Journal of Animal & Plant Sciences, 35(3): 2025, Page: 701-712
ISSN (print): 1018-7081; ISSN (online): 2309-8694
https://doi.org/10.36899/JAPS.2025.3.0059

REPRODUCTIVE CONSTRAINTS AND CONSERVATION STRATEGIES FOR THE ENDANGERED *Liquidambar orientalis*: IMPLICATIONS FOR RESTORATION ECOLOGY

V. Eroğlu

Department of Botany, Faculty of Science, Ege University, İzmir, Turkiye
Corresponding author's e-mail: volkan.eroglu@ege.edu.tr
ORCIDs: https://orcid.org/0000-0003-4868-5988

ABSTRACT

Liquidambar orientalis, a relict and endangered species endemic to the Mediterranean region, is of significant conservation concern. Understanding the reproductive biology of this species is crucial for developing effective conservation strategies. This study investigated the reproductive strategy of *L. orientalis*, focusing on sex ratios, flower developmental stages, stigma receptivity, pollen viability, phenology, and ovule and seed production success. It was determined that *L. orientalis* had a trimonoecious reproductive system with male, female and hermaphrodite flowers on the same individual. It was discovered that approximately 85% of the inflorescences on the individual had only male flowers, 15% had both male and female or male and hermaphrodite flowers, and that in pistillate inflorescences with both sexes; 30% had female and hermaphrodite flowers at the terminal part of the inflorescence, while 70% had female flowers at the basal part of the inflorescence. In the reproduction of the *L. orientalis*, the first problem identified as the disadvantage caused by the flowers exhibiting stigma-pollen interaction for a very limited time during the 2.5-month flowering period. Another reproductive challenge was the low success rate of ovule-to-seed conversion, with only 2.25% success in terminal pistillate flowers and 5.8% in basal pistillate flowers, primarily due to the constraints of maternal resource allocation. Consequently, the low reproductive success of this rare species suggested that *L. orientalis* would struggle to regenerate naturally if its habitats are compromised. As a solution, we emphasized the importance of identifying populations with the highest reproductive success in their natural habitats, establishing gene conservation areas for seed breeding, and prioritizing restoration efforts using seeds from these genetically diverse populations, rather than relying on monoclonal propagation techniques, to preserve the genetic pool of the species.

Key words: *Liquidambar orientalis*, Reproductive biology, breeding system, forest conservation.

Published first online May 09, 2025 Published final June 26, 2025

INTRODUCTION

Liquidambar orientalis Mill. (Oriental Sweetgum) belongs to the Altingiaceae family and is a Mediterranean species of the *Liquidambar* L. genus, represented by 15 different species worldwide (IPNI, 2024). This species is only distributed in South-western Anatolia and the Island of Rhodes and is important as an endemic species (Govaerts, 2024). Oriental Sweetgum is the only species among other sweetgum species that is in the endangered (EN) category on the IUCN Red List (Kavak and Wilson, 2018). This situation requires urgent and effective work to protect the species.

Numerous studies have been conducted on the ecological requirements and micropropagation of this wind pollinated endangered species (Mercan *et al*., 2022; Kenar, 2024; Ürker and Günlü, 2024). However, studies focusing on its floral characteristics and reproductive system of are relatively scarce (Ickert-Bond *et al*., 2015; Klocko *et. al.*, 2020; Smilyanets *et al*., 2024). Existing research is predominantly centered on the morphology and anatomy of infructescence. The only specific study on the reproductive biology of *L. orientalis* was conducted by Ickert-Bond *et al*. (2015), which examined only the morphology and anatomy of flowers. In this context, lack of information about L. *orientalis* regarding reproductive success and reproductive strategy has the potential to lead to the development of incorrect strategies in planning the conservation strategies of this rare species.

Sexual dimorphism in angiosperms occurs in different morphotypes, including dioecy (unisexual individuals), gynodioecy (populations with both hermaphroditic and female individuals), and androdioecy (populations with both hermaphroditic and male individuals) (Barrett, 2002). A rarer system, trioecy, involves the coexistence of males, females, and hermaphrodites within the same population (Godin, 2022). Despite its rarity, trioecy provides valuable insights into the evolution of sexual systems, sex

allocation strategies, and the genetic basis of sex determination (Pannell and Jordan, 2022; Takahashi *et al*., 2023). Sexual allocation is quite common in Altinginaceae and is seen in many species (Croizat, 1947). In some female flowers of *Liquidambar* species in this family, the stamens are completely absent, in others they are reduced to staminode-like structures, and in male flowers the pistils are completely absent (Endress, 1993; Wisniewski and Bogle, 1982). However, since such a conclusion was reached without conducting pollen viability and stigma receptivity tests, the sex distribution of *Liquidambar* species needs to be comprehensively reconsidered. Research on reproductive biology can provide critical insights for the conservation, breeding, and restoration of biodiversity (Moza and Bhatnagar, 2007). Such studies are also essential for developing strategies to preserve the genetic potential of rare species, contributing significantly to restoration and regeneration efforts (Ramírez, 2006).

The evaluation of pollen viability and stigma receptivity is crucial in understanding the reproductive success of flowering plants (Dafni and Firmage, 2006). - Pollen viability tests, such as the dehydrogenase activity, provide insights into the metabolic activity and health of pollen grains, indicating their potential to successfully germinate and fertilize the ovule (Rodriguez-Riano and Dafni, 2000). Stigma receptivity tests, including esterase and reactive oxygen species (ROS) detection assays, reveal the readiness of the stigma to receive and support pollen germination (Shivanna and Sastri, 1981; Foreman *et. al.,* 2003, Zafra *et al*., 2010). These tests are vital for identifying the optimal conditions and timing for successful pollination, thereby enhancing our understanding of plant reproductive biology and aiding in the development of strategies for improving crop yield and biodiversity conservation (Harder *et al*., 2016).

By comparing the potential reproductive output to the actual reproductive success, researchers can identify factors that influence fertility, such as environmental conditions, genetic diversity, and the presence of pollinators (Gutiérrez *et al*., 2015; Ellegren and Galtier, 2016; Bauer *et al*., 2017). Additionally, this information helps in assessing the reproductive strategies of the plant, determining reproductive fitness, and making informed decisions for conservation and agricultural practices (Bouttier and Morgan,1992). Understanding these dynamics is essential for managing plant populations, and preserving biodiversity.

This study aims to determine the most appropriate conservation strategy by evaluating the reasons behind the limited distribution of the endangered and rare *L. orientalis* through its reproductive system. To achieve this, we (1) determined the sexual distribution of flowers, (2) examined the functional floral developmental stages, (3) assessed pollen-pistil interactions across different floral stages using enzymatic activity tests, and (4) investigated the reproductive potential in different floral infloresence.

We hypothesize that (H_1) *L. orientalis* does not exclusively produce unisexual flowers, as sex separation is commonly observed among members of the Altingiaceae family, suggesting the possible presence of both unisexual and bisexual flowers within the species; (H_2) pollen viability and stigma receptivity may impose significant limitations on reproduction, since in narrowly distributed species, reduced pollen viability or limited stigma receptivity often act as key constraints on successful fertilization and seed formation; and (H_3) seed production in *L. orientalis* is expected to be low, as relict and narrowly distributed species typically exhibit reduced reproductive output, likely due to genetic bottlenecks, or environmental constraints affecting reproductive success.

MATERIALS AND METHODS

Material and study area: Specimens of *Liquidambar orientalis* were chosen from the Günnücek National Park located in Muğla, Marmaris, Türkiye. Studies were conducted on 50 mature wild individuals. The flower and fruit samples to be studied were carefully selected, labeled and their flowers photographed, and collected for testing at various stages of development with a total of 6 field studies carried out every 2 weeks during January and March 2024. Fruit samples were collected and photographed between June and July 2024 (accession numbers Ege 44318, 36.8498N, 28.286941666E WGS84; approximately 10 m above sea level). The study areas have a warm and temperate climate; The average annual temperature, sunshine duration, rainy day and rainfall amount are 15.1 °C (max: 21.3 °C, min: 9.6 °C), 7.2 hours, 93.6 days and 1206.1 mm, respectively (Turkish State Meteorological Service, 2024).

Experimental environment: To obtain standard data, all experiments were carried out in the ecology laboratory of the Ege University Botanical Garden and Herbarium Research and Application Center, where temperature, light and humidity values were constant. The temperature of the laboratory where the experiment was carried out was fixed at 25°C, the illumination amount was 1500 lumens and the humidity rate was 50%. In each of the tests (pNPA, MTT, DAB, TMB, X-Gal and Aniline blue), 30 anther and 30 stigma samples were taken for each developmental flower stage (in the X-gal (4th stage) and Aniline blue test (3rd and 4th stage), samples were selected based on activity).

Functional floral development and Phenology: Flower samples were randomly selected and a total of 50 flower samples from each flower stage were collected and transported to the laboratory (Figure 4). To examine the

structural features of the flowers, the samples were examined under an Olympus SZ61 stereomicroscope and measurements were made with a Dino-eye AM7025X camera. To understand the functional characteristics of flowers, phenological stages were observed in their natural environment. The changes in the flowers from bud to full bloom were carefully monitored with Nikon Z9 camera. In particular, pistillate and staminate flowers characteristics were examined at each stage. Flower phenology observations were made between January 2024 and November 2024 to determine the flower axis, flower bud, flowering period, capsule and seed dispersal times.

p-NPA test for detection of esterase: To detect esterase activity in pistils, 63.4 mg p-Nitrophenyl Acetate (Sigma, N 8130) was dissolved in 1 mL methanol and stored in the refrigerator. Prior to use, the p-NPA solution was diluted with ultra-pure water (at a ratio of 1:100) to obtain a working solution with a concentration of 3.5 mM (Haagen and Brock, 1992; Kowacz and Warszyński, 2019). Pistils were incubated in the working solution for an average of 5 minutes and then photographed under a microscope. Stigmas showing yellow or dark coloration were considered positive for esterase activity.

MTT Test for Dehydrogenase Detection: MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Sigma M-2128, 10mg) was dissolved in a 5% sucrose solution and stored as a stock solution in a dark environment at 2-8°C. The stigma sample was directly immersed in the reagent within an Eppendorf tube. For pollen samples, 5-10 µl of reagent droplets were placed on a slide, allowed to dry, and the process was repeated. After 10 minutes, viable pollen and receptive stigmas turned purple. (Rodriguez-Riano and Dafni, 2000).

TMB Test for ROS/H_2O_2 Detection: ROS/H_2O_2 localization was performed by immersing stigmas and pollen in a solution containing the ROS indicator dye TMB (3,39,5,59-tetramethylbenzidine-HCl Sigma T-8768, TRIS-acetate (Tris(hydroxymethyl)-aminomethane Acetate) TCI T-3294 at 0.1 mg/ml, pH 5.0) until a blue color developed (McInnis *et al*., 2006). Blue staining indicated H_2O_2 production in stigmas and pollen.

DAB Test for Peroxidase Detection: The DAB test was prepared by mixing 1% DAB (3,3′-Diaminobenzidine Sigma D-12384), 3% H_2O_2, and water in 60% ethanol in a V:V:V ratio of 4:11:22. The stigma sample was directly immersed in the reagent within an Eppendorf tube, while the pollen sample was placed in 5-10 µl reagent droplets on a slide, mixed thoroughly to prevent clumping, allowed to dry, and the process was repeated (Dafni, 1992). The viability of pollen grains and the receptivity of stigmas were determined by brown coloration.

X-GAL Test for Galactosidase Activity Detection: 1 mg X-Gal (5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside) Sigma B-4252 was mixed with 5 µl N.N-dimethyl formamide Serva 39756 and1 ml acetate buffer (50 mmol, pH-4.89) to prepare the incubation medium. Pollen grains were placed in the incubation medium until they turned blue, indicating galactosidase activity (Trognitz and Schmiediche, 1993). MTT, TMB, and DAB tests were conducted at the 4th stage of the flower with the highest activity.

Aniline Blue Epifluorescence Test for Detecting Pollen Behavior on Stigma Surfaces: Fixation: Pistils extracted from flower samples were fixed in a Formaldehyde Alcohol Acetic Acid (FAA) solution containing 95% ethanol, acetic acid, 37% formalin in a 10:1:2 (v/v/v) ratio. Samples were stored in this solution for at least 36 hours, then rinsed three times with distilled water for 30 minutes before examination.

Softening and Cleaning Tissues: The washed samples were transferred to a 4 N sodium hydroxide (NaOH) solution immediately. After four to six days, when the tissues were softened, they were rinsed three times with distilled water for 30 minutes.

Staining: A 0.1% K_3PO_4 buffer was prepared and adjusted to pH 11 A 0.1% aniline blue solution was prepared in the buffer and left at 25°C for about 12 hours until it became clear. The softened and cleaned pistils were incubated in the clear aniline blue solution for approximately 10-12 days.

Examination: The pistils were then placed on a slide with 1-2 drops of 50% glycerol, squashed with a cover slip, and examined under a fluorescence microscope for photography (Martin, 1959).

Reproductive success: To determine the number of seeds and ovules under natural conditions, 30 terminal and 30 basal pistillate flowers were randomly selected. 5 months after flowering, all mature fruits were collected and the number of seeds and ovules in each fruit were counted using light microscope. Reproductive success was calculated from the average number of seeds produced per flower and the average ovule production.

Statistical analysis: Differences between pollen viability in the developmental stages of the flower were evaluated with one-way ANOVA. Statistical results of flower, ovule and seed counts were performed using Multiple t test. All statistical analyses were performed using GraphPad Prism 8.

RESULTS

Functional floral development and Phenology: *Liquidambar orientalis* has a spirally and pyramidally arranged inflorescence. The flowers are fused into tight heads arranged in a conical, complex inflorescence

structure. Each inflorescence contains approximately 10-15 flower heads. The flower heads are naked, with each head having two small and two large bracts at its base. Most of the inflorescences (about 85%) consist only of staminate flower heads, while a smaller portion (about 15%) contains both pistillate and staminate flower heads (Figure 1). Inflorescences with pistillate flower heads typically begin with a long-stalked pistillate head, followed by the remaining male heads arranged from short-stalked to sessile. Pistillate flower heads can occasionally be found at the top of the inflorescence, but these are usually stalkless or have very short stalks, and are sometimes fused together. Approximately 30% of the inflorescences with pistillate flower heads have these heads emerging from the top of the inflorescence, while 70% have them emerging from the bottom. The anthers in the pistillate flower heads at the bottom of the inflorescence are sterile, whereas those at the top can be either sterile or fertile. Typically, there is one (rarely two) pistillate flower head at the bottom of the inflorescence, while there are about 1-4 pistillate flower heads at the top (Figure 1, Figure 2). In both cases, when examining the pistillate flower heads, the ones at the bottom of the inflorescence are found to produce larger fruits compared to those at the top. Staminate flower heads shed pollen shortly before falling off, starting from the distal part.

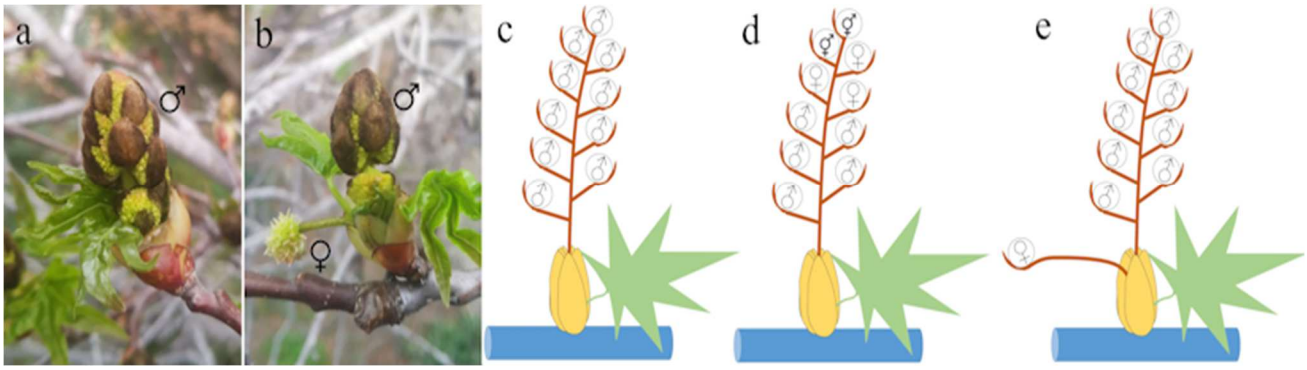


Figure 1. Inflorescences types according to gender distribution in *Liquidambar orientalis* a) staminate inflorescence b) pistillate inflorescence c) staminate type d) terminal pistillate type e) basal pistillate type

Figure 2. Fruits from the pistillate inflorescence of *Liquidambar orientalis* a) terminal pistillate b) single from the basal pistillate c) double from the basal pistillate

Each basal pistillate flower head contains a total of 48.6 (±3.95) and terminal pistillate ones 23,2 (±3.49) flowers, with each flower surrounded by six extrorse anthers arranged around the pistil. In these flowers, the carpels are covered by a sterile fleshy phyllome. Although the ovary appears to be two-chambered in the fruit, it is actually a single chamber because the upper parts of the carpels merge with centrally facing openings. In unpollinated flowers, these openings are clearly visible, but after pollination, they close tightly, giving the ovary the appearance of being two-chambered in the fruit. (Figure 3).

In the first stage of flower development, bracts initially develop. Following the bract development in pistillate heads, the stigmas begin to elongate (Figure 4a). In staminate flowers, after bract development, the anthers become prominent (Figure 4e). During the second stage, the stigmas of pistillate flowers continue to elongate and start to curl (Figure 4b). Meanwhile, in staminate flowers, anther development progresses (Figure 4f). In the third stage, anthers start to develop in pistillate flowers, and it is observed that the elongation of the style slows down (Figure 4c). In staminate flowers, the anthers have fully matured (Figure 4g). In the fourth stage, anther

maturation in pistillate flowers ceases, and the stigmas continue to elongate until they reach their final size (Figure 4d). In staminate flowers, the anthers dehisce longitudinally, releasing their pollen (Figure 4h).

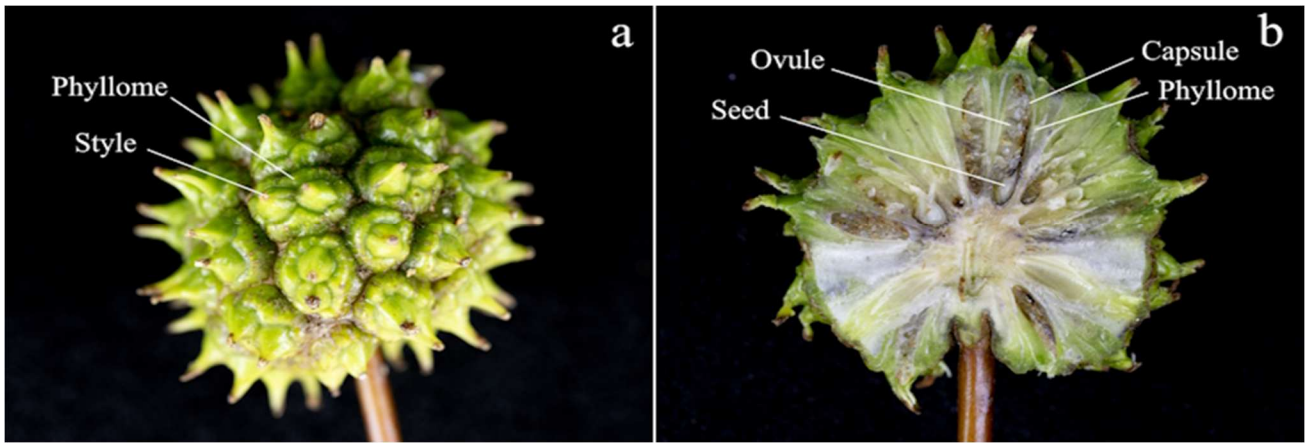


Figure 3. Fruit structure of *Liquidambar orientalis* a) external view b) longitudinal section

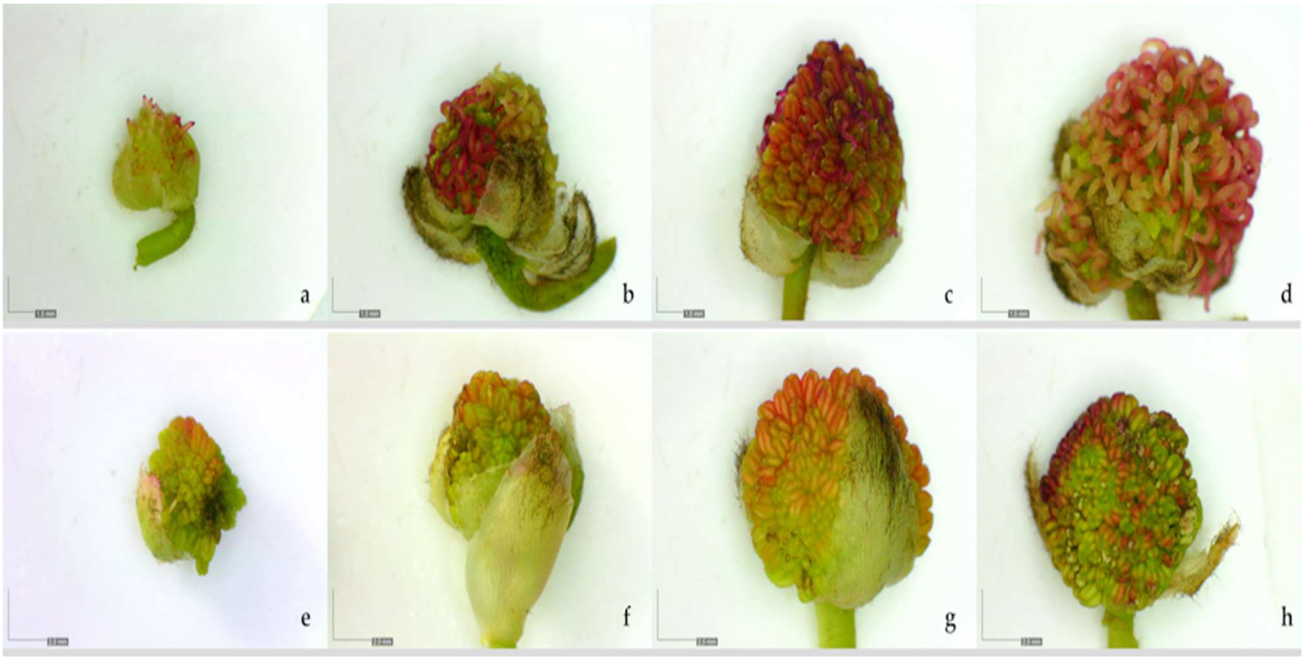


Figure 4. Developmental stages of staminate and pistillate flowers. a-d) pistillate flowers e-h) staminate flowers. a) bud stage (pistillate) b) style elongation stage c) anther growth stage (female phase) d) anthers opening stage (male phase) e) bud stage (staminate) f) anthers development stage 1 g) anthers development stage 2 h) anthers opening stage (male phase)

Based on the flower phenology observations, it was determined that the duration from the flowering stage to the completion of seed dispersal for the species spans 10 months (Table 1). During this period, flower axes begin to develop in mid-January and continue until the end of March. Following the formation of flower axes, flower buds are observed from February, with bud formation continuing until mid-April. The maturation and blooming of flowers start by mid-February and persist until the end of April. Following pollination and fertilization, fruit development occurs from the first week of April until mid-September. The fruits begin to dry, open and disperse their seeds in early September. Although this process occurs throughout the year, the majority of the seeds are dispersed by mid-November.

Stigma receptivity, pollen viability and pollen germination tests: Esterase activity has been detected in the stigmas throughout all developmental stages of the flower. In the first stage of the flower, activation is observed at the distal parts of the stigma and style, while

from the second stage onwards, esterase activity has been detected throughout the entire style until the final stage. In the second and third stages of the flower, not all styles exhibit esterase activity. As the flower develops, esterase activity gradually increases, and in the fourth stage, all stigmas exhibit esterase activity (Figure 5, Table 2).

Table 1. Flower phenology observations of *Liquidambar orientalis*.

	January		February		March		April		May		June		July		August		September		October		November	
Weeks	1-2	3-4	1-2	3-4	1-2	3-4	1-2	3-4	1-2	3-4	1-2	3-4	1-2	3-4	1-2	3-4	1-2	3-4	1-2	3-4	1-2	3-4
Flower axis																						
Flower bud																						
Flowering period																						
Unopened Fruit																						
Seed dispersal																						

In the MTT test, dehydrogenase activity was detected in the stigmas at all stages of the flower. During developmental stages 1, 2, and 3, activity occurred at a moderate level, while in the fourth stage, the large portion of the styles turning purple is an indicator of increased activity in the stigmas. Activity is observed in the entire stigma and style throughout all flower stages (Table 2). However, in the pollen, no dehydrogenase activity was observed in the first and second stages of the flower, with activity beginning in the third stage, when the flower transitions to presentation, and reaching its peak in the fourth stage (Figure 5). In this context, activities of 75.6% (±3.8) in the third stage and 94.4% (±2.3) in the fourth stage were calculated (Table 3).

In the TMB test, endogenous H_2O_2 was detected only in the third stage of the flower (Table 2). In the stage where activity was observed, not all stigmas turned blue, with activity detected in only a few stigmas. No activity was observed in the pollen at any flower development stage (Figure 5).

Using the DAB test, peroxidase activity in stigmas was observed to gradually increase with flower development. Activity that begins at the tips of the stigmas in the first stage of the flower extends towards the style in the third stage and is observed throughout the stigma and style in the fourth stage (Table 2). In the pollen, peroxidase activity was observed to increase progressively from the flower bud stage to the maturity stage (Figure 5). In this context, peroxidase activity of 63.4% (±5.3) in the first stage, 71.3% (±3.6) in the second stage, 96.4% (±1.7) in the third stage, and 96.1% (±2.3) in the fourth stage was detected (Table 3).

The highest stages of MTT and DAB test results coincide with the fourth stage when the flower has finished shedding its pollen, and the X-Gal test was applied to the pollen, revealing 85.9% (±4.5) galactosidase activity (Figure 5, Table 3). It was noted that pollen with deformed cell structure also showed activity.

In the Aniline Blue test, pollen tubes were observed to begin germinating in the third stage of the flower. In the fourth stage, newly germinating pollen and pollen tubes extending into the style were observed (Figure 5).

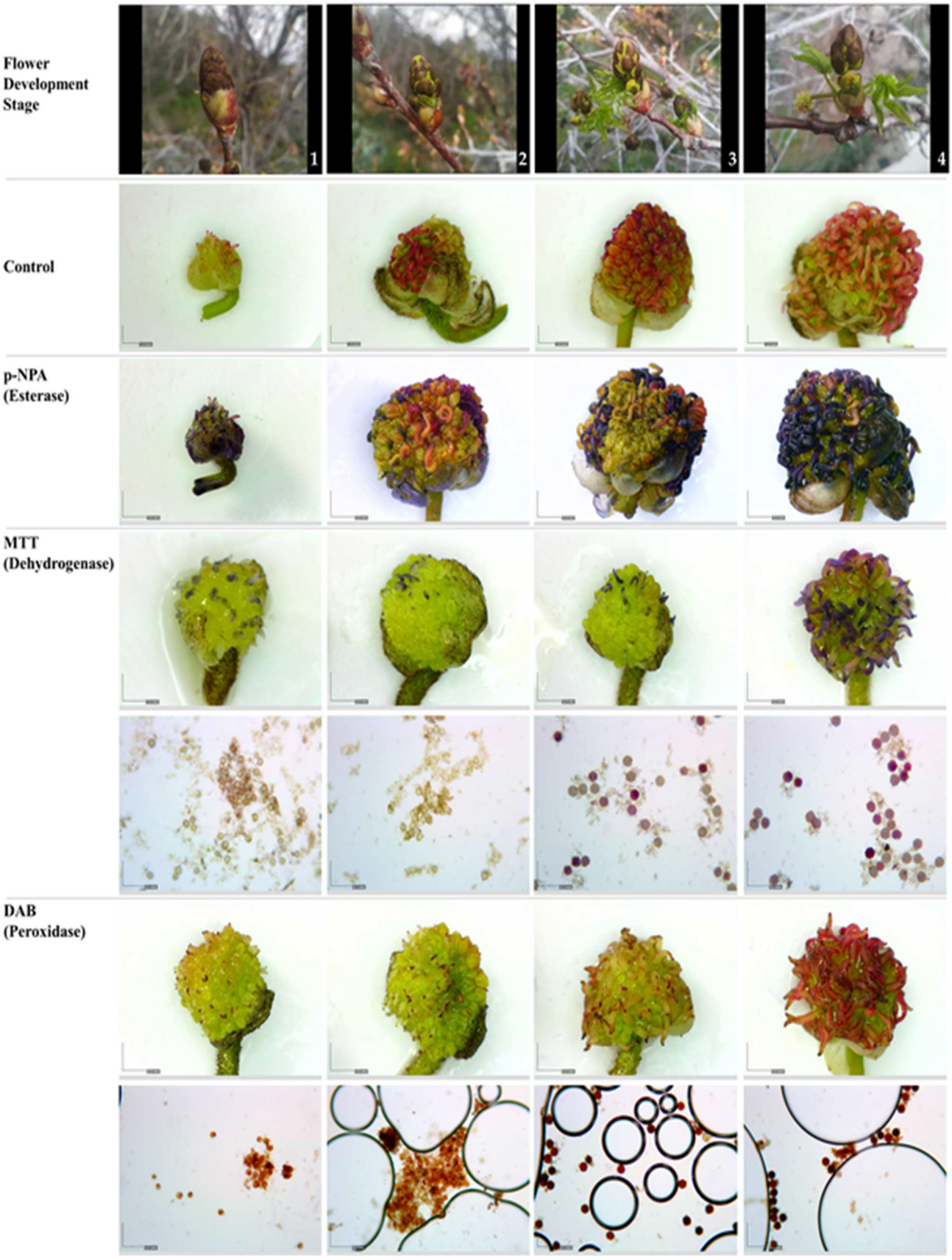
Table 2. Chromogenic enzyme activity test results applied to stigma depending on flower development stages.

Flowerdevelopmentstage	p-NPA (Esterase)	MTT (Dehydrogenase)	DAB (Peroxidase)	TMB (H_2O_2)
1st stage	+	++	+	-
2nd stage	++	++	++	-
3rd stage	+++	++	+++	+++
4th stage	++++	++++	++++	-

(-) no reaction; (+) weak reaction; (++) medium reaction; (+++) strong reaction; (++++) very strong reaction.

Table 3. Chromogenic enzyme activity test results applied to pollen depending on flower development stages. Boldface indicates significant effects (p<0.05).

	Flower Developmental Stages				ANOVA results	
Test	1st stage (Mean±SD)	2nd stage (Mean±SD)	3rd stage (Mean±SD)	4th stage (Mean±SD)	F value	P value
MTT (%)	0 ± 0	0 ± 0	75.6 ± 3.8	94.4 ± 2.3	15006	<0.001
DAB (%)	63.4 ± 5.3	71.3 ± 3.6	96.4 ± 1.7	96.1 ± 2.3	704	<0.001
TMB (%)	0 ± 0	0 ± 0	0 ± 0	0 ± 0		
X-Gal (%)	-	-	-	85.9 ± 4.5		


Flower Development Stage
1
2
3
4
Control
p-NPA (Esterase)
MTT (Dehydrogenase)
DAB (Peroxidase)

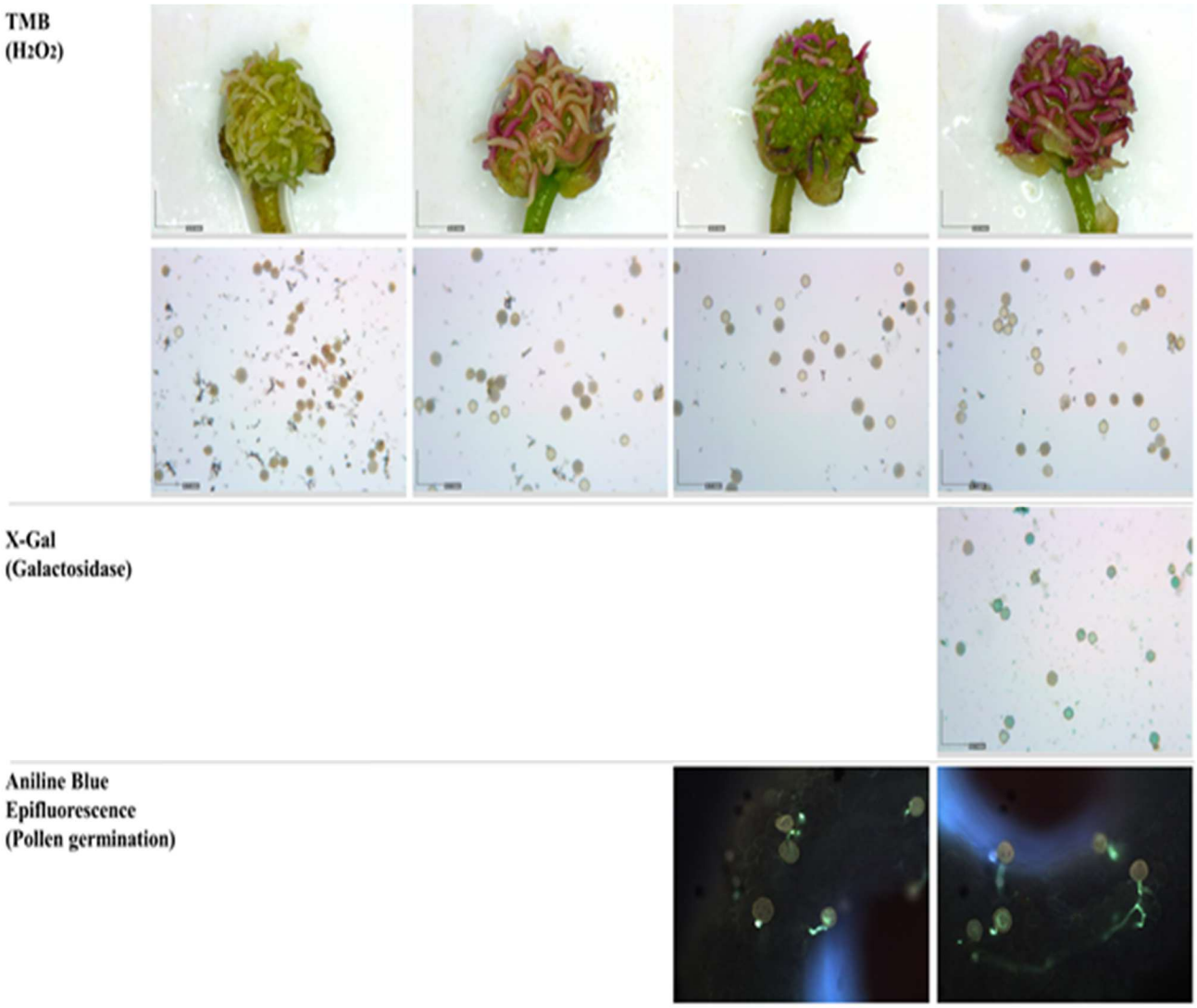


Figure 5. Patterns of chromogenic enzyme activity tests and polen tube observation tests in stigma and pollen depending on flower development stages in *Liquidambar orientalis*.

Reproductive success: Pistillate flowers from the terminal position produced an average of 23,2 (±3.49) flower and 35.5 (±3.41) ovules per flower, with a range of 30 to 40 ovules. These flowers produced an average of 0.8 (±0.92) seeds per flower and demonstrated a seed production success rate of 2.25%. In contrast, pistillate flowers from the basal position had a slightly lower average of 48.6 (±3.95) flower and 34.5 (±3.54) ovules per flower, ranging from 28 to 38 ovules. However, these basal flowers had a higher seed production, averaging 2 (±0.82) seeds per flower, and a notably higher seed production success rate of 5.80%. These results indicate a significant difference in reproductive success based on the flower's position within the inflorescence (Table 4).

Table 4. Reproductive success of *Liquidambar orientalis.* Boldface indicates significant effects (P < 0.05).

Observed Variables	Terminal pistillate head			Basal pistillate head			Statistical analysis	
	Mean	SD	Number of samples	Mean	SD	Number of samples	t ratio	P value
Flowers per head	48.6	3.8	30	23.2	5.3	30	21.46	**<0.001**
Ovules per flower	35.5	3.3	30	34.5	3.4	30	1.16	0.25
Seeds per flower	0.8	0.9	30	2	0.8	30	5.59	**<0.001**
Seed Production succes (%)		2.25			2.8			

DISCUSSION

The reproductive strategy of *Liquidambar orientalis* is characterized by a complex and highly structured inflorescence, arranged in a spiral and pyramidal pattern. The inflorescences can consist exclusively of male flowers or include a combination of male, hermaphroditic, and female flowers. This phenomenon, referred to as trimonoecy, is rarely observed among angiosperms (Torices *et al*., 2011; Renner, 2014).

Phenological observations reveal that *L. orientalis* undergoes an extensive reproductive cycle lasting approximately 10 months, from the initial development of the flower axis in mid-January to the completion of seed dispersal in mid-November. This extended period allows for the sequential development and maturation of reproductive structures, optimizing the timing for pollination and subsequent seed development. The length of the flowering process is known to increase the seed production rate in plants (Rodríguez-Pérez and Traveset, 2016). In this context, the 2.5-month flowering period of *L. orientalis* provides a significant advantage for the species' reproduction.

It has been frequently emphasized that understanding pollen-stigma interactions requires the use of multiple tests (Ferreira *et al*., 2021; Hao *et al*., 2022; Sharma *et al*., 2023; Taylor and Williams, 2009). Therefore, in our study, we employed multiple assays to investigate pollen viability and stigma receptivity. Among the tests we used, MTT indicated the presence of respiration through dehydrogenase activity, DAB revealed whether ROS produced as a result of respiration were scavenged by peroxidases, TMB detected the presence of H_2O_2, which serves as a signaling molecule in pollen germination on stigmas, and X-GAL demonstrated galactosidase activity, which reflects the degradation of polysaccharides and glycoproteins as an indicator of cellular activity. These four assays are directly related to cellular functions and cell viability. Recent studies have demonstrated that for pollen grains to germinate on stigmas, H_2O_2 must be present at higher levels than other ROS (Ali and Muday, 2024; Anjum *et al*., 2022; Breygina *et al*., 2021, 2023). We observed that dehydrogenase activity in the stigmas of *L. orientalis* gradually increased throughout all floral stages, and that peroxidase activity, which functions to scavenge ROS generated during respiration, also increased in parallel. At the third floral stage, the TMB assay indicated that peroxidases were insufficient to scavenge H_2O_2, suggesting that stigmas could accept pollen only at this stage due to the presence of H_2O_2. In pollen grains, dehydrogenase and peroxidase activities were detected exclusively in the third and fourth floral stages, indicating that only pollen from flowers at these stages could germinate. However, even if the pollen was viable, it was observed that only in the 4th stage of the flower did the anthers split and spread the pollen into nature. To confirm whether pollen germination occurred specifically at this stage, we performed an Aniline Blue test, which demonstrated that pollen grains germinated exclusively at the third floral stage. The progressively increasing esterase activity in stigmas suggested that the degradation of lipids and other esters on the stigma surface facilitated a suitable environment for pollen tube growth, particularly during the third and fourth floral stages. The distribution of viable pollen and the presence of pistils with receptive stigmas only during a short period of the flower indicate a negative effect on the reproduction of *L. orientalis*. This restrictive timeframe for pollen-stigma interaction is known to negatively impact seed production (González et al., 1995).

The positional variation in reproductive success within the inflorescence underscores the differential allocation of resources and potential selective pressures. Ovule limitation occurs when a plant's ovules are fertilized, yet only a few zygotes survive genetic death and predation to compete for maternal resources. This constraint results in minimal resource investment in ovule production during flowering, potentially due to improved resource availability between floral initiation and seed production (Lloyd, 1992; Schoen *et al*., 1996; Morgan *et al*., 2005). In our study, we identified a disparity in the number of flowers and seeds produced by the pistillate heads located at the terminal and basal portions of the inflorescence. Terminal pistillate heads produced fewer flowers and ovules, whereas basal pistillate heads produced more flowers and ovules, indicating a progressive depletion of maternal resources from the base to the top of the peduncle. This finding demonstrates that basal pistillate flowers have more than twice the seed production success compared to terminal ones. Considering the number of terminal and basal pistillate flower heads produced by an individual, the seed production potential of basal pistillates is over twelve times greater. Overall, when comparing the potential number of seeds if all ovules were fertilized to the actual number produced, the species achieves only about 5% of its potential seed output. The reduction in seed production attributed to maternal resource limitation is emphasized in numerous studies, which highlight its dependence on the plant's genetic pool and organ positioning within the individual (Diggle, 2003; Cao *et al*., 2011; Liu and Huang, 2012).

In conclusion, our study elucidates the reproductive system and success of *L. orientalis*, presenting the first comprehensive investigation of the reproductive mechanisms within the *Liquidambar* genus. Our findings indicate that the seed production success of *L. orientalis* is notably low. This limitation is primarily due to the brief periods of pollen viability and stigma receptivity, compounded by low seed yield resulting from

maternal resource allocation constraints. The low seed yield may hinder the species' ability to recolonize its natural habitat following disturbances.

Conclusion: Considering its restricted pollination period and low seed production success, the use of clonal propagation methods, such as cutting propagation or meristematic tissue culture, should be discontinued in population restoration efforts for *Liquidambar orientalis*. Plantations established using clones derived from these methods may lead to a reduction in the species' genetic diversity, ultimately causing the homogenization of populations in wetland habitats within its natural distribution range. As part of conservation strategies for *L. orientalis* (Oriental Sweetgum), we recommend identifying the populations with the highest reproductive success in their natural habitats and shifting conservation efforts from clonal propagation to seed-based propagation through the establishment of gene conservation areas.

Acknowledgment: The present study was supported by the Ege University Scientific Research Projects Coordination (Project No: 28463), the Scientific and Technological Research Council of Türkiye (TUBITAK 1002-A Project No: 222Z235).

REFERENCES

Ali, M.F. and G.K. Muday (2024). Reactive oxygen species are signaling molecules that modulate plant reproduction. Plant Cell Environ. 47(5): 1592-1605. https://doi.org/10.1111/pce.14837

Anjum, N.A., S.S. Gill, F.J. Corpas, C. Ortega-Villasante, L.E. Hernandez, N. Tuteja, A. Sofo, M. Hasanuzzaman and M. Fujita (2022). Recent insights into the double role of hydrogen peroxide in plants. Front. Plant Sci. 13: 843274. https://doi.org/10.3389/fpls.2022.843274

Bauer, A.A., M.K. Clayton and J. Brunet (2017). Floral traits influencing plant attractiveness to three bee species: Consequences for plant reproductive success. Am. J. Bot. 104(5): 772-781. https://doi.org/10.3732/ajb.1600405

Barrett, S.C.H. (2002). The evolution of plant sexual diversity. Nat. Rev. Genet. 3: 274–284.

Breygina, M., E. Klimenko, E. Shilov, A. Podolyan, A. Mamaeva, V. Zgoda, and I. Fesenko (2021). Hydrogen peroxide in tobacco stigma exudate affects pollen proteome and membrane potential in pollen tubes. Plant Biol. 23(4): 592-602.

Breygina, M., O. Luneva, O. Schekaleva, N. Lazareva, K. Babushkina and I.A. Kirilyuk (2023). Pattern of ROS generation and interconversion on wet stigmas in basal and divergent angiosperms. Plant Growth Regul. 101(2): 463-472.

Bouttier, C. and D.G. Morgan (1992). Ovule development and determination of seed number per pod in oil seed rape (*Brassica napus* L.). J. Exp. Bot. 43(5): 709-714. https://doi.org/10.1093/jxb/43.5.709

Cao, G., L. Xue, Y. Li and K. Pan (2011). The relative importance of architecture and resource competition in allocation to pollen and ovule number within inflorescences of *Hosta ventricosa* varies with the resource pools. Ann. Bot. 107: 1413–1419. https://doi.org/10.1093/aob/mcr085

Croizat, L. (1947). Trochodendron, Tetracentron, and their meaning in phylogeny. Bull. Torrey Bot. Club. 60-76.

Dafni, A. (1992). Pollination ecology: a practical approach. Oxford University Press; New York (USA).

Dafni, A. and D. Firmage (2000). Pollen viability and longevity: practical, ecological and evolutionary implications. In: Dafni, A., M. Hesse and E. Pacini (eds.), Pollen and Pollination. Springer; Vienna (Austria) https://doi.org/10.1007/978-3-7091-6306-1_6

Diggle, P.K. (2003). Architectural effects on floral form and function: a review. In: Stuessy, T., V. Mayer and E. Hörandl (eds.) Deep morphology: Toward a renaissance of morphology in plant systematics. Koeltz; Königstein (Germany). 63–80 p.

Ellegren, H. and N. Galtier (2016). Determinants of genetic diversity. Nat. Rev. Genet. 17(7): 422-433. https://doi.org/10.1038/nrg.2016.58

Endress, P.K. (1993). Hamamelidaceae. In K. Kubitzki (ed.), The families and genera of vascular plants, 2. Springer-Verlag; New York (USA) 322-331 p.

Ferreira, J.A., C.A. Ledo, F.V. Souza, J.Q. Conceicao, M.L. Rossi and E.H. Souza (2021). Stigma structure and receptivity in papaya (*Carica papaya* L.). An. Acad. Bras. Ciênc. 93(1): e20190605.

Foreman, J., V. Demidchik, J.H. Bothwell, P. Mylona, H. Miedema, M.A. Torres, P. Linstead, S. Costa, C. Brownlee, J.D.G. Jones, J. M. Davies and L. Dolan (2003). Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature. 422(6930): 442-446. https://doi.org/10.1038/nature01485

Godin, V.N. (2022). Trioecy in flowering plants. Dokl. Biol. Sci.. 507: 301–311.

González, M.V., M. Coque and M. Herrero (1995). Stigmatic receptivity limits the effective pollination period in kiwi fruit. J. Am. Soc. Hortic. Sci. 120(2): 199-202. https://doi.org/10.21273/JASHS.120.2.199

Govaerts, R. (ed.) (2024). WCVP: World Checklist of VascularPlants. Facilitated by the Royal Botanic

Gardens, Kew. [online] Website http://sftp.kew.org/pub/data-repositories/WCVP/ [accessed 21 May 2024].

Gutiérrez, H.F., J.F. Pensiero and J.M. Zabala (2015). Effect of population combinations on the reproductive success and germination of seeds of *Bromus auleticus* (Poaceae). Grass Forage Sci. 70(1): 176-184. https://doi.org/10.1111/gfs.12096

Haagen L. and A. Brock (1992). A New Automated Method for Phenotyping Aryl esterase (EC 3.1.1.2) Based Upon Inhibition of Enzymatic Hydrolysis of 4-Nitrophenyl Acetate by Phenyl Acetate. Clin. Chem. Lab. Med. 30(7): 391-396. https://doi.org/10.1515/cclm.1992.30.7.391

Hao, Q., L. Xu, H. Wang, Q. Liu and K. Wang (2022). Evaluation of pollen viability, stigma receptivity, and the cross barrier between tropical and hardy water lily cultivars. Flora. 290: 152046. https://doi.org/10.1016/j.flora.2022.152046

Harder, L.D., M.A. Aizen and S.A. Richards (2016). The population ecology of male gametophytes: the link between pollination and seed production. Ecol. Lett. 19(5): 497-509. https://doi.org/10.1111/ele.12596

Ickert-Bond, S.M., K.B. Pigg and J. Wen (2005). Comparative infructescence morphology in *Liquidambar* (Altingiaceae) and its evolutionary significance. Am. J. Bot. 92(8): 1234-1255. https://doi.org/10.3732/ajb.92.8.1234

IPNI (2024). International Plant Names Index [online]. Website http://www.ipni.org, The Royal Botanic Gardens, Kew, Harvard University Herbaria and Libraries and Australian National Herbarium. [accesed 02 July 2024].

Kavak, S. and B. Wilson (2018). *Liquidambar orientalis*. The IUCN Red List of Threatened Species 2018: e.T62556A42326468 [online]. Website https://dx.doi.org/10.2305/IUCN.UK.2018-1.RLTS.T62556A42326468.en. [accessed 26 July 2024].

Kenar, N. (2024). Predicting the effect of climate change on the geographic range of the Mediterranean relict tree *Liquidambar orientalis* Mill. Nord. J. Bot.. 2024: e04250. https://doi.org/10.1111/njb.04250

Klocko, A.L., A.M. Brunner, C. Ma, E. Etherington, K. Rosenstiel, A. Magnuson, B.J. Taylor, J. Cappellazzi, T. Lockwood, N. Covarrubias, M. Bao, J.J. Morrell, S.H. Strauss, M. Bao and N. Covarrubias (2020). RNAi of AGAMOUS genes in sweetgum alters reproductive organ identity and decreases fruit persistence. Plant Direct. 4(5): e00225. https://doi.org/10.1002/pld3.225

Kowacz, M. and P. Warszyński (2019). Beyond esterase-like activity of serum albumin. Histidine-(nitro) phenol radical formation in conversion cascade of p-nitrophenyl acetate and the role of infra red light. J. Mol. Recognit. 32(8): e2780. https://doi.org/10.1002/jmr.2780

Liu, C.Q. and S.Q. Huang (2012). Does the relative importance of resource competition and architectural effect in floral variation vary with stages of floral ontogeny?. J. Syst. Evo. 50(2): 119-124. https://doi.org/10.1111/j.1759-6831.2011.00175.x

Lloyd, D.G. (1992). Self- and cross- fertilization in plants. II. The selection of self-fertilization. Int. J. Plant Sci. 153: 370–80. https://doi.org/10.1086/297041

Martin, F.W. (1959). Staining and observing pollen tubes in the style by means of fluorescence. Stain Technol. 34(3): 125-128. https://doi.org/10.3109/10520295909114663

McInnis S.M., R. Desikan, J.T. Hancock and S.J. Hiscock (2006). Production of reactive oxygen species and reactive nitrogen species by angiosperm stigmas and pollen: potential signalling crosstalk?. New Phytol. 172(2): 221-228. https://doi.org/10.1111/j.1469-8137.2006.01875.x

Mercan, T., S. Galatalı, D.E. Özkaya, O. Çelik and E. Kaya (2022). Effects of different boron salt treatments on micropropagation and genetic stability in in vitro cultures of *Liquidambar orientalis* Miller. J. Boron. 7(4): 521-527. https://doi.org/10.30728/boron.1140926

Morgan, M.T., W.G. Wilson and T.M. Knight (2005). Plant population dynamics, pollinator foraging, and the selection of self-fertilization. Am. Nat. 166: 169–83.

Moza, M.K. and A.K. Bhatnagar (2007). Plant reproductive biology studies crucial for conservation. Curr. Sci. Bangalore. 92(9): 1207. https://doi.org/10.1086/431317

Pannell, J.R. and C.Y. Jordan. (2022). Evolutionary transitions between hermaphroditism and dioecy in animals and plants. Annu. Rev. Ecol. Evol. Syst. 53: 183–201.

Ramírez, N. (2006). Reproductive biology and plant species selection for habitat restoration in the Venezuelan Gran Sabana plateau. Interciencia. 31(5): 114-124.

Renner, S.S. (2014). The relative and absolute frequencies of angiosperm sexual systems: dioecy, monoecy, gynodioecy, and an updated online data base. Am. J. Bot. 101(10): 1588-1596. https://doi.org/10.3732/ajb.1400196

Rodríguez-Pérez, J. and A. Traveset (2016). Effects of flowering phenology and synchrony on the

reproductive success of a long-flowering shrub. AoB PLANTS. 8: plw007. https://doi.org/10.1093/aobpla/plw007

Rodriguez-Riano, T. and A. Dafni (2000). A new procedure to asses pollen viability. Sex. Plant Reprod. 12: 241-244. https://doi.org/10.1007/s004970050008

Schoen, D.J., M.T. Morgan and T. Bataillon (1996). How does self-pollination evolve? Inferences from floral ecology and molecular genetic variation. Phil. Trans. R. Soc. Lond. Series B. 351: 1281–90. https://doi.org/10.1098/rstb.1996.0111

Sharma, B., G. Kalra and H. Verma (2023). Evaluation of stigma receptivity and its properties in *Helianthus annuus* L.(Asteraceae). Vegetos. 36(2): 474-483.

Shivanna, K.R. and D.C. Sastri (1981). Stigma-surface esterase activity and stigma receptivity in some taxa characterized by wet stigmas. Ann. Bot.. 47(1): 53-64. https://doi.org/10.1093/oxfordjournals.aob.a086000

Smilyanets, N., T. Vakulenko and I. Svitylko (2024). Morphological Structure of Infructescence, Fruits, and Seeds of Some Species of the Genus *Liquidambar* L. in Species Identification. Agrobiodiver. Improv. Nutr. Health Life Qual. 8(1): 108-121. https://doi.org/10.15414/ainhlq.2024.0012

Takahashi, K., S. Suzuki, H. Kawai-Toyooka, K. Yamamoto, T. Hamaji, R. Ootsuki, H. Yamaguchi, M. Kawachi, T. Higashiyama and H. Nozaki (2023). Reorganization of the ancestral sex-determining regions during the evolution of trioecy in *Pleodorina starrii*. Commun. Biol. 6: 590.

Taylor, M.L. and J.H. Williams (2009). Consequences of pollination syndrome evolution for post pollination biology in an ancient angiosperm family. International J. Plant Sci. 170(5): 584-598. https://doi.org/10.1086/597269

Torices, R., M. Méndez and J.M. Gómez (2011). Where do monomorphic sexual systems fit in the evolution of dioecy? Insights from the largest family of angiosperms. New Phytol. 190(1): 234-248. https://doi.org/10.1111/j.1469-8137.2010.03609.x

Trognitz, B.R. and P.E. Schmiediche (1993). A new look at incompatibility relationships in higher plants. Sex. Plant Reprod. 6: 183-190. https://doi.org/10.1007/BF00228647

Turkish State Meteorological Service (2024). Analysis, official statistics [online]. Website https://www.mgm.gov.tr/veridegerlendirme/il-ve-ilceler-istatistik.aspx?k=A&m=IZMIR [accessed26 July 2024]

Ürker, O. and A. Günlü (2024). Identification of plantation areas for the endangered oriental sweetgum tree (*Liquidambar orientalis* Miller, 1768) in Türkiye. Int. J. Environ. Sci. Technol. 21(1): 153-168. https://doi.org/10.1007/s13762-023-05243-7

Wisniewski, M. and A.L. Bogle (1982). The ontogeny of the inflorescence and flower of *Liquidambar styraciflua* L.(Hamamelidaceae). Am. J. Bot. 69(10): 1612-1624. https://doi.org/10.1002/j.1537-2197.1982.tb13414.x

Zafra, A., M.I. Rodríguez-García and J.D.D. Alché (2010). Cellular localization of ROS and NO in olive reproductive tissues during flower development. BMC Plant Biol. 10: 1-14. https://doi.org/10.1186/1471-2229-10-36.