

## THE REPRODUCTIVE BIOLOGY OF *MARATHRUM RUBRUM* NOVELO & C. T. PHILBRICK: MORPHOLOGICAL AND ANATOMICAL CHANGES DURING FLOWER DEVELOPMENT

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### Abstract

**Background:** Podostemaceae is the largest family of strictly aquatic angiosperms. Species of this family possess unusual morphological and reproductive characters, grow attached to rocks in rivers with strong currents, and have a life cycle tightly linked to seasonality. Due to its reduced floral structures, which characterizes most species, the analysis of developmental stages and events such as anthesis or stigma receptivity has proven difficult.

**Question:** How floral morphology, floral development and stigma receptivity of different stages of flower development of *Marathrum rubrum* Novelo & C. T. Philbrick are related among them?

**Studied species:** *Marathrum rubrum* Novelo & C. T. Philbrick.

**Study site and dates:** Horcones River, Jalisco, Mexico. January, 2014 and 2015.

**Methods:** We made an *in situ* and lab analyses of flower morphology to assess the relationships between flower morphology, stages of development, reproductive structures, and stigma receptivity, during different stages of flower development.

**Results:** When flowers emerge from the spathe, both the ovules and pollen grains are fully developed, implying that sporogenesis and gametogenesis take place within the stem while the plant is still submerged.

**Conclusions:** The morphological changes observed in the flowers of *M. rubrum* during the analyzed stages are related only to events associated with fertilization and fruit development. The time-course of morphological changes in the flowers, from anthesis to fruit formation, lasts 20 to 30 days. The detailed observation of the aerial reproductive biology of *M. rubrum* bears important implications to the genetic structure of populations, plant fitness and conservation biology in threatened environments.

**Keywords:** Aquatic plants, flower anatomy, flower phenology, *Marathrum*, Mexican Pacific, Podostemaceae.

### Resumen

**Antecedentes:** Las especies de la familia Podostemaceae poseen rasgos inusuales en angiospermas. Crecen sumergidas en ríos de corriente fuerte y su ciclo de vida está ligado a la estacionalidad. Debido a su estructura floral reducida, el análisis de las etapas de desarrollo y eventos como la antesis o la receptividad del estigma ha sido complicado.

**Pregunta:** ¿Cuál es la relación entre la morfología, el desarrollo y la receptividad del estigma en diferentes etapas del desarrollo de la flor de *Marathrum rubrum*?

**Especie de estudio:** *Marathrum rubrum* Novelo & C. T. Philbrick.

**Sitio y años de estudio:** Río Horcones, Jalisco, México. Enero 2014 y 2015.

**Métodos:** Se realizó un análisis *in situ* y en laboratorio de la morfología floral de *M. rubrum* para evaluar la relación entre la morfología y desarrollo floral, y la receptividad del estigma en distintas etapas.

**Resultados:** Cuando las flores emergen de la espata, los óvulos y el polen están completamente desarrollados, por ello, la esporogénesis y la gametogénesis ocurren dentro del tallo, estando las plantas aún sumergidas.

**Conclusiones:** Los cambios morfológicos observados en las flores de *M. rubrum* durante las etapas analizadas se relacionan únicamente con eventos asociados con la fecundación y el desarrollo del fruto. Las observaciones detalladas de la biología reproductiva de *M. rubrum* en el ambiente aéreo tienen importantes implicaciones para el conocimiento básico de la biología de las plantas de esta familia, la estructura genética de las poblaciones, el éxito reproductivo y la biología de la conservación en ambientes amenazados.

**Palabras clave:** Anatomía floral, fenología floral, *Marathrum*, Pacífico mexicano, plantas acuáticas, Podostemaceae.

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**P**odostemaceae is the largest family of strictly aquatic angiosperms (Cook 1990) recognized by the APG IV classification (Chase *et al.* 2016). The family (order Malpighiales; Kita & Kato 2001) includes 49 genera and 280 species (Koi *et al.* 2012). Plants of this family live in unusual habitats for angiosperms (Philbrick & Novelo 1995, Philbrick & Novelo 1998), such as river's rapids and waterfalls (Cheek & Haba 2016).

Species can be annual or perennial (Cook & Rutishauser 1997, Cheek & Haba 2016), occurring in tropical and subtropical rivers around the world and, less frequently, in rivers of temperate regions of Japan and North America (Kato 2016). The vegetative structures of podostemads (roots, shoots, and leaves) are green and photosynthetic (Cook & Rutishauser 1997), displaying significant morphological variation (Jäger-Zürn *et al.* 2016). The plants grow attached to submerged rocks (Philbrick & Novelo 1995) and their life cycle is tightly linked to seasonality. Vegetative growth increases during the rainy season, when plants are submerged. In contrast, in the dry season when the river's water level descends, the flowers emerge, and reproduction occurs outside water (Kato 2016) (Figure 1A, B). Seeds are released and germinate at the onset of the rainy season (Rutishauser 1997, Castillo *et al.* 2017, Flores-Enríquez *et al.* 2019).

The Podostemoideae subfamily of Podostemaceae do not conform to the classic root-shoot model of a typical angiosperm, presenting roots and shoots that overlap in form and function (Leleeka *et al.* 2017). The plant's vegetative body is a cylindrical structure, flattened in the dorsoventral axis that grows attached, via adhesive hairs, to rocks in the riverbed (Jäger-Zürn *et al.* 2016), fallen logs and other materials (Cook & Rutishauser 2007). A sticky polysaccharide biofilm is produced in interaction with cyanobacteria (Jäger-Zürn & Grubert 2000).

Podostemaceae species generally produce small flowers, with reduced tepals, solitary or in inflorescences (Kato 2016). The small seeds are dispersed by wind and by animal vectors (e.g., birds). However, species can be restricted to a single river (Philbrick *et al.* 2010), suggesting low long-distance dispersal capability. Once seeds get hydrated, the epidermal cells released a sticky mucilage that enhances fast adhesion to the substratum (Jäger-Zürn & Grubert 2000).

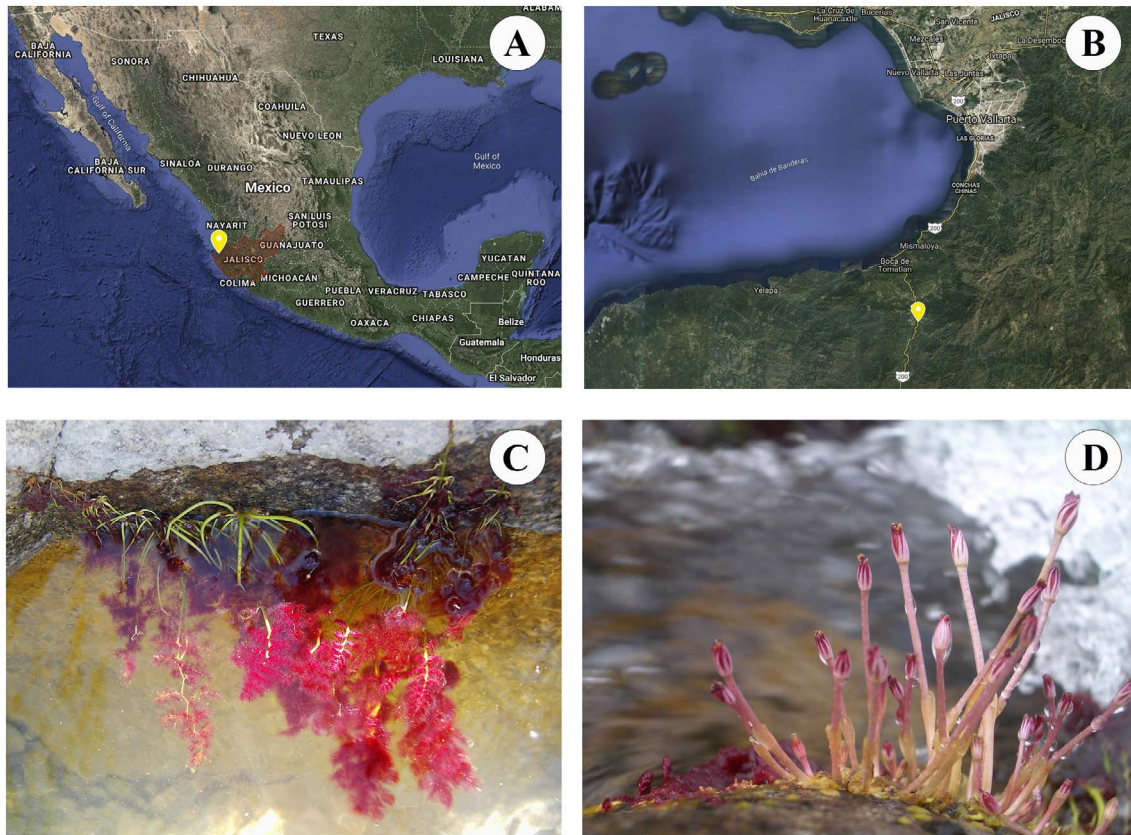
Podostemaceae have distinctive morphology, embryology, and development in relation to other angiosperms, making it an evolutionary enigma (Philbrick & Novelo 1995). Plants of this family show a significant reduction structures and developmental processes (Rutishauser 1997). Specifically, plants display unique embryological characteristics, such as a reduced embryo sac composed of four cells and no antipodals (Sehgal *et al.* 2011), the absence of double fertilization (Battaglia 1971, Sikolia & Onyango 2009, Chaudhary *et al.* 2014, Khanduri *et al.* 2016, Jiménez-Durán *et al.* 2021), and the absence of endosperm formation (Battaglia 1971, Mukkada & Chopra 1973, Jäger-Zürn 1997, Leleeka *et al.* 2017). Instead, Podostemaceae form a structure known as pseudo embryo sac or nucellar plasmodium, which might take the function of the endosperm during embryo development and germination (Razi 1949, Arekal & Nagendran 1977, Jäger-Zürn 1997).

The aim of this study was to analyze the floral reproductive morphology of *Marathrum rubrum* Novelo & C. T. Philbrick and the relationships between reproductive structures at different stages of flower-fruit development.

## Materials and methods

*Site of study.* This study took place at the Horcones River, Jalisco (Mexico), located in the Municipality of Cabo Corrientes, 27 kilometers south of Puerto Vallarta (20° 27' 45" N and 105° 17' 30" W), at an altitude of 350 m asl (Figure 1C, D).

In this locality, we studied the population of *M. rubrum* during the dry season, during five consecutive years (December-January), when the water level drops and flowering, fruiting and seed dispersal take place. In each year, 100 flowers sampled at random were tagged and followed for ten days to record changes in floral morphology and the different stages of development (Figure 2). Stigma receptivity was assessed, according to Zeisler (1938), by adding a 6% hydrogen peroxide solution for a minute to the stigmatic area and recording the presence/absence of a reaction (production of bubbles). Positive or negative receptivity was assessed at different floral stages.



**Figure 1.** A. *Marathrum rubrum* Novelo & C. T. Philbrick in vegetative phase; B. *M. rubrum* flowers in aerial phase; C. Study site in the State of Jalisco, Mexico (yellow tag); D. Bahía de Banderas, city of Puerto Vallarta, and the site of study (modified from Google, 2023)

*Development of reproductive structures.* Approximately 15 floral buds and flowers of *M. rubrum* were collected each year at different developmental stages, fixed in FAA, and dehydrated in a series of increasingly concentrated ethyl alcohol (30, 50, 70, 85, 96, and 100 %). These samples were gradually infiltrated with Paraplast (xilol 1, Paraplast 1:1 xilol, and Paraplast 1) and included in pure Paraplast for later processing in a rotatory microtome.

We obtained sections of 8  $\mu\text{m}$  wide, dyed with fast green safranin in methylcellosolve, and mounted in a synthetic resin. Micrographs were obtained with an Olympus Provis AX70 camera using a Kodak Ektachrome film and later digitized.

To avoid subjectivity in assigning color names to describe the different stages of the *M. rubrum* flower, a color table was used. The table shows the hexadecimal code that corresponds to each color identified in the different stages (Figure S1). For the ease of interpretation, we have divided flower development into five stages depicted and explained in Figure 2A-E.

*Flower length per stage.* We sampled 10 different rocks along the Horcones river to record the flower stage and length of the flower, taken from the base of the pedicel (contact point with the stem) to the apex of the flower stigmas, with a digital caliper ( $\pm 0.05$  mm).

## Results

*Flower stages.* Five discrete stages of flower morphology and development were identified (Figure 2A-E).



**Figure 2.** Flower's developmental stages: A. Stage 1: flower enveloped by the spathe; B. Stage 2: rupture of the spathe; C. Stage 3: reproductive structures are completely freed from the spathe; D. Stage 4: anthesis; E. Stage 5: filaments can be found adhered around the ovary or they can be lost.

Stage 1.- Flower bud is completely surrounded by a light green spathe, a translucent tissue bag-shaped structure that surrounds it (Figure 3A). Removing the spathe reveals fuchsia-colored anthers, that are arrow-shaped and pointing towards both stigmas. A light green color is still visible in the area near the filaments. Filaments are pale pink; stamens are adhered to the ovary; the ovary is magenta, and the two stigmas, which are close together, are purple. The elongation of the pedicel is highly variable, as there are small and large floral buds.

Stage 2.- Rupture of the spathe in the apical zone (Figure 3B). This stage starts with the rupture of the spathe and finishes when the flower is completely outside of this tissue. Anthers' filaments are still pale pink. The gynoecium is formed exclusively by the ovary and the bifurcated stigma; no style is present.

Stage 3.- Elongation of the pedicel leaves the spathe below the ovary or around the pedicel. Both anthers and ovary are completely freed from the spathe (Figure 3C). Anther's filaments are still pale pink and anthers are fuchsia; stigmas, purple in color, still close to each other (Figure 3C). The ovary is magenta and hard to observe since it's completely surrounded by the stamens.

Stage 4.- The stamens are separated from the ovary (Figure 3D). The anthers, now purple, change their arrow-like shape and become perpendicular to the filament. Anthesis occurs as anthers start to dehisce and release pollen. Stigmas are still purple, separated from each other and now receptive, according to the Zeisler's test. The ovary is still magenta.

Stage 5.- Both the pedicel and the anthers' filaments become pale brown. Filaments can be found adhered around the ovary or they can be lost (Figure 3E). Anthers turn ochre brown and can either remain in place or be lost. Stigmas are separated and show a dark brown color. The ovary becomes pale green but eventually starts turning ochre brown.

*Interpretation of the flower stage into their life cycle.* While the plants of *M. rubrum* are submerged, flower buds are not noticeable on the stem and are covered by the spathe. Once the water level drops and the vegetative structures become exposed to the dry environment, flower buds protrude from the stem and remain covered by the spathe (Figure 3A). The next event corresponds to the rupture of the spathe (Figure 3B), which is triggered by the pedicel growth. The pedicel elongation provokes the spathe to remain as a rudiment around the base of the pedicel. The emerging flower presents 6 to 8 stamens growing close to the ovary and located around the stigma (Figure 3C).

The most noticeable changes in the flowers of *M. rubrum* occur during Stage 4 (see Figure 2D), which corresponds to anthesis. The flowers have tepals, like rudiments at the base of the ovary, between the stamens. Filaments are separated from the ovary, and the anthers position themselves perpendicular to the filaments. Anthers dehisce, and it is possible to observe them covered by a powder that corresponds to pollen (Figure 3D). Anthers change from fuchsia to purple, like the color of the stigmas. Then, the stigmas separate and become receptive during this stage



**Figure 3.** Description of the different stages of flower development in *Marathrum rubrum* Novelo & C. T. Philbrick. During the dry season the flowers of *M. rubrum* are exposed to the dry environment. A. Stage 1: the reproductive organs are completely surrounded by a green-colored spathe; B. Stage 2: this structure breaks at the apex due to the pedicel elongation, marking the start of this stage which lasts up until; C. Stage 3: the ovary is completely released from the spathe; the pedicel continues its elongation, and the flower leaves the spathe completely behind. During this first stages the filaments are pale pink and completely surround the ovary; anthers are magenta, with an arrow-like shape, and also surrounding the ovary; both stigmas are purple and are closed together; D. Stage 4: anthesis occurs; at this point, filaments separate from the ovary, anthers dehisce, and the stigmas separate from each other and become receptive; E Stage 5: corresponds to post anthesis; in this stage, the whole flowers start turning ochre brown, the filaments wrap around the ovary and anthers fall down or remain in place. an = anther; fi = filament; ov = ovary; pe = pedicel; sp = spathe; st = stigma.

(Figure 3D), which lasts about five minutes. There's less than an hour between anther dehiscence and stigma receptivity. Pollination starts, and pollen grains can be observed on the stigma's surface. During this stage, pollen grains germinate over the stigma, and pollen tubes begin to grow, followed by fertilization.

After anthesis, during Stage 5, filaments wrap around the ovary (Figure 3E) or they can fall apart. Anthers turn ochre, anther filaments become pale, and the ovary turns ochre as well. Stigmas are dark brown, completely separated (Figure 3E) and are no longer receptive. The main changes in the stages of floral development of *M. rubrum* are summarized in Table 1.

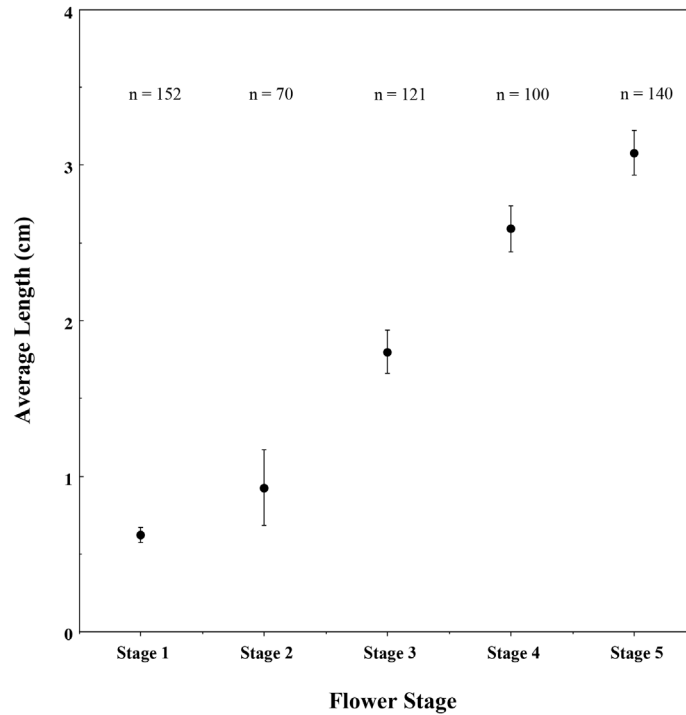
**Table 1.** Stages of flower development in *Marathrum rubrum* Novelo & C. T. Philbrick.

Stage	Spathella	Anthers	Stigmas
Stage 1	Present; completely surrounding the flower; pale green tissue.	Filaments are pale pink; anthers are fuchsia; arrow-shaped.	Closed together; purple in color; not receptive.
Stage 2	Present; broken at the apex; reproductive organs unexposed.	Filaments are pale pink; anthers are fuchsia; arrow-shaped.	Closed together; purple in color; not receptive.
Stage 3	Absent or present; reproductive structures outside the spathella.	Filaments are pale pink; anthers are fuchsia; arrow-shaped.	Closed together; purple in color; not receptive.
Stage 4	Absent spathella.	Filaments away from the ovary, and anthers are purple and perpendicular to the filaments. Anthers dehisce, releasing pollen over their surface.	Stigmas are separated; purple in color, and receptive.
Stage 5	Absent spathella.	Anthers are ochre brown, degenerating, or absent. Filaments are pale brown, present or absent.	Stigmas are separated, brown in color, and not receptive.

*Flower length per stage.* A random sampling was carried out, in which all the flowers in each stage of ten different rocks were recorded, adding a total of 583 flowers (Table 2).

As can be seen in Table 2, the length of the flower increases as the stage of development becomes more advanced. However, there is a considerable variation between the flowers of the same stage. In the field, it was observed that this variation is more related to the position of the flower on the rock than to the stage of development of the flower. Commonly, the flowers that grow on slopes of the rock tend to have long pedicels, while flowers that grow on flat areas of the rock may have shorter pedicels. As shown in Figure 4, there is a clear tendency for the most advanced stages to be longer. However, there is a lot of variation in the length of the flower within each stage, observable through the error bars. Flowers of *M. rubrum* attain an average length of ca. 3 cm at Stage 5 (Figure 4). The confidence interval (at 95 %) allows to discriminate flower size between stages. However, Stage 2 displays an enormous variation rendering it indistinguishable from Stage 1.

*Anatomy of the reproductive structures.* When the flower emerges from the spathella, both the pollen grains and ovules are fully developed (Figures 5A-D and 7A-B). From Stage 1, pollen grains are bicellular and ovules show a fully developed embryo sac and nucellar plasmodium (Figure 7A). The biggest changes in the gynoecium during the



**Figure 4.** Average values of flower of *Marathrum rubrum* Novelo & C. T. Philbrick of different stages. Confidence interval (at 95 %) and number of flowers measured of each stage are given.

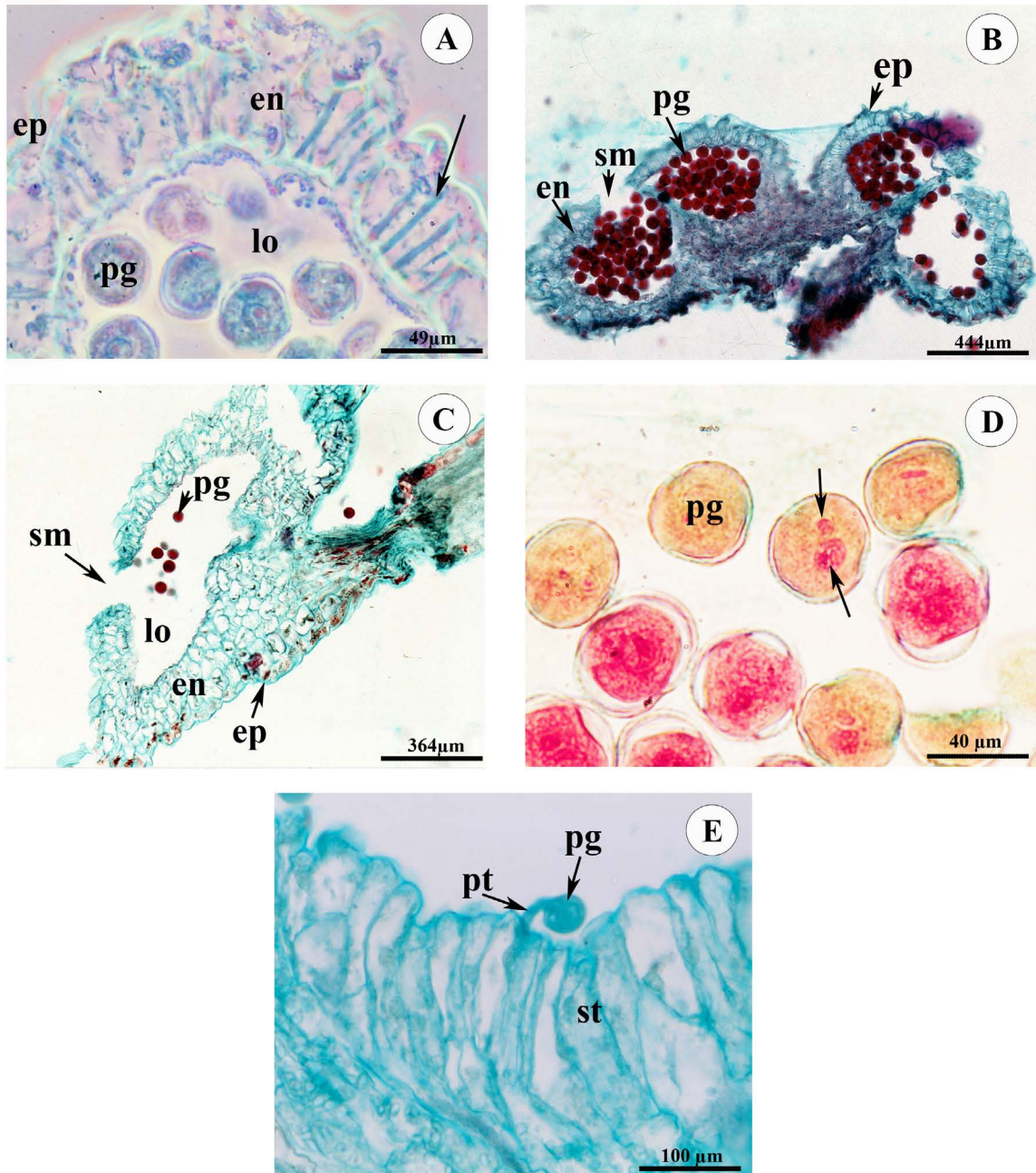
stages occur in the stigma and in the ovary wall (Figure 6). Once the flower breaks the spathella, there is only growth of the pedicel.

**Anther.**- During Stage 1, the anther wall is comprised of the epidermis and the endothecium (Figure 5A); both the middle layers and the secretory tapetum are gone; at this stage the endothecium starts thickening; this thickening precedes the dehiscence occurring in Stage 4 (Figure 5B). During Stage 5, at a time when pollen germination has presumably occurred, pollen has been completely released from the anther (Figure 5C). Pollen grains are completely developed from Stage 1; pollen grains are tricolpate and are formed by two cells, the generative and the vegetative cells (Figure 5D); we can assume microsporogenesis occurs when the plants are submerged, and the flowers are inside the stem. We observed germinating pollen grains on Stage 4 stigmas (Figure 5E); this corroborates the results obtained by receptivity tests performed *in situ*.

**Ovary wall.**- The ovary wall of *M. rubrum* is formed by seven to eight cellular layers during Stage 1. The inner epidermis cells exhibit lipid accumulation, while cells from other layers only accumulate starch (Figure 6A); there is also a high concentration of tannins (in red), especially in the outer epidermis; this becomes even more evident throughout development (Figure 6B-D5). The inner and outer cuticles of the ovary wall are both thick, with the inner cuticle being thicker than the outer cuticle. Histochemical tests were performed to identify the chemical constituents

**Table 2.** Measurements of flower length during the different stages of development in *Marathrum rubrum* Novelo & C. T. Philbrick.

Measure	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
Mean length	0.62	0.926	1.806	2.591	3.088
	n = 152	n = 70	n = 121	n = 100	n = 140

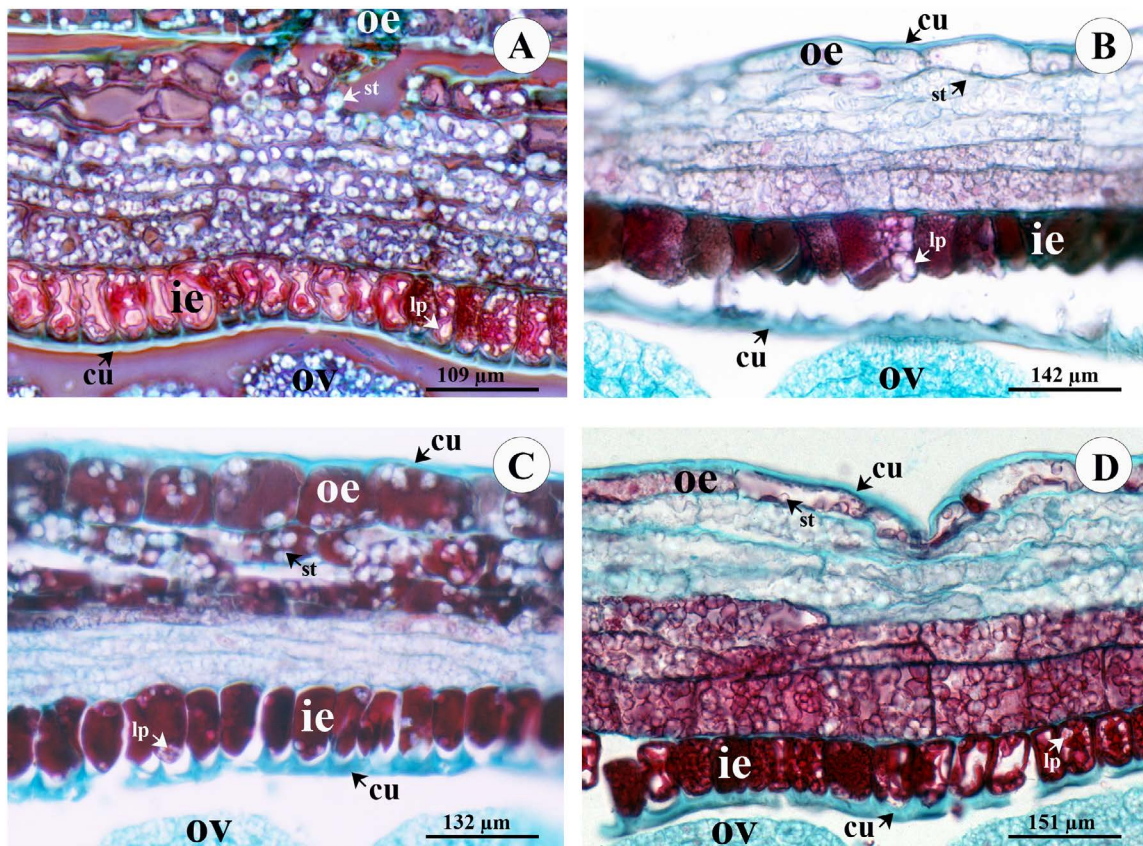


**Figure 5.** Anatomy of the anthers in *Marathrum rubrum* Novelo & C. T. Philbrick A. Section of a microsporangium (phase contrast) in Stage 2: the anther contains the mature pollen grains, and two layers can be distinguished in the anther wall -the epidermis and the endothecium-; thicker sections start appearing during this stage that will later provoke small ruptures in the anther wall; some orbicules (Ubisch bodies) are visible in the remaining tapetum, between the loculus and the endothecium; B. Section of a microsporangium (light field) in Stage 4: anthers have four loculi; C. Section of a microsporangium (light field) in Stage 5: it can be seen the stomium through which pollen grains were liberated, and degenerating tissue is easy to identify; D. Two-celled pollen grains at Stage 4 (phase contrast): pollen grains are tricolpate and have a generative and a vegetative cell; E. Pollen grains germinating over a Stage 4 stigma with a visible pollen tube (light field): during this stage, the stigmas become receptive, and pollen grain germination occurs. en = endothecium; ep = epidermis; lo = locule; pg = pollen grain; pt = pollen tube; sm = stomium; st = stigma.

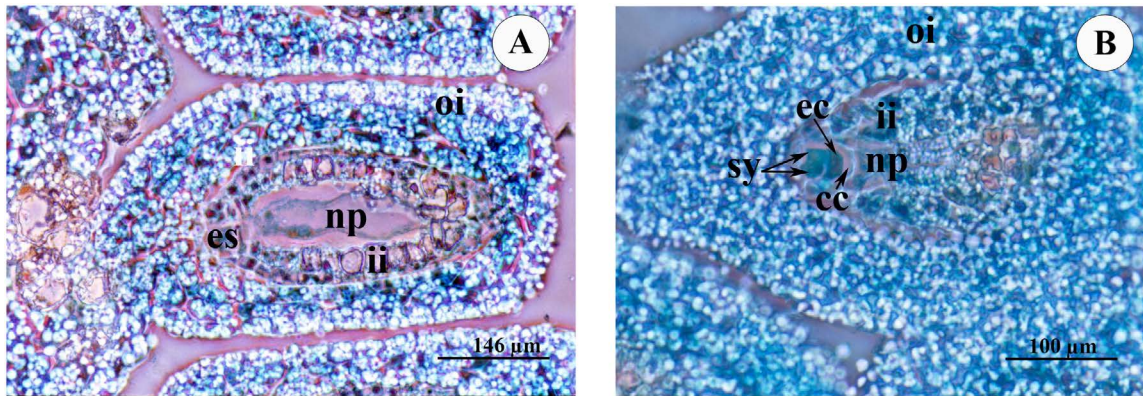


in the tissues, as follows: to confirm the presence of starch, the Lugol's iodine staining was performed, for the presence of insoluble lipids, present in the cuticles, the neutral red staining, and for the presence of soluble lipids, the Sudan III staining.

Ovule.- Ovules are fully developed from Stage 1 (Figure 7A) and do not undergo further changes during the stages analyzed in this study. The ovules of *M. rubrum* are tenuinucellate and bitegmic. They consist of an outer integument, which contains several layers filled with reserve substances like starch, and an inner integument that also accumulates starch to a lesser extent, along with other substances of unknown chemical nature (Figure 7A-B). The ovules have a four-celled embryo sac (7B) and the nucellar plasmodium that characterizes the Podostemaceae family (Figure 7A-B).



**Figure 6.** Anatomy of the ovary wall in *Marathrum rubrum* Novelo & C. T. Philbrick. A. Ovary wall in Stage 1 (phase contrast): presence of starch is evident in all layers, as well as lipid inclusions in the inner epidermis; tannins, shown in red, are present in the inner epidermis, and adjacent layers; B. Ovary wall in Stage 2 (light field): starch continues to be present in all layers, as well as lipid inclusions in the inner epidermis, the presence of tannins is larger than in the first stage, since it is identified in all layers, especially in the inner epidermis; C. Ovary wall in Stage 3 (light field): starch remains observable in all layers, as do lipid inclusions in the inner epidermis, tannins are more evident in the inner epidermis and in the outer epidermis, as well as in the layers adjacent to it. The presence of thick cuticles that separate the ovary wall from the environment and the ovules is evident; D. Ovary wall in Stage 5 (light field): the presence of starch, in all layers, and lipid inclusions in the inner epidermis remains unchanged. The presence of tannins is more evident in all layers, especially in the external and internal epidermis, and in the layers adjacent to it. cu = cuticle; ie = internal epidermis; lp = lipids; st = starch; oe = outer cuticle; ov = ovule.



**Figure 7.** Anatomy of the ovules of *Marathrum rubrum* Novelo & C. T. Philbrick. A. Ovule in Stage 1 (phase contrast): ovules are fully developed since the first stage and not undergo further changes during the states analyzed in this study. This means that they are completely developed before the water level drops and flowers are exposed to the dry environment. The ovule is bitegmic with both an inner and an outer integument and possess the nucellar plasmodium, characteristic of the Podostemaceae family. The nucellar plasmodium is fully developed in all stages analyzed; B. Ovule in Stage 4 (phase contrast): The ovules contain a four-celled embryo sac, consisting of two synergids, a central cell, and the egg cell. cc = central cell; ec = egg cell; es = embryo sac; ii = inner integument; np = nucellar plasmodium; oi = outer integument; sy = synergid.

## Discussion

The life cycle of *Marathrum rubrum* Novelo & C. T. Philbrick shows a seasonal pattern of vegetative-reproductive events. During the rainy season (May to November in the northern hemisphere), most vegetative growth occurs while the plant is completely submerged (Philbrick & Novelo 1995). Like other species of Podostemaceae, *M. rubrum* initiates its gametophytic phase when the flower is submerged and immersed in the stem. Anthesis, pollination, fertilization, and seed and fruit formation are processes that always occur once the reproductive parts of the plant are aerial; these processes take around thirty days.

When the flower bud is still inside the spathe and becomes exposed to the dry environment, both the ovules and pollen grains are fully developed. That is, the ovules have developed the embryo sac, and the pollen grains are in a bicellular stage, tricolpate, covered with exine. This aligns with observations made on *Vanroyenella plumosa* Novelo & C. T. Philbrick (Murguía-Sánchez *et al.* 2001), a species that shares habitat with *M. rubrum* in the Horcones river and is closely related to *Marathrum* Bonpl. (Tipperry *et al.* 2011). Species in the family Podostemaceae exhibit unusual characteristics in terms of morphology, anatomy, ecology, and embryology, not known in any other family of angiosperms (Nagendran *et al.* 1980, Philbrick & Novelo 1995, Sikolia & Ochora 2008). Therefore, they have been considered an evolutionary enigma (Jäger-Zürn 1997, Rutishauser 1997, Chaudhary *et al.* 2014, Leleeka *et al.* 2017). Regarding embryology, they are characterized by the formation of a tetracellular and tetranucleate female gametophyte, with the absence of antipodes (Battaglia 1971, Mukkada & Chopra 1973, Arekal & Nagendran 1975, 1977, Jäger-Zürn 1997, Murguía-Sánchez *et al.* 2002, Sikolia & Ochora 2008, Chaudhary *et al.* 2014, Sehgal *et al.* 2014, Sikolia 2015). The absence of double fertilization (Chopra & Mukkada 1966, Battaglia 1971, Mohan Ram & Sehgal 1992, Sikolia & Onyango 2009, Sehgal *et al.* 2011, Chaudhary *et al.* 2014, Sikolia 2015, Khanduri *et al.* 2016, Jiménez-Durán *et al.* 2021) has been documented within the family. This is related to the absence of endosperm (Battaglia 1971, Nagendran *et al.* 1976, Jäger-Zürn 1997, Leleeka *et al.* 2017) and the development of a unique structure known as the nucellar plasmodium. (Went 1908, Razi 1949, Mukkada 1969).

Anthers in *M. rubrum* are tetrasporangiate and dithecous, showing only the epidermis and endothecium from Stage 1. The endothecium exhibits thickenings that later allow dehiscence through the stomium. The anthers release pollen grains just before the stigma becomes receptive. Since Stage 1, pollen grains are bicellular, corresponding to the developmental stage in which Podostemaceae usually release their pollen grains (Bedinger *et al.* 1994) through

anther dehiscence. This means that the only changes occurring in the anthers during the stages are related to the process of dehiscence.

The ovules of *M. rubrum* are axillary, anatropous, bitegmic, and tenuinucellate, coinciding with the ovules present in the family (Cook & Rutishauser 2007). Its internal integument consists of two cellular layers, and the external integument has three layers, supporting previous reports for the family (Mukkada 1969). The reduced embryo sac, typical in the family, is formed by two synergids, an egg cell, and a central cell, which, based on its appearance, might be undergoing degeneration. Studies revealing the ultrastructure of the central cell in the five described stages and subsequent stages are necessary to assess if programmed cell death exists in this cell, as the observations made in this work lack the resolution to establish whether this cell is degenerating.

The absence of double fertilization in Podostemaceae is an intriguing characteristic, especially considering it has been established as a defining feature for angiosperms. This has been attributed to both the inability of the male gamete to fuse with the central cell (Mukkada & Chopra 1973), one of the sperm cells not being discharged from the pollen tube (Chopra & Mukkada 1966, Mukkada 1969, Sikolia 2015), and the early degeneration of the central cell (Battaglia 1971, Nagendran *et al.* 1980, Friedman 2006). Recent evidence suggests that double fertilization does not occur due to the degeneration of the central cell before the pollen tube entry. Therefore, it is irrelevant whether the second sperm cell is released or not, as it would not find a counterpart for fusion if released.

The ovule of *M. rubrum*, as well as in all investigated species of the Podostemaceae, develops a structure called the nucellar plasmodium, found to be fully developed from the first considered stage and remains unchanged until the last studied stage, occurring before fertilization. This corresponds to the nucellar plasmodium's developmental type in the Podostemoideae subfamily (Jäger-Zürn 1997, Kita & Kato 2001).

The nucellar plasmodium -originally termed pseudo-embryo sac- derives from the breakdown of nucellar cells adjacent to the embryo sac (Arekal & Nagendran 1977, Jäger-Zürn 1997). Initially described as “an empty space containing many nuclei in a state of degeneration” (Went 1908, Razi 1949), subsequent revisions mentioned that its nuclei are healthy and constitute a multinucleate protoplast (Jäger-Zürn 1967, Arekal & Nagendran (1975) suggested it has a nutritive role, proposing the term change to “nucellar plasmodium” as it is not homologous to the ontogeny, organization, or appearance of an embryo sac. This sporophytic tissue has been proposed to substitute the endosperm in nourishing the embryo (Arekal & Nagendran 1975, Jäger-Zürn 1997, Sikolia & Onyango 2009, Chaudhary *et al.* 2014) and holds significant taxonomic importance within the group, as its development timing distinguishes the Tristichoideae, Podostemoideae, and Weddellinoideae subfamilies (Arekal & Nagendran 1977, Jäger-Zürn 1997, Chaudhary *et al.* 2014).

In the strata constituting the ovary wall, the presence of tannins, starch, and lipids was detected, which accumulate due to the lack of endosperm (Battaglia 1971, Mukkada & Chopra 1973, Jäger-Zürn 1997, Leleeka *et al.* 2017). The latter could provide nourishment to the developing embryo since communication between the fruits and the rest of the plant is interrupted in the early stages of fruit development by the obliteration of vascular tissues in the pedicel. This is fundamentally important considering that species of the family do not develop endosperm, which is a nutritive tissue for the developing embryo. Cells in the inner layer of the ovary wall show a significant accumulation of lipids. This layer, along with the cuticles, could act as an impermeable barrier, preventing the developing seeds from coming into contact with the water of the surrounding environment.

The unique morphology present in Podostemaceae, in contrast with the rest of the angiosperms, their restricted geographic distribution, the environmental conditions of their habitats, and their dependence on seasonal environmental rhythms to complete their life cycle, render them very susceptible to extinction given their rarity (*sensu* Rabinowitz 1986). We consider that understanding the biology of Podostemaceae will contribute to their conservation.

*M. rubrum* is an endemic plant species of rivers from the Pacific Coast of Mexico, in the States of Jalisco and Guerrero (Tippery *et al.* 2011, Mora-Olivo *et al.* 2013, Hassler 2017). The species was cataloged as vulnerable by the IUCN (1997) and subject to special protection (Mexican Official Norm, NOM-059 (SEMARNAT 2010). Hence, efforts to better understand its biology, especially reproductive biology for its connection to population genetic structure, are of utmost importance to propose conservation areas. This is especially urgent for Podostemaceae, whose habitats are re-

duced, threatened by pollution, and affected by the construction of hydroelectric dams (Kato 2016, Zuluaga-Gómez *et al.* 2016, Castillo *et al.* 2017). This work contributes to understanding the species, particularly laying the groundwork to determine which stages are most suitable for conducting ecological and embryological studies. It encompasses the development of ovules and pollen grains, as well as the moments of stigma receptivity and anther dehiscence.

### Supplementary material

Supplemental data for this article can be accessed here: <https://doi.org/10.17129/botsci.3409>

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