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Revista Facultad Nacional de Agronomía

Rubus glaucus Benth.: morphology and floral biology aimed at plant breeding processes

UNIVERSIDAD NACIONAL DE COLOMBIA

Rubus glaucus Benth.: morfología y biología floral dirigida a procesos de fitomejoramiento

doi: 10.15446/rfnam.v72n1.75910

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ABSTRACT

Keywords:
Andean blackberry
Androecium
Gynoecium
Viability

Rubus glaucus is widely distributed throughout the three mountain ranges of Colombia, where the blackberry growers have highlighted the need to standardize the supply of planting material, starting with plant breeding schemes that lead to more productive varieties with morphological characteristics that ease agricultural activities. Plant breeding activities have improved by considering the pollination mechanisms of plants. The implementation of controlled sexual hybridization depends on these pollination mechanisms, and several plant breeding methods have been adapted to crop pollination patterns. Morphological characteristics and studies on the floral biology of *R. glaucus* Benth were conducted to improve plant breeding processes. In addition, a study on pollen viability and stigma receptivity were performed. The reported morphological characteristics of *R. glaucus* enabled characterization of its flowers as complete and perfect with a regular, actinomorphic, perianth heterochlamydeous, dialipetalous, and dialisepalous structure. Meanwhile, the evaluation of different collection times for pollen viability revealed significant differences. The highest pollen viability occurred at 10:00 am, followed by 9:00 am. Qualitative evaluation of stigma receptivity led to the conclusion that the highest stigma receptivity is at anthesis at 12:00 m.

RESUMEN

Palabras clave:
Mora de los Andes
Androceo
Gineceo
Viabilidad

La especie *Rubus glaucus*, está distribuida en las tres cordilleras de Colombia, donde los productores asociados de mora en Colombia, han destacado la necesidad de formalizar la oferta de materiales de siembra, iniciando con esquemas de fitomejoramiento que conduzcan a obtener variedades más productivas, con características morfológicas que faciliten las actividades agrícolas. El tener en cuenta los mecanismos de polinización en plantas, ha hecho más eficiente las actividades de fitomejoramiento. La implementación de una hibridación sexual controlada depende de los mecanismos de polinización, incluso varios métodos de mejoramiento en plantas se han ajustado a los patrones de polinización del cultivo. Con el fin de avanzar en un futuro, en procesos de fitomejoramiento para la especie *Rubus glaucus* Benth, se realizaron las descripciones morfológicas y estudios sobre la biología floral de la especie mencionada, además del estudio de viabilidad del polen y la receptividad del estigma. Las descripciones morfológicas realizadas para *R. glaucus* en esta investigación, permitieron caracterizar la flor de *R. glaucus*, como completa y perfecta, con una estructura actinomorfa regular y periantada, heteroclamídea, dialipétala y dialisépala. Entre tanto, se encontraron diferencias significativas al evaluar diferentes horas de viabilidad polínica. Encontrando que, la mayor viabilidad del polen se obtenía a las 10:00 horas, seguido por el tratamiento de las 9:00 horas. Las evaluaciones cualitativas de receptividad del estigma permitieron concluir que, el estado de mayor receptividad del estigma es el de antesis y la hora de mayor receptividad es a las 12 horas del día.

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Received: October 31, 2018; Accepted: July 4, 2019
Rev. Fac. Nac. Agron. Medellín 72(3): 8909-8915. 2019. ISSN 0304-2847 / e-ISSN 2248-7026

Rubus is a widely distributed genus. Its species are found on all types of cultivable soils ranging from tropical to subarctic regions. However, the exact number of species remains unknown because its classification was last done by Focke (1910-1914), and since then, many species have been described. It is estimated that the number of species of this genus ranges from 600 to 800. Difficulties in its taxonomic classification, particularly of *Rubus* subgenus in Europe and North America, are due to the prevalence of interspecific hybridizations, polyploidy, and various forms of apomixis (Thompson, 1995). The Andean blackberry (*Rubus glaucus* Benth.) is distributed in the three mountain ranges of Colombia and has combined characteristics of *Idaeobatus* and *Rubus* subgenera. It is a fertile amphiploid or allotetraploid that has resulted from the genome fusion of two species (Jennings, 1988).

In Colombia, blackberry production and cultivated area, but not yield, have increased, and it is considered a fruit with cultivation opportunities in Colombia, both for supply in the domestic market and export. The Colombian National Fruit Plan (PFN, Spanish acronym; 2006–2026) expects a 94.1% increase in cultivated area in Colombia by 2026 (MADR, 2006), i.e., from 10,743 ha in 2008 to 20,631 ha in 2026, which would generate approximately 6,917 direct jobs. This species exhibits great variability in terms of size, color, and fruit quality, which was possibly produced through selection breeding from wild plants in ancient times (Rativa *et al.,* 2016).

The associated producers of blackberry in Colombia have highlighted the need to standardize the supply of planting material, starting with plant breeding schemes that lead to more productive varieties with morphological characteristics that ease agricultural activities; the desired characteristics include absence of spines, tolerance to diseases, and increase in productivity. Therefore, it is necessary to collect, multiply, and characterize possible parent plants and conduct crossing. The latter step warrants studies on the morphology and floral biology of this species for improvement.

In plant breeding activities, the desired characteristics are obtained using existing variability. The understanding of pollination mechanisms in plants is critical for improvement processes. Sexual hybridization, essential in plant breeding, constitutes controlled pollination and implementation of such pollination mechanisms (Frankel and Galun, 2012).

In this study, for advancements in breeding processes for *R. glaucus*, morphological characteristics and studies on floral biology of this species were conducted as well as pollen viability and stigma receptivity were studied.

MATERIALS AND METHODS

Vegetal material

Experimental plant material (*R. glaucus* flowers) was obtained in August 2017 from the Botanical Garden of Universidad Tecnológica de Pereira, located at 1,467 masl (4°47'28.2" N 75°41'24.5" W).

Floral morphology

Flowers collected at anthesis (fully opened flowers) were inspected using a Leica EZ4 stereoscope. The morphological characteristics of the flowers, as suggested by Strasburger (1994), were evaluated and classified for external (chalice and corolla) and internal verticils (androecium and gynoecium). Finally, the floral formula of *R. glaucus* was constructed.

Pollen viability

A total of 25 flowers were collected at anthesis from different plants, and their anthers were detached under a stereoscope to collect pollen grains. To obtain a random sample, 100 pollen grains per sheet were counted for evaluation at each collection time. Pollen collection started at 8:00 am and continued until 1:00 pm with periodic collection conducted every hour (6-hour collection, treatments 1 to 6).

Pollen viability analysis

Pollen viability analysis was conducted using coloration with 2% acetocarmine glycerol as described by Alexander (1969). This procedure can detect non-aborted pollens and indirectly evaluate their viability. Pollens were visualized under a Leica DM750 optical microscope with a photographic head. Pollens were considered viable when they presented reddish coloration and a hyaline halo (Figure 1A). Viable pollens were quantified in percentage by determining viability percentage in 100 pollen grains from 5 flowers;

this procedure was performed in triplicates. Statistical analysis was performed using ANOVA after determining normal distribution of data using Shapiro–Wilk test with at 5% significance. Differences among treatments were determined using Tukey test. All statistical analyses were performed using the R3.4.4 software.

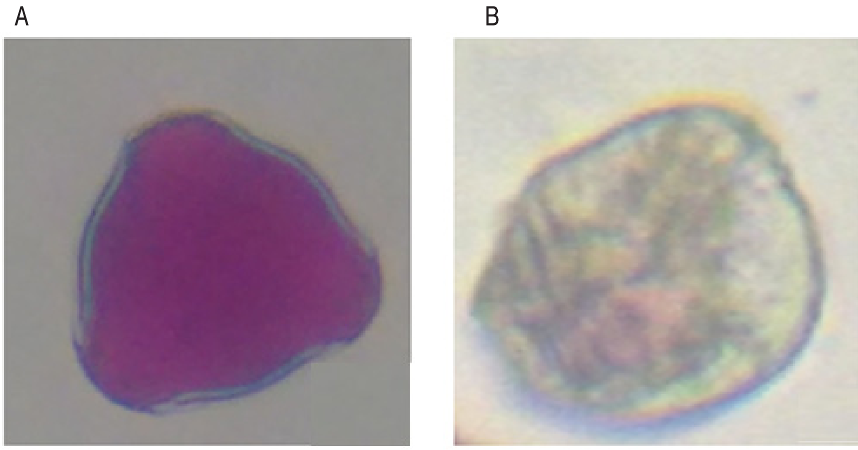

Figure 1. Pollen grains of *R. glaucus*. A. Viable pollen grain; B. Non-viable pollen grain.

Scanning electron microscopy (SEM)

To visualize pollen grains using SEM, flowers were collected at anthesis, and their anthers were detached under a stereoscope. The detached anthers were fixed in a 3% glutaraldehyde solution overnight at 4 °C. Subsequently, the samples were washed three times for 10 min in a 0.1 M PIPES solution (pH 7.2), followed by serial washes in ethanol solutions (50%, 60%, 70%, 80%, 90%, 95%, and 100%; v/v). Subsequently, the samples were transferred into a drying equipment. The specimens, previously visualized by optical microscopy, were observed under a FEI scanning electron microscope (version Quanta 250; Thermo Fisher Scientific).

Stigma receptivity

Stigma receptivity was chemically evaluated with 3% hydrogen peroxide (H_2O_2). This evaluation involved depositing H_2O_2 in the stigma of flowers by considering receptive stigmas that rapidly formed bubbles (Zambon *et al.*, 2018). Evaluation of stigma receptivity was performed at pre-anthesis and anthesis stages using a periodicity schedule, which started at 7:00 am and continued until 2:00 pm.

RESULTS AND DISCUSSION

Floral morphology

External verticils. *R. glaucus* flowers were complete and perfect and had a regular and perianth, actinomorphic structure, with 5 petals and 5 differentiated sepals (heterochlamydeous). The corolla had free petals and sepals (dialipetal and dialisepal, respectively; Figure 2).

Internal verticils. The flowers were hypogynous with pluricarpelar and apocarpous gynoecium and numerous stigmas and intrusive anthers (Figure 2). Based on the previously described characteristics, the following floral formula was constructed:

Floral formula for *R. glaucus* = * K_5 C_5 A_αG5 (1)

The morphological characteristics of *R. glaucus* reported in this study are consistent with those described by Monasterio-Huelin (1992), who reported the taxonomic characteristics of *Rubus* in the Iberian Peninsula and Balearic Islands. Monasterio-Huelin (1992) described *Rubus* species as heterochlamydeous and hermaphrodites wherein the chalice is formed by 5 imbricate pieces with whole lanceolate sepals. *Rubus* from the Iberian Peninsula and Balearic Islands have white, pink, or red petals depending on the species; *R. glaucus* petal is white. On the other hand, Cancino-Escalante *et al.* (2011) described *Rubus* flowers in Colombia as pentamer flowers with welded sepals at the base; deltoid to ovate-acuminate, usually with obovate; and free; and white, pink, or red petal trichomes. These characteristics completely coincide with those found in the present study.

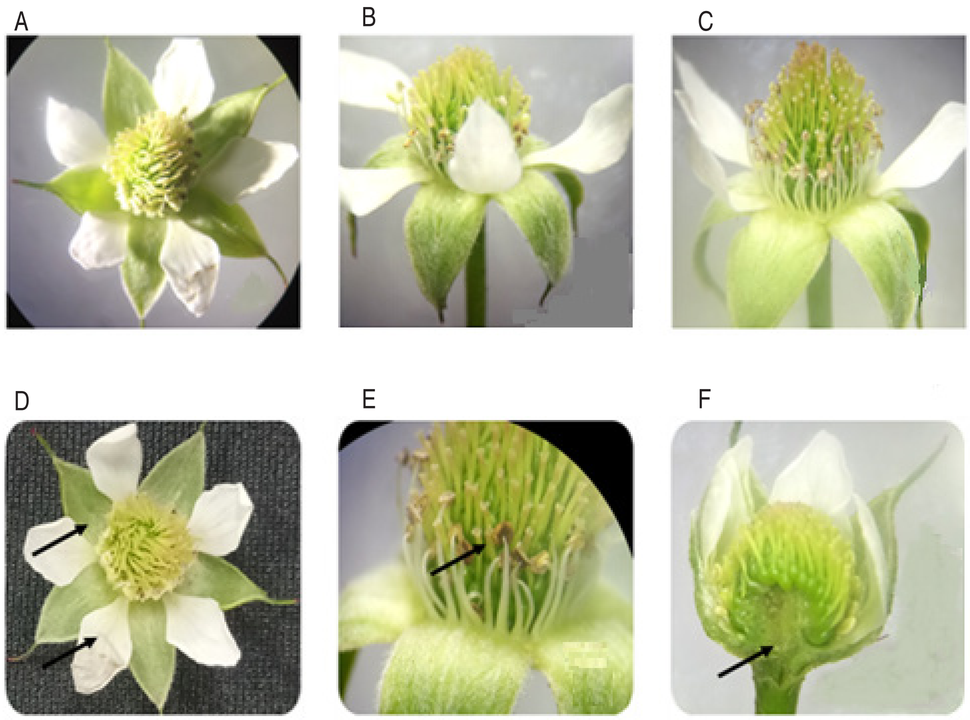

Figure 2. Floral morphology of *Rubus glaucus*. A. Regular actinomorphic flower; B. Perianth flower; C. Heterochlamydeous; D. Dialisepal–Dialipetal; E. Intrusive anther; F. Hypogynous ovary

Pollen viability

Pollen viability data showed a normal distribution and coefficient of variation of 6.77%. Analysis of variance showed highly significant differences in terms of pollen viability according to the collection time. Regarding differences in pollen viability according to the collection time, Tukey's test showed that the highest pollen viability was obtained at 10:00 am, followed by at 9:00 am and then 11:00 am. Pollen viability percentages at 9:00 and 10:00 am were significantly different from each other and different from other collection time. However, pollen viability was similar at 8:00 and 11:00 am. Likewise, pollen viability at 12:00 and 1:00 pm were similar, with the latter having the lowest pollen viability percentage (Table 1).

Table 1. Tukey test for setting differences among treatments.

Treatment (Pollen collection time)	Viability percentage (average)
8:00	0.3784 c
9:00	0.4901667 b
10:00	0.5769 a
11:00	0.3803667 c
12:00	0.2913333 d
13:00	0.2693333 d

Different letters indicate significant differences among treatments (P<0.05).

Evaluation of other *Rubus* species (Nybom, 1985) has shown 8% viability for triploid species and 54% for tetraploid species, compared with 81% for apomictic diploid species. These results coincide with those found in the present study for *R. glaucus*, which is a tetraploid species. Other studies, which evaluated pollen viability percentages from first-generation progeny individuals of interspecific crosses of *Rubus* genus, have reported values between 66.7% and 28.8%, with most frequently reported values being 45% and 60% depending on the parents of each progeny (Nybom, 1995). The previously reported results coincide with those found in the present study.

The relationship between pollens and fruits and seed formation has been recognized since the beginning of agriculture. This information has allowed for artificial pollination and double pollination in angiosperms. Darwin's contribution is also known, and his theory of evolution states that cross-fertilization is not only beneficial in evolutionary terms but is also necessary for maintaining a species' vigor and fertility. Understanding the viability of these gametes is vital for plant breeding.

SEM

R. glaucus pollen grains showed a tricolored structure (Sáenz Laín, 2004), with equatorial diameters ranging between 20 and 25 µm (Figure 3). According to Soejarto and Fonnegra (1972), this pollen size is small; they have reported that most species have a diameter between 15 and 50 µm and that almost all plant species have pollen diameters between 8 to 150 µm. In general, there is a correlation between pollen size and pollinating agents. Similar studies conducted on pollen grains from 9 European *Rubus* species have described pollen grains to be small, isopolar, and tricolporate (Tomlik-Wyremblewska, 1995) such as those found in *R. glaucus*.

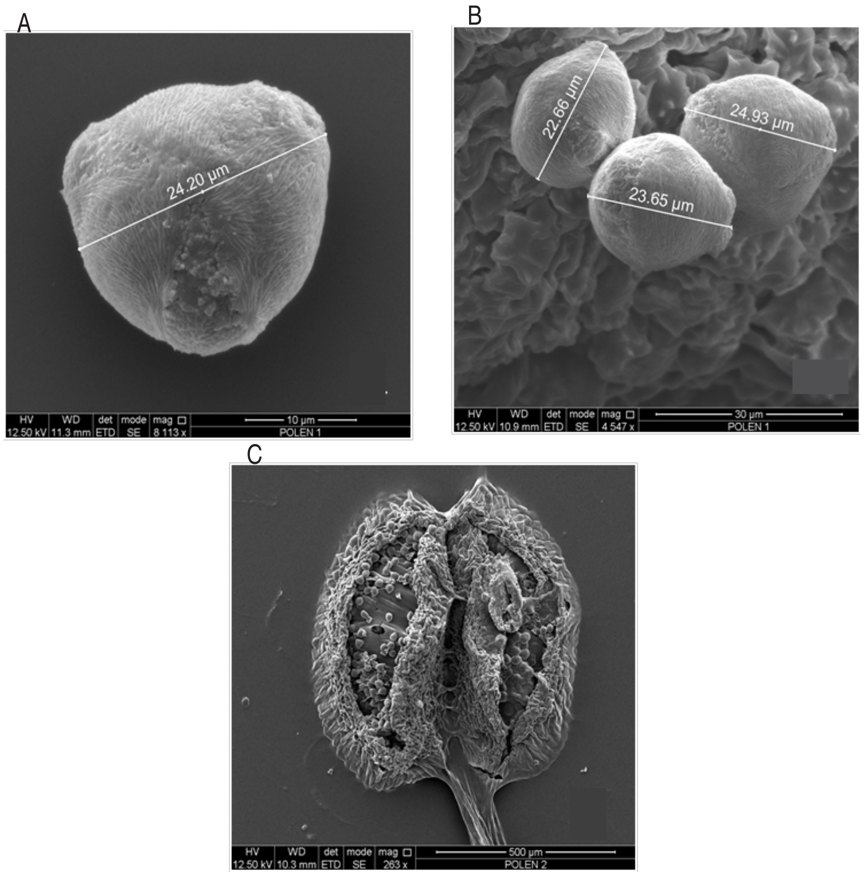

Figure 3. Scanning electron microscopy of pollen grains and *Rubus glaucus* anthers. A. Tricolpored pollen grain; B. Pollen grains; C. Anther.

Stigma viability

Qualitative assessments of stigma receptivity led to the conclusion that most stigmas were receptive at anthesis (fully open flower; Figure 4) and the time of highest stigma receptivity was 12:00 m when the highest and most frequent bubble production on stigma exposure to H_2O_2 (3%) was observed.

Studies on the fertility aspects of other *Rubus* species have been conducted on American "blackberries" in breeding programs (Ruple *et al.,* 2010), which aimed to evaluate the effects of self-pollination and cross-pollination and the subsequent fruiting in different genotypes of *Rubus*. They have evaluated different fertility components of these genotypes in controlled conditions, particularly before and after being emasculated. This study led to the conclusion that flowers do not change their stigma receptivity as opposed to being emasculated or not.

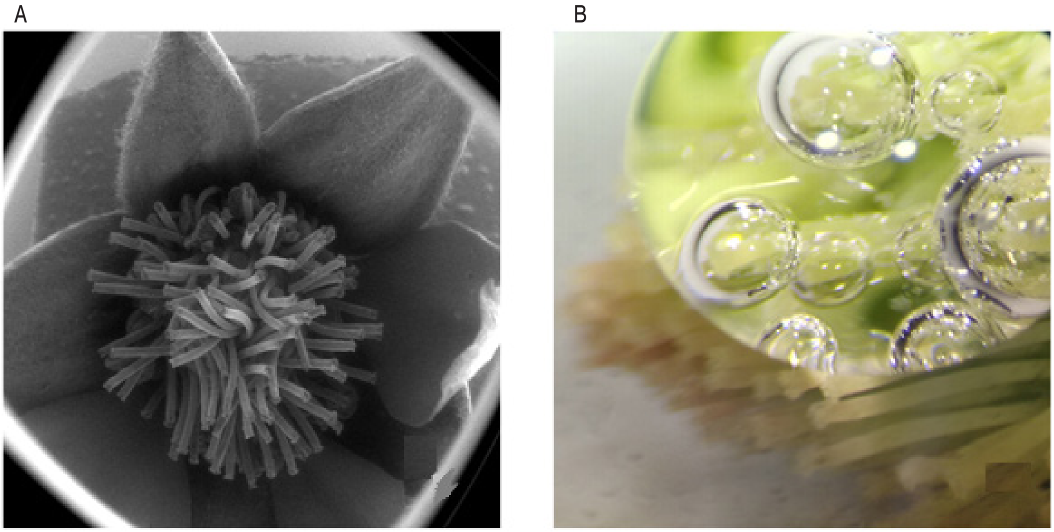

Figure 4. Appearance of stigma and production of bubbles during stigma receptivity test. A. *Rubus glaucus* multiple stigma (30×); B. Production of bubbles in *R. glaucus* stigma on reaction with hydrogen peroxide.

A previous study (MacPhail and Kevan, 2005) on ecological aspects related to the frequency of insect visits to the wild species of *Rosa* genus, a species highly related to *Rubus*, found that the largest period of insect visits to flowers occurs between 9:00 am and 12:00 m, consistent with the findings of the present study. For *R. glaucus*, the highest insect activity is observed in the morning, with the highest frequency of *Apis mellifera* (Hymenoptera) in commercial crops; this denoted that stigma is more receptive at 12:00 m.

Understanding *R. glaucus* pollination mechanisms is vital to improve breeding processes with controlled pollination. Floral morphology indicates pollination mechanisms of this species and helps to infer its most frequent type of pollinators. In addition, determination of the fertility behavior of internal verticils (androecium and gynoecium) allows improved protocol designing for crosses that formally initiate an improvement program for this species. The last aspect has not been previously reported for *R. glaucus* and would allow establishment of a new variety, leading the productive sector to be more competitive.

CONCLUSIONS

Regarding floral morphology, *R. glaucus* is an actinomorphic, perfect, and complete pentamer flower, similar to other flowers of *Rubus*. Evaluation of its pollen grains led to the conclusion that the highest pollen viability occurs at 10:00 am. Meanwhile, the highest stigma receptivity occurs at 12:00 m, i.e., at anthesis. Based on gamete behavior, controlled pollination protocols can be standardized to initiate breeding programs for this species.

ACKNOWLEDGEMENTS

Authors express their gratitude to Gustavo Bolaños (Universidad de Caldas) for the support with SEM processing, Universidad Tecnológica de Pereira (Pereira, Colombia), and the General System of Royalties (National Planning Department) for financing the research program “Development of scientific and technologies in biotechnology applied to the sectors of health and agro-industry in the department of Risaralda” (BPIN code 2012000100050).

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