KEMINY RIBETT BAUTZ

EFFECTS OF THE FUNGUS *Syncephalastrum* (MUCORALES: SYNCEPHALASTRACEAE) IN NESTS OF LEAFCUTTER ANTS (FORMICIDAE: ATTINI)

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Magister Scientiae*.

Orientador: Simon Luke Elliot

Coorientador: André Rodrigues

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ABSTRACT

BAUTZ, Keminy Ribett, M.Sc., Universidade Federal de Viçosa, February, 2020. **Effects of the fungus *Syncephalastrum* (Mucorales: Syncephalastraceae) in nests of leafcutter ants (Formicidae: Attini).** Advisor: Simon Luke Elliot. Co-advisor: André Rodrigues.

Leafcutter ants form a symbiotic nutritional mutualism with a basidiomycete fungus, growing in fungus gardens. This symbiosis interacts naturally with a range of microrganisms including some fungi that may be antagonist. Among these is the fungus *Syncephalastrum*, which is considered a pathogen of the fungus garden. Studies of the effects of potentially antagonist fungi on the ant-fungus mutualism are mostly performed in colonies in the absence of the queen, which may result in unrealistic conclusions about the negative effects of the fungi on the mutualist. Thus, the present study aimed to investigate the effects of *Syncephalastrum* sp. in the fungus garden of colonies of the leafcutter ant *Acromyrmex subterraneus subterraneus* in two levels of colony complexity (presence and absence of the queen). We tested the following hypothesis: that although *Syncephalastrum* sp. is found naturally in healthy colonies, it can cause negative effects to colonies when we apply a high concentration of its spores to the colony. Further, we hypothesized that the negative effects increase with reduced colony complexity. Thus, colonies of leafcutter ants of the species *Ac. subterraneus subterraneus* were divided into two levels of complexity: queenright colonies (fungus garden, queen and workers) and queenless colonies (fungus garden and workers). They were inoculated with spores of *Syncephalastrum* sp. or blank controls. Dependent variables measured were weight – leaves cut by ants; midden production; colony weight; presence of *Syncephalastrum* sp. in the midden; and survival of colonies. Our results showed that, in general, *Syncephalastrum* sp. negatively affected leaf cutting, waste production and the weight of colonies, with effects greater in queenless colonies. These effects, however, were not enough to compromise the survival of these colonies – all queenright and queenless colonies survived the experimental period of 28 days and indeed 96 days after that when maintained in the laboratory. To conclude, the isolate of *Syncephalastrum* sp. used in the present study caused negative effects to colonies of the leafcutter ant *Ac. subterraneus subterraneus* but did not compromise the survival of the colonies. However, our results highlight the importance of each element (fungus garden, queen and workers) in the symbiosis of leafcutter ants, mainly in studies that investigate the effect of antagonist microrganisms on leafcutter ant’s fungal cultivars.

Keywords: Leafcutter ant. Fungus garden. Microrganisms. Host-parasite interactions. *Acromyrmex*. *Leucoagaricus gongylophorus*.

RESUMO

BAUTZ, Keminy Ribett, M.Sc., Universidade Federal de Viçosa, fevereiro de 2020. **Efeitos do fungo *Syncephalastrum* (Mucorales: Syncephalastraceae) em ninhos de formiga cortadeiras (Formicidae: Attini).** Orientador: Simon Luke Elliot. Coorientador: André Rodrigues.

As formigas cortadeiras formam um mutualismo simbiótico nutricional com um fungo basidiomiceto, cultivando-o em jardins de fungos. Essa simbiose interage naturalmente com uma variedade de microrganismos, incluindo alguns fungos que podem antagonistas. Entre estes, está o fungo *Syncephalastrum*, considerado um patógeno do jardim do fungo. Estudos dos efeitos de fungos potencialmente antagonistas no fungo mutualista das formigas cortadeiras são realizados principalmente em colônias na ausência da rainha, o que pode resultar em conclusões irrealistas sobre os efeitos negativos dos fungos no mutualista. Assim, o presente estudo visou investigar os efeitos causados pelo *Syncephalastrum* sp. no jardim de fungo de colônias de formigas cortadeiras *Acromyrmex subterraneus subterraneus* em dois níveis de complexidade de uma colônia (presença e ausência da rainha). Portanto, testamos a seguinte hipótese: que apesar de *Syncephalastrum* ser encontrado naturalmente em colônias saudáveis, pode causar efeitos negativos nas colônias quando aplicamos uma alta concentração de seus esporos nas colônias. Além disso, avaliamos a hipótese de que o efeito negativo aumenta com a complexidade da colônia reduzida. Dessa forma, colônias de formigas cortadeiras da espécie *Ac. subterraneus subterraneus,* foram divididas em dois níveis de complexidade: colônias com rainha (jardim de fungo, rainha e operárias) e colônias sem rainha (jardim de fungo e operárias) cada nível foi inoculado com esporos de *Syncephalastrum* sp. ou (controles em branco). Os parâmetros avaliados foram pesos – folhas cortadas pelas formigas; lixo produzido; peso da colônia; presença do *Syncephalastrum* sp. no lixo e sobrevivência das colônias. Nossos resultados mostraram que, em geral, *Syncephalastrum* sp. afetou negativamente as atividades de corte de folhas, produção de lixo e peso das colônias com efeitos maiores nas colônias sem rainha. Esses efeitos, no entanto, não foram suficientes para comprometer a sobrevivência dessas colônias - todas as colônias com e sem rainha sobreviveram ao período experimental de 28 dias e ainda, foram mantidas por 96 dias no laboratório. Para concluir, o isolado de *Syncephalastrum* sp. utilizado no presente estudo causou efeitos negativos nas colônias de formigas cortadeiras *Ac. subterraneus subterraneus*, mas não comprometeu a sobrevivência dessa colônias.

Além disso, nossos resultados destacam a importância de cada elemento (jardim de fungo, rainha e operárias) na simbiose de formigas cortadeiras, principalmente em estudos que investigam o efeito de microrganismos antagonistas no cultivar fúngico das formigas cortadeiras.

Palavras-chave: Formigas cortadeiras. Jardim de fungo. Microrganismos. Interações parasita-hospedeiro. *Acromyrmex. Leucoagaricus gongylophorus.*

SUMMARY

INTRODUCTION

No organism lives in isolation, and one organism can physically associate with another and thus live together. This physical association is known as symbiosis. A symbiosis can be mutualistic, wherein organisms of different species interact for their mutual benefit, that is, a reciprocal exploration (Begon et al., 2008). However, other organisms can also interact with these organisms, being able to influence aspects of the life history of symbionts, such as reproduction and survival, positively or negatively.

Eusocial insects, such as fungus-growing ants (Formicidae: Attini: Attina) form a symbiotic nutritional mutualism with different fungi and are well-known to interact with a diversity of microrganisms that are present in their colonies, including mutualists and parasites (Fisher et al., 1996; Currie et al., 1999a; Pagnocca et al., 2012). Due to this, many studies have investigated the effects that these microrganisms cause on the host. However, the effects can vary with the interaction and the ecological context in which both organisms are inserted.

The subtribe Attina comprises 18 genera with approximately 230 species of ants described that specialize in the cultivation of fungus. These ants have developed this ability to grow fungi to use as food (Mueller et al., 2001). The genera *Atta* and *Acromyrmex* occur primarily in the Neotropical region and are popularly known as leafcutter ants (Brandão et al., 2011). They stand out for being considered pests for agriculture due to the damage they cause to crops and forestry (Brandão et al., 2011), because they use fragments of leaves and flowers that serve as substrate for the growth of their symbiotic fungus (Fisher et al., 1994; Mueller et al., 2005), *Leucoagaricus gongylophorus* (Basidiomycota: Leucocoprini, Agaricales) (Fisher et al., 1994). They use the fungus as food, while providing nutrients, protection, and dispersion in return. The fungus is grown in underground chambers and is known as the fungus garden. In addition, the fungus garden can harbour a variety of bacteria, filamentous fungi, and yeasts (Fischer et al., 1996; Rodrigues et al., 2005a, b, 2008; Meirelles et al., 2016). These microrganisms probably originate from the soil and plant material that ants use to maintain the symbiotic fungus (Fisher et al., 1996; Rodrigues et al., 2008).

The fungus *Syncephalastrum* (Mucoromycota: Mucorales) is commonly found in leafcutter ants' nests (Rodrigues et al., 2005a, 2008, 2009; Barcoto et al., 2017; Bizarria et al., 2018). Barcoto et al. (2017) performed Koch's postulates and determined that *Syncephalastrum* sp. is a pathogenic fungus in the fungus garden of the leafcutter ants of genus *Atta*, which can develop infections in the symbiotic fungus of ants. Studies have also shown that *in vitro* this fungus is an antagonist of *L. gongylophorus* capable of inhibiting the growth of the fungal cultivar in culture medium (Rodrigues et al., 2009; Barcoto et al., 2017; Bizarria et al., 2018). In addition, in the study *in vivo* performed by Barcoto et al. (2017) in colonies of *Atta sexdens rubropilosa* in the absence of the queen, demonstrated that *Syncephalastrum* sp. caused deterioration and death of the fungus garden of ants. However, the effect of *Syncephalastrum* sp. in colonies of leafcutter ants of the genus *Acromyrmex* in the presence of the queen is unknown.

Currently, most experimental studies that investigate the effects of potentially antagonist fungi on the ant-fungus mutualism mostly are conducted either *in vitro* (Folgarait et al., 2011a, b) or *in vivo* with colonies in the absence of the queen (Wallace et al., 2014; Barcoto et al., 2017; Rocha et al., 2017), which may result in unrealistic conclusions about the negative effects of the fungi on the symbiosis of leafcutter ants. Furthermore, the absence of the queen in a colony can alter some activities and behaviours performed by workers, as demonstrated by Sousa-Souto and Souza (2006) who observed that the absence of the queen in *Atta sexdens rubropilosa* colonies led to decreased waste disposal and an increase in worker mortality. Another study with colonies of *A. sexdens* *rubropilosa* showed that the absence of the queen led workers to decrease the caring behaviour of the young, and many workers escaped from the nest with a consequent decrease in nesting activities (Della Lucia et al., 2003). Based on these studies, it is clear that the queen is especially important to maintain the interaction between the nestmates and the cohesion of the colony, mainly related to the colony's activities.

Thus, in our study, we investigated the effect of the fungus *Syncephalastrum* sp. in colonies of leafcutter ants, *Acromyrmex subterraneus subterraneus,* in one group considering all the elements of the colony together and in another group considering a colony without the queen. We based our study on that of Mendonça (2018) in which considered that a higher level of colony complexity involves the interaction between the queen, fungus garden and specialized workers (queenright colony). On the other hand, a colony without queen (queenless colony) includes the fungus garden and specialized workers but does not contain an essential component of the colony, the queen and, thus the level of complexity of this interaction decreases. We tested the following hypothesis: that although *Syncephalastrum* sp. is found naturally in healthy colonies, it can cause negative effects to colonies when we apply a high concentration of its spores to the colony. Further, we hypothesized that the negative effects increase with reduced colony complexity. Our findings document the effects of *Syncephalastrum* sp. on the leafcutter ant colonies of genus *Acromyrmex* in the presence and absence of the queen. In addition, we demonstrated the importance of each element that makes up a colony, mainly in studies that investigate the effect of microrganisms on leafcutter ant’s fungal cultivar.

MATERIAL AND METHODS

We conducted an experiment to investigate the effect of the fungus *Syncephalastrum* sp. on the leafcutter ant-fungus symbiosis. We evaluated this, *in vivo,* on *Acromyrmex subterraneus subterraneus* leafcutter ants colonies with two different complexity levels: (i) queenright colonies (fungus garden + queen + workers; Fig. 1A) and (ii) queenless colonies (fungus garden + workers; Fig. 1B) (method adapted from Mendonça 2018). For this, the fungus garden of each colony was inoculated the following treatments (a) spores suspension of *Syncephalastrum* sp. + 0.01% Tween 80$^{®}$ solution (Biotec Reagentes Analíticos) and (b) blank control of 0.01% Tween 80$^{®}$ solution.

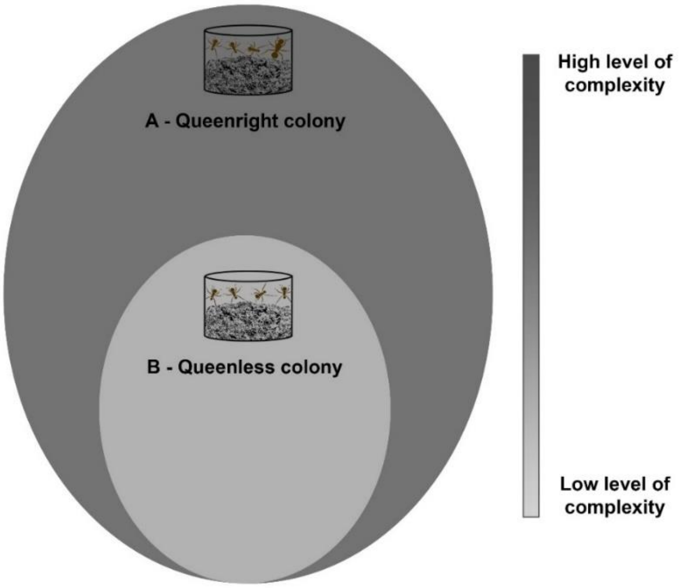

Fig 1. Schematic representation of the experiment showing the two complexity levels of leafcutter ant-fungus symbiosis. **(A)** queenright colony and **(B)** queenless colony. From these observations, we evaluated the effects of the fungus *Syncephalastrum* sp. on *Acromyrmex subterraneus subterraneus* colonies and their fungal cultivar in two different levels of complexity.

Organisms

Twenty colonies of *Ac. subterraneus subterraneus* (ca. 1 year old) were collected in two areas on the campus of Universidade Federal de Viçosa (UFV), Viçosa, Minas

Gerais at mean altitude of 649 m and mean temperature of 20.6 °C, southeastern Brazil: *Horto Botânico* (20º 45'25"S 42º52'23"W) and *Recanto das Cigarras* (20º 45'26"S 42º51'45"W). The first area is a living plant collection comprising native plant specimens while Recanto das Cigarras is a fragment of secondary Atlantic forest. The fungus garden of each colony was transferred to a plastic pot (500 ml) and then placed in a plastic tray (43 x 29 x 7cm). In the base of the pots, we made a 2 cm diameter exit hole to allow the passage of workers to the foraging arena in the tray. The inner sides of each tray were covered with neutral talcum powder (magnesium silicate) to prevent the ants from escaping. We offered fresh leaves of *Acalypha wilkesiana* daily as forage for the ants. The colonies were maintained under controlled temperatures (25±2°C) and humidities (75±3% RU) with a 12-hour photoperiod.

The genus *Syncephalastrum* sp. has rapid growth in culture medium and produces a large number of spores, facilitating experimental work. The isolate of the *Syncephalastrum* sp. used in this study was collected from the fungus garden of the colony of *Ac. subterraneus subterraneus* (collected in *Recanto das Cigarras*, 20º 45'26"S 42º51'45"W) (isolated by M.C.S.C., 2018). Subsequently, the purity of the isolate was verified through the technique of monosporic culture. From this pure culture, we isolated the fungus in Malt Extract Agar (MEA) culture medium (20 g malt extract, 20 g dextrose, 5 g peptone, and 15 g agar l^{-1}), at 25ºC in the dark for 12 days. The isolate of *Syncephalastrum* sp. was identified through its morphological characteristics, such as the presence of spores. For morphological identification, we made slides and examined these with a Nikon (Eclipse E200) light microscope.

In order to verify if *Syncephalastrum* sp. is found naturally in the collected colonies, we conducted a sample of the fungus gardens from each of the 20 colonies collected and plated these in growth media. For each colony, we collected 30 fragments from different regions of the fungus garden. The fragments of the fungus garden were plated on to Petri dishes containing PDA (potato, dextrose, and agar l^{-1}) + chloramphenicol following incubation at 25ºC in the dark for 15 days. The presence of that fungus was recorded in the results.

Experimental set-up

The twenty colonies were allocated randomly (by drawing lots) to the two complexity levels (Fig. 2A): ten queenright colonies (Fig. 2B) and ten queenless colonies (Fig. 2C). Three days before the experiment start, the queens of the colonies considered queenless were located in the fungus garden and removed using tweezers (Fig. 2D). We observed that the fungus garden suffered a change in its physical structure when the queen was removed from each colony, due to manipulation during the procedure. In order to apply the same conditions to all the colonies, the fungus gardens of the 10 queenright colonies were fragmented similarly, without damaging the queens or the workers. After this, each queenright and queenless colony was placed in 250 ml plastic pots then placed a piece tray (30×20×6cm) to allow movement of ants. On top of each plastic pot was placed a piece of cotton, which was moistened daily.

To prepare our suspension, we first grew the isolate *Syncephalastrum* sp. on plates containing MEA, then incubated it at 25ºC in the dark for 12 days. After this period, the spores were removed from the plates and individually inserted into a Falcon tube containing sterile distilled 0.01% of Tween 80® solution. The suspension was stirred for 3 min and then filtered using sterile gauze. This procedure allowed the separation of the spores from hyphal fragments, resulting in suspension containing only spores. The maximum concentration obtained of the suspension was determined using a Neubauer chamber as 10^6 spores ml^{-1} (*ca.* 100,000 spores/gram of fungus garden).

For treatment with the fungus, we inoculated 4 ml of the suspension of *Syncephalastrum* sp., containing *ca.* 100,000 spores/gram of fungus garden for each colony. (Fig. 2E). For the blank control, we inoculated 4 ml of blank 0.01% Tween 80® solution. Six queenright colonies were inoculated with *Syncephalastrum* sp. and four with blank control, while of the ten queenless colonies, five were inoculated with *Syncephalastrum* sp. and five with blank control (drawing lots as above). We sprayed the suspensions on the surface of fungus gardens. The trays that contained each treatment were placed randomly on four shelves and trays were rotated every three days, so that they could get the same conditions.

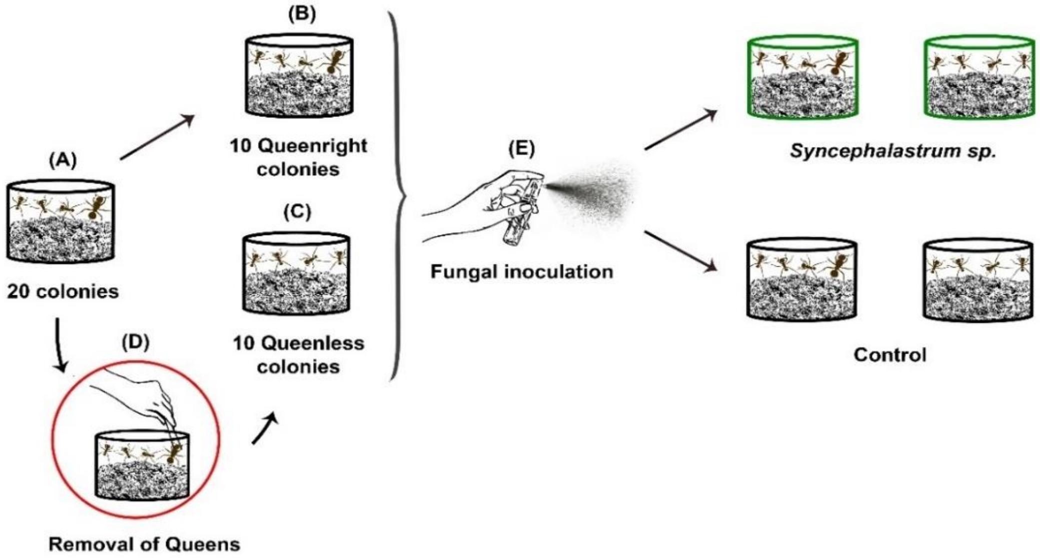

Fig. 2 Schematic representation of experimental set-up. Twenty colonies of the leafcutter ants *Acromyrmex subterraneus subterraneus* were necessary to assemble the experiment. **(A)** Twenty complete colonies. **(B)** Ten queenright colonies (fungus garden + queen + workers) and **(C)** ten queenless colonies (fungus garden + workers). **(D)** Removal of queens with the help of a tweezers. **(E)** Inoculation fungal of spore's suspension of the fungus *Syncephalastrum* sp. and blank control (0,01% Tween 80® solution).

The data collected were the weights of leaves cut by ants (wet weight), midden weights (wet weight), weights of queenright and queenless colonies (wet weight), and presence or absence of *Syncephalastrum* sp. in the midden. These data were chosen because they are part of the daily activities performed by ants (K. R. Bautz, personal observation). All weighing was done using an analytical balance (Bel Engineering). The weights of leaves were measured daily while the midden weights, the weights of queenright and queenless colonies and presence or absence of *Syncephalastrum* sp. in the midden were measured 12 hours after inoculation. This procedure was repeated then every 72 hours for 28 days with 10 collections.

Weights of leaves cut by ants

The weights of leaves offered for each queenright and queenless colony were measured to determine if inoculation with *Syncephalastrum* sp. might alter the amount of cut leaves supplied to the ant's symbiotic fungus. For this, we offered, daily, 3 g of *A. wilkesiana* fresh leaves and after 24 hours, we weighed the leaves that were not cut (leaves remaining in the trays). To account for water loss from the leaves, we placed a tray containing only *A. wilkesiana* fresh leaves (3 g) on each shelf that had the treatment every day and weighed the leaves at the same times as wet weights were taken of leaf material offered to the ants. Therefore, we calculated the weight of leaves that were cut according to the formula adapted from Antunes & Della Lucia (1999) and Gandra (2014):

$Cr = QFi - QFf - \%PA$
Cr= Weight of cut leaves
QFi= Quantity of leaves that we offered
QFf= Quantity of leaves that were not cut by ants
%PA= Percentual weight of water loss

Midden weights

The midden weight from each queenright and queenless colony was measured to analyse if the colonies inoculated with *Syncephalastrum* sp. might alter the amount of midden produced by the ants. The midden of each colony was removed with the aid of a sterile spoon and placed in plastic pots and subsequently weighed.

Weights of queenright and queenless colonies

The weight from each queenright and queenless colony was measured to analyse if the colonies inoculated with *Syncephalastrum* sp. might alter the weight of these colonies. We weighed the queenright and queenless colonies one day before the experiment starts, to obtain the initial weights of the colonies. The initial weight of each queenright colony presented some variation, and this was also observed between the queenless colonies. Therefore, we compared the initial weights of the colonies via ANOVA and from this, the colonies were considered viable to start the experiment.

Presence of Syncephalastrum sp. *in the midden*

The sampling from middens from each queenright and queenless colony was measured to check if the ants were removing *Syncephalastrum* sp. from their fungus gardens. After weighing the middens of the colonies, five fragments of midden from each queenright and queenless colony were plated on Petri dishes (90×15mm) containing PDA + chloramphenicol and then incubated at 25ºC in the dark for 15 days. We checked the plates daily to verify the presence of *Syncephalastrum* sp.

Survival of queenright and queenless colonies

The survival of queenright and queenless colonies was checked every day to analyse if the colonies inoculated with *Syncephalastrum* sp. can cause the death of these colonies. We considered colonies dead when there was no fungus garden in the plastic pot, accompanied by a dead queen (for queenright colonies) and dead workers.

Data analysis

We evaluated the effects of *Syncephalastrum* sp. on the leafcutter ant colonies in two different complexity levels. For this, we use the Linear Mixed Models (LMM) for which the response variables were: (i) weight of leaves cut by ants, (ii) midden weight produced by ants (iii) weight of queenright and queenless colonies. Explanatory variables consisted of two levels: the colonies complexity levels (queenright or queenless) and treatments (fungus or control). We considered each sample as a repeated measure and the significance was evaluated using χ^2 test ($P < 0.05$). Significance was evaluated using the F test ($P <0.05$). All analyses were conducted in the statistical software R (version 3.5.1).

RESULTS

In total, *Syncephalastrum* sp. was found in the fungus garden of seventeen of the 20 colonies, representing 85% of the colonies collected.

Weights of leaves cut by ants

Queenright colonies cut more leaf in the experimental period than queenless colonies independent of inoculation of the fungus (Fig. 3). The mean weights of leaves cut by ants for queenright colonies were greater when inoculated with *Syncephalastrum* sp. (1.663 ± 0.028 g day^{-1}; mean ± S.E.M.) than queenless colonies when inoculated (1.497 ± 0.036 g day^{-1}). Additionally, the mean weight of leaves cut by ants for queenright colonies were greater when uninoculated with *Syncephalastrum* sp. (1.786 ± 0.028 g day^{-1}) than queenless colonies when uninoculated (1.159 ± 0.056 g day^{-1}). There was an interaction between the treatments and the levels of complexity of the colonies ($\chi^2_{(6)}$ = 45.23, P = < 0.001; Fig. 3).

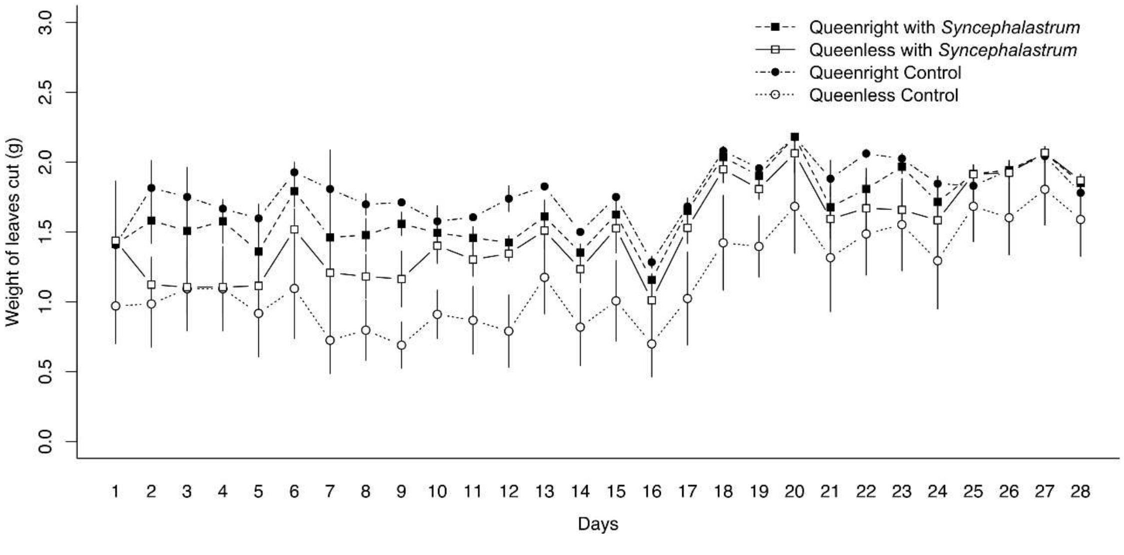

Fig. 3 Weights of leaves cut daily by *Acromyrmex subterraneus subterraneus* leafcutter ants in different levels of complexity inoculated and uninoculated with the fungus *Syncephalastrum* sp. The suspensions were inoculated on Day 1 and the queenright and queenless colonies were evaluated for 28 days. Each dot represents the mean and the bars

represent the standard error for each treatment over time.

Midden weights

Queenright colonies produced more midden in the experimental period than queenless colonies independent of inoculation of the fungus (Fig. 4). The mean weights of midden produced by the ants from queenright colonies were greater over three days when inoculated with *Syncephalastrum* sp. (1.151 ± 0.049 g) than queenless colonies when inoculated (0.936 ± 0.073 g). Additionally, the mean weights of midden produced by the ants from queenright colonies were greater over three days when uninoculated with *Syncephalastrum* sp. (1.210 ± 0.074 g) than queenless colonies uninoculated (1.020 ± 0.072 g). There was no effect of inoculation with *Syncephalastrum* sp. ($\chi^2_{(4)} = 1.5706$, $P = 0.2101$; Fig. 4) on the weight of midden. The weight of midden produced by ants from queenright colonies was greater than from queenless colonies ($\chi^2_{(3)} = 11.402$, $P = 0.0007338$; Fig. 4) Thus, There was no interaction between the fungal treatments and the levels of complexity of the colonies ($\chi^2_{(6)} = 0.0567$, $P = 0.8118$; Fig 4).

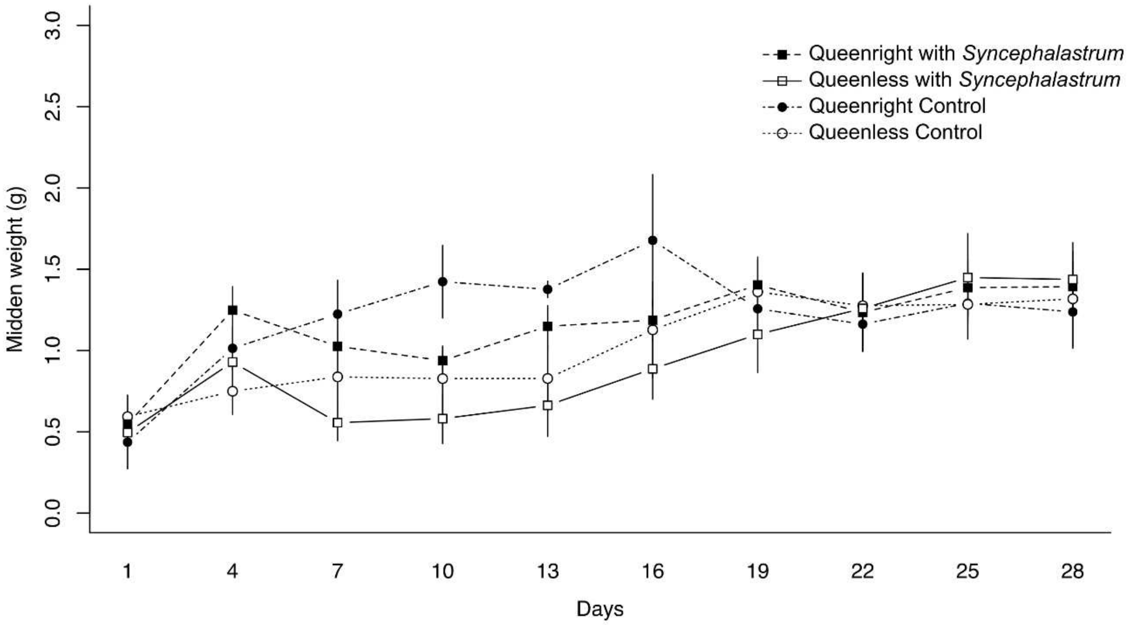

Fig. 4 Weights of midden produced by *Acromyrmex subterraneus subterraneus* leafcutter

ant in different levels of complexity inoculated and uninoculated with the fungus *Syncephalastrum* sp. The suspensions were inoculated on Day 1 and the queenright and queenless colonies were evaluated for 28 days. Each dot represents the mean and the bars represent the standard error for each treatment over time. To conduct the analysis, we used linear mixed models (LMM). Significance was evaluated using a χ^2 test ($P<0.05$).

Weights of queenright and queenless colonies

The initial weights of colonies inoculated and uninoculated with *Syncephalastrum* sp. were no different ($F_{(17)} = 0.0783$, $P = 0.7830$) and the same was observed for queenright and queenless colonies ($F_{(18)} = 1.0312$, $P = 0.3241$).

Queenright colonies were heavier in the experimental period than queenless colonies independent of inoculation of the fungus (Fig. 6). The mean weights of the queenright colonies were greater over three days when inoculated with *Syncephalastrum* sp. (40.572 ± 0.796 g) than queenless colonies when inoculated (38.122 ± 0.650 g). Additionally, the mean weights of the queenright colonies were greater over three days when uninoculated with *Syncephalastrum* sp. (36.545 ± 0.757 g) than queenless colonies when uninoculated (34.442 ± 1.549 g). In addition, the colonies when inoculated with *Syncephalastrum* sp. were heavier than colonies when uninoculated with *Syncephalastrum* sp. ($\chi^2_{(4)} = 15.796$, $P = 0.001$; Fig. 5). In addition, queenright colonies uninoculated with *Syncephalastrum* sp. maintained a similar weight over time. Additionally, the queenless colonies were lighter than queenright colonies ($\chi^2_{(4)} = 4.4758$, $P = 0.03438$; Fig. 5). There was no interaction between the fungal treatments and the levels of complexity of the colonies ($\chi^2_{(6)} = 0.1414$, $P = 0.7069$; Fig 5).

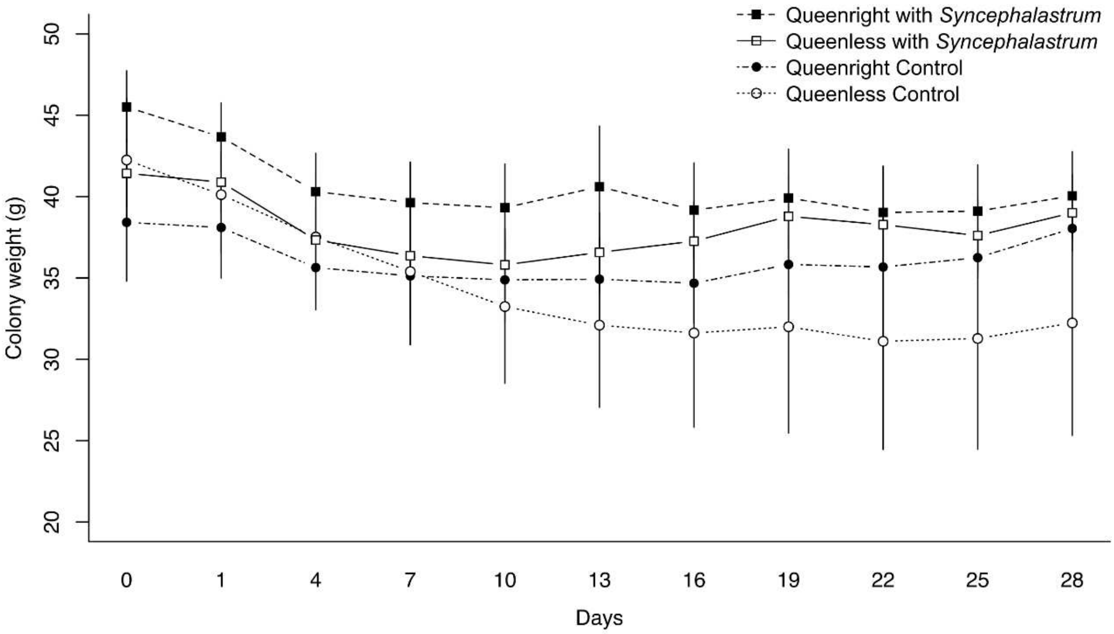

Fig. 5 Weights of *Acromyrmex subterraneus subterraneus* leafcutter ant colonies in different levels of complexity inoculated and uninoculated with the fungus *Syncephalastrum* sp. The suspensions were inoculated on Day 1 and the queenright and queenless colonies were evaluated for 28 days. Time zero represents the initial weight of the colonies. Each dot represents the mean and the bars represents the standard error for each treatment over time. To conduct the analysis, we used linear mixed models (LMM). Significance was evaluated using a χ^2 test ($P<0.05$).

Presence of Syncephalastrum sp. in the midden

We observed that queenright and queenless colonies inoculated with *Syncephalastrum* sp. removed fragments from the fungus garden containing *Syncephalastrum* sp. and placed them in the midden (Fig. 6). On the other hand, *Syncephalastrum* sp. was found growing in the midden from the queenright and queenless colonies uninoculated with *Syncephalastrum* sp., because this fungus was found naturally in the colonies collected.

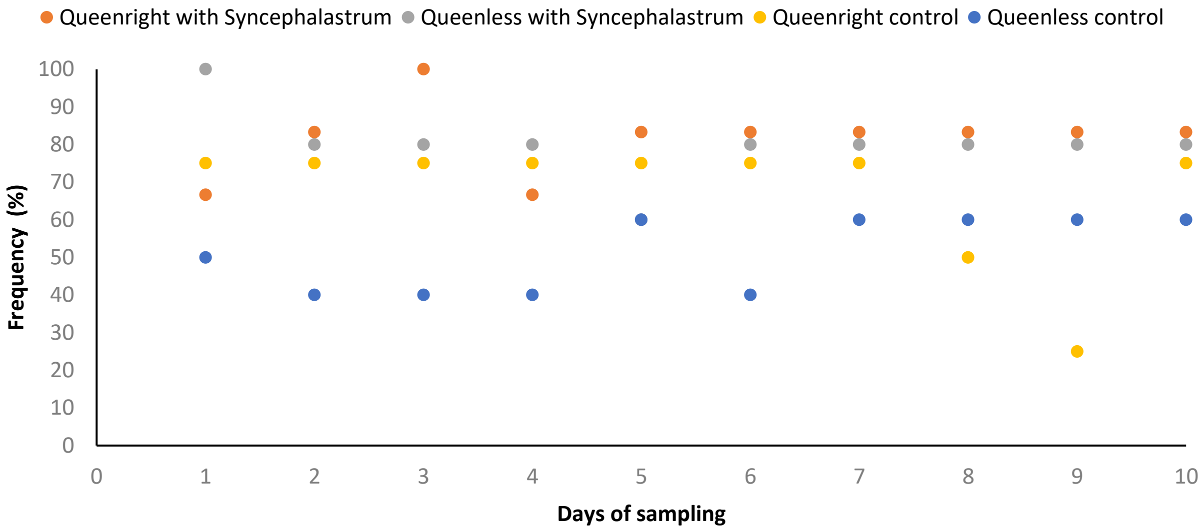

Fig. 6 Frequency in percentage to the fungus *Syncephalastrum* sp. found in midden of *Acromyrmex subterraneus subterraneus* leafcutter ant colonies in different levels of complexity inoculated and uninoculated with *Syncephalastrum* sp. We plated five fragments of midden from each queenright and queenless colony on Petri dishes containing PDA + chloramphenicol and then incubated at 25°C for 15 days. We checked the plates daily to verify the occurrence of *Syncephalastrum* sp. in the midden. Each dot represents of the frequency (%) of the fungus *Syncephalastrum* sp. present in each colony along the 10 days of sampling.

Survival of queenright and queenless colonies

All colonies, whether queenright or queenless, survived whether inoculated with *Syncephalastrum* sp. or not in the experimental period of 28 days and indeed 96 days after that when maintained in the laboratory.

DISCUSSION

The *Syncephalastrum* sp. is commonly present in the fungus garden of colonies of *Acromyrmex* collected, as has been previously observed in *Atta* and *Acromyrmex* (Rodrigues et al., 2005a, 2008, 2009; Barcoto et al., 2017; Bizarria et al., 2018). It is worth noting that the non-detection of the fungus in some of the colonies is not proof of its absence. The explanation for why this happened can be related to the fact that this fungus is found in the soil (Domsch et al., 1980). Thus, in nature, colonies of leafcutter ants that nest inside the soil may be in direct contact with this fungus. Furthermore, we observed that the activities developed by the ants after exposition of the fungus *Syncephalastrum* sp., such as leaf cutting, waste production, and weight of colonies were affected, with effects greater in queenless colonies. Based on our results, there was a pattern for these activities (Figs. 3, 4 & 5) in which the ants in the first days of the evaluations cut fewer leaves, produced more waste, and suffered weight loss from the colony, however after the 16th day of evaluation these activities stabilized.

The mean leaf weights cut by ants from queenright colonies uninoculated with *Syncephalastrum* sp. was greater than from inoculated queenright colonies. Therefore, we suggest that after the inoculation of *Syncephalastrum* sp., workers decrease leaf cutting activity to invest in the protection from the fungus garden against the alien microrganism. However, we expected colonies inoculated with *Syncephalastrum* sp. to produce more midden due to the removal of contaminated fungus garden fragments, which did not occur. However, colonies inoculated with *Syncephalastrum* sp. on the 4th evaluation days, they produce more waste than other colonies. Thus, we presume that in the first days there was a greater removal of the fungus garden contaminated with *Syncephalastrum* sp. and subsequently, we suspect the ants used other tactics to combat the negative effects of Syncephalastrum sp., such as secretion of antimicrobial compounds by the metapleural gland (Poulsen et al., 2002; Fernández-Marín et al., 2006, 2009) and association with filamentous bacteria containing antiseptic compounds (Currie et al., 1999a). Mendonça (2018) observed when queenright colonies were exposed to *Escovopsis moelleri* and *Trichoderma longibrachiatum* the workers cut less leaves. Accordingly, the workers invested in efforts to remove the parasites from their colonies rather than cutting leaves to be incorporated into fungus gardens, but after this period, the workers increased the amount of cut leaves, promoting the growth of the fungal cultivar.

The ants controlled the growth of *Syncephalastrum* sp. in a way that did not compromise the survival of the fungus gardens. For this, ants can perform two hygienic behaviours to control the development of alien microrganisms in the fungus garden, such as grooming and weeding (Currie & Stuart 2001). These behaviours are characterized by the "licking" the contaminated fungus garden fragment and the removal of infected garden fragments or of vegetal material contaminated, respectively (Currie & Stuart 2001), and all this infected material is discarded in the midden (Hölldobler & Wilson 2009).

In the current study, the behaviour of weeding, that is, the removal of the infected fragment of fungus garden in queenright and queenless colonies was observed about 1 hour after inoculation of *Syncephalastrum* sp. (Bautz, K. R. personal observation), while in the study developed by Barcoto et al. (2017), it was observed that the first fragment of fungus garden was removed about 5 hours and 30 minutes after inoculation to the *Syncephalastrum* sp. Moreover, as demonstrated here, *Syncephalastrum* sp. was found in the midden produced by ants from queenright and queenless colonies inoculated and did uninoculated with *Syncephalastrum* sp., because this fungus was present naturally in the colonies collected. Thus, we suspect that ants recognize that their fungus garden is contaminated with alien microrganisms, remove these infected fragments, and dispose of them in the midden through the hygiene behaviours mentioned above. Similar results were found by Mendonça (2018) however, only *Trichoderma* was found growing in the midden of the queenright and queenless colonies, independent of the treatment to which they were exposed. This author presumes that, *Escovopsis* and *Escovopsioides* could be present in the midden, nevertheless, other microrganisms that are also present may have inhibited their growth in the culture medium.

In relation to the weight of colonies, firstly we saw that the initial weight of queenright and queenless colonies, that is before the starting the experiment, did not differ between them. In addition, throughout the evaluations, we observed that the queenright colonies when inoculated with *Syncephalastrum* sp. were heavier than uninoculated queenright colonies although we expected the opposite. However, we suspect that the spores of the *Syncephalastrum* sp. changed the weight of these colonies, although the ants removed a large part of the spores of the *Syncephalastrum* sp. from the fungus garden and deposited in the midden, and or used other tactics to fight the fungus, the inoculated concentration was high of ca. 100,000 spores/gram of the fungus garden. Meanwhile, the other colonies uninoculated with the fungus received the blank control with only the Tween solution.

Although the queenright and queenless colonies inoculated with *Syncephalastrum* sp. did suffer negative effects, this was not enough to compromise their survival as we expected. This contrasts with a previous study by Barcoto et al. (2017) in which they tested the pathogenicity of an isolate of the *Syncephalastrum* sp. in queenless colonies of *At. sexdens rubropilosa* and observed that these colonies died on the 13th day after inoculation of fungus. Based on our results, we presume that the different isolates of *Syncephalastrum* sp. and the different ant genera used (from different parts of Brazil) may be the factors that justify the difference between our results. Previous studies have shown in vitro that different isolates of fungi show different virulence against the *L. gongylophorus* (Rodrigues et. al., 2009; Folgarait et. al., 2011a; Wallace et. al., 2014). Furthermore, *Acromyrmex* species host more abundant Pseudonocardia layers on their integuments than the *Atta* species or are practically absent (Currie et al., 2006). Thus, to understand the effects of antagonist's fungi that interact with leafcutter ants, we must be aware that minor differences in experimental design can lead to contradictory results (Gange et. al., 2019).

This current study showed that *Syncephalastrum* sp. caused negative effects on the queenright and queenless colonies, with effects greater in queenless colonies and demonstrated how ants react to the *Syncephalastrum* sp. through activities and behaviours carried out daily by ants to combat the negative effects caused by the fungus. Thus, our results highlight the importance of each element such as fungus garden, queen, and workers in the symbiosis of leafcutter ants, mainly in studies that investigate the effect of antagonist microrganisms on leafcutter ant's fungal cultivars. Furthermore, we emphasize the need of studies (i) identifying new species of *Syncephalastrum* sp., (ii) investigate the virulence of different isolates of this fungus, and (iii) and evaluate the different behavioural responses of *Atta* and *Acromyrmex* in the presence of the queen when they are under microbial threat. To conclude, this study provides insights into the effects of *Syncephalastrum* sp. in the symbiosis of the leafcutter ants and these findings could impact the planning of biological control strategies.

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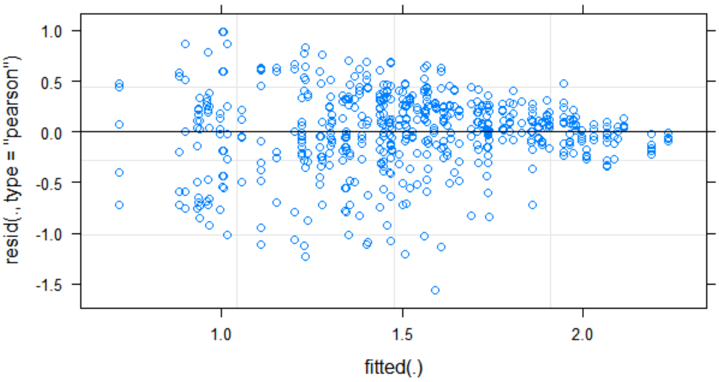
APPENDIX

APPENDIX S1 - Script: Weights of leaves cut by ants

```
dados1<-read.table("corte_folha.txt",h=T)
dados1
summary(dados1)
attach(dados1)
names(dados1)
library(lme4)

m1=lmer(Cr~Level*Treat+(1|Avalia))
par(mfrow=c(2,2))
names(dados1)
plot(m1)
```

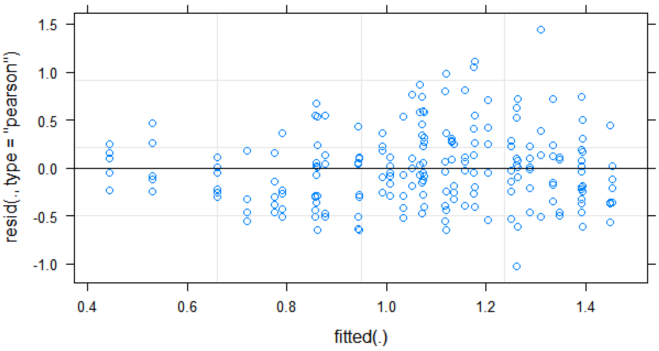


```
summary(m1)
Linear mixed model fit by REML ['lmerMod']
Formula: Cr ~ Level * Treat + (1 | Avalia)

REML criterion at convergence: 619.2

Scaled residuals:
    Min      1Q  Median      3Q     Max
-3.9674 -0.4575  0.0943  0.6357  2.5065

Random effects:
 Groups   Name        Variance Std.Dev.
 Avalia   (Intercept) 0.06322  0.2514
 Residual             0.15452  0.3931
Number of obs: 560, groups:  Avalia, 28
Fixed effects:
Estimate Std. Error t value
(Intercept)         1.78051    0.06032  29.518
```

```
Levelwork          -0.62629    0.04984 -12.565
Treatsync          -0.12193    0.04796  -2.542
Levelwork:Treatsync  0.46001    0.06714   6.851

Correlation of Fixed Effects:
(Intr) Lvlwrk Trtsyn
Levelwork   -0.459
Treatsync   -0.477  0.578
Lvlwrk:Trts  0.341 -0.742 -0.714

m2=lmer(Cr~Level+Treat+(1|Avalia))
summary(m2)
anova(m1,m2,test="Chi")

Data: NULL
Models:
m2: Cr ~ Level + Treat + (1 | Avalia)
m1: Cr ~ Level * Treat + (1 | Avalia)
Df    AIC    BIC  logLik deviance Chisq Chi Df Pr(>Chisq)
m2  5 656.75 678.39 -323.37   646.75
m1  6 613.52 639.48 -300.76   601.52 45.23      1  1.752e-11 ***
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

APPENDIX S1 - Script: Midden weights

```
dados2<-read.table("peso_lixo.txt",h=T)
dados2
summary(dados2)
attach(dados2)
names(dados2)
library(lme4)

m1=lmer(Midden~Level*Treat+(1|Day_n))
summary(m1)
par(mfrow=c(2,2))
names(dados2)
plot(m1)
```



```
m2=lmer(Midden~Level+Treat+(1|Day_n))
anova(m1,m2,test="Chi")

Data: NULL
Models:
m2: Midden ~ Level + Treat + (1 | Day_n)
m1: Midden ~ Level * Treat + (1 | Day_n)
Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
m2  5 235.53 252.02 -112.77    225.53
m1  6 237.47 257.26 -112.74    225.47 0.0567      1     0.8118

m3=lmer(Midden~Level+(1|Day_n))
anova(m2,m3,test="chi")

Data: NULL
Models:
m3: Midden ~ Level + (1 | Day_n)
m2: Midden ~ Level + Treat + (1 | Day_n)
```

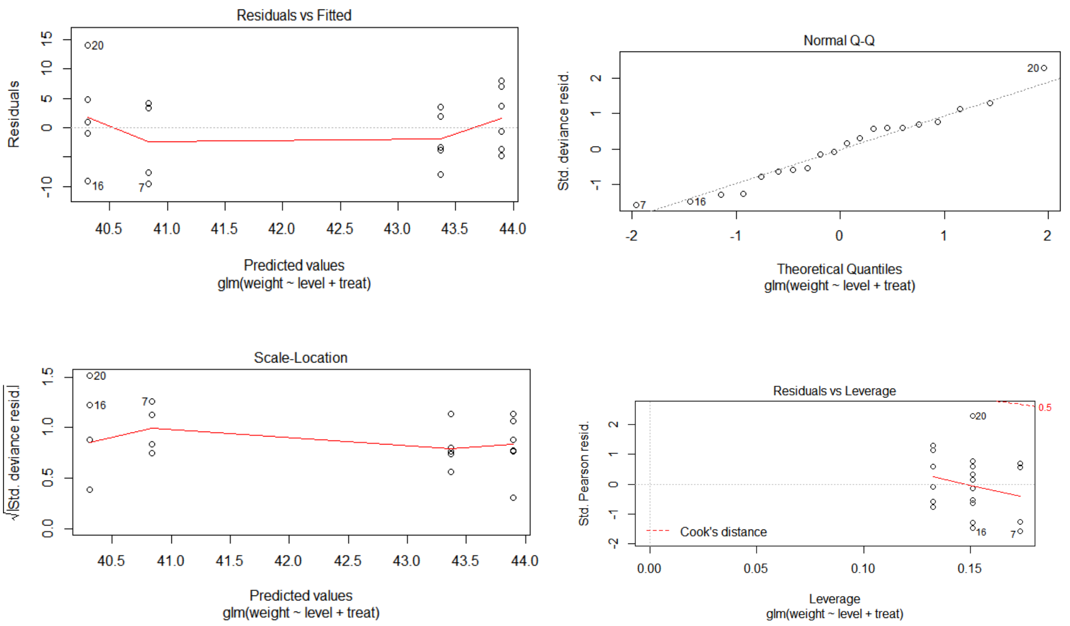
```
Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
m3  4 235.10 248.30 -113.55   227.10
m2  5 235.53 252.02 -112.77   225.53 1.5706      1     0.2101

m4=lmer(Midden~1+(1|Day_n))
anova(m3,m4,test="chi")

Data: NULL
Models:
m4: Midden ~ 1 + (1 | Day_n)
m3: Midden ~ Level + (1 | Day_n)
Df   AIC   BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
m4  3 244.5 254.4 -119.25    238.5

m3  4 235.1 248.3 -113.55    227.1 11.402      1  0.0007338 ***

Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

APPENDIX S1 - Script: Initial weights of the colonies

```
dados <- read.table("peso_colonias_antes_do_experimento_Test.txt", header = TRUE)
mod1 <- glm(weight ~ level+treat, data = dados)
mod2 <- glm(weight ~level*treat, data=dados)
anova(mod1, mod2, test="F")
```

```
Analysis of Deviance Table
Model 1: weight ~ level + treat
Model 2: weight ~ level * treat
Resid. Df Resid. Dev Df Deviance      F Pr(>F)
1       17     755.63
2       16     679.03  1    76.596 1.8048 0.1979
```

```
plot(mod1)
```

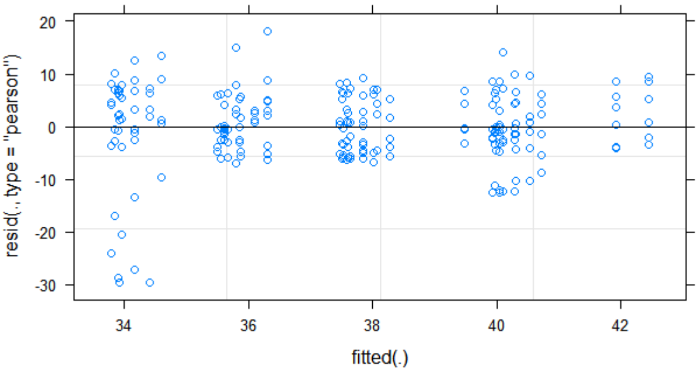


```
anova(mod1, test = "F")
```

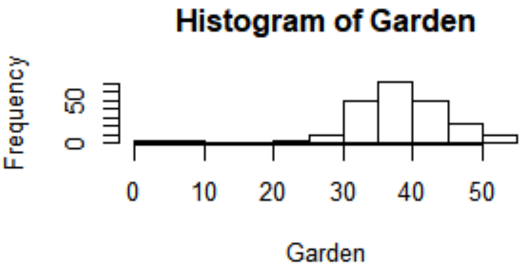
```
Df Deviance Resid. Df Resid. Dev      F Pr(>F)
NULL                    19     804.94
level  1    3.480       18     801.46 0.0783 0.7830
treat  1   45.835       17     755.63 1.0312 0.3241
```

APPENDIX S1 Script: Weights of queenright and queenless colonies

```
dados<-read.table("peso_jardim.txt",h=T)
dados
summary(dados)
attach(dados)
library(lme4)
library(Matrix)

m1=lmer(Garden~Level*Treat+(1|Day_n))
par(mfrow=c(2,2))
names(dados)
plot(m1)
```



```
hist(Garden)
```



```
summary(m1)

m2=lmer(Garden~Level+Treat+(1|Day_n))
anova(m1,m2,test="Chi")
```

```
Models:
m2: Garden ~ Level + Treat + (1 | Day_n)
m1: Garden ~ Level * Treat + (1 | Day_n)
Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
m2  5 1515.7 1532.7 -752.87   1505.7
m1  6 1517.6 1538.0 -752.79   1505.6 0.1414      1      0.7069
```

```
m3=lmer(Garden~Treat+(1|Day_n))
anova(m2,m3,test="Chi")

Data: NULL
Models:
m3: Garden ~ Treat + (1 | Day_n)
m2: Garden ~ Level + Treat + (1 | Day_n)
Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
m3  4 1518.2 1531.8 -755.10   1510.2
m2  5 1515.7 1532.7 -752.87   1505.7 4.4758      1    0.03438 *
  ---
  Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

m4=lmer(Garden~Level+(1|Day_n))
anova(m2,m4,test="Chi")

Data: NULL
Models:
m4: Garden ~ Level + (1 | Day_n)
m2: Garden ~ Level + Treat + (1 | Day_n)
Df    AIC    BIC  logLik deviance  Chisq Chi Df Pr(>Chisq)
m4  4 1529.5 1543.1 -760.76   1521.5
m2  5 1515.7 1532.7 -752.87   1505.7 15.796      1  7.054e-05 ***
  ---
 Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

APPENDIX S1 - Script: Graphic

```
folha<-read.table("corte_folha.txt",h=T)
summary(folha)

folha$Rep<-as.factor(folha$Rep)
is.factor(folha$Rep)
head(folha)
folha$Avalia<-as.factor(folha$Avalia)
is.factor(folha$Avalia)

#plot todos os tratamentos - corte de folha
lineplot.CI(Avalia,Cr, group, xlab='Days', ylab='Weight of leaves cut (g)',
leg.lab=c('Queenright with Syncephalastrum','Queenless with
Syncephalastrum','Queenright control','Queenless control'),
ylim=c(0,3),xlim=c(1,28),x.leg=18,y.leg=3,type="b", data=folha, bty='l')

#_________________
lixo<-read.table("peso_lixo.txt",h=T)
summary(lixo)

lixo$Rep<-as.factor(lixo$Rep)
is.factor(lixo$Rep)

lixo$Day_n<-as.factor(lixo$Day_n)
is.factor(lixo$Day_n)
summary(lixo)

#plot todos os tratamentos -peso do lixo
lineplot.CI(Day_n,Midden,group, xlab='Days', ylab='Midden weight (g)',
ylim=c(0,3),xlim=c(1,10), x.leg=7,y.leg=2.9, leg.lab=c('Queenright with
Syncephalastrum','Queenless with Syncephalastrum','Queenright control', 'Queenless
control'), type="b", data=lixo, bty='l')

#_________________
jardim<-read.table("peso_jardim.txt",h=T)
summary(jardim)
jardim$Rep<-as.factor(jardim$Rep)
is.factor(jardim$Rep)
jardim$Day_n<-as.factor(jardim$Day_n)
is.factor(jardim$Day_n)
install.packages("sciplot")
library(sciplot)
dados
```

```
#plot todos os tratamentos - peso da colônia
lineplot.CI(Day_n,Garden, group, xlab='Days',
ylim=c(20,50),xlim=c(1,11),x.leg=7.4,y.leg=52,leg.lab =c('Queenright with
Syncephalastrum','Queenless with Syncephalastrum','Queenright control','Queenless
control'), ylab='Colony weight (g)',type="b", data=jardim, bty='l')
```