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# IN-VITRO GERMINATION AND CLONAL PROPAGATION OF ENDEMIC *TILLANDSIA CALIFANII* RAUH (BROMELIACEAE) FROM MEXICO

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ABSTRACT. *Tillandsia califanii* is an endemic epiphytic bromeliad from the Tehuacán-Zapotitlán-Cuicatlán region, Puebla, México. Its basic reproductive patterns and distribution are poorly documented. The authors studied its distribution in two natural populations in the Zapotitlán Valley and performed a series of laboratory experiments to determine the basic culture conditions to determine a propagation strategy. In nature, *T. califanii* commonly propagates asexually producing up to four plantlets per mother plant. Most *T. califanii* individuals (90%) use *Beaucarnea gracilis* as phorophyte, and only 2% live on the columnar cacti *Cephalocereus columna-trajani.* Almost 50% of the *T. califanii* populations include individuals measuring 20–40 cm in height, considered as adult individuals in reproductive stage. Flowering occurs August–October. Inflorescence, formed by a spike, reaches 60 cm in height and produces an average of 20 capsules and up to 2000 seeds per spike. Seeds disperse late February–May. Viability tests show that most seeds contained a highly damaged embryo, which contributed to low survival. The low germination and survival observed in the field may be associated to the endemism of *T. califanii,* thus resulting in a genetically vulnerable species.

*Key words:* micropropagation, in-vitro seed germination, *Tillandsia,* epiphyte

## INTRODUCTION

*Tillandsia califanii* Rauh (Bromeliaceae) is an endemic species of the semiarid Tehuacán-Zapotitlán-Cuicatlán region of Puebla, México. This epiphytic monocot mainly grows in the crowns of the succulent endemic tree *Beaucarnea gracilis* Lem. (Nolinaceae), which is the main component of the Izotal plant community. At the Cardonal plant community, smaller populations are established on the columnar cacti *Cephalocereus columna-trajani* (Karw.) K.Schum. and *Neobuxbaumia tetetzo* (J.M.Coult.) Backeb. (Cactaceae). Thus their abundance and distribution depend on the spatial arrangement and distribution pattern of their hosts as well as germination within the sites on their hosts (Bennett 1986, 1987; Benzing 1990; García-Suárez et al. 2003).

Anatomical features of *Tillandsia califanii* allow its growth and development in this arid environment as a drought-resistant tank bromeliad. Xerophytic adaptations include water-absorbing scaly trichomes on the epidermis of its leaves and sclerified roots that work mainly as holdfasts. Rauh described *T. califanii* in 1971 (Smith & Downs 1977), and Gardner (1984) demonstrated the viability of its pollen.

*Tillandsia califanii* is the only endemic bromeliad among the 600 endemic plant species growing within the Zapotitlán Valley (Dávila et al. 1995). The distribution and abundance of these endemics has been described by García-Suárez et al. (2003).

The physiology of epiphyte seeds has not been studied intensively, except for some well known orchids and bromeliads (Benzing 1980). In-vitro germination can be used for rapid propagation, not only of epiphytic species with commercial value, but also as an alternative method for conserving species that are part of the rich

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flora of Mexico. This method of micropropagation also might help in studies of ecological and physiological processes characteristic of such species.

The study was carried out in the Zapotitlán de las Salinas valley of Puebla, México, a mountainous area in the southeast portion of the state of Puebla (18°20′N, 97°28′W at 1400–1600 m), close to the northeastern limits of the state of Oaxaca. The climate is dry with a rainy season May–August (occasionally September). Total annual precipitation is ca. 350–400 mm, and mean annual temperature is ca. 20°C (Zavala-Hurtado 1982). Many parts of the following major vegetation associations or communities in the valley lack clear ecotones.

**Thorny scrub.** In the "matorral espinoso," as thorny scrub also is known, can be found *Acacia cochliacantha* Humb. & Bonpl. ex Wild., *Cercidium praecox* (Ruiz & Pav. ex Hook) Harms, *Ipomoea pauciflora* M.Martens & Galeotti, *Mimosa luisiana* Brandegee, and *Prosopis laevigata* (Humb. & Bonpl. ex Wild.) M.C.Johnst.

**Cardonal.** In this plant association are *Cephalocereus columna-trajani, Neobuxbaumia tetetzo, Agave karwinskii* Zucc., *Mimosa luisana, Caesalpinia melanadenia* (Rose) Standl., *Calliandra capillata* Benth., *Gymnosperma glutinosum* (Spreng) Less., *Mammillaria haageana* Pfeiff, *M. sphacelata* Mart., and *M. carnea* Zucc. ex Pfeiff.

**Izotal.** Among the Izotal plants are *Yucca periculosa* Baker, *Agave marmorata* Roezl, *Beaucarnea gracilis* Lem, *Echinocactus platyacanthus* Link & Otto, *Castela tortuosa* Liebm., *Caesalpinia melanadenia,* and *Hechtia podantha* Mez.

**Tropical dry forest.** This plant community has *Bursera biflora* (Rose) Standl, *Ceiba parvifolia* Rose, *Senna holwayana* (Rose) H.S.Irwin & Barneby, and *Plumeria rubra* L.

The valley has been considered as a unique region, in terms of its floristic richness, as one of the major centers of plant diversity in Mexico (IUCN 1990) with a 30% level of endemism and as an important nuclei to be preserved (Dávila et al. 1993, Dávila et al. 1995, Jaramillo & González-Medrano 1983, Rzedowski 1978, Villaseñor et al. 1990, Vite et al. 1992). Several species of *Tillandsia* (Bromeliaceae) are found within the valley: *T. atroviridipetala* Matuda, *T. circinnatoides* Matuda, *T. makoyana* Baker, *T. pueblensis* L.B.Sm., *T. califanii, Tillandsia* sp., and *T. recurvata* (L.) L. *Tillandsia califanii* Rauh is an endemic species of the Tehuacán-Cuicatlán-Zapotitlán valley system (Smith & Downs 1977).

## Materials and Methods

Fieldwork was accomplished at the Valley of Zapotitlán within the Valley of Tehuacán, where we established four 50 × 50 m quadrants (as demonstrated by Zavala-Hurtado 1982). Two quadrants were in the Cardonal plant community, and two in the Izotal. We marked the estimated plant size of the *Tillandsia califanii* plants, measuring length and diameter and plant orientation (using a pocket compass). The numbers of offshoots at the base of mother plants were recorded for such use as evaluating clonal propagation. Phenology of *T. califanii* was observed year round. Seeds were collected from closed and open capsules in the Izotal in February–May 1998 and 1999. Seeds were counted per capsule, and the infruitescence spike was measured.

### In-Situ Seed Germination

The few seedlings observed at 1–2.5 cm in height were often confused with plantlets coming from *Tillandsia* species. Larger plantlets 3–10 cm in height were not observed. A failure may occur in their later establishment; or as we observed, goat and squirrel depredation may prevent further growth.

### Laboratory Work

Seeds were separated, washed thoroughly with tap water followed by two washes with distilled sterile water; immersed in sodium hypochlorite 5% and Tween 20 as surfactant for 5 min, and again washed with sterile distilled water. Seed germination was tested with scarified and non-scarified seeds. The scarification treatment consisted of immersing seeds in concentrated sulfuric acid, followed by an immediate and thorough washing with distilled water; seeds were then sown in appropriate media.

Our first approach to identifying an appropriate germination medium was to compare two liquid or agar-gel solidified media at different strengths, using distilled water as control. The basic media were Murashige-Skoog (Murashige & Skoog 1962; Sigma Chemical Co., St. Louis, Missouri) and Knudson C media (Sigma Chemical Co.). Seeds were sown later on Murashige-Skoog (M-S) solid media with or without gibberellic acid (0.5 g/l), plus 3% (w/v) sucrose in all media, which was then exposed to either light or dark conditions. Seeds were tested on M-S solid media supplemented or not with activated charcoal and activated charcoal plus coconut milk, plus 3% (w/v) sucrose in all media. Finally germination was tested on M-S solid media sup-

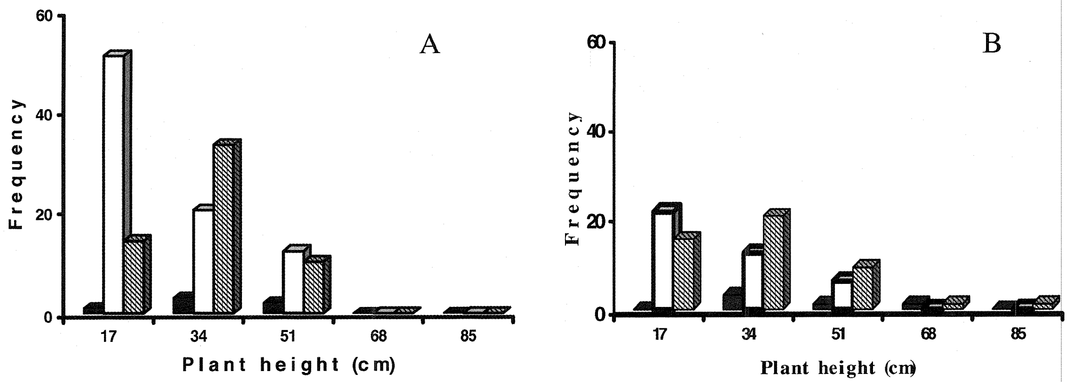



FIGURE 1. Age structure of *Tillandsia califanii* estimated by plant height. Plant orientations, determined with a pocket compass, were grouped in size classes: **A.** Plants facing to the north. **B.** Plants facing to the south. More individuals were observed on the northern side, and 50% of individuals were 20–40 cm in height. Solid bars = mother plants. Open bars = plantlets growing at the base of mother plants. Dashed bars = individual plants.

plemented or not with sucrose (1.5% or 3%). For each treatment, 50 seeds were sown with five replicates. All seed treatments were placed in a chamber for 16/8 hours of a light/dark cycle, with 45 μmol $m^2$ s illumination and 29 ± 1°C temperature. Seed viability was tested using 2,3,5 triphenyl tetrazolium chloride 1% (TTC) supplemented with drops of Tween 20, a colorless solution used as an indicator of metabolic activity within the embryo (van Waes & Debergh 1986). Embryo viability was assessed by TTC and classified according to Kuo et al. (1996); only category I (fully viable seeds) were used.

## RESULTS

### Plant Population and Clonal Propagation

The population density of *Tillandsia califanii* changes depending on the phorophyte. At the Izotal, *T. califanii* is present on 90% of the *Beaucarnea gracilis* trees, which thus are considered the bromeliad's main phorophyte. At the Cardonal, only 2% of the columnar cacti *Cephalocereus columna-trajani* had *T. califanii,* yet it is the main host in this plant community. These populations of *T. califanii* are mainly ramets distributed in clumps. Each clump arises from vegetative shoots of the mother plant, which produces up to four plants on the host side of its base. These offsets have a high probability of reaching adult phase. Most individuals within the 20–40 cm height range are practically in reproductive stage and account for nearly 50% of the population at the Izotal. Younger individuals produced by asexual reproduction are oriented mainly to the north (FIGURE 1). At the Cardonal, the *T. califanii* population consists of only seven clumps in one quadrant and six in the other, with a preferred orientation to the NE, on the opposite side of the characteristic woolly cephalio of its phorophyte, *C. columna-trajani.*

At the Izotal community, 353 *Tillandsia califanii* individuals were found living on 24 *Beaucarnea gracilis* trees, giving a 14.7 ± 13.62 individuals occupancy for the phorophyte. At the Cardonal, this number was less than one individual 0.41 ± 0.58 per *Cephalocereus columna-trajani* cacti.

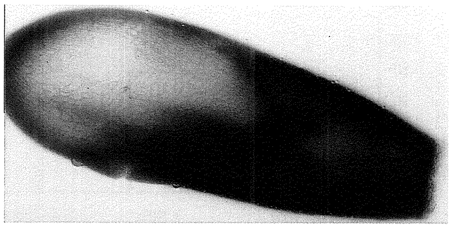
### Seed Production

*Tillandsia califanii* flowers August–October. Inflorescences reach 20.38 ± 5.22 cm in height (60 cm total plant plus inflorescence); each produces 21 ± 6.9 capsules and up to 2000 seeds/spike. Seeds, compressed within the capsule, are liberated 14 months later, when the capsule opens. Ripening and seed dispersal occurs late February–May.

Viability tests showed that 90% of embryos had white patches, mainly in their apical region. This metabolic capacity enabled them to germinate (FIGURE 2).

### In-Vitro Germination

Seeds of *Tillandsia califanii* started germinating under in-vitro conditions, but not before 15 days when the seeds increased in size, turned green, and remained rootless, as do other *Tillandsia* species (Benzing 1978b). At this stage, growth and development halted and rarely continued to form a seedling with leaves and roots. After 15 days, seeds under distilled water ger-



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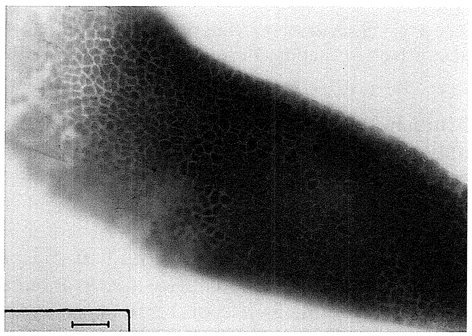

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FIGURE 2. Microscopic determination of seed viability. **A.** *Tillandsia califanii* embryo showing an apical zone with white patches (Light microscope 10×). **B.** Close detail of embryo cells of *Tillandsia califanii* (Light microscope 40×). Scale bar, 25 μm.

minated with roots; 45 days later, 68% of the seeds grew into swollen greenish seedlings. Sudden death, however, occurred after just 5 more days because of tissue oxidation.

### Artificial Media and Sterile Techniques

In-vitro germination was obtained using either liquid or solid media. The former yielded a germination rate of 72% of planted seeds after 45 days (FIGURE 3). Again, seedlings stopped growing and started a secondary latent phase, in which tissues oxidized, leading to whole tissue and seedling death. These seeds were photoblastic-indifferent (initiating germination in the presence or absence of light). Seedlings germinated in the dark were etiolated; but, when exposed to light, turned green. Bromeliads are able to produce chlorophyllic seedlings that synthesize sugars required for plantlet growth (Slack 1992). Gibberellic acid neither increased seed germination nor diminished the apparent secondary latent state (FIGURE 4).

The addition of coconut milk and activated charcoal with 3% sucrose to solid M-S media

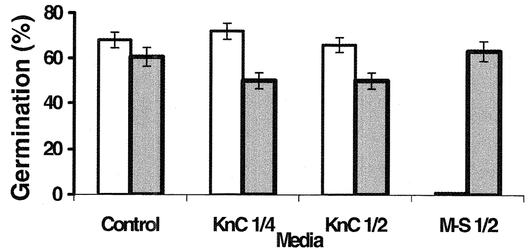


FIGURE 3. Effect of different media on the germinative capacity of *Tillandsia califanii* seeds. Seeds were sown in liquid (open bars) or solid (solid bars) conditions. The media culture used was Knudson C (KnC) at quarter or half strength and Murashige-Skoog (M-S) at half strength. Lines above bars = standard error.

had a negative effect on germination capacity; the control behaved the same way with both additives (FIGURE 5). The use of lower sugar concentrations increased seed germination to 79% (FIGURE 6), but as in other media, seedling growth and development stopped. Scarified seeds did not germinate at all. Only 12 out of the 3000 seeds tested developed to full plantlets. Survival and vigor of this species thus are difficult to evaluate.

## DISCUSSION

Similar to other bromeliads, propagation of *Tillandsia califanii* is based on clonal growth. This reproductive strategy may have genetic consequences for the population. Sexual reproduction in the field was difficult to evaluate, because of the morphological similarity between the plantlets of *T. califanii* and a *Tillandsia* sp. that was a sympatric bromeliad sharing the same phorophytes (García-Suárez et al. 2003). Even

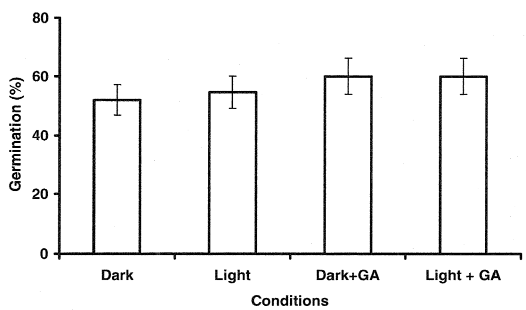


FIGURE 4. Effect of light and gibberellic acid (GA) on the germinative capacity of *Tillandsia califanii* seeds. Seeds were sown on Murashige-Skoog solid media with or without GA (0.5 g/l). Some seeds were then exposed to light, and others to dark conditions. Lines above bars = standard error.

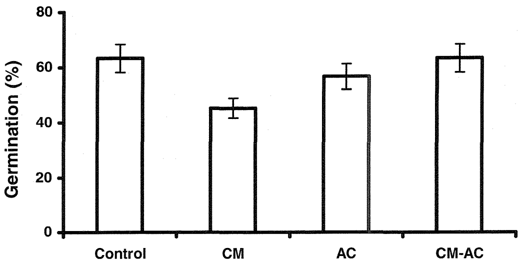


FIGURE 5. Effect of coconut milk (CM) and activated charcoal (AC) on the germinative capacity of *Tillandsia califanii* seeds. Seeds were sown on solid Murashige-Skoog media with CM, AC, or both added; they were maintained under standard conditions as described in Materials and Methods. Lines above bars = standard error.

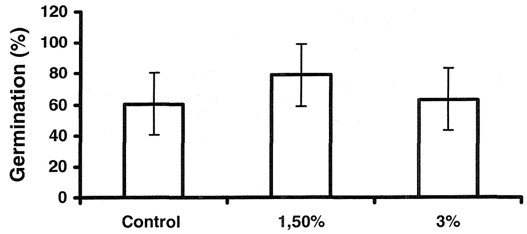


FIGURE 6. Effect of sucrose on the germinative capacity of *Tillandsia califanii* seeds. After being sown on solidified Murashige-Skoog medium with sucrose added at 1.5% (W/V) or 3% (W/V), seeds were incubated as described in Materials and Methods. Lines above bars = standard error.

when nearly 50% of its individuals are in reproductive stage, *T. califanii* may exhibit a population decrease similar to that of *T. brachycaulus,* another monocarpic species, in which the population decreases every time an individual reproduces sexually (Mondragón et al. 1999). Low seed viability (no embryo or non-viable ones) of *T. califanii* is similar to other endemic plants in arid environments (Clark & Molina 2003). Furthermore, autogamy has not been observed, and outcrossing is probable among genetically closely related individuals (sibling crosses).

Sufficient surface for establishment is a critical factor for epiphytes, where competition may play a major role in constraining distribution (Bazzaz 1991). Competition, however, is not a factor where establishment is low and disturbance, high (Bennett 1987, Benzing 2000). *Tillandsia califanii* in the Izotal may solve this problem by using the rough-barked and multibranched *Beaucarnea gracilis* trees, as principal phorophyte with a preferential orientation to the north (FIGURE 1), rather than other phorophytes such as *Cephalocereus columna-trajani,* where *T. califanii* uses the few spaces available. Seedling establishment did occur, but low survival was not evaluated in the study. The accomplishment of the sexual reproductive phase suggests similarity to *T. circinnata* (Benzing 1978a, 1981).

Even with low germination and embryo viability, the population of *Tillandsia califanii,* has been sustained in the Valley. Crossing between close relatives may occur and needs to be further evaluated. Gardner (1984) demonstrated that 96% of the *T. califanii* pollen is viable, suggesting it is not of recent hybrid origin.

As can be seen in FIGURE 5, no difference exists between the media employed for inducing seed germination, with the exception of liquid Murashige-Skoog ½ strength where no germination was registered. As expected, the use of a sterile technique diminished seed loss by fungi and bacteria contamination. The artificial media tested proved that 1) availability of water is important for germination, 2) liquid media permit a good germination but also allow a later fast tissue oxidation, 3) use of 1.5% sucrose obtains more favorable results than higher concentrations, and 4) seeds appear to be photoblastic-indifferent. The media additives used did not enhance seed germination nor promote the growth and development of seedlings. One possible explanation for this low germination may be a low correlation between the metabolic state of the embryo and its developmental potential. Further studies are needed to explore this hypothesis.

At the Valley of Tehuacán, aridity plays a crucial role. Low precipitation is characteristic during the dry season, as is high solar irradiation year round. In-situ seed germination occurs. Scarce seedlings on the *Beaucarnea* trees had the capacity to reflect the sunrays with epidermic scales and developed the morphology of adult plants, including a tank impoundment that allowed water maintenance over long periods.

The genetic diversity of *Tillandsia califanii* populations needs to be evaluated, and research is needed to determine if asexual propagation has affected species diversity. The reproductive strategies of this species, sexual and asexual, have allowed its survival in a harsh environment. Even though seed germination and seedling establishment in *Tillandsia* are sporadic, seldom-observed events, the reproductive status and recruitment in the species studied pose no direct, apparent threat to its survival. Epiphyte survival, however, is indirectly and strongly affected by host survival; and both the columnar cacti (*Cephalocereus columna-trajani*) and the host tree (*Beaucarnea gracilis*) are slow-growing and low-recruitment species (Cardel et al. 1997).

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