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Article

Morpho-Anatomy of the *Echium plantagineum* L. (Boraginaceae) Diaspores in Relation with Water Uptake and Germination

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Abstract: *Echium plantagineum* (Boraginaceae) is native of the Mediterranean regions, has been introduced and become widespread within the American continent (North to South), South Africa, New Zealand and Australia. This plant has seed dormancy, aggressively spreads to infest vast areas of predominantly agricultural land and is considered a toxic weed to livestock. The objectives of this research were: (i) to study the morpho-anatomy of the diaspores of *E. plantagineum*; (ii) to identify the pathway of water uptake; and (iii) to characterize the germination and the seedling. The morpho-anatomical studies were carried out analyzing semi-permanent slides of transverse and longitudinal sections of the fruits, seeds and seedlings. Histological, histochemical and conventional staining techniques by using stereoscopic, optical and scanning electron microscopy were applied. In the diaspores, the water uptake pathway was determined by fast green staining, germination tests were performed and the morpho-anatomy of seedlings was analyzed. The diaspores showed acrescent calyx surrounding the fruit, composed by 4 rough tuberculate mericarpids. Each indehiscent mericarpid encloses an exalbuminous seed with a thin coat and a spatulate embryo with folded cotyledons. Water uptake took place through the vascular trace "protuberance" in the cicatrix of the mericarpid, which triggers the germination process. Germination was epigeal, the cotyledons were photosynthetically active at the emergence, and a rudimentary developed gemmula was observed. Germination percentage was 66.5%. The macrosclereids of the pericarp functions as an obstacle to water uptake, which is overcome when the mericarpid is detached from the gynobase, and the protuberance that acts as a water uptake path is exposed, leading to germination. The morpho-anatomical characteristics of diaspore, explain the successful dispersal for this aggressive weed, and provide important information in relation to the necessary care for its control.

Keywords: *Echium plantagineum*; fruit; germination; imbibition; mericarpid; seed

1 Introduction

Echium plantagineum L. (Boraginaceae) is a European species native to the Mediterranean basin, but also introduced in the south of the United States, México, Honduras, Brazil, Chile, Uruguay and Argentina, known as "borraja cimarrona", "borraja del campo", "flor morada", or "viper's bugloss" [1–6].

The species has been cited for its melliferous, medicinal and ornamental qualities [7,8]. The medicinal properties were emphasized by Núñez and Cantero [9], Barboza et al. [10] and Molinelli et al. [11]; while the ornamental potentials were highlighted by Planchuelo et al. [12]. However, the species has been considered an invasive weed [13] and has become important as a highly toxic plant for livestock [14,15]. In addition, *E. plantagineum* is present in South Africa, Australia and New Zealand, where it is known as "Paterson's curse" and "salvation Jane". Particularly in Australia, this species was introduced for ornamental purposes, but is very aggressive and consequently considered a weed [16].

On the one hand, due to the nutritional benefits of *E. plantagineum*, it has been proposed in Chile to implement field production programs [17]. On the other hand, in order to develop systematic control strategies for this invasive weed, several authors have studied and described the effects of various factors that affect the germination of this species. Piggin [18], documented that germination is favored by high temperatures and the seeds showed innate dormancy. Other studies have shown that while *E. plantagineum* has the capacity to germinate in a wide range of temperatures, it is negatively affected by sowing depth and favored by the presence of light [19]. The studies that analyzed the effect of fire and smoke on germination concluded that neither showed a noticeable effect [20]. Regarding the characteristics of the fruit and the seed of the Boraginaceae, there are previous morphological descriptions carried out by Niembro Rocas [21] and Zomlefer [22]. The seed, seed coat, endosperm and the embryo have been described in the work of Corner [23], while the transfer cells presence was investigated by Diane et al. [24]. However, the study of the diaspora as a whole remains uninvestigated although the development of the Boraginaceae fruit has been characterized by various authors. The fruit of the Boraginaceae is derived from a two-carpel gynoecium with a common style, and at maturity each half-carpel is disarticulated forming four one-seeded mericarpids, known as schizocarpic fruit [25]. Each indehiscent mericarpid of a schizocarpic fruit is a diaspore [26,27], in addition it has been pointed out that the clusa (nutlet) is the typical fruit of this family [28].

In the literature of the Boraginaceae there are misunderstandings and a contradictory terminology applied to the gynoecium and the resulting fruit [29]. For this reason, the author defined the gynobase as the part of the gynoecium and later the fruit, which remains on the mother plant. The dispersal unit was referred as eremocarps (morphologically are the mericarpids = half carpels), and the abscission scar that remains on the side of the gynobase, was named the areole, while the one on the side of the emerocarp, the cicatrix.

The majority of the research carried out on this invasive species, besides the descriptive studies of the fruit and the seed, have considered aspects related to the factors that affect its germination. Nevertheless, both the morpho-anatomical characteristics that explain the initial water uptake that triggers germination, and its behavior as an invasive weed remain unidentified. A more detailed description of the diaspore morpho-anatomy, germination process, and seedling structure is essential not only for the recognition of seeds in the soil bank but also for the identification of seedlings, which enables practical recommendations for the control of this weed. The aim of this research was to identify connections with the morpho-anatomy of the diaspores, the water uptake and the germination of *Echium plantagineum*.

2 Materials and Methods

2.1 Plant Material

Echium plantagineum L. plants were collected in both flowering and fructifying stages from a population grown on the railroads and on adjoining land in the city of San Francisco, Córdoba, Argentina (31°25'59.4"S 62°06'43.1"W), during 2013 and 2014. The accessions were placed in the Herbarium ACOR as vouchers specimens LMC 1106 and 1107, and the diaspores were deposited in the seed collection. Additionally, 1500 mericarpids were harvested in December 2016 to perform the germination test and stored in paper bags at (20 ± 5)°C, until the time of the test. The present study was carried out

consulting herbarium collection specimens ACOR (*Echium plantagineum* 228) and CORD (*Echium plantagineum* 515).

2.2 Diaspore and Morpho-Anatomy of Seedlings

The fruits were examined dry or fixed both externally and internally, the color of the pericarp was determined using the Munsell color chart [30].The length and diameter of the mericarpids were recorded from two repetitions of 25 units with a digital caliper. The weight of one thousand mericarpids was determined according to the ISTA Rules [31], with a weight of eight repetitions of 100 units.

Fruit morphology was described following Hilger [29] terminology. The seed sensu stricto was analyzed according to the criteria of Corner [23], and for internal characteristics, the nomenclature of Martin [31] and Werker [26] was followed. The description of the seedling was based on the terminology proposed by De Vogel [33] and Ye [34].

For the anatomical studies, the mericarpids, the seeds and seedlings, were fixed in FAA (10% of 40% formaldehyde, 50% of 96% ethanol, 5% of 96% acetic acid, and 35% of distilled water). Handmade cuts, of fresh and preserved material were made in different planes with razor blades, in order to make temporary slides. The sections were dyed with safranin (1 g of safranin diluted in 100 ml of 50% ethanol) and Astral blue (0.5 g of Astral blue dissolved in 100 ml of distilled water with the addition of 2 g tartaric acid) for about 3–5 min, and then were mounted in glycerin 50%.

Histochemical techniques were accomplished to determine different cellular contents: lugol for starch, ferric sulfate solution (10% in formol) for tannins, floroglucine solution (1% in 96° ethanol) for lignin, and Coomassie blue for proteins. All these techniques were performed according to Zarlavsky [35]. The seedlings for morpho-anatomical studies were obtained from the germination test.

Morphological and anatomical observations were made with a Zeiss Stemi DV4 stereomicroscope and Nikon Eclipse E 400 optical microscope respectively, in the laboratory of the Agronomic Botanical Transfer Center of the Faculty of Agricultural Sciences, National University of Córdoba, Argentina. For the scanning electron microscopy (SEM) analysis, the mericarpid sections were mounted on tabs with double-sided carbon tape, and covered with a 12 nm layer of gold. They were observed in a Zeiss Microscope, Sigma model at the Lamarx Laboratories, Faculty of Mathematics, Astronomy and Physics, National University of Córdoba, Argentina. In all cases, photographs were taken with a color digital camera.

2.3 Initial Pathway of Water Uptake

To determine the initial pathway of water uptake, 20 mericarpids were immersed in fast green saturated in 96% ethanol saturated solution at room temperature [36]. Staining observations were made by removing the mericarpids at 24-hours intervals, and up to 15 days after the start of the test. Then, they were washed with water and dried between paper towels in order to absorb the superficial dye excess. Ten mericarpids were cut by free hand in longitudinal and ten in cross sections, and the pathway of the dye was observed with a stereomicroscope Zeiss Stemi DV4 and a Nikon Eclipse E400 optical microscope. The technique above described is an adaptation of the methods used by Voyiatzis [37] and Mattana et al. [38]. Photographs were taken as explained above.

In order to establish possible connections between the morpho-anatomical characteristics of the fruit and the seed with water entry, the information obtained from these studies was linked with the germination test results.

2.4 Germination Test

The germination test was performed following ISTA Rules [31], which establish that for *Echium*, the germinative unit has to be the mericarpid (nutlet). Four replicates of 50 mericarpids were placed on

18 cm × 16 cm × 5 cm plastic trays on top of Valot® paper moistened with distilled water, in closed plastic bags. They were taken to a germination chamber at alternating temperature 20°C–30°C, with a photoperiod 8/16 h (light/dark). Germination was considered succeeded when the radicle reached to 2 mm long, from the mericarpid apex [39]. The number of germinated seeds was noted down at 3, 5, 10, 14 and 20 days after sowing (DAS); and the final germination percentage (GP) was registered 20 DAS. The morphological characteristics were analyzed from the emergence of the radicle to the emission of the first true leaves.

At the end of the germination test, mechanical scarification was performed on the pericarp of the ungerminated seeds, removing a small section with a histological needle. Then, they were tested for viability with the tetrazolium assay. The mericarpids were immersed in 0.5% tetrazolium (2, 3, 5, triphenyl tetrazolium Sigma®) solution at 30°C for 24 h in darkness. After rinsing with water, the pericarps were removed and the embryos were observed. According to the intensity and location of the dye, viable, and non-viable seeds were discriminated, following Bulan [31] and ISTA [40] criteria. The mericarpids showing the absence of an embryo were considered "empty seeds".

To evaluate the normal seedling, the essential structures formed in the trial period were examined, according to the Handbook on Seedling Evaluation [41].

3 Results and Discussion

3.1 Diaspore Morpho-Anatomy

It was established that the diaspore (sensu lato) of *E. plantagineum*, comprises the persistent calyx, longer than the fruit, and the four ovoid-trigonal mericarpids with the truncated base and the acute apex (Fig. 1A). The persistent calyx is a morphological adaptation to the anemochory dispersal strategy. These aerodynamic properties allow diaspores to remain for a relatively long period in the air, therefore, carried by the wind, they move away from the mother plant. These could explain the success of the dispersion to new environments in an efficient way. Even though each mericarpid is monochrome, two color patterns for the pericarp were established: brown (7.5YR4/2) and light gray (2.5Y7/2) (Fig. 1B). Each mericarpid, also a diaspore (sensu stricto), measured 2.6 mm in length and 2.23 mm in the mean diameter; and the weight of 1000 mericarpids was 3.098 g. These characteristics are consistent with those mentioned as aquenes by Parodi [1], clusas according to Pérez-Moreau [2] and Ariza Espinar [3] for the species. In addition, they are related to the description of Spjut [25], which classified the fruit of boraginaceae as microbasarium, as well as, the achaenium (small achene) and the schchizocarpic nutlets among others, as synonyms. Eventhough Hilger [29] prefers the term eremocarp, in this study we consider that mericarpid is a morphological more accuracy term.

In each mericarpid the abscission scar with the gynobase (from now named cicatrix), and its counterpart the areola in the gynobase was observed (Figs. 1C and 1D). The areola was characterized by its triangular shape, and surrounded by the annular nectarium. Because the columella was not lengthened, the areola remained horizontal, this feature was also described for *Arnebia decumbens* (Vent.) Coss. and Kralik by Hilger [29].

The pericarp surface pattern was rough-tuberculate with ridges (Fig. 1E). These crests are sharp projections sting-shaped, with an extremely thick cuticular layer (Fig. 1F). The superficial roughness of the pericarp, together with the above mentioned features (the light weight and the persistent calyx) could explain, by aerodynamic considerations, the success of the diaspora by wind action.

The histological distribution pattern in the developing pericarp was as follows: the epicarp showed a unistrata cell layer, the mesocarp 9-12 cell layers, and the endocarp 6-10 cell layers (Fig. 2A). In the epicarp of the young fruit, the cells were rectangular with thin, straight walls. As the fruit development progressed, some of these cells retained the isodiametric shape, although most showed significant changes. Anticlinal elongations (Fig. 2B), and irregular thickenings in the radial and tangential walls were

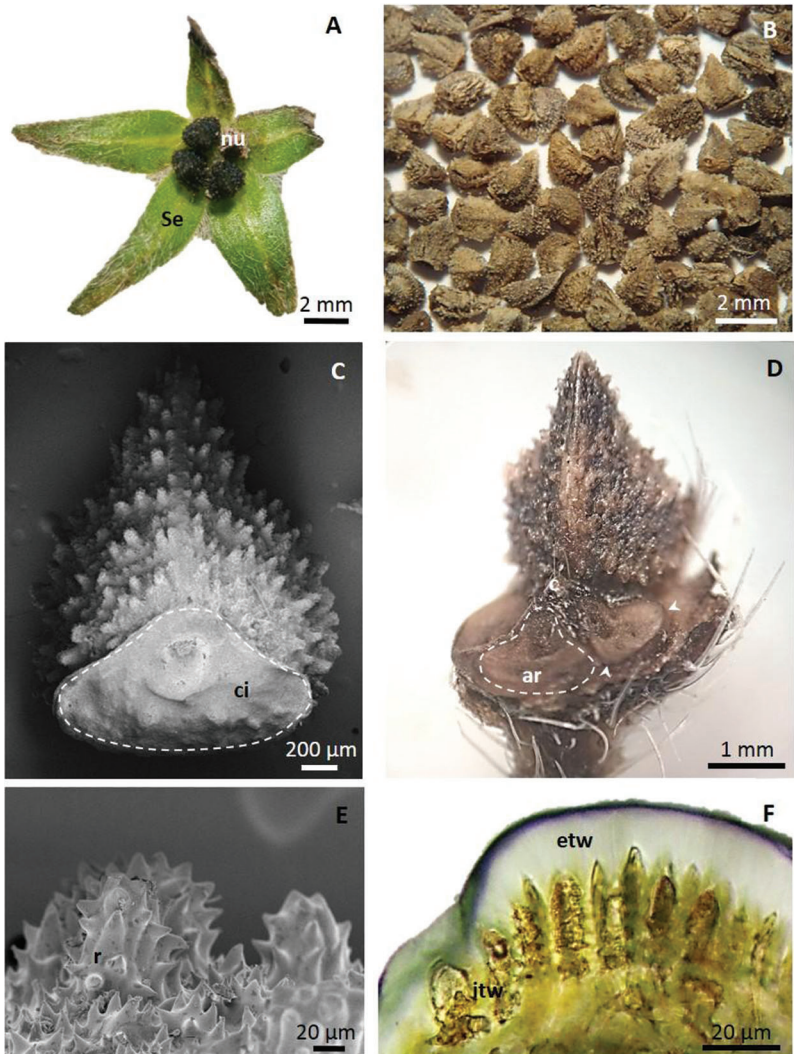

Figure 1: Diaspores of *Echium plantagineum*: A. General aspect of the schizocarpic fruit accompanied by the calyx; B. Mericarpids, brown and light gray; C. Detail of the mericarpid cicatrix (dashed line); D. Gynobase with three out of four mericarpids detached, dashed line indicate the areole, the arrowheads indicate annular nectary; E. Detail in surface view of the tuberculate pericarp with ridges (SEM); F. Longitudinal section of the ridge with macrosclereids and thick external tangential wall. Abbreviations: ar, areole; c, columella; ci, cicatrix; etw, external tangential wall; itw, internal tangential wall; nu, nutlet; r, ridge; se, sepal

observed, mainly in the external tangential with the conspicuous cuticle (Fig. 2C). As a consequence of these changes, the macrosclereids of the epicarp differentiate and are characterized by forming the rough tuberculate surface of the pericarp in the mature fruit. Macrosclereids with brown contents in the lumen were observed, which reacted positively with the ferric sulfate staining that detected the presence of tannins (Fig. 2D). In the mesocarp the initially rounded cells thickened their walls, and lengthened in the periclinal direction (Fig. 2D). In the development of the rough-tuberculate pattern of the pericarp, the mesocarp was also involved with the contribution of cells that grew in the anticlinal direction. Following this region, which is innervated by vascular bundles, the endocarp formed by thin-walled globose cells

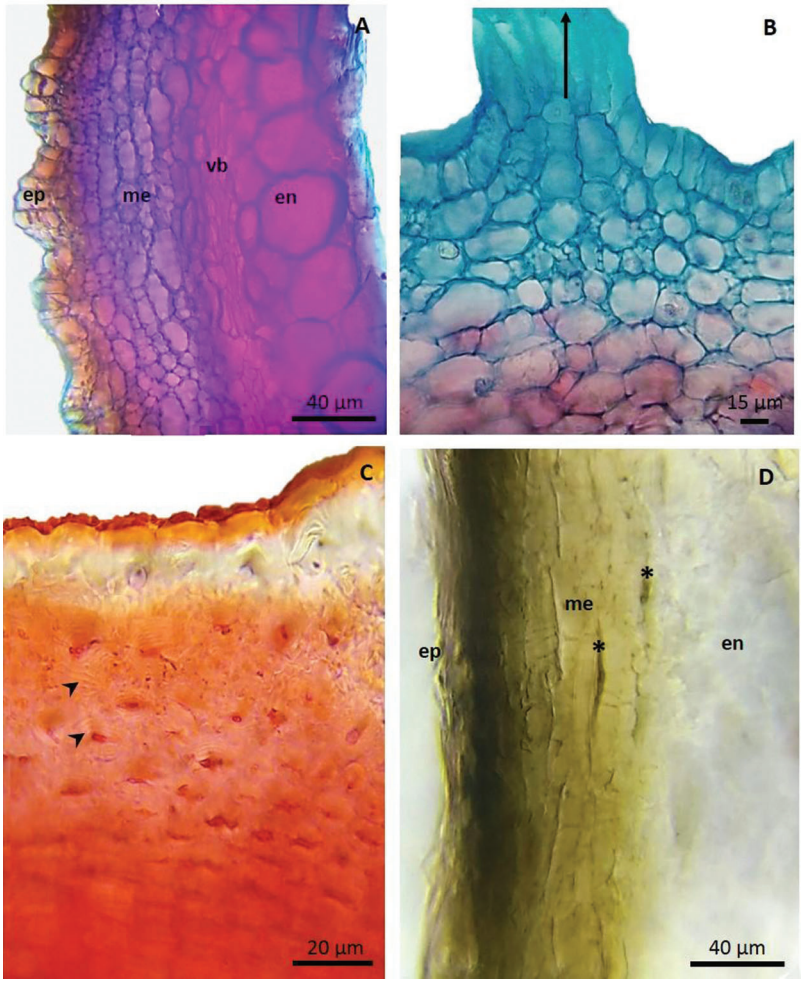

Figure 2: Anatomy of the pericarp of *Echium plantagineum*: A. Longitudinal section of the developing pericarp; B. Detail of the epicarp cells with anticlinal lengthening (arrow) and mesocarp cells with periclinal lengthening, in longitudinal section; C. Detail of the epicarp and the mesocarp in cross section, arrow heads: concentric layers of the secondary wall; D. Detail of the mature pericarp, (*) tannins. Abbreviations: en, endocarp; ep, epicarp; me, mesocarp; vb, vascular bundle

was observed (Fig. 2A). As the ripening of the fruit progresses, the endocarp collapsed, while their remains persisted in the pericarp of the mature fruit. The abscission region of the mericarpids was placed between the nectary and the fruit base (Fig. 3A). This was matched with the abscission cicatrix in the mericarpid, and with the areola in the gynobase. This abscission region presented parenchymatous cells of isodiametric shape, which became differentiated in macrosclereids later than the other tissues of the pericarp. The splitting force in these mericarpids, is due to the development of the two different types of tissues placed in the abscission zone. Parenchymatous cells of the lower side have relatively thin cell walls and isodiametric shape, whereas sclerenchymatous cells of the upper side have remarkably thicker walls and they are transversely elongated. The sclerenchymatous tissue on the upper side of the mericarpid directly continues with the parenchymatous tissue of the inner side of the mericarpid, except in the area of the vascular strand of the latter. It was also observed that the vascular bundle sheath that continues to the funiculus, remains with its thin-walls cells, flanked by the macrosclereids (Fig. 3B). In this way, the

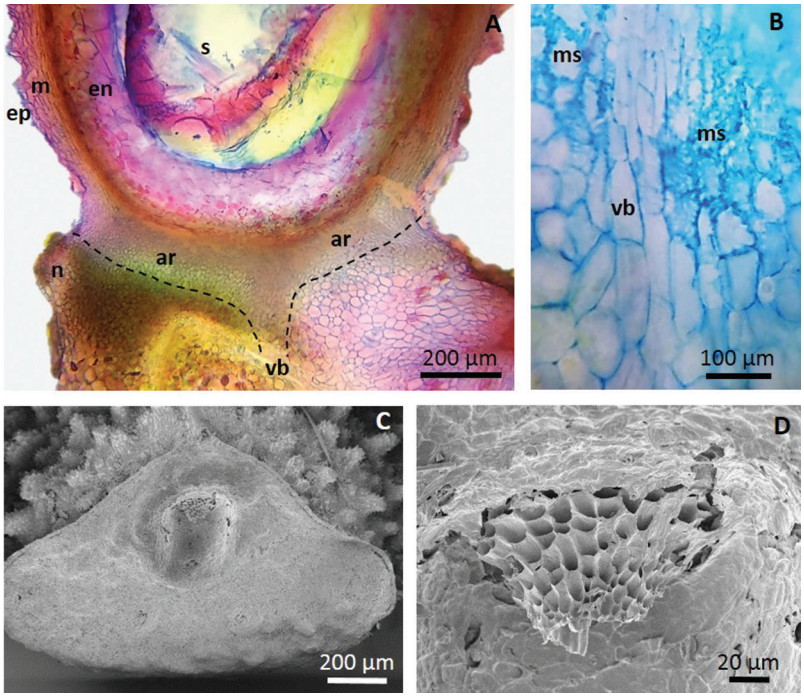

Figure 3: Anatomy of the abscission region of the mericarpid of *Echium plantagineum*: A. Portion of the mericarpid and receptacle in longitudinal section, the abscission region indicated by dashed lines; B. Detail of the vascular bundle of the gynobase surrounded by sclereids; C. Abscission scar with the protuberance formed by the remains of the vascularization; D. Detail of the remains of the vascularization. Abbreviations: ar, abscission region; en, endocarp; ep, epicarp; m, mesocarp; ms, macrosclereids; n, nectary; s, seed; vb, vascular bundle

vascular bundle and the perivascular tissue functioned as a continuous thread, and the mericarpid remained connected to the gynobase in the region of the vascular strand, while the progressive division took place in the axial plane. This disruption is due to differential shrinkage of its tissues while the pericarp shrinks upon drying. Due to the presence of the persistent calyx, this detachment became effective when the mericarpid was still enclosed within the sepals, a mechanism that was also described in *Asperugo procumbens* by Hilger [29].

The development of macrosclereids at the base of each mericarpid in *E. plantagineum*, can be related to the same type of tissue that it was described by Silva et al. [42] as one of the most important events in the fruit dehiscence of *Dalechampia stipulacea*, although in *E. plantagineum*, the mericarpids are indehiscent. We agree with these authors in the sense that macrosclereids constitute a resistant layer of the fruit, which gives protection to the seed, besides in this study, it is also demonstrated that they are responsible for the abscission mechanism, which results in the complete detachment of the mericarpids from the gynobase. In the middle area of the mericarpid scar (cicatrix), and in correspondence with the remains of the vascular system and perivascular tissue of the pericarp, a protuberance formed by a group of well differentiated cells was observed (Figs. 3C and 3D). With this event, the initial pathway of water uptake is enabled, which is necessary to trigger the next germination stage.

3.2 Seed Morpho-Anatomy

The seed was characterized by its ovoid form, funicular remains and small vascular bundles were observed in the thin, light brown seed coat. (Fig. 4A). The embryo was slightly curved, spatulate, with

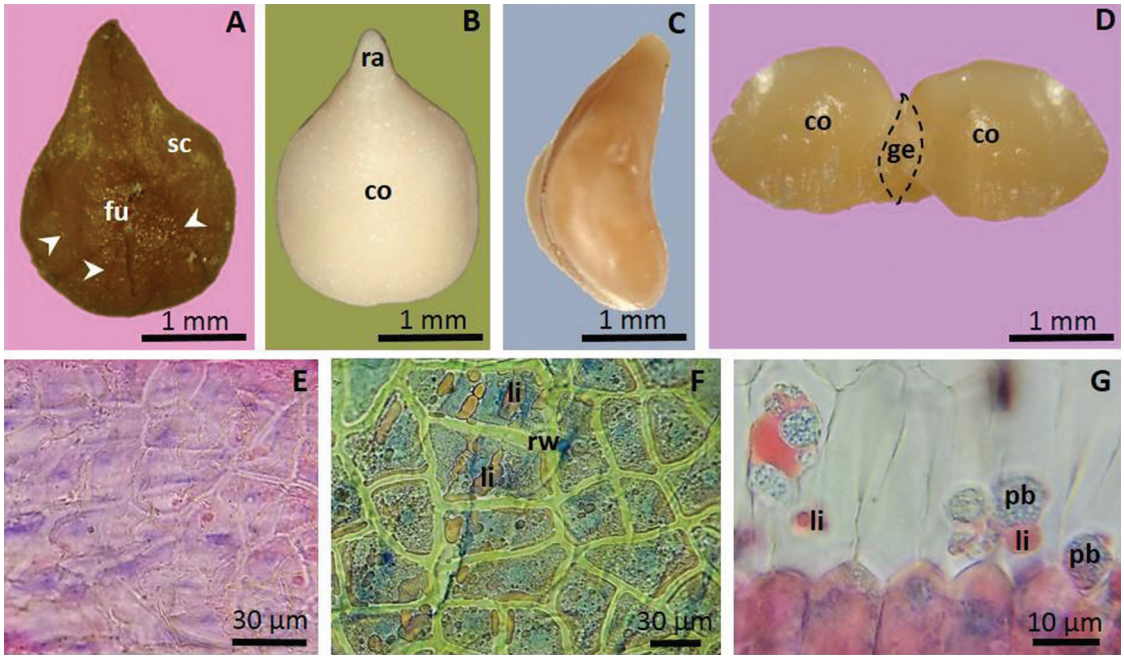

Figure 4: Seed of *Echium plantagineum*: A. Seed showing the seed coat with funiculus and minor vascular bundles (arrowheads); B. Frontal view of the embryo; C. Embryo in lateral view; D. Surface view of the gemmula; E. Paradermal view of the seed coat, the endosperm is seen by transparency; F. Paradermal view of the endosperm; G. Cross section of the cotyledon, mesophyll cells with protein bodies and lipids; Abbreviations: co, cotyledon; fu, funiculus; ge, gemmula; li, lipids; pb, protein bodies; ra, radicle; rw, radial wall; sc, seed coat

cotyledons wider than the hypocotyl-radicular axis, and the gemmula showed to be rudimentarily developed (Figs. 4B and 4D). The type of seed was classified within the axial division, spatulated subdivision and with total embryo, which are similar to the general characteristics given by Martin [32] for *Echium*.

The seed coat is formed by a compact epidermal layer of irregular, thin-walled cells, the radial walls being shorter than the tangential ones (Fig. 4E). This account for the single seminal integument that undergoes differentiation and become in the seed coat of the Boraginaceae [23]. Besides, the thin seed coats are typically found amongst indehiscent fruits, the presence of tannins is related to impermeability and to the ability to prevent violent water inrush to the embryo during imbibition [27]. In this way it could be explained the relationship between the structure and function of the *E. plantagineum* seed coat. Moreover, this author also adds that the thick wall cells as well as their compact distribution serve as a protective layer. All these peculiarities were not observed in the seed coat of *E. plantagineum* but they do coincide with the pericarp descriptions of the indehiscent fruit. In the example quoted here the indehiscence explains how seeds could not only persist viable inside the fruit but also overcome different environmental stresses. In this respect, therefore, show the close connection with the diaspora morpho-anatomy and the protection function of the pericarp for *E. vulgare*. Although a remnant layer of endosperm was observed with isodiametric, thick-walled, and lipid cells (Fig. 4F), the seed is instead exalbuminous, in agreement with the general characteristics of the family cited by Corner [23]. However, there were not detected transfer cells either in the seed or within the fruit of *E. plantagineum*, in a manner similar to that reported by Diane et al. [24]. Histochemical tests indicated the presence of lipids and protein bodies in the storage cotyledons (Fig. 4G).

3.3 Initial Pathway of Water Uptake

The mericarpids after 24 h of immersion in the fast green solution, showed that water uptake took place through the protuberance of the vascular bundle and the perivascular tissue, which functioned as a water pathway (Fig. 5A).

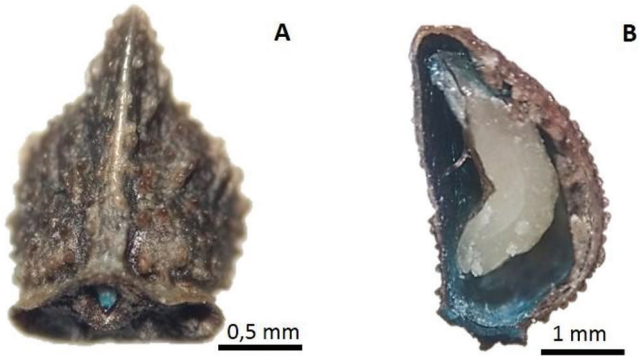

Figure 5: Initial pathway of water uptake in the diaspore of *Echium plantagineum*: A. mericarpid with the protuberance formed by the remains of the vascularization stained with fast green; B. Longitudinal section of the mericarpid with the locule wall and seed coat colored with fast green

In the pericarp, the macrosclereids became an impermeable barrier, which is only interrupted in the area of the vascular bundle, which forms a morpho-anatomical functional region for passive access of water into the mericarpid. This is so, since the vascular bundles extend continuously from the base of the pericarp and along the funiculus to the seed coat. These observations agree with those made by Diane et al. [24], for *Heliotropium erosum* Lehm., who explained that the transport of water to the embryo is enabled by the funicular perivascular tissue. Although, these authors described the entrance of water in *H. erosum* due to the presence of transfer cells in the funicular region, this was not the case of *E. plantagineum*, since the species lacks this type of specialized cells. The seed coat was also colored with the fast green solution after 24 h of immersion, (Fig. 5B). Furthermore, the embryo remained unstained, even after 15 days of the start of imbibition in the fast green solution, although it did show visual evidence of water entry in its tissues, indicating that water diffuses through the seed coat to the embryo. Similar results were reported by Mattana et al. [38] and Dias et al. [36]. This is so, because the staining method is an indirect approach, as the permeability of the seed coat may be different for water, with a molecular weight of 18.015 g/mol, than for other larger molecules, e.g., fast green of 808.843 g/mol [38].

3.4 Germination Test

The test showed a final germination percentage of 66.5% ± 4.6%, 20 days after sowing (Fig. 6). The viability tetrazolium test stated that 30% of seeds that did not germinate were unviable. The lack of color reaction in the embryos, showed vital injuries, both at hypocotyl-radicle axis or at cotyledons level (Fig. 7); and allowed to explain, as it does, the ungerminated seed percentage.

These results were due to the meristems, along with the axis and the cotyledons are essential for the development of normal seedlings, and their damage determined the inability to reach a successful germination. In this sense, as noted by ISTA [31], the whitish color is due to dead cells that do not breathe; and according to Bulan [40], the cherry-red color indicates the loss of integrity of the membranes that facilitates the entry and reaction of tetrazolium. Taking into account the changes associated during the germination process, according to Bewley et al. [39], the seeds imbibe in phase 1, but cannot

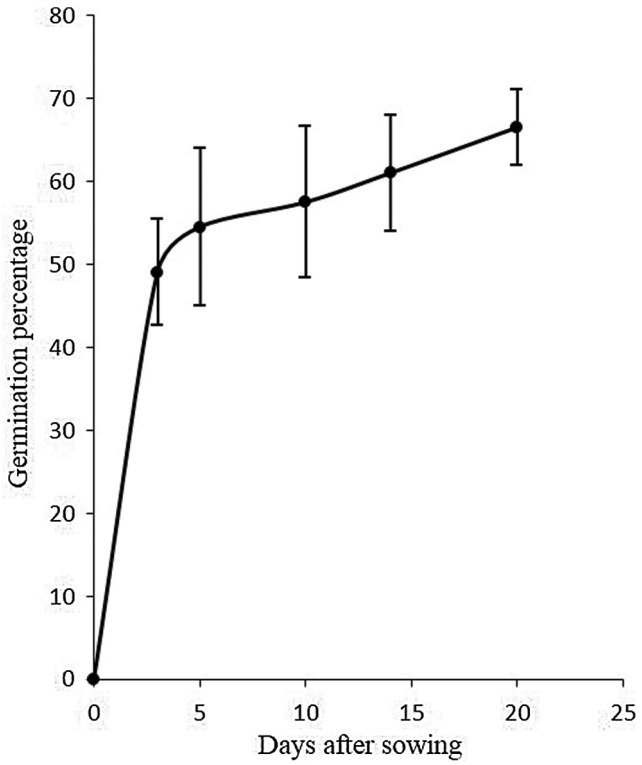

Figure 6: Accumulated germination percentage 20 days after sowing of *Echium plantagineum* with alternating temperature 20°C–30°C and a photoperiod 8/16 h. Vertical bars indicate the standard deviation.

overcome phase 2 of metabolic activation; and therefore do not go to phase 3, where the greatest reserve mobilization occurs.

On the other hand, it was recorded that 1.5% of the seeds were hard and 2% were empty. All this supports the results of the germination test of *E. plantagineum*; and are consistent with the ones of Piggin [18], who concluded that the species is well adapted to persist in a Mediterranean climate and its germination is in advantage during years with marked seasonality. However, the germination percentages of our study contrast significantly with those reported by Roso et al. [19], who stated out that the diaspores of this species showed "intrinsic dormancy" and that this was overcome with the combination of a pretreatment of 12 h KNO_3 (0.2%) plus 48 h of AG3 (500 ppm). In doing this, germination reached 86% in comparison with 0% of germination obtained in the control treatment. Moreover, they concluded that the seeds are photoblastic positive and that their germination was more efficient at 20°C. The differences quoted here can be explained, if one takes into account that these authors carried out the germination tests of the control not only at a 25°C constant temperature, but also with 24 h of continuous light, regardless of the conditions for *E. plantagineum* given by ISTA [31]; that is to say, 20°C–30°C and a photoperiod of 8-16 h. Additionally, these authors evaluated viability with tetrazolium according to the homogeneity of the endosperm coloration, instead of observing the coloration pattern of the embryo.

Furthermore, the results of the present work confirm the previous studies carried out by Florentine et al. [20], who obtained the highest percentages of germination, with alternating temperature (20°C–30°C) and a photoperiod of light-darkness (12 h). It is emphasized that the 93% of germination obtained by these authors contrasts with the 66.5% obtained by the current investigation. These discrepancies can be attributed to the

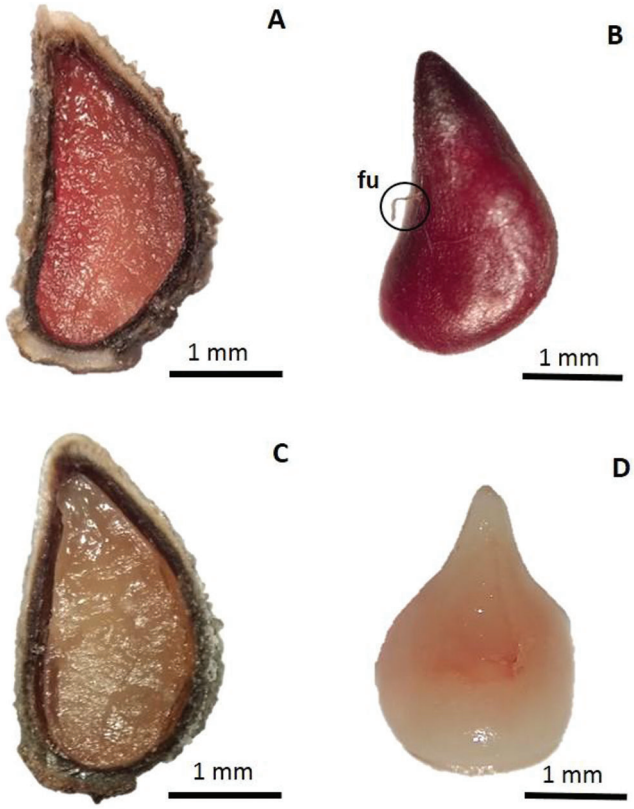

Figure 7: Tetrazolium stain on *Echium plantagineum* diaspores: A. Seed with pericarp, viable with fully stained embryo; B. Seed with seed coat, not viable with the cotyledons and the end of the radicle stained deep red, the circle shows the funiculus remains; C. Seed with pericarp, not viable with the embryo completely brown; D. Seed without pericarp, with the largest area of undyed cotyledons and with the central area of the slightly pink cotyledon. Abbreviations: fu, funiculus

use of seeds with different storage periods, four and eight months respectively for each research; and so, storage deterioration became evident in the non-viable seeds detected in the tetrazolium assay.

3.5 Seedling Morpho-Anatomy

The radicle emerged through the seed coat and the apical end of the mericarpid (Fig. 8A). In the early stages of germination, the hypocotyl with the characteristic hook-shape was observed and by means its elongation, lifted the cotyledons together with the gemmula above ground (Fig. 8B). Up to this point, cotyledons acted as a reserve source, even though a few endosperm remains, stayed attached to the abaxial face of the cotyledons (Fig. 8C). Then, when the pericarp became detached, the cotyledons began to fulfill a photosynthetic function, and the seedling started its heterotrophic phase (Fig. 8D).

These typical characteristics of an epigeal germination allowed to define the seedling type Sloanea, subtype Sloanea, according to the classification of De Vogel [33]. Besides, it was correlated with the classification proposed by Ye [34] with the seedling type Sophora. However, the results of the present work did not agree with the Magnolia or Chimonanthus type, which Ye [34] assigned to the Boraginaceae. This is because this author defined the Magnolia type, for its seeds with conspicuous endosperm, small embryo, and cotyledons with both haustorial and photosynthetic functions.

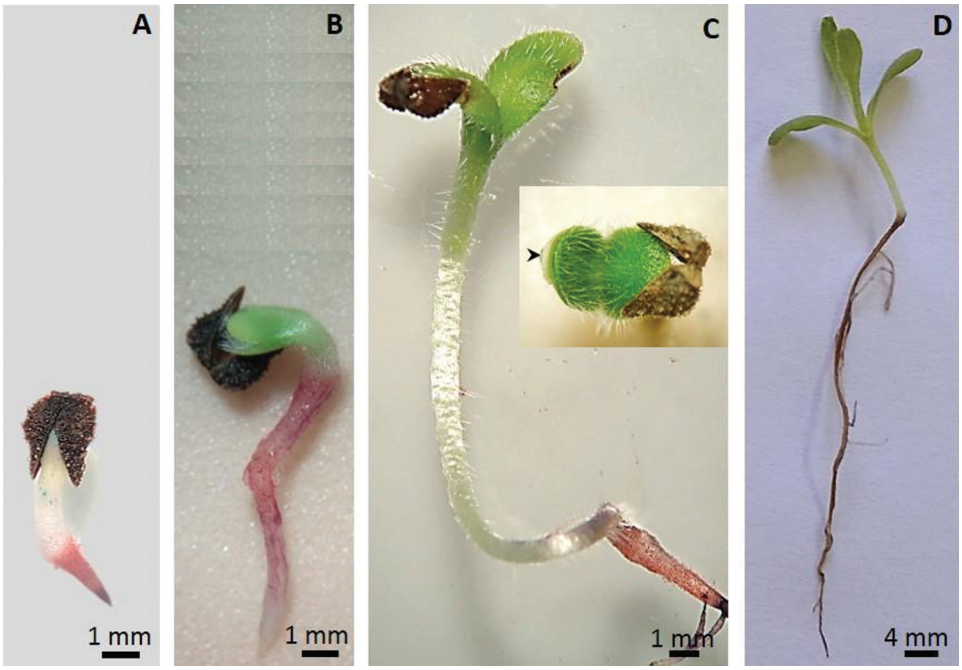

Figure 8: Germination and seedling structure of *Echium plantagineum*: A. Radicle protrusion through the pericarp; B. Elongation of the hypocotyl; C. Cotyledons unfolded and detachment of the pericarp, box: detail of cotyledons with endosperm remnants (arrowhead); D. First pair of nomophiles unfolded

Moreover, Ye [34] described the Chimonanthus seedling-type, with exalbuminous seeds, thin, leaf-like cotyledons and with only photosynthetic function. On the contrary, the *E. plantagineum* seedling was characterized by its fleshy cotyledons with storage and photosynthetic functions, features similar to the Sophora type.

Related to the characteristics of the normal seedling, it was confirmed that the seedlings agree with the category A, seedling type E, group A 2-1-1-1, according to the handbook for seedling evaluation [41] indicated for *Echium*. The anatomical studies of the seedlings, showed in the primary root, a uni-stratified rhizodermis with abundant trichomes and the parenchyma cortex with the endodermis in primary state. In the central cylinder, the pericycle (the outermost layer) was observed in contact with protoxylem vessels (exarch position) that constitute a diarch protostele. The centripetal differentiation of the primary vessel members, with the metaxylem conducting elements in the center of the axis, flanked by the procambium and the primary phloem, complete the vascular system (Fig. 9A). Both the hypocotyl and epicotyl showed shaggy appearance due to a hirsute indumentum of the epidermis, where the long eglandular trichomes described for this species by Molinelli et al. [11] were distinguished. The cortex of the hypocotyl presented a subepidermal layer of collenchymatous cells and in the rest fundamental parenchyma. The primary vascular system of the seedling axis is continuous and the vascular transition encompassing a short portion of the seedling axis (Fig. 9B). The endarch condition is achieved at the level of the epicotyl, characterized by four open collateral bundles that determine a typical eustele. The unfolded photosynthetic cotyledons showed the same type of indumentum already mentioned (Fig. 9C), besides a dorsiventral mesophyll with two-three layers of palisade parenchyma and five-six layers of spongy parenchyma, and in a central position, the vascular bundle corresponding to the midrib was recognized (Fig. 9D).

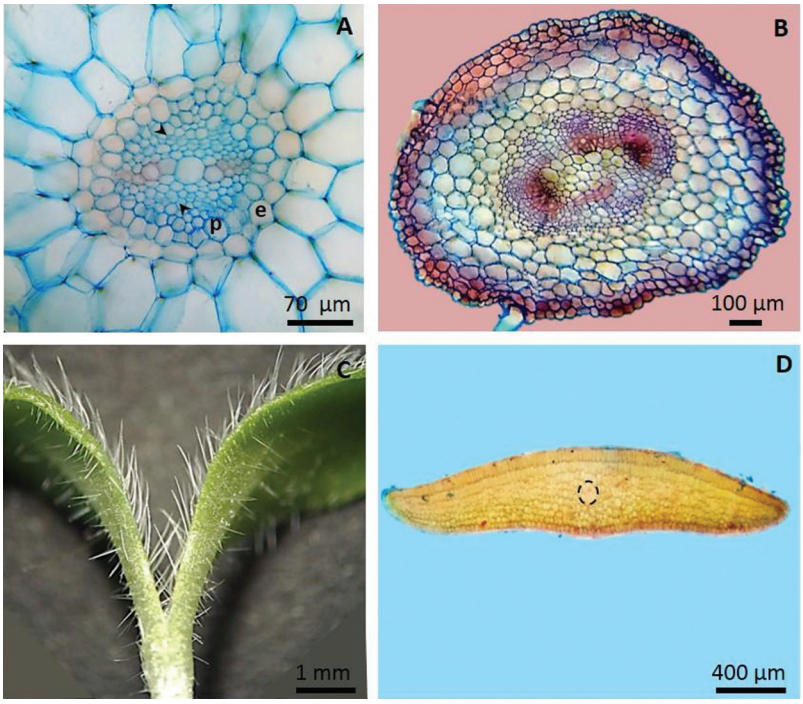

Figure 9: Seedling morphology of *Echium plantagineum*: A. Detail of the vascular cylinder in the cross section of the root, arrowheads indicate procambium; B. Cross section of the hypocotyl showing the eustele; C. Detail of eglandular trichomes on both cotyledon surfaces; D. Tissue distribution in cross section of the cotyledon, the central vascular bundle highlighted with a dashed line. Abbreviations: e, endodermis; p, pericycle

4 Conclusions

This work describes the morpho-anatomy of the *E. plantagineum* diaspores, where the characteristics of the pericarp reflect the close connection with its seed protective function. In this respect, this study reveals that the tissues of the pericarp along with the morpho-anatomical characteristics of the gynobase modulate water uptake during imbibition.

In addition, this research bring about new contributions on account the morpho-anatomical features of the sensu stricto seed, i.e., the unistrata seed coat, the remains of endosperm together with protein bodies and lipids in the storage cotyledons. It is shown the water pathway from the protuberance in the scar abscission of the mericarpid, and finally across the entire seed coat surface to the embryo.

Regarding germination, it is demonstrated that *E. plantagineum* does not possess dormancy, and according to the characteristics corresponding to the morphology of the normal seedling, the category A, seedling type E group A 2-1-1-1, for its evaluation in germination tests is confirmed.

This work provides valuable information for future research on weed control, and suggests that for the efficient management of *Echium plantagineum*, control during the early stages, avoiding flowering, is critical since it allows reducing seed production and its subsequent dissemination. Moreover, the descriptions of the morphological characteristics of the seed and the seedling provide a workable system for the identification of this species, either in the seed bank or in the early stages of development, in order to select the most suitable herbicide to control this weed.

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