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In-Vitro Clonal Propagation and EMS-Induced Mutagenesis in *Tillandsia fasciculata*: Optimization of Shoot Proliferation and Recovery of Chlorophyll-Deficient Phenotypes

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Abstract

Tillandsia fasciculata, a commercially valuable epiphytic bromeliad recognized for its ornamental aesthetics and air purifying attributes is increasingly threatened by overexploitation and inherently protracted growth cycles. The present study establishes a novel *in-vitro* micropropagation protocol designed to expedite propagation efficiency in both wild type and EMS-induced mutant seedlings of *T. fasciculata*. The protocol encompasses four sequential phases: aseptic seed sterilization, induction of axillary bud clusters, iterative shoot multiplication via subculturing, and establishment of vigorous, transplantable plantlets. EMS mutagenesis at differential concentrations successfully generated chlorophyll-deficient phenotypes, including variegated, chlorine, xantha, and albina mutants. Germinated seedlings were cultured on MS basal medium fortified with equimolar BA and IBA, eliciting a concentration dependent morphogenic response with optimal shoot proliferation observed at 0.1-10.0 µM BA/IBA. Variegated mutants exhibited enhanced regenerative competence, though phenotypic instability in sectorial and mericlinal chimeras constrained clonal fidelity. The protocol achieved a fourfold enhancement in seedling emergence rates with each seed yielding 4-5 plantlets and a substantial reduction in growth cycle duration from 13-15 months to 36-45 months. This *in-vitro* system provides a scalable, reproducible platform for the mass propagation of *T. fasciculata*, facilitating both sustainable commercial exploitation and ex-situ germplasm conservation, while enabling the generation of novel ornamental cultivars through targeted mutagenesis.

Keywords Micropropagation, Mutagenesis, Variegation, Organogenesis, Propagation and Conservation.

Introduction

Tillandsia species, commonly referred to as air plants, constitute one of the most taxonomically diverse genera within the epiphytic members of the Bromeliaceae family renowned for their capacity to purify indoor air (Pittendrigh 1948; Kellert *et al*., 2013). The genus *Tillandsia* represents the largest group within Bromeliaceae, comprising approximately 600 to 650 species of perennial, evergreen, and flowering plants (Brighigna *et al*., 1997; Estrella-Parra *et al*., 2019). Scientific studies have demonstrated that *Tillandsia* can absorb volatile organic compounds (VOCs) such as formaldehyde compounds during both photoperiods and scotoperiods (Li *et al*., 2024). In addition, these plants exhibit efficient carbon dioxide uptake, contributing to their classification as environmentally beneficial organisms. Due to their minimal substrate requirements, unique aesthetic appeal, and

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adaptability, *Tillandsia* species are widely employed in interior and office landscaping for purposes of air purification and ornamental enhancement (Sadhu and Naika 2021). However, most *Tillandsia* species are inherently slow growing (Chukwujekwu *et al*., 2003), and their increasing demand in the horticulture market has led to overharvesting from natural habitats, placing significant pressure on wild populations. Micropropagation technologies have been established as indispensable methodologies for the clonal propagation of genetically homogeneous plantlets, facilitating the rapid and scalable production of elite germplasm for commercial horticulture applications (Mehbub *et al*., 2022). Based on assessments compiled in the IUCN-World Conservation Monitoring Centre's red list of Threatened Plants (Walter and Gillett 1998), nearly one-quarter of Bromeliaceae species face significant risk of extinction, highlighting the urgent conservation challenges within this family. Conventional propagation techniques have proven inadequate to meet commercial needs or to support conservation efforts due to their inefficiency and low multiplication rates. The genus *Tillandsia* exhibits a broad geographic range, primarily spanning Mexico, the southeastern United States, and extensive regions of Central and South America-including countries such as Argentina, Brazil, Venezuela, Colombia, Peru, Ecuador, Bolivia, Uruguay, Paraguay, and Guatemala-as well as the West Indies and select areas of the Himalayan region. Despite this wide native distribution, *Tillandsia* species have seen minimal introduction and cultivation within India to date. To enhance its availability and conservation, there is a pressing need to adopt effective propagation strategies. Traditionally, *Tillandsia* is propagated through seed germination and vegetative division. However, large-scale propagation remains a challenge due to the absence of advanced and efficient multiplication systems in India. In this context, the present study investigates - utilization of *in-vitro* tissue culture approaches for *Tillandsia fasciculata* to establish a sustainable and efficient alternative propagation strategy.

Induced mutagenesis has been widely utilized to generate plant variants with enhanced agronomic and economic traits (Broertjes and van Harten 1988; Ahloowalia 1998). In addition to their commercial applications, certain mutants have significantly contributed to advancing our understanding of plant genetics, developmental biology, and physiological processes (Koh and Davies 2001). Propagation of *Tillandsia* species is primarily achieved through traditional techniques such as vegetative division and stem cuttings. While alternative methods like seed germination and *in-vitro* tissue culture are viable, each approach is accompanied by inherent challenges that constrain large-scale or efficient multiplication. While seed propagation exhibits a relatively high success rate, the process from germination to the development of mature, market-ready plants typically requires 2 to 3 years. Despite the rapid growth potential of vegetative explants, tissue culture remains challenging due to the difficulty of explant sterilization; *Tillandsia* leaves are densely covered with trichomes (Papini *et al*., 2010; Herppich *et al*., 2018), which can harbor microbial contaminants. Moreover lateral buds, although rapidly developing are highly susceptible to fungal contamination, often resulting in culture failure. Additionally, the high level of cellular differentiation in *Tillandsia* tissues make callus induction particularly difficult. Tissue culture methodologies for *Tillandsia* remain largely in the experimental and developmental phases. As a result, the majority of commercial producers continue to depend on conventional division-based propagation systems. Given the limited introduction and cultivation of this genus in India, the present study represents the first documented effort to initiate propagation of *Tillandsia* through seed-derived seedlings under Indian conditions. This research further delineates the integration of *in-vitro* tissue culture methodologies as a strategic framework for establishing an efficient and scalable propagation platform for *T. fasciculata*.

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A robust, reproducible, and optimized *in-vitro* regeneration protocol was established for *T. fasciculata* ‘‘providing a reliable framework for large scale propagation and experimental studies,’’ enabling accelerated seed germination kinetics and vigorous axillary meristem activation leading to sustained shoot organogenesis throughout a 17 month continuous culture regime. The protocol is delineated into four sequential phases: (1) stringent aseptic seed decontamination, (2) induction of axillary bud cluster morphogenesis, (3) exponential shoot proliferation via iterative subculturing cycles, and (4) *ex-vitro* acclimatization of physiologically competent, transplantation-ready plantlets. The meticulously optimized sterilization procedures significantly curtailed microbial colonization and endogenous contamination, thereby surmounting a critical constraint traditionally impeding the large-scale micropropagation of *Tillandsia* species and other epiphytic Bromeliaceae, and ensuring high culture viability and reproducibility.

Material and methods

Propagation via Mother Plants and Controlled Seed Production: The stock plants were cultivated under controlled conditions in a glasshouse environment, with a maximum photosynthetic photon flux (PPF) of 380-400 μmol m^{-2} s^{-1}, while the recorded thermal range extended form 25ºC (minimum) to 34ºC (maximum), while the relative humidity ranged from 62% to 95%. At the anthesis stage, flowers were manually self-pollinated to ensure genetic uniformity. Each successfully pollinated plant developed approximately 20 to 35 seed capsules, which required nearly 14 months to reach full maturity. Mature capsules exhibited a characteristic brown coloration and were harvested prior to natural dehiscence to prevent seed loss. Post-harvest, capsules were disinfected with 10% (v/v) sodium hypochlorite (commercial Clorox) for 10 minutes, rinsed thoroughly with sterile water, and dried under ambient conditions before being transferred to perforated plastic containers for controlled dehiscence. Each capsule typically harbored 100-200 viable seeds.

Ethyl Methanesulfonate (EMS) Mutagenesis Treatment: For mutagenesis, seeds with excised trichomes (coma) were enclosed within sterilized, tightly woven autoclaved muslin fabric, with the ends firmly secured to form bundles. The seed clusters underwent disinfection with 10% (v/v) NaOCl treatment for 10 minutes, followed by transfer into a laminar airflow cabinet to ensure aseptic handling. Under aseptic conditions, the bundles underwent five sequential washes using sterile distilled water, each lasting 2-3 minutes. After disinfection, the seeds were immersed in sterile distilled water inside a laminar airflow chamber for approximately 24 hours. Post-imbibition, the seeds were subjected to the following EMS treatments: (i) 0% EMS for 6 hours (negative control), (ii) 1.2% (v/v) EMS exposure for 4 hours, and (iii) 0.4% (v/v) EMS exposure for 6 hours. Each treatment was replicated four times (n = 1500 seeds for each replicate). All EMS applications were carried out in sterile 0.1 M phosphate buffer at pH 7.2 under a fume hood to ensure safe handling of the mutagen. Prior to use, all glassware along with magnetic stir bars was sterilized using 70% ethanol. For each experimental condition, the required volume of EMS was dispensed into a 30 mL aliquot of phosphate buffer contained in an aseptic beaker positioned on a magnetic stirring hot plate. The EMS-buffer solution was continuously agitated throughout the treatment duration to maintain homogeneity (Bretagne-Sagnard *et al*., 1996). The imbibed seed bundles were submerged in the EMS solution for the specified time periods. Following EMS exposure, the mutagen solution was carefully

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decanted, and each seed bundle was immediately rinsed in 30 mL of sterile distilled water under continuous agitation to ensure complete removal of residual EMS. The rinse water was replaced every 20 minutes over a 3 hour period to ensure thorough removal of residual EMS and minimize toxicity.

Plant Material and General Culture Methods: The cultivation of *Tillandsia* species was carried out under controlled glasshouse conditions and seeds were obtained from a natural population maintained at Sparrow Design Nursery, Mumbai, India. Mature inflorescences were carefully harvested, and seeds were collected immediately following capsule dehiscence. The seeds, measuring approximately 2-3 mm in length, possess prominent coma hairs that facilitate wind-mediated dispersal. Prior to sterilization, coma hairs were carefully removed. The seeds of *Tillandsia* were subjected to a preliminary sterilization protocol, beginning with a 5 minutes immersion in a 5% sodium hypochlorite (NaOCl) solution, followed by a 10 minutes treatment in a 5% Tween 20 surfactant solution (Tween 20, Sigma-Aldrich) to effectively eliminate surface impurities and microbial contaminants. Thereafter, seeds samples underwent four successive washes using sterile distilled water, each sterile distilled water in five successive washes, and each lasting 5 minutes, to eliminate residual chemical agents. Subsequently, a standardized surface sterilization procedure was implemented to ensure aseptic handling of the samples. Seeds were treated with 70% ethanol for 2-3 minutes to disrupt superficial microbial membranes. Subsequently, the material underwent three sequential washes using sterile distilled water to ensure complete removal of residual ethanol, after which it was immersed in a 0.1% (w/v) $HgCl_2$ solution supplemented with SDS (sodium dodecyl sulfate) for 4-5 minutes to enhance microbial decontamination. Final decontamination was performed through two sequential rinses in sterile distilled water, each lasting 5 minutes, to ensure the complete elimination of any residual sterilizing agents.

Culture Medium and Growth Conditions: Basal culture media were prepared following the Murashige and Skoog (MS) formulation (Murashige and Skoog 1962), comprising full-strength MS, half-strength MS, and a modified formulation consisting half-strength micronutrients in combination with full-strength micronutrients. Each culture medium was enriched with 25mg L^{-1} NaFeEDTA and supplemented with 3% (w/v) sucrose, then gelled using 0.8% (w/v) agar. The culture medium pH was carefully calibrated to 5.7-5.8 with 0.5 M NaOH, and subsequently sterilized by autoclaving at 121°C for 25 minutes under standard pressure conditions. For the initiation of *in-vitro* cultures, 12 mL aliquots of the sterilized culture medium was dispensed into sterile 100 mL borosilicate culture tubes and hermetically sealed using autoclavable polycarbonate caps. Under sterile laboratory conditions, groups of 4-5 surface sterilized *T. fasciculata* seeds were aseptically transferred onto each of the three different basal media formulations. The cultures were incubated in a controlled sterile environment at 24±2°C with a light-dark cycle of 16 hours and 8 hours, respectively, while photosynthetically active radiation at an irradiance of ~45 µmol m^{-2} s^{-1} was provided by white fluorescent tubes. All manipulations and culture maintenance procedures were carried out within a laminar airflow workstation located in a sterile culture facility to ensure aseptic integrity throughout the experimental period.

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***In-vitro* propagation of wild-type *T. fasciculata*:** In the subsequent experimental set, 5 week old *Tillandsia* seedlings were established on a germination medium formulated with modified MS basal salts. Prior to sterilization, the trichomes of each seed were carefully trimmed to enhance disinfection efficiency. Seed material underwent surface disinfection with NaOCL (1:10, v/v) for a duration of 10 minutes, followed by transfer into a laminar airflow cabinet, where it received three successive washes with sterile distilled water. Subsequently, seeds were aseptically inoculated into 25 mm x 160 mm culture tubes filled with 12 mL basal formulation. Each treatment consisted of 50 seeds (n = 50), with two seeds included per test tube. A basal formulation composed of ½ strength MS salts supplemented with vitamins (4.4 g L^{-1}) was used, to which sucrose (15 g L^{-1}) was incorporated, and the matrix was gelled with agar (8 g L^{-1}). Medium pH was calibrated to 5.7-5.8 before sterilization at 121°C under 2.9 MPa (29 psi) pressure for 25 minutes. In a parallel trial, two seeds were inoculated into 25 x 160 mm culture tubes filled with 12 mL of the same basal formulation.

T. fasciculata seeds treatments consisted of equimolar concentrations of BA (N^6-benzyladenine) and IBA (indole-3-butyric acid), tested at 0 (control), 0.01, 0.03, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.50, 1.0, 3.0, 5.0 and 10.0 µM.

***In-vitro* propagation of Irradiated and EMS-treated Mutant Seeds Material:** Following mutagenic treatment, the propagules were inoculated onto 90 x 15 mm aseptic polystyrene petri plates, each containing 25 mL of a semi-solid nutrient medium. The culture medium was prepared using half-strength MS salts with added vitamins, enriched with sucrose (15 g L^{-1}), solidified using agar (8 g L^{-1}), and supplemented with equimolar concentrations (0.15 µM) of BA and IBA. These specific concentration of BA and IBA were intentionally selected based on preliminary experiments indicating that they do not promote shoot organogenesis in non-mutagenized *T. fasciculata* seedlings. This strategy was employed to prevent early shoot proliferation in mutant populations, which could potentially interfere with the phenotypic identification of variegated variants. The pH of the plant growth medium was calibrated to 5.7-5.8 prior to sterilization through autoclaving at 121°C for a duration of 25 minutes.

Under rigorously maintained aseptic conditions, surface sterilized seeds were meticulously inoculated at a density of 10 seeds per sterile Petri dish (Dimensions: 90 mm x 15 mm), with 20 biological replicates constituted per treatment cohort, thereby establishing a cumulative experimental population of 200 seeds per treatment (n = 200). Seeds subjected to EMS-induced mutagenesis underwent identical disinfection and culture protocols; however, these were sown at a standardized density of 10 seeds per Petri dish, with 50 biological replicates allocated per treatment, culminating in an aggregate sample size of 500 seeds per treatment group (n = 500). All cultures were maintained under standardized *in-vitro* environmental conditions for a duration of two months prior to morphological and phenotypic assessment. After five weeks of initial *in-vitro* culture, germinated seedlings were aseptically transferred to sterile glass culture vessels (dimensions: 200 mL; standard tissue culture glass jars), each containing 50 mL of full-strength MS medium to facilitate continued morphogenic development. Unless otherwise specified for experimental variables such as light quality or temperature, all explants were sustained within a regulated environment inside a plant growth cabinet adjusted to 25 ± 1°C, provide with a 16 hours light regime and an incident photon density of 125 µmol m^{-2} s^{-1}, supplied through white fluorescent illumination. To maintain

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optimal physiological activity and prevent nutrient depletion, subculturing onto freshly prepared medium was performed at three week intervals throughout the duration of the experiment.

Micropropagation of Mutagenized *T. fasciculata* Seedlings: Following an initial *in-vitro* incubation of two months using MS basal formulation enriched with equimolar levels (0.20 µM) of BA together with indole-3-butyric acid, mutagenized *T. fasciculata* seedlings were subjected to phenotypic screening to identify somaclonal variants. Visual assessments focused on foliar characteristics, particularly the presence of variegation or aberrant pigmentation patterns indicative of deviations from wild-type morphology. Seedlings exhibiting presumptive mutant traits were excised separately and transferred into culture containers (25 mm x 160 mm glass tubes) filled with 12 milliliters solidified formulation composed of diluted MS macro plus micro nutrients, standard vitamin supplementation and equimolar concentrations 0.03 µM of BA and IBA. In cases where no shoot organogenesis or proliferation was observed within a subsequent three month incubation period, the explants were relocated into an optimized organogenic medium supplemented by equimolar levels (0.20 µM) of BA together with IBA to trigger morphogenic responses and facilitate clonal multiplication.

Results and Discussion

Ethyl Methanesulfonate Treatment: Three months following *in-vitro* culture initiation, the surviving *T. fasciculata* seedlings from each EMS treatment group were assessed to determine lethality rates (Fig. 1). Phenotypic classification was based on visible foliar traits; seedlings were categorized as variegated if they exhibited one or more leaves with variegation, regardless of whether this characteristic was maintained in subsequent foliage or lateral shoots. The mean viability of seeds in the untreated control group was approximately 20%, a reduction attributed to seed senescence, as the seed lot used was approximately 15 months old. For EMS treated groups, approximately 80 viable seedlings were obtained from an initial population of 500 seeds, and this value was employed as the standard denominator for percentage calculations. Each EMS treatment was replicated four times, yielding a total of 320 viable seedlings per treatment group. Across all EMS treatments, a total of 120 viable seedlings ware recovered. Despite the inherently low seed viability, the results clearly demonstrated the mutagenic potential of EMS, as evidenced by phenotypic alterations and differential survival rates among treated seedlings. It is also plausible that additional EMS induced seedlings may have germinated but failed to survive until the two month evaluation point, thereby contributing to an underestimation of the true extent of EMS-induced lethality and mutagenesis.

For the *in-vitro* propagation of mutant seedlings, EMS-induced protocols broadly analogous to those described by (Koh and Davies 2001) were employed. However, the present investigation differs from the approach of (Koh and Davies 2001) in that it applied a distinct and systematically varied range of EMS concentrations. This methodological refinement not only generated diverse survival responses but also produced novel phenotypic variants that had not been reported earlier. Consequently, the findings of this study advance current understanding by showing that concentration-dependent optimization of EMS treatments can substantially enhance mutagenesis

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efficiency in *T. fasciculata*. Thus, this work establishes both the novelty and the incremental value of the present research compared to earlier reports.

Treatment Group	Seed Population	Seedlings (at 2 months)	Seed Viability%	Seedlings Identified	Estimated Lethality%
Control	500	100	20%	0	80% (seed senescence)
EMS treated (Replicate 1)	500	80	16%	Data pooled	84%
EMS treated (Replicate 2)	500	80	16%	Data pooled	84%
EMS treated (Replicate 3)	500	80	16%	Data pooled	84%
EMS treated (Replicate 4)	500	80	16%	Data pooled	84%
Total (EMS treated)	2500	320	16%	120 (Mutant Phenotypes)	-

Table 1. Summary of Seed Viability, Phenotypic Classification, and Lethality Rates Post-EMS Treatment in *T. fasciculata*

***In-vitro* Regeneration and Multiplication of Wild-Type *T. fasciculata* Seedling:** Considering the naturally slow *in-vitro* growth of *T. fasciculata* seedlings, periodic subculturing onto fresh medium was deemed unnecessary over the course of the 6-8 month experimental period. No indication of shoot multiplication appeared in cultures enriched with equimolar doses of BA together with IBA at control (0), 0.01, 0.03, 0.05, 0.10, and 0.15 µM (Table 2). In contrast, explants maintained on media containing equimolar concentrations of 0.20 and 0.25 µM BA and IBA exhibited the initiation of multiple adventitious shoot primordia in approximately 40% to 55% of explant, indicating a threshold morphogenic response to auxin-cytokinin synergism. Increasing phytohormone concentrations to 0.30 and 0.50 µM induced the formation of compact, spheroidal shoot clumps in 60% and 75% of cultured explants, respectively. An additional elevation in the levels of benzyladenine together with indole-3-butyric acid to 1, 3, 5, and 10 µM induced a uniform organogenic response across 80-100% of the explants, characterized by the proliferation of spherical shoot aggregates whose dimensional expansion was directly proportional to the escalating phytohormonal gradient (Table 2). These observations delineate a clear dose-dependent morphogenetic trajectory modulated by auxin-cytokinin homeostasis. To evaluate the regenerative capacity and stability of induced shoot clumps, two representative shoot clusters from each treatment group 0.1-10.0 µM BA/IBA, were excised, bisected longitudinally, and subcultured onto hormone-free, half-strength MS basal medium. All resulting explants successfully regenerated into morphologically normal shoot clumps, thereby confirming the proliferative potential of the initial organogenic response under auxin-cytokinin synergism. Although spontaneous rhizogenesis was observed during both *in-vitro* cultivation and subsequent *ex-vitro*

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establishment, the primary objective of this study was to optimize induction and proliferation within shoot aggregates as a foundation for large-scale micropropagation of *T. fasciculata*.

BA + IBA concentration (µM)	**Shoot Proliferation Response**	**Shoot Initiation %**	**Morphogenetic Response Description**
0 (Control)	No Response	0%	No shoot proliferation observed
0.01	No Response	0%	No shoot proliferation observed
0.03	No Response	0%	No shoot proliferation observed
0.05	No Response	0%	No shoot proliferation observed
0.10	No Response	0%	No shoot proliferation observed
0.15	No Response	0%	No shoot proliferation observed
0.20	Initiation of adventitious shoots	~ 40%	Multiple adventitious shoot primordia initiated
0.25	Initiation of adventitious shoots	~ 55%	Multiple adventitious shoot primordia initiated
0.30	Formation of shoot clumps	~ 60%	Compact, spheroidal shoot clumps
0.50	Formation of shoot clumps	~ 75%	Compact, spheroidal shoot clumps
1.00	Uniform organogenic response	~ 80%	Uniform organogenic response
3.00	Uniform organogenic response	~ 87%	Uniform organogenic response
5.00	High Frequency Shoot Clumps	~ 90%	Spherical shoot aggregates proliferated; size increases with concentration
10.0	High Frequency Shoot Clumps	90-100%	Spherical shoot aggregates proliferated; size increases with concentration

Table 2. Effect of Equimolar BA and IBA Concentrations on Shoot Proliferation in *T. fasciculata* Seed-Derived Explants

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Figure 1. Developmental Progression of *T. fasciculata* Seedlings during *In-vitro* Regeneration and Multiplication under Auxin-Cytokinin Enriched Conditions

Determination of Shoot-initiation Percentage (General Methodology): Shoot-initiation percentages were quantified by direct enumeration of responsive seed-derived explants cultured on MS basal medium (Murashige and Skoog 1962) supplemented with equimolar concentrations of BA and IBA, in line with established micropropagation protocols (George *et al*., 2008; Thorpe 2007). For each hormonal treatment a fixed cohort of fifty explants (n = 50; two seeds per culture tube) was inoculated and maintained under controlled *in-vitro* conditions for 6-8 months, following procedures comparable to those reported in tissue culture studies of bromeliads and related monocots (Pierik 1997). Cultures were inspected at defined intervals using both macroscopic observation and, where required, low-magnification stereomicroscopy (Koh and Davies 2001). An explant was scored as responsive when one or more visible adventitious shoot primordia or compact shoot clumps were present; merely increased tissue swelling without organogenic differentiation was not scored as initiation (Thorpe 2007). The percentage of responsive explants for each treatment was calculated as:

$$\text{Shoot initiation (\%)} = \frac{\text{Number of explants exhibiting shoot initiation}}{\text{Total number of explants inoculated}} \text{ x } 100$$

For example, if 25 of 50 inoculated explants produced adventitious shoots, the shoot-initiation percentage was calculated as $\frac{25}{50}$ x 100 = 50%. This standardized scoring criterion was applied uniformly across all hormone concentrations; the derived percentages are presented in Table 2 and are visually corroborated by the developmental stages illustrated in Figure 1, thereby permitting construction of the dose-response relationship reported in the Results.

***In-vitro* Propagation of EMS-Induced *T. fasciculata* Mutant Seedlings:** Following EMS-induced mutagenesis, a total of 250 variegated seedlings (8.0% of viable seeds) were recovered, alongside 15 chlorina (0.8%), 11 xantha

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(0.6%), 7 albina (0.4%), and 3 sectorial variegated seedlings (0.2%). These phenotypic variants reflect the mutagenic spectrum induced by EMS, targeting chlorophyll biosynthesis pathways. The *in-vitro* regenerative responses of these mutants varied significantly when cultured on auxin-cytokinin enriched media. Variegated seedlings exhibited a shoot proliferation rate of 82% and shoot clump formation in 68% of explants. Shoot proliferation was moderate in chlorine 66.7% and xantha 63.6% mutants, whereas albino 42.9% and sectorial 33.3% variants exhibited comparatively lower regeneration potential. Shoot clump formation was most prominent in variegated mutants, indicating their higher morphogenic potential. The overall data confirm that EMS-induced variegated mutants of *T. fasciculata* possess enhanced regenerative competence, offering a reliable source for clonal propagation and potential ornamental cultivar development.

Mutant Phenotype	**No. of Seedlings recovered**	**Viable Seeds%**	**Shoot Proliferation%**	**Shoot Clump%**	**Remarks**
Variegated	250	8.0%	82.0%	68.0%	Highest morphogenic potential
Chlorina	15	0.8%	66.7%	Data not specified	Moderate regeneration capacity
Xantha	11	0.6%	63.6%	Data not specified	Moderate regeneration capacity
Albina	7	0.4%	42.9%	Data not specified	Limited regeneration capacity
Sectorial Variegated Chimeras	3	0.2%	33.3%	Data not specified	Very low regeneration capacity

Table 3. *In-vitro* Regenerative Responses of EMS-Induced Mutants of *T. fasciculata* on Auxin-Cytokinin Enriched Medium

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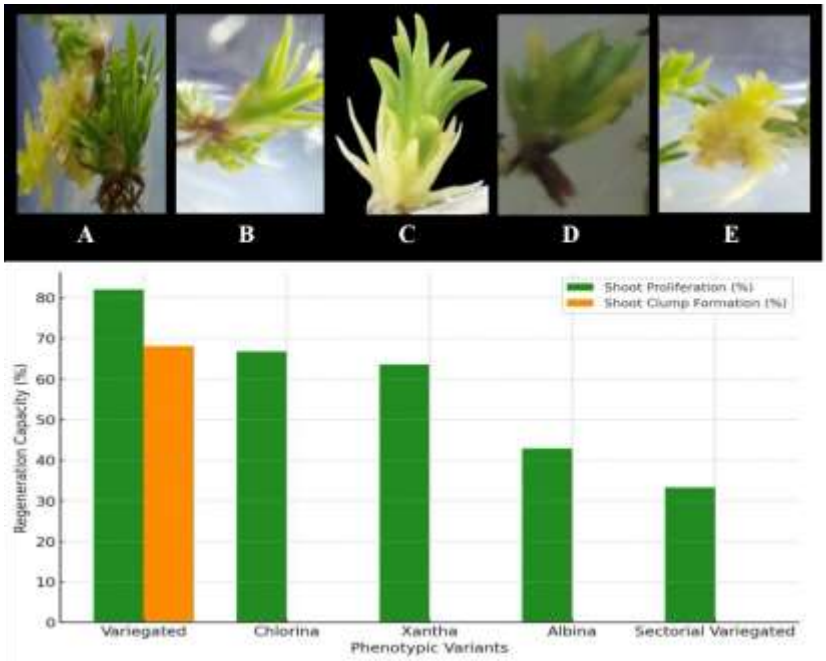

Figure 2 *In-vitro* Regeneration Responses and Capacities of EMS-Induced Chlorophyll-Deficient Mutant Seedlings of *T. fasciculata*. (A) Variegated mutants showing the highest shoot proliferation capacity (82%); (B) Chlorina mutants exhibiting moderate regeneration (66.7%); (C) Xantha mutants with reduced chlorophyll content and moderate regeneration (63.6%); (D) Albina mutants demonstrating poor survival and regeneration capacity (42.9%); (E) Sectorial variegated chimeras showing the lowest regeneration capacity (33.3%)

Graph 1. A Comparative Analysis of Shoot Proliferation and Clump Formation

***In-vitro* Clonal Propagation of Wild-Type *T. fasciculata* Seedlings:** Zygotic-origin tissues from *T. fasciculata* displayed vigorous organogenic activity, evident through the development of compact spherical shoot aggregates under culture conditions employing diluted MS formulation enriched with BA and IBA were applied in equimolar amounts at concentration ranging from 0.1 to 10.0 µM. The shoot proliferation efficiency and temporal responsiveness achieved under these conditions were comparable to, and in several instanced exceeded, previously established *in-vitro* regeneration protocols for bromeliads belonging to the subfamily *Tillandsioideae* derived from seed explants. Notably, half strength MS formulation enriched with 1 to 10 micromolar (µM) equimolar BA and IBA consistently induced the development of more than 20 shoots per seedling over an 8 month culture period, highlighting the medium's efficacy in promoting high frequency shoot multiplication. Furthermore, medium containing 1.0 to 10.0 µM equimolar BA and IBA also demonstrated substantial morphogenic competence, rendering it a cost effective and efficient alternative for the large scale clonal propagation of *T. fasciculata* seedlings. These findings underscore the versatility and robustness of the optimized hormonal regime in facilitating reliable micropropagation of this slow growing epiphytic species.

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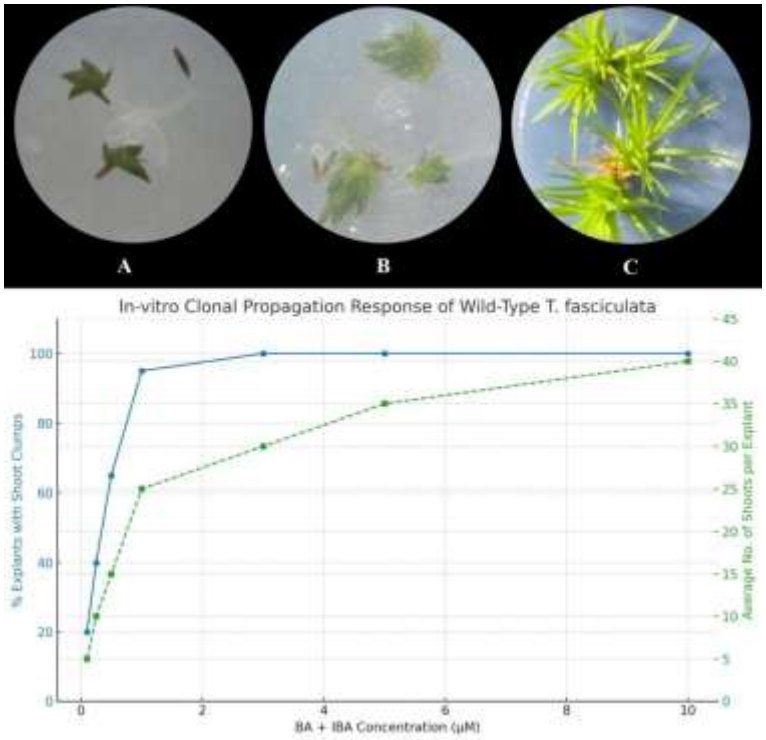

Figure 3 Efficient *In-vitro* Clonal Propagation of Wild-type *T. fasciculata* via Organogenic Shoot Clump Formation with BA + IBA: (A) Control seedlings showing no morphogenic response 0 µM (0%); (B) Moderate concentration (0.25-0.50 µM BA+IBA) inducing compact shoot primordia (~55-75%), (C) High concentration (1.0-10.0 µM BA+IBA) producing large shoot clumps with high frequency proliferation (~80-100%)

Graph 2. Influence of BA with IBA Concentrations on Shoot Multiplication plus Clump Formation

***In-vitro* Clonal Propagation of Chlorophyll-Deficient Mutants in *T. fasciculata*:** Chlorophyll-deficient mutant seedlings of *T. fasciculata* were considered to exhibit successful clonal propagation when the explants gave rise to five or more adventitious shoots under *in-vitro* conditions. Although shoot induction was achieved in certain variegated phenotypes, the hallmark variegation failed to manifest in the regenerated propagules. This phenotypic instability is likely attributable to the sectorial or mericlinal chimeric composition of the primary explants, which inherently limits the transmission fidelity of cytological mosaics during meristematic regeneration. Due to the infrequent emergence of chlorophyll-deficient individuals across experimental treatments, all resultant phenotypic classes within each treatment condition were consolidated and analyzed as discrete experimental units to ensure statistical robustness and mitigate the confounding effects of low-frequency variants. The mutant seedlings demonstrated pronounced growth retardation, with each explant typically producing fewer than 8-10 shoots during controlled *in-vitro* conditions. Seedlings maintained in formulations enriched with 1.0 µM BA and IBA retained a morphologically stable phenotype, implying that a moderate escalation in the concentrations of these phytohormones could potentially enhance morphogenic responsiveness and shoot proliferation.

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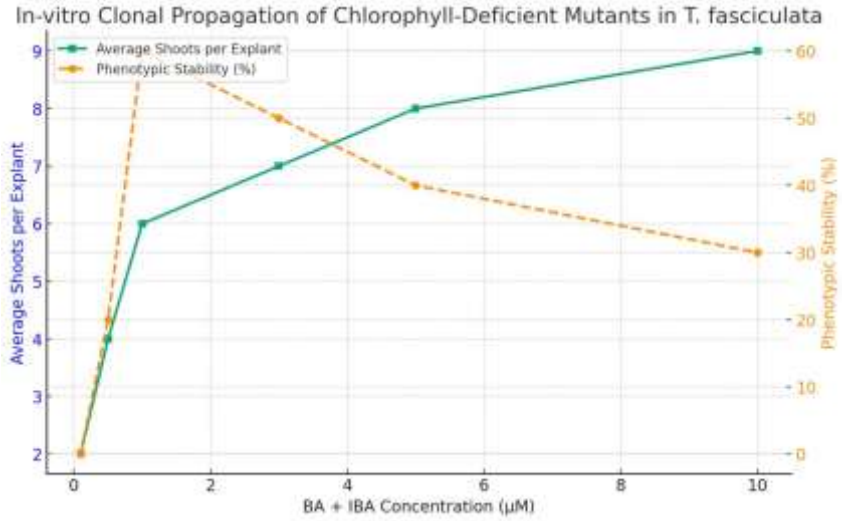

Graph 3. Hormonal Modulation of Shoot Proliferation and Phenotypic Stability in Chlorophyll-Deficient *T. fasciculata* mutants under *In-vitro* Conditions

The present investigation demonstrates the feasibility of generating variegated phenotypes in *T. fasciculata* through seed-based mutagenesis. Given the prolific seed production characteristic or many *Tillandsia* species, the application of mutagenic treatments to seeds represents a practical and scalable approach for expanding phenotypic diversity within this genus for ornamental purposes. The induction of stable variegation in *Tillandsia* species via controlled mutagenesis warrants further investigation, especially considering the rarity of such phenotypes under natural conditions and the increasing market demand for variegated bromeliads. A principal aim of this investigation was to enhance the production efficiency of *T. fasciculata* through optimized *in-vitro* propagation strategies. Although a substantial number of plantlets were successfully regenerated, the majority of these regenerants, manifested as phenotypically unstable sectorial and mericlinal chimeras. Such chimeric phenotypes, while capable of being maintained through the development of lateral shoots, present inherent limitations in the establishment of genetically stable and horticulturally desirable variegated lines. Consequently, the attainment of stable variegated cultivars would necessitate successive cycles of vegetative propagation coupled with stringent phenotypic selection across multiple clonal generations to ensure the fixation of desirable traits.

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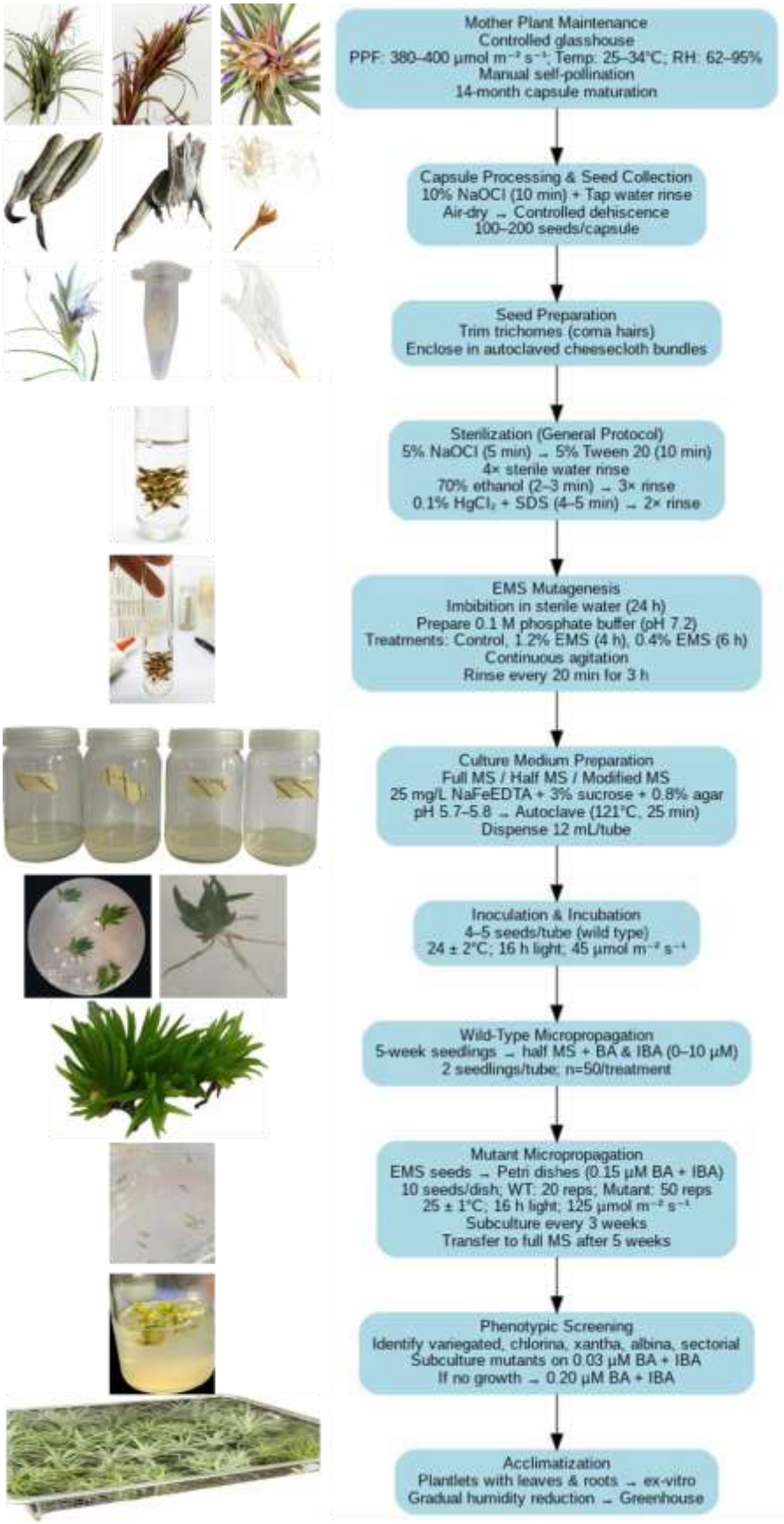

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Figure 4. Stepwise Protocol for EMS-Induced Mutagenesis and *In-vitro* Propagation of *T. fasciculata* (The scheme highlights major stages – seed sterilization, EMS exposure, inoculation on MS medium, identification of chlorophyll-deficient mutants, and their subsequent clonal propagation on hormone-enriched medium - demonstrating the integration of mutagenesis with tissue culture to enhance phenotypic diversity and regeneration efficiency)

Conclusion

The present investigation establishes a meticulously optimized and reproducible *in-vitro* propagation system for *T. fasciculata*, synergistically integrating classical micropropagation methodologies with EMS-induced chemical mutagenesis to augment clonal propagation efficiency and expand phenotypic variability. Despite the inherent ontogenetic recalcitrance and extended juvenile phase of seed-derived explants, the exogenous application of phytohormones, specifically within an optimized concentration matrix of 0.10-10.0 µM BA in combination with IBA, elicited a pronounced morphogenic response characterized by the proliferation of compact, meristematically active shoot clumps. This direct organogenetic pathway effectively circumvented the temporal constraints of natural growth cycles, facilitating high frequency shoot multiplication with enhanced propagation throughput. The application of EMS mutagenesis induced a spectrum of chlorophyll-deficient phenotypes, including variegated, chlorine, xanthan, and albina morphotypes. Among these, variegated lines exhibited superior regenerative competence and shoot proliferation dynamics, rendering them particularly valuable as elite ornamental cultivars. Nonetheless, the phenotypic instability observed in sectorial and mericlinal chimeras underscores the necessity for subsequent selection cycles aimed at achieving genetic fixation and phenotypic uniformity.

Given the limited representation of *Tillandsia* germplasm within Indian floricultural systems, the propagation protocol elucidated herein provides a scalable and commercially viable platform for ex-situ conservation, large scale production and the generation of novel phenotypic variants through targeted mutagenesis. Furthermore, this study substantiates the strategic utility of EMS-mediated chemical mutagenesis as a potent tool for expanding the ornamental genetic repertoire and fostering trait innovation within *Tillandsia* plant breeding programs.

Future Prospects

The successful establishment of a scalable and genetically tractable *in-vitro* regeneration system for *T. fasciculata* constitutes a pivotal advancement, providing a versatile platform for integrative biotechnological interventions encompassing genetic improvement, germplasm conservation, and industrial-scale propagation. Future research imperatives should be strategically aligned with the following critical domains:

1. **Comprehensive Molecular Analysis using high-throughput Approaches:** Such as whole-genome resequencing, transcriptome profiling, and epigenomic mapping is crucial for elucidating the mutation patterns and regulatory alterations induced by EMS treatment.
2. **Optimizing *Ex-Vitro* Physiological Acclimatization Protocols:** Through precise microenvironmental control and incorporation of arbuscular mycorrhizal fungi (AMF) can markedly improve plant physiological resilience and transplant success.

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3. **Comprehensive Metabolomic and Phytochemical Profiling of Mutant Lines:** Advanced metabolomic platforms (LC-MS/MS, GC-MS) should be harnessed to characterize the secondary metabolite spectra of EMS-derived variants, facilitating the discovery of bioactive molecules with potential uses in pharmacognosy, nutraceutical formulation, and phytoremediation.
4. **CRISPR/Cas-Mediated Genome Engineering and Synthetic Biology Integration:** Precision genome editing tools, coupled with synthetic regulatory networks, will enable targeted modulation of key metabolic and developmental pathways, fostering the generation of cultivars with enhanced pigment profiles, stress resilience, and architectural novelty.
5. **Development of Autoluminescent Genotypes via Synthetic Gene Circuitry:** The stable integration of fungal bioluminescence operons (e.g., lux, luz, hispS) through transgenic or cisgenic methodologies holds transformative potential for engineering self-sustained bioluminescent *Tillandsia* cultivars offering distinctive aesthetic attributes and high commercial promise and significant market potential.
6. **Temporary Immersion Bioreactor (TIB) Driven Mass Propagation:** The transition from semi-solid culture systems to automated TIB platforms will facilitate high-throughput, cost efficient multiplication of elite genotypes, bridging the translational gap between laboratory innovations and industrial scale horticultural production.

The synergistic integration of micropropagation, chemical mutagenesis, precision genome editing, and synthetic biology paradigms will catalyze the repositioning of *T. fasciculata* as a next generation ornamental and functional horticultural entity of substantial commercial, ecological, and scientific significance. Concurrently, progressive advancements in plant tissue culture protocols, precision genetic engineering, and integrative molecular breeding approaches have unlocked substantial opportunities for the development of novel cultivars endowed with superior ornamental phenotypes, including augmented floral architecture, prolonged post-harvest longevity, and distinct chromatic variations (Partap *et al*., 2023). Collectively, these technological innovations constitute a robust framework that underpins the accelerated evolution of *Tillandsia* species as a premium ornamental genus with significant economic and aesthetic valuation.

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References

Ahloowalia BS (1998) In-vitro Techniques and Mutagenesis for the Improvement of Vegetatively Propagated Plants. Somaclonal Variation and Induced Mutations in Crop Improvement 293-309. https://link.springer.com/chapter/10.1007/978-94-015-9125-6_15

10.48047/jocaaa.2024.33.05.53

Bretagne-Sagnard B, Fouilloux G, Chupeau Y (1996) Induced albina mutations as a tool for genetic analysis and cell biology in flax (Linum usitatissimum). Journal of Experimental Botany 47:189-194. https://doi.org/10.1093/jxb/47.2.189

Brighigna L, Ravanelli M, Minelli A, Ercoli L (1997) The use of an epiphyte (Tillandsia caput-medusae morren) as bioindicator of air pollution in Costa Rica. Science of The Total Environment 198(2):175-180. https://doi.org/10.1016/S0048-9697(97)05447-8

Broertjes C, ven Harten AM (1988) Applied Mutation Breeding for Vegetatively Propagated Crops. Elsevier Science Publishers ISBN 0-444-42786-4 (Vol. 12). https://api.pageplace.de/preview/DT0400.9781483289991_A23885015/preview-9781483289991_A23885015.pdf

Chukwujekwu JC, Staden J. van, Bornman CH (2003) Tissue culture enhances the propagation potential of some Tillandsioideae. South African Journal of Botany 69(2):214-216. https://doi.org/10.1016/S0254-6299(15)30350-1

Estrella-Parra E, Flores-Cruz M, Blancas-Flores G, Koch SD, Alarcon-Aguilar FJ (2019) The Tillandsia genus: history, uses, chemistry, and biological activity. Blacpma 18(3):239-264. https://www.researchgate.net/publication/336567279

George EF, Hall MA, De Klerk GJ (2008) Plant Propagation by Tissue Culture (3rd ed.). Springer 1-28. https://link.springer.com/chapter/10.1007/978-1-4020-5005-3_1

Herppich WB, Martin CE, Totzke C, Manke I, Kardjilov N (2018) External water transport is more important than vascular transport in the extreme atmospheric epiphyte Tillandsia usneoides (Spanish moss). Plant Cell Environment 42(5):1645-1656. https://doi.org/10.1111/pce.13496

Kellert SR, Heerwagen J, Mador M (2013) Biophilic Design: The Theory, Science and Practice of Bringing Buildings to Life. Wiley ISBN: 978-0-470-16334-4 1-400. https://www.wiley.com/en-us/Biophilic+Design%3A+The+Theory%2C+Science+and+Practice+of+Bringing+Buildings+to+Life-p-9780470163344

Koh YC, Davies FT (2001) Mutagenesis and in vitro culture of Tillandsia fasciculata Swartz var. fasciculata (Bromeliaceae). SCIENTIA HORTICULTURAE 87(3):225-240. http://dx.doi.org/10.1016/S0304-4238(00)00166-7

Li J, Pang S, Tu Q, Li Y, Chen S, Lin S, Zhong J (2024) Endophyte-assisted non-host plant Tillandsia brachycaulos enhance indoor formaldehyde removal. Journal of Biotechnology 393:149-160. https://doi.org/10.1016/j.jbiotec.2024.07.022

Mehbub H, Akter A, Arjina Akter M, Mandal MSH, Hoque MA, Tuleja M, Mehraj H (2022) Tissue Culture in Ornamentals: Cultivation Factors, Propagation Techniques, and Its Application. Plants 11(23)3208:1-33. https://doi.org/10.3390/plants11233208

10.48047/jocaaa.2024.33.05.53

Murashige T, Skoog F (1962) A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Culture. Physiologia Plantarum 15(3):473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Papini A, Tani G, Falco PD, Brighigna L (2010) The ultrastructure of the development of Tillandsia (Bromeliaceae) trichome. Flora – Morphology, Distribution, Functional Ecology of Plants 205(2):94-100. https://doi.org/10.1016/j.flora.2009.02.001

Partap M, Verma V, Thakur M, Bhargava B (2023) Designing of future ornamental crops: a biotechnological driven perspective. Horticulture Research 10(11):uhad192. https://doi.org/10.1093/hr/uhad192

Pierik RLM (1997) In Vitro Culture of Higher Plants (2nd ed.). Springer 1-348. https://www.google.co.in/books/edition/In_Vitro_Culture_of_Higher_Plants/eUWe9894KzwC?hl=en&gbpv=1&dq=Pierik,+R.+L.+M.+(1997).+In+Vitro+Culture+of+Higher+Plants+(2nd+ed.).+Springer.&pg=PA1&printsec=frontcover

Pittendrigh CS (1948) The bromeliad-Anopheles-malaria complex in Trinided; the bromeliad flora. Evolution (1):58-89. https://doi.org/10.1111/j.1558-5646.1948.tb02732.x

Sadhu DS, Naika MBN (2021) Nature's Own Miracle: Air Plants an Emerging Ornamental Wonder. Research Biotica 3(1):69-73. https://doi.org/10.54083/ResBio/3.1.2021.69-73

Thorpe TA (2007) History of plant tissue culture. Molecular Biotechnology 37(2):169–180. https://doi.org/10.1007/s12033-007-0031-3

Walter KS, Gillett HJ (1998) 1997 IUCN Red List of Threatened Plants. The IUCN Species Survival Commission 1-934. https://portals.iucn.org/library/sites/library/files/documents/RL-1997-001.pdf