



## Facilitators or predators? The role of seed-feeding beetles in the germination of the ornamental and invasive plant *Leucaena leucocephala* in an urban environment

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### ABSTRACT

*Leucaena leucocephala* is a forage legume with a dangerous allelopathic and invasive potential. Its main natural enemy is the endophytic beetle *Acanthoscelides macrophthalmus* (Chrysomelidae: Bruchinae), whose larvae penetrate seeds and can consume the entire endosperm and embryos. However, there is much to explore about how the abundance of larvae inside the seed affects plant germination, resulting in an antagonistic (predation) or mutualistic (facilitation) interaction. Our goal was to test how this beetle affects the germination of *L. leucocephala* seeds from an urban environment in Uberlândia-MG. We hypothesized that a single larva inside the seed facilitates germination since the exit holes left break the physical dormancy and enable water imbibition. On the other hand, two or more larvae inside the same seed result in predation, as the amount of endosperm is not enough for beetle satiation before they attack the seed embryo. We collected 100 pods, dissected them, and quantified and qualified the seeds. In the germination experiment, we used healthy seeds (control), scarified healthy seeds (physical treatment), and attacked seeds (one, two, or more larvae). We found that beetles infested 74% of the sampled seeds. Independently of the abundance of larvae inside the seed, attacked seeds did not germinate. The scarified seeds germinate first and in greater quantity. Therefore, the predation hypothesis was confirmed, and the facilitation hypothesis was rejected. *A. macrophthalmus* acts as a predator in an antagonistic interaction that decreases the germination rate of *L. leucocephala*.

**Keywords:** *Acanthoscelides macrophthalmus*, biological control, plant health, urban ecology, seed damage.



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### Introduction

*Leucaena leucocephala* (Lamarck) de Wit is a shrub or tree plant belonging to the family Fabaceae. It is an invasive exotic plant in many countries (Campbell et al., 2019), as in Brazil, and recently, it has been described as having an allelopathic potential (Kato-Noguchi & Kurniadie, 2022). This potential can be harmful in agroecosystems, reforested urban areas, or even in natural environments, where populations of *L. leucocephala* can reduce the abundance of other plants with economic and/or native purposes (Mauli et al., 2009). The leaves of *L. leucocephala* can also contain toxins, which can poison herds of goats and sheep (Peixoto et al., 2008). In this sense,

it is necessary to elucidate strategies to reduce the abundance of this invasive species, and one of the ways to do this is to get to know its natural enemies and use them in biological control programs.

One of the foremost natural enemies of *L. leucocephala* is the endophytic seed-feeding beetle in pre- and post-dispersal phases, *Acanthoscelides macrophthalmus* (Schaeffer, 1907; Chrysomelidae: Bruchinae). The death of seeds by bruchines occurs when these beetles feed their embryos, leaving them hollow inside, or even when they feed on the entire endosperm or a significant fraction, leaving the embryos without energy reserves for their development (Ribeiro-Costa & Almeida, 2012; Sousa-Lopes et al., 2019). This is

the type of damage that *A. macrophthalmus* can inflict on *L. leucocephala* seeds (Rodrigues et al., 2012), in addition to leaving the possibility of fungal attack through the exit holes left when the adult emerges (Silva & Rossi, 2019).

However, it has been hypothesized that the role of this seed beetle may be different, as instead of killing the embryo, it may facilitate the germination of *L. leucocephala* seeds (Silva & Rossi, 2019). Facilitation occurs when the beetles penetrate the seed and feed on only a few parts of the endosperm, thus leaving the embryo free from damage and with an energy reserve. Then, the beetles leave exit holes that allow water to enter (breaking the physical dormancy), increasing seed germination (Fox et al., 2012).

Although some studies record the damage inflicted by *A. macrophthalmus* on *L. leucocephala* seeds, there is much to explore on the impact it causes on the survival and germination of the host plant in urban environments. For instance, there is no study on the germination and attack rate by *A. macrophthalmus* on *L. leucocephala* for the Triângulo Mineiro, highlighting the need for studies. After all, seed traits, such as size, can change among different plant populations and affect the final result of the beetle-seed interaction (Silva & Rossi, 2019; Sousa-Lopes et al., 2020). Considering the economic and ecological relevance of *L. leucocephala*, and the doubtful role of the associated seed beetle (facilitator or predator), this study was designed to show the role played by *A. macrophthalmus* in this interaction with broad implications for biological control and plant nurseries.

Our goal is to test how *A. macrophthalmus* affects the germination of *L. leucocephala* seeds in an urban environment. Our principal hypothesis is that a single larva inside the seed facilitates germination since the exit hole left breaks the physical dormancy and enables water imbibition. On the other hand, we hypothesized that two or more larvae inside the same seed result in predation, as the amount of endosperm is not enough for beetle satiation before they attack the seed embryo.

## Material and Methods

In September 2019, 100 mature pods were collected from twelve individuals of *L. leucocephala* found in the urban area of Uberlândia, Minas Gerais (18°56'08.2" S, 48°15'58.0" W, and altitude of 843 m). The pods were dissected, and the seeds were packaged separately in 200 mL transparent plastic containers or 50-milliliter Falcon tubes, covered with voile fabric to prevent the proliferation of fungi.

Considering that the seed beetles can emerge six months after the pods mature (Silva & Rossi, 2019), the seeds were kept in the same containers at room temperature (12h of light, 20-30°C) until August 2020. On August 10, 2020 (~11 months after collection), the attacked (with exit holes) and unattacked (healthy) seeds from 52 pods were separated and counted to determine the percentage of attacked and healthy seeds.

The number of seed beetles and other insects found per pod was also counted. The identification of the seed beetles was made by comparing the specimens obtained with the photos and descriptions available in Vassilou & Papadoulis (2008) and on the website of the Population Ecology Laboratory of the Federal University of São Paulo (Rossi, 2019).

Considering germination tests, 100 healthy seeds and 100 attacked seeds were randomly separated. Half of the healthy seeds were scarified with sandpaper n. 80, until it superficially reaches the endosperm to break physical dormancy and allow water to enter (Sousa-Lopes et al., 2019).

The other half of the healthy seeds were the control, that is, subjected to the germination test without any treatment. The attacked seeds were not scarified, as the beetles leave exit holes, which allow water to enter. In these seeds, it was noted that there were one (n = 50), two (n = 29), three (n = 18), or four exit holes (n = 03). Each exit hole represents the number of seed beetles that attacked the seed and reached the adult stage. Therefore, the germination tests were divided into the following groups: (1) Control (healthy seeds), (2) Scarified (healthy seeds subjected to scarification), (3) One exit hole (seeds attacked by a seed-beetle), (4) Two or more exit holes (seeds attacked by two, three or four seed-beetles). Seeds with two or more exit holes were grouped into one treatment because the sample number was too small to separate them.

Finally, on August 18, 2020, under room temperature (~28°C) and a 12h light/dark photoperiod, the seeds were placed inside 500 mL transparent plastic containers, covered with a paper towel moistened with 10 milliliters of water. Five seeds were placed in each pot, totaling 10 containers (replicates) for each of the four groups of seeds. The seed was considered to have germinated when the primary root of at least two millimeters appeared (Rodrigues et al., 2012). The number of germinated seeds was counted daily for each group for 14 days (see Silva & Rossi, 2019).

Data on seeds attacked and not attacked by pods are presented in absolute numbers, percentages, mean, and standard deviation. After carrying out the normality test, Lilliefors, it was decided to use parametric tests. To compare

whether there was a difference in seed germination rates between the groups delimited in this study, an Analysis of Variance (ANOVA) was run for repeated measures since during the 14 days of seed observation, the seeds that germinated first were retold daily until the end of the experiment. A multiple comparison test was carried out using the Tukey Test to compare the germination among the groups. The significance level for all tests was 5% (Zar, 2010).

## Results

The pods of individuals of *L. leucocephala*, complete (Figure 1a) and dissected (Figure 1b), showed seeds attacked (with exit holes; Figure 1c-e) and no attacked (healthy).

Some containers showed separated seeds (healthy and attacked with one, two, or more exit holes, Figure 1f) and were used for subsequent germination testing. The germination was indicated by a primary root (radicle) exposed (Figure 1g).

Some seeds are attacked by the beetle *Acanthoscelides macrophthalmus* (Figure 1h-i).

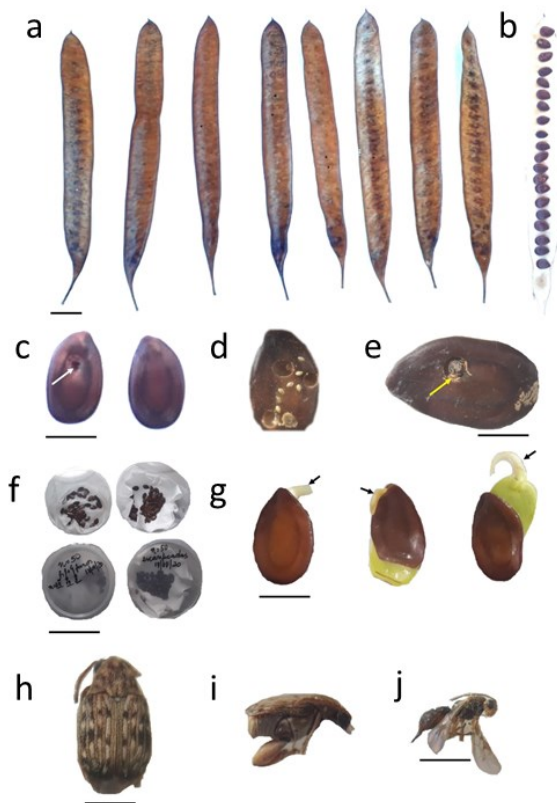


Figure 1. Study system: a. some *Leucaena leucocephala* pods; b. *L. leucocephala* seeds after pod dissection; c. seed attacked by the beetle *Acanthoscelides macrophthalmus* (on the left, with a white arrow indicating the exit hole left by the beetle) and healthy seed (on the right); d. seed with more than one attack and also showing the beetle's

posture, with seven eggs; e. adult beetle leaving the seed after its development (yellow arrow); f. container with the separated seeds (healthy and attacked with one or two or more exit holes) for subsequent germination testing; g. seeds that were considered to have germinated (black arrows indicate the primary root); h. dorsal view of the *A. macrophthalmus* beetle; i. lateral view of the beetle *Acanthoscelides macrophthalmus*; j. probable parasitoid of the beetle Hymenoptera. Bars: a,b = 15 mm; c-e, g = 8 mm; f = 95 mm; h = 1 mm; j. 0.5 mm.

The number of seeds per pod varied from 10 to 29, with a mean of 21.07 and a standard deviation of 4.22. The number of seeds attacked per pod ranged from none to 26, with a mean of 14.25 and a standard deviation of 7.37. Finally, the number of healthy seeds without damage varied from none to 28, with a mean of 6.42 and a standard deviation of 6.98.

Considering the total, 74% (n = 818) of the seeds sampled were attacked, while 26% (n = 284) were healthy. Along with the attacked seeds, 814 individuals of *A. macrophthalmus* were found (Figure 1h-i). An unidentified species of wasp (Hymenoptera) was also found, possibly a parasitoid of *A. macrophthalmus* (n = 20, Figure 1j). The seed beetles continued reproducing under laboratory conditions, with females laying eggs (Figure 1d) and adults emerging (Figure 1e).

The control and scarified seeds were the only ones to germinate, with the latter having the highest germination percentage (Figure 2). There was a significant difference between the mean number of seeds that germinated among the four evaluated groups ( $F_{(3,33)} = 20.51, P < 0.0001$ ), with the control and scarified seeds having greater germination compared to those from the two groups of attacked seeds. However, no significant difference in germination rates was found when comparing the number of control and scarified seeds (Table 1).

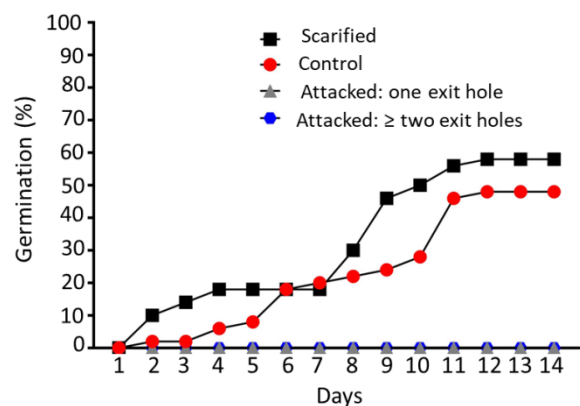


Figure 2. Cumulative percentage of germination of *Leucaena leucocephala* seeds from the urban area of Uberlândia-MG, which were divided into control (n = 50) and three treatments: (1) scarified

(n = 50), (2) with one exit hole (n = 50), and (3) with two or more exit holes (n = 50) caused by *Acanthoscelides macrophthalmus*.

Table 1. Multiple comparisons, using the Tukey test, of the number of *Leucaena leucocephala* seeds germinated in control and treatments (scarified, attacked with one hole, and attacked with two or more holes). P values < 0.05 are statistically significant. C. I. = Confidence Interval. Font: Rodrigues & Sousa-Lopes (2023).

Variables	Mean difference	C.I. 95%	Adjusted P-value
Control vs. Scarified	-4.583	-10.47 a 1.303	0.17
Control vs. one exit hole	9.333	3.447 a 15.22	0.0008
Control vs. $\geq$ two exit holes	9.333	3.447 a 15.22	0.0008
Scarified vs. one exit hole	13.92	8.031 a 19.80	<0.0001
Scarified vs. $\geq$ two exit holes	13.92	8.031 a 19.80	<0.0001
One exit hole vs. $\geq$ two exit holes	0.000	-5.886 a 5.886	>0.99

No attacked seed germinated in this study; we outlined a flowchart with the hypotheses and predictions for the system (Figure 3).

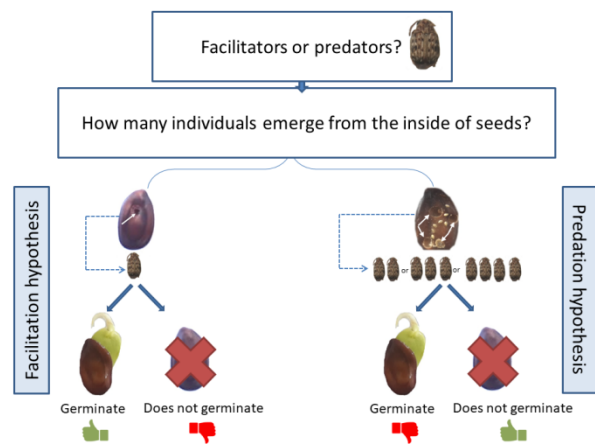


Figure 3. Flowchart to elucidate the hypotheses and predictions for the system *Acanthoscelides macrophthalmus* and *Leucaena leucocephala*. The white arrows in the seeds indicate the exit holes. Font: Rodrigues & Sousa-Lopes (2023).

On the left side of Figure 3, the facilitation hypothesis predicts that a single individual attacking the seed may result in germination. The hypothesis is rejected when the seed does not germinate after the attack.

On the right side of the same figure, the predation hypothesis predicts that the attacked seed does not germinate due to insufficient endosperm for two or more larvae to grow. The hypothesis is rejected whether the seed germinates when attacked by two or more larvae.

However, in this study, the result is always predation, independent of the number of beetles (one or more) that emerge from the inside seeds.

## Discussion

The mean number of seeds per pod found here (n = 21) is like that described in previous studies, with 20 seeds per pod (Stone, 1970; Tuda et al., 2009). Furthermore, like Effowe et al. (2010) and Wu et al. (2012), it was shown that females can oviposit eggs directly on seeds, and their offspring can infest them under laboratory conditions (Figure 1d-e).

The percentage of seed predation found in this study was high (74%) compared to other studies on the same system in Australia (~11%, Raghu et al., 2005) and southeastern Brazil (~42%; Rodrigues et al., 2012). In the cited studies, the percentage of predation was observed only on seeds in the pre-dispersal phase. Here, as the seeds were stored for a long period under laboratory conditions, and copulation and posture behaviors were also observed, it is plausible to say that there was a post-dispersal infestation of the seeds in the containers, making the percentage of attacked seeds to increase with the different generations of the seed-beetle over the time they remained stored. Therefore, the studied seed beetle can damage stored seeds, increasing its potential as a biological control agent for *L. leucocephala*.

Oliveira et al. (2023) showed that *A. macrophthalmus* is responsible for most of the seed predation in *L. leucocephala* in Lavras, Minas Gerais state, Brazil. They elucidated that when *A. macrophthalmus* feeds on this plant, two native parasitoid species, *Paracrias pluteus*, and *Stenocorse suldamericanos*, shift from herbivores on native plants to *A. macrophthalmus* on *L. leucocephala* (Oliveira et al., 2023). Thus, although *A. macrophthalmus* acts as a potential biological control agent for *L. leucocephala*, it can also promote a cascading change in the trophic web since attracting natural enemies from native plants

can change the structure and composition of the community.

We show that scarified healthy seeds germinated first and with a higher amount than healthy seeds without any treatment. The hard surface of the seeds is a factor that can delay germination. Thus, when scarified, water enters more easily and probably improves germination (Sousa-Lopes et al., 2019). This information may be important for nurseries, vegetable gardens, or people who want to obtain *L. leucocephala* seedlings, suggesting that mechanical abrasion using water sandpaper until it superficially reaches the endosperm is relevant to germination. However, whether these seeds grow faster in the population studied remains to be seen. In this sense, Silva & Rossi (2019) showed that the seeds that germinated faster also developed larger seedlings in populations in São Paulo, even though these seeds were attacked by the beetle *A. macrophthalmus*.

Here, the same beetle also left exit holes that allowed water to enter, but none of the attacked seeds germinated, regardless of the number of attacks (one, two, three, or four) (Figure 1). It is suggested that, in the population studied, the beetles feed on the entire endosperm and remove the necessary supply for developing the seed embryo. The seed embryos can also be directly preyed upon by the beetle.

However, studying this same system in other populations of *L. leucocephala* from São Paulo demonstrated that seed beetles can facilitate germination (Silva & Rossi, 2019; Figure 3). Silva and Rossi (2019) showed that in 14 of the 26 populations studied, the germination of *L. leucocephala* seeds was facilitated by *A. macrophthalmus*. The authors suggested that the seed size can influence the outcome of this interaction, as smaller seeds would have fewer resources for seed beetle development. It is likely, then, that the seeds from Uberlândia are smaller in size when compared to those from São Paulo and, therefore, *A. macrophthalmus* acts as a predator in the plant population studied here.

Oliveira et al. (2023), Sousa-Lopes et al. (2020), and Haga & Rossi (2016) also highlighted that large seeds of Fabaceae attract a great number of seed beetles and their natural enemies. Here, we recorded parasitoids but in low abundance (20 individuals). The open question now is whether populations of *L. leucocephala*, mainly those with larger seeds, are causing changes in the topography of parasitoid-herbivore-plant networks where it is present. Further studies are needed to elucidate how the seed size of *L. leucocephala* affects the

community of herbivores, natural enemies, and other plants.

## Conclusion

The hypothesis that a single larva inside the seed would facilitate germination was rejected. The hypothesis that two or more larvae inside the same seed would result in predation was corroborated.

Regarding germination, the scarified seeds germinate first and with a higher amount than those without scarification and are attacked by the seed beetle.

Therefore, we highlighted the predatory role of *A. macrophthalmus* on *L. leucocephala* seeds in an urban environment. This seed beetle infests seeds under laboratory conditions and is an alternative for biological control of the plant. However, this must be seen with caution since natural enemies may shift from herbivores on native plants to *A. macrophthalmus* on *L. leucocephala*, negatively affecting the dynamics of food webs and communities.

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