

Enhanced Seed Germination and Seedling Growth of *Tillandsia Eizii* In Vitro

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Abstract. *Tillandsia eizii* is an epiphytic bromeliad that due to over-collection, habitat destruction, and physiological constraints has declined to near threatened status. This species exhibits high mortality in the wild, and seed are characterized by low percentages of germination. As a means to conserve this species, in vitro culture protocols were developed to enhance seed germination and seedling growth. A sterilization protocol using 70% ethanol for 2 minutes followed by 2.6% NaOCl for 40 minutes disinfested seed and promoted seedling growth. Sucrose incorporated into the culture medium had no effect on germination or growth, while NAA inhibited growth, but not germination. Cultures maintained under a 16-hour photoperiod at 22 °C exhibited greater growth than those grown at 30 °C. Seed that germinated in the dark remained etiolated and failed to develop even after transfer to light conditions. Plants grown in vitro were successfully acclimatized and transferred to the greenhouse. Over 86% survival and rapid growth were obtained with either an all-pine-bark medium, or a mixture of 2 redwood bark : 2 fir bark : 2 potting mix : 1 perlite. This demonstrated that in vitro culture of seed may be used to rapidly produce large numbers of *T. eizii*, and thus can be used for the conservation and reintroduction of this species.

Tillandsia eizii is one of the largest and most conspicuous members of the Tillandsioideae subfamily of bromeliads. It has important cultural significance to the people of southern Mexico for its use in ceremonial and religious functions. Thus, it has been collected to near threatened status in some areas. *T. eizii* is particularly susceptible to over-collection in the wild because of its specific biological characteristics. Offset formation around the base of mature plants on *T. eizii* is rarely observed in the wild (J. Wolf, personal com.), unlike most other members of this genus. Its reproduction is thus wholly dependent upon seed. However, propagation by seed is inefficient, with ~5% germination reported under field conditions (Toledo-Aceves, 1998). Another characteristic of *T. eizii* is that it is monocarpic (i.e., the plant flowers and then dies), which in combination with the absence of side branch production makes the species particularly vulnerable. A slow growing species, *T. eizii* is estimated to take 6–10 years to reach reproductive maturity in the wild. The slow growth seen in *T. eizii* may be related to its crassulacean acid metabolism (CAM) which is an adaptive response to drought conditions during the dry season (Medina and Dias, 1986).

Habitat destruction also threatens this rare and exceptional species. The highland forests of southern Mexico are diminishing because

of human activity that includes a greater need for resources, such as lumber and farming land (Ochoa-Gaona and Gonzalez-Espinosa, 2000). The sloping terrain of the highlands, coupled with a long rainy season, lead to soil erosion, resulting in further land clearing for farming. Deforestation has diminished valuable niches in pine-oak forests thus resulting in loss of species.

In vitro propagation is a useful tool for many aspects of plant biology, such as germplasm preservation, conservation of species, collection, and mass clonal propagation (Dodds, 1991; Fay, 1992; Munoz, 1999). In vitro propagation has been used for preservation of rare or hard to propagate species including bromeliads. For example, *T. dyeriana*, a highly localized species endemic to western Ecuador, was successfully cultured to prevent over-collection for commercial demands (Rogers, 1984). *T. cyanea* has also been propagated in vitro to preserve selected lines and hybrids (Pierik and Sprengels, 1991). In vitro methods were found to be an excellent method for producing large numbers of the endangered Brazilian bromeliad *Vriesea hieroglyphica* (Mercier and Kerbaudy, 1995). Further, in vitro propagation of bromeliads also has been used when propagation by conventional means of cuttings or seed is slow or difficult to achieve. For example, bud proliferation was induced in the ornamental species *Neoregelia cruenta*, (Carneiro et al., 1999), *Vfosteriana*, and *Dyckia macedoi*. (Mercier and Kerbaudy, 1993; 1995).

The cultivation of seed in vitro may be a means to increase germination and enhance growth rates of *T. eizii*. For conservation purposes, seed as a source of plant material as compared to vegetative material is preferable because it has a wider genetic base. When conventional methods produce low or no germination, in vitro techniques can greatly enhance germination (Fay 1992). Moreover, in vitro techniques can optimize culture conditions and media to enhance growth rates. The development of protocols to produce large numbers of *T. eizii* plants would reduce the pressure for collection in natural habitats and present the possibility of reintroducing plants back into their native habitat. The objectives of this study were to develop methods to regenerate plantlets of *T. eizii* from aseptically germinated seed, and to assess growth and survivability of plants during growth in vitro and after planting in soil. Specific parameters evaluated included the effects of surface sterilization method, carbohydrate source, NAA, light and temperature conditions, and potting substrates for ex-vitro planting.

Materials and Methods

Plant material and general culture methods. Seeds of *Tillandsia eizii* were obtained from a natural population growing in Chiapas, Mexico. Inflorescences were removed from plants and seed were collected at dehiscence of the capsule. *T. eizii* seeds are ~2 mm long and have extensive coma hairs that aid in seed dispersal. The coma hairs were excised and seeds were wet by immersion in 1% NaOCl plus Tween 20 (Sigma-Aldrich, St. Louis, Mo.) for 20 min, then rinsed with H₂O for 5 min. Except where otherwise indicated, seeds were disinfested using 70% ethanol for 2 min, followed by 2.6% NaOCl + Tween 20 for 40 min and rinsed in sterile water two times for 5 min each. Seeds were placed on germination medium consisting of a modified Knudson's medium (KND) composed of Knudson's basal salts (Knudson, 1946) plus myo-inositol (0.1 mg·L⁻¹), nicotinic acid (5 mg·L⁻¹), thiamine HCl (5 mg·L⁻¹), glycine (4 mg·L⁻¹), pyridoxine (5 mg·L⁻¹) and solidified with 0.4% (w/v) Gelgro (ICN Biochem., Irvine, Calif.). Except in carbohydrate studies, seeds were germinated and grown in medium without sucrose until 12 weeks after initiation, then transferred to the same medium but with 2% sucrose. Media were adjusted to pH of 5.5 prior to sterilization, and autoclaved at 121 °C for 20 min at a PSI of 2.9 MPa. Two seeds were placed per 25 × 50 mm test tubes with 20 mL medium. After 8 weeks, seedlings were transferred into 5.5 × 5.5 × 6-cm baby food jars containing 50 mL of KND medium. Except in light and temperature studies, cultures were maintained in a growth room at 25 °C under a 16-h photoperiod with illumination of 125 μmol·m⁻²·s⁻¹, and subcultured every 4 weeks to fresh medium.

Sterilization treatments. Seeds were wet in 1% NaOCl plus Tween 20 as described in general methods, then submitted to one of four surface sterilization procedures listed in Table 1. The experiment was conducted twice using

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25 and 40 seed per treatment. Sterilization was assessed by the appearance of visible contamination. Uncontaminated seeds were rated for germination (emergence of the radicle from the seed coat) and growth over 12 weeks.

Sucrose. To evaluate the effect of carbohydrate, seeds were placed on germination medium with or without sucrose [i.e., 0% or 2% sucrose (w/v)], with 100 seed per treatment. Seeds were evaluated every 3 weeks for 12 weeks for percent contamination and percent germination. At 12 weeks after culture initiation, seedling height was measured.

NAA amendments. Studies to evaluate the effect of NAA on growth and germination were conducted by incorporating NAA at either 0, 0.25 mg·L⁻¹ (1.35 μM), 0.5 mg·L⁻¹ (2.64 μM), 0.75 mg·L⁻¹ (4.04 μM), 1.0 mg·L⁻¹ (5.37 μM), or 2.0 mg·L⁻¹ (10.74 μM) into KND medium. Seeds, 50 per treatment, were evaluated for germination and growth at 3-week intervals for 12 weeks.

Light and temperature. The effects of temperature and light on seed germination were investigated under different environmental conditions. Two temperatures (i.e., 30 °C or 22 °C) and two light regimes (i.e., 16-h photoperiod or continuous dark) were evaluated. Seeds were placed on KND media and maintained on their respective environments for 4 weeks, at which time continuous dark treatments were transferred to a 16-h photoperiod. Each treatment was evaluated for germination, growth, and developmental changes for 12 weeks from initiation. Sixty seeds were used per treatment.

Greenhouse vs. in vitro germination. Germination was compared in seed placed under greenhouse vs. in vitro culture conditions. For greenhouse studies, seeds were planted in 72-cell plug trays filled with Fafard Seed Germination Mix. Flats were maintained under a 16-h photoperiod, at 22 °C under a mist bed. For in vitro experiments, seeds were sterilized and placed in culture media as described under general culture methods. Forty-eight seed were evaluated per treatment.

Planting studies. In vitro-grown seedlings were removed from culture and plants were washed with distilled water to remove agar. Plant height was measured and seedlings were transplanted into one of four potting substrates (see Table 3) with 36 plants per substrate. Plants were placed in a growth room at 22 °C under a 16-h photoperiod, covered with polytene bags, and acclimated by gradually removing the coverings. Plantlets were evaluated for 32 weeks for percent survival, growth, and developmental changes.

Statistical analysis. Experiments employed a randomized block design and were subjected to analysis of variance procedures (ANOVA) of the Statistical Analysis System (SAS Institute, 1995).

Results and Discussion

Developmental stages during seed germination are shown in Fig. 1A. Following sterilization, seeds imbibed water, swelled and enlarged. Seed required 3–4 weeks to germinate,

Table 1. Contamination, germination percentages, and growth of *Tillandsia eizii* seed sterilized using different methods.^a

Sterilization method	Contamination at 6 wk (%)	Germination at 6 wk (%)	Cultures + true leaves at 12 wk (%)	Mean height of cultures + true leaves at 12 wk (mm)
I. 70% EtOH (2 min) 2.6% NaOCl (40 min)	10 a ^b	94 a	83 a	7.6 a
II. 95% EtOH (30 sec) 1% NaOCl (60 min)	6 ab	92 a	85 a	7.5 a
III. 95% EtOH (30 sec) 6% Ca (OCl) ₂ (40 min)	1.5 b	69 b	58 b	7.6 a
IV. 70% EtOH (3 min) 1% NaOCl (40 min)	0 b	80 ab	63 b	7.0 a

^aData are pooled from two sterilization studies; n = 58–72 seed per sterilization treatment.

^bMean separation within columns by Tukey's Studentized range test at P = 0.05.

at which time emergence of leaf primordia was observed. After 6 weeks, seedlings had emerged from the seed coat and young leaves were ≈4 mm long. At 12 weeks, leaves were elongated and plants were 6–12 mm in height. At this time they were transferred into baby food jars. At 15 weeks, plants had a mean height of 14 mm (Fig. 1A and B). At 20 weeks, plantlets were ≈20 mm in height (Fig. 1A) and could be transferred either into Magenta GA7 vessels (Magenta Corp.) or planted into soil.

Sterilization. All treatments were effective in disinfesting seed. Contamination was ≤10% regardless of sterilization method. Seed germination under the different sterilization regimes varied from 69% to 94% (Table 1). This was a dramatic increase over the 5% germination rate reported for seeds in the wild (Toledo-Aceves, 1998). The increased germination obtained under in vitro conditions was consistent with the performance observed for some other bromeliads in culture. *Vriesea heiroglyphica* exhibited a 90% germination of seeds after 6–12 d on

Knudson's medium (Mercier and Kerbauy, 1994). Seeds showing low germination due to dormancy or other conditions can increase germination in vitro (Fay et al., 1999).

Sterilization method had a significant effect on percent germination. Treatment III [95% (30 s) ethanol and Ca(OCl)₂ (40 min)] resulted in significantly lower germination than treatments I [70% EtOH (2 min) plus 2.6% NaOCl (40 min)] and II [95% EtOH (30 sec) and 1% NaOCl (60 min)]. In addition, seedling development was markedly affected by sterilization treatment. A significantly higher percentage of cultures reached the true leaf stage by 12 weeks from initiation in cultures sterilized with methods I and II vs. methods III and IV. However, of those cultures that reached the true leaf stage, mean height was not significantly affected by sterilization method. The major difference observed among the sterilization treatments was on seedling development. All sterilization treatments were adequate for disinfesting seeds, however treatments III and IV

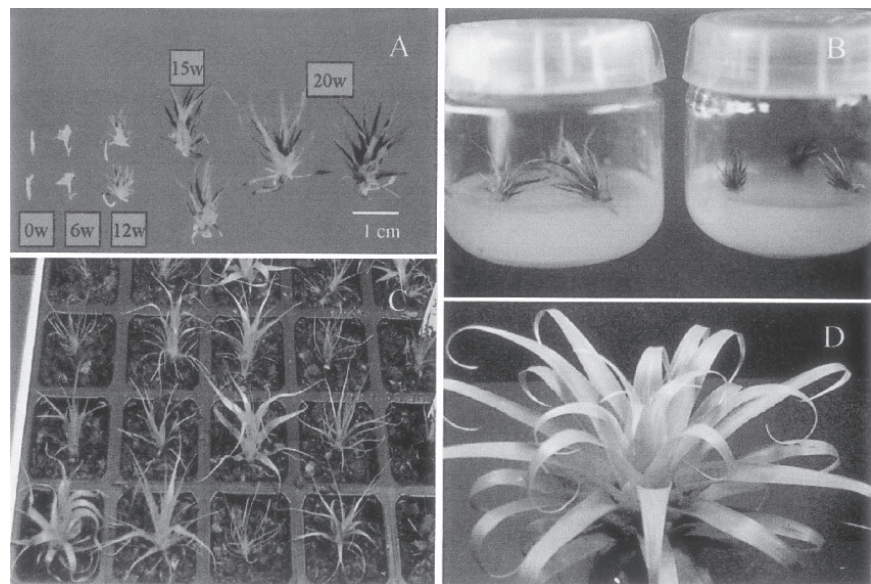


Fig. 1. Seed germination, seedling growth, and outplanting of in vitro-grown *Tillandsia eizii*. (A) The development of plants in culture is shown (from left to right) at 0, 6, 12, 15, and 20 weeks after culture on KND medium. (B) 15-week-old seedlings in baby food jars. (C) Acclimatized plants were planted into flats. (D) A plant initiated from in vitro-germinated seed. Plant is ≈60 cm high and exhibits characteristic morphology.

inhibited seedling growth. Because treatment I was less time consuming, it was adopted for use in subsequent studies.

Carbohydrate. Sucrose had no significant effect on seed germination or seedling growth. Germination was 88% and 86% for treatments with and without sucrose, respectively. The percentage that reached the true leaf stage was 80% or more, and, likewise, not significantly different. This is in contrast to other bromeliad species, where seed germination and survival were inhibited with 2% and 3% sucrose in *T. polystachya*, and with 2%, 3%, and 4% sucrose in *Vriesea heliconioides* (Mekers, 1977). In the current study, the amendment of an additional energy source into the germination medium had no promoting effect, suggesting that the seed contain adequate energy reserves to support germination and early seedling growth.

Effect of NAA. NAA had no significant effect on seed germination, which ranged from 56% to 78% in all treatments (Fig. 2). However, at all concentrations tested, NAA significantly inhibited seedling development. At 12 weeks from sowing, significantly fewer seedlings reached the true leaf stage, and none of the plants grew to over 3 mm if NAA was present at any concentration. Expansion of young leaves occurs when seedlings attain a height of 3–4 mm. This is in contrast to the treatment with no NAA, where over 80% of the plants attained this height. Plants grown in media with the three higher concentrations of NAA (i.e., 0.5 mg·L⁻¹–1.0 mg·L⁻¹) exhibited arrested growth, had browning of leaves and in many cases died. The effects of NAA on seed germination in other bromeliad species are variable. Seeds of *V. splendens* germinated greater than controls when 1 mg·L⁻¹ NAA was added to the media (Mekers, 1977). With *Guzmania minor*, *Guzmania lingulata*, and *V. splendens*, Pierik et al. (1984) found that NAA incorporated in the medium had no effect on seed germination at levels up to 5 mg·L⁻¹, but inhibited germination at higher levels. In contrast to effects on germination, NAA enhances growth in some members of the Bromeliaceae. Shoot, root, and plant fresh weights of *G. minor*, *G. lingulata*, and *V. splendens* increased with addition of NAA (Pierik et al., 1984). Strong stimulation of root and shoot growth occurred, with an optimum response at 0.5–0.8 mg·L⁻¹. Mekers (1977) found improved growth in *V. splendens* when either 1 mg·L⁻¹ NAA or GA₃ were incorporated into the medium.

Environment. The effects of light and temperature on in vitro seed germination and seedling growth are shown in Table 2. At 30 °C, there was no significant difference in percent germination between light vs. dark treatments. However at 22 °C, germination in the dark resulted in significantly lower germination. Subsequent seedling growth was severely inhibited at 30 °C. At 6 weeks after initiation, no cultures maintained at 30 °C developed to the true leaf stage. In contrast, 30% or more of the cultures maintained at 22 °C attained this stage. The light regime had no significant effect on the percentage of cultures reaching the true leaf stage at 6 weeks. However, subsequent growth was markedly

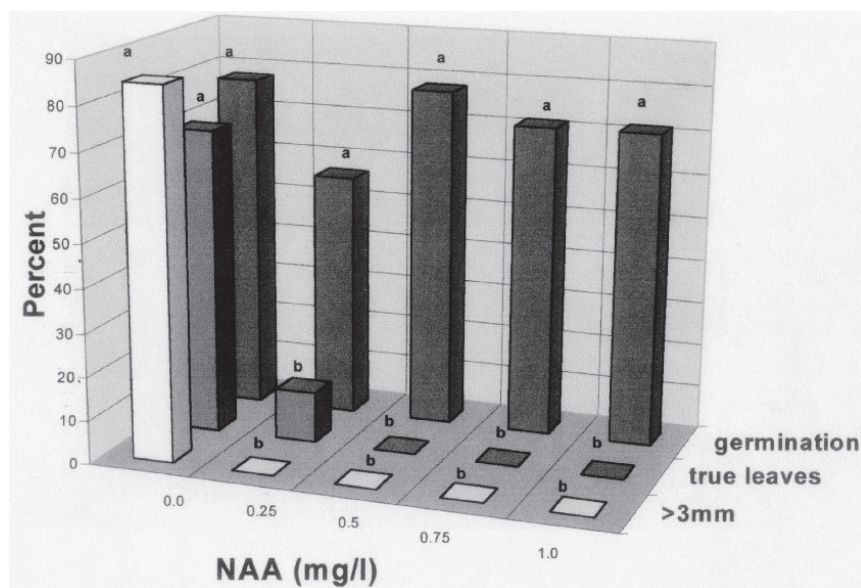


Fig. 2. Seed germination and plant growth of tissue-cultured *Tillandsia eizii* with and without NAA incorporated into the medium. Percentages for seed germination, plants that reached the true leaf stage, and plants that grew 3 mm tall are shown. Mean separations by Tukey's Studentized range test at $P = 0.05$.

affected. At 12 weeks, no plants reached 6 mm in height in dark germination treatments. It should be noted that cultures were subjected to the dark treatments for only the first 4 weeks, and thereafter transferred to a 16-h photoperiod. At 4 weeks, germination is limited to swelling of seed and partial emergence from the seed coat. Thus, initial dark-germination conditions severely inhibited further seedling growth even though subsequent development occurred after cultures were transferred to a 16-h photoperiod. Cultures initiated in the dark remained etiolated and failed to become green even after transfer to light conditions.

Greenhouse vs. in vitro germination. Percent germination was markedly higher for seeds grown under in vitro vs. greenhouse

conditions. Percent germination was 78% and 31% for in vitro- and greenhouse-grown seed, respectively. Furthermore, seedling growth was enhanced in tissue culture. At 8 weeks after sowing, 48% of the seedlings grown in vitro had shoots with expanding leaves; none of the plants in the greenhouse had attained that stage of development.

Potting substrate. The potting substrate had a marked effect on plantlet survival (Table 3). Survival was extremely low in plants transferred onto substrate IV (Fafard : perlite). Highest survival percentages were obtained for substrates I (redwood : fir : Fafard : perlite) and II (pine bark). Survival was five times greater than that with substrate IV. Plants grown in substrate III (redwood : fir) exhibited

Table 2. Seed germination and seedling growth of *Tillandsia eizii* at four temperature/light combinations.

Temperature and light	Germination at 3 weeks (%)	Cultures + true leaves at 6 weeks (%)	Cultures ≥ 6mm at 12 weeks (%)
30 °C temperature			
16-h photoperiod	88 a ²	0 b	0 b
Continuous dark, 4 weeks ³	76 a	0 b	0 b
22 °C temperature			
16-h photoperiod	78 a	48 a	40 a
Continuous dark, 4 weeks ³	55 b	30 a	0 b

²Mean separation within columns by Tukey's Studentized range test at $P = 0.05$.

³Seed were maintained under the different light regimes for 4 weeks, after which time a 16-h photoperiod was used for all cultures.

Table 3. Percent survival and growth of surviving in vitro-grown seedlings transplanted into four different potting substrates. Data were taken 24 weeks after outplanting.

Potting Substrate	Survival (%)	Mean height (cm) ²	Height increase (%) ³
I. Redwood bark : fir bark : fafard 3B : perlite (2:2:2:1)	86 a ²	5.7 a	139 a
II. Pine bark	89 a	6.0 a	152 a
III. Redwood bark : fir bark (1:1)	72 b	4.4 b	82 b
IV. Fafard 3B : perlite (2:1)	17 c	3.9 c	62 b

²Mean height is of surviving plants.

³Percentage of height increase is for surviving plants; mean height at the time of planting was 2.4 cm.

⁴Mean separation within columns by Tukey's Studentized range test at $P = 0.05$.

intermediate survival rates. Plants grown in substrates I and II exhibited a significantly higher final height and greater percentage height increase than those grown in substrates III and IV. Substrate IV retained excessive moisture, and plants incurred disease and high mortality.

Morphological differences were also noted in plants grown in the different substrates. Plants grown in substrates I and II had elongated leaves which were spirally arranged and overlapping at their basal leaves to form a well-shaped cup. The leaves in plantlets grown in substrate III and IV had thinner, less overlapping leaves and were smaller overall than with substrates I and II. The development of a "tank" morphology provides a reservoir for water and is important for survival of plants in the wild (Benzing, 1980). In subsequent experiments, plants were planted successfully into flats (Fig 1C) and then transferred into 6-inch pots in the greenhouse (Fig 1D). More mature plants retain the characteristic tank morphology necessary for water retention in this species.

Rapid seedling growth was obtained by germinating *T. eizii* seed in vitro with subsequent transfer into a pine bark medium. At 5 and 12 months after culture initiation, plants were obtained with mean heights of 20 and 75 mm, respectively. This is in contrast to Toledo-Aceves (1998), who followed the growth of *T. eizii* seedlings in an oak tree canopy in the highlands of Chiapas, Mexico. Growth rates in situ were considerably slower, with seedlings attaining mean heights of only 5 mm at 6 months, and 9 mm at 18 months after germination.

Conclusions

These are the first studies reported for the in vitro culture of *T. eizii*. Protocols were developed that provide high percentages of seed germination and rapid seedling growth. Results show that in vitro propagation via seed is a feasible means to propagate this species, which exhibits poor seed germination and high mortality in the wild. These protocols can be useful in conservation of this species as well as to produce plants for repopulation in the wild.

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