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Evaluation of scarification and *in vitro* culture methods for callus production in the genus *Erythrina*

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ABSTRACT. The genus *Erythrina* (Fabaceae) has been researched due to its tetracyclic alkaloids that have pharmacological properties. Species of *Erythrina* have the potential to act on the central nervous system, presenting anxiolytic and anticonvulsant properties. Among them, *Erythrina verna* is particularly known for its anxiolytic and sedative effects, being widely used in traditional medicine. Studies indicate that the species *E. speciosa* has gastroprotective properties besides its high ornamental value. *In vitro* culture involves techniques for growing cells, tissues or organs under aseptic conditions, based on culture media containing essential nutrients for development. In addition, different seed scarification techniques and aseptic methods can be employed to achieve better results, which may vary for each species. In this context, the present work aimed to standardize the methodology of 'callus culture' in two species of *Erythrina*, as well as to evaluate the behavior of these plants under *in vitro* conditions. The methodology employed for *in vitro* germination of *Erythrina verna* and *E. speciosa* seeds was successful, resulting in seedlings with well-developed stem, cotyledons and root, which served as a source of explants for 'callus culture'. The scarification techniques used were effective in breaking dormancy in both species. Additionally, the sterilization, disinfection, and cleaning processes played a crucial role in the satisfactory *in vitro* development of the species.

Keywords: tissue culture; germination; *Erythrina verna*; *Erythrina speciosa*, plant tissue.

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Introduction

The genus *Erythrina* (Fabaceae) comprises approximately 115 species and has been investigated due to the pharmacological properties of the tetracyclic alkaloids it contains. *Erythrina* species have the potential to act on the central nervous system, exhibiting anxiolytic and anticonvulsant properties (Rambo et al., 2019). Species of the genus have been examined for the presence of biologically active compounds in calluses and intact plants, and typical tetracyclic alkaloids have been identified in both species listed in this study (Amaral et al., 2019). The authors suggest that tissue culture technique represents an alternative for the production of pharmacologically important alkaloids in this genus.

The studied species are native to Brazil and can be found in various regions encompassed by the Atlantic Forest biome. They are angiosperms belonging to the legume family (Fabaceae/Leguminosae) and Faboideae subfamily. These species exhibit robust trunk and vibrant flowers, with deciduous behavior during the flowering and fruiting season, which gives them ornamental value, in addition to their significant medicinal potential.

Seed dormancy is a widely employed strategy in Cerrado species, given the region's characteristics of high rainfall and pronounced seasonality. However, cultivating species with dormant seeds becomes challenging due to the prolonged germination period, which delays seedling growth (Bewley et al., 2013). Additionally, there is concern that when these seeds remain in the soil for extended periods after planting, they may become susceptible to fungal infections, resulting in significant losses, both in terms of production and finances. This issue can be addressed through scarification, a process that involves creating superficial damage to the seed coat (Bewley et al., 2013). In nature, scarification occurs through factors such as acidic soil pH, wildfires, and the action of microorganisms. To achieve these results artificially, specialized tools known as scarifiers are employed (Lorenzi et al., 2003).

In vitro cultivation involves techniques for cultivating cells, tissues, or organs under aseptic conditions, based on nutrient-rich media containing the necessary factors for development, such as water, carbon

sources, vitamins, minerals, and growth regulators (Oliveira, 2016). The process begins with explants, which are fragments of a plant organism, which can originate from stems, leaves or roots. With a correct formulation of the culture medium composition, explants proliferate into calluses containing undifferentiated cells, which can undergo a screening process for one or more products of interest. This work intended to standardize an *in vitro* germination methodology for species of the genus *Erythrina,* incorporating a scarification step, and to perform callus culture from *in vitro* germinated seedlings.

Material and methods

Plant material

Seeds of *Erythrina verna* from the 2021 harvest, from an unknown batch with a purity of 81%, and *Erythrina speciosa* from the 2022 harvest, from batch 169 with a purity of 90%, were obtained from the following partners: Mundo das sementes® and Sementes Caiçara®, respectively.

Scarification of *Erythrina*'s seeds

Two different methods were evaluated for scarification of *Erythrina* seeds: scarification with concentrated sulfuric acid (PA) for 30 minutes at 25°C, and mechanical scarification performed manually with 80-grit sandpaper. For sulfuric acid scarification, 50 seeds of *E. speciosa* were immersed in H_2SO_4 for 30 minutes in a beaker, stirred with a glass rod. In the manual scarification with sandpaper, 50 seeds of *E. speciosa* were manually sanded with 80-grit sandpaper for approximately 2 minutes (per seed), on the opposite side to the embryo, until the cotyledon was exposed. After both scarification methods, the seeds were placed in a beaker with autoclaved distilled water for 24 hours, followed by disinfection before inoculation onto the culture medium, as detailed bellow.

Sterilization, cleaning and disinfection

This stage followed García-Mateos et al. (1999), with modifications. After scarification, the seeds were washed with water and soap, and then disinfected by immersion in a 70% ethanol solution for 20 minutes, followed by a 15-minute treatment with a 0.75% chlorine solution. The seeds were then rinsed five times with autoclaved distilled water. The workbenches were sterilized with 70% ethyl alcohol, and all instruments were thoroughly washed with water, soap, and concentrated commercial hypochlorite.

After the disinfection procedure, the seeds were immediately inoculated on solid WPM medium, and all instruments and glassware used for inoculation were also properly sterilized. Inoculation was performed in a laminar flow cabinet, cleaned with 70% ethyl alcohol and general-purpose disinfectant (composition: 0.45% Benzyl Alkyl Dimethyl Ammonium Chloride/Didecyl Dimethyl Ammonium Chloride [Formaldehyde], fragrance, and carrier). The airflow and the UV lamp were turned on 15 minutes before use (with all instruments and materials inside the cabinet, including the seeds).

Seed inoculation for seedling development

In the laminar flow cabinet, the seeds were inoculated in test tubes (1 seed per tube/20 replicates), each containing 20 mL of solid WPM culture medium. The WPM (Lloyd & Mc Cown, 1980) culture medium was supplemented with 30 g L^{-1} sucrose, 8 g L^{-1} agar (Himedia®), and the pH was adjusted to 5.8 before autoclaving. The medium was autoclaved at 121ºC and a pressure of 1.05 kg cm^{-2} for 20 minutes.

The tubes were kept in a growth room at 25ºC, with a 16-hour photoperiod and photosynthetic active radiation of 45-60 µmol m^{-2} s^{-1}, for approximately 20 days. The same inoculation was also done in 200 mL glass flasks (5 seeds per flask/10 replicates), containing about 50 mL of medium. The seedlings resulting from *in vitro* germination were used as a source of aseptic stem, leaf, and root explants for callus culture.

MS medium was also evaluated for germination of *E. verna* seeds; however, germination was unsuccessful.

Preparation of cotyledonary explants and establishment of callus culture

Under aseptic conditions, nodal segment, cotyledonar node, and hypocotyl explants from 12 to 15-day-old seedlings were excised and transferred to a Petri dish with dimensions of 90 x 15 mm (5 explants $dish^{-1}$) with 35 mL of solidified WPM medium using 8 g L^{-1} agar (Himedia®), supplemented with 3% sucrose and 2.0 mg L^{-1} 2,4-D (2,4-Dichlorophenoxyacetic acid). They were incubated at 30ºC in the absence of light for 10 to 30 days, following Fidemann et al. (2017).

Statistical analysis

For comparison and statistical evaluation, visual assessments and measurements with a ruler were utilized. The germination index for the two scarification methods was statistically computed, with means compared using ANOVA followed by Tukey's test, all performed using the software 'Sisvar' (Ferreira, 2019).

Results and discussion

Seeds scarification

In the germination tests, seeds were considered germinated if they developed a primary root with a length equal to or greater than 2 mm. The germination test evaluations followed the protocols described in the literature for each species (Pereira et al., 2014). Statistical analysis was performed using paired ANOVA and Tukey's Test in the Sisvar® software (Table 1). Two sample groups of *E. speciosa* seeds were compared regarding the seedling growth, measured in centimeters.

Tab1e 1. Results of the application of the Tukey's test on the germination index of *Erythrina speciosa*, with seeds scarified with sulfuric acid and seeds scarified with 80-grit sandpaper.

Treatment	Repetitions	Average	Pr > Fc
80-grit sandpaper	50	1.537a[1]	0.0334
Sulfuric acid	50	2.182a[2]	
C.V. (%)	80.36		

Two methods were tested for their efficiency in breaking dormancy in *E. speciosa* seeds. The results indicated that sulfuric acid (H_2SO_4) scarification was the superior method, showing the highest efficacy in breaking dormancy for this species, as determined by the germination tests. This was also the only method in which seed heating (thermal scarification) was observed. After scarification with sulfuric acid, 50 seeds of *E. speciosa* resulted in an average of 15 seedlings, which grew to approximately 3-5 cm within 15 days (20% germination rate). In contrast, after scarification of 50 seeds with 80-grit sandpaper, only an average of 8 seedlings reached approximately 3-5 cm in growth within 15 days (Figure 1).

(a) (b)

Figure 2. Seedlings with approximately 15 days of growth treated with manual sandpaper of grit 80 (a) and sulfuric acid (b).

The same scarification method through sulfuric acid was also used on 15 seeds of *E. verna*, resulting in 3 seedlings reaching approximately 3-5 cm in 11 days of growth (Figure 2).

Plants of the *Erythrina* genus are known in the literature for their high physical resistance, and previous studies have demonstrated that scarification with manual sandpaper is effective for breaking seed dormancy (Magalhães et al., 2021). The results obtained in this study are consistent with the literature, although in this case, acid scarification was more effective for both treated species. The use of thermal scarification with sulfuric acid has been recommended for species with seed coats that are impermeable to water and gases (Moreira et al., 2017). However, this type of scarification can lead to contradictory results in overcoming dormancy (Ferreira et al., 2014).

Figure 3. Sulfuric acid scarified seed (a) and *in vitro* germinated seedling (b) of *Erythrina verna*, with 11 days of growth.

Standardization of the *in vitro* cultivation methodology of seedlings and callus culture

In all experiments, the Woody Plant Medium (WPM), formulated by Lloyd and Mc Cown (1980) was used. This medium was specifically developed for shoot culture in woody plants and has ¼ the concentrations of nitrate, ammonia, and potassium ions compared to the MS medium, while having twice the levels of phosphate (PO_4^{3-}). This medium has been recommended for woody species due to its low total ion concentrations (Silva et al., 2019). Both species showed satisfactory results in the callus culture stage, with young hypocotyls exhibiting higher dedifferentiation potential. In other words, stem explants of approximately 0.5-1 cm resulted in a large quantity of compact and undifferentiated cell mass (Figure 3). Additionally, cotyledonary explants of both species showed good dedifferentiation potential, although more time was required for this process. Finally, root explants showed no dedifferentiation potential in both species, and in all callus cultures conducted, these explants were discarded.

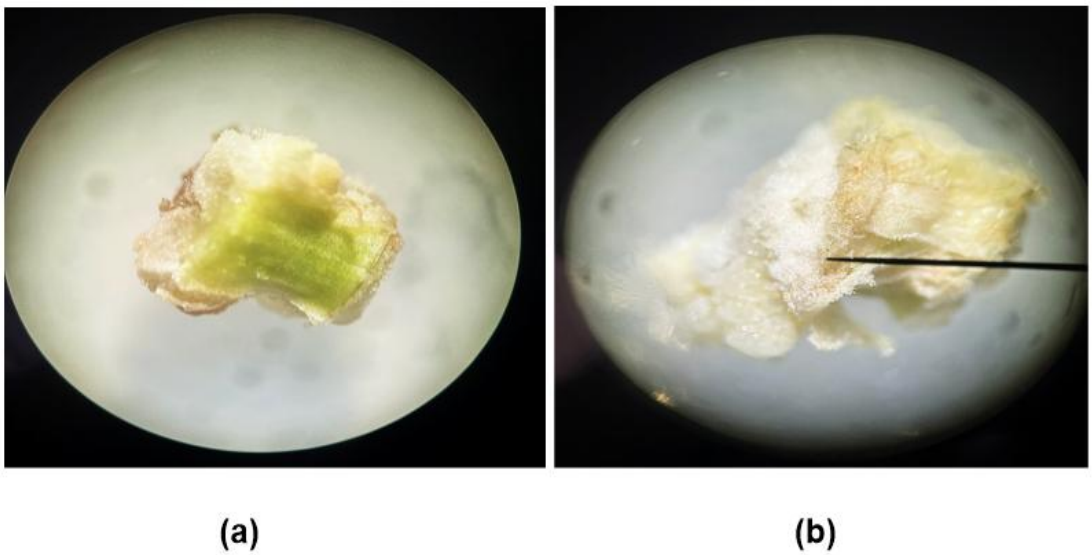

Figure 4. Calli obtained from stem explants of *Erythrina speciosa*. Callus with 13 days of growth (a), and callus with 20 days of growth (b).

Conclusion

The methodology employed for *in vitro* germination of *Erythrina verna* and *Erythrina speciosa* seeds was successful, resulting in seedlings with well-developed stems, cotyledons, and roots, which served as a source of explants for callus culture. The culture medium used in the stages (WPM medium supplemented with sucrose, agar, and plant hormone in the case of the callus medium) demonstrated high efficiency. Regarding seed dormancy breakage for both species, it can be concluded that the most satisfactory alternative was sulfuric acid scarification (H_2SO_4), making it the most advantageous for the *in vitro* cultivation methodology

of *Erythrina* genus species. The methods for obtaining calli involved rigorous aseptic and sterile procedures. Calli from both species exhibited satisfactory cell mass growth with a great potential for dedifferentiation, with hypocotyl and cotyledon explants showing the highest dedifferentiation potential, respectively.

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