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Scientific Article

ANATOMY AS A TOOL TO UNDERSTAND THE CAUSES OF SEED FAILURE: A STUDY OF CROSSES *Eucalyptus* SPECIES

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ABSTRACT

Seminal propagation of eucalypt plays a crucial role in breeding programs, serving internal consumption and commercial applications. This study aimed to develop a method for examining anatomical changes in seeds obtained from *Eucalyptus* crosses to elucidate the causes of seed viability loss and low seed production observed in certain mating pairs. Flower buds/fruits were collected from four different genetic materials (*E. grandis*, *E. urophylla*, *E. urophylla* × *E. grandis*, and self- pollinated *E. urophylla*). Crosses classified as good or poor seed producers were sampled at 15-day intervals from anthesis to maturity. After collection, samples were fixed in FAA50 solution (1:1:18 v/v formaldehyde, acetic acid, and 50% ethanol) and stored in 70% ethanol. Subsequently, the samples were subjected to six different preparation methods, involving standard procedures, combinations of high and/or low temperatures, seed softening substances, and vacuum treatment. Flower buds producing unformed seeds exhibited a functional ovary with reduced locules and increased lignification. Aditionally, unformed seeds were covered by thick integuments and displayed abnormal endosperm cells. In cases where the developing ovary was visible, highly lignified tissues with numerous sclereids were observed. By contrast, flower buds with lower lignification had ovaries with well-developed locules, containing seeds with a normal appearance, characterized by a single-layered integument and well-delimited nucleated endosperm cells. Also, self-pollinated materials produced a few seeds, some of which were unformed. Thus, we conclude that anatomical alterations, likely influenced by genetic factors, lead to incompatibility, resulting in limited seed production or the production of unformed seeds in certain *Eucalyptus* crosses.

Keywords: Seed viability; Anatomical analysis; Controlled crosses

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ANATOMIA COMO FERRAMENTA PARA COMPREENDER AS CAUSAS DE FALHAS NA FORMAÇÃO DE SEMENTES: UM ESTUDO DE CRUZAMENTOS ENTRE ESPÉCIES DE *Eucalyptus*

RESUMO - A propagação seminal do eucalipto desempenha um papel crucial em programas de melhoramento, atendendo ao consumo interno e a aplicações comerciais. Este estudo teve como objetivo desenvolver um método para examinar alterações anatômicas em sementes obtidas de cruzamentos de *Eucalyptus*, a fim de elucidar as causas da perda de viabilidade e baixa produção de sementes observadas em determinados pares de cruzamento. Botões florais/frutos foram coletados de quatro diferentes materiais genéticos (*E. grandis*, *E. urophylla*, *E. urophylla* × *E. grandis* e *E. urophylla* autopolinizado). Cruzamentos classificados como bons ou maus produtores de sementes foram amostrados em intervalos de 15 dias, desde a antese até a maturidade. Após a coleta, as amostras foram fixadas em solução de FAA50 (formaldeído, ácido acético e etanol 50% na proporção 1:1:18 v/v) e armazenadas em etanol 70%. Posteriormente, as amostras foram submetidas a seis diferentes métodos de preparo, envolvendo procedimentos padrão, combinações de altas e/ou baixas temperaturas, substâncias para amolecimento das sementes e tratamento a vácuo. Botões florais que produziram sementes inviáveis apresentaram ovário funcional com lóculos reduzidos e aumento da lignificação. Além disso, sementes inviáveis estavam cobertas por um tegumento espesso e exibiam células do endosperma anormais. Nos casos em que o ovário em desenvolvimento era visível, foram observados tecidos altamente lignificados com numerosos esclereídeos. Por outro lado, botões florais com menor lignificação apresentavam ovários com lóculos bem desenvolvidos, contendo sementes com aparência normal, caracterizadas por um tegumento de camada única e células do endosperma nucleadas e bem delimitadas. Além disso, materiais autopolinizados produziram poucas sementes, algumas das quais inviáveis. Assim, concluímos que alterações anatômicas, provavelmente influenciadas por fatores genéticos, levam à incompatibilidade, resultando em baixa produção de sementes ou na produção de sementes inviáveis em determinados cruzamentos de *Eucalyptus*.

Palavras-Chave: Viabilidade de sementes; Análise anatômica; Cruzamentos controlados

1. INTRODUCTION

It is necessary to understand the reproductive biology of a species for the development of effective breeding strategies (Bolton et al., 2022). Breeding programs have used a variety of methods for *Eucalyptus* species, including controlled pollination (Ramalho et al., 2022; Castro et al., 2021). However, there is a lack of comprehensive information on the floral biology of *Eucalyptus*. To date, only eight species have been studied for Pollen–pistil interactions: *Eucalyptus morrisbyi* (Ouma, 2022), *E. regnans* (Sedgley et al., 1989), *E. woodwardii* (Sedgley, 1989; Sedgley & Smith, 1989), *E. spathulata*, *E. cladocalyx*, *E. leptophylla* (Ellis & Sedgley, 1992), *E. globulus* (Pound, 2002), and *E. nitens* (Pound et al., 2003).

The genus *Eucalyptus* has been reported to exhibit high outcrossing rates (0.69 and 0.84), facilitated by protandry (Dabral et al., 2024; Pryor, 1976). Most species within the genus exhibit a marked reduction in seed yield when self-pollinated compared to cross-pollination (Potts & Savva, 1988; Ellis & Sedgley, 1992). In fact, there is evidence

that more than one mechanism of self-incompatibility operates in *Eucalyptus*, acting at both pre- and post-zygotic levels (Pauldasan et al., 2022). The observed variability in outcrossing rates in *Eucalyptus* could be attributed to the existence of an enzymatic system of self-incompatibility. Such a mechanism commonly acts during the growth of the pollen tube and leads to faster development of pollen tubes relative to the stigma (Nagle et al., 2023), thereby preventing self-pollination.

Understanding reproductive barriers between species is also essential to gain insight into speciation processes (Abbott et al., 2013; Baack et al., 2015; Binks et al., 2021). Hybridization is common in nature and has wide-ranging effects on differentiation of individuals within and between species (Abbott et al., 2013; Morgan-Richards et al., 2022). Hybridization is historically important, as it might have influenced the evolution of many species existing today (Wang et al., 2015; Stull, 2023). The consequences of hybridization depend on several factors, including the degree of genetic divergence between taxa and the strength of intrinsic and extrinsic reproductive barriers (Seehausen et al., 2014; Peñalba et al., 2024). Hybridization can facilitate gene flow, leading to the homogenization of divergent populations and potentially decreasing speciation (Ellstrand & Elam, 1993; Barrera-Guzmán et al., 2024). Conversely, it can also generate unique adaptive phenotypes that contribute to species divergence (Rieseberg et al., 2003). In breeding programs, hybridization strategies are used to produce new materials by combining specific characteristics of interest. However, the successful application of this approach in *Eucalyptus* breeding programs requires a comprehensive understanding of the mechanisms that can cause certain crosses to produce unformed seeds.

The genus *Eucalyptus* is native to Australia and comprises about 800 species (Bayle, 2019), represented by trees that dominate forests and woodlands. It has become one of the most ecologically important plant groups in the Southern Hemisphere (Hopper, 2021). Although natural hybridization is often observed in the genus, there are strong barriers to its occurrence (Robins, 2021). For example, hybridization is not possible between the ten largest subgenera (Griffin et al., 1988), even among phylogenetically related subgenera, with the rate of spontaneous crossings decreasing markedly with increasing genetic distance between species (Larcombe et al., 2015).

Studies using artificial hybridization have confirmed the existence of both pre- and post-zygotic barriers (Binks et al., 2021). Post-zygotic barriers are the most difficult to overcome, as they tend to be related to genetic mechanisms aimed at preserving the distance between materials. The importance of pre-zygotic barriers in *Eucalyptus* species is exemplified by the unidirectional incompatibility observed between *E. globulus* and *E. nitens* (Gore et al., 1990). Although taxonomically these species are placed in the same series (*Globulares*, subgenus *Symphyomyrtus*, section *Maidenaria*; Nicolle, 2015), the validity of this grouping has been increasingly questioned by phylogenetic analyses (Steane et al., 2011).

Differences in flower size are likely to produce two pre-zygotic barriers. Variation in overall flower size can result in distinct pollinators (Ibarra et al., 2023), creating a pre-mating barrier. Additionally, differences in style length can create a unilateral post-mating barrier that prevents F1 hybridization. This is exemplified by the pollen–pistil size mismatch that prevents *E. nitens* pollen from pollinating flowers of *E. globulus* (Gore et al., 1990). Pollination of *E. nitens* flowers by *E. globulus* pollen is still possible. However, this crossing results in a reduced number of

seeds due to incompatibility between the size of the pollen tube of *E. globulus* and the size of the ovaries of *E. nitens* (Gore et al., 1990).

Anatomical investigations of *Eucalyptus* flower buds and seeds are scarce due to methodological challenges related to sample preparation. These plant materials undergo significant lignification from the middle to the end of their development stages. Because of the foregoing, this study aimed to develop a comprehensive method for processing *Eucalyptus* flower buds, fruits, and seeds throughout their entire developmental timeline. Subsequently, the goal is to use the method created to investigate the causes of seed failure and low seed production in certain crosses of *Eucalyptus* spp.

2. MATERIAL AND METHODS

Flower buds were collected from anthesis to maturity from six genotype materials of *E. grandis*, two of *E. urophylla*, and two of *E. urophylla* × *E. grandis*. Additionally, the two *E. urophylla* genotypes were self-pollinated and the resulting flower buds were collected. Flower buds from the crossing of *E. urophylla* and *E. grandis* were produced by artificially induced protogyny. Flower buds from pure species were obtained by open pollination between selected breeders of *E. grandis* and *E. urophylla* in the breeding orchard. Each sample consisted of 15 flower buds. Samples were collected every 15 days for 6 months or until no more flower buds and fruits were observed on the branches of breeders selected for this study (Table 1).

After collecting, flower buds were immediately stored in the FAA50 fixative (1:1:18 v/v formaldehyde, acetic acid, and 50% ethanol) and incubated under vacuum

Table 1. Collection schedule of *Eucalyptus* flower buds after pollination. Two breeders were selected from *E. grandis* (GRA), *E. urophylla* (URO), and *E. urophylla* × *E. grandis* (URO × GRA). The two *E. urophylla* genotypes were also self-pollinated

Tabela 1. Cronograma de coleta de botões florais de *Eucalyptus* após a polinização. Dois genitores foram selecionados de *E. grandis* (GRA), *E. urophylla* (URO) e *E. urophylla* × *E. grandis* (URO × GRA). Os dois genótipos de *E. urophylla* também foram autopolinizados

Date	GRA 1	GRA 2	URO 1	URO 2	URO × GRA 1	URO × GRA 2	Self-pollinated URO 1	Self-pollinated URO 2
January 10, 2018	C1	-	C1	C1	C1	-	-	-
January 25, 2018	C2	-	C2	C2	C2	-	Self-pollination	-
February 6, 2018	C3	-	-	C3	C3	-	SPC1	-
February 28, 2018	C4	C1	C3	C4	C4	C1	SPC2	-
March 14, 2018	C5	C2	C4	C5	C5	C2	SPC3	Self-pollination
June 6, 2018	C11	C8	C10	C11	C11	C8	SPC9	SPC6
June 20, 2018	C12	C9	C11	C12	C12	C9	SPC10	SPC7
July 4, 2018	C13	C10	C12	C13	C13	C10	SPC11	SPC8
July 18, 2018	C14	C11	C13	C14	C14	C11	SPC12	SPC9
August 7, 2018	C15	C12	C14	C15	C15	C12	SPC13	SPC10
August 15, 2018	C16	C13	C15	C16	C16	C13	SPC14	-
August 29, 2018		C14	C17	C17	C17	C14	SPC15	-
September 12, 2018	C18	C15	C17	C18	C18	C15	SPC16	-
September 26, 2018	C19	C16	C18	C19	C19	C16	SPC17	-
October 10, 2018	C20	C17	C19	C20	C20	C17	SPC18	-

for 48 h. Subsequently, the material was stored in 70% ethanol (Johansen, 1940). Samples were dehydrated in an ethanol series and embedded in methacrylate (Leica HistoResin). Different embedding and processing methods were tested to identify which afforded optimal penetration of the resin into the ovary and seeds. These organs are covered by several layers of lignified tissue (Figure 1).

2.1 Processing methods

The first method (Method 1) tested was the standard method recommended by the manufacturer of acrylic resin. Resin needs to penetrate well into the biological material to make it hard enough to resist the pressure of anatomical sectioning without affecting the natural characteristics of tissues.

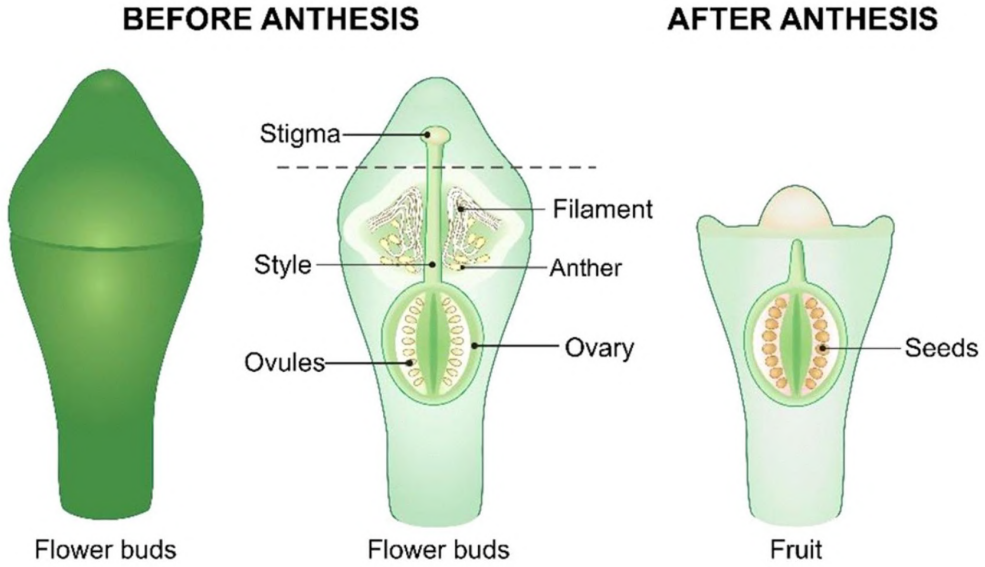
Method 1. Samples were dehydrated in an increasing ethanol series (80%, 90%, 95%, and 100%), being incubated for 2 h under vacuum in each solution. Then, samples were immersed in pure HistoResin and stored in a refrigerator for 2 months, with a vacuum applied for 2 h twice a day. After this period, samples were blocked according to the manufacturer's instructions (1 mL of hardener for every 15 mL of pure HistoResin) and cut to visualize resin penetration.

Five other methods based on the standard method were tested, as described below.

Method 2. Flower buds were placed in a 1:1 (v/v) glycerin and water solution in an oven at 35 °C for 2 months. The solution was changed every 7 days. After this period, samples were dehydrated in an increasing ethanol series (80%, 90%, 95%, and 100%), being incubated for 2 h under vacuum in

Figure 1. Illustration of a *Eucalyptus* flower bud with its constituent structures before and after anthesis, when the ovules can be fertilized. The dashed line indicates the height where a cut is performed artificially induce protogyny or one-stop pollination (Assis et al., 2005)

Figura 1. Ilustração de um botão floral de *Eucalyptus* com suas estruturas constituintes antes e após a antese, quando os óvulos podem ser fertilizados. A linha pontilhada indica a altura onde é realizado um corte para induzir artificialmente a protoginia ou realizar uma polinização única (one-stop pollination, Assis et al., 2005)



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each solution. Finally, samples were transferred to pure HistoResin and stored in a refrigerator for 2 months. During this period, vacuum was applied for 2 h twice a day. Samples were embedded according to the manufacturer's instructions and cut to visualize resin penetration.

Method 3. Flower buds were first treated with glycerin solution as described above for Method 2. Then, samples were trimmed in the region opposite to the insertion site of the stylet and were cut in half along the longitudinal axis. After these procedures, samples were treated with HistoResin for about 2 months, being kept 5 days under vacuum and 2 days under non-vacuum conditions during this period, always stored in a refrigerator. Samples were embedded according to the manufacturer's instructions and cut to visualize resin penetration.

Method 4. Flower buds were trimmed around the longitudinal axis to remove most of the lignified outer shell. Care was taken to ensure that the procedure did not reach the ovary chambers. After this, samples were immersed in a 1:1 (v/v) glycerin and water solution in a water bath at 75 °C for 2 h and dehydrated in an increasing ethanol series (80%, 90%, 95%, and 100%). Samples were then immersed in HistoResin solution and kept under constant vacuum for 2 months. After this period, samples were embedded according to the manufacturer's instructions. Blocks were incubated in an oven at 35 °C for 4 days and cut to visualize resin penetration.

Method 5. Flower buds were trimmed as described in Method 4, placed in a 1:1:1 (v/v/v) solution of 100% ethanol, water, and glycerin, and subjected to three 30 min autoclave cycles spaced 72 h apart. After the procedure, samples were dehydrated in an increasing ethanol series (80%, 90%, 95%, and 100%), immersed in HistoResin solution, and kept under constant vacuum for 2 months. Samples were embedded according to the manufacturer's instructions, incubated in an oven at 35 °C for 4 days, and cut to visualize resin penetration.

Method 6. Samples were dehydrated in an increasing ethanol series (70% 80, and 95%), being kept under vacuum for 2 h in each solution. Then, samples were transferred to pure HistoResin and kept under constant vacuum in a refrigerator for 30 days. The resin solution was changed every 5 days or earlier if the solution color changed from translucent to light yellow. Samples were embedded on ice using 1.2 mL of hardener for every 15 mL of resin. After this, samples were rapidly transferred to −20 °C for 7 days, then stored at 4 °C for another 7 days and incubated in an oven at 35 °C for 7 days for polymerization. Subsequently, blocks were mounted on wooden stubs and cut.

In all methods, after resin penetration, samples were cut to 5 µm thick sections using an automatic rotary microtome (RM2155, Leica Microsystems Inc., Deerfield, USA). This made it possible to assess resin penetration and embedding quality. Sections were stained with toluidine blue in phosphate buffer, pH 6.5 (O'Brien & McCully, 1981). Glass slides were mounted using synthetic resin (Permount®).

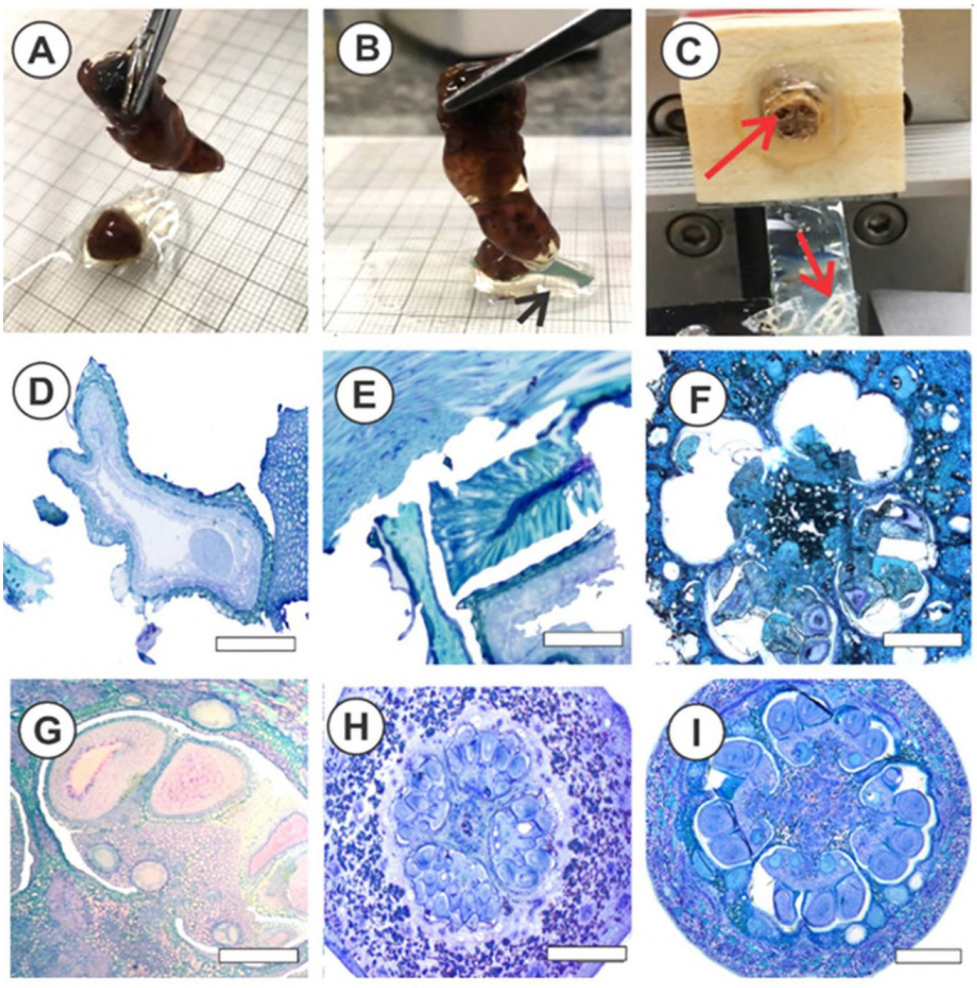
3. RESULTS

Methods 1 and 2 were ineffective in facilitating resin penetration into the flower bud, with clear evidence of inadequate resin infiltration. Notably, the resin began polymerizing even in the absence of the hardener (Figure 2A and B). Method 3 demonstrated improved resin penetration, enabling the formation of blocks and subsequent sectioning of the flower bud (Figure 2C, D, and E). The arrows in these images highlight areas of insufficient HistoResin penetration, resulting in the

degradation of internal structures within the flower bud. Methods 4 and 5 also showed enhanced resin penetration, though they still did not allow for complete visualization of the ovary (Figure 2F, G, and I). In contrast, Method 6 achieved full resin penetration, providing clear visualization of the ovary and seeds (Figure 2H and I). Anatomical sections were obtained from samples treated using Method 6.

Figure 2. Results of different sample preparation methods. (A and B) Initially, resin polymerization occurred during infiltration. (C and D) Method 3 also resulted in polymerization, but with some resin penetration into the flower bud. Methods 4, 5, and 6 afforded a remarkable improvement in (G) resin infiltration, (H) in younger and (I) mature flower buds. The black arrow indicates resin hardening and red arrows indicate absence of resin penetration inside the flower bud. Scale bars: D–F, 100 µm; G, 300 µm; H and I, 200 µm

Figura 2. Resultados dos diferentes métodos de preparo das amostras. (A e B) Inicialmente, a polimerização da resina ocorreu durante a infiltração. (C e D) Método 3 também resultou em polimerização, mas com alguma penetração de resina no botão floral. Os Métodos 4, 5 e 6 proporcionaram uma melhoria notável na (G) infiltração da resina, (H) em botões florais mais jovens e (I) em botões florais maduros. A seta preta indica o endurecimento da resina, e as setas vermelhas indicam a ausência de penetração da resina dentro do botão floral. Barras de escala: D–F, 100 µm; G, 300 µm; H e I, 200 µm



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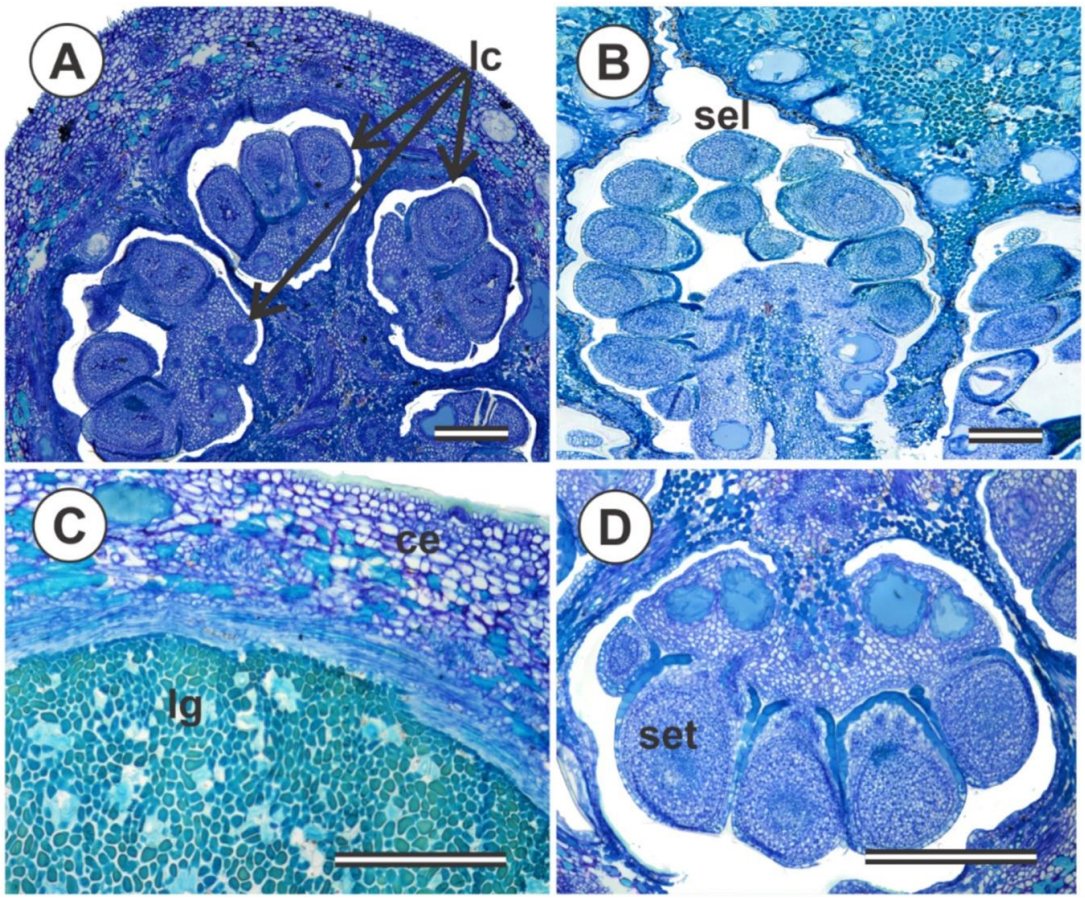
The application of Method 6 facilitated the acquisition of high-quality anatomical sections, enabling adequate visualization and the division of seeds into two distinct groups. The first group was characterized by the formation of seeds exhibiting a normal appearance, with a well-developed, circular ovary (Figure 3). Notably, the integument tissue was observed, consisting of a single layer of cells, and the seeds displayed well-organized, clearly delineated cellular structures (Figure 3).

Figure 3. Anatomical cuts of flower buds of *Eucalyptus* showing the formation of viable, normal seeds. (A) Cross-section, (B) longitudinal section, (C) lignified tissues of the flower bud, (D) seeds in formation, (E) integument and endosperm, and (F) enlarged image of the seed showing normal cells. Arrow heads indicate cells undergoing division. Scale bars: A–D, 300 µm; E and F, 100 µm. cb, flower bud capsule; lc, cross-section of ovary locules; sel, longitudinal section of ovary locules; set, developed seeds; tg, integument; lg, highly lignified tissue; ed, endosperm

Figura 3. Cortes anatômicos de botões florais de Eucalyptus mostrando a formação de sementes viáveis e normais. A) Corte transversal, (B) corte longitudinal, (C) tecidos lignificados do botão floral, (D) sementes em formação, (E) tegumento e endosperma, e (F) imagem ampliada da semente mostrando células normais. As pontas de seta indicam células em divisão. Barras de escala: A–D, 300 µm; E e F, 100 µm. cb, cápsula do botão floral; lc, corte transversal dos lóculos do ovário; sel, corte longitudinal dos lóculos do ovário; set, sementes desenvolvidas; tg, tegumento; lg, tecido altamente lignificado; ed, endosperma



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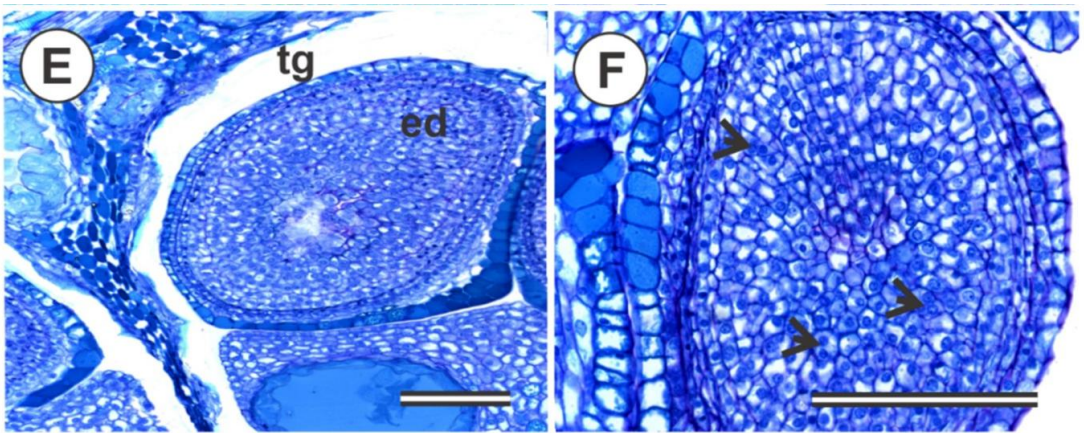


The second group was characterized by flower buds exhibiting poorly expanded, narrow, and underdeveloped ovaries (Figure 4). The structures within the locules, where seeds are typically located, did not resemble normal seeds. Contrary to expectations, these structures displayed disorganized cellular arrangements, and the tissue that would typically correspond to the integument was thickened and abnormal, showing signs of excessive hardening. Additionally, there was a complete absence of normal, well-structured cells.

4. DISCUSSION

We developed an effective method to obtain anatomical cuts of *Eucalyptus* flower buds. This plant material has high lignification and regions with sclereids, which increase hardening. This factor hindered resin penetration, necessitating various adaptations to the tested methods, such as the use of elevated temperatures and softening substances (glycerin).

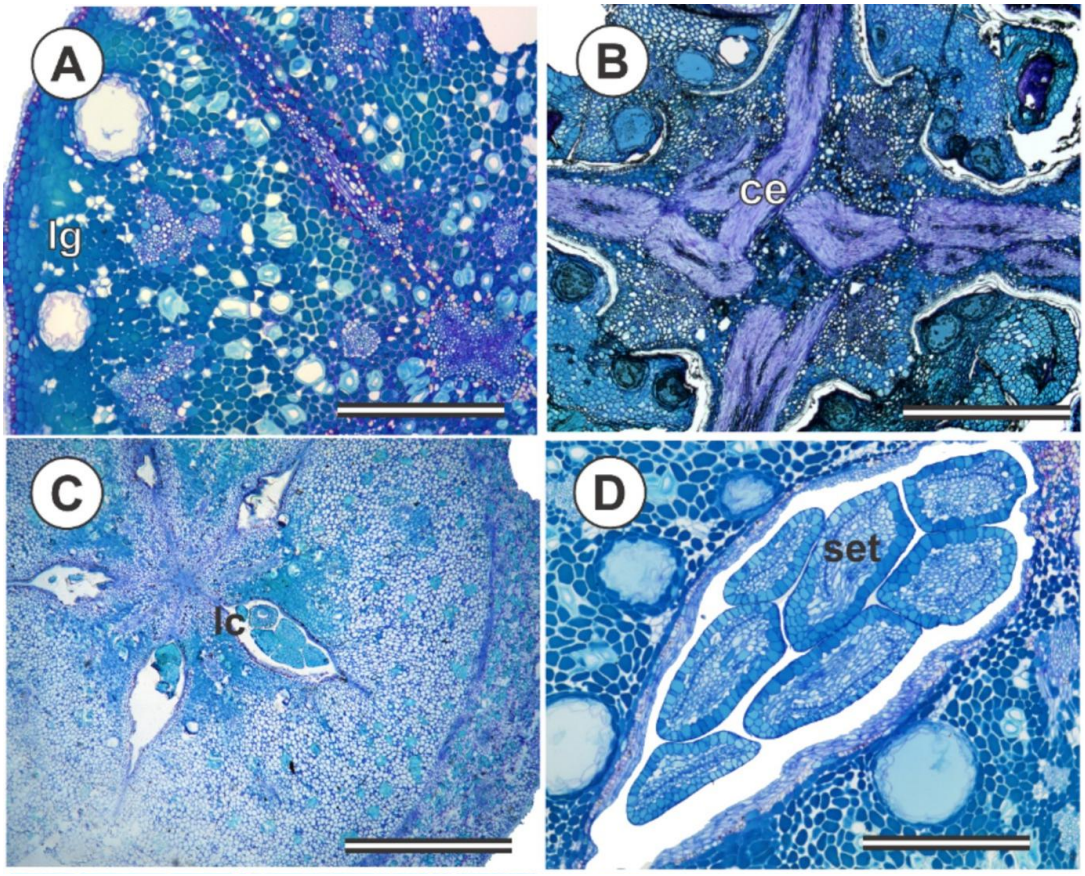
The superiority of the developed method over the others has direct implications for the anatomical analysis of formed and unformed seeds, enabling a more precise distinction between the morphological characteristics associated with normal seed formation and the structural alterations that lead to reproductive inviability in certain crosses. This methodological approach can, therefore, be adopted as a reference for future investigations involving plant species with similar challenges in histological preparation, expanding analytical possibilities in genetic improvement programs and plant reproduction studies (Binks et al., 2021).

The causes of resin polymerization in Methods 1 and 2 are unclear. It might have been due to the high temperatures at the time of the experiment or exudation of phenolic compounds by the sample, which may have altered resin composition. For these reasons, samples subjected to Methods 1 and 2 were discarded.

Low temperatures combined with constant vacuum provided the best results for *Eucalyptus* flower buds. Low temperatures prevented resin polymerization, allowing its penetration into samples. This finding suggests the lignification of tissues and, most likely, the negative relationship between material constituents (in this case, phenolic compounds) and high temperatures. It is likely that methods using high temperatures promoted the extravasation of phenolic compounds and alteration of their chemical composition. Studies have shown that *Eucalyptus* oil is mainly composed of oxygenated monoterpenes and monoterpene hydrocarbons and has a yellowish color

Figure 4. Anatomical cuts of flower buds of *Eucalyptus* showing the formation of non-viable, abnormal seeds. (A) Cross-section and (B) longitudinal section of the flower bud demonstrating high lignification of tissues; (C) ovary locules; (D) close-up of a locule showing the seeds; (E) longitudinal section showing the formation of non-viable seeds; (F) enlarged image of the seed showing abnormal cells and thickening of the endosperm layer. It is not possible to observe cells undergoing division. Scale bars: A–D, 300 µm; E and F, 100 µm. lg, lignified tissue; ce, cells with sclerifications; lc, cross-section of ovary locules; set, seed-like structures or non-viable seeds; tg, integument; ed, endosperm; sel, non-viable seeds inside the locule. Arrows indicate the cell nucleus, suggesting viability

Figura 4. Cortes anatômicos de botões florais de *Eucalyptus* mostrando a formação de sementes inviáveis e anormais. (A) Corte transversal e (B) corte longitudinal do botão floral demonstrando alta lignificação dos tecidos; (C) lóculos do ovário; (D) close-up de um lóculo mostrando as sementes; (E) corte longitudinal mostrando a formação de sementes inviáveis; (F) imagem ampliada da semente mostrando células anormais e espessamento da camada do endosperma. Não é possível observar células em divisão. Barras de escala: A–D, 300 µm; E e F, 100 µm. lg, tecido lignificado; ce, células com esclereídeos; lc, corte transversal dos lóculos do ovário; set, estruturas semelhantes a sementes ou sementes inviáveis; tg, tegumento; ed, endosperma; sel, sementes inviáveis dentro do lóculo. As setas indicam o núcleo celular, sugerindo viabilidade



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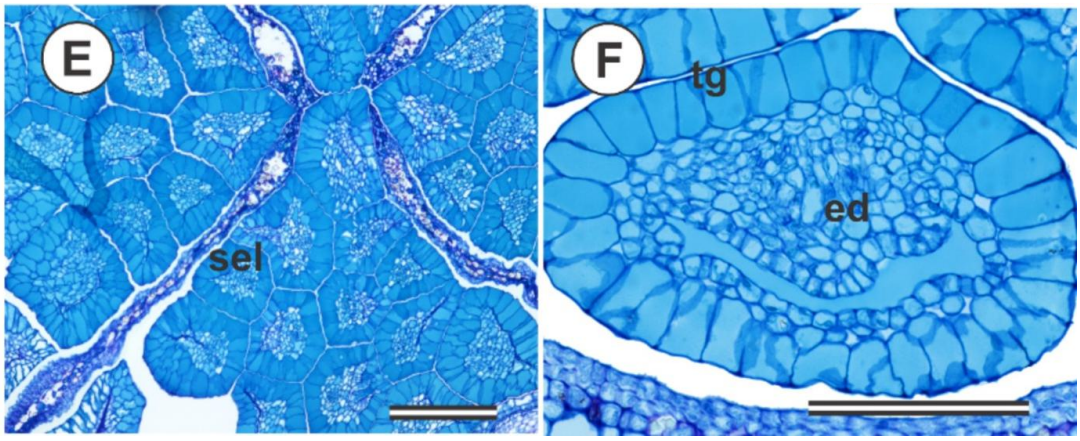

(Shala & Gururani et al., 2021; Harkat-Madouri et al., 2015). The oil also contains tannins, saponins, terpenoids, glycosides, alkaloids, phenolic compounds, steroids, cardiac glycosides, terpenes, reducing sugars, carbohydrates, resins, acidic compounds, and flavonoids (Ajilore et al., 2021; Jamil et al.,. 2017). These compounds might have affected HistoResin composition, inducing polymerization, thereby impairing its penetration. The resin is known to harden quickly in the absence of the hardening agent. Thus, for analysis of *Eucalyptus* flower buds, it is recommended to use low temperatures and constant vacuum for good resin penetration (Castro et al., 2021)

Samples prepared using optimum conditions (Method 6) were classified into two groups with different anatomical findings, corresponding to good and poor crosses. Seeds from poor crosses did not germinate because of the formation of unformed seeds resulting from anatomical changes that hinder or do not allow germination. The causes of the formation of unformed seeds from poor crosses have not yet been elucidated. There is still no evidence supporting a possible self-incompatibility in the phase of pollen adhesion to enzymatic exudate, as has been demonstrated in other *Eucalyptus* species. In these cases, the pollen tube does not grow sufficiently, resulting in a reduction in self-pollination rate compared with cross-pollination rate (Ascher, 1976). This delay in pollen tube growth suggests the existence of an unknown form of gene alteration associated with self-incompatibility (Mcguire & Rick, 1954; Hardon, 1967; Ascher, 1976).

In the current study, the malformation or non-formation of seeds might have been related to a deficiency in pollen tube growth during crosses. Direct measurements of the pollen tubes of *Amsinckia grandiflora* (Weller & Ornduff, 1989), *Erythronium grandiflorum* (Cruzan, 1989), and *Delphinium nelsonii* (Waser et al., 1987) revealed differences in pollen tube growth between self- and cross-pollinating individuals. A similar phenomenon occurs in *E. urophylla* and *E. grandis*. These species are considered critically self-incompatible because the growth of incompatible pollen tubes is slower than that of compatible ones, rather than being completely inhibited.

Late-acting self-incompatibility may also be considered, given the low seed formation in self-crosses, in line with what occurs between *E. urophylla* and *E. grandis* and in *E. nitens* (Pound et al., 2003). In the cited study, yields were reduced after self-pollination; even though pollen tubes had grown, most of the ovaries of *E. nitens*

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originating from self-pollination began to degenerate in the first weeks (Pound et al., 2003). The authors suggested that ovule degeneration was a self-incompatibility response.

It is difficult to experimentally determine which mechanism of self-incompatibility operates within a species. Uniform ovule abortion may indicate a self-incompatibility response, whereas abortion of some ovules at various stages of embryonic development may be indicative of inbreeding depression (Seavey & Bawa, 1986). However, there are questions about the ability of inbreeding to cause high levels of ovule abortion (Waser & Price, 1991).

Because of the relevance of *Eucalyptus* species in the forestry sector, it is of great importance to develop a rapid method to assess malformation and formation of unformed seeds arising from specific crosses. Genetic improvement programs can be time-consuming, and many *Eucalyptus* plantations are decades old (Ramalho et al., 2022). Furthermore, pure line production, which is a novel method in *Eucalyptus* breeding (Castro et al., 2022), requires 5 to 6 generations of self-pollination (inbreeding). Such processes may be long and costly if not well planned, if flowering acceleration techniques are not used, or if fertilization rates are unknown. Thus, the method proposed may contribute to obtaining new and better *Eucalyptus* cultivars.

Understanding reproductive barriers is fundamental to unravel the processes that cause seed non-viability (Baack et al., 2015). Although natural outcrossing is often reported in *Eucalyptus*, there are strong barriers to its occurrence (Griffin et al., 1988). For example, outcrossing is not possible between the ten major subgenera. Even within phylogenetic subgenera, the probability of outcrossing decreases rapidly with increasing genetic distance between species (Larcombe et al., 2015). Mechanical mismatch in size between the pollen of one species and the stigma of another is known to be a strong post-mating reproductive barrier in plants (Grant, 1994). In this study, anatomical and morphological analyses of the floral biology of breeders were not conducted; however, it is likely that such an asymmetry would be observed.

The proportions of viable and unformed seeds may differ. It is possible that there is a variation in the length of pollen tubes. Early acting post-zygotic barriers may also be acting in this case, as already reported in *Eucalyptus* (Potts & Dungey, 2004), including at the embryo stage (Dickinson et al., 2012). This phenomenon may explain the formation of seeds lacking endosperm and showing abnormal cells, possibly without a viable embryo. The existence of seeds lacking their normal constituents reinforces the hypothesis of post-zygotic mechanisms. This type of seed can be visualized and collected but will not germinate.

Crossings in which the pollen tube reaches the ovules result in fertilization and formation of normal seeds. This hypothesis is supported by the fact that, during seed fertilization, double fertilization occurs. A sperm nucleus from pollen fertilizes the oosphere, generating an embryo (2n tissue), and another sperm nucleus fertilizes the synergid cell, originating the endosperm (3n tissue). This explains the existence of endosperm in seeds from good crosses and its absence in crosses that produce unformed seeds.

The methodological approach developed in this study represents a significant advancement in plant anatomy applied to *Eucalyptus* breeding. The adaptation of the resin embedding process under vacuum and low temperatures proved to be an effective solution for obtaining high-quality histological sections, enabling detailed

observation of seed morphogenesis. Furthermore, this method allows for a substantial reduction in the time required to obtain high-quality histological sections. Unlike traditional methods, which may require several months for the embedding and processing of lignified tissues, the approach proposed here reduces this time to just thirty days. This efficiency represents a major advantage for research in the field, enabling faster analyses and facilitating decision-making in genetic improvement programs and reproductive viability studies in *Eucalyptus*.

5. CONCLUSION

The best method for resin penetration into *Eucalyptus* flower buds consists of using constant vacuum at low temperatures, as applied in Method 6.

Seeds from poor crosses lack endosperm with viable cells. There is abnormal thickening of the integument in unformed seeds.

This study provides a novel and important approach to anatomical studies of *Eucalyptus* fruits not yet addressed in the literature.

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AUTHOR CONTRIBUTIONS

Souza, GA de: Conceptualization; Data curation; Formal analysis; Writing – original draft; Writing – review & editing. Caiafa KF: Formal analysis; Writing – original draft; Writing – review & editing. Santos GA dos: Conceptualization; Writing – review & editing. Gandra K do C: Formal analysis; Methodology. Castro CA de O: Formal analysis; Methodology; Writing – original draft. Pantuza IB: Formal analysis; Methodology; Writing – original draft. Ladeira J dos S: Formal analysis; Methodology; Writing – original draft.

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