossRef]
3. Inglis, G.D.; Goettel, M.S.; Butt, T.M.; Strasser, H. Use of hyphomycetous fungi for managing insect pests. In *Fungi as Biocontrol Agents: Progress, Problems and Potential*; Butt, T.M., Jackson, C., Magan, N., Eds.; CABI Publishing: Wallingford, UK, 2001; pp. 23–69. [CrossRef]
4. Vega, F.E. The use of fungal entomopathogens as endophytes in biological control: A review. *Mycologia* **2018**, *110*, 4–30. [CrossRef] [PubMed]
5. Klieber, J.; Reineke, A. The entomopathogen *Beauveria bassiana* has epiphytic and endophytic activity against the tomato leaf miner *Tuta absoluta*. *J. Appl. Entomol.* **2016**, *140*, 580–589. [CrossRef]
6. Rocha, J.P.L.; Nunes, T.V.; Rodrigues, J.N.; Lima, N.M.P.; Rocha, P.A.L.; Pinto, I.D.O.; Sarmento, M.I.; Araújo, W.L.; de Moraes, C.B.; Sarmento, R.A. Morphophysiological responses in *Eucalyptus* demonstrate the potential of the entomopathogenic fungus *Beauveria bassiana* to promote resistance against the galling wasp *Leptocybe invasa*. *Forests* **2023**, *14*, 1349. [CrossRef]
7. Cotes Ramal, B.; Thoming, G.; Amaya Gomez, C.V.; Novak, O.; Nansen, C. Root-associated entomopathogenic fungi manipulate host plants to attract herbivorous insects. *Sci. Rep.* **2020**, *10*, 22424. [CrossRef]
8. Wang, H.; Peng, H.; Li, W.; Cheng, P.; Gong, M. The toxins of *Beauveria bassiana* and the strategies to improve their virulence to insects. *Front. Microbiol.* **2021**, *12*, 705343. [CrossRef] [PubMed]
9. Lohse, R.; Jakobs-Schönwandt, D.; Vidal, S.; Patel, A.V. Evaluation of new fermentation and formulation strategies for a high endophytic establishment of *Beauveria bassiana* in oilseed rape plants. *Biol. Contr.* **2015**, *88*, 26–36. [CrossRef]
10. Rosado, A.M.; Rosado, T.B.; Alves, A.A.; Laviola, B.G.; Bhering, L.L. Simultaneous selection of *Eucalyptus* clones according to productivity. stability and adaptability. *Braz. Agric. Surv.* **2012**, *47*, 964–971. [CrossRef]
11. Barbosa, L.R.; de Queiroz, D.L.; Nickele, M.A.; de Queiroz, E.C.; Reis Filho, W.; Iede, E.T.; Penteado, S. Pests of Eucalyptus. In *Eucalyptus and Embrapa: Four Decades of Research and Development*, 1st ed.; de Oliveira, E.B., Pinto Junior, J.E., Eds.; Embrapa Forestry: Brasilia, DF, Brasil, 2021; pp. 751–780.
12. Sarmento, M.I.; Pinto, G.; Araújo, W.L.; Silva, R.C.; Lima, C.H.O.; Soares, A.M.; Sarmento, R.A. Differential development times of galls induced by *Leptocybe invasa* (Hymenoptera: Eulophidae) reveal differences in susceptibility between two Eucalyptus clones. *Pest Manag. Sci.* **2021**, *77*, 1042–1051. [CrossRef]
13. Yousuf, M.; Singh, S.; Ikram, M.; Khan, S. Re-description and morphometric analysis of Eucalyptus gall wasp. *Leptocybe invasa*. *Int. J. Agric. Environ. Biotechnol.* **2019**, *12*, 93–99. [CrossRef]
14. Köppen, W.; Geiger, R. (Eds.) *Handbuch der Klimatologie*; Gebrüder Borntraeger: Berlin, Germany, 1930; Volume 1.
15. Souza, A.R.; Barbosa, L.R.; Souza Passos, J.R.; Castro, B.M.D.C.; Zanuncio, J.C.; Wilcken, C.F. Longevity and survival of *Leptocybe invasa* (Hymenoptera: Eulophidae) an invasive gall inducer on Eucalyptus with different diets and temperatures. *PeerJ* **2018**, *6*, e5265. [CrossRef]
16. Calazans, C.C.; Souza, J.L.; Silva-Mann, R. Innovating *Eucalyptus* resistance: A meta-analysis of gene associations for combatting *Leptocybe invasa*. *Res. Sq.* **2023**, *2*, 1–19. [CrossRef]
17. Pinto, O.I.; Sarmento, M.I.; Martins, A.O.; Rocha, J.P.L.; Pinto, G.; Araújo, W.L.; Soares, A.M.; Sarmento, R.A. Cell death and changes in primary metabolism: The onset of defence in *Eucalyptus* in the war against *Leptocybe invasa*. *Pest Manag. Sci.* **2022**, *78*, 1721–1728. [CrossRef]
18. Sinno, M.; Ranesi, M.; Di Lelio, I.; Iacomino, G.; Becchimanzi, A.; Barra, E.; Molisso, D.; Pennacchio, F.; Digilio, M.C.; Vitale, S. Selection of endophytic *Beauveria bassiana* as a dual biocontrol agent of tomato pathogens and pests. *Pathogens* **2021**, *10*, 1242. [CrossRef] [PubMed]
19. Volkova, N.; Levchenko, M.; Uspanov, A.; Lednev, G. Screening of fungal strains of the genus *Beauveria* for their ability to form endophytic relations with *Phaseolus* plants. *EDP Sci.* **2020**, *18*, 00029. [CrossRef]
20. Kuzhuppillymyal-Prabhakarankutty, L.; Tamez-Guerra, P.; Gomez-Flores, R.; Rodriguez-Padilla, M.C.; Ek-Ramos, M.J. Endophytic *Beauveria bassiana* promotes drought tolerance and early flowering in corn. *World J. Microbiol. Biotechnol.* **2020**, *36*, 1–10. [CrossRef]
21. Levchenko, M.; Gerus, A.; Malysh, S.; Orazova, S.; Lednev, G. The effect of endophytic colonization of wheat plants by the fungus *Beauveria bassiana* on the development of the nymphs of the migratory and desert locusts. *EDP Sci.* **2020**, *18*, 00018. [CrossRef]
22. Amobonye, A.; Bhagwat, P.; Pandey, A.; Singh, S.; Pillai, S. Biotechnological potential of *Beauveria bassiana* as a source of novel biocatalysts and metabolites. *Crit. Rev. Biotechnol.* **2020**, *40*, 1019–1034. [CrossRef]
23. Aayushi, G.; Laxmi, B.; Sunita, K. Interaction between Plants and Microorganisms: Microbial Biological Control Agents against Plant Pathogens. *Res. J. Biotech.* **2023**, *18*, 134–144. [CrossRef]
24. Tan, R.X.; Zou, W.X. Endophytes: A rich source of functional metabolites. *Nat. Prod. Rep.* **2001**, *18*, 448–459. [CrossRef]
25. Ren, C.G.; Dai, C.C. Jasmonic acid is involved in the signaling pathway for fungal endophyte-induced volatile oil accumulation of *Atractylodes lancea* plantlets. *BMC Plant Biol.* **2012**, *12*, 128. [CrossRef]

26. Zhu, H.; Fu, J.; Wang, H.; Bidochka, M.J.; Duan, M.; Xu, W.; Sui, L.; Ren, B.; Li, Q.; Zhang, Z. Fitness consequences of oviposition choice by an herbivorous insect on a host plant colonized by an endophytic entomopathogenic fungus. *J. Pest Sci.* **2023**, *96*, 745–758. [CrossRef]
27. Behie, S.; Jones, S.J.; Bidochka, M.J. Plant tissue localization of the endophytic insect pathogenic fungi *Metarhizium* and *Beauveria*. *Fung. Ecol.* **2015**, *13*, 112–119. [CrossRef]
28. Otieno, B.A.; Salminen, J.P.; Steinbauer, M.J. Resistance of subspecies of Eucalyptus camaldulensis to galling by *Leptocybe invasa*: Could quinic acid derivatives be responsible for leaf abscission and reduced galling? *Agric. For. Entomol.* **2022**, *24*, 167–177. [CrossRef]