

## Research Paper

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# Seed traits favouring dispersal and establishment of six epiphytic *Tillandsia* (Bromeliaceae) species

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**Abstract**

There are a number of studies describing the gross range of morpho-anatomical variability in epiphytic *Tillandsia* species, but the interspecific variation in seed traits remain largely unexplored, although these play an important role in determining dispersal and establishment success. In order to evaluate interspecific variation in seed morphology, anatomy and germination, we sampled six *Tillandsia* species from the Yucatan peninsula, Mexico, distributed along a precipitation gradient. We studied morpho-anatomical traits (seed length, seed mass, ratio of coma to seed, ratio of embryo to endosperm), seed terminal velocity in still air, and performed histochemical analyses and germination trials under controlled conditions. *Tillandsia recurvata* differs from the other five species in the structure of the plumose coma; it was the only species lacking an endosperm and showed distinct seedling development. Among the species, bigger seeds were related to longer comas, and had higher germinability. Overall, seed terminal velocity was invariably slow, compared with reports of other anemochorous species, suggesting a high dispersal potential. Taxonomical and ecological implications of our results are discussed.

**Introduction**

Seed traits play a vital role in the successful dispersal and establishment of plant species. For example, traits may be related to dispersal mode: anemochorous seeds, i.e. seeds dispersed by wind, tend to be small, light and may have appendices that favour buoyancy (Magalhães and Mariath, 2012; Barfuss *et al.*, 2016). However, although selective pressures are exerted on seed traits to adapt to local environments, seed traits are more conserved than other plant traits such as leaf size and plant height (Ackerly, 2009), and thus have been used to define taxonomic relationships (Smith and Downs, 1974; Magalhães and Mariath, 2012). Thus, seed traits can provide valuable insights into the ecology of a species and its evolutionary history, and this information may also be useful to predict possible effects of changing environmental conditions.

The epiphytic habitat presents a number of challenges as epiphytes establish in a discontinuous mosaic of suitable trees. Among these challenges are effective dispersal and the colonization of a suitable environment for germination and establishment. There is a significant convergence across families, where about 84% of the epiphytic species are anemochorous, a much higher percentage than among terrestrial species (Madison, 1977). Published studies on seed traits are sufficient to identify this general strategy, but, for most genera, the studies are not detailed enough to characterize seed trait diversity among species that may show specific traits that favour the colonization of particular habitats.

*Tillandsia* consists of about 730 species and is the biggest genus of the family Bromeliaceae (The Plant List, 2013). The genus encompasses a wide range of vegetative forms that allowed the colonization of diverse terrestrial and epiphytic habitats (Benzing, 2000). The diversity of forms described in the adult phase is not concomitant with diversity described in seeds. Most of the studies including *Tillandsia* species have been performed with adults. A few studies have described seed dispersal ability (García-Franco and Rico-Gray, 1988; Mondragón and Calvo-Irabien, 2006; Cascante-Marín *et al.*, 2009), seed morphology and anatomy (Cecchi-Fiordi *et al.*, 2001; Palací *et al.*, 2004; Magalhães and Mariath, 2012), while there are more studies on germination (Bernal *et al.*, 2005; Scatena *et al.*, 2006; Mondragón and

**Table 1.** Ecological characteristics of six *Tillandsia* species from the Yucatan peninsula

Species	Inflorescence type*	Ecophysiological type**	Vegetation types***	Precipitation regime****
<i>Tillandsia</i> subg. <i>Tillandsia</i>				
<i>Tillandsia brachycaulos</i> Schtdl.	Compound, 1–4 purple flowers tubular, hidden scape	Atmospheric	Deciduous forest, semi-deciduous forest, semi-evergreen forest, lowland inundated forests (Tintal) and secondary vegetation	Ample distribution
<i>Tillandsia juncea</i> (Ruiz and Pav.) Poiret vel sp. aff.	Compound digitate, 3–5 yellow flowers in spikes, scape 13–30 cm	Nebulophyte	Semi-evergreen forest, lowland inundated forests (Tintal)	Wet environments
<i>Tillandsia polystachia</i> (L.) L.	Compound pinnate, 5–15 purple sessile flowers, scape 10–25 cm	Tank	Semi-evergreen forest	Wet environments
<i>Tillandsia schiedeana</i> Steud	Simple, 1–2 yellow tubular flowers, scape 4–9 cm	Atmospheric	Deciduous forest, semi-deciduous forest, semi-evergreen forest, lowland inundated forests (Tintal) and secondary vegetation	Ample distribution
<i>Tillandsia yucatanana</i> Baker	Simple to digitate composite, 6–9 purple sessile flowers, scape 4–18 cm	Atmospheric	Coastal sand dune scrub, deciduous forest, scrub mangrove, semi-deciduous forest, lowland inundated forests (Tintal)	Dry environments
<i>Tillandsia</i> subg. <i>Diaphoranthema</i>				
<i>Tillandsia recurvata</i> (L.) L.	Simple, 1–2 purple tubular flowers, scape 5–13 cm	Atmospheric	Deciduous forest, coastal sand dune scrub, semi-deciduous forest, lowland inundated forests (Tintal)	Dry environments

\*Davidse *et al.* (1994).

\*\*Pittendrigh (1948); Benzing (2000); classification of nebulophytes were defined as in Reyes-García *et al.* (2012).

\*\*\*Ramírez *et al.* (2004); Cach-Pérez *et al.* (2013).

\*\*\*\*Precipitation regime defined according to Supplementary Fig. S2.

Calvo-Irabián, 2006; Valencia-Díaz *et al.*, 2010; Sosa-Luría *et al.*, 2012; Marques *et al.*, 2014; Duarte *et al.*, 2017; Müller *et al.*, 2017).

Seed morphology has been used in taxonomic studies in the Bromeliaceae for a long time; Smith and Downs (1974) chose the presence and position of appendices in the seeds among the primary characters of distinction to delimit three subfamilies. Within Tillandsioideae, *Tillandsia* seeds have a plumose coma formed by numerous whitish hairs growing from the micropilar region; the apical end consists of trichomes derived from seed coat cells (external integument; Benzing, 2000). This coma, attached to a small seed increases drag force, maintains the seed in the air for a longer time and potentially increases dispersal (Greene and Quesada, 2005). The seed coat is also composed of dead cells filled with air, which further increases buoyancy (Madison, 1977). There are few studies that describe in detail the characteristics of the plumose coma in epiphytic bromeliads (Palací *et al.*, 2004; Scatena *et al.*, 2006; Wester and Zotz, 2011; Magalhães and Mariath, 2012; Corredor-Prado *et al.*, 2014). Considering the diversity of anemochorous species, information on the falling velocity of their seeds is also scarce (Sheldon and Burrows, 1973; Augspurger, 1986; Matlack, 1987; Greene and Quesada, 2005).

*Tillandsia* seeds are  $\leq 5$  mm in length and possess a small embryo and starchy endosperm (Scatena *et al.*, 2006; Sosa-Luría *et al.*, 2012), which can be variable in size (Martin, 1946; Magalhães and Mariath, 2012; Montes-Recinas *et al.*, 2012). The development of the endosperm relative to the embryo can vary across plant lineages. More basal lineages tend to have a small embryo and large endosperm (Stebbins, 1974). In seeds with bigger embryos germination tends to be faster and dormancy reduced; bigger embryos are favoured in environments with short growing seasons, where rapid emergence and establishment may

be crucial (Vivrette, 1995; Finch-Savage and Leubner-Metzger, 2006; Vandeloos *et al.*, 2012).

Anemochorous *Tillandsia* seeds tend to be small, which does not preclude the possibility for some species-specific variation in seed size, a phenomenon that remains unexplored. In general, increased germination and establishment success has been reported for heavier seeds in exchange for reduced dispersal ability (Dalling, 2002).

In the present study, we set out to evaluate seed, germination and dispersal traits of six species of *Tillandsia* collected in the seasonally dry forests of the Yucatan peninsula, Mexico. Our objective was to describe the seed form and relate it to its function in the six *Tillandsia* species, and to emphasize the divergence and convergence among them, which may be taxonomically relevant. We expected the species limited to the dry sites of the peninsula to invest more in seed resources (seed size, seed mass and coma length) in order to survive under higher stress and to have a higher proportion of embryo to endosperm coupled with increased germination rate to promote faster establishment.

## Materials and methods

### Study species

The six species in this study are grouped in the Tillandsioideae complex; the majority corresponding to the subgenus *Tillandsia* except for *Tillandsia recurvata*, which is in the subgenus *Diaphoranthema* (see Table 1 and Supplementary Fig. S1; Smith and Downs, 1974). *Tillandsia* species are distributed in different vegetation types (Table 1, Supplementary Fig. S2) which present a distribution according to the rainfall gradient from north to south in the Yucatan peninsula, Mexico. Specimens were collected each year from 2012 to 2015 at three sites in the Yucatan peninsula:

*T. brachycaulos*, *T. recurvata* and *T. yucatanana* were collected in the tropical dry deciduous forest at the Dzibilchaltún National Park (21°05'N; 89°35'W), *T. brachycaulos* was also collected in the tropical semi-deciduous forest at the Kaxil-Kiuic Biocultural Reserve (20°05'N; 89°32'W) and *T. juncea*, *T. polystachia* and *T. schiedeana* were collected in the tropical semi-evergreen forest of the Calakmul Biosphere Reserve (18°06'N; 89°48'W; Supplementary Fig. S2). The three collection sites have very small differences in temperature, with most environmental heterogeneity being derived from the differences in precipitation. The species represent ample distribution species (*T. brachycaulos* and *T. schiedeana*), and species limited to either the dry (*T. recurvata* and *T. yucatanana*) or moist (*T. juncea* and *T. polystachia*) areas of the peninsula (Supplementary Fig. S2). From each species, at least five plants with seed capsules were collected and brought to a greenhouse at the Centro de Investigación Científica de Yucatán (CICY). Seeds were collected once the capsules opened naturally.

### Seed anatomy and morphology

Length of seed and plumose coma of a total of 50 seeds per species were measured with a Vernier caliper. Seed mass (without coma) was determined with a digital analytical balance (AND GR 200, Bradford, MA, USA). The outer morphology of seed and coma was studied through photomicrographs using a digital camera coupled to a stereomicroscope (Canon EOS 100D-Stemi SV6, Carl Zeiss, NY, USA). Three seeds with plumose coma per species were given a gold bath for its subsequent observation in scanning electron microscopes (MEB; Jeol, JSM-6360LV, Illinois, USA and Jeol JSM-5310 LV, Utah, USA).

To characterize anatomical structures, five seeds per species were sanded to wear down the hard seed coat and prevent the soft inner seed from collapsing under the microtome. The sanded seeds were fixed in FAA (10% formaldehyde, 5% acetic acid, 50% alcohol ethyl and 35% distilled water) for 24 h and then progressively dehydrated through an ethanol series (30, 50, 70, 85, 96 and 100%, v/v) for subsequent inclusion in paraffin (Merck, Darmstadt, Germany) at 60°C for another 24 h (Márquez-Guzmán *et al.*, 2016). Seeds were cut longitudinally into sections of 8 µm with a rotary microtome (American Optical 820, NY, USA) and a Reichert Jung razor (American Optical and Reichert, NY, USA). Longitudinal sections were stained with Safranin-Fast Green, and photographed under a light microscope (Olympus Provis AX-70; Olympus, Tokyo, Japan, software Q capture Pro). The cross-sectional area of the embryo and endosperm was determined using ImageJ software (Rasband, 2014).

### Seed terminal velocity

We determined the terminal velocity ( $V_{\text{term}}$ ) of falling seed in still air for 50 seeds of each *Tillandsia* species. Each seed was dropped in a closed chamber with no air currents from a height of 2 m and the time of fall was timed. Care was taken to ensure that the plumose coma was deployed.

### Histochemical characterization

Longitudinal sections were also used to perform histochemical tests for protein, starch and lipids in six seeds of each *Tillandsia* species. For the best structure visibility, the objective was to obtain the central longitudinal section of each seed, but this was not achieved every time. Sections close to the central one, with an acceptable visibility of the anatomy were sometimes used. For staining, the

sections were dehydrated in an ethanol series from 96% alcohol (v/v) to water. To observe proteins and polysaccharides, sections were stained with periodic acid-Schiff reagent and then later with Naphthol Blue Black for 5 min, which dyes proteins in blue and carbohydrates in magenta. To detect starch, sections were stained with Lugol, which dyes starch grains in purple or black. Lipid reserves were stained with Oil Red O (for 25 min), which dyes lipids in an orange or reddish colour. It is noteworthy that the reaction product should be insoluble, which prevents diffusion or migration of the reagents in the solution (Márquez-Guzmán *et al.*, 2016). Photomicrographs were obtained with a Photomicroscope (Olympus Provis AX-70; software Q capture Pro).

### Germination trials

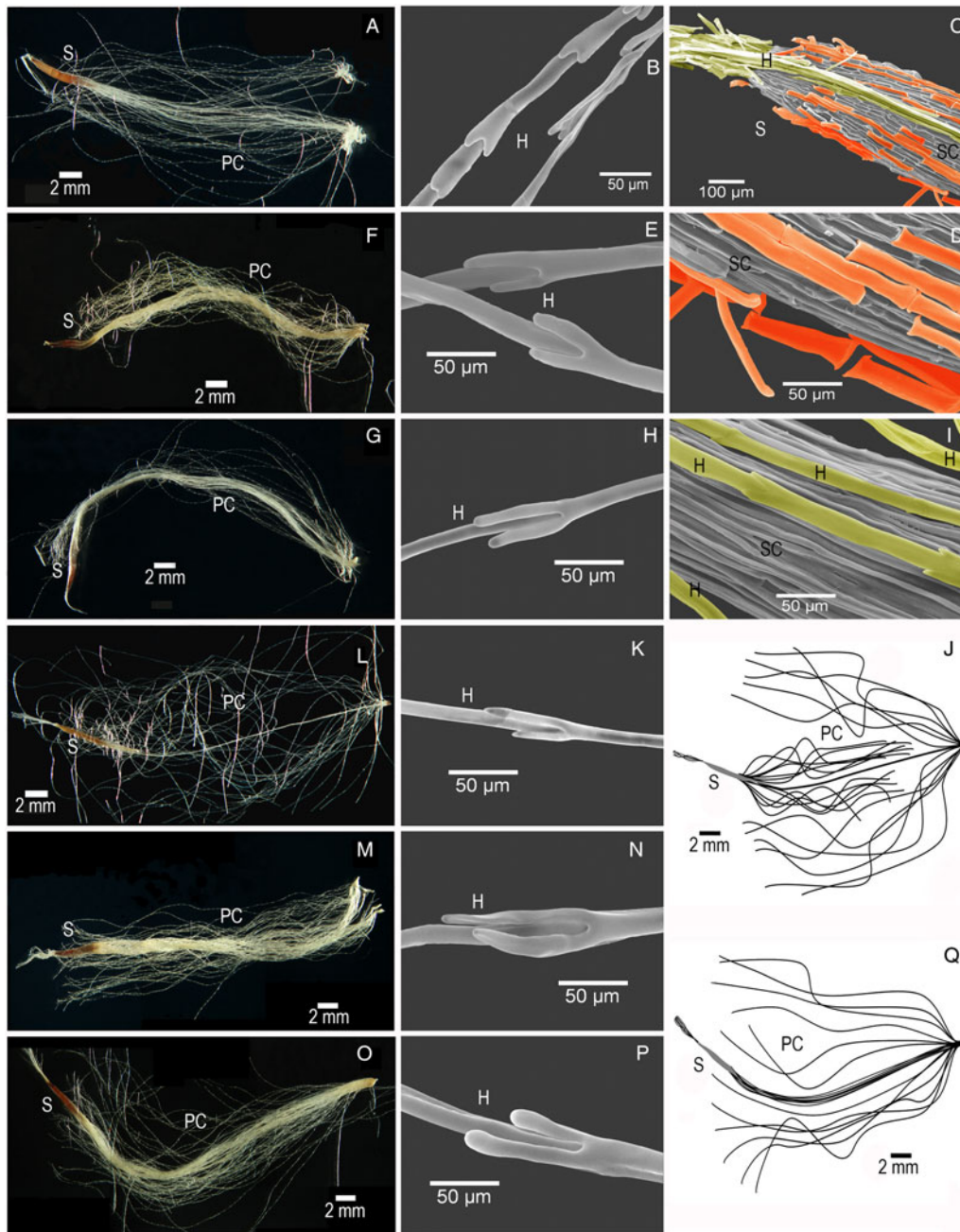
To quantify germination, we collected 90 seeds per species, except for *T. recurvata* and *T. juncea* for which we only had 60 and 35 seeds, respectively. After plumose coma removal, seeds were disinfected in a solution of ethanol (70% v/v) for 2 min, followed by immersion in a solution of sodium hypochlorite (NaClO, 2.5% v/v) for 40 min. Subsequently, the seeds were rinsed in distilled water twice for 5 min to completely remove the remnants of NaClO (Wester and Zotz, 2011). The seeds were evenly distributed in 9 cm Petri dishes (three per species) on filter paper (Whatman No. 1) with 4 ml of sterile distilled water. We selected environmental conditions for germination that were similar to those in the field, and to previously published studies (Valencia-Díaz *et al.*, 2010; Montes-Recinas *et al.*, 2012). Petri dishes were kept closed and placed inside a growth chamber (E-30BHO, Percival Scientific Inc., Perry, IA, USA) for 16 days under a photoperiod of 12 h light/12 h dark, at  $30 \pm 0.5^\circ\text{C}$  and relative humidity of  $60 \pm 0.5\%$  (Wester and Zotz, 2011). We also performed previous germination trials on some of the species to increase the chances of high germinability in our final experiment. Previous trials involving the optimal germination temperature were carried out in *T. brachycaulos* and *T. yucatanana*, germinating the seeds at 18, 28, 35 and  $42^\circ\text{C}$  ( $n = 90$  seeds per species per treatment, distributed evenly in three Petri dishes), and in *T. polystachia*, *T. recurvata* and *T. schiedeana* under the last three temperatures ( $n = 20$ – $90$  seeds per species per treatment, distributed evenly in three Petri dishes). High germination was observed in the range of 28 to  $35^\circ\text{C}$ . To determine the effect of seed age on germination, seeds from *T. brachycaulos*, *T. recurvata* and *T. yucatanana* ( $n = 90$ , 30 per Petri dish) were germinated at 0, 3, 6, 9, 12 and 18 months after the release from capsule, at  $26^\circ\text{C}$ . A decrease in germination was observed after 6 months. Results were not conclusive for *T. recurvata* given the consistently low germinability. We did not use seeds older than 6 months in the current study. The Petri dishes were periodically randomly re-arranged inside the chamber.

Germination was quantified according to Ranal *et al.* (2009). The percentage of germination is reported as germinability and calculated by dividing the number of germinated seeds by the number of seeds sown and multiplying the result by 100. Mean germination rate is calculated as the reciprocal of the mean germination time. Mean germination time ( $\bar{t}$ ) is calculated as:

$$\bar{t} = \frac{\sum_{i=1}^k n_i t_i}{\sum_{i=1}^k n_i}, \quad (1)$$

where  $t_i$  is the time from the start of the experiment to the  $i^{\text{th}}$  observation ( $t$  measured in days);  $n_i$  is the number of seeds germinated in the





**Figure 1.** Photomicrographs of seeds of six *Tillandsia* species. (A–D) *Tillandsia brachycaulos*. (A) Entire seed. (B) Detail of the hair in plumose coma. (C) Detail of seed with dead cells that cover the seed coat (indicated in orange) and coma hairs attached to the seed coat (indicated in yellow). (D) Detail of seed showing the dead cells covering the seed coat (indicated in orange). (E–F) *Tillandsia juncea*. (E) Detail of coma hairs. (F) Entire seed. (G–H) *Tillandsia polystachia*. (G) Entire seed. (H) Detail of coma hairs. (I–L) *Tillandsia recurvata*. (I) Detail of hairs attached to seed. (J) Schematic representation of the *T. recurvata* seed with plumose coma deployed. (K) Detail of coma hairs, *recurvata* seed coat. (L) Entire seed. (M–N) *Tillandsia schiedeana*. (M) Entire seed. (N) Detail of coma hairs. (O–Q) *Tillandsia yucatana*. (O) Entire seed. (P) Detail of coma hairs. (Q) Schematic representation of the seed of *T. yucatana* with plumose coma deployed (the coma structure is similar to that of *T. brachycaulos*, *T. juncea*, *T. polystachia* and *T. schiedeana*). H, hair; PC, plumose coma; S, seed; SC, seed coat. The scale bar in the schematic representation corresponds to 2 mm for the seeds.

$i^{\text{th}}$  time (not the accumulated number, but the number corresponding to the  $i^{\text{th}}$  observation), and  $k$  is the last time of germination.

We define seed germination as ‘visible germination’, i.e. the rupture of the testa and the protrusion of the hypocotyl.

### Data analyses

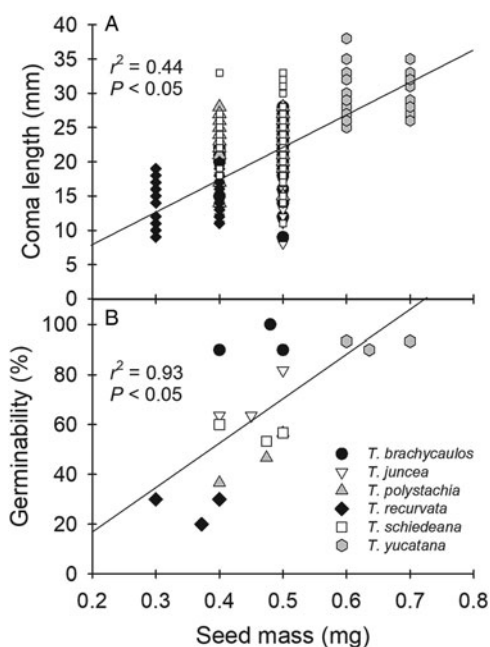
To evaluate the interspecific differences in seed traits, seed length, seed mass, average total seed area, seed terminal velocity and

germinability, one-way ANOVAs were used. A nested ANOVA was performed to assess differences in the proportion of embryo and endosperm within the seed. Percentages and ratios were arcsine transformed prior to analysis. Data transformations were performed for the variables that did not fulfil the assumptions of normality and homoscedasticity, for seed terminal velocity decadal logarithms were used. A non-parametric Wilcoxon test was applied when the data did not fulfil the assumptions even when transformed, as was the case for coma length, ratio of coma/seed and

**Table 2.** Means and standard error of seed and coma length, ratio of coma/seed, seed mass, embryo and endosperm percentage of six *Tillandsia* species from Yucatan peninsula. Ratio of coma/seed refers to the ratio of coma length to seed length. Error terms are not shown for seed mass because they were all < 0.01 mg.

Species	Plumose coma length (mm)	Seed length (mm)	Ratio coma/seed	Adult plant size (cm)*	Seed mass (mg)	Seed projected area (mm <sup>2</sup> )	Embryo (%)	Endosperm (%)
<i>Tillandsia</i> subg. <i>Tillandsia</i>								
<i>Tillandsia brachycaulos</i>	20.7 ± 0.5 <sup>c</sup>	5.0 ± 0.1 <sup>a</sup>	4.2 ± 0.1 <sup>c</sup>	20	0.5 ± 0.0 <sup>b</sup>	0.9 ± 0.2 <sup>a</sup>	42.0 ± 1.3 <sup>c</sup>	48.0 ± 1.3 <sup>a</sup>
<i>Tillandsia juncea</i>	14.6 ± 0.4 <sup>d</sup>	4.8 ± 0.1 <sup>b</sup>	3.1 ± 0.1 <sup>d</sup>	20–40	0.5 ± 0.0 <sup>c</sup>	0.9 ± 0.1 <sup>a</sup>	51.7 ± 2.9 <sup>bc</sup>	38.3 ± 2.9 <sup>bc</sup>
<i>Tillandsia polystachia</i>	22.8 ± 0.4 <sup>b</sup>	4.9 ± 0.1 <sup>ab</sup>	4.7 ± 0.1 <sup>b</sup>	20–65	0.5 ± 0.0 <sup>bc</sup>	0.8 ± 0.2 <sup>a</sup>	47.4 ± 2.8 <sup>bc</sup>	42.6 ± 2.8 <sup>bc</sup>
<i>Tillandsia schiedeana</i>	23.3 ± 0.7 <sup>b</sup>	4.9 ± 0.1 <sup>ab</sup>	4.8 ± 0.2 <sup>b</sup>	20–40	0.5 ± 0.0 <sup>bc</sup>	0.7 ± 0.2 <sup>a</sup>	44.0 ± 0.6 <sup>c</sup>	46.0 ± 0.6 <sup>ab</sup>
<i>Tillandsia yucatanana</i>	30.8 ± 0.4 <sup>a</sup>	5.0 ± 0.04 <sup>a</sup>	6.1 ± 0.1 <sup>a</sup>	10–45	0.6 ± 0.0 <sup>a</sup>	0.9 ± 0.2 <sup>a</sup>	55.6 ± 2.9 <sup>b</sup>	34.4 ± 2.9 <sup>c</sup>
<i>Tillandsia</i> subg. <i>Diaphoranthema</i>								
<i>Tillandsia recurvata</i>	15.4 ± 0.4 <sup>d</sup>	5.0 ± 0.1 <sup>a</sup>	3.1 ± 0.1 <sup>d</sup>	4–23	0.4 ± 0.0 <sup>d</sup>	0.6 ± 0.3 <sup>a</sup>	81.2 ± 2.3 <sup>a</sup>	3.5 ± 0.6 <sup>d</sup>

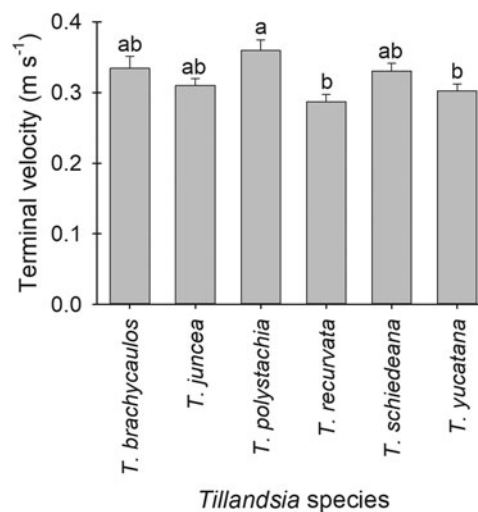
Different letters within columns of plumose coma length, ratio coma/seed ( $n=50$ ) indicate significant differences: Wilcoxon test,  $P < 0.05$ . Different letters within columns of seed length, seed mass ( $n=50$ ), average total seed area ( $n=4$ ) indicate significant differences: one-way ANOVA,  $P < 0.05$ . Different letters within columns of embryo and endosperm percentage ( $n=4$ ) indicate statistically significant differences within columns: nested ANOVA,  $P < 0.05$ . \*Data obtained from Ramirez et al. (2004).



**Figure 2.** Relation between (A) Coma length (mm) and (B) Germinability (%) and the seed mass (mg) of six *Tillandsia* species. Data are presented as means ± SE (in some cases SE bars are smaller than the symbol) of seed mass ( $n=50$  seeds), coma length ( $n=50$  seeds) and germination ( $n=11-30$  seeds).

mean germination rate. Significant group differences ( $P < 0.05$ ) were evaluated using Tukey’s honestly significant difference test.

Simple regressions were performed to assess the relationship between percentage of germination or coma size and seed mass, as well as for seed mass or coma length and seed terminal velocity.



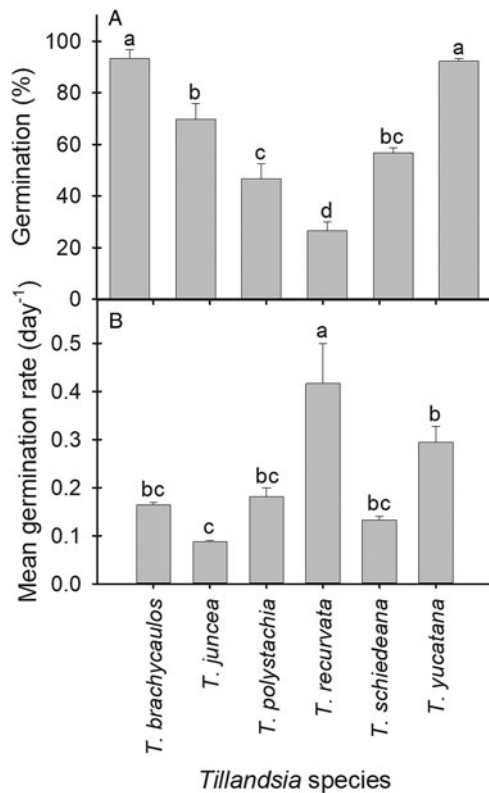
**Figure 3.** Terminal velocity of seeds ( $V_{term}$ ) of six *Tillandsia* species. Data are means ± SE ( $n=50$  seeds). Different letters denote significant differences among species (one-way ANOVA, Tukey’s test,  $P < 0.05$ ).

All analyses were performed using the software STATISTICA 7 (Tulsa, OK, USA).

## Results

### Morphology and anatomy

The coma structure of *Tillandsia recurvata* differed from the five studied congeners (Fig. 1A,F,G,L,M,O,Q). Instead of a single umbrella-like structure in the distal part of the coma, it exhibits two umbrellas, located in the proximal and distal sections



**Figure 4.** Germination of seeds of six *Tillandsia* species. (A) Germinability (%) over 16 days. (B) Mean germination rate ( $\text{day}^{-1}$ ). Seed number varied between species according to seed availability at the time of the experiment,  $n=11\text{--}30$  per Petri dish ( $n=3$ ). Data are means  $\pm$  SE; in one case the SE bar is not seen due to its reduced size. Different letters denote significant differences among species (one-way ANOVA and Wilcoxon test,  $P < 0.05$ ).

(Fig. 1L,J). Imbrications of the individual coma hairs differed among the species (Figs. 1B,E,H,K,N,P). The indentations in the junctions between cells of *T. brachycaulos* and *T. juncea* were shallow (Fig. 1B and E), while the other four species exhibited more pronounced indentations (Fig. 1H,K,N,P). The symmetry of the indentations also varied. The extended plumose coma covered the seed and the seed coat was covered by dead cells and flakes, creating air pockets (Fig. 1C,D,I).

The plumose coma length ranged from  $14.6 \pm 0.4$  mm (mean  $\pm$  SE,  $n=50$ ) in *Tillandsia juncea* to  $30.8 \pm 0.4$  mm in *T. yucatanana* (Table 2,  $P < 0.05$ ). Significant differences were found in seed size, even though the range of variation was small. Seeds of *T. juncea* were the smallest with a length of  $4.8 \pm 0.1$  mm, with the largest seeds (*T. brachycaulos*, *T. yucatanana* and *T. recurvata*) averaging 5.0 mm (Table 2,  $P < 0.05$ ). A positive relationship between coma length and seed mass was found among the six species ( $r^2 = 0.44$ ,  $P < 0.05$ ; Fig. 2A), which was most strongly affected by the light seeds and small coma of *T. recurvata*, and the heavy seeds and large coma of *T. yucatanana*, with the other four species showing intermediate values.

Seeds of *T. recurvata* were notably different from those of the other five species. Seed mass was lowest, averaging 0.4 mg, with an embryo that covers the entire seed, without an endosperm (Table 2,  $P < 0.05$ ). *Tillandsia yucatanana* seeds were the heaviest, averaging almost 50% more at 0.6 mg (Table 2,  $P < 0.05$ ). The embryo in the species of the subgenus *Tillandsia* covered  $42.0 \pm 1.3$  to  $55.6 \pm 2.9\%$  of the area within the seed (Table 2,  $P < 0.05$ ).

### Seed terminal velocity

Seed terminal velocity ( $V_{\text{term}}$ ) varied by ca 15%, ranging from a mean of 0.29 to  $0.36 \text{ m s}^{-1}$  (Fig. 3). The lowest  $V_{\text{term}}$  values were observed in *T. recurvata* and *T. yucatanana* seeds, while *T. polystachia* seeds fell fastest ( $P < 0.05$ ). Surprisingly, terminal velocity did not show a significant correlation with seed mass or coma length (Supplementary Fig. S3).

### Germination

Germinability was highest in *Tillandsia brachycaulos* and *T. yucatanana* at  $93.3 \pm 3.3$  and  $92.2 \pm 1.1\%$ , respectively, after 16 days (Fig. 4A,  $P < 0.05$ ). The lowest values were observed in *T. recurvata* with  $26.7 \pm 3.3\%$ . A positive relationship was obtained between germinability and seed mass among the six species ( $r^2 = 0.93$ ,  $P < 0.05$ ; Fig. 2B).

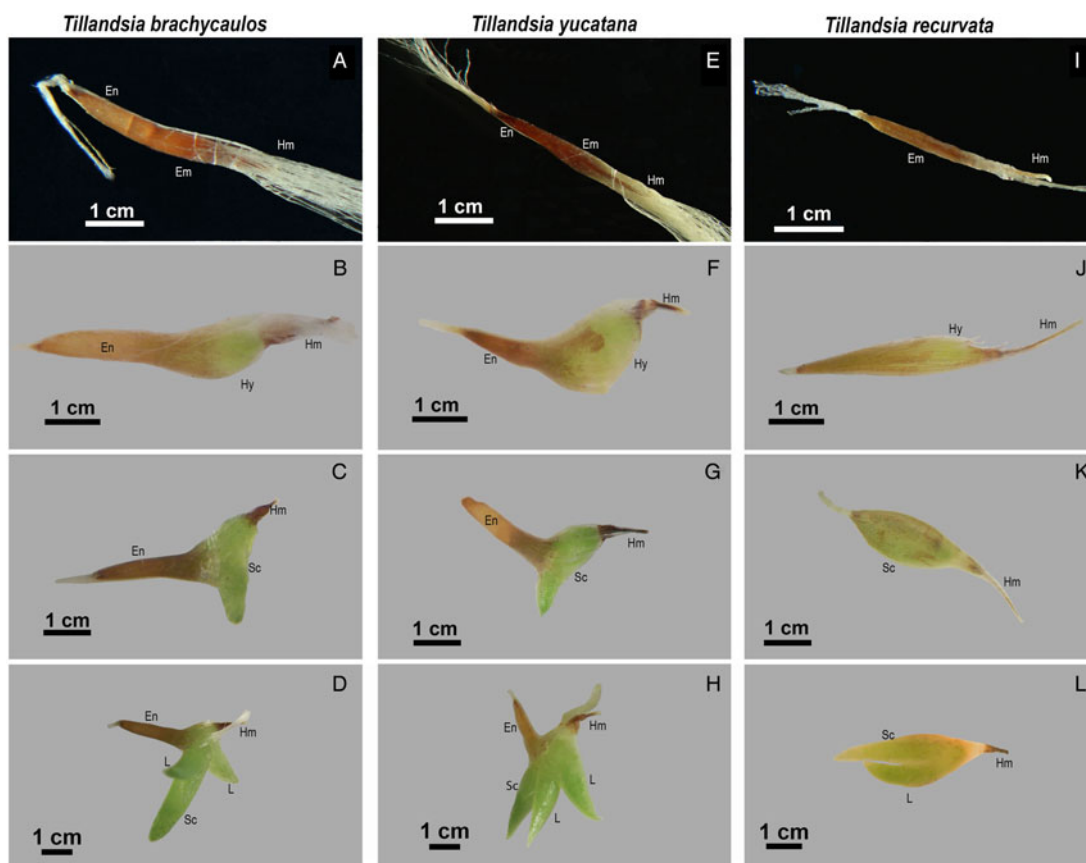
Mean germination rate ( $\text{day}^{-1}$ ) was generally fast, but differed among species (Fig. 4B,  $P < 0.05$ ). *Tillandsia juncea* had the slowest mean germination rate of  $0.1 \text{ day}^{-1}$ , while germination was four times faster in *T. recurvata* ( $0.4 \pm 0.1 \text{ day}^{-1}$ ) followed by *T. yucatanana*, averaging  $0.3 \text{ day}^{-1}$ .

We observed differences in the visible germination and post-seed development of *Tillandsia* species: in *T. recurvata* the seed coat seems to tear longitudinally as the embryo elongates (Fig. 5J,K) and there was a progressive thickening of the hypocotyl, which subsequently developed the scutellum. In the other five species, the seed coat broke transversely and the protruding hypocotyl located between the haustorium and the embryo could be observed within 8 days (Fig. 5B,F); after 15 days, the developed scutellum was observed, and the seed coat containing the endosperm remained visible at the side of the scutellum (Fig. 5C,G). The scutellum grew more slowly in *T. recurvata* and the first leaf developed after 80 days (Fig. 5K,L), while in the other species, we observed two to three leaves after 35 days (Fig. 5D,H).

In the five species with an endosperm, the seed coat protected the reserves, which decreased progressively in size after germination (Fig. 5A–D and E–H). During the first 2 months, the endosperm remained visibly turgid and apparently served as a source of reserves. At that point the seedling had produced up to five leaves and seemed less dependent on the shrinking endosperm. Under controlled experimental conditions the seed coat appeared completely dry after approximately 6 months, and in the second or third month an adventitious root system developed. For *T. recurvata*, which has no endosperm, the scutellum had a predominant role for a longer time.

### Histochemical components in seeds

The photomicrographs of the stained cross-sections showed that all six species stored similar biomolecules, either in the endosperm or, for *T. recurvata*, within the embryo. Starch granules were observed in all species stained in purple (Fig. 6A,B) or using polarized light, as the typical Maltese cross (Fig. 6D). Crystals were ubiquitous within the endosperm, most likely of calcium oxalate (Fig. 6F). Similarly ubiquitous were proteins stained in blue localized in the embryo (Fig. 6C) and endosperm (Fig. 6G). Insoluble polysaccharides (stained in magenta) were found in the endosperm (except for *T. recurvata*) and seed coat (Fig. 6G). Lipids (stained in red) were observed in the hypocotyl–radicle axis that is differentiated as a zone of constriction separating the radicle and the embryo



**Figure 5.** Differences in the visible germination process of three *Tillandsia* species. (A–D) *T. brachycaulos*. (E–H) *T. yucatana*. (I–L) *T. recurvata*. (A) Dry seed *T. brachycaulos*. (E) Dry seed *T. yucatana*. (B,F) Protrusion of the hypocotyl, seed coat is ruptured transversally (8 days). (C,G) Development of scutellum (18 days). (D,H) Leaf growth (35 days). (I) Dry seed *T. recurvata*. (J) Protrusion of the hypocotyl, seed coat is ruptured longitudinally while the scutellum grows. (K) Developed scutellum (18 days). (L) First leaf appears after ca 80 days. Em, embryo; En, endosperm; Hm, haustorium; Hy, hypocotyl; L, leaf; Sc, scutellum.

(Fig. 6E); lipids were also present in the aleurone layer (Fig. 6H), which was found between the embryo and seed coat.

## Discussion

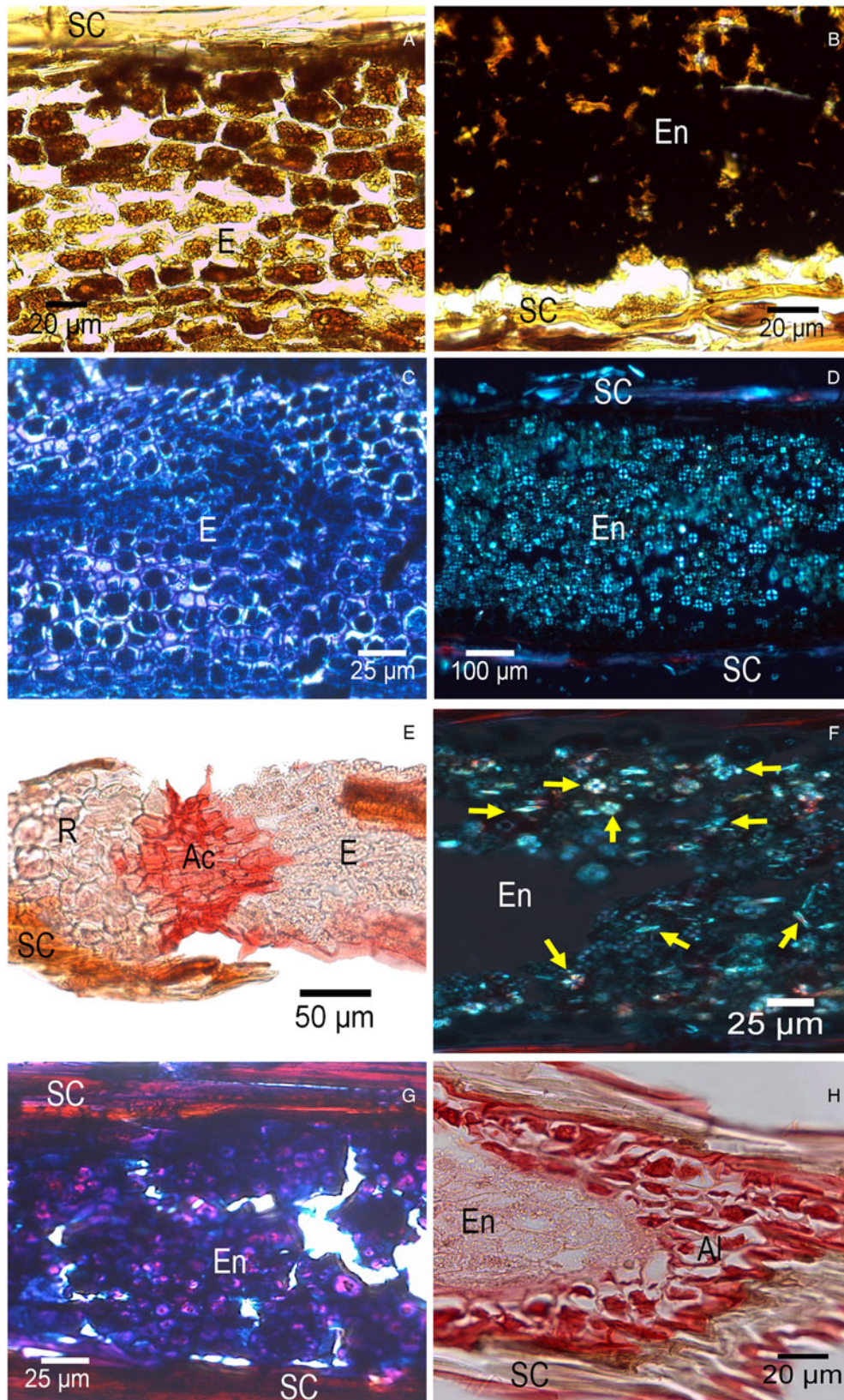
In recent years, the genera and subgenera within the subfamily Tillandsioideae have undergone controversial redefinitions (Spencer and Smith, 1993; Grant, 1995; Espejo-Serna, 2002; Barfuss *et al.*, 2016). The definition of this clade remains a work in progress, partly due to the large number of species it encompasses. In the present study we found relevant seed traits which may be useful for cladistics analyses, such as the imbrications in the hairs that make up the coma. We also found a highly divergent seed anatomy and coma structure in *Tillandsia recurvata*, compared with the other *Tillandsia* species. These differences may reflect the phylogenetic distance between the subgenera.

The structure of the coma imbrications (Fig. 1B,E,H,K,N,P) differed between the studied *Tillandsia* species, and further, unpublished observations suggest that this variation may extend to other species. Unfortunately, no other study has analysed this particular structure. Hence, the function of the imbrications and their usefulness for taxonomic studies (Benzing, 1982) are unresolved and await further study.

A significant divergence in seed traits was observed between *T. recurvata* and the other five species studied. In *T. recurvata* the coma forms a double umbrella (Fig. 1J,L), unlike the single

umbrella, which is observed in the other species (Fig. 1A,F,G,M, O,Q). There is a wide taxonomic distance between *T. recurvata* and the other species, the former being in the more derived subgenus *Diaphoranthema* while the latter belong to the subgenus *Tillandsia* (Barfuss *et al.*, 2016). Magalhães and Mariath (2012) compared the coma structure of eight *Vriesea* species and six *Tillandsia* species, which belonged to the subgenus *Diaphoranthema* (two species) and to the sister subgenus *Anoplophytum* (four species). They describe the double umbrella in all the *Tillandsia* species in their study and infer that the double umbrella can be used to discern *Tillandsia* from *Vriesea* which has a single umbrella. Our study suggests that the single umbrella can be a shared character among *Vriesea* and some *Tillandsia* species, and the double umbrella seems to be a derived character present in species such as *T. recurvata* and *T. stricta*. Furthermore, the character (one or two umbrellas) seems to be more correlated to the phylogeny (Barfuss *et al.*, 2016) than to the environment, as those species included in the Magalhães and Mariath (2012) study are located in more temperate and humid environments compared with the species sampled in the present study, which are found in seasonally dry forests. While many other studies may mention the coma, and may provide illustrations or photographs (Palací *et al.*, 2004; Scatena *et al.*, 2006; Magalhães and Mariath, 2012; Corredor-Prado *et al.*, 2014), the degree of detail is usually not enough to analyse the differences mentioned here, and thus more species surveys are needed to define the extent





**Figure 6.** Photomicrographs of seeds of six *Tillandsia* species. (A) Starch granules in the embryo of *Tillandsia recurvata*. (B) Starch granules in endosperm of *T. schiedeana*. (C) Proteins in the embryo of *T. yucatanana*. (D) Starch granules under polarized light in endosperm of *T. brachycaulos*. (E) Lipid reserves (red) in the constriction area in the radicle-hypocotyl axis of *T. recurvata*. (F) Starch granules and crystals (yellow arrows) in endosperm of *T. juncea*. (G) Structures containing proteins and polysaccharide (proteins in blue and polysaccharides in magenta) in endosperm of *T. polystachia*. (H) Lipid reserves in the aleurone layer of *T. yucatanana*. Al, aleurone layer; Ac, constriction area; E, embryo; En, endosperm; R, root. Panels A, B, C, E, G and H are with light microscopy; polarized light was used in panels D and F.



of the double or single umbrella within both *Vriesea* and *Tillandsia*.

Anatomically, the seed of *T. recurvata* is the most divergent of the species studied. As previously reported by Montes-Recinas *et al.* (2012), the species lacks an endosperm (Table 2), which diverges from all other congenics studied to date (Cecchi-Fiordi *et al.* 2001; Scatena *et al.*, 2006; Magalhães and Mariath, 2012; Montes-Recinas *et al.*, 2012; Sosa-Luría *et al.*, 2012). Evolutionarily, there seems to be a progression from seeds with a large endosperm in *Vriesea* species (73–77%), with a reduced endosperm in the subgenus *Tillandsia* (33–55%) and a further reduction up to a complete loss in the subgenus *Diaphoranthema*, even though this lack of endosperm was not found in another *Diaphoranthema* species, *T. usneoides* (35%; Magalhães and Mariath, 2012). Noteworthy, an evolutionary trend towards a reduced endosperm for the sake of larger embryos has been observed in the angiosperms in general (Finch-Savage and Leubner-Metzger, 2006).

The anatomical differences in *T. recurvata* are accompanied by differences in germination and early seedling development. These developmental differences may also relate to the lack of endosperm. In the species with an endosperm, the endosperm stays turgid for at least 6 months (Fig. 5B,C,D,F,G,H). This suggests that the endosperm is still supporting seedling growth during this time and may explain the faster leaf development compared with *T. recurvata* (Fig. 5D,H,L).

Vandelook *et al.* (2012) found that the species lacking an endosperm in the family Apiaceae exhibited faster germination, compared with those with an endosperm. This same pattern was found in the species in this study, where *T. recurvata* had the highest mean germination rate (Fig. 4). Vandelook *et al.* (2012) suggested that endosperm reduction may have evolved as an adaptation to dry sites where the period of favourable conditions for growth and establishment is shorter than in wetter environments.

There was also a non-significant trend for *T. yucatanana*, the other species from the drier part of the peninsula, to show high germination rates and a small endosperm. These patterns may be worth studying with a larger number of *Tillandsia* species and a wider range of habitats in order to discern a trend between the seed traits and the environment.

Seed terminal velocity was lower in the two species from dry sites, compared with the wet forest species, *T. polystachia* (Fig. 3). A reduced velocity may increase the possibility for dispersal in patchy drier sites, where distances between suitable hosts may be larger than in closed moister forests. The higher buoyancy was achieved by a larger coma in *T. yucatanana*, and a short but more ornamented coma and a lower seed mass in *T. recurvata* (Table 2). Overall, terminal velocity was not related to either coma length or seed mass (Supplementary Fig. S3).

The terminal velocity reached by seeds is highly relevant to the distances at which the seed can disperse. Matlack (1987) studied seed terminal velocity under still air for 38 anemochorous species and found that plumed seeds were in general faster than winged or samara seeds, although value ranges sometimes overlapped. Plumed seeds had velocities ranging from 1.66 to 0.07 m s<sup>-1</sup>, which places the *Tillandsia* seeds in the lower range of the spectrum, with values of 0.36 to 0.29 m s<sup>-1</sup> (Fig. 3). In *Tillandsia*, besides small seed sizes and large comas, the seed coat is covered by dead cells filled with air (Fig. 1C,F) that aid in their flotation (Madison, 1977). It is worth noting that none of the 38 species included in the Matlack study was an epiphyte, thus it would be interesting to test the range shown by epiphytic species, for

which dispersal to the top canopy may be crucial for establishment. Field studies have found that even though *Tillandsia* species produced many seeds, very few seeds can reach distances greater than 15 m from the source (García-Franco and Rico-Gray, 1988; Mondragón and Calvo-Irabián, 2006).

On some of the attributes measured, the two species from the dry sites seemed to have opposite strategies, as *T. yucatanana* had longer comas, heavier seeds and higher germinability, in contrast to the shorter comas, lighter seeds and lower germinability in *T. recurvata*, while the rest of the species clumped at intermediate values (Table 2, Fig. 4). The positive correlations we found between both coma length and germinability in relation to seed mass are highly driven by the differences between *T. yucatanana* and *T. recurvata* (Fig. 2). In general, heavier seeds increase germination and establishment success (Mazer, 1989; Westoby *et al.*, 2002; Paz and Martínez-Ramos, 2003; Peco *et al.*, 2009), yet the applicability of this pattern for *Tillandsia* would need further study, including more species. Heavier seeds in *Tillandsia* may implicate reduced dispersal ability, and thus variation in this trait was very small. Seed size varied by only 0.2 mm (4%) among the species, while mass varied by only 0.2 mg (33%, Table 2).

There were large differences in germinability among species, from germination close to 90% in *T. brachycaulos* and *T. yucatanana*, to values <50% in *T. polystachia* and *T. recurvata*. The causes behind these differences in germinability are unknown, as seed viability and dormancy would have to be further evaluated. Dormancy has not been reported in *Tillandsia*; generally, seeds show rapid germination, often with high germinability under controlled conditions in an interval of 5–15 days after watering (Bader *et al.*, 2009; Cascante-Marin *et al.*, 2009; Montes-Recinas *et al.*, 2012; Valencia-Díaz *et al.*, 2010), unless they are severely stressed (Bader *et al.*, 2009). In general, epiphyte seeds do not seem to form seed banks, but germinate immediately after dispersal at the end of the unfavourable season (reviewed in Mondragón *et al.*, 2015). A study performed on six *Tillandsia* species from Oaxaca, Mexico, found that germinability differences were largely explained by seed viability, determined through seed X-rays (Sosa-Luría *et al.* 2012). Loss of viability was explained by incomplete embryo formation, which was found in 11–39% of the seeds, depending on species. Environmental stress, such as limited water availability, may be a cause for the incomplete seed formation (Zotz and Asshoff, 2010; Zotz *et al.*, 2010); alternatively, morphological dormancy could also explain this observation.

## Conclusions

We report differences in the coma structure between the species in our study with a double umbrella in *T. recurvata* and a single umbrella in the other five *Tillandsia* species. We confirmed that *T. recurvata* lacks an endosperm, a trait so far only described for this species within the genus. These morphological differences are concomitant with functional differences such as fast germination, but slower initial growth of *T. recurvata* and an enhanced dispersal capacity (low seed terminal velocity with a small coma). In general, increases in seed mass were associated with increases in coma size, thus maintaining seed low terminal velocity compared with other anemochorous species. Variation in seed size and mass were very small. Nevertheless, seed mass was also positively related to germinability. Our results highlight the need to characterize the seed structure and function of more *Tillandsia* species in order to quantify the range of trait variation as well as their importance for taxonomical studies and/or ecological relationships.

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**Supplementary material.** To view supplementary material for this article, please visit <https://doi.org/10.1017/S0960258518000247>

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