DOI: https://doi.org/10.29312/remexca.v14i4.3060

Article

Brosimum alicastrum: sexing, flower, seed production and growth regulators

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Abstract

Brosimum alicastrum Swarts (known locally as ramón) is a tropical forest species with importance for the food sector; therefore, studying its physio-technical characteristics is essential. The sex ratio was determined in three adult populations of the state of Yucatán, Mexico. The first plantation comprised a total of 312 individuals, 208 males and 104 females: the second, 45 male and 29 female trees (74 trees) and the third, 41 males and 29 females (70 trees). On average, 64% of the trees were male and 34% female. In a separate plantation (50 trees) established *ad hoc*, 30% of the trees began to produce flowers 7 years after transplantation, 38% did so after 8 years, 28% at nine years and 4% at 10 years, 33 (66%) of the trees were males and 17 (34%) females. The experiment lasted from 2009 to 2019 and no sex changes were recorded during this time. The average seed production of adult trees was 145.6 kg tree^{-1} year^{-1}. A parallel experiment was conducted to monitor retention of leaves, they remained in the crown for more than 40 months (1 217 days). Additionally, the content of plant growth regulators that could be used as molecular markers to select females was measured. Female trees showed higher content of gibberellic acid and cytokinins than male trees. The difference in cytokinin content between the sexes reached 500%. The relevance of these results for the establishment of commercial plantations is discussed.

Keywords: cytokinins, gibberellic acid, sexual expression, trees.

Reception date: February 2023
Acceptance date: April 2023

Introduction

Brosimum alicastrum (known locally in Mexico as ramón) is a species of tropical evergreen forest, very abundant and widely distributed in the tropical rainforests of Mesoamerica (CONABIO, 2014), where it is known locally by more than 50 common names, including ramón, ojite, ojoche, capono, uhi, apomo and others (Pardo-Tejeda and Sánchez-Muñoz, 1980). This species has attracted great interest due to the environmental services it provides, including climate change mitigation and high potential for food production (Subiria-Cueto *et al*., 2019).

Sexual expression in *B. alicastrum* has not been fully clarified and is an open research topic. Studies carried out in the twentieth century describe it as a monoicous species that presents anemophilous and geitonogamous pollination, so cross-fertilization is favored (López-Mata, 1987). Puleston (1968) reported that *B. alicastrum* flowers twice a year, with a seed production of 32.6 kg year^{-1}.

Other authors have reported that each tree can produce 50 to 75 kg of fruit, with approximately 32.6 kg year^{-1} of seed (Martínez, 1936; González, 1939; Puleston, 1968; Peters and Pardo-Tejeda, 1982). In this research it was found that trees with more than 25 years cultivated in the state of Yucatán produce about 163 kg year^{-1} of foliage and 95 kg year^{-1} of seed, and 300 to 350 seeds per kilogram. Therefore, a plantation of 250 trees ha^{-1} can produce between 20 and 25 t ha^{-1} year^{-1} of seed. It was also found that trees begin to produce flowers seven years after planting (Hernández-González *et al*., 2015).

Peters (1989) examined 51 *B. alicastrum* trees, of which 23 (45%) were females and 28 (55%) males; they produced 30 kg tree^{-1} year^{-1} of seeds. He noted that female trees change sex as they grow, so that medium-sized trees are mostly female, while taller trees are male. Sexual expression in plants is known to be regulated by plant growth regulators (PGRs) and other environmental factors (Khryanin, 2002). PGRs are a group of substances that influence the physiological processes of plants at very low concentrations (Davies, 2010). For example, gibberellins promote the suppression of male reproductive parts in corn flowers (Chen and Tan, 2015).

Abscisic acid (ABA) regulates the beginning of flowering, senescence and abscission (Wang *et al*., 2002). Another PGR is ethylene gas, which regulates the opening and senescence of flowers. Studies conducted on cucumber, melon and watermelon show that increasing ethylene concentration stimulates the development of female flowers (Martín *et al*., 2009; Guo *et al*., 2010; Manzano *et al*., 2014). Given the interest in establishing commercial plantations of *B. alicastrum*, this study was conducted in order to quantify the sex ratios in adult trees in plantations established in the State of Yucatán, detect sex change processes, determine levels of PGRs that could influence sexual expression and estimate the potential for seed production and the period of leaf retention.

Materials and methods

The first part of the study was conducted in three plantations of *B. alicastrum*. The first plantation comprised a total of 312 adult trees and was located on Paseo de Montejo Avenue in the city of Mérida (20° 59’ 23.86” north latitude, 89° 36’ 59.02” west longitude). The second plantation was made up of 74 adult trees and was located on the entrance road to Hacienda Chichi Suárez (20° 59’ 57.12” north latitude, 89° 33’ 7.92” west longitude), near the city of Mérida. Through the observation of the texture and diameter of the trunks, an age of 40 years in the trees was estimated. The third plantation was located at Hacienda Xoccheila in the municipality of Sacalum (20° 33’ north latitude, 89° 34’ west longitude); composed of 69 trees of different ages, all at least 10 m tall and more than 25 years old, according to reports from the owners.

The trees were sexed by direct observation: the male trees have the typical inflorescence described by (López-Mata, 1987), while the female trees bear fruit. These trees grow in a Leptosol-type soil (according to the WRB classification; Ek-lu’um according to the Mayan soil classification), in a subhumid tropical climate with a summer rainy season (climate type Aw, according to the classification of Köppen and Geiger). A sample consisting of 30 previously sexed trees (20 trees in the Xoccheila plantation and 10 on the campus of the Scientific Research Center of Yucatán) was followed for 10 years to identify any sex changes.

Sampling

To evaluate seed production, six adult trees (over 30 years old, according to local information) were selected in the city of Mérida, specifically on the campus of the Scientific Research Center of Yucatán (21° 01’ 54.8” north latitude and 89° 37’ 21.2” west longitude). Plastic nets were placed around the trees to collect the fruits and seeds produced. The pericarp was extracted from the fruits and the seeds were weighed using an Ohaus triple beam balance.

To estimate the time of first flowering and determine the sex of each tree, an *ad hoc* experimental planting of 50 young trees was established in 2009. The seeds were sown in bags with vegetable compost; the seedlings were grown for a year under 50% shade in field conditions and subsequently established at the planting site.

The seedlings 50 cm tall were planted on 40 cm holes with a spacing between plants of 5 m from each other and received supplementary irrigation once a week in the summer season for the first two years to ensure their survival. From the third year after transplantation, growth occurred under rainfed conditions. The plantation was monitored regularly; both flowering and sex of each tree were recorded by direct observation. To estimate the period of leaf retention, four plants were selected and marked for 40 months. All leaves produced (between 9 and 10 leaves per tree; 38 leaves in total) were marked as they unfolded. The leaves were counted weekly for 40 months. Survival data were converted to arcsine and then subjected to a survival analysis, using the software SPSS version 23.

Instruments for PGR analysis

The concentration of PGRs was determined according to the method described by Salazar *et al*. (2021) and an Agilent liquid chromatograph model 1 100 equipped with an automatic sampler model 1200 and a diode array detector was used. A Zorbax SB-C8 column of 4.6 x 75 mm was used, the mobile phase system was water with trifluoroacetic acid (A) at 0.1% and acetonitrile with trifluoroacetic acid (B) at 0.1%. The analysis was performed using 22 to 50% B in a 1.5 min gradient. The flow was 3 ml min^{-1} at 60 ºC, with the detector set to 254 nm. Commercial Sigma-Aldrich standards were used as a reference to construct calibration curves for PGRs: gibberellic, indoleacetic, abscisic indolebutyric acids and kinetin.

Extraction and total content of plant growth regulators (PGRs)

A sample of 20 trees of similar size, previously sexed, from 20 to 30 years old were selected from the Hacienda Xoccheila plantation in the municipality of Sacalum (20° 32’ 43. 1” north latitude, 89° 33’ 27. 5” west longitude). A 5 g leaf sample was collected from each tree and immediately frozen in liquid nitrogen for transport to the laboratory. Leaf tissue samples were freeze-dried, finely ground and immediately stored in the dark at 4 °C until PGR extraction.

The extraction method proposed by Xiangqing *et al*. (2010) was used. Subsamples of 100 mg were weighed in triplicate and then transferred to 2 mL conical polypropylene tubes containing 500 µl of an extraction solution of 2-propanol: water: concentrated HCl (2:1:0. 002, v/v). The tubes were stirred at 100 rpm for 30 min at 4 °C, then 1 ml of HPLC-grade methylene chloride was added and the tubes were stirred again for 30 min. The tubes were centrifuged at 13 000 rpm for 5 min. An aliquot of 900 µl of the lower phase was collected, the solvent was evaporated to 100 µl with a nitrogen stream and 500 µl of HPLC-grade methanol was added. A 100 µl aliquot of the sample was injected into the chromatograph.

Statistical analysis

PGR contents were expressed as the mean (± standard error) of three repetitions. Analyses of variance (Anova) were carried out, followed by Tukey’s multiple comparison tests to assess differences between the means, using a significance level of $p< 0.05$.

Results

Sexing of adult trees in plantations

The trees of the first plantation established in Paseo de Montejo (González, 2011) continued with the production of flowers more than 100 years later (2019), which confirms the great fruiting capacity of *B. alicastrum*, making this study a first reference for this species. Records on the 312 trees of this plantation also showed that 208 (66.6%) trees produced no fruit and were recorded as male trees, while 104 (33.3%) trees produced fruit and were recorded as female trees.

In the plantation of 74 trees in Chichi Suarez, 45 (60.8%) trees were males and 29 (39.2%) were females. These trees over 40 years old also maintained their full capacity to produce male and female inflorescences. The Xoccheila plantation comprises trees over 20 years old; the results showed that 40 (58%) of the 69 trees monitored were males and the other 29 (42%) females. The fact that the sex ratio in this population of unequal age was very similar to that found in the other plantations stands out.

When data from the three plantations are combined, 293 (64.4%) of the 455 trees examined produced no fruit and were classified as male trees, while 162 (35.6%) were female. Therefore, one in three trees is female (Cuadro 1). This information is relevant and should be considered when establishing a new plantation for seed production purposes, as methods such as micropropagation or grafting to propagate seed-producing trees will need to be implemented.

Table 1. Sex ratio of adult trees of *B. alicastrum* in three localities of the state of Yucatán.

Locality	Males	Females	Total
Paseo Montejo	208 (66.7%)	104 (33.3%)	312
Chichí Suárez	45 (60.8%)	29 (39.2%)	74
Xoccheila	41 (58%)	29 (42%)	70
Total	294 (64.4%)	162 (35.6%)	456

Time required for first flowering

The time needed for the production of the first flower was recorded in an experimental plantation of 50 trees established for this purpose. The trees were produced from seeds and then planted to the field site; this condition did not affect the sexual expression of the trees. The juvenile period ended when the trees began to produce flowers, with time varying markedly between trees, as shown in Figure 1, 15 (30%) of the 50 trees began to produce flowers seven years after their establishment, 19 (38%) did so after 8 years, 14 (28%) after nine years and 2 (4%) after ten years.

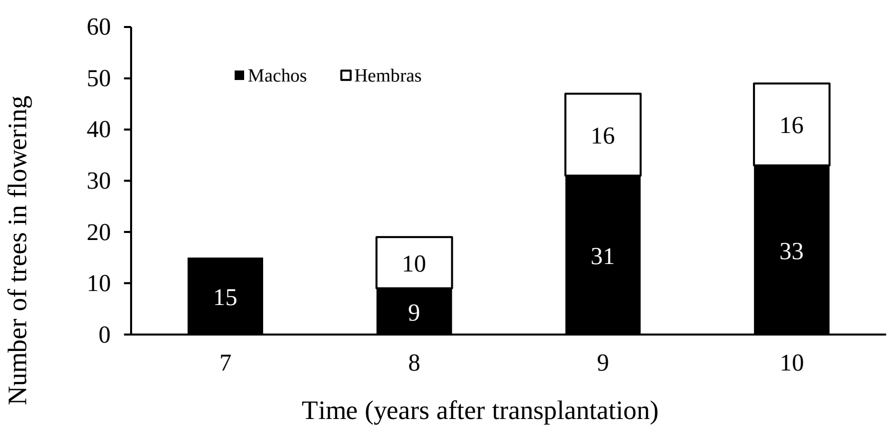

Figure. 1. Sex ratio and beginning of flowering in ramón (*Brosimum alicastrum*) trees that grow in field conditions. The initial sample comprised a total of 50 trees.

The trees that flowered earlier were all males; of those that flowered after eight years, ten were females and nine were males. In summary, 33 (66%) of the trees were males and 17 (34%) females. An important finding in this study is that male trees begin to produce flowers earlier than female trees; this allowed trees to be sexed from the first flowering event. It is difficult to explain why the beginning of flowering was not simultaneous but occurred at different times after the establishment of the plantation, even though all the trees had been produced in nurseries under the same conditions.

It is hypothesized that, being trees produced from seed of wild trees, there is a high genetic variation between them. In addition, the origin of the seeds used to produce the tree was not documented. An additional cause may be the high variability of the soil at the plantation site, evidenced by its high stone content and the heterogeneous availability of water and nutrients. The results obtained in an *ad hoc* plantation differ from those reported by Peters (1989) for the natural populations studied in 1981 and 1982 in tropical rainforests.

The observations showed that, after 10 years of monitoring: 1) no sex change was observed in the trees; 2) the definition of sex took place from the first flowering event, with no sex changes observed in the following years; and 3) male trees were recorded since they were seven years old, 4 m tall and 7 cm in diameter. Based on these data, the sex of trees is considered to be independent of their age and height.

Seed production

Figure 2 shows the seed harvest values for a sample of six adult trees. The average seed production was 145.6 kg tree^{-1} year^{-1}. Three of the trees produced more than 150 kg of seed, the least productive tree yielded 117 kg. These results confirm those reported by Hernández-González *et al*. (2015) of 100 kg tree^{-1} year^{-1}. This information is valuable for planning the use of *B. alicastrum* as a seed source for use as food. Previous studies had reported seed production values lower than those obtained in the present research.

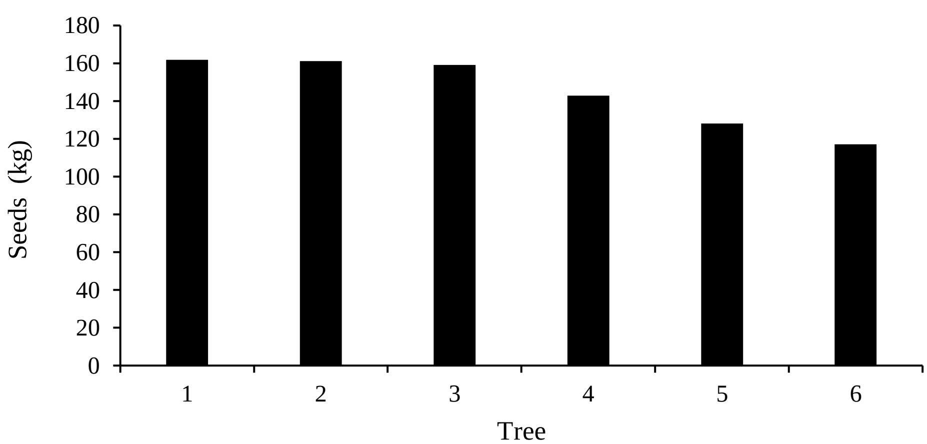

Figure 2. Seed production in six adult trees of *B. alicastrum*, on the campus of the Scientific Research Center of Yucatán.

González (1939); Pardo-Tejeda and Sánchez (1980); Peters and Pardo-Tejeda (1982) indicate values ranging from 32 to 95 kg tree^{-1} year^{-1}, well below the volume of production (145 kg tree^{-1} year^{-1}) recorded in this study. This value is high compared to the volume of edible seed production recorded for other tree species such as: pecan [*Carya illinoinensis* (Wangenh.) K. Koch] that produces 100 kg year^{-1} (Tarango, 2012), olive tree (*Olea europea* L.) with 22 kg year^{-1} (Grijalva *et al*., 2009), pistachio (*Pistacia vera* L.) 15 kg year^{-1} (De León *et al*., 2020), Mexican pinyon (*Pinus cembroides* Zucc.) 0.9 kg year^{-1} (Sánchez *et al*., 2005) and sweet chestnut (*Castanea sativa* Mill.) 50 kg year^{-1} (Enrique, 2022).

Leaf retention

As *B. alicastrum* is an evergreen tree, it was considered important to examine leaf retention, as leaves are likely to be the plant organ showing the greatest plasticity, which allows them to adapt morphologically, anatomically and physiologically to interactions with different environments. For example, leaves that grow in low light have been shown to retain their leaves longer on the tree (Coste *et al*., 2011; Craven *et al*., 2011; Kitajima *et al*., 2013).

The results show that the median of the leaf retention period in *B. alicastrum* was 25.82 months (787 days). There were no significant differences in the mean span of leaf retention between the four monitored plants [Log Rank (Mantel-Cox), p= 0.229]. Figure 3 shows the pattern of leaf retention in the four trees examined. As can be seen, the first three leaves (8%) shed after 15 months (457 days), and the last ones remained on the trees until the end of the experiment (40 months or 1 217 days). These results are consistent with those reported for the same species in a forest on the Buena Vista Peninsula, Panama, that is, an average retention span of 614 days for the leaves of trees growing in forest clearings and 1 028 days for those in closed forest (Kitajima *et al*., 2013).

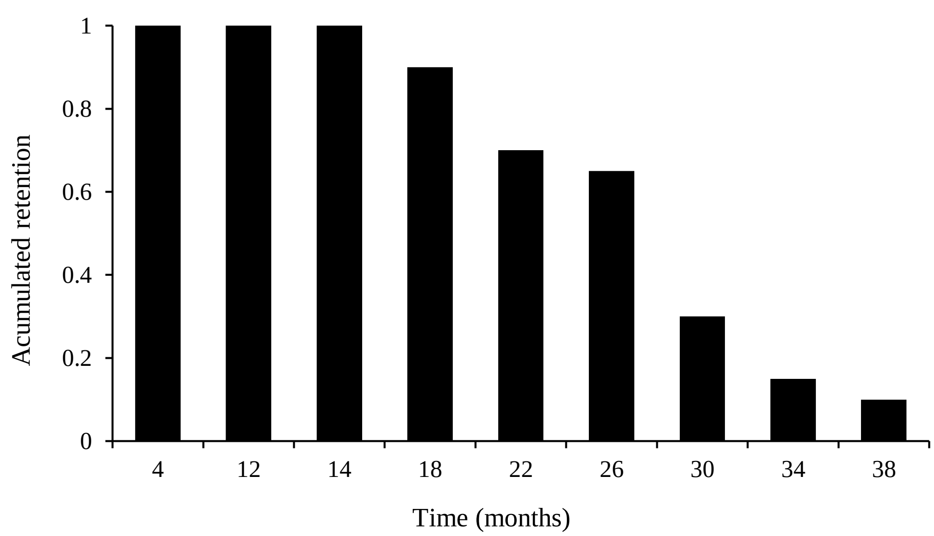

Figure 3. Leaf retention pattern in *B. alicastrum* trees. The values shown are the average of 38 leaves on four trees.

Although in this study the leaves were retained for up to 40 months, a plant is considered evergreen even if its leaf retention period is shorter, but they are continuously replaced. That is, the perennial condition depends on the entire canopy of the plant and not on the individual leaves (Kikuzawa and Lechowicz, 2011). This information is useful for the establishment of commercial plantations, especially since leaf biomass can be used as food for cattle (Peters and Pardo-Tejeda, 1982).

PGR levels

PGR accumulation in foliage was studied to determine endogenous levels in male and female trees. In general, the foliage of female trees had a higher content of gibberellins (GA) relative to males (Table 2). There were no significant differences between sexes in the content of indoleacetic acid (IAA) auxin. Abscisic acid (ABA) is another PGR identified as a participant in sexual differentiation in plants (Wang *et al*., 2002). However, no significant differences were found in the level of ABA between sexes (Table 2). Where highly significant differences were detected was in the cytokinin (CT) content, since the content in female trees was 8.7 times higher compared to that of male trees (Table 2).

Table 2. Levels of plant growth regulators in female and male trees of *B. alicastrum* from the locality of Xoccheila, Mérida, Yucatán.

Sex	GA	CT	IAA	ABA
Female	93.11 ±7.57 a	465.12 ±79.29 a	5.12 ±2.63 a	0.509 ±0.25 a
Male	66.07 ±7.83 b	53.33 ±14.23 b	4.15 ±3.87 a	0.274 ±0.04 a

Values in ng mg^{-1} dry weight. GA= gibberellic acid; CT= cytokinins; IAA= indoleacetic acid; ABA= abscisic acid. Different letters in the same column denote significant differences ($p< 0.05$) between male and female trees.

Discussion

Sex determination of *B. alicastrum* trees that are part of forest plantations for seed production is crucial. Ramón is a monoicous plant, so females are the ones that produce and contain fruits. Recent studies on sex assignment mechanisms in plants have clearly demonstrated that angiosperms, including crop plants, possess sex assignment mechanisms involving several genetic (sex chromosomes) and epigenetic factors such as: PGR, light intensity, photoperiod, among others (Khryanin, 2002; Golenberg and West, 2013).

Regarding the sex that the trees expressed for the first time after transplantation, 66% of male and 34% of female trees were found in the sample of 50 trees, similar to the overall sex ratio found in the 456 trees of the three plantations examined: 64% males and 36% females. Observations show that the sex of trees does not change with age or plant height (Hernández *et al*., 2015). The determination of male or female trees occurred when reaching at least seven years, 4 m in height and more than 7 cm in diameter at breast height (dbh).

The sex registered for the first time did not change during the following six years, at which time this study ended. These results contradict what was reported by Peters (1989), who did not observe short young or male trees in natural populations. The trees studied grew under natural conditions, which did not appear to have affected sex assignment. According to Khryanin (2002), the balance between cytokinins and gibberellins is known to play a central role in sex assignment in plants. It has been cited that gibberellins favor the differentiation of male plants, while cytokinins stimulate the differentiation of female plants (Golenberg and West, 2013), this pattern, however, does not occur in all species.

In this study, female trees had a significantly higher gibberellin content than male trees (Table 2). This trend is contrary to that observed by Khryanin (2002), who points out that a higher content of gibberellins promotes sexual differentiation towards the male gender in annual plants. Evidence collected since the 1980s shows that the application of cytokinins in species such as grapes (*Vitis vinifera* L.) favors the development of female inflorescences. More recently, high cytokinin levels have been shown to have a feminizing effect on *Arabidopsis* (Riefler *et al*., 2006). In this study, highly significant differences in cytokinin content were found between sexes.

The leaves of female trees had much higher concentrations of cytokinins than those of male trees. This would suggest that cytokinins have a feminizing role in *B. alicastrum*, a fact consistent with the findings of Khryanin (2002) for other species. The information gathered in this 10-year study will be very useful in planning in the future the establishment of commercial plantations for seed or biomass production, thus contributing to improved food security.

Conclusions

In the *B. alicastrum* tree there is a greater predominance of male trees, which is linked to the contents of growth regulators present in trees, mainly cytokinins, and that there is no change of sex over time as has been reported. The beginning of flowering occurs between seven and ten years after establishment at the planting sites, with higher and lower occurrence between eight and ten years respectively, the average seed production of adult trees was 145.6 kg tree^{-1} year^{-1} and the leaves on the trees can last up to a period of time of 40 months.

The results of the study also show that *B. alicastrum* has a high productivity, therefore, commercial plantations can be established for the production of fodder and seeds that can be used for human consumption.

Acknowledgements

To Roger Sulub, Oscar Eduardo Calderón and Israel García Sesheña for their support in field collections and sampling.

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