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Occurrence of *Amblycerus* species in *Cordia trichotoma* seeds and their influence on germination

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ABSTRACT

Forest species can have their seeds damaged by granivorous insects, especially by those in their larval stage. In this context, this study aims to report the occurrence of *Amblycerus* species in *Cordia trichotoma* seeds, to describe their main damage to seeds and effects on germination, as well as their associated hymenopteran parasitoids. Therefore, seven trees were selected in the municipality of Taquaruçu do Sul, RS, Brazil. Fruits were collected weekly from the medium third of the tree crown, from the beginning of their formation until total dehiscence. To examine the damage caused by granivorous insects within the fruits, 15 fruits from each tree were sectioned with a scalpel. Furthermore, 10 fruits from each tree were stored individually in clear plates to verify the occurrence and identification of granivorous insect species. Evidence of the damage caused to seeds was verified through the germination test by comparing pre-served and damaged seeds, with four repetitions of 25 seeds each. The species *Amblycerus longesuturalis* and *Amblycerus profaupar* (Chrysomelidae: Bruchinae) were found associated with fruits of *C. trichotoma*. Female insects predominantly laid eggs on the superior part between the marcescent calyx and the fruit, and larvae perforated the fruit tegument to start consuming seed embryos and reserves. Bruquine larvae are parasitized by Hymenoptera of *Bracon*, *Mirax*, *Omeganastatus* and *Triapsis* genera. In conclusion, the germination of *C. trichotoma* seeds is significantly affected by emergence orifices caused by granivorous species.

Introduction

Cordia trichotoma (Vell.) Arrab. ex Steud. (Boraginaceae), is considered a priority species for forest plantings, mainly due to its economic viability, high volumetric growth, quality and multifunctionality of wood, as well as its potential in environmental recovery strategies (Grings and Brack, 2011). These deciduous trees can reach 35 m (Reitz et al., 1988) and 90 cm diameter at breast height in their adult stage (Lorenzi, 1992). This species occurs in secondary vegetation (Carvalho, 2002), being one of the only arboreal species that flourishes during autumn (Grings and Brack, 2011) in South America, and standing out as an apiculture tree species.

The natural occurrence of this species extends from northeastern to southern Brazil, eastern Paraguay, northern Argentina and southern Bolivia (Reitz et al., 1988; Sobral et al., 2006). In Rio Grande do Sul (Brazil), *C. trichotoma* is commonly found in Alto Uruguay forests, open forests and plateau grassland mounds, especially in the Central Depression (Reitz et al., 1988).

The species presents small white hermaphrodite flowers, with paniculate, cymose inflorescences. Fruits are dry, indehiscent, monocarpic, brownish, and fully enclosed by the tube of the flower corolla and calyx. The seed is attached to the fruit wall at the base of the stigma, dispersing long distances due to the calyx and floral corolla marcescentes (Felippi et al., 2012), which surround the ripe fruit and collaborate with the anemochoric dispersal (Souza et al., 2006). Internally, the seed is exalbuminous, with a cotyledonary, basal, invaginated embryo, with short and thick plicate cotyledons of white coloration, attached around the hypocotyl root axis (Felippi et al., 2012).

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Cordia trichotoma provides resources to entomofauna and its flowers are visited by bees, as well as several other small insects that help pollinize the species (Carvalho, 2003). However, animal-plant interactions are harmful when the internal seed content is consumed either by birds, such as *Leptotila verreauxi* (Bonaparte, 1855) (Pereira et al., 1995), or larvae of granivorous insects from the Chrysomelidae (Bruchinae) (L'argentier, 1983) family.

The subfamily Bruchinae Latreille currently comprises 70 genera distributed worldwide, with *Amblycerus* Thunberg being one of the most diverse genera in the Western Hemisphere, comprising more than 120 species. In Brazil, there are 61 species described for *Amblycerus*, which consume seeds of at least 13 families of host plants, among them Boraginaceae. For *C. trichotoma*, there have been records of parasitism *Amblycerus longesuturalis* (Pic, 1954) with distribution reported for Brazil (Rio Grande do Sul) and Argentina, *Amblycerus profaupar* Ribeiro-Costa, 2000, with limited distribution in the states of Mato Grosso, São Paulo, Paraná and Rio Grande do Sul, Brazil and *Amblycerus unimaculatus* (Pic, 1955) in São Paulo (Ribeiro-Costa et al., 2018).

The species of *Amblycerus* are important since, among seed beetles, they present the highest number of host plants. None of the host plants are considered major agricultural crops, but when their seeds are destroyed by bruchids, their potential for regeneration is reduced (Johnson et al., 2001).

When consuming seed reserves and embryos, the larvae of seed beetles obtain the energy needed for several activities, such as flying and reproduction when they reach adult stage (Ribeiro-Costa and Almeida, 2009). However, seed consumption by larvae can cause varied damage to embryos, hindering their germination process (Duarte et al., 2014).

In addition to insects, fungi may also influence the germination percentage of *C. trichotoma* seeds, and certain measures in fruit processing could reduce the incidence of pathogens. According to Berghetti et al. (2015), removing the entire calyx and floral corolla marcescentes of the fruit increases the germination percentage of seeds, compared to fruits in which only petals are removed, associated with reducing the external seed material.

Thus, the aims of this study were to report the occurrence of *Amblycerus* species in *C. trichotoma* seeds to describe the main damage caused to seeds and their effects on germination, as well as to describe the associated hymenopteran parasitoids.

Material and methods

Fruits were collected in the municipality of Taquaruçu do Sul (27°23′48″S and 53°29′55″W), located in the Alto Uruguay region in Rio Grande do Sul, Brazil. The main vegetation cover in this region is Deciduous Seasonal Forest, due to leaf fall periods with restrictive climate factors, specifically in winter with intense cold, which can affect more than 50% of forest species from the upper emerging canopy (IBGE, 2012). The climate in the region is classified as humid subtropical with hot summers (Cfa) and well-distributed precipitation throughout the year (Alvares et al., 2013).

Seven visually healthy trees with well-formed crowns were selected in forest fragments that varied between 0.5 and 5 ha. Collections of *C. trichotoma* fruits were carried out weekly between April and July (fruiting period) for two consecutive years (2015 and 2016), totaling 14 weeks of collection in each study year. For laboratory tests, approximately 30 fruits were randomly collected from the crown of each tree using a trimmer.

The collections were carried out by tree; thus, in order to differentiate them in this text they were denominated by the letters A, B, C, D, E, F and G. In the year 2015, 10, 13, 14, 14, 6, 8 and 13 collections were performed from trees A, B, C, D, E, F and G,

respectively. In 2016, zero (A), 12 (B), 13 (C), 13 (D), 14 (E), 14 (F) and 13 (G) collections were carried out.

Ten fruits per tree/per collection/per year were randomized and stored in clear plates (8.0 × 12 × 1.5 cm) containing 24 cells with 16 mm diameter each. Filter paper was cut with a perforator and sterilized in a Pasteur oven for one hour at 150 °C to prevent fungi proliferation. In total, 1570 fruits were stored, 780 in 2015 and 790 in 2016.

During the assessment period, containers were kept at room temperature (mean temperature = 18 °C and relative humidity = 80.5%) in the laboratory. To avoid excessive desiccation of fruits, 0.0015 mL of distilled water were applied weekly to filter paper with micro pipetting equipment, according to the methodology adapted by Dorneles (2014). The emergence of adult insects was monitored weekly for ∼ three months. Emerged adult insects were removed from the plates and kept in *eppendorf* microtubes containing alcohol 70% for subsequent quantification, assembly and identification by specialists.

Additionally, 15 fruits of each tree/collection/year were sectioned for observation of damage caused by insects. Fruits were sectioned using a scalpel. Subsequently, they were observed under a 4.2× binocular microscope, for better visualization of seed damage and for evidence of insect presence, *i.e.* egg-laying. Larval parasitism was recorded during fruit sectioning, verifying cephalic capsules and remaining seed beetle larval with the larvae of hymenopteran parasitoids. 1170 fruits were sectioned in 2015, and 1185 in 2016, totaling 2355 sectioned fruits.

The number of stored and sectioned fruits among the years varied due to differences in fruit maturation of the trees selected in this study.

Insects of the order Coleoptera, family Chrysomelidae, subfamily Bruchinae, were identified by Professor Dr. Cibele S. Ribeiro-Costa, at the Universidade Federal do Paraná (UFPR) and received identification registry n° 75. Hymenopteran parasitoids were identified by the fourth author at the Instituto Nacional de Pesquisas da Amazônia (INPA).

For the germination test, ripe fruits were collected from five trees on July 3rd, 2016. To verify whether the damage caused by granivorous insects leads to decreased germination, fruits were separated into three treatments: T1 – without traces of insects, being preserved without the marcescent calyx; T2 – without traces of insects, being preserved with the marcescent calyx; T3 – seeds with orifice caused by seed beetle emergence.

The germination test was performed with four repetitions of 25 seeds, inside a plastic box (*gerbox*) that was previously disinfected with a 1% sodium hypochlorite solution and alcohol 70%. The asepsis of seeds was performed by immersing them in 1% sodium hypochlorite for 30 s and in alcohol 70% for 30 s, followed by two washes with distilled water. Sand was used as substrate (fine and sieved in a net of 0.84 mm), which was sterilized for 4 h in a Pasteur oven at 170 °C.

The "in sand" procedure was used where 200 g of sand were placed at the bottom of a plastic box and 100 g were used to cover the seeds, which was then wet with 45 mL of distilled and autoclaved water. To calculate the amount of water to be added to the substrate, the methodology proposed by Brasil (2009, 2013) was adopted.

Throughout the assessments, the sand was wet with distilled and autoclaved water whenever necessary. Samples were put in a BOD (Biological Oxygen Demand) incubator at 25 °C and a photoperiod of 12 h under white light to promote seed germination. Test installation occurred one day after fruit collection and lasted for 44 days.

Germination data (G%) was submitted to the Kolmogorov–Smirnov normality test and Bartlett's homogeneity test, and when these presumptions were not met, data was

transformed into arcsine $\sqrt{x/100}$ with the statistical supplement Action installed in Microsoft Office Excel® 2007. Afterwards, data was submitted to analysis of variance and means were compared by Tukey's test at a 5% error rate, with the Sisvar software (Ferreira, 2008), and zero-value treatment was not included in the analysis.

Results and discussion

The species of granivorous insects that emerged from *C. trichotoma* fruits were *Amblycerus longesuturalis* (Pic, 1954) and *Amblycerus profaupar* Ribeiro-Costa, 2000 (Chrysomelidae, Bruchinae). Of the total number of emerged seed beetles, 156 specimens belonged to *A. profaupar* (75%) and 52 to *A. longesuturalis* (25%).

In Rio Grande do Sul, insects from the same genus have already been reported damaging *C. trichotoma* seeds, including *Amblycerus maculicollis* Ribeiro-Costa, 2000 and *A. profaupar* (Ribeiro-Costa,

2000). In the present study, we expanded the occurrence of *A. longesuturalis* to Rio Grande do Sul (Ribeiro-Costa et al., 2018).

Amblycerus longesuturalis has already been reported in Argentina, where egg laying was visualized in *C. trichotoma* fruits, whose larval stages feed on the embryo (L'argentier, 1983) and can severely damage fruit, interfering with *in vitro* germination (Duarte et al., 2014).

By analyzing the longitudinally sectioned fruits throughout the two consecutive study years, there were 1354 (57.5%) fruits with indications of insect presence. It was possible to verify that seed beetle eggs were predominantly found attached to the superior part of seeds, between the marcescent calyx and the fruit (Fig. 1). Eggs were also found on the marcescent petals, mainly on the abaxial part and reproductive parts of the flower such as the stigma. Thus, females did not lay eggs endophytic in the seed, like in some *Acanthoscelides* (Skaife, 1926; Larson and Fisher, 1938). One to four eggs were verified per fruit in 2015, and one to nine eggs were visualized per fruit in 2016.
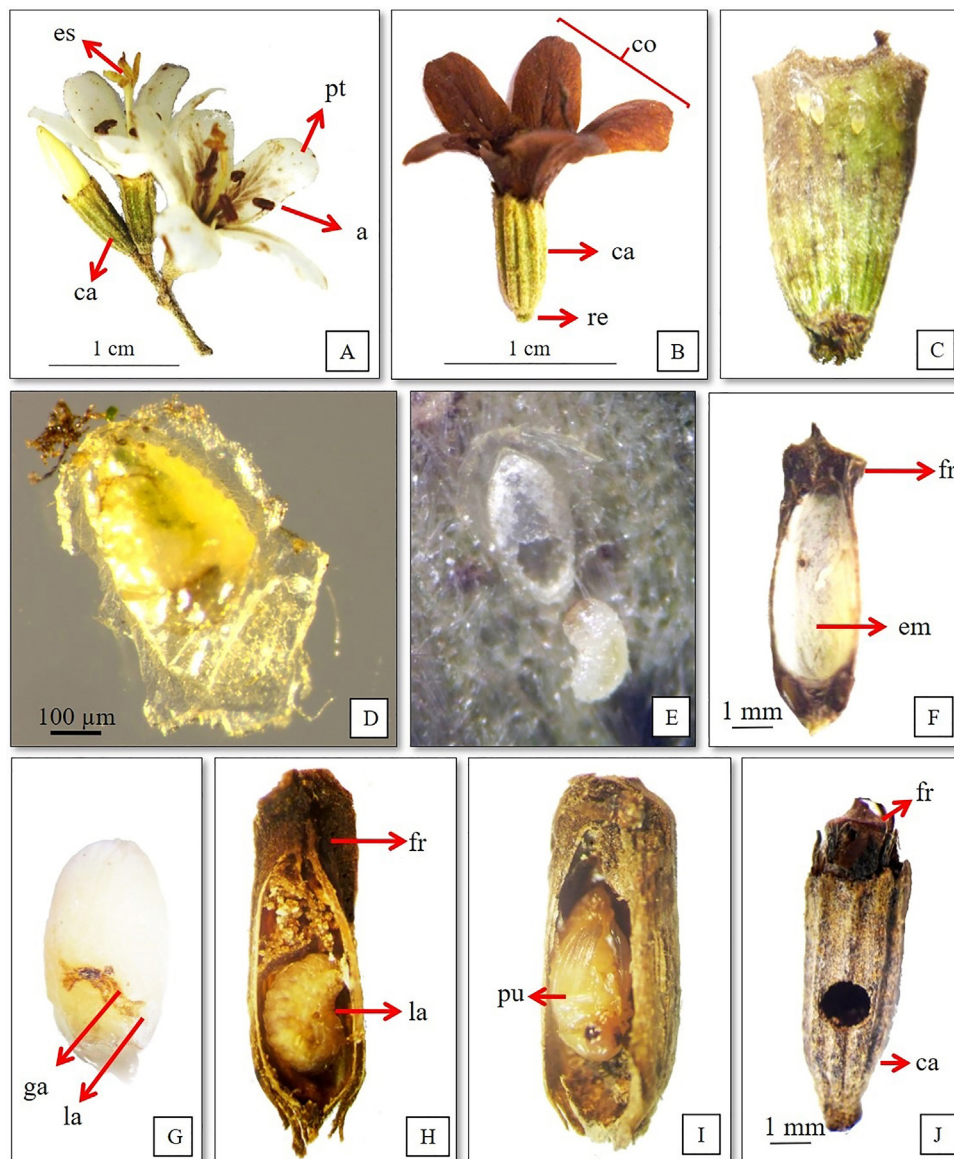

Fig. 1. A: Floral bud and open flowers of *Cordia trichotoma*. B: Ripe fruit with marcescent calyx and corolla. C: Eggs attached under the fruit calyx. D: Detail of the seed beetle egg. E: Outbroken larva of first instar and egg exuviae under the fruit calyx. F: Fruit with developed embryo and detail of the orifice in the embryo made by the first instar larva. G: Detail of the gallery made by the seed beetle larva in the embryo. H: Absence of embryo (totally consumed by larva) with only the fruit tegument remaining. I: Pupa of seed beetle inside the fruit. J: Detail of adult emergence orifice. (a: anther; ca: calyx; co: corolla; em: embryo; es: stigma; fr: fruit; ga: gallery; la: larva; pt: petals; pu: pupa; re: receptacle).

Nevertheless, seed beetles have several oviposition behaviors that demonstrate different ways of overlapping barriers imposed by host plants, or even strategies to prevent nonviability of their eggs through the action of natural enemies (Ribeiro-Costa and Almeida, 2009). The visualized eggs were attached to the fruit structures only at the peripheral part (Fig. 1C) and, according to Johnson et al. (2001), this prevents eggs from detaching from the fruit while developing and during first instar larva outbreak, as well as protects eggs against mechanical lesions or abiotic factors such as high insolation and low relative humidity, which cause egg desiccation (Kingsolver, 2004).

It was observed that larvae nutrition occurred exclusively in the seed interior, as they perforated the fruit tegument to start consuming the seed embryo and reserve. Similarly, Ribeiro-Costa and Almeida (2009) emphasize that seed beetle nutrition is different in the larval and adult stages, since adults feed on nectar and pollen, whereas larvae develop by consuming the internal seed contents.

When breaking out of eggs, larvae perforate the seed tegument and penetrate its interior to start consuming the reserve (Fig. 1F), subsequently pupating and emerging in the adult stage. According to Ribeiro-Costa and Almeida (2009), the mandibles of larvae scrape the tegument and endosperm of seeds, generally with four instars in seed beetles, and in the last instar the larva makes a deep demarcation in the internal seed wall and pupates. Afterwards, this demarcation becomes the emergence orifice of the adult with a round shape. The occurrence of orifices in fruits and seeds is referred to by Vanin and Gaiger (2005) as an indication of damage caused by insects.

Throughout the two consecutive study years, only one adult specimen was found in the seeds during fruit sectioning, which suggests that seed beetle development from pupa to adult and subsequent emergence occurs when the fruit has already dispersed from the tree.

Larval parasitism was recorded during fruit sectioning. Such result was similar to that found by Ribeiro-Costa (1992) for Amblyc-erus hoffmanseggi (Gyllenhaal, 1833).

Of the 1570 stored C. trichotoma fruits, there were six hymenopteran parasitoid specimens, identified as Bracon sp.1 Fabricius, 1804; Bracon sp. 2 Fabricius, 1804 (Ichneumonoidea, Braconidae, Braconinae); Triapsis sp. 1 (Ichneumonoidea, Braconidae, Brachistinae); Mirax sp. 1 Fabricius, 1804 (Ichneumonoidea, Braconidae, Miracinae); and Omeganastatus sp. 1 Gibson, 1995 (Chalcidoidea, Eupelmidae, Eupelminae). These results match those found by Ribeiro-Costa and Almeida (2009), who point out the superfamilies Chalcidoidea and Ichneumonoidea as the main seed beetle parasitoids.

Despite the small number of specimens identified, the present study increases the number of hymenopteran parasitoid records for Amblycerus species, as well as the genera. Of the 61 species reported by Ribeiro-Costa et al. (2018), only five Amblycerus species have occurrence records of parasitoid wasps belonging to the families Braconidae, Eulophidae (Horismenus sp.) and Chalcididae (Spilochalcis sp.).

By comparing the seeds with and without damage, it was observed that damaged seeds did not germinate due to total consumption of seed embryo and reserve by seed beetle larvae (Table 1). The low germination rate of seeds with marcescent calyx could have also been caused by the high occurrence of associated fungi (Berghetti et al., 2015) in seeds. Another factor that might have interfered with seed germination was possible egg laying or even larvae of seed beetles in the upper part between the calyx and the fruit (Fig. 1C), which was not observed due to the adhered calyx.

Donato et al. (2010) observed that the germination of Enterolobium contortisiliquum (VELL.) Morong. (Fabaceae) seeds is influenced by damage caused by seed beetles. Loureiro et al. (2004) verified that seeds of Apuleia leiocarpa Vog. Macbride (Fabaceae)

Table 1

Germination of Cordia trichotoma seeds with no indications of insect occurrence and seeds with adult emergence orifice. Taquaruçu do Sul, RS, Brazil, 2016.

Treatment	Germination (%)
Seeds without marcescent calyx	23[a]
Seeds with marcescent calyx	1[b]
Seeds with adult emergence orifice	0

Values with different subscript letters are statistically different according to Tukey's test ($p > 0.05$).

damaged by insects did not germinate, and undamaged fruits do not safely indicate undamaged seeds, as they can actually contain damaged seeds.

In this sense, studies involving germination of C. trichotoma seeds can be performed by removing marcescent calyx and petals, because in addition to decreasing the incidence of fungi (Berghetti et al., 2015), such action can decrease the occurrence of granivorous beetles that prefer to lay eggs in the upper region between the marcescent calyx and the fruit.

Conclusions

Cordia trichotoma seeds damaged by emergence holes of Amblycerus longesuturalis and Amblycerus profaupar present significantly impaired germination.

The procedure of removing the entire calyx and floral corolla marcescent of C. trichotoma fruits for germinative tests constitutes a control tactic.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

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