Adventitious Bud Development and Regeneration in Tillandsia Eizii

Introduction

Bromeliads are an extremely diverse group that includes 50 genera and over 2000 species that live in habitats ranging from tropical jungles to savannahs or rocky cliffs. Within this group is the genus *Tillandsia*, which alone has over 450 species (Oliva-Esteve, 2000), and includes members with very divergent morphological and physiological characteristics. One species, *Tillandsia eizii*, is highly valued as an ornamental. It has striking, pendulous inflorescences reaching 1 m in length that characteristically have colorful pink-to-red persistent involucral bracts. Because *T. eizii* is of great cultural and religious importance to Mayan populations, this has led to its over-collection in the wild. Several problematic physiological characteristics have led to declines in natural populations, including slow growth and maturation, and monocarpic flowering. In addition, seed viability is ephemeral and germination rates in the wild are low. Of further consequence to the survival of

T. eizii is the loss of suitable habitat due to deforestation and habitat destruction. These factors have positioned this species to near threatened status.

A number of bromeliad species have been clonally propagated

via in vitro culture. Some species have been propagated for their ornamental value, including *Aechmea fasciata* (Zimmer and Pieper,1976), *Aechmea fulgens* (Pierik and Sprenkels, 1989), *Cryptanthus bromelioides* var. *tricolor* (Matthews and Rao, 1982), and *Tillandsia cyanea* (Pierik and Sprenkels, 1991). Some tissue culture protocols employ organogenic systems using leaves, shoot tips, and axillary buds as explants, which are of advantage particularly when the objective is to clonally propagate selected genotypes. For example, shoot tips were used in the micropropagation of *T. cyanea* where there was an interest in preserving characteristics of individuals selected from seedling populations (Pierik and Sprenkels, 1991). Likewise,

C. bromelioides was propagated *in vitro* via lateral buds to preserve leaf color and variegation patterns (Matthews and Rao, 1982).

Other bromeliad species have been tissue cultured because of their rare or endangered status, such as *Puya tuberosa* (Varadarajan et al., 1993), *Dyckia macedoi* (Mercier and Kerbauy, 1993),

T. dyeriana (Rogers, 1984), *Vriesea fosteriana*, and *Vriesea hieroglyphica* (Mercier and Kerbauy, 1995). A number of these species are threatened due to habitat destruction and over-collection because of their ornamental value. In such cases, due to scarcity of explant material, the development of organogenic systems may be warranted even when selection of unique individuals has not been made. Reintroduction of micropropagated plants and the development of sustainable harvesting strategies (Wolf and Konings, 2001) could contribute to both plant conservation and community economic development.

The success of using clonal micropropagation to induce multiple shoots in other species of bromeliads suggests that this method may

be an effective way to propagate *T. eizii*. Our studies have shown that the *in vitro* germination of seed can be a means to efficiently produce large numbers of seedlings for conservation purposes. Seed can be axenically germinated and seedling growth is enhanced using *in vitro* culture (Pickens et al., 2003). However, culture protocols using an organogenic approach would be useful in this species at locations or with germplasms that produce limited viable seed. Furthermore, our evaluations of tissue-cultured seedlings and plants in the wild indicate that there are wide variations in plant morphology (including leaf size, arrangement, and flower charac- teristics). As superior genotypes are identified, clonal propagation for ornamental purposes becomes increasingly desirable.

The objectives of this study were to develop methods to induce adventitious bud proliferation in *T. eizii*. Culture parameters investigated included the plant growth regulator composition of the bud induction medium, the effect of explant age on bud proliferation, and the time of treatment on induction medium. The induction and development of adventitious buds was evaluated using light and scanning microscopy. The development of successful micropropagation protocols can be used to further increase the numbers of this near-threatened species to fulfill ceremonial and commercial demands and to provide plants for reintroduction into native stands.

Materials and Methods

Plant material. Seeds of *Tillandsia eizii* were obtained from a natural population growing in Chiapas, Mexico during the same fruiting season. Inflorescences were removed from plants and seeds were collected at dehiscence of the capsule.

General culture methods. Seeds or seedling explants were prepared as described in Pickens et al. (2003). *T. eizii* seeds

are small (,2 mm) and have extensive coma hairs which are outgrowths of the seeds coat that aid in seeds dispersal. Hairs were excised and seeds were initially wet by immersion in 1% NaOCl plus Tween 20 for 20 min, and then rinsed with H_2O for 5 min. Seeds were disinfested using 70% ethanol for 2 min, followed by 2.6% NaOCl þ Tween 20 for 40 min, then rinsed in sterile water two times each for 5 min. For germination, seeds were placed on a medium consisting of a modified Knudson's medium (KND) composed of Knudson's basal salts (Knudson, 1946) plus myo-inositol (0.1 g l^{21}), nicotinic acid (5 mg l^{21}), thiamine HCl (5 mg l^{21}), glycine (4 mg l^{21}), pryridoxine (5 mg l^{21}), and gelled with 4% (w/v) Gelgro (ICN Biochem., Irvine, CA, USA). Media were

adjusted to pH 5.5 prior to sterilization and autoclaved at 1208C for 20 min at 2.9 MPa. Seeds were dispensed into 25 £ 50 mm test tubes with 20 ml medium and subcultured every 4 wk into fresh medium. After 8 wk, seedlings were transferred into 5.5 £ 6 cm baby food jars containing 20 ml medium. Cultures were maintained in a growth room at 258C under a 16-h photoperiod with an illumination of 125 mmol m^{22} s^{21}.

Explant age and induction period. To evaluate the effect of explant age, seeds of *T. eizii* were initiated and grown on basal KND medium for various times (i.e. 0, 3, or 12 wk) and then transferred onto bud induction medium containing KND medium plus 1 mg l^{21} (5.37 m*M*) NAA, 0.5 mg l^{21} (2.22 m*M*) BA, and 2% sucrose. Seeds at 0 wk were transferred to bud induction medium directly after sterilization. Seedling explants that were 3 wk old had emerging leaf primordia that appeared as green protuberances. The 12-wk-old seedlings were fully emerged from the seed coat, *c.* 7 mm long and had several well-differentiated leaves. The effect of time on induction medium on explants of different ages was evaluated by comparing continuous exposure versus 30-d exposure followed by transfer

to basal medium. Cultures were evaluated for growth, presence of buds, and number of buds per explant. Each treatment was replicated four times with 6 seeds per replication.

Concentration of plant growth regulators. Seedling explants were grown for 12 wk on basal KND medium and then transferred to one of the four bud induction media containing different levels of the plant growth regulators NAA and BA. The plant growth regulator treatments were: (1) 2 mg l^{21}

(8.88 m*M*) BA þ 0.1 mg l^{21} (0.54 m*M*) NAA; (2) 2 mg l^{21} (8.88 m*M*) BA þ 0.5 mg l^{21} (2.69 m*M*) NAA; (3) 1 mg l^{21} (4.44 m*M*) BA þ 1 mg l^{21} (5.37 m*M*) NAA; and (4) 1 mg l^{21} (5.37 m*M*) NAA þ 0.5 mg l^{21} (2.22 m*M*)

BA. Cultures were evaluated for 24 wk for the presence of buds and number of buds per explant. Each treatment was replicated four times with 6 seeds per replication.

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

Light microscopy. Plant material at different bud developmental stages was fixed in a solution of 3% paraformaldehyde plus 2% glutaraldehyde in

0.2 *M* cacodylate buffer, pH of 7.2 at 48C. Tissue was dehydrated through a series of methyl cellusolve (2 £), absolute ethanol (2 £), propanol (2 £), butanol (2 £), butanol/infiltration medium (1:1), and infiltration medium, and then embedded in Historesin (Leica Instruments, Heidelberg, Federal

Republic of Germany), which consisted of hydroxyethylmethacrylate. Material was sectioned with a rotary microtome (Microm, Heidelberg, Federal Republic of Germany) into 8 – 10 mm sections and stained in an aqueous solution of

2% analine blue (w/v). Serial sections were analyzed so that accurate interpretations of structures could be made.

Scanning electron microscopy (SEM). Plant material was fixed in 4% glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.2 at 48C. Tissue was then dehydrated in an ethanol series and critical point dried through carbon dioxide using a Samdri-780 critical point drier (Tousimis Research Corporation, Rockville, MD, USA). Samples were mounted on aluminum

stubs and sputter coated with 60 nm gold/palladium in a SPI-Modulee (SPI

Supplies Division of Structure Probe Inc.) sputter coater. Samples were evaluated for morphological characteristics and origin of adventitious buds using a LEO 982 SEM/FEG scanning electron microscope (Carl Zeiss, Oberkochen, West Germany).

Results and Discussion

Induction period and age of explant. The time period that explants were exposed to induction medium did not significantly affect the percent of cultures with buds, but did impact the number of buds produced (Table 1). Explants that were placed on a 30-d induction period had significantly higher numbers of buds per explant and significantly more buds per responding culture than those given a continuous induction. Moreover, bud cultures that were continually maintained on induction medium had less developed buds with fewer differentiating into shoots. The frequency of callusing, chlorosis, and browning was also higher in continuous versus pulsed treatments. This is consistent with findings in *Tillandsia*, *Vriesea*, and *Guzmania*, where cytokinin- induced axillary shoots failed to develop unless transferred to a medium devoid of cytokinin and amended with auxin. Cytokinins caused proliferation of axillary shoots, but strongly inhibited apical growth (Mekers, 1977). Transfer of axillary shoots to a medium containing auxin was also required for

further shoot development in *Vriesea* (Mekers and Van Onsem, 1983; Mercier and Kerbauy, 1992). In several species of *Bromeliaceae*, Zimmer and Pieper (1976) found that the clusters of buds induced from stem and leaf explants would grow into plants only in a cytokinin-free medium.

Explant age did not significantly affect the percent of cultures with buds or the mean number of buds (Table 1). However, adventitious bud development was most rapid with the oldest seedling explants where bud proliferation was evident at 4 wk after placement on induction medium (data not shown); the percentage of cultures initiating buds reached maximum levels at 20 wk after initiation. In 0-wk-old and 3-wk-old explants, bud production was delayed and did not occur until 16 – 20 wk after placement on induction medium. In addition, qualitative, developmental differ- ences among the explants of different ages were observed. Buds

TABLE 1

EFFECT OF EXPLANT AGE AND INDUCTION PERIOD ON ADVENTITIOUS BUD PRODUCTION FROM *T. EIZII*. CULTURES WERE RATED 32 WK AFTER PLACEMENT ON INDUCTION MEDIUM

	Cultures with buds (%)	Mean buds per culture	Mean buds per responding culture
Induction period Continuous	23.7 a[z]	0.67 b	1.8 b
30 d	29.2 a	1.47 a	4.0 a
Explant age			
0 wk	14.6 a	0.46 a	1.9 a
3 wk	29.2 a	1.25 a	3.7 a
12 wk	35.5 a	1.48 a	3.1 a
Induction period NS	*	*	

Explant age NS NS NS

Induction £ age NS NS NS

NS, Not significant; *, significant.

[z] Mean separation in *columns* by Tukey's Studentized range (HSD) at

P # 0.05.

between induction period and age of explant for percent cultures with buds, mean buds per culture, or mean buds per responding culture (Table 1).

Concentration of plant growth regulators. The effects of different plant growth regulator combinations on bud proliferation are shown in Table 2. In general, a higher percentage of cultures produced buds when placed on media with higher concentrations of BA. Cultures on media with 2 mg l^{21} (8.88 m*M*) BA plus 0.1 mg l^{21} (0.54 m*M*) NAA had significantly higher levels of bud induction 2 – 4 times greater than in cultures with either 1 or 0.5 mg l^{21} (4.44 or 2.22 m*M*) BA. In contrast, the number of visible buds per explant was little affected by growth regulator concentrations. The response of bromeliad species to plant growth regulator formulations is variable. In *Vriesea splendens*, the maximum number of axillary shoots obtained was 1.8 shoots per explant on a medium with

0.1 mg l^{21} (0.44 m*M*) BA and 1 mg l^{21} (5.37 m*M*) NAA (Mekers,

1977). Mercier and Kerbauy (1995) compared bud induction media in two endangered bromeliads. A medium containing 2 mg l^{21} (8.88 m*M*) BA alone or in combination with 0.5 mg l^{21} (2.69 m*M*)

initiated from 12-wk-old explants expanded and developed leafy shoot-like structures (Fig. 1*A*). In contrast, buds formed on

3-wk-old seedlings had inhibited growth, and often were associated with callus proliferation. Most of these buds remained small, and few elongated into shoots (Fig. 1*B*). Seeds that were placed directly on induction medium germinated, but exhibited limited growth and became chlorotic or dark. Small adventitious buds grew, but more slowly than those induced from older explants; typically, they did not exhibit further development (Fig. 1*C*). No interaction was found

NAA produced a multiplication rate of seven shoots per seedling in

V. hieroglyphica. In *V. fosteriana*, 22.5 buds per explant were produced (Mercier and Kerbauy, 1995).

Microscopy. Light microscopic evaluations revealed that bud proliferation in *T. eizii* was adventitious in origin. Exposure of explants to induction media promoted regions of cell division. Localized mitotic activity occurred in areas within the outermost 4 – 5 subepidermal cell layers and formed rounded protrusions (Fig. 2*A*). Cells proliferated to form globular meristematic regions with meristemoid-like organization (Fig. 2*B*). Meristematic regions had cells with high cytoplasmic content. Protrusions developed on the surface of explants and had epidermal layers continuous with

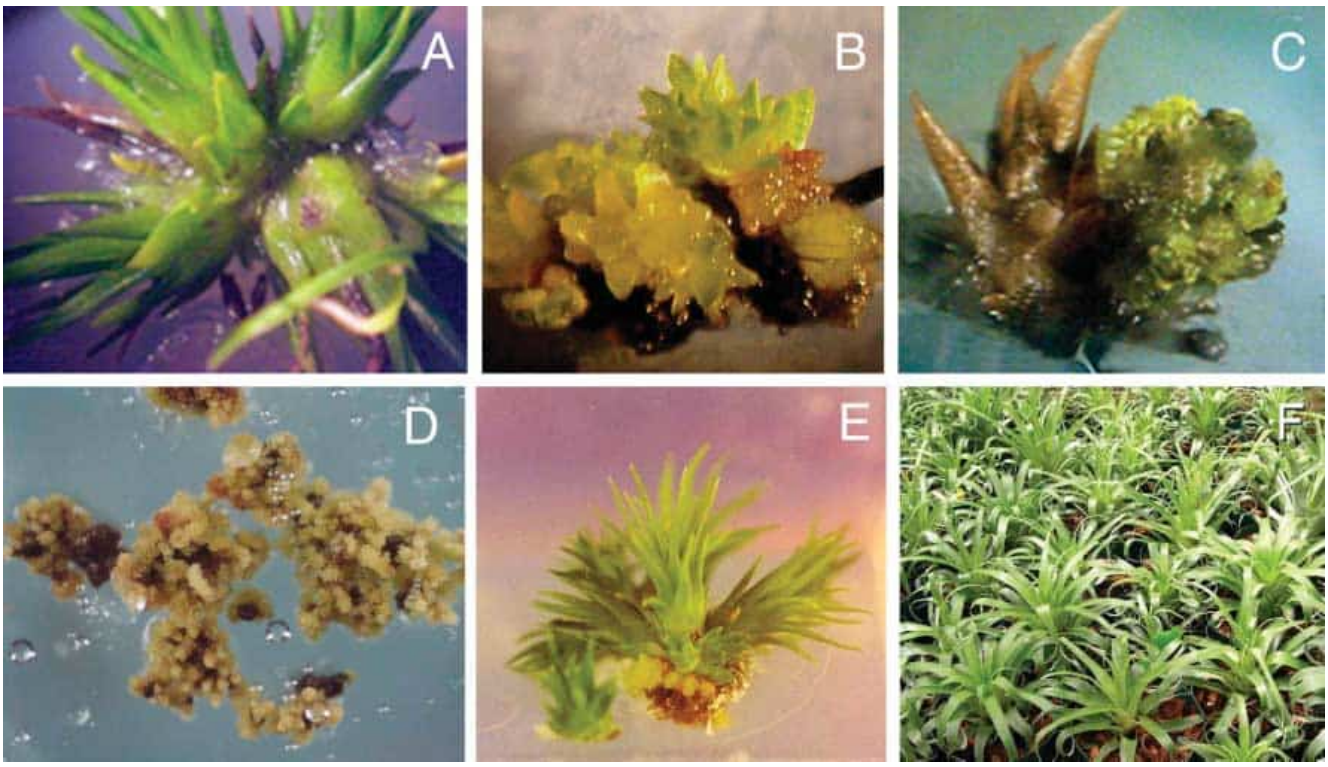

FIG. 1. *T. eizii* cultures or plants derived from explants placed on bud induction medium. *A*, Expanding shoots in a culture initiated from a 12-wk-old seedling explant. *B*, Culture initiated from a 3-wk-old seedling explant; buds exhibited inhibited growth, chlorosis, and some browning. *C*, Seed explants placed directly on induction medium produced green adventitious buds; however, buds failed to develop into shoots. *D*, Highly proliferative culture obtained after repeated subculture on basal medium; numerous adventitious buds are present. *E*, Repetitive culture with expanding shoots. *F*, Plants growing in the greenhouse.

TABLE 2

EFFECT OF PLANT GROWTH REGULATORS ON ADVENTITIOUS BUD DEVELOPMENT OF *T. EIZII*. EXPLANTS WERE 12-WK-OLD SEEDLINGS WHEN PLACED ON INDUCTION MEDIUM FOR 4 WK

Medium[z]	*n*	Cultures with adventitious buds (%)	No. buds per responding explant
I. 8.88 m*M* BA 0.54 m*M* NAA	48	40 a[y]	2.2 ab
II. 8.88 m*M* BA 2.69 m*M* NAA	72	26 ab	2.5 a
III. 4.44 m*M* BA 5.37 m*M* NAA	72	10 b	1.7 b
IV. 2.22 m*M* BA 5.37 m*M* NAA	33	20 b	2.0 b

[z] No response was seen in the absence of PGRs.

[y] Mean separations in *columns* by Duncan's multiple range test, *a* ¼ 0.5.

the original explant. More differentiated structures had a well- defined apical meristem and leaf primordia (Fig. 2*C*). More developed buds had numerous leaf primordia, leaves with defined mesophyll, epidermal regions, and differentiated vascular tissue (Fig. 2*D*).

The origin of bud proliferation in some bromeliads appears to be affected by the level of cytokinin in the bud induction media. Using an induction medium of similar concentration to our study, callus formation and adventitious bud proliferation was induced in

A. fasciata, Neoregelia carolinae var. *tricolor*, and *Guzmania* hybrids (Pierik, 1984). Lower concentrations of cytokinin (i.e., media with BA concentrations in the range of 0.2 – 0.8 mg l^{21} or 0.89 – 3.55 m*M*) induced axillary proliferation in *T. cyanea* in the absence of callus proliferation and adventitious shoots (Pierik and Sprenkels, 1991).

Higher concentrations of cytokinin are likely to produce

adventitious budding whereas lower concentrations can stimulate axillary shoots from meristematic regions already present within tissues (George, 1996).

Scanning electron micrographs of developing adventitious buds are shown in Fig. 3. Numerous adventitious buds developed on explants and bud development was asynchronous (Fig. 3*A*, *B*). Development ranged from buds that had 3 – 4 small leaf primordia to elongated shoots that had well-developed leaves with differentiated trichomes (Fig. 3*A*). Callus proliferation was observed in some explants; however, it was independent of organized meristematic regions and was not observed to form adventitious buds. Surface protrusions were evident that resembled meristematic regions seen in light microscopy sections (Fig. 3*B*). Enlargement of axillary buds

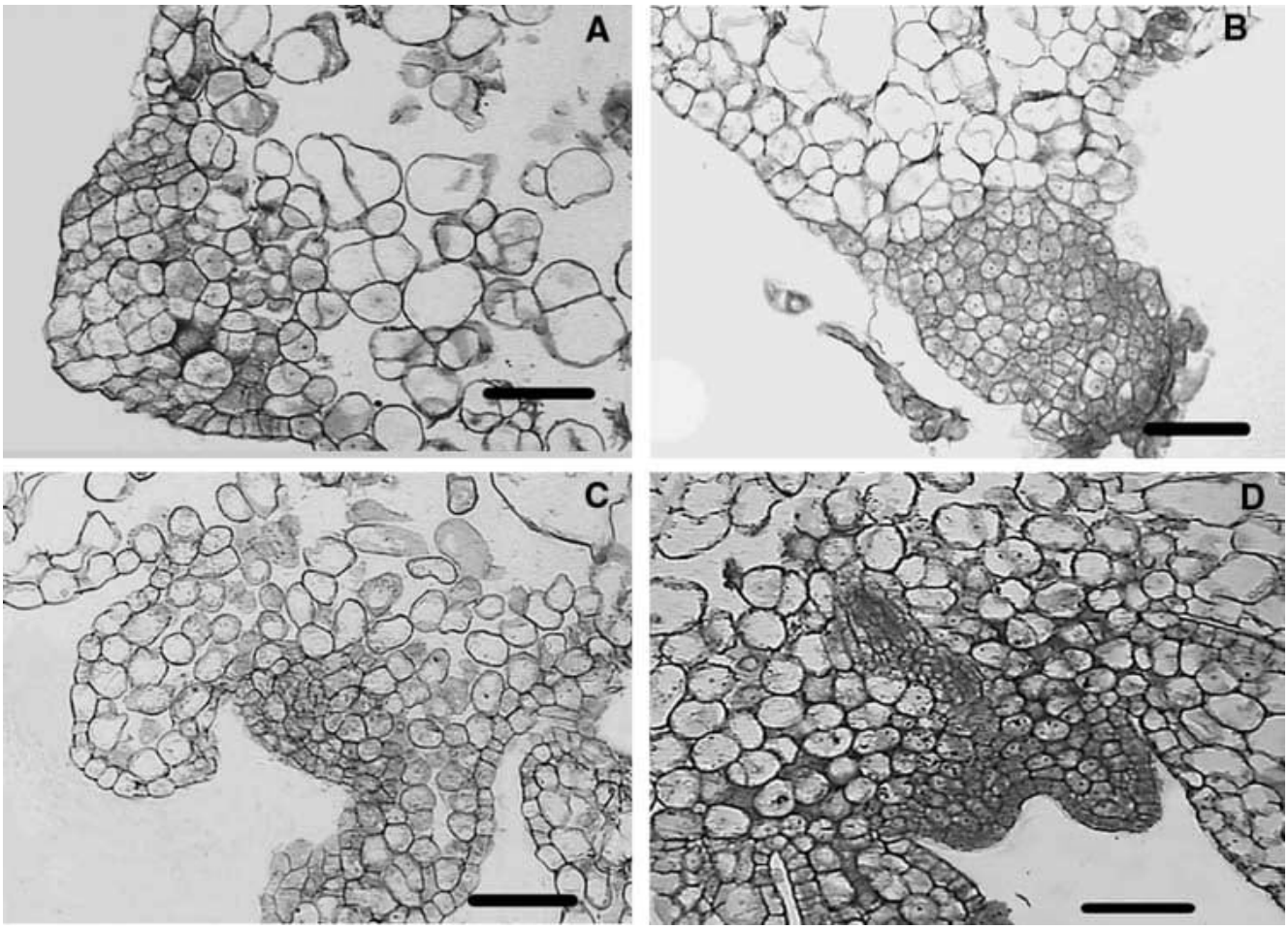

FIG. 2. Light microscopic sections of adventitious bud initiation and development. *A*, Localized mitotic activity

occurred in areas within the outermost 4 – 5 subepidermal cell layers that formed rounded protrusions. *B*, Cells proliferated and formed globular meristematic regions. *C*, An apical meristem with small leaf primordia is evident. *D*, A more developed bud with numerous leaf primordia and differentiated vascular tissue. *Scale bars* ¼ 1 mm.

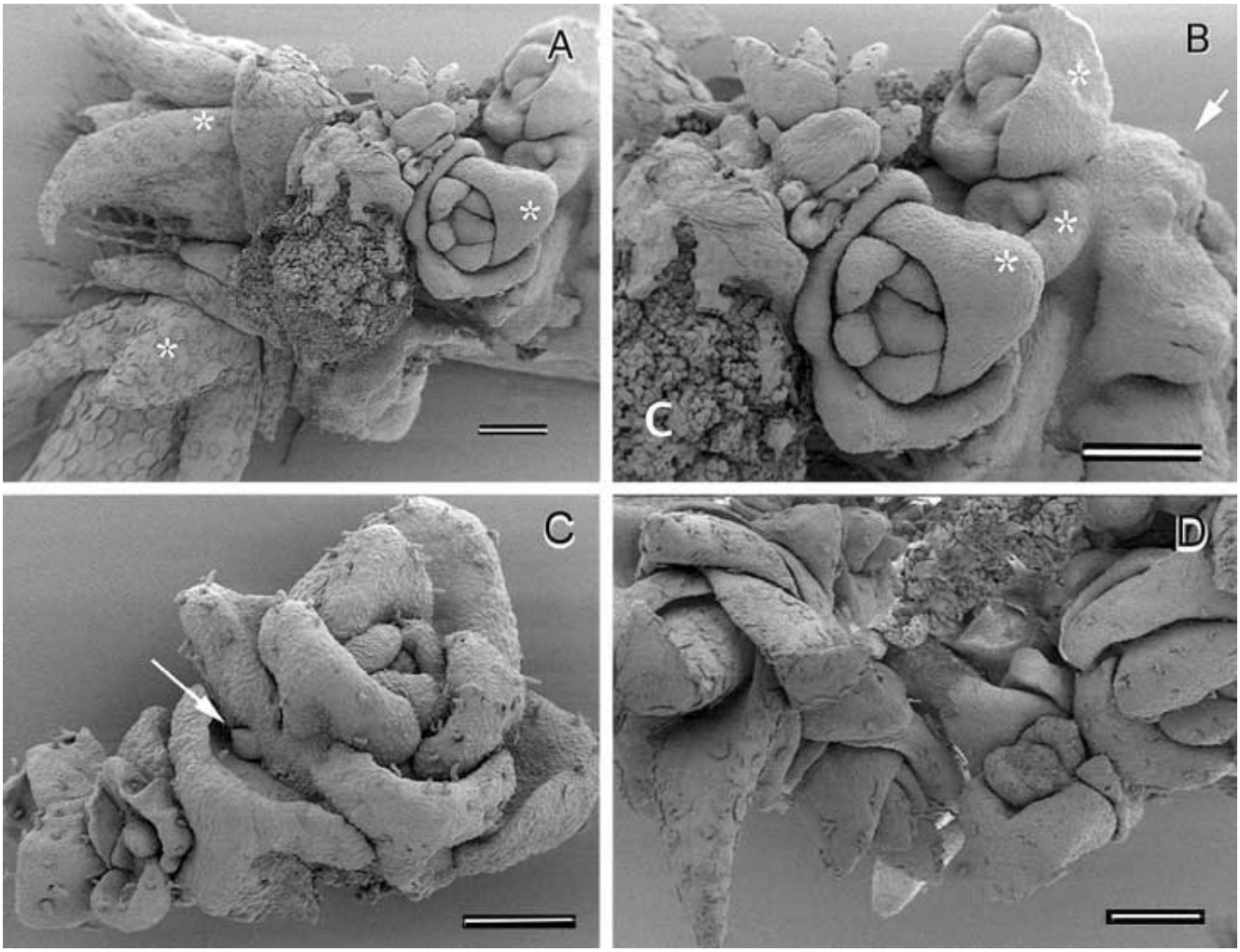

FIG. 3. Scanning electron micrographs of adventitious bud development. *A*, Buds (*) at different stages of maturity showing asynchronous development. *B*, Higher magnification revealing callus (c), adventitious buds (*), and protrusions (*arrow*) that may form into adventitious buds. *C*, Micrograph showing an axillary bud (*arrow*) at the base of an adventitious bud. *D*, Numerous adventitious buds of differing stages of development. *Scale bars* ¼ 0.5 mm.

on leaf bases of some adventitious buds was observed (Fig.

3C). SEM (Fig. 3A, D) showed that adventitious bud production was more proliferative than indicated by visible bud counts (Table 2).

Under short-term culture conditions, many of these microscopic buds failed to elongate into shoots, even after maintenance on basal media. Cytokinin effects have been observed to have a prolonged effect in *Tillandsioideae*, even after transfer onto a cytokinin-free medium (Mekers, 1977). Some cultures were maintained for extended periods (over 6 mo.) and became highly proliferative (Fig. 1D). Cultures were characterized by the presence of numerous adventitious buds, some of which would elongate, exhibit greening and expand into shoots (Fig. 1E). Following elongation, shoots could be acclimated and grown in the greenhouse (Fig. 1F). Methods to enhance bud development and shoot elongation are areas for further study.

This study reports the successful clonal propagation of *T. eizii in vitro* using an adventitious shoot culture system. Twelve-week- old seedling explants rapidly initiated adventitious buds after a 30-d induction period on shoot initiation medium. Adventitious buds were induced in 40% of the explants placed on media with 2 mg l^{21} (8.88 m*M*) BA plus 0.1 mg l^{21} (0.54 m*M*) NAA, with some cultures becoming highly prolific after repeated subculture. The results indicate that tissue culture may be used as a means to propagate this epiphytic bromeliad species that is being seriously affected by deforestation and habitat destruction. In addition, adventitious bud proliferation can provide a means to propagate superior genotypes.

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