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A new seed coat water-impermeability mechanism in *Chaetostoma armatum* (Melastomataceae): evolutionary and biogeographical implications of physiophysical dormancy

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Abstract

Determining the phylogenetic and biogeographic distribution of physical dormancy remains a major challenge in germination ecology. Here, our goal was to describe a novel water-impermeable seed coat mechanism causing physical dormancy (PY) in the seeds of *Chaetostoma armatum* (Melastomataceae). Although seed coat permeability tests indicated a significant increase in seed weight after soaking in distilled water, anatomical and dye-tracking analyses showed that both water and dyes penetrated the seed coat but not the embryo, which remained in a dry state. The water and dye penetrated the lumen of the exotestal cells, which have a thin outer periclinal face and thickened secondary walls with U-shaped phenolic compounds. Because of this structure, water and dye do not penetrate the inner periclinal face of the exotestal cells, indicating PY. Puncturing the seeds increased germination more than tenfold compared to that of the control, but GA_3 did not increase germination further. A significant fraction of the seeds did not germinate after puncturing, indicating that embryos are also physiologically dormant (PD). This paper constitutes the first report of the water-impermeable seed coat in the Myrtales and the first report of physiophysical (PD+PY) dormancy in a shrub from a tropical montane area.

Keywords: *campo rupestre*, germination, Microlicieae, Myrtales, physical dormancy, physiological dormancy, seed anatomy

Introduction

Water-impermeable seed- or fruit-coats give seeds with physical dormancy (PY). PY is absent in gymnosperms but occurs in 18 angiosperm families that are distributed throughout several clades across all of the biogeographical regions on Earth, and is particularly important in tropical savannas (Baskin and Baskin, 2014). The phylogenetic distribution of PY strongly suggests its independent evolution during angiosperm history (Willis *et al.*, 2014). The recent discovery of water-impermeable fruits in basal angiosperms (Mahadevan and Jayasuriya, 2013) indicates that PY has been overlooked in angiosperms and suggests that our understanding of the phylogenetic and biogeographical distribution of seed dormancy increases as additional taxa are studied (see a complete list of taxa with PY seeds in Baskin and Baskin, 2014).

PY is caused by a multitude of seed and fruit structures in different plant lineages. Water-repellent substances can be found throughout the pericarp, only in the endocarp, the exotestal or exotegmic palisade cells, or endotestal cells of seeds across different botanical families (Baskin *et al.*, 2000; Baskin and Baskin, 2014). In addition to physical dormancy, seeds of many species may also have physiologically dormant embryos (PD). The combination of PY and PD is termed physiophysical dormancy and is the rarest seed dormancy type (Willis *et al.*, 2014). Physiophysical dormancy evolved only in eight families, most of which are restricted to Rosids (Baskin and Baskin, 2014).

Based on seed anatomy, Baskin *et al.* (2000) predicted the evolution of water impermeability in several eudicot lineages, including Melastomataceae, but empirical data on germination and seed coat permeability are still lacking for this family. Melastomataceae (Myrtales) comprises nearly 4500 species in *c.* 150 genera (Renner, 2004) and attains its highest

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diversity in the Neotropics (Clausing and Renner, 2001). This pantropical family comprises shrubs, climbers, herbs, epiphytes and trees that dominate montane to lowland forests, savannas, grasslands and disturbed areas throughout Central and South America (Silveira *et al.*, 2013). The seeds of Melastomataceae are morphologically diverse (Corner, 1976; Groenendijk *et al.*, 1996), with six basic forms – cochlear or subcochlear, obpyramidal, winged, ovate, oblong-obovate, orbicular plano-convex and clavate (Baumgratz, 1985) – and have a wide range of cell structures, forms and appendages (Ocampo and Almeda, 2013).

Seeds of Melastomataceae are either non-dormant or physiologically dormant (PD), but there are no reports on water-impermeable seeds in plants from this family (Willis *et al.*, 2014). PD has been described in species from temperate areas (Baskin *et al.*, 1999), tropical seasonal vegetation (Silveira *et al.*, 2013) and tropical rainforests (Garwood, 1983). Although we have previously demonstrated PD in melastomes from tropical montane grasslands (Silveira *et al.*, 2012), preliminary seed anatomy studies suggest a new mechanism determining water-impermeable seed coats in at least one species. In this study, we evaluated the seeds of *Chaetostoma armatum* using dye tracking, germination experiments, embryo imbibition and detailed seed coat development and anatomical data. We provide evidence that *C. armatum* produces seeds with a water-impermeable seed coat and dormant embryo, and discuss the implications of our findings to the understanding of the biogeographical and phylogenetic distribution of physiophysical dormancy (PD+PY).

Materials and methods

Study site and species

Seeds of *C. armatum* D.C. (Microlicieae: Melastomataceae) were collected in 2010 from 25 individuals from rupestrian grasslands (*campos rupestres*), Serra do Cipó, south-eastern Brazil (19°16′53.3″S, 43°35′31.8″W; 1200 m above sea level). The *campos rupestres* are fire-prone seasonal grasslands in which sclerophyllous, ericoid-like plants establish on quartzite-derived, nutrient-poor, acidic and shallow soils (Giulietti *et al.*, 1997; Benites *et al.*, 2007). The climate in Serra do Cipó is mesothermic, with rainy summers from October to March and dry winters from April to September. The mean monthly temperatures range from 7.8°C in July to 32°C in February (Madeira and Fernandes, 1999).

C. armatum is a subshrub that is distributed in rupestrian grasslands and open savannas from Bahia (north-eastern Brazil) to Paraná (southern Brazil) (Koschnitzke and Martins, 2006). The sampled population of *C. armatum* occurs in sandy grasslands and produces dry capsules that open at the beginning of the dry season, when the soil moisture is still high (Silveira, 2011). The seeds of *C. armatum* are small (average 0.63 mm in length and 0.0013 mg in weight), and nearly half of them are embryoless (Silveira *et al.*, 2012). Increased seed weight after soaking, and well-developed and differentiated embryos, has led to the conclusion that seeds of *C. armatum* are physiologically dormant (Silveira *et al.*, 2012). Vouchers were deposited at the Herbarium of the Federal University of Minas Gerais (BHCB), accession number 154026.

Seed coat anatomy, development and histochemistry

The data on seed coat anatomy and development were assessed in individuals under natural field conditions. We tagged flower buds of 20 randomly chosen individuals at Serra do Cipó and sampled ovules and developing seeds at 15, 30 and 50 d after anthesis (DAA), and immediately fixed these in formalin–acetic acid–50% ethanol (Johansen, 1940) for 48 h, including 24 h under vacuum. The samples were dehydrated in an increasing ethanol series (50, 60 and 70%, plus 10% glycerin), stored for 3 d and dehydrated once more in a subsequent ethanol series (80, 90 and 95%). The samples were infiltrated in the refrigerator for 1 week and embedded in (2-hydroxyethyl)-methacrylate (Leica™) according to Paiva *et al.* (2011). The mature seeds were fixed and softened according to Ribeiro and Oliveira (2014) using Franklin solution (Franklin, 1945), glycerin and heating in a water bath; the embedding was performed as previously described (Paiva *et al.*, 2011). Longitudinal and transverse sections (6 μm thickness) were stained with toluidine blue 0.05%, pH 4.7 (modified from O'Brien *et al.*, 1964) and mounted in Entellan™. Toluidine blue is a metachromatic dye that stains phenolic compounds with a blue to green colour and pecto-cellulosic substances with red to purple colours (O'Brien and McCully, 1981).

Histochemical tests were performed in 8-μm-thick sections of ovules and seeds (15 DAA, 30 DAA and mature). We used 10% aqueous ferric chloride plus sodium carbonate to detect phenolic compounds (Johansen, 1940); phloroglucinol in HCl for lignin; Sudan IV in 70% ethanol for lipids (Jensen, 1962); lugol for starch; ruthenium red for pectic substances (Jensen, 1962); and mercuric bromphenol blue for proteins (Mazia *et al.*, 1953). The sections were analysed under a Zeiss light microscope (Zeiss, Oberkochen, Germany) and described using the terminology of Corner (1976) and Werker (1997).

Dye tracking and embryo imbibition

To determine the seed coat permeability, some seeds were imbibed in 1% methylene blue (modified from

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Johansen, 1940) and others in 0.01% Lucifer yellow (modified from Briggs *et al.*, 2005) for 12 h. Due to the small size of *C. armatum* seeds, scarification was performed through puncturing the radicular lobe; the punctured seeds were also exposed to the methylene blue solution. Methylene blue is a polar compound, and Lucifer yellow is a double-negatively charged molecule, both of which are frequently used as an apoplastic tracker in seeds (Egerton-Warburton, 1998; Briggs *et al.*, 2005; Mahadevan and Jayasuriya, 2013). Sections were obtained by cryomicrotomy using a 5030 Bright cryomicrotome (Bright Instruments Co. Ltd, Huntingdon, United Kingdom) and observed under a Zeiss light microscope (methylene blue) and under an epifluorescent Olympus microscope (Olympus Optical Co. Ltd, Southall, United Kingdom) with blue excitation (Lucifer yellow). Photomicrographs were taken using a Nikon digital camera (Nikon Corporation, Tokyo, Japan) coupled to the microscope.

Seed coat permeability tests were performed in control and punctured seeds in the micropylar region. Six replicates of 50 seeds were used for each treatment. We ensured that only those seeds containing embryos were used in this experiment, by separating brown and dark-yellow seeds (seeds containing embryos) from the pink or light-yellow seeds, which were embryoless. The seeds were weighed on a digital balance, soaked in tap water for 4, 12 and 24 h at room temperature and reweighed. The seed permeability was determined by the seed mass increase, and differences in the percentage increase between seed mass of dried and soaked seeds were determined by a paired *t*-test or Wilcoxon test (Silveira *et al.*, 2012).

Germination experiments

The seeds were checked for embryo presence under a Zeiss stereomicroscope, and embryoless seeds were excluded from the germination experiments. Six experimental treatments were created: control, punctured seeds, punctured + 500 mM GA_3, and control + GA_3 in three standard concentrations – 250, 500 and 1000 mM. These concentrations cover a wide range of germination responses to gibberellins. Six replicates of 25 fresh seeds were used for each treatment. The seeds were set to germinate in Petri dishes that were layered with a double sheet of filter paper, moistened with 1% Nistatin solution to prevent fungal growth. For gibberellin treatments, the seeds were exposed to gibberellin for 3 d and then transferred to Petri dishes. The germination was monitored at daily intervals for 56 d, and the germinated seeds that showed root protrusion were removed from the dishes. The dishes were incubated at 25°C under a 12/12-h photoperiod (Silveira *et al.*, 2013). We calculated final germination percentage and the mean germination time (MGT) for each replicate, following Ranal and Santana (2006):

$$\mathrm{MGT} = \sum_{i=1}^{k} n_i t_i / \sum_{i=1}^{k} n_i$$

where n_i is the number of seeds that were germinated in the time i, t_i is the time from the start of the experiment to the ith observation, and k is the time of the last germination.

At the end of the germination trials, the seed viability was estimated for four replicates of 25 seeds, using the tetrazolium test (Delouche *et al.*, 1962). The seeds were punctured at the chalazal end, imbibed in distilled water for 24 h to soften the seed coat, and then incubated in 1% tetrazolium solution for 24 h at 30°C under dark conditions (Silveira *et al.*, 2012).

The data were checked for the assumptions of parametric analyses (Sileshi, 2012), and because the residual normal distribution was not met, we ran the non-parametric Kruskal–Wallis test. To determine the statistical significance among the groups, the Mann–Whitney test with the Bonferroni correction was used.

Results

Seed coat anatomy, development and histochemistry

The ovules of *C. armatum* are anatropous and bitegmic (Fig. 1A). The outer integument is two-layered and thicker at the micropyle end (Fig. 1B). The inner integument is single-layered and slightly thicker in the micropyle. In the micropyle, the exostome (Fig. 1B) and endostome are not in the same line, compounding a zigzag.

Few changes were observed in seed size during development (compare Fig. 1A with Fig. 1K). The structure remains bitegmic (Fig. 1C–L), and the formation of the endosperm can be observed (Fig. 1D). The testa is non-multiplicative, and only the exotesta differentiates during development (Fig. 1C–D, F–G); the endotesta is reabsorbed, remaining in the micropylar end (Fig. 1E) and in the raphe as lignified cells (Fig. 1H). The exotestal cells have a thin and pectic–cellulosic outer periclinal face and thick secondary walls in a U-shaped pattern in the other faces; the secondary walls are impregnated with phenolic compounds (Fig. 1H–J).

The tegmen is also non-multiplicative, remaining one-layered during seed development (Fig. 1E, I–L). At 15 and 30 DAA, the seeds have only a few cells in the embryo, changing from a globular (Fig. 1D) to a cordate state (Fig. 1G); at 50 DAA, the embryo is nearly cylindrical (Fig. 1H).

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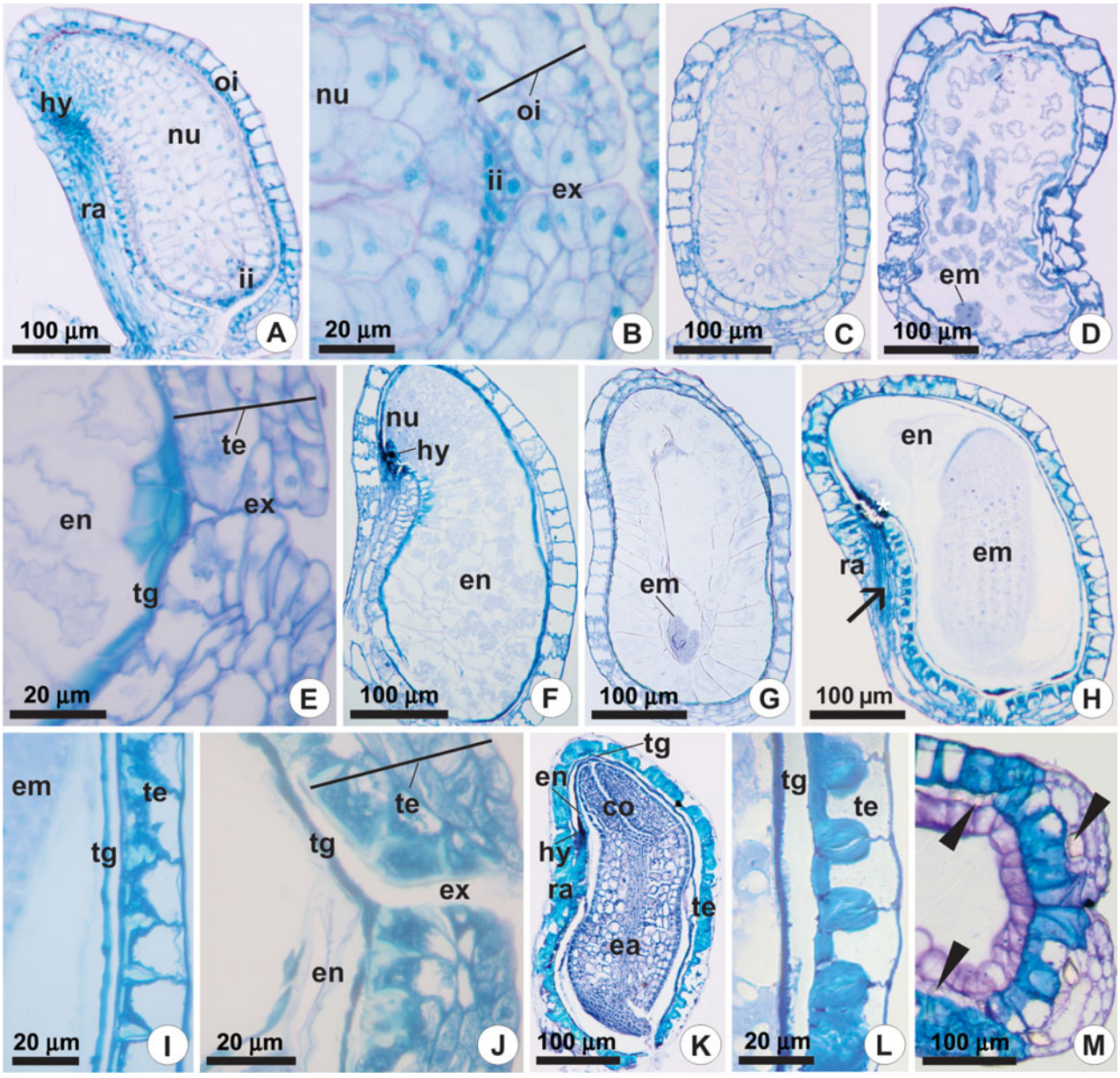

Figure 1. Longitudinal sections of *Chaetostoma armatum*. (A) Ovule of a pre-anthesis floral bud. (B) Detail of the previous figure at the micropylar end. (C) Young seed in the immediate post-anthesis stage. (D) Seed, 15 DAA; note the globular embryo. (E) Detail of the micropylar end at 15 DAA; note the cell elongation. (F) Seed, 30 DAA; still showing remnants of nucellar cells in the chalazal end. (G) Seed, 30 DAA in a transmedian section, showing a cordate embryo. (H) Seed, 50 DAA, with embryo; note the lignified cells in the xylem of the rapheal bundle (arrow). (I) Detail of the seed coat of a seed 50 DAA, showing the exotesta. (J) Detail of the micropylar end of a seed at 50 DAA. (K) Mature seed. (L) Detail of the testa and tegmen of a mature seed. (M) Detail of the micropylar end of a mature seed, showing the presence of calcium oxalate crystals in the testa and tegmen (arrowheads). co, Cotyledon; ea, embryo axis; em, embryo; en, endosperm; ex, exostome; hy, hypostase; ii, inner integument; nu, nucellus; oi, outer integument; ra, raphe; te, testa; tg, tegmen.

In mature seeds, the phenolic deposition on the inner periclinal face and thickened anticlinal faces is conspicuous (Fig. 1L). The testa and tegmen have calcium oxalate crystals at the micropylar end, which is multistratified (Fig. 1M). Except in the raphe (Fig. 1K), all of the other regions of the seed coat are one-layered. The embryo is well developed and differentiated, with fleshy, slightly curved cotyledons filling most of the seed (Fig. 1K). The mature seed is exalbuminous (Fig. 1K), and the embryo is rich in protein. A summary of the results of the histochemical tests is shown in Table 1 and in the supplementary Fig. S2.

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Table 1. Results of histochemical tests on the ovule of a flower during anthesis, a young seed immediately post-anthesis, a seed 30 DAA and a mature seed of *Chaetostoma armatum* (see also supplementary Fig. S2). –, Reaction not detected; +, detected reaction

	Lugol	Ruthenium red	Ferric chloride + sodium carbonate	Mercuric bromphenol blue	Phloroglucinol	Sudan IV
Ovule (during anthesis)	–	+ (nucellus, integument)	+ (hypostase)	+ (nucellus)	+ (raphe)	+ (cuticles: integument, nucellus)
Young seed (after anthesis)	–	+ (endosperm, integument)	+ (hypostase)	+ (endosperm)	+ (raphe)	+ (cuticles: testa)
Seed 30 DAA	–	+ (embryo, endosperm, tegmen, testa)	+ (hypostase, testa)	+ (embryo)	+ (raphe)	+ (cuticles: testa, embryo)
Mature seed	–	+ (embryo, testa, tegmen)	+ (hypostase, testa)	+ (embryo)	+ (raphe)	+ (cuticles: testa, embryo)

Dye tracking and embryo imbibition

We found a significant increase in seed weight, after soaking, in both control ($W = 21$; $P = 0.036$) and punctured seeds ($t = 4.7$; $P = 0.018$). The percentage mass increase was 14.3 and 15.7%, respectively, for control and punctured seeds. Both of the dyes penetrated the seed coat of *C. armatum* in the control and punctured seeds. However, in the control seeds, the dyes penetrated until the inner periclinal face of the exotestal cells, but never beyond it (Figs 2A–E). The dyes remained confined within the lumen of the exotestal cells (Fig. 2C, E) and never reached the embryo (Fig. 2B, D; supplementary Fig. S1A). In contrast, dye staining was observed in the embryos and inner seed coat layers in the raphe of the punctured seeds (supplementary Fig. S1B).

Germination experiments

We found statistically significant differences in the germination of *C. armatum* seeds after several treatments (using the Kruskal–Wallis test, $H = 23.74$, $P < 0.0001$; Fig. 3). Despite being viable, the control seeds germinated less than 5%. Puncturing resulted in a more than tenfold increase in the germination percentage (52 ± 5.3%). Adding gibberellins did not increase the germination of the control or punctured seeds (Fig. 3). The punctured seeds took on average 13.8 (± 0.45) d to germinate. The MGT of the gibberellin-exposed seeds ranged from 25.3 d to 51.8 d, depending on the GA_3 concentration.

Discussion

This work provides the first unequivocal report of the occurrence of PY in Melastomataceae and Myrtales. In the dye tracking and embryo imbibition experiment, our data indicate that both of the dyes stained the lumen of the exotestal cells but not the embryo, which remained in a dry state (dormant) even after a significant increase in the total seed weight after soaking in distilled water. After puncturing, both of the dyes penetrated the seed coat and stained the well-developed and differentiated embryo. Nearly half of the punctured seeds germinated, suggesting that the non-germinating seeds are also physiologically dormant (Willis *et al.*, 2014). Therefore, the seeds of *C. armatum* have physiophysical dormancy (PY+PD), increasing to nine the number of families with this dormancy type.

The report of a water-impermeable seed coat in *C. armatum* increases the number of families with PY to 19 (for the other 18 families, see Baskin and Baskin, 2014). As far as we are aware, *C. armatum* is the only

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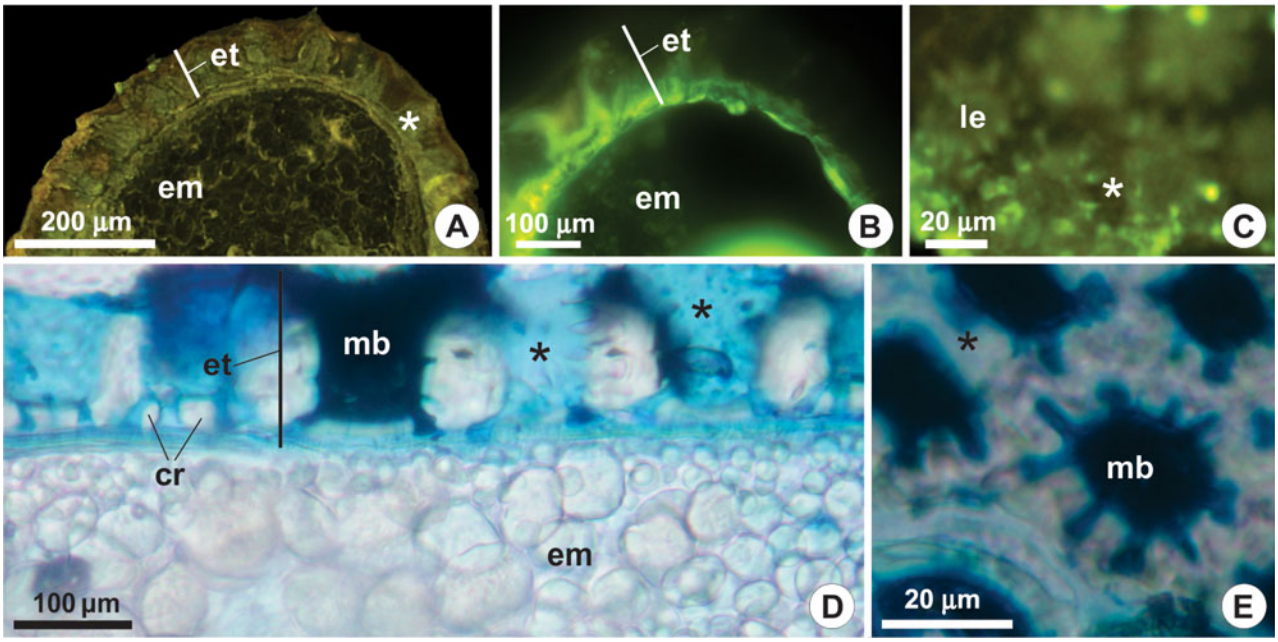

Figure 2. Permeability of the seeds of *Chaetostoma armatum* with an intact seed coat (A, B, D, cross-sections; C, E, paradermic sections). (A–C) Permeability test using epifluorescence microscopy. (A) Control (white) of the permeability test; note the absence of autofluorescence. (B) Penetration of Lucifer yellow into the testa; note the barrier provided by the inner periclinal face of exotesta. (C) Lucifer yellow retained in the lumen of the exotestal cells; note the secondary walls with phenolic compounds acting as barriers. (D–E) Permeability test with methylene blue using light microscopy. (D) Penetration of methylene blue into the exotestal cells; note the barrier provided by the inner periclinal face of the exotesta. (E) Methylene blue retained in the lumen of the exotestal cells; as in (C), note the secondary walls with phenolic compounds acting as barriers. Asterisks, secondary walls impregnated by phenolic compounds; cr, crystal; em, embryo; et, exotesta; le, Lucifer yellow; mb, methylene blue.

species in the Myrtales with a water-impermeable seed coat; the closest clade to Myrtales (*sensu* APG III; Angiosperm Phylogeny Group, 2009) with PY (and also PY+PD) is Geraniaceae (Geraniales) (Willis *et al.*, 2014). The phylogenetic distribution of the seed dormancy types (Baskin and Baskin, 2014; Willis *et al.*, 2014) suggests that PD+PY in *C. armatum* is likely derived from PD, because PD is common in melastomes (Silveira *et al.*, 2013) and PY was absent in the Myrtales (Willis *et al.*, 2014) until this study. Physiophysical dormancy is a derived state in angiosperms and is the less common type of dormancy across all vegetation; our results represent the first report of physiophysical dormancy in a shrub from

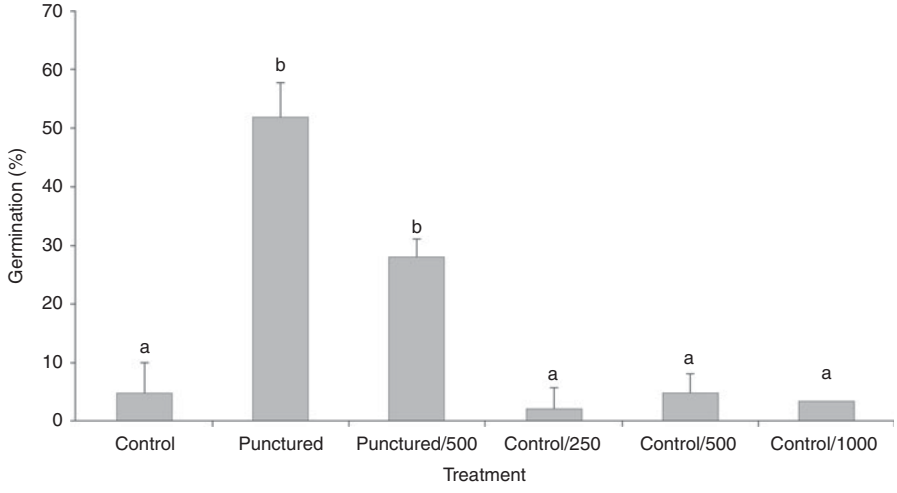

Figure 3. Mean (± SE) germinability (%, bars) of fresh mature seeds of *Chaetostoma armatum* after 56 d of germination. Pre-germination treatments include control vs. punctured seeds, and GA_3 concentrations 250 mM, 500 mM and 1000 mM. The same letters indicate no significant differences.

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tropical montane vegetation (see Baskin and Baskin, 2014). Further investigation may reveal other species with physiophysical dormancy, particularly those with higher phylogenetic affinities with *C. armatum* (Fritsch *et al.*, 2004).

The seeds of *C. armatum* are dispersed abiotically without the assistance of dispersal agents. Preliminary data indicate that dispersal distance for this species is within the range of a few centimetres (R.C. Ribeiro *et al.*, unpublished data). Thus, a likely adaptive significance of PY+PD is to prevent germination below the mother plant. A horizontal run-off-mediated seed displacement would increase the seed dispersal distance, spreading germination in space and time and, therefore, decreasing the density-dependent mortality and sibling competition. In addition, the studied population of *C. armatum* shed seeds at the beginning of the dry season, when the conditions for establishment are unfavourable (Silveira *et al.*, 2012). Physiophysical dormancy would also postpone germination until the next rainy season and maximize fitness when the conditions for establishment become more favourable. PY species have a specific germination season (Baskin and Baskin, 2014), but a dormancy break in *C. armatum* under natural conditions is currently unknown.

PY appears to have originated during periods of increased aridity (Baskin and Baskin, 2014). The core Microlicieae (Melastomataceae) originated in the Miocene, nearly 8 million years ago (Simon *et al.*, 2009), during the expansion of C_4 grasses and, therefore, savannas and grasslands (Beerling and Osborne, 2006). PY attains its greater geographical importance in tropical savannas, where nearly 34% of the species have this type of dormancy (Baskin and Baskin, 2014). Although fire-triggered germination and dormancy are assumed to be important in seeds that have a physical seed coat barrier to uptake of water in fire-prone environments (Bond and Keeley, 2005), caution should be taken in sustaining a fire-induced argument. Fire-triggered germination data are scarce for tropical shrubs and herbs (for trees, see Ribeiro and Borghetti, 2014), and when they are available, they suggest otherwise (Le Stradic, 2012). Second, PY can be broken by fluctuating temperatures without fire (Baskin and Baskin, 2000). PD, in turn, may be broken by cold stratification during dry winters, when temperatures can drop to 7°C (Brito *et al.*, unpublished data).

In the water-permeable seeds of *Miconia ferruginata* (Miconieae, Melastomataceae), water uptake occurs through the raphe (Mendes-Rodrigues *et al.*, 2010). Unlike other regions, the micropyle of *C. armatum* has outer cell layers with pectic–cellulosic walls and an inner layer impregnated by phenolic compounds, suggesting that it is the water gap. The pore is evident, and anatomical analyses also indicate the absence of obstructions to the water, but further studies are necessary to locate the water gap precisely in *C. armatum*.

In species with PY, it is common to find water-impermeable macrosclereids, because these structures are impregnated with water-repellent substances. Although macrosclereids are absent in the seed coat of Melastomataceae species, the exotestal cells of *C. armatum* seeds have secondary walls with phenolic compounds in certain faces that are thought to cause water impermeability, as described for numerous species (Baskin and Baskin, 2014). However, the outer periclinal faces of these cells are thin and not impregnated, i.e. they are permeable. The U-shaped impregnation pattern explains why *C. armatum* seeds can acquire water and remain dormant: the accumulation of water is restricted to the lumen of the exotestal cells, but water passage to the embryo is prevented by the phenolic compounds of the inner face of the exotestal cells. If this new reported mechanism occurs in other species, simple imbibition experiments may not be sufficient to determine the seed dormancy types. To accurately determine the mechanisms accounting for dormancy, it may be necessary to perform dye-tracking analyses and to determine whether embryos imbibe water rather than determining whether seeds acquire water. In this sense, ontogenetic and anatomical studies are necessary to understand the seed coat and to interpret unexpected results in germination tests.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0960258515000070

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Conflicts of interest

None.

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