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Biotic and Abiotic Interactions Shape Seed Germination of a Fire-Prone Species

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Abstract: Both biotic and abiotic environmental filters drive the occurrence, distribution, and persistence of plant species. Amongst drivers that influence the distribution of plants in harsh environments, seed predation and temperature are particularly important in habitats that are prone to fire. In this study, we highlight the combined effects of predation and high temperature simulating fire to understand its effects on the germination percentage and germination speed of the fire prone species *Copaifera oblongifolia*. Groups of seeds attacked by the beetles *Rhinochenus brevicollis* and *Apion* sp., seeds manipulated by the ant *Atta laevigata*, and seeds left intact were put to germinate in controlled environments. To evaluate the effects of abiotic filters, seeds with intact elaiosomes and seeds with elaiosomes removed by the ant *Atta laevigata* were exposed to temperatures of 27, 60, 100, and 200 °C. The results showed that only 2.8% of the seeds attacked by *R. brevicollis* germinated. Seeds attacked by *Apion* sp. germinated faster, followed by seeds with their elaiosomes removed and seeds with intact elaiosomes. Seeds attacked by *Apion* sp. had the lowest germination percentage. The temperature of 200 °C killed seed embryos, whereas seeds exposed to 100 °C took longer to germinate than seeds exposed to other temperatures. Our results reveal that fire intensity and seed damage are important drivers of seed germination of *C. oblongifolia*.

Keywords: *Copaifera oblongifolia*; environmental filters; fire prone ecosystems; seed dormancy; seed predation

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1. Introduction

Biotic and abiotic environmental filters are able to regulate the recruitment of different plant species and shape the organization of natural communities [1–4]. However, some seed traits allow these environmental filters to be overcome, giving them a high capacity to colonize and be established in specific habitats [5,6]. Identifying the mechanisms that regulate seedling recruitment in different habitats is key to understanding the patterns of distribution and abundance of plant species and establish strategies for the conservation and management of terrestrial ecosystems [7]. This is particularly important in an anthropized world characterized by global and accelerated changes in abiotic factors such as temperature and fire regimes, with serious impacts on plant and animal communities [8].

One of the main biotic filters that determine the seedling recruitment success of many plant species in natural communities is seed consumption by different organisms [9–13]. The relationship between herbivores and seeds can range from negative [14–16] to neutral [13,17] to positive [18,19]. Some insects lay their eggs directly on fruits and the development of immature individuals take place inside the seeds [20]. These insects, which feed on the endosperm and embryo (often causing their death), act as true predators [12,21].

In other cases, insects partially consume the seeds without causing direct damage to the embryo, producing cracks in the tegument that can facilitate the imbibition and accelerate seed germination [13,19]. On the other hand, endosperm consumption can affect the availability of resources for the seed embryo and negatively affect germination and seedling establishment [22,23]. Finally, some organisms can also feed on the external seed structures without causing apparent damage to the embryo [24–26].

In fact, many plant species produce seeds with an appendage (elaiosome) rich in nutrients such as lipids, carbohydrates, proteins, and vitamins [27,28] that are attractive food resources for many animal species, including ants, birds, and small primates [12,24]. For example, it has been reported that the dispersion of seeds and fruits in more than 3000 plant species belonging to 80 families of plants is made by ants [29]. In this mutualistic relationship, the elaiosomes are key structures as ants collect seeds to use elaiosomes as a food resource [24,28]. Additionally, the dispersion of these propagules to distant habitats from the parental plants can promote predation-free spaces, favoring the recruitment of new seedlings [25,27,30,31]. Furthermore, some studies suggest that the manipulation of seeds by ants during elaiosome removal can cause cracks in the seed coat, which facilitate imbibition and accelerate germination [32–34]. Thus, myrmecochory (i.e., seed dispersal by ants) can positively affect seed germination, seedling survival, and plant community dynamics [35,36].

Many plant species of fire-prone ecosystems produce seeds that are adapted to resist high temperatures as a result of occasional fires [37–40]. Previous studies suggest that high temperatures can break seed dormancy, favoring the germination of fire-adapted species [37,41–44]. However, seed responses to different temperatures can vary within and between plant species [42,44,45]. For example, in *Mimosa leiocephala*, seed germination was positively related with the increase of temperature, whereas germination rates and seed viability of *Mimosa pteridifolia* and *Harpalyce* sp. were negatively associated to high temperatures [44]. Because the intensity and frequency of fires depends on the levels of drought and temperature in a particular habitat [46], it is possible to expect important consequences for the plant communities prone or adapted to fire. Therefore, understanding the effects of fire on expected future climate change scenarios is crucial to assess how species will respond to altered climatic conditions, as well as changes in the availability of mutualistic and antagonistic interacting species.

The Cerrado is the second largest biome in Brazil and represents one of the world's biodiversity hotspots [47]. This biome harbors about 13,400 plant species whose phenology is related to local climatic seasonality and soil quality [39,48,49]. The vegetation of the Cerrado has its own characteristics, which probably arose in response to local stressors, such as fire and herbivory [39,50]. As such, many plants in the Brazilian Cerrado produce seeds with adaptations to resist high temperatures resulting from wild fires [40] or seeds with nourishing appendages that favor dispersal and recruitment [51,52]. Furthermore, leaf cutting ants belonging to *Atta* and *Acromyrmex* genera are the principal defoliating agents of Cerrado vegetation [53]. These ant species can also collect and transport seeds of myrmecocoric plants to their nests, impacting seed germination of many Cerrado plants [54,55] and influencing ecosystem services.

Copaifera oblongifolia Mart. ex Hayne (Fabaceae: Caesalpinioideae) is a shrub species that occurs in the central region of the Brazilian Cerrado. The seeds of *C. oblongifolia* have a hard coat and are partially covered by a fleshy, lipid-rich elaiosome [56]. The shrubs occur in dense patches and often invade pastures and cultivated areas, becoming dominant and negatively affecting the productivity of agricultural systems [57]. Therefore, characterizing the role of local environmental filters in the variation of seed germination success of this plant species is essential to understand its population dynamics and its invasiveness potential. In this study, we evaluated the effects of both biotic and abiotic environmental filters on seed germination of *C. oblongifolia.* The specific questions addressed were: (i) is seed germination of *C. oblongifolia* affected by the variation of the different insect damage levels? and (ii) how does seed germination of *C. oblongifolia* respond to thermal variation?

2. Materials and Methods

2.1. Study System

Copaifera oblongifolia popularly known in Brazil as pau d'olinho, is a shrub growing up to 2.5 m [56]. It is endemic to the Cerrado areas of central Brazil, occurring in open areas and disturbed environments such as abandoned pastures, roadsides, and edges of Cerrado fragments. Flowering occurs from February to May and fruits ripen from August to October. When the fruit is opened, an ellipsoid, black, and shiny seed, partially covered by a yellow–orange fat-rich elaiosome, is exposed. This propagule represents a food resource for birds, small marmosets, and different groups of insects [57,58].

Among insects, *Rhinochenus brevicollis* Chevrolat (Curculionidae: Cryptorhynchinae) represents the main insect predator of *C. oblongifolia* seeds. Females lay their eggs directly on the fruit in initial stages of seed formation and the larvae develop inside the seeds, feeding on the cotyledons and often causing damage to the embryo [12]. Different species of *Apion* (Brentidae: Apioninae) also attack the seeds of *C. oblongifolia*. This group of insects feeds upon the outermost parts of the seeds, such as the seed coat and the surface of the cotyledons [59,60]. Ants of the genus *Atta* also collect these seeds and use their elaiosomes to cultivate fungi inside their nests [61]. This ant species removes seeds from plants and transports them to the nest. Once the elaiosome is removed, the ants discard the seeds without elaiosomes onto the loose soil around the anthill [62].

2.2. Obtaining Seeds

Seeds were collected from a population of *C. oblongifolia* located in an abandoned pasture (−17.215, −44.414 UTM) in a rural area of the municipality of Jequitaí, northern Minas Gerais State, Brazil. This region has a semi-arid climate with well-defined dry and rainy seasons. The average annual temperature is 23 °C, and precipitation is around 1000 mm/year [63]. The region is located in a transition zone between the Cerrado and Caatinga biomes, evidencing a mosaic of phytophysiognomies such as campo rupestre, cerrado *sensu stricto*, semi-deciduous seasonal forests, and riparian forests [64].

Prior to the dispersion of fruits (August 2018), 40 reproductive-stage individuals of *C. oblongifolia* were identified and tagged in the field. These individuals were periodically monitored to ensure the seed collection only from the open fruits or in the process of opening. We collected at least 30 fruits with seeds for each plant sampled for the two germination experiments. All fruits collected were taken to the laboratory and stored individually in test tubes for a period of 30 days for the emergence of adult insects, which allowed the identification of predators and the type of damage caused to the seeds. The seeds were subsequently grouped into three classes of damage: (i) healthy seeds (intact seeds with no evidence of damage), (ii) seeds attacked by *R. brevicollis*, and (iii) seeds attacked by *Apion* sp. We evaluated a total of 70 seeds predated by *R. brevicollis*, 70 seeds attacked by *Apion* sp., and 350 intact seeds. In addition to collecting fruits from the plants, six nests of *Atta laevigata* Smith. (Hymenoptera: Formicidae) were identified in the study area to collect seeds manipulated by individuals of this ant species. We collected 350 non-elaiosome seeds (about 60 seeds per *A. laevigata* nest) located on the soil mounds of the six *A. laevigata* nests to be used in two germination bioassays.

2.3. Bioassay 1: Effects of Predation on Seed Germination

To evaluate the effects of different levels of seed predation by different insects (Figure 1) on the germination of *C. oblongifolia* seeds, 70 seeds from each of the four treatments (seeds without predation with intact elaiosome; seeds manipulated by *A. laevigata* without elaiosome; seeds attacked by *Apion* sp.; and seeds attacked by *R. brevicollis*) were placed to germinate in individual cells of four Styrofoam trays (one tray per treatment). This experimental design assumes that each seed represents a statistically independent experimental unit and allows comparing germination percentages among treatments using the binomial distribution [6,65]. Approximately 3.0 g of sterile vermiculite was used as the germination substrate in each tray cell. The four trays were placed to germinate in a germination

chamber with controlled photoperiod, temperature, and light intensity (12 h/light at 28 °C and 12 h/dark at 28 °C, 47.5 µmol.m.$^{-2}$s^{-1} irradiation). The humidity of the germination substrate was kept constant by the daily addition of 3 mL of distilled water to each germination cell. Seeds were monitored daily to determine germination percentage and the time required for germination. Seeds were considered germinated when they presented primary root protrusion.

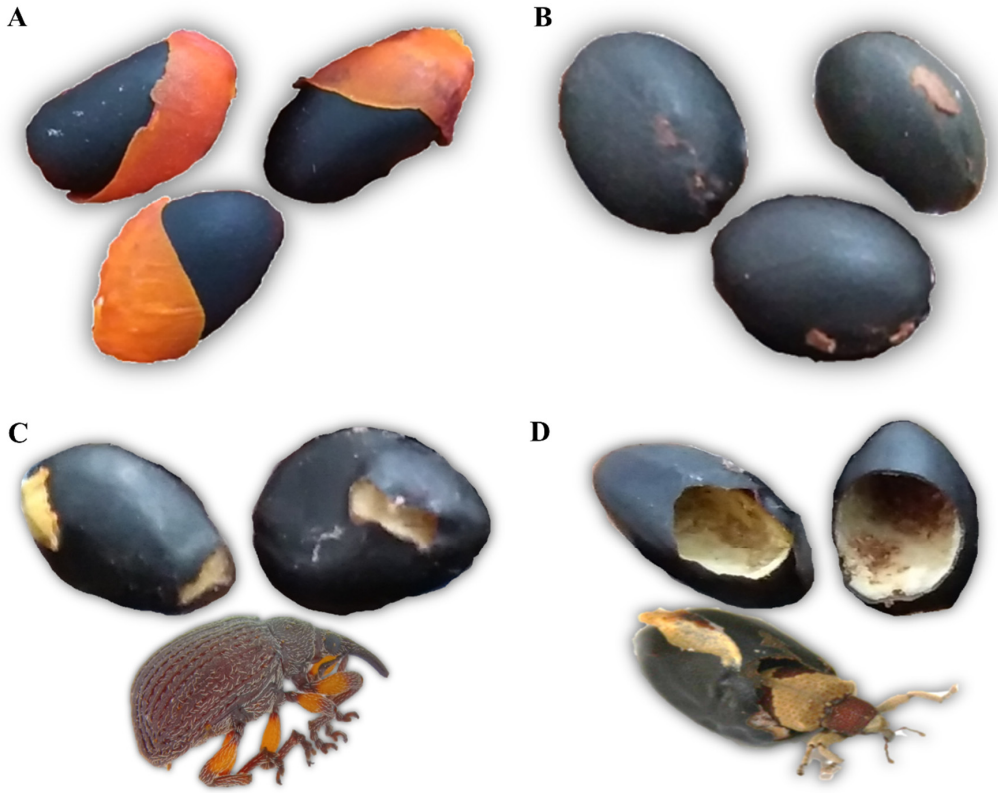

Figure 1. Images of seeds of *C. oblongifolia* showing different predation treatments: (**A**) unmanipulated seeds with elaiosome intact, (**B**) seeds with elaiosome removed by *Atta laevigata*, (**C**) seeds damaged by *Apion* sp., and (**D**) seeds preyed upon by *Rhinochenus brevicollis.*

Generalized linear models (GLM) were used, followed by analysis of variance (ANOVA) to assess whether germination percentage varied among predation treatments. In this model, seed treatments were used as the explanatory variable, and seed germination (0 or 1) was used as the response variable, with a binomial distribution (corrected for quasibinomial). Survival analysis with the Weibull distribution was used to test whether mean germination time differed among seed treatments [66]. In this analysis, seed treatments were used as the explanatory variable and seed germination time as the response variable. Residual analyses were performed to check the suitability of all models. After ANOVA tests, differences among treatments were assessed using contrast analysis, grouping levels that were similar and separating levels that were statistically different. These analyses were performed using R software version 3.5.0 [67].

2.4. Bioassay 2: Effects of Heating and Manipulation by Atta Laevigata on Seed Germination

To evaluate the interactions between abiotic and biotic factors on seed germination, an initial batch of 560 seeds of *C. oblongifolia* was divided into two groups. The first group consisted of 280 intact seeds (elaiosome present) collected directly from the plants. The second group consisted of 280 seeds manipulated by *Atta laevigata* (elaiosome removed) that were collected near the loose soil of *A. laevigata* nests. Each group of seeds was evenly divided into four subgroups of 70 seeds and subjected to four different heat treatments:

(27, 60, 100, and 200 °C) using a heating oven (QUIMIS Q314M) to simulate the effects of seed exposure to different intensities of fire [68]. Thus, the oven was preheated to the desired temperature (27, 60, 100, or 200 °C) and immediately turned off, and 70 seeds from each treatment (seeds manipulated by ants and seeds with elaiosome) were placed inside. The oven door remained open until room temperature was reached, at which point the seeds were removed from the oven. This procedure was repeated for the four heating treatments. After fire simulation, each seed of the eight treatments were placed in individual cells of eight Styrofoam trays and placed to germinate in a B.O.D., as described in the previous bioassay.

GLMs, followed by ANOVA tests were used to assess whether germination percentage varied between heating and seed manipulation treatments. In this model, heating, seed manipulation by ants, and the interaction between them were used as explanatory variables and seed germination (0 or 1) as the response variable, with the binomial distribution (corrected for quasibinomial). The effects of different temperatures and of seed manipulation by *A. laevigata* on seed germination time were evaluated by survival analysis using the Weibull distribution [66]. In this analysis, treatments (heating and seed manipulation by ants) were used as the explanatory variables, and seed germination time was considered as the response variable. Residual analyses were performed to check the suitability of all models. Contrast analyses were used to group levels that were similar and separate levels that were statistically different when ANOVA indicated significant differences among treatments. These analyses were performed using R software version 3.5.0 [67].

3. Results

3.1. Bioassay 1

Our results showed that the percentage of seed germination of *C. oblongifolia* seeds significantly varied among the different treatments of predation (Table 1). Contrast analysis showed that germination percentage did not vary between non-manipulated seeds (with elaiosome) and seeds manipulated by ants. However, seeds attacked by *Apion* sp. had a lower germination percentage than intact seeds or seeds manipulated by ants. Only two seeds attacked by *R. brevicollis* germinated (Figure 2).

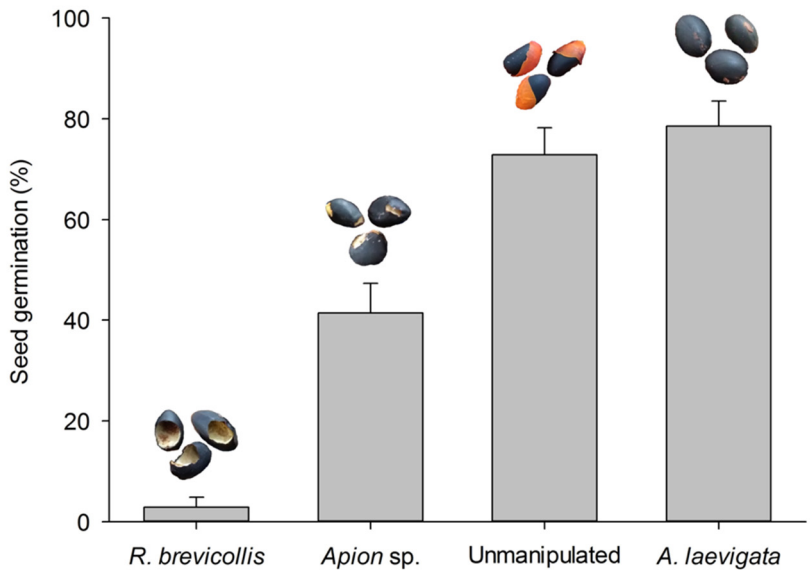

Figure 2. Variation in the percentage of seed germination for *C. oblongifolia* seeds in relation to different types of predation. Seeds preyed upon by *Rhinochenus brevicollis* had the lowest germination percentage followed by seeds attacked by *Apion* sp., non-manipulated seeds, and seeds with elaiosomes removed by *Atta laevigata*.

Table 1. Generalized linear models used to evaluate the effects of different type of predators on germination time and percentage for *C. oblongifolia* seeds. (d.f. = degrees of freedom, Dev = deviance). Values in bold indicate statistically significant values at alfa = 5%.

Response Variable	Explanatory Variable	Error Distribution	D.f.	Residual Deviance	Residual Df	Dev	F	*p*
Time for germination	Predation treatments	Weibull	2	—	131	28.278	—	**<0.001**
Germination percentage	Predation treatments	Quasibinomial	2	249.57	207	24.169	11.912	**<0.001**

The time required for germination of *C. oblongifolia* seeds varied among the different predation treatments (Table 1). Particularly, contrast analysis indicated that seed germination time varied among the three treatments analyzed, with seeds attacked by *Apion* sp., ants and unmanipulated seeds requiring, respectively, 11.2 ± 0.5, 16.9 ± 0.8, and 22.3 ± 0.9 days for germination of 50% of seeds (Figure 3). Finally, it is important to emphasize that only two seeds (2.8%) attacked by *R. brevicollis* germinated, and this treatment was not included in the survival analyses.

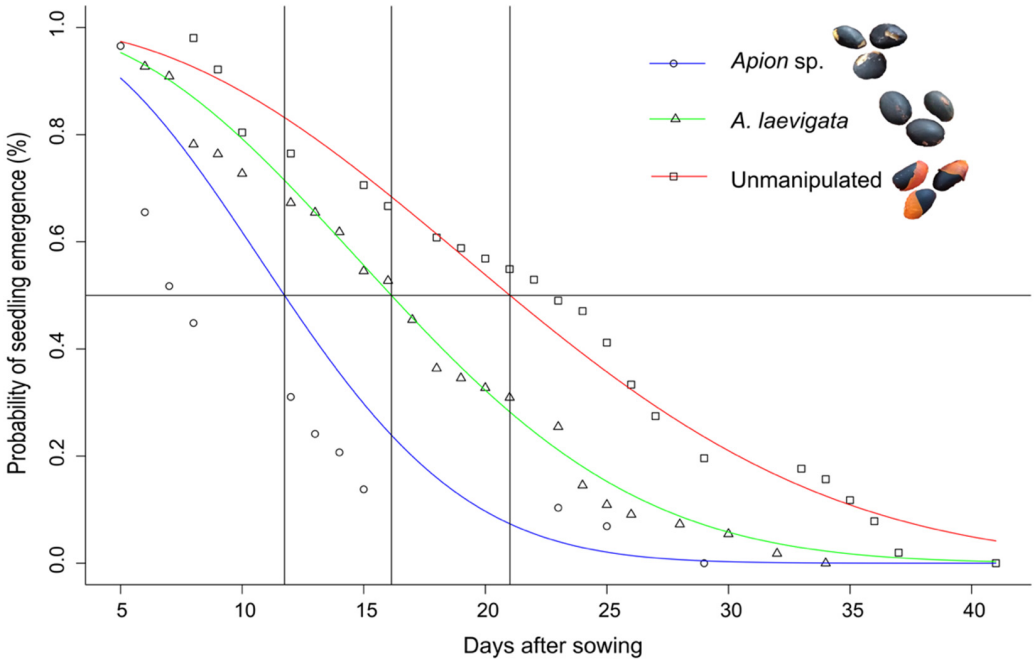

Figure 3. Variation in the probability of germination for *C. oblongifolia* seeds in relation to germination percentage for seeds in each treatment. The intersection of vertical lines with the horizontal line indicates the probability of 50% germination for seeds of each treatment (in days).

3.2. Bioassay 2

Initially, it should be emphasized that no seed of the 200 °C treatment germinated, and this treatment was excluded from statistical analysis. The seed germination percentage of *C. oblongifolia* did not vary as a function of temperature (i.e., temperatures of 27, 60, and 100 °C), seed manipulation by ants, and their interaction (Table 2, Figure 4).

The time required for the germination of *C. oblongifolia* seeds varied statistically among treatments involving manipulation by ants, temperature, and their interaction (Table 2). Contrast analysis showed that seeds subjected to the 100 °C treatment had a slower germination speed than the seeds subjected to the 27 and 60 °C treatments. Seeds manipulated by ants required less time to germinate than non-manipulated seeds. However, a statistically significant interaction showed that the effect of seed manipulation by ants was evident only at the temperature of 27 °C, when seed manipulation reduced germination time by 5.3 days compared to non-manipulated seeds (Figure 5).

Table 2. Generalized linear models used to evaluate the effects of predation (seeds that had elaiosome removed by ant manipulation or seeds with an intact elaiosome) and temperature treatments on germination time and percentage for *C. oblongifolia* seeds. (d.f. = degrees of freedom, Dev = deviance).

Response Variable	Explanatory Variable	Error Distribution	D.f.	Residual Deviance	Resid. Df	Dev	F	*p*
Time for germination	Elaiosome	Weibull	2	—	293	4.135	—	**<0.001**
	Temperature		1	—	294	24.372	—	**0.0419**
	Elaiosome * temperature		2	—	291	6.987	—	**0.0303**
Germination percentage	Elaiosome	Quasibinomial	1	506.12	418	0.04621	0.0456	0.8311
	Temperature		2	503.17	416	2.95142	1.4546	0.2347
	Elaiosome * temperature		2	501.99	414	1.17516	0.5792	0.5608

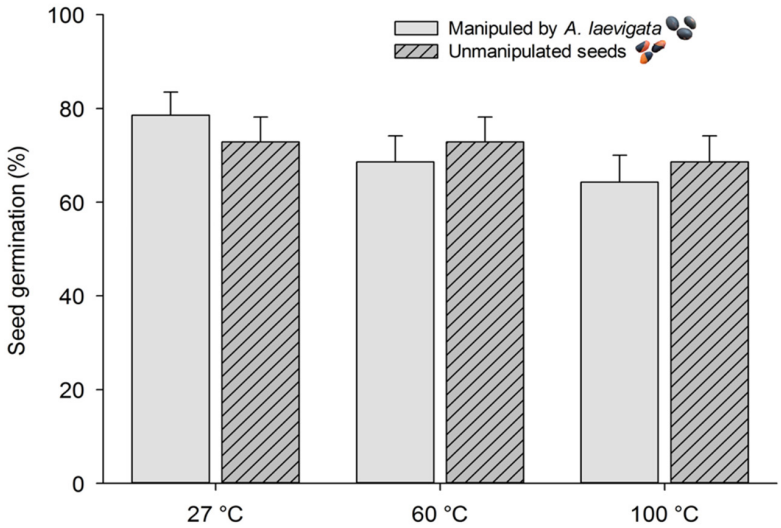

Figure 4. Germination percentage for *C. oblongifolia* seeds in relation to temperature and manipulation by *Atta laevigata*.

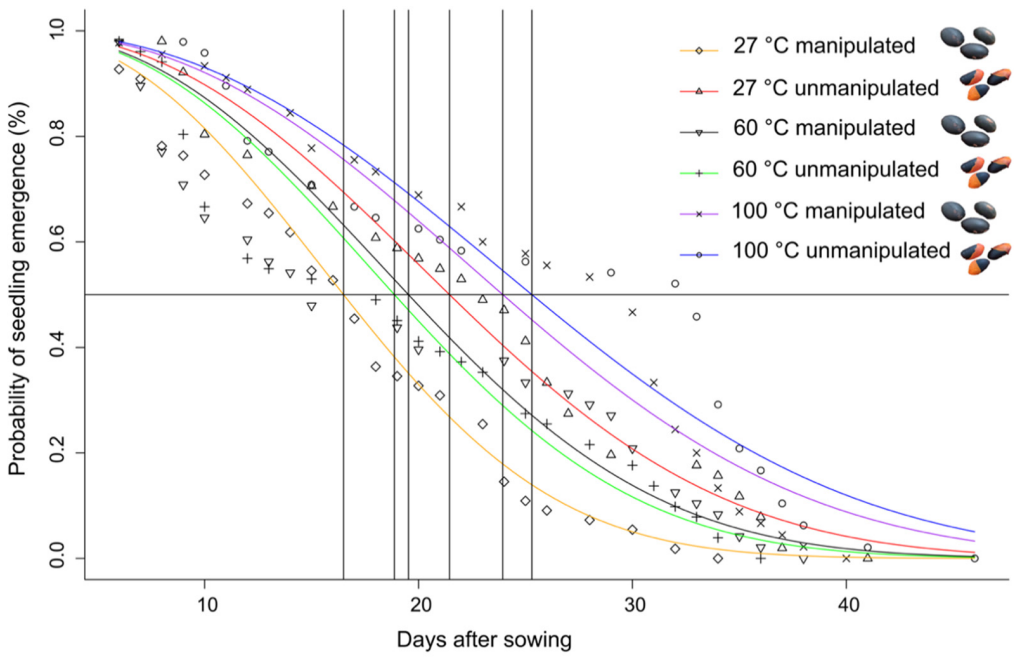

Figure 5. Variation in germination time for *C. oblongifolia* seeds as a function of manipulation by *Atta laevigata* and temperature. The intersection of vertical lines with the horizontal line indicates the probability of 50% germination for seeds of each treatment (in days).

4. Discussion

The results of the bioassay of insect damage to seeds showed that seed predation by *R. brevicollis* drastically reduced the probability of germination of *C. oblongifolia* seeds. Females of these beetles lay their eggs inside the newly formed seeds where the immature insects complete their development, feeding on reserves and the embryo [12]. Seed predation by this beetle can reach 30% for seeds produced by *C. langsdorffii* [10]. If this same pattern occurs with *C. oblongifolia*, *R. brevicollis* can be responsible for the death of a high proportion of seeds produced by *C. oblongifolia* under natural conditions.

Seeds attacked by *Apion* sp. had a significant and strong reduction in germination compared to seeds of the control group. These beetles are external predators that feed on only part of the seeds, and, in many cases, do not cause death to the embryo. Despite the negative effect on germination percentage, seeds attacked by *Apion* sp. exhibited higher germination speed than seeds of the control treatment. This difference is probably related to cracks that these beetles causes in the seed pods, which increase imbibition speed [13]. Rapid germination is an efficient strategy for colonizing less competitive habitats, such as those in the early stages of succession [69]. In this scenario, seeds attacked by *Apion* sp. that still retain their elaiosomes could be expected to be dispersed by other animals and obtain some germination success away from the mother plants.

Our results showed that seeds manipulated by ants had a higher germination speed than non-manipulated seeds with elaiosomes intact. When ants transport seeds and remove the elaiosome, they cause wear to the integument, which promotes greater water absorption and accelerates germination [33,70]. Increased water absorption capacity is especially important under natural conditions because it allows a better use of a resource that is often scarce in seasonal environments such as the Brazilian Cerrado. Decreased germination time can also limit the amount of time seeds are in contact with pathogenic microorganisms in the soil, thereby increasing their germination probability. Therefore, seed transport and manipulation by ants may positively affect the dispersal and colonization of new habitats by *C. oblongifolia*. However, the directional dispersion performed by *A. laevigata* (i.e., from the plant towards the ant hill) could help to explain the clustered distribution of *C. oblongifolia* often observed in the field.

The germination percentage for seeds manipulated by the ant *A. laevigata* did not differ from that for non-manipulated seeds (with elaiosomes). Previous studies have shown that seed manipulation by ants can positively [35,36,71] or negatively [55] affect the probability of seed germination. Different mechanisms have been suggested to explain this variation. For instance, ants can release antibiotic substances during seed manipulation that protect the seeds against microorganism infection, thereby increasing the germination percentage [71]. On the other hand, removal of the elaiosomes by ants can expose the seed micropyle, which represents a window for microorganisms to enter the interior of the seed and cause its death [55,72]. Our experiment was conducted under aseptic conditions, and no fungal growth was observed in the seeds. Thus, variation in germination related to the action of microorganisms between treatments did not interfere with the germination results of *C. oblongifolia*. Furthermore, it is also important to emphasize that although some studies suggest elaiosomes can produce substances that inhibit seed germination, our results do not support the evidence that these structures can act as a germination inhibitor of *C. oblongifolia* seeds.

The results of the heating bioassay showed that seeds of *C. oblongifolia* did not resist high temperatures, as no seed submitted to the highest temperature of 200 °C germinated. Generally, fire in the Cerrado is characterized by high frequency (occurring every 3–4 years), superficial occurrence, and low intensity, with rapid consumption of the dry biomass of the subshrub stratum [68,73]. Soil surface temperatures during fires in the Cerrado range from 74 to 768 °C [74], but temperatures of the soil layers immediately below the surface do not exceed 80 °C [68]. Thus, considering that the germinability of *C. oblongifolia* did not vary among temperatures from 27 to 100 °C, and that this species is restricted to the Cerrado, it is reasonable to consider that seeds of *C. oblongifolia* have resistance to moderate temperatures.

These results also indicate that fire does not directly stimulate the germination of these seeds. However, it has been observed that gases released during fires, such as ethylene and ammonia, can stimulate the germination of species of fire-prone ecosystems [41,75–77]. Thus, further studies testing this hypothesis of the indirect effects of fire on the germination of *C. oblongifolia* are still needed.

Moderately high temperatures (100 °C) slowed the germination rate. Seeds exposed to this temperature are likely to have a reduction in moisture content. Reduced moisture inhibits the production of gibberellin [78], which is important in the process of breaking seed dormancy [79,80]. Furthermore, we observed that, despite heating, seeds manipulated by ants had a higher germination speed. Thus, seeds manipulated by ants, even when subjected to fire, must absorb water more quickly and have a higher germination speed. Elaiosomes are lipid-rich appendices that partially involve the seed aril [55] and, therefore, could act as a thermal insulator, protecting the embryo from thermal oscillations. However, seeds manipulated and not manipulated by ants differed in the time for germination only at the temperature of 27 °C. These results, associated to fact that germination percentage was not affected by moderate temperatures, suggest that the elaiosome did not protect seeds of *C. oblongifolia* against thermal fluctuations. Thus, the resistance of the *C. oblongifolia* seeds to fire would be mainly associated with the hardness of the seed tegument.

Finally, our results show evidence that ants do not influence seed germinability, whereas beetle damage has serious negative consequence on seed germination of *C. oblongifolia*. We also highlight that *C. oblongifolia* seeds have a high tolerance to moderate temperatures typical of Cerrado environment. Leaf cutting ants and low intensity fire are two important biotic and abiotic filters capable of driving community organization in Cerrado areas, and we suggest that *C. oblongifolia* seed is adapted to overcome these environmental filters. Thus, these seed traits should be considered in future management strategies of this plant species in Cerrado areas.

Author Contributions: M.F. and P.C.-R. designed the study; H.T.d.S. collected data and performed experiments; and all authors contributed equally to data analysis, manuscript preparation, and editing. T.C. is the corresponding author. All authors have read and agreed to the published version of the manuscript.

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