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The origin of new roots from cut seeds of *Eugenia* species

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ABSTRACT: Studies on seed germination in *Eugenia* species after embryo fractionation have demonstrated that cotyledon cells have capacity for de-differentiation and consequent production of roots. However, there is no information about the origin of those new roots. Thus, the aim of this study was to characterize anatomically the cotyledon regions of seeds of five species of *Eugenia* to elucidate the tissue that originates such roots. Seeds were sectioned across the hillum region and immediately placed to germinate. As soon as the fractions of these cut seeds develop roots they were fixed and processed to the usual techniques for light microscopy. The adventitious roots originated only when the seed was fractioned, never occurring spontaneously in uninjured seeds. Adventitious roots were formed from perivascular parenchyma cells, located close to the injured region of the cotyledons. These cells divided periclinally and proliferated, giving rise to a root meristem. Therefore, new seedlings probably have a different genome than the mother plant, but they would be identical to the embryo that was a result of fertilization.

Index terms: cell de-differentiation, fractionation, perivascular cells, plant anatomy.

RESUMO: Estudos de germinação de sementes em espécies de *Eugenia* após fracionamento de embriões demonstraram que as células dos cotilédones têm grande capacidade de desdiferenciação e consequente produção de raízes. No entanto, não há informações sobre a origem dessas novas raízes. Assim, o objetivo deste estudo foi caracterizar anatomicamente as regiões dos cotilédones de sementes de cinco espécies de *Eugenia* para elucidar o tecido que origina tais raízes. As sementes foram seccionadas na região do hilo e imediatamente colocadas para germinar. Assim que as frações desenvolveram raízes, elas foram fixadas e processadas com as técnicas usuais de microscopia de luz. As raízes adventícias originaram-se apenas quando a semente foi fracionada, nunca ocorrendo espontaneamente em sementes não danificadas. Raízes adventícias foram formadas a partir de células do parênquima perivascular, localizadas próximas à região lesada dos cotilédones. Essas células se dividiram periclinalmente e proliferaram, dando origem a um meristema radicular. Dessa forma, as novas mudas provavelmente têm genoma diferente da planta mãe, mas seriam idênticas ao embrião resultante da fecundação.

Termos de indexação: desdiferenciação celular, fracionamento, células perivasculares, anatomia vegetal.

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INTRODUCTION

Eugenia L. is among the most important genus of Myrtaceae, a family that comprises species of commercial, nutritional, and pharmacological values. Also, these species have characteristics of landscape and food industry interest, and great ecological importance because their seeds are dispersed by animals and, for this reason, suitable for the recovery of degraded areas (Camilo et al., 2016; Amorim et al., 2020, and references therein; Girardelo et al., 2020; Lamarca et al., 2020; Araujo et al., 2021; Guedes et al., 2021; Lazzarotto-Figueiró et al., 2021; Justino et al., 2022).

Plant commercial production can not be done, for most of the species, without obtaining seeds, which can be difficult for those species which produces few amounts of seeds. In spite of having no seed dormancy and short or no delay in seed shoot emergence after root protrusion (Baskin and Baskin, 2021), several species of *Eugenia* produce a few number of fruits and seeds, or produce a great amount of fruits and seeds but from few individuals, sometimes with high natural phenotypic variation (Tonetto et al., 2013; Novaes et al., 2018; Amorim et al. 2020, and references therein). Also, these seeds are desiccation sensitive, thus difficult to store for long periods (Delgado and Barbedo, 2012; Scalon et al., 2012; Alegretti et al., 2015; Lamarca et al., 2016; Calvi et al., 2017a; Silva et al., 2017; Barbedo, 2018; Fernandes et al., 2019). Therefore, for a commercial scale production, the source of seeds could be a considerable problem which could be solved, for example, by regenerating shoots *in vitro*, as shown in *E. myrtifolia* (Blando et al., 2013). However, some studies have shown the possibility to obtain more than one seedling from the same seed of *Eugenia* species by cutting them *in vivo* (Amorim et al., 2020, and references therein).

Studies on seed germination in *Eugenia* species after cutting the embryo have demonstrated that cotyledon cells have great capacity for de-differentiation and, consequently, production of roots and / or shoots (Anjos and Ferraz, 1999; Silva et al., 2005; Amador and Barbedo, 2011; Delgado et al., 2010; Prataviera et al., 2015; Alonso et al., 2019). This regenerative ability could produce several roots, but frequently there is the formation of only one root and/or shoot per fraction, which suggests some seed internal control to avoid the germination of more than one root at the same time. Thus, when a root meristem begins to develop, there could be an inhibition of the formation of new roots (Delgado and Barbedo, 2011; Amador and Barbedo, 2015).

The origin of these new roots is unknown. One of the possibilities could be the presence of several embryos. Therefore, anatomical studies are of fundamental importance to know both, the possibility of polyembryony in *Eugenia* species and the origin of the various roots from cut seeds.

Thus, the aim of this study was to analyze the embryos of five *Eugenia* species of Brazil and to evaluate the morphological changes resulting from the germination in cut seeds, identifying the tissue that originates the new roots.

MATERIAL AND METHODS

Ripe fruits of *Eugenia brasiliensis* Lam., *E. cerasiflora* Miq*., E. involucrata* DC*., E. pyriformis* Cambess and *E. uniflora* L. were collected in two different locations: *E. uniflora* from a private property (23° 27 ‘S and 46° 36’ W) and *E. cerasiflora, E. brasiliensis, E. pyriformis* and *E. involucrata* from the São Paulo Botanic Garden (23° 38’ S, 46° 37’ W), both in São Paulo city, Brazil. The seeds were removed from the fruits by manual washing in running water with the aid of a sieve and the excess of humidity was immediately removed (Delgado and Barbedo, 2011).

Aiming to observe changes in embryo tissues during germination in cut seeds, the seeds were split in half, taking care to separate them by the hillum, as described by Delgado et al. (2010). Then, these seeds, as well as the intact ones, were placed to germinate in a germination box (gerbox) containing pre-moisted germitest paper, with two sheets for the base and one for cover (Brasil, 2009), and incubated at 25 °C with alternating light (12 hours of light / 12 of darkness), using four replications of ten seeds.

Samples of 3 seeds for anatomical analysis were taken daily from 1-15 days of incubation, in addition to the control (zero days). Throughout the development after fractionation at all times analyzed, seed samples were prepared for anatomical observation, aiming to identify, in intact and cut seed embryos, when and which cells / tissues have the capacity to originate new roots. For this, samples were fixed in neutral buffered formalin (Lillie, 1954) for 48 hours and placed in a vacuum pump for at least 2 hours, to maintain the integrity of the tissue after death without altering the cell structure. Subsequently, they were transferred to 10% ethanol, 30% and 50% for 12 hours for each treatment and then stored in 70% ethanol (Johansen, 1940) until the beginning of the preparation of histological slides.

Samples of both whole and cut seeds was gradually dehydrated in an ethanolic series, softened in terpeniol oil for five days and embedded in paraffin (Johansen, 1940). The sectioning was performed in series, transversely (8 µm thick) and longitudinally (10 µm thick); the sections obtained were stained with 0.05% toluidine blue (O'Brien et al., 1964), and 0.1% safranin and 1% astra blue (Bukatsh, 1972) and mounted on synthetic resin (Gerlach, 1969). Photomicrographs with the appropriate scales were obtained under the same optical conditions in a Leica DM 4500 photomicroscope coupled to the Leica DFC 320 digital camera and to a Leica MZ 75 stereomicroscope and in a photomicroscope equipped with Olympus model BX41-BF-III digital camera, software analysis of images Image – Pro Express version 4.0.1, by Media Cybernetics.

Samples of cut seeds from zero to 15 days of germination was fixed in FAA 50 (formaldehyde: acetic acid: 50% alcohol) and stored in 70% ethanol. Afterwards, it was included in plastic resin (Historesin® Leica), mounted on wooden blocks and sectioned transversely and longitudinally (8 µm) in a manual rotary microtome with a disposable knife. The sections were stained with 0.05% toluidine blue in acetate buffer, pH 4.7 (O'Brien et al., 1964), for 5 minutes, and the slides temporarily mounted in water. The sections were observed and photographed in an Olympus BX51 photomicroscope, and the scales were taken under the same optical conditions.

RESULTS AND DISCUSSION

The seeds of *Eugenia brasiliensis, E. cerasiflora, E. involucrata, E. pyriformis* and *E. uniflora* are exalbuminous, exhibit a brown, thin, membranous seed coat and a semiglobous to reniform, massive, colored embryo (Figure 1). The embryo is whitish in *E. brasiliensis, E. cerasiflora, E. involucrata, E. pyriformis* and greenish in *E. uniflora* (Figures 1C, G, K, O, S), completely filling the seminal cavity. The embryo has a rudimentary plumule, a short hypocotyl-radicle axis (Figures 2G, H, I, J) and two fleshy cotyledons (Figures 3A, B). The hypocotyl-radicle axis is distinguished in the embryo in *E. cerasiflora* and *E. involucrata*, in which the apex of the radicle is formed by a small protuberance, being surrounded by a circular depression; in *E. brasiliensis, E. pyriformis* and *E. uniflora* this distinction of the embryo was not evident.

Cotyledons exhibit uniseriate epidermis, whose cells contain phenolics in the region of cotyledonar epidermis (Figure 3); stomata were not observed; only in *Eugenia cerasiflora* there is a thin cuticle (Figure 3D). The cotyledon mesophyll is parenchymatic, with cells containing large amounts of starch grains (Figure 3G). High amounts of starch were also found in seeds of *E. inundata* (Melo et al., 2015). The vascular bundles (Figures 2D, F) are collateral and delimited by parenchyma cells that form a sheath. Cells adjacent to the epidermis and close to the vascular bundles have phenolic content. Vascular bundles start from the same point on the hypocotyl-radicle axis towards the apical region of the cotyledons where they join.

The occurrence of new roots and/or shoots occurred only when the seed was fractioned. Around the 14th day of germination after fractionation, in all studied species, an adventitious root was formed near the vascular bundles in the injured areas just below the wound-healing tissue (Figures 1D, H, L, P, T). Perivascular parenchyma cells divide periclinally and proliferate (Figure 4), giving rise to a root meristem. Eventually, several adventitious roots originate in the region where the fractionation occurred (Figure 2E), but this was a rare event. In these cases, there is no formation of normal seedlings, probably due to traumatic injury caused to the vascular bundle.

In the injured regions of the cotyledons, either by fractionation or by the attack of coleoptera, subepidermal parenchyma cells differentiate and form a meristematic band compactly arranged, without intercellular spaces, with suberized cell walls, not very dense cytoplasm, and usually containing phenolics (Figures 3E, F). This band originates a healing protuberant tissue that consists of elongated cork cells.

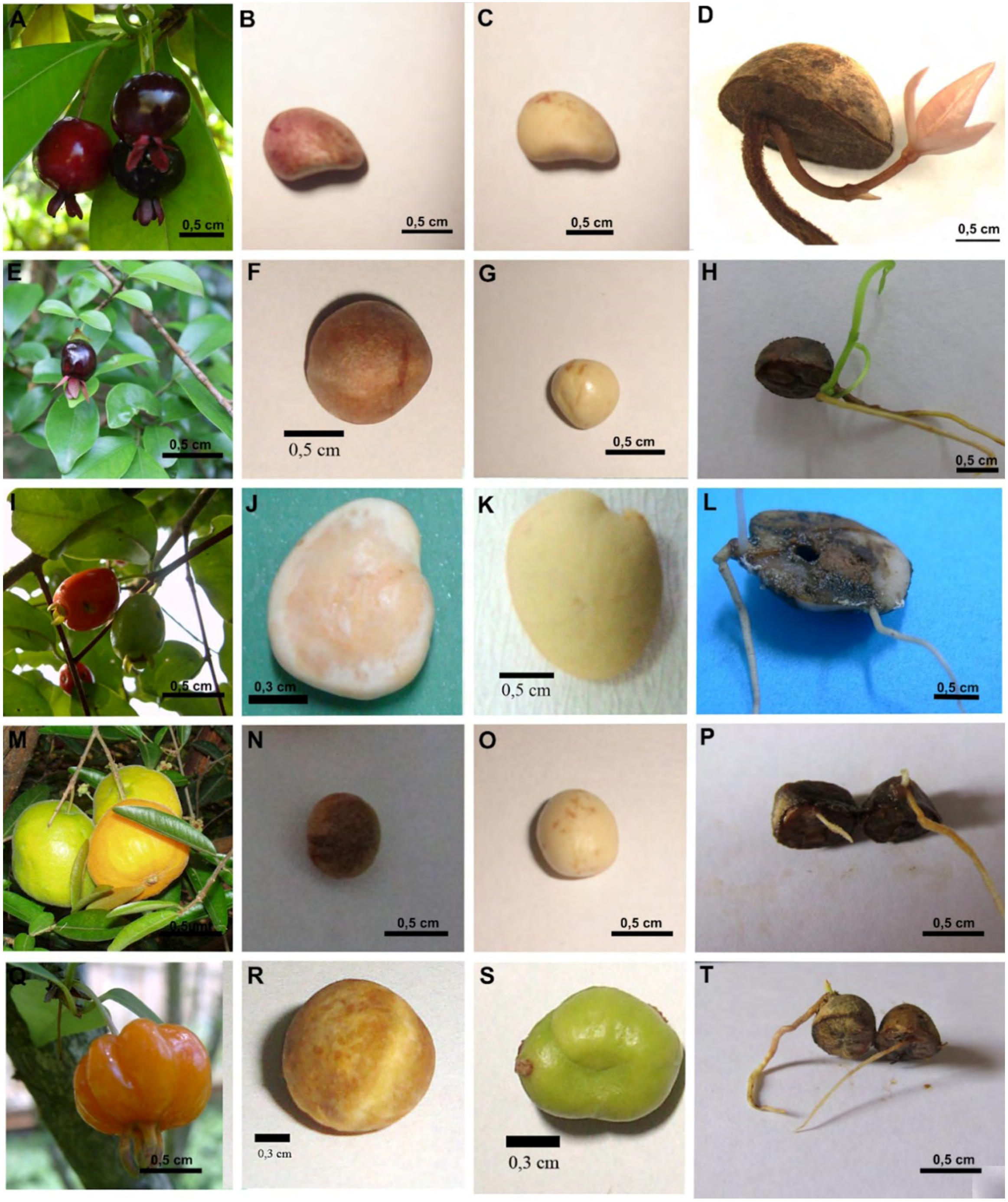

Figure 1. Fruits, seeds, embryos and aspects of seed fraction germination of *Eugenia* species. A-D - *Eugenia brasiliensis,* A – Fruit, B – Seed, C- Embryo, D – germinated seed fraction; E-H - *E. involucrata,* E – Fruit, F – Seed, G - Embryo, H – germinated seed fraction; I-L - *E. cerasiflora,* I – Fruit, J – Seed, K- Embryo, L – germinated seed fraction; M-P - *E. pyriformis,* M – Fruit, N – Seed, O - Embryo, P – germinated seed fraction; Q-T - *E. uniflora,* Q – Fruit, R – Seed, S - Embryo, T – germinated seed fraction.

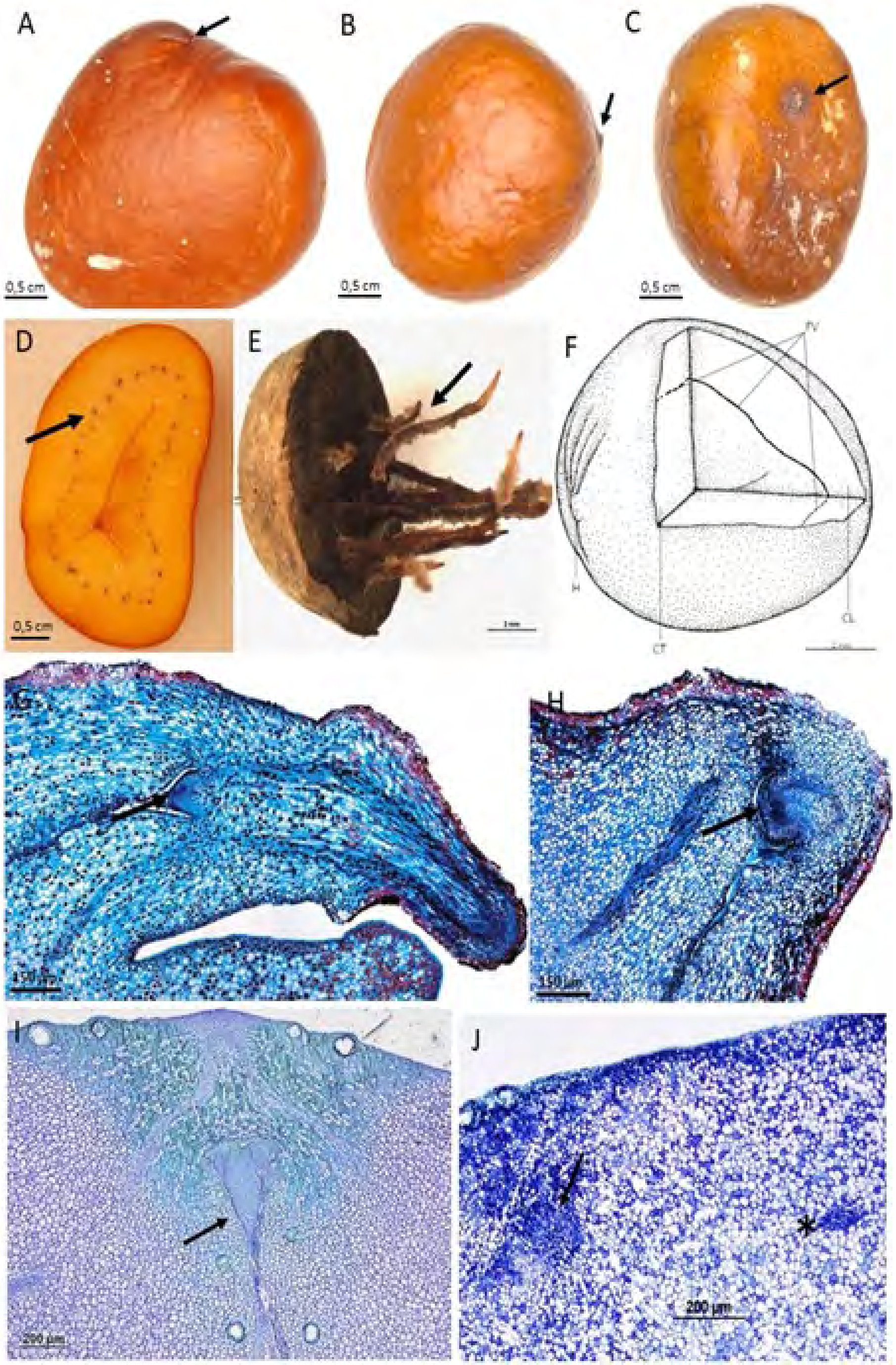

Figure 2. Whole seeds of *Eugenia cerasiflora* (A) and *E. involucrata* (B) with a small protrusion (arrow) surrounded by a circular depression which allows to distinguish the hypocotyl-radicle axis by naked eye, not so evident in *E. uniflora* (C). D: Vascular bundles (arrow) in the peripheral area of cotyledons showed by a transversal section of *E. involucrata* embryo from cut seed. E: Multiple adventitious roots from the cut embryo of *E. uniflora* from cut seed. F: Schematic illustration of transversal (CT) and longitudinal (CL) sections of *E. involucrata* seed showing the vascular bundles (FV) and hillum (H). Anatomical transversal sections of the hypocotyl-radicle axis (arrows) of seeds from immature fruits of *E. cerasiflora* (G), *E. involucrata* (H) and *E. uniflora* (I). J: Transversal section of cut *E. uniflora* seed after four days from the beginning of germination (the arrow shows the greater celular proliferation of the vascular bundle than the other marked by an asterisk).

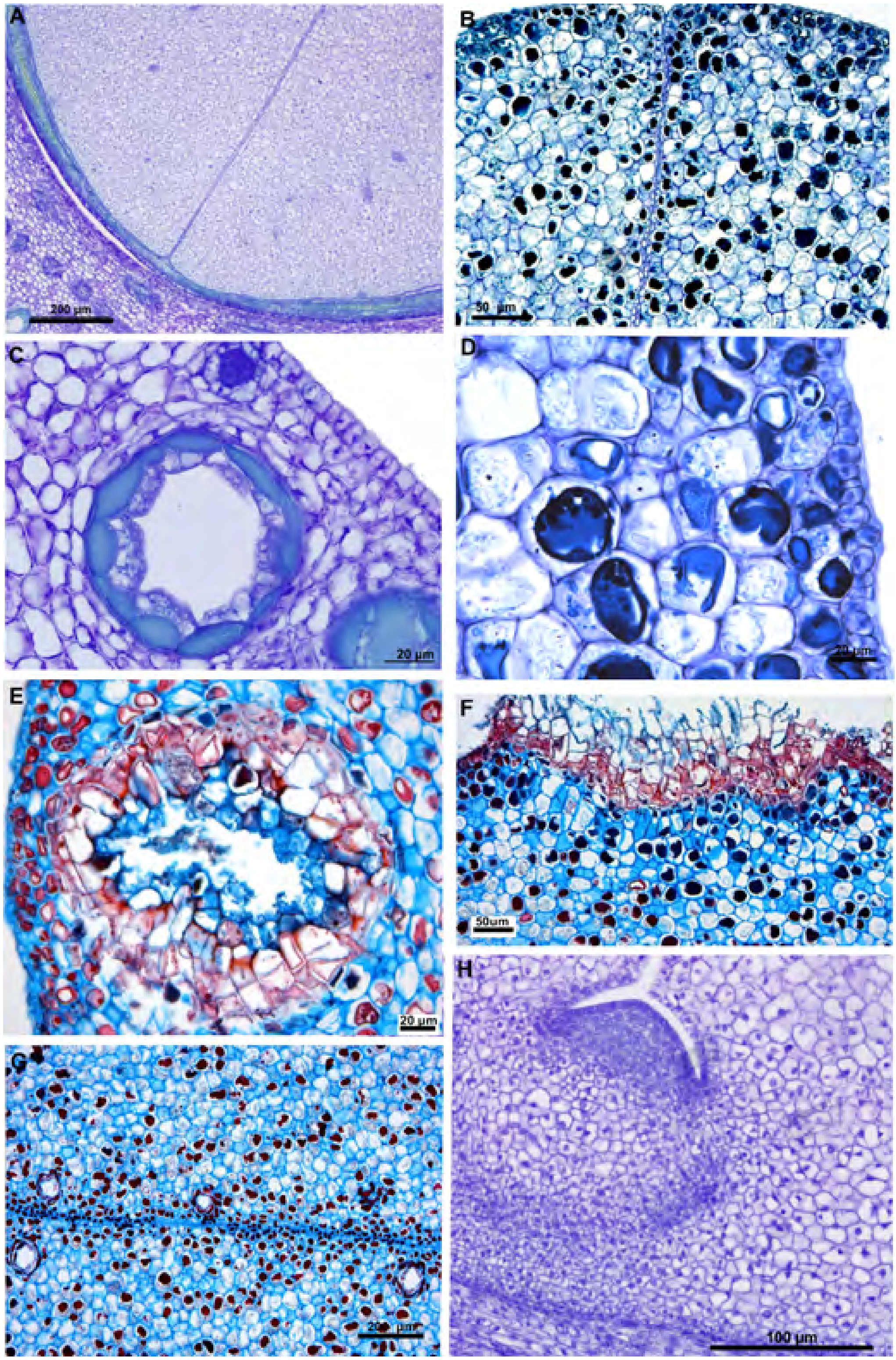

Figure 3. Anatomical sections of cotyledons of *Eugenia* embryos*.* A: Two cotyledons of *E. uniflora* embryo identified by their uniseriate epidermis; B: Uniseriate epidermis and union of cotyledons of *E. involucrata* embryo; C: Detail of a secretory cavity with the secretory multiseriate epithelium in *E. uniflora*. Note that the cotyledon epidermis is uniseriate; D: Uniseriate epidermis with cuticle in *E. cerasiflora* embryo*;* E: Wound-healing tissue after larval predation in *E. cerasiflora* embryo; F: Wound-healing tissue after cut in *E. cerasiflora* embryo; G: *S*tarch in the cotyledon of *E. cerasiflora;* H: Rudimentary embryonic axis in seed from mature fruit of *E. uniflora.*

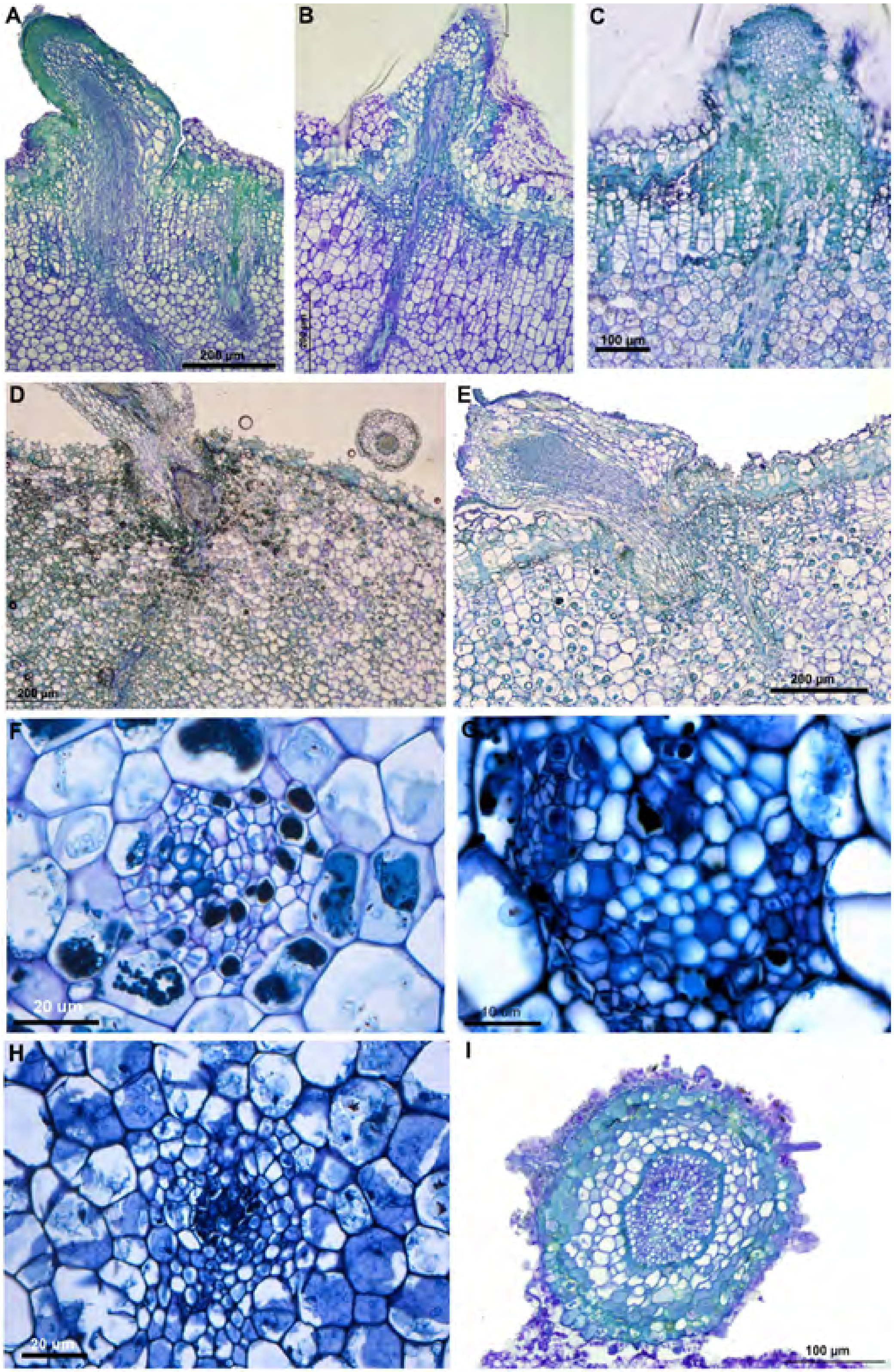

Figure 4. Anatomical sections of cotyledons of *Eugenia* embryos. A-E: Adventitious root from the perivascular parenchyma cells after cutting the embryo of *Eugenia involucrata* (A), *E. uniflora* (B), *E. brasiliensis* (C), *E. cerasiflora* (D) and *E. pyriformis* (E). F-H: Periclinal divisions and proliferation of perivascular cells in embryos of *E. cerasiflora* (F), *E. uniflora* (G), *E. involucrata* (H) after nine days of cutting seeds. I: Adventitious root in *E. uniflora* embryo after seed cut.

The embryo has numerous secretory cavities distributed both in the hypocotyl-radicle axis and in the cotyledons. Such cavities are subepidermal and consist of a multiseriate secretory epithelium that delimits a lumen. A greater number of larger secretory cavities occurs in *Eugenia uniflora* (Figure 3C).

Secretory cavities and phenolic idioblasts are the main secretory structures found in the embryo of *Eugenia cerasiflora, E. involucrata* and *E. uniflora*. The presence of phenolic compounds in embryos has been associated with plant and animal interaction mechanisms, acting as a food deterrent, and reducing herbivory (Castro and Machado, 2006). Indeed, in *E. uniflora*, in which the density of secretory cavities is higher, the herbivore attack was lower.

Regarding the ability of producing new roots, it has been shown, in 17 different studies, that seeds of at least nine species of *Eugenia* have a high regenerative capacity, producing new roots and even whole seedlings (Table 1). The efficiency in producing new roots and shoots, however, depends on some factors like seed maturation degree, type and number of cuts, number of seeds per fruit, size of the seed, species, stage of germination, among other possible ones, therefore resulting in different levels of successful by cutting these seeds (Table 1). Since the seeds of the five species analyzed in this work are monoembryonic, as previously reported by Justo et al. (2007) for *E. pyriformis*, the new roots are clearly not a result of polyembryony. In this work it was demonstrated that the production of new roots occurred from the differentiation of cells of perivascular tissues located in the apical region of cotyledons. It means that the new roots and shoots from cut seeds have a different genome than the mother plant, but they are identical to the embryo that was a result of fertilization. This result is different from that obtained for other species of Myrtaceae that also showed the ability to regenerate new somatic embryos such as *Syzygium jambos*, *S. cuminii*, *Myrciaria trunciflora*, *M. cauliflora*, species in which new plants were produced from nucellar adventitious embryos (Gurgel and Soubihe Sobrinho, 1951) and *Garcinia indica, G. xanthochymus* and *G. cambogia* (Malik et al., 2005), that present facultative agamospermy.

Table 1. Regenerative ability described for seeds of *Eugenia* species.

Species	Information	Source
E. pyriformis	Seeds cut to leave 1/4 of their original size can germinate and produce normal seedlings	1
	The embryo fills the entire space delimited by the integument and the axis is less than 1.0 mm long; the fleshy cotyledons vary from 1.0 to 2.0 cm	2
	Fragmented seeds show potential for root and seedling regeneration, but germination inhibits the formation of new roots and shoots	3
	Seeds can germinate and produce normal seedlings after the removal of 3/4 of their mass, even for immature and germinating ones	4
	The capacity to produce new seedlings reduces with the reduction in seed size	5
	Germination and normal seedlings are greater for entire seeds than the cut ones	6
	Seeds have high capacity to producing normal seedlings even from fruits at the beginning of development and after some seed reserve material is lost	7
E. involucrata	A batch in which seeds are cut into two parts can germinate more than 100%, but do not produce more than 100% of normal seedlings	8
	Seeds can germinate and produce normal seedlings after the removal of 3/4 of their mass, even for immature and germinating ones	4
	The uncut seeds have higher germination percentage than the cut ones	9
	Seeds have high capacity to producing normal seedlings even from fruits at the beginning of development and after some seed reserve material is lost	7
	Seeds are able to produce new roots and seedlings until the third radicle/seedling produced is removed	10
	Roots and seedlings continued to develop from apparently water stress necrotic tissue on the seed surface	11

Continue...

Table 1. Continuation.

Species	Information	Source
E. brasiliensis	A batch in which seeds are cut into two parts can germinate more than 100%, but do not produce more than 100% of normal seedlings	8
	Seeds can germinate and produce normal seedlings after the removal of 3/4 of their mass, even for immature and germinating ones	4
	Seed germination inhibits the formation of new roots or seedlings in the seed	12
	Seeds have high capacity to producing normal seedlings even from fruits at the begining of development and after some seed reserve material is lost	7
	Seeds are able to produce new roots and seedlings until the third radicle/seedling produced is removed	10
E. uniflora	A batch in which seeds are cut into two parts can germinate more than 100%, but do not produce more than 100% of normal seedlings	8
	Seeds can germinate and produce normal seedlings after the removal of 3/4 of their mass, even for immature and germinating ones	4
	Seed germination starts the inhibition of the formation of new roots or seedlings in the seed and seed incision can block the action of these inhibitors	12
	Seeds have high capacity to producing normal seedlings even from fruits at the beginning of development and after some seed reserve material is lost	7
E. stipitata	Seeds show a high capacity for regeneration; even when cut in the middle or in the meristematic zone, they still form seedlings	13
	Cut seeds cut at the meristematic protuberance make normal seedlings, with the same characteristics of intact seed germination	14
	When seedlings are detached from the seed, the ‘resown’ seeds produce a second, normal seedling within 9 months	15
E. cerasiflora	A batch in which seeds are cut into four parts can germinate more than 200%	16
	Seeds can germinate and produce normal seedlings after the removal of 3/4 of their mass, even for immature and germinating ones	4
	Seeds have high capacity to producing normal seedlings even from fruits at the beginning of development and after some seed reserve material is lost	7
E. candolleana	It is possible to obtain more than one normal seedling from each seed; after root and shoot removal, regeneration of new roots and seedlings occurred	17
E. umbelliflora	The seeds have high capacity of resume germinability after being cut	16
E. pruinosa	The seeds have high capacity of resume germinability after being cut	16

1Silva et al. (2003); 2Justo et al. (2007); 3Amador and Barbedo (2011); 4Teixeira and Barbedo, (2012); 5Prataviera et al. (2015); 6Costa et al. (2017); 7Delgado and Barbedo (2020); 8Silva et al, (2005); 9Gomes et al. (2016); 10Alonso and Barbedo (2020); 11Inocente and Barbedo (2021); 12Amador and Barbedo (2015); 13Anjos and Ferraz (1999); 14Mendes and Mendonça (2012); 15Calvi et al. (2017b); 16Delgado et al. (2010); 17Alonso et al. (2019).

Considering that the production of new plants did not occurre spontaneously in intact seeds, it is possible that the embryo itself produces substances, such as phytoregulators, that inhibit the development of these new individuals, as reported by Amador and Barbedo (2015). The reserves, then, are concentrated only on the hypocotyl-radicle axis. The unusual ability of *Eugenia* seeds to produce new plants, even after removing more than half of their storage tissue, may be related to the survival strategies of these species when affected by external agents, like herbivory or anthropic action (Teixeira and Barbedo, 2012), as well as to successive germinations since they are able to produce new roots and shoots until the third root/shoot produced is removed, or even when half of the seed is initially removed (Alonso and Barbedo, 2020). The internal control allows the reserves to be used in every unique germination, *i.e.*, when a root starts to develop there is inhibition of the formation of new roots (Amador and Barbedo, 2015).

CONCLUSIONS

The origin of the new roots produced after cutting *Eugenia* species seeds is the differentiation of cells of perivascular tissues located in the apical region of cotyledons.

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