Botanical Sciences 103 (1): 43-59. 2025
DOI: 10.17129/botsci.3522

Received: April 26, 2024, Accepted: September 4, 2024
On line first: November 27, 2024

Ecology / Ecología

Reproductive biology and propagation of *Magnolia caricifragrans* (Lozano) Govaerts, an endangered species from Colombia

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Abstract:
Background: *M. caricifragrans* is an endangered tree species. Around 50 isolated trees grow in highly altered landscapes in the cloud forests of the states of Cundinamarca and Tolima (Colombia).
Questions: How is the reproductive phenology of *M. caricifragans* in individuals under *ex situ* conditions? Can *M. caricifragrans* produce seeds with the aid of hand pollination? Are seeds issued form hand pollination viable? Who are the flower visitors of *M. carcifragans* under *in situ* conditions? What is the survival rate of the plantlets in the field after reintroduction?
Sites and years of study: Bogotá Botanical Garden "José Celestino Mutis" from 2019 to 2022 and in the municipalities of San Francisco and Silvania.
Methods: We monitored reproductive events one year in *ex situ* conditions, including manual pollination, fruit development, and seed germination. We also captured floral visitors from wild *M. caricifragrans* flowers. Seedlings reintroduced into nature reserves and survival was evaluated.
Results: Trees *ex situ* exhibit constant flower bud and flower production throughout the year and do not produce fruit. Fruits with viable seeds that germinated were obtained through hand-pollination. *In situ* flower visitors consisted mainly of roving beetles; twenty-seven seedlings were successfully reintroduced.
Conclusions: A peak of flower production was determined between September and November, successful fruit development by controlled cross-pollination. Seed germination was achieved using a closed container with a substrate with high moisture retention and good drainage. Trees reintroduced in nature present a healthy development and 100 % survival.
Keywords: Hand-pollination, *Magnolia*, Neotropics, phenology, pollinator.

Resumen
Antecedentes: *M. caricifragrans* es un árbol en peligro de extinción. Se conocen alrededor de 50 árboles aislados en paisajes alterados en bosques nubosos de los departamentos de Cundinamarca y Tolima (Colombia).
Preguntas: ¿Como es la fenología reproductiva de *M. caricifragans* en individuos bajo condiciones *ex situ*? ¿Puede *M. caricifragrans* producir semillas mediante polinización manual? ¿Son viables las semillas obtenidas mediante polinización manual? ¿Quiénes son los visitantes florales de *M. caricifragans* en condiciones *in situ*? ¿Cuál es la tasa de supervivencia de las plántulas reintroducidas?
Sitios y años de estudio: Jardín Botánico de Bogotá José Celestino Mutis del 2019 al 2022 y en los municipios de San Francisco y Silvania.
Métodos: Monitoreamos eventos reproductivos durante un año, incluyendo polinización manual, el desarrollo de frutos y la germinación de semillas. Capturamos visitantes florales de flores silvestres de *M. caricifragrans* y se reintrodujeron plántulas en reservas naturales evaluando la sobrevivencia.
Resultados: Producción annual constante de botones florales y flores, y árboles que no desarrollan frutos bajo condiciones *ex situ*. La polinización manual genero frutos y semillas que germinaron. Los visitantes florales *in situ* fueron principalmente escarabajos errantes. Veintisiete plántulas fueron reintroducidas exitosamente.
Conclusiones: Se determinó un pico de producción de flores entre septiembre y noviembre, un desarrollo exitoso de frutos mediante polinización cruzada controlada. Se logró germinación de las semillas utilizando un recipiente cerrado, sustrato con alta retención de humedad y buen drenaje. Las plántulas reintroducidas se mantuvieron sanas y tuvieron 100 % de supervivencia.
Palabras Clave: Fenología, *Magnolia*, Neotrópico, polinización manual, polinizador.

Magnolia caricifragrans is a woody species with a pyramidal crown and dense dark green foliage that naturally grows in cloud forests between 1,800 and 2,600 m asl. Its specific epithet, *caricifragrans,* was designed because the flowers have a pleasant aroma, like one of some *Carica* species fruits that can be felt at a great distance (Lozano 1983). Currently, it is categorized as an endangered species (EN) according to the IUCN criteria mainly by habitat loss and logging, and it was included in the Red List of Threatened Species (Calderón *et al.* 2016), and also in the Red Book of Plants of Colombia (Calderón *et al.* 2007). *M. caricifragrans* is endemic to Colombia, the second country with the highest diversity of species of *Magnolia* (40 species), most of them considered endangered (Calderón *et al.* 2007, Vázquez-García *et al.* 2017, Aguilar-Cano *et al.* 2018, Rivers *et al.* 2016, Rodríguez-Duque *et al.* 2022). Open flowers all year round, low seed production and scarce recruitment are reported for all the studied Colombian species (López-A *et al.* 2008, Gómez 2011, Toro & Gómez 2011, Serna-González & Urrego-Giraldo 2016, Serna-González *et al.* 2022). The last published fieldwork confirms at least 50 isolated adult trees in its distribution area at Cundinamarca province in the Eastern Andean Cordillera. The noticeable scarcity of juveniles found in nature turns the alarm on its real extinction risk (Samper 2010). The assemblage of pollinators and floral visitors networks of *Magnolia* have involved mainly beetles of the families such as Cantharidae, Cerambycidae, Chrysomelidae, Curculionidae, Elateridae, Melyridae, Mordellidae, Nitidulidae, Scarabaeidae and Staphylinidae, which have been observed mating, hiding, and feeding on pollen and other floral resources (*e.g*., stigmatic exudates). Other strategies that diverse Magnoliidae species offer to attract them are thermogenic flowers and floral scents emission during floral anthesis (Piegler 1988, Wang *et al.* 2014, Ruohan & Zhang 2015). Although traits of Magnoliaceae flowers are mainly adapted to beetle pollination (cantharophily), bees and flies are also recognized as part of their flower visitor networks as effective pollinators (Thien 1974, Thien *et al.* 1998, Sayer *et al.* 2019). Little is known about floral visitors and pollinators of Colombian and neotropical species. However, it is essential to highlight that the role of plant pollination by beetles (Coleoptera) has been majorly underestimated and unquantified (Sayer *et al.* 2019).

Although pollination, propagation, and phenological research have been conducted in other *Magnolia* species (Gardiner 2000, Toro & Gómez 2011, González & Montoya 2014, Calderón *et al.* 2016, Wang *et al.* 2016, Bakhramov *et al.* 2021, Serna-González *et al.* 2022), no advances are reported on *M. caricifragans*. Besides, there is an increasing call for action related to conservation of *Magnolia* species through propagation, translocation and reintroduction (Calderón *et al.* 2007, Oldfield 2009, Monks *et al.* 2012, Sánchez-Velásquez *et al.* 2016), living collections of botanical gardens can make big contributions in this matter mainly by conserving individuals and generating plant material for reintroduction programs (Wang *et al.* 2014, Magdalena 2018). This study evaluates *M. caricifragans* phenology, hand-pollination, sexual propagation (*ex situ*) and its floral visitors in the wild (*in situ*). Also describes the reintroduction of the species in nature reserves. All this knowledge is fundamental for making decisions regarding the conservation of *M. caricifragans*.

Materials and methods

Study area. Floral development, reproductive phenology, hand-pollination, and propagation experiments were performed at Bogota's Botanical Garden (4º 40' 0.5268'' N, 74º 05' 56.0616'' W; 2,570 m asl), which has a bimodal rainfall pattern typical of the intertropical convergence zone (Sarmiento 1986). Three adults of this species are within Bogotá Botanical Garden's living collection, approximately 20, 30 and 40 years old. Two of them were collected in the municipalities of Granada and Silvania, both in Cundinamarca province, 46-49 km from Bogota's Botanical Garden and were established in the living collection. The third one and youngest is from an unknown origin.

As floral visitors and pollinators of *M. caricifragans* were not observed in the Botanical Garden, we visited the following localities in search for them, "Vereda San Raimundo", Granada municipality, Cundinamarca province, Colombia (4° 29' 7.86594" N, 74° 23' 25.99085" W), "Vereda Subia Central", Silvania municipality, Cundinamarca province, Colombia (4° 24' 11.45799" N, 74° 26' 9.05574" W; 4° 24' 9.67684" N, 74° 26' 10.18072" W; 4° 24' 7.28035" N, 74° 26' 15.9249" W; 4° 24' 7.00529" N, 74° 26' 20.10698" W), Nature Reserve "Guaira" (5° 0'9.036" N; 74° 15' 50.646" W) and Nature Reserve "Anami" in San Francisco municipality, Cundinamarca province, Colombia (4° 59’ 46.26” N; 74° 15’ 18.354” W), all with isolated adult trees. The trees located at Granada and Silvania had produced fruits in the past, suggesting the presence of pollinators (Samper 2010), but that was not the case for San Francisco province trees (nature reserves owners, H. Garcia & J. Salazar pers. comm.).

Seedlings obtained from this work were reintroduced with owners of five nature reserves and/or healthy forest in their properties (Nature Reserve “Anami”, 4°59’46.26” N; 74°15’18.354” W, Nature Reserve “Guaira”, “Finca El Descanso” 4° 59’ 7.428” N; 74°16’ 54.588” W, Nature Reserve “Los Mochuelos” 4° 59’ 6.168” N; 74° 16 ’30.858” W, and “Paradiso Perduto” farm 4° 57’ 19.098” N; 74° 19’ 8.382”W).

Reproductive phenology. Monthly direct observations of floral buds and opening flowers of the two biggest adults from the living collections of Bogotá Botanical Garden were performed between November 2019 and December 2020. Observations were always performed after 03:00 pm so that we could include flowers that were opening on that day. Each phenophase was analyzed separately using Oriana - Circular Statistics for Windows (Version 4). Months were converted into angles with intervals of 30º wide to calculate the mean angle (μ) or mean date, referring to the time of the year around which the phenological activity is most concentrated, and vector r, which indicates the intensity of concentration (0 to 1) around the mean angle. Vector r can be considered as a measure of the seasonality degree. The Rayleigh test was applied to indicate the significance ($P < 0.05$) of the mean angle (Serna-González *et al.* 2022).

Floral development. Observations of three flowers belonging to two trees were performed in Bogotá Botanical Garden to describe the external morphology and anthesis process every 30 minutes from 02:00 pm to 08:30 pm on the first day and from 07:00 am to 03:00 pm on the second day. At 05:00 pm three opening flowers from two trees were cut with a 10 cm branch portion, the branch was maintained submerged under water and the whole branch and flower was introduced in a humid chamber in order to observe the stigmatic movement during the night, preventing branch/flower dehydration. We took special care that flowers did not receive artificial light to minimize its possible influence on flower movements. Observations during the morning of the second flower stage were made in the field too, in order to contrast with *ex situ* observations.

Pollination and propagation experiments. Crossed and self-pollination experiments were conducted by hand in 63 flowers of the two oldest trees, from October 2019 until November 2022. Thirty-nine flowers were auto-pollinated and 24 flowers were crossed-pollinated. Fresh pollen of the same day was always used, and cross pollination was made when possible. Fresh pollen was collected between 02:00 pm - 03:00 pm and high flowers were obtained with a pole pruner always before 03:00 pm after 03:00 pm it is harder to find any pollen because anthers shed. Information such as day of pollination event, crossed or auto pollination, fruit abortion, day of fruit opening was recorded for each flower.

A general morphological description of fruits and seeds was made from fruits obtained by hand-pollination at Bogota Botanical Garden. Fruit weight, length and width (n = 5), morphometric seed measurements, seed weight with and without sarcotesta, sarcotesta thickness, seed length, and width (n = 43) were measured. Weight measurements were taken using an Ohaus analytical balance (Parsippany, New Jersey) and morphometric measurements with a Vernier digital caliper (China, Jiavarry). Total seeds/fruit, number of formed seeds, number of unformed or aborted seeds, number of empty follicles, and number of follicles with two, one or any seed were gathered for each formed fruit.

A seed imbibition test was carried out to determine the amount of water absorbed by the seeds with and without scarification (sanding of no more than 1 mm). For this purpose, seven seeds were used for each treatment, recording their fresh biomass. Then, seeds were immersed in distilled water, and their weight was recorded on an Ohaus analytical bascule (Parsippany, New Jersey) after removing the external water excess. Monitoring was done at one-hour intervals, for 12 h in a row and at 24, 25, and 26 h; the data of mass increase over time was converted into a percentage using the formula:

$$W_i = (W_i - W_d) \div W_d$$

where *Wi* is the assimilated mass and Wd the imbibition mass (Baskin & Baskin 2004).

Open fruits were collected, seed sarcotesta was removed, and seeds were planted the same day or the following day to a depth not greater than 1 cm. Seven experimental trials were carried out and five treatments for availability and fruit size with 5 to 17 seeds (79 seeds in total) at different times of the year during 2020 and 2022 and each trial was followed up for 7 to 14 months. The substrate mixtures were sterilized by heat in an oven for more than four hours at 120 °C. In some trials, a biological product based on a complex of *Trichoderma* species (Fitotripen WP), was added after substrate cooling. The three types of substrates were prepared as follows: a) loam and sand at a ratio of 2:1, b) loam and burnt husk at 1:1. c) Peat and burnt husk at 2:1 more 2 g of *Trichoderma* per substrate kg. The substrates were chosen because they are easily available in Colombia and could be then replicated by anyone. The proportions of the substrate mixture were chosen in order to have a 30 % of air as recommended for general seed propagation purposes (Toogood & Anderson 1999). In addition, superficial scarification (slight sanding of the testa), deep scarification (testa rupture 1mm), and hydration for two hours were tested.

We determined that a seed had germinated when the hypocotyl emerging from the substrate was first observed. Follow-up was made every 8 to 15 days; with this data, the Germination Percentage (GP) was calculated as:

$$GP = (N \div NS) \times 100$$

where N is the number of germinated seeds and NS the total number of seeds sown (William 1991).

A high percentage of the trials were made during pandemic emergency time, which difficult detailed follow-up; the Mean Germination time (MGT) was then calculated with the trials that obtained more follow-up data by the formula:

$$MTG = \Sigma_i = 1n_i \times t_i \div \Sigma_i = 1n_i$$

where *ti* is the time in days elapsed for germination on the i-th day; ni is the number of seeds germinated on the i-th day and k, the last germination (Tompsett & Pritchard 1998).

Survival Percentage (SP) was established by the formula:

$$SP = (NT \div NE) \times 100$$

where NT is the number of transplanted seedlings and NE, the number of established seedlings. A general description of the seedlings obtained was made.

Floral visitors in the wild. Thirteen trees were sampled in Granada, San Francisco, and Silvania municipalities, each with three flowers recorded during anthesis on average. Floral visitors were sampled on different flowers between 08:00 am and 03:00 pm on two consecutive days. With the help of a pole pruner and an entomological net, we carefully collected flowers in the stage where the floral chamber is closed and pollinators are presumed to be trapped in. Then, we manually opened the flowers, trying to quickly collect insects inside the floral chamber with the help of an entomological aspirator and entomological forceps. Insects were deposited and transported in vials with ethanol (75 %) for their identification in the laboratory. One specimen of each morphospecies collected was mounted and prepared for photography. Stacks of photographs were taken using a Zeiss Discovery.V12 stereomicroscope (Jena, Alemania, Zeiss). Specimens were identified at the subfamily and tribe level using the keys by Navarrete-Heredia *et al.* (2002). José Ramírez-Salamanca and Margaret Thayer, Staphylinidae specialists from the Entomology Laboratory of the Argentine Institute for Research on Arid Zones (Mendoza, Argentina), and The Field Museum of the Chicago University, respectively, confirmed specimens determination. The specimens were deposited in the Museo Javeriano de Historia Natural "Lorenzo Uribe S.J." (MPUJ_ENT).

Reintroduction. The reintroduction program was designed following IUCN/SSC guidelines (IUCN 2013). The goal is to establish viable populations of *M. caricifragrans* within its natural and historical range through a long-term program. This program uses individuals propagated from seeds obtained through hand pollination at the Bogotá Botanical Garden. Although there are other complementary actions for conserving *M. caricifragrans*, this reintroduction program is a strategic initiative that empowers nature reserve owners in their conservation efforts. It is a cost-effective strategy that may inspire additional local conservation actions. So far, 27 individuals have been planted with the owners of five nature reserves and/or healthy forests on their properties and were visited to check its survival. These owners are committed to caring for the plants long-term and expanding the propagation and reintroduction efforts. We began reintroducing plants in the municipalities of San Francisco and La Vega in Cundinamarca province due to the strong collaboration among local nature reserves, unlike other areas where reserves are sparse and distant. We planted one individual in the main park of San Francisco village with the mayor to raise public awareness about the species and its extinction risk. We also shared other individuals with the Jardín Botánico Joaquín Antonio Uribe in Medellín and the Jardín Botánico del Quindío.

Results

Reproductive phenology. The production of floral buds and open flowers occurred year-round in this study. On a monthly average, floral buds reached up to 24.9 in November 2019, and open flowers reached up to 3.6 in September and November 2020. There is a suggested seasonality in flower bud abundance in November and for open flowers in September and October, although with no statistical support (Figure 1).

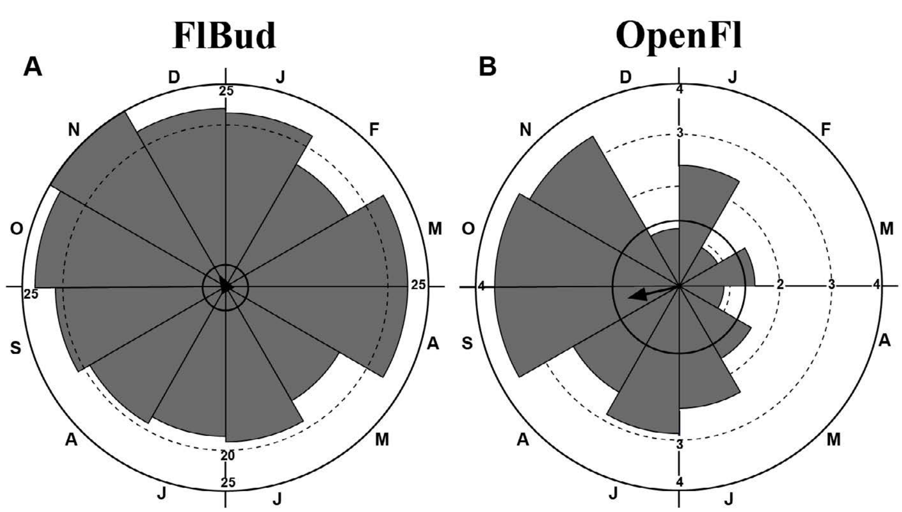

Figure 1. Variation in the temporal occurrence of two phenophases of *M. caricifragrans* A. Floral buds (FlBud). B. Open flowers (OpenFl). The direction of vector r represents mean angles; the length (0 to 1) indicates the concentration around the mean angle. Mean angles are significant when vector r crosses the solid line (Rayleigh test $p < 0.05$)

Floral development. According to the *ex situ* observations, *M. caricifragrans* flowers have three central functional moments (Figure 2). Day one, 03:00 pm - 07:30 pm, Anthesis: Sepals and petals of the second whorl open, letting an entrance for pollinators to the floral chamber; stigmas are open and receptive, and the flower releases a strong aroma scent. Day one 07:30 pm - Day two 03:00 pm: sepals reflect backward, petals of the second whorl close, creating a floral chamber, light scent, stigmas close at midnight (12:00 am), thecae dehisce introrsely, and anthers detach at 02:00 pm Day two 03:00 pm: outer and inner petals open. This pattern was consistent with flowers from trees in the wild located in the Granada and Silvania municipalities but different in trees visited in San Francisco municipality, where flowers were opened earlier in the morning of the second day (Figure 3I and 3J).

Pollination and propagation experiments. 12 of 39 flowers auto-pollinated flowers formed a fruit with viable seeds, for a 30.76 % success in fruit development rate. 12 of 24 crossed pollinated formed a fruit with viable seeds, for a 50 %

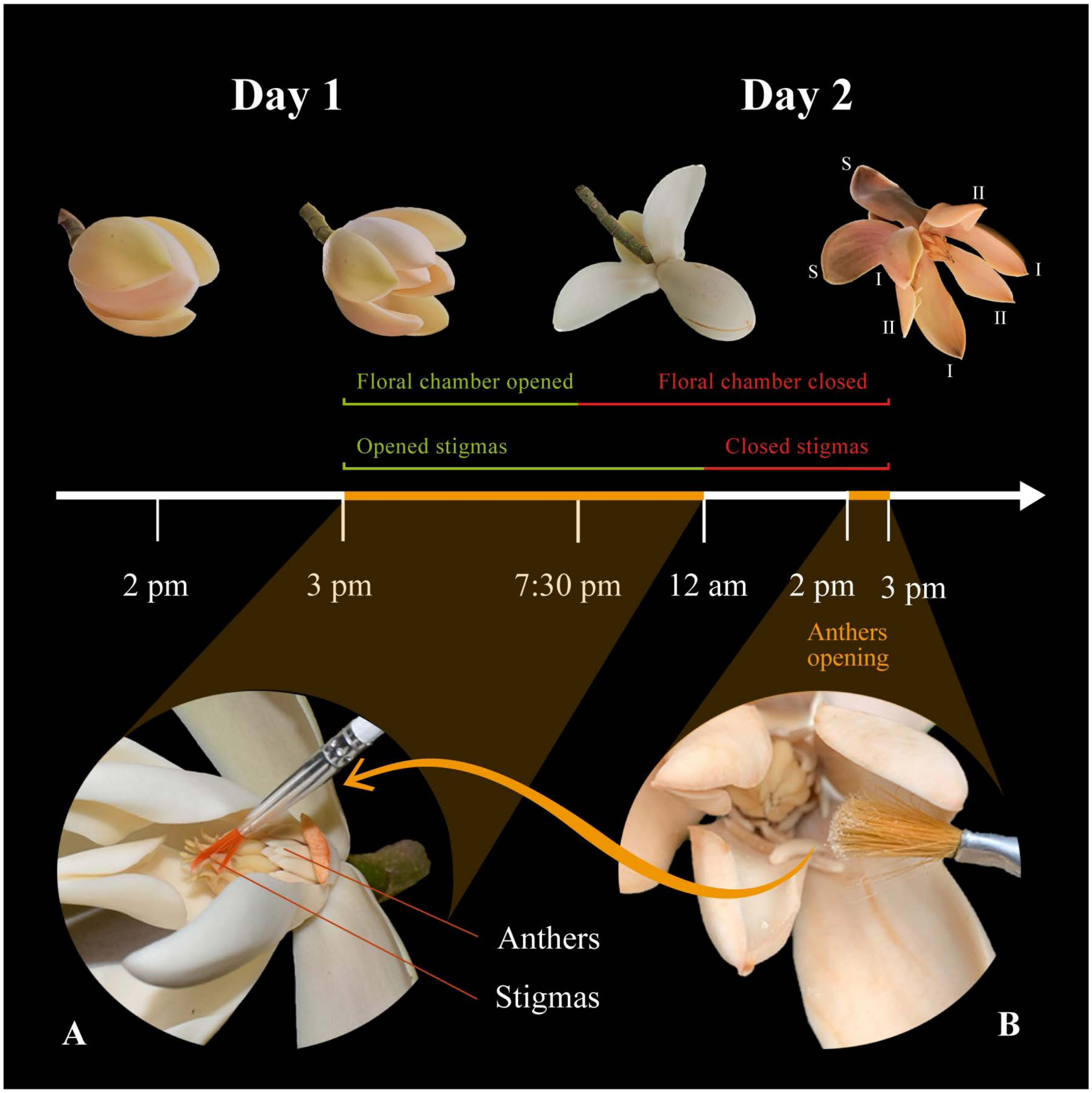

Figure 2. *M. caricifragrans* floral development diagram. In the upper section are illustrated the major flower movements and stages in relation to a timescale. In the lower section hand-pollination details are illustrated A. Taking pollen from anthers with a brush; B. Pollen deposition brushing the stigmas surface. S: Sepals; I: First whorl of petals; II: Second whorl of petals.

success in fruit development rate and a general 38.10 % success in fruit development rate. After hand-pollination, fruits took on average 301 ± 17 days (n = 24) and a range of 265-337 days to completely develop. Historically, just one spontaneously formed fruit had been observed in individuals of the *ex situ* living collection, these seeds were not viable.

The fruits of *M. caricifragans* obtained by hand pollination are aggregate, dry, woody, elliptical fruits with truncated base and acute apex, 2.5-3.4 cm wide, 8.2-6.8 cm long and weighing 17.1-25.4 g. Their color changed during development from light green to reddish and finally to dark green at maturity (Figure 3A, 3B, and 3C). The central axis or woody receptacle is beige, with glabrous cavities where the follicles contain the seeds. Seeds are irregular obovate, with very aromatic orange sarcotesta, black testa, a length of 7.29-18.87 mm, a width of 8-11 mm, thickness between 3.97-8.37 mm, weight with sarcotesta 0.124-0.614 g, and without sarcotesta 0.072-0.460 g. Sarcotesta thickness of 0.3-0.96 mm (Figure 3D and 3E). 100 seeds could weigh 12.4-46 g. Fruits presented 12 to 14 follicles containing between 7 and 23 fully formed seeds, each follicle acquiring between one and three developed seeds, thus having follicles with one or two unformed seeds of size less than 1-3 mm, a situation that generates between 1 and 12 unformed seeds of size less than 1 mm in the whole fruit, in addition between two and four completely empty follicles were observed; then, 41.2 - 95.8 % of the seeds were found fully developed (Figure 3D).

In the first hour of imbibition, the scarified seeds had a more significant weight increase than the non-scarified seeds and stabilized at ten hours with a 3.14 % mass increase. The seeds with endocarp or without scarification at seven hours reached the mass increase of the scarified seeds in the first hour, and no stability was observed in the mass increase at 26 hours, which was 2.64 % (Figure 4).

Germination is epigeal. Seedlings were characterized by well-developed roots, white to light green hypocotyl, obovate cotyledons with entire margins, smooth green epicotyl, alternate true leaves, elliptical shape, smooth margin, smooth texture, and shiny beam. The onset of this event was observed between 92 and 122 days after sowing (Figure 3F).

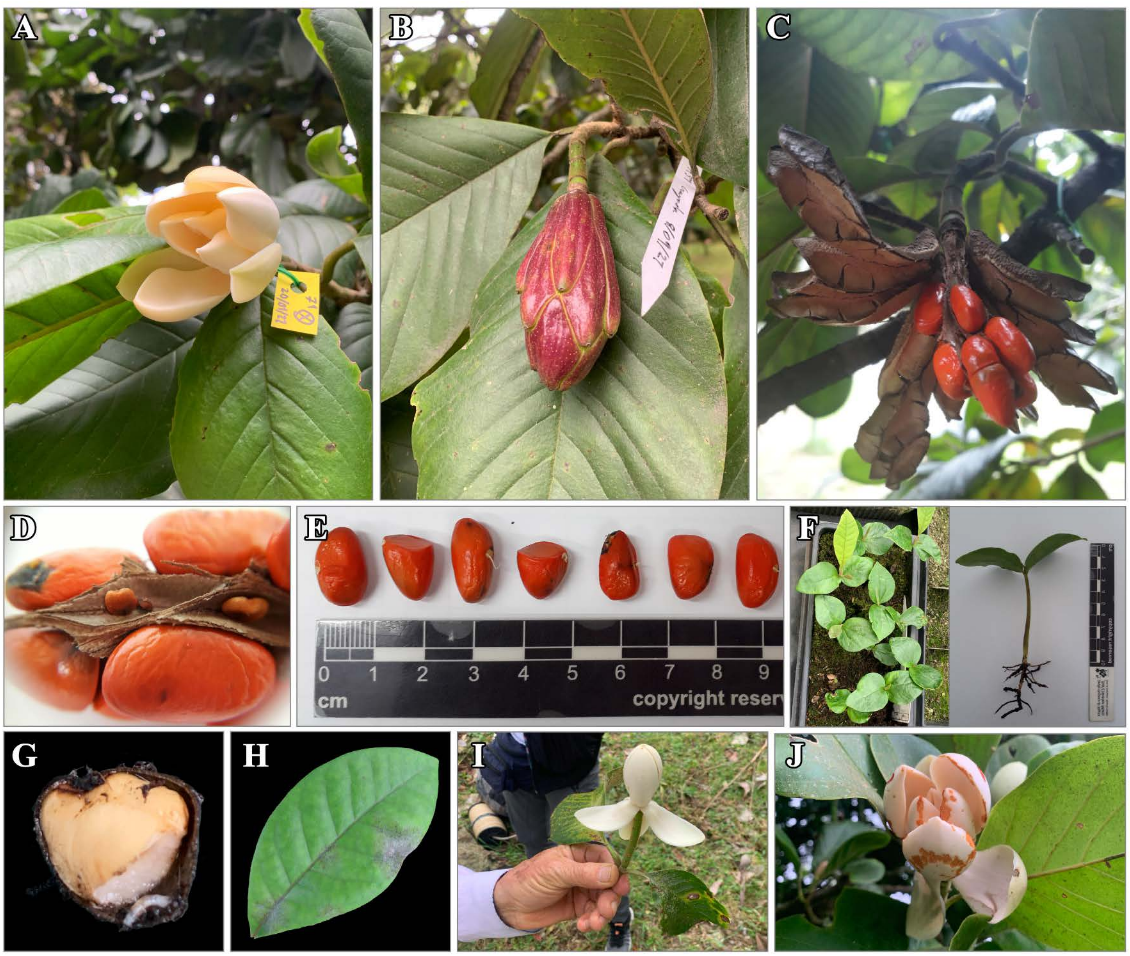

Figure 3. Picture of the process from pollination to reintroduction. A. Flower pollinated and marked; B. Unripe fruit; C. Ripe fruit; D. Follicles with formed and unformed seeds; E. Seeds of a single fruit; F. Young plantlets with leafy cotyledons; G. Sciaridae fly larvae predating seed endosperm; H. Leaf affected by mildew; I. Flower on its second day collected at Granada municipality at 9:00 am J. Flower on its second day collected at San Francisco municipality at 10:00 am.

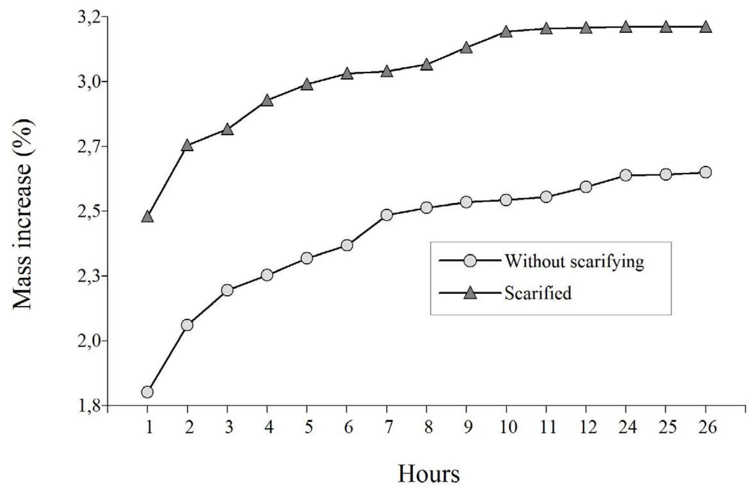

Figure 4. Mass increase (weight) of unscarified and scarified *M. caricifragans* seeds for 26 hours.

Germination percentages varied between 0 and 100 % (Table 1), being 0 on seeds with deep scarification. The highest percentage of germinated seeds was presented in the substrate peat, husk at a ratio of 2:1, and *Trichoderma* (2 g/kg) using unscarified seeds. Mean germination time (MGT) was between 110 and 122.5 days. The survival of germinated seedlings in the different trials was between 50 and 100 % after 7 to 14 months of follow-up. The main reason for seedling loss was downy mildew (*Peronospora sparsa*) infections in the early stages (Figure 3H).

The seedlings presented in the first 12 months, a growth between 0.94 and 1.32 cm/month reaching between 11.28 and 16.32 cm a year, still having cotyledons and between 3 and 5 true leaves. When they got 2 years old, they had between 34.5 and 39.5 cm and between 8 and 10 true leaves. Seedlings were characterized by well-developed roots, white to light green hypocotyl, obovate cotyledons with entire margin, smooth green epicotyl, alternate true leaves, elliptical shape, smooth margin, smooth texture and shiny beam. Plantlets were transplanted into containers with a mixture of 2 soil: 1 husk: 1 compost, 1 to 2 months after germination with the two cotyledons well opened (Figure 3F).

Floral visitors in the wild. Five morphospecies of beetles belonging to the families Cerambycidae, Coccinellidae, Latridiidae, and Staphylinidae (commonly known as rove beetles) visiting flowers of *M. caricafragrans*, were found in the localities of Granada and Silvania. However, only rove beetles were found trapped within the flowers on the second day (Figure 5). In terms of abundance, just one individual of Cerambycidae, Coccinellidae, and Latridiidae were collected, whereas seven rove beetles were sampled in total, including a couple mating inside a floral chamber. We also captured some flies (Diptera) of Drosophilidae (2 ind), Psychodidae (12 ind), and Phoridae (1) visiting *M. caricafragrans* flowers.

Flowers visited by beetles rarely were observed with their outer petals damaged and eaten; likewise, we did not observe inner petals eaten, stinking detritus, or beetles' excrement inside flower chambers. The number of coleopterans visiting flowers climaxed at about 9:00 am-11:00 am when we collected all of them (Figure 3I). By contrast, we did not find any insects interacting with the flowers of the three adults found in the San Francisco municipality. However, the petals and even stamens exhibited evident damage. The petals and thecae of the observed flowers were prematurely opened (Figure 3J).

Reintroduction. A 100 % survival rate of the 27 reintroduced seedlings for the moment was recorded. Seedlings were planted in full sunlight or partial shade at forest edges, where nature reserve owners can easily monitor their development and potentially continue pollinating and propagating them in the future. They have reached an average of 6.46 meters after 7 years of planted. The reserve owners are very enthusiastic about the reintroduction program and are assisting us in planning future plantings in the area. Many plantlets are currently growing at Bogotá Botanical Garden and will be reintroduced in the coming years. Additionally, six individuals were shared with both the Medellín and Quindío Botanical Gardens to support national *ex-situ* conservation efforts (Figure 6).

Discussion

Reproductive phenology. Even in tropical latitudes, the constant flowering pattern of *M. caricifragrans* is unusual in angiosperms (Borchert 1983, Borchert *et al.* 2005) and might suggest that pollinators' life cycle or pollinators' community feeding depends on flower availability. *Magnolia* flowering periods in northern hemisphere species generally synchronize in spring (Kikuzawa & Mizui 1990, Dieringer *et al.*1999, Setsuko *et al.* 2008). Flowering correlation with climatic factors might been diffuse in Colombian *Magnolia* species because of their constant flowering patterns all year round, even though flowering peaks are reported in different months of the year (López-A *et al.* 2008, Gómez 2011, Serna-González & Urrego-Giraldo 2016, Serna-González *et al.* 2022). This species tends to have more buds and open flowers from September to November. Nevertheless, flower buds and opened flowers can be found in all developmental stages all year round.

All year-round flower production seems to be the reason why ripe and unripe fruits can be found in different months in natural isolated specimens of *M. caricifragrans* and other *Magnolia* species (Samper 2010, Gómez 2011, Serna-González *et al.* 2022).

Floral development. M. caricifragrans follow a sequence commonly observed in other *Magnolia* species (Chen *et al.* 2016, Serna-González *et al.* 2022) and in many other tropical protogynous species pollinated by beetles like Araceae (*Philodendron, Xanthosoma, Dieffembachia*), Nymphaeaceae (*Nuphar, Victoria*), and Annonaceae (*Annona, Duguetia, Xylopia*) (Valla & Crino 1972, Prance & Arias 1975, Gottsberger 1990, Lippok *et al.* 2000, Ratnayake *et al.* 2007, Magdalena 2018, Saravy *et al.* 2021). The sequence consists of a phase where the flower opens or partially opens and attracts pollinators with both scents and/or heating. The second phase is where the flower closes or partially closes, trapping pollinators. In the third phase, anthers open, and the flower liberates pollinators; these three phases always

Table 1. Details of germination trials and results

No.	Substrate and pretreatment	No. seeds sown	Beginning of germination (days)	% Germination	% Survival	Month reading survival
1	Loam and sand (2:1)	9	97	20	0	7
2	Loam and sand (2:1)	5	92	40	50	14
3	Loam and burnt husk (1:1)	9	105	44	100	14
4	Peat, burnt husk (2:1) + *Trichoderm*a 2g/kg	14	110	100	78.6	12
5	Peat, burnt husk (2:1) + *Trichoderma* 2g/kg	6	122	100	83.3	8.5
6	Peat, burnt husk (2:1) + *Trichoderma* 2g/kg (Surface scarification)	6	94	100	100	8.5
7	Peat, burnt husk (2:1) + *Trichoderma* 2g/kg	13	116	100	100	7
8	Peat, burnt husk (2:1) + *Trichoderma* 2g/kg (Deep scarification + hydration 2 h)	17	N/A	0	N/A	N/A

last 24 hours, synchronizing flowers at phases one and three. From field observations, damage to flowers might shorten the second phase, making a premature transition to the third phase, further research will be made to resolve this observation. Moreover, since heat was not assessed in the field or detected in the closed flowers where insects were collected, evaluating this factor will be important in future studies.

Understanding these cycles helps us to capture possible pollinators trapped inside the flower and program artificial pollination focusing on the moment of thecae dehiscence before anthers are shed.

Pollination and propagation experiments. Hand-pollination is a common practice in botanical gardens, especially in rare species (Wang *et al.* 2016, Magdalena 2018). In nature and botanical gardens, *M. caricifragrans* can be easily hand-pollinated all year round by anyone with a brush between 02:00 and 03:00 pm, ideally (Figure 2). This hand-pollination process allowed us to obtain fruits with a 38 % success rate, less than in *Magnolia sinica* hand-pollination (58 - 69 %) (Chen *et al.* 2016), but still very useful for *ex-situ* seed production. On the contrary, *M. caricifragans* was more successful in ovule formation because it managed to obtain between 41.2 and 95.8 % of formed seeds, a higher range than that reported in *ex-situ* pollination trials conducted on *M. sinica* where they obtained 14.29 - 60.42 % (Wang *et al.* 2016). Similar rates of seed formation occur naturally in *M. espinalii* with 50 - 80 % and *M. hernandezii* with around 50 % (Gómez 2011). Cross-pollination is clearly more effective than auto-pollination in fruit formation and should always be favored. Results demonstrate that *ex-situ* hand-pollination is a viable methodology that allows the formation of fruits and viable seeds to propagate *M. caricifragans*.

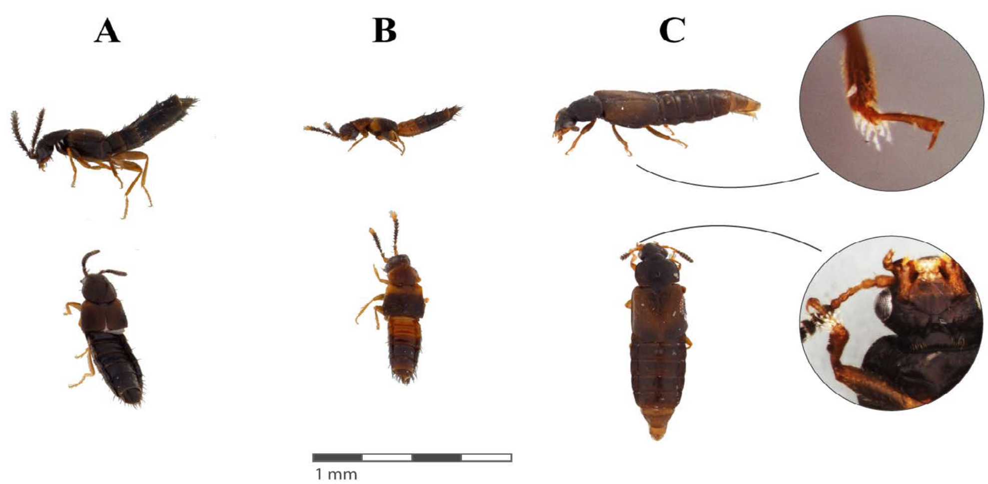

Figure 5. Morphospecies of Staphylinidae as flower visitors of *M. caricafragrans*. Lateral and dorsal view of: A. Athetini, *Atheta* cf. sp.1; B. Athetini, *Atheta* cf, sp.2; C. *Phloeonomus* sp.1 (male). We show in this last figure teneral setae (or spatulate setae) found on tarsus in all legs both on males and females.

Some *Magnolia* species present reproductive limitations in part due to small populations with isolated individuals, which results in low fruit and seed production (Chen *et al.* 2016, Serna-González *et al.* 2022). *M. caricifragans* may be another example of this situation, which has made fruit collection in the wild challenging for researchers and locals.

Fruits of *M. caricifragrans* can be considered undersized for the genus, with between 3 and 23 seeds per fruit; a quantity similar to that reported for *M. guatapensis*, between 13 and 20 seeds, and *M. polyhypsophylla* between 10 and 19 seeds per fruit, which contrasts with the large number of seeds that can be obtained from other species such as *M. hernandezii* with 105 to 219 seeds per fruit and *M. grandiflora* with 30 to 155 seeds per fruit (Toro & Gómez 2011, Bakhramov *et al.* 2021). This characteristic makes it hard to obtain large amounts of seed for propagation trials of *M. caricifragans*.

Scarified seeds of *M. caricifragans* showed a more significant increase in weight in the first hour of the imbibition test, an increase that was not reached after 26 h in unscarified seeds. Similar results were obtained in seeds of *M.*

champaca, where there is a differential increase in mass of scarified and non-scarified seeds up to 24 hr. However, after 48 hours, the difference disappears (Fernando *et al.* 2013). According to these results, there is no physical latency in the seeds because seeds without scarification also show water entry; furthermore, if a faster water entry process is desired, the deep scarification technique can be considered a viable alternative in *M. caricifragans* during the first 2 to 3 h of hydration. However, it should be taken into account that seed hydration for some species such as *M. hernandezii* and *M. dealbata* seems to improve germination (Corral-Aguirre & Sánchez-Velásquez 2006, González & Montoya 2014), for *M. champaca* it is indifferent (Fernando *et al.* 2013). For others, it generates delays in germination (*M. guatapensis*) and even no germination when using hot water in different periods (Gómez 2011, Corral-Aguirre & Sánchez-Velásquez 2006).

With very few exceptions, it is well known that seeds of Magnoliaceae species are recalcitrant, which means that they do not withstand prolonged storage or drying. Therefore, the longer the seeds are dried, either exposed to the sun, even in the fruit or in the soil, can reduce their internal moisture content and thus reduce the germination percentage (Bonner & Russell 1974, Kha *et al.* 2005, José *et al.* 2009, Fernando *et al.* 2013, González & Montoya 2014, SID 2023). However, Fernando *et al.* (2013) established that *M. champaca* has seeds with storage behavior that can be classified as intermediate, that means seeds tolerate dehydration at an intermediate moisture content of 7 to 10 % (Hong & Ellis 2002). Different types of dormancies have been reported in the seeds of some species of *Magnolia* (Baskin & Baskin 2004, Fernando *et al.* 2013, Pereira *et al.* 2016, Borah *et al.* 2023). In order to avoid viability loss, the sowing of fresh seeds, ideally from the same day of fruit collection or at most the day after fruit opening is recommended in any *Magnolia* germination trial.

The germination percentage of magnolias may vary significantly from 1 to 100 % depending on the species and/or treatment (Serna-González & Urrego-Giraldo 2016). It is hard to explain the reasons for these differences, even though *M. caricifragans* seed germination success seemed to be influenced by three main factors in this study, moist retention of substrate, moderate irrigation and pathogen control. High humidity retention with good aeration and drainage can be provided with different substrate combinations, peat and carbonized husk mixture is one of them and demonstrated good germination results in this study. The use of *Trichoderma* and carbonized rice husk was intended to avoid seedling pathogens and seemed to work better in combination with peat more than with loam. Excluding the entrance of Sciaridae flies in propagation trays is recommended, this can be made with proper cap sealing of trays or even with a clear plastic bag (Figure 3G). *M. caricifragrans* initially requires one to two more months of care before germination compared to other colombian *Magnolia* species such as *M. guatapensis* (54-64 days), *M. hernandezii* (56-61 days), *M. polyhypsophylla* (56-63 days) and *M. yarumalensis* (starts between 39-55) (Gómez 2011), that is why special care for fungus control is required in this species.

Scarifying and accelerating water entry does not generate faster germination results, probably because germination depends on the morphological state reached by the seed at the time of fruit collection; seeds might not be fully formed when the fruit opens (Le Page-Degivry 1970). Therefore, scarifying is not recommended because it might instead favor pathogen infection of endosperm, consistently reducing seed germination.

Floral visitors in the wild. Under natural conditions, it was identified that *M. caricifragrans* flowers are visited mainly by rove beetles which have been recognized as an important pollinators of diverse early diverging angiosperms, including *Magnolia* (Sayer *et al.* 2019, Bernhardt 2000). Although, we could not record beetle licking or gnawing of floral parts or making other foraging behaviors while visiting flowers on the trees, it could be possible that at least one of the three morphospecies identified as *Atheta* cf. sp.1 (3.7 mm length), *Atheta* cf sp.2 (2.3 mm length) (Aleocharinae ,Staphylinidae), and *Phloeonomus* sp.1 (4.2 mm length) (Omaliinae, Staphylinidae) acts as pollinators of this species, considering that all individuals were collected inside the floral chamber from picked flowers including two individuals mating of *Phloeonomus* sp.1 and that only in those localities formed fruits where seen. This last rove beetle have a tarsal setae called “tenant setae”, purely descriptive as an “spatulate and elongate setae” present on all tarsi and especially well-define in the tarsus of the first leg (Figure 5), which seem to be involved in aiding the beetles’ clinging to flowers (Thayer 2005, M. Thayer, pers. comm.), which are the place for nourishment, hiding and mating of diverse beetle-pollinated magnoliids during flowering period (Thien 1974,

Figure 6. Reintroduction. Tree planted in "Finca el Descanso", San Francisco municipality, Cundinamarca province, 1,600 m asl. with 7 years of planted.

Gottsberger *et al.* 2012, Chen *et al.* 2016). New studies will be required to identify if *Phloeonomus* sp.1 or the other identified rove beetles are effectively pollinators of this species or if they are only flower visitors, as pollen was not detected in these insects.

On the other hand, we found *M. caricifragrans* flower visitors in the wild include moth flies (Psychodidae) which were numerous in the samplings. These flies are commonly known as pollinators of sapromyophilous plants which emitted odours of dung or fermentation mimicking moth flies ovipositions sites (Larson *et al.* 2001, Chartier *et al.* 2013). Contrastingly, *M. caricifragrans* releases a sweet floral fragrance, as previously described. The owners of San Francisco reserves have been looking for fruits in the three adults for the past ten years and have never seen one, another animal might be interacting with the flowers during nighttime making damage to the petals and anthers but seems non effective as pollinator. Future research should focus on assessing other aspects of flower- visitor interactions of *M. caricifragrans*, such as chemical compositions of the floral blend, thermogenesis aspects and its role in insect attraction.

Botanical gardens should focus their efforts on collecting species that are less representative and/or threatened to ensure their survival and subsequent reintroduction into the wild (Wang *et al.* 2016, Zhao *et al.* 2022). *M. caricifra-*

grans is a threatened species in the wild, mainly because of land cover change (Calderón *et al.* 2007). As expected from the last evaluation of the species state in the field (Samper 2010) in the visited populations, adults are found mainly isolated in pasture fields with very few opportunities of dispersion to a healthy forest and null opportunities for seedlings to be established. Squirrels eat the seeds before the fruits completely ripen, making the dispersal and recruitment even more difficult in their natural habitats. The state of these natural populations has not changed at all since the last update in 2010 (Samper 2010). Plant propagation, translocation and reintroduction in healthy forest in its original distribution range, is an urgent need of most Magnolia species from Colombia (Samper & Garcia 2001, Calderón *et al.* 2007, López-A *et al.* 2008, Samper 2010, Toro & Gómez 2011, Serna-González *et al.* 2022). Even though the adult trees from Bogota Botanical Garden were not chosen with genetical diversity criteria, we decided to start reintroducing the plants propagated in the same parental province due to its endangered condition and the urgent call for action (Oldfield 2009, Cires *et al*. 2013). All the individuals have been planted in full sunlight exposure or semi shade, as recommended by colleagues, other studies and by our own experience with other Magnolia species (Toro & Gómez 2011, Sánchez-Velásquez *et al.* 2016), with a 100% survival rate for now. More natural reserve owners want us to plant in their fields, demonstrating a promising future for the species establishment with their help. This experience demonstrates as mentioned in the National Strategy for Plant Conservation in Colombia (Garcia *et al*. 2010) that the integration of *in situ* and *ex situ* actions is essential to ensure the success of conservation.

In conclusion this work offers an example of how *Magnolia ex situ* living collections at botanical gardens can complement *in situ* conservation initiatives through hand pollination, propagation and reintroduction. These results give us a positive perspective for the future conservation of the species if we continue the hand-pollination and propagation program complementing it with reintroduction in a long-term work. However, these efforts should be combined with new research to better understand the network structure (including composition) of flower visitors and pollinators of *M. caricifragrans* in the wild; the conservation of this species is strongly dependent on its own plant-pollinator interactions.

Acknowledgements

We would like to thank the Scientific Department of the Botanical Garden of Bogotá "José Celestino Mutis" for funding the research. To J. Ramírez-Salamanca and Margaret Thayer for corroboration of the taxonomic identity of the collected Staphylinidae. To L.A. Rojas-Araque and I. Aguilar for their contributions to phenological monitoring. To D. Moreno for his support in the revision and debugging of databases. To M. Briceño for the layout and design of figures and graphs. To the anonymous reviewers, and editors for their time and constructive comments.

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Associate editor: Pedro Valverde

Author contributions: MBR, research conceptualization, data collection and analysis, writing, discussion and manuscript revision; JERM, data collection and analysis, writing, discussion and manuscript revision; JDP, data collection and analysis, writing, discussion and manuscript revision; MSG, analysis, writing, discussion and manuscript revision; GML, data collection, writing, discussion and manuscript revision.

Supporting agencies: Jardín Botánico de Bogotá José Celestino Mutis-JBBJCM (Proyecto de inversión 7679: Investigación para la conservación de los ecosistemas y la flora de la Región y Bogotá, y proyecto BPIN 2023000050005 del Sistema General de Regalías SGR) and Tecnológico de Antioquia.

Conflict of interest: The authors declare that there is no conflict of interest, financial or personal, in the information, presentation of data and results of this article.