Journal of Applied and Natural Science
18(1), 73 - 79 (2026)
ISSN : 0974-9411 (Print), 2231-5209 (Online)
journals.ansfoundation.org

Research Article

Enhancing the survival of cryptogams through *ex-situ* acclimatization under controlled conditions

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Article Info
https://doi.org/10.31018/jans.v18i1.7080
Received: August 09, 2025
Revised: January 28, 2026
Accepted: February 08, 2026

How to Cite
Samanta, S. *et al.* (2026). Enhancing the survival of cryptogams through *ex-situ* acclimatization under controlled conditions. *Journal of Applied and Natural Science*, 18(1), 73 - 79. https://doi.org/10.31018/jans.v18i1.7080

Abstract
The hilly regions of West Bengal are recognized as rich repositories of cryptogams, but they face threats from pollution, overexploitation, and other anthropogenic factors. Many cryptogams, which are generally used as laboratory specimens, are also facing threats in their natural habitats. An attempt has been made to cultivate various types of cryptogams in an artificial system, thereby supporting research and experimentation in botanical sciences. Nutrient supplementation for the cultivated specimens has been formulated based on the NPK ratio of the *in-situ* soil sample, characterized by low nitrogen, moderate phosphate, and elevated potassium levels (3:1:6). The system provides ideal growth conditions (light intensity 150-170 lux, humidity 85-95%, temperature 24-28ºC) for cryptogams. The macro- and micro-morphological and dry-weight assessment of system-grown plants exhibit attributes indicative of healthy physiological development. The moisture content of each selected species increased by 0.304 g, 0.047g, 0.05 g and 0.248 g per gram weight in *Equisetum* sp., *Selaginella* sp., *Funaria* sp. and *Lunaria* sp. respectively. The present study contributes significantly to the preservation of biodiversity and the safeguarding of endangered flora.

Keywords: Acclimatization, Conservation, Control environment, Cryptogams, Growth chamber

INTRODUCTION

Acclimatization is a physiological process by which an organism adapts to survive in a novel environment that differs from its native habitat (Mazess, 1975; Peck, 2011; Khlebovich, 2017). Environmental factors such as rainfall, humidity, and temperature play a critical role in facilitating the adjustment of organisms to new conditions (Parkhurst and Loucks, 1972; Bhatt *et al*. 2024). Adaptation and acclimatization are interconnected processes, with adaptation often occurring as a result of acclimatization (Billings, 1952; Mallet, 2023). Physiological changes may be required for survival in a different environment (Andrews, 1996; Wani *et al*., 2022), and in some cases, an organism's physiological mechanisms can overcome the challenges posed by envi-

ronmental factors. This process enables the introduction of new species into distinct or extreme climates, where they may exhibit altered morphological features as part of their acclimatization (Carey, 2005; Jardeleza *et al*., 2022).

Organisms capable of acclimatizing to new environments often demonstrate vigorous growth (Preece and Sutter, 1991; Mishra, 2022). However, extreme weather conditions can sometimes lead to mortality in susceptible species, as not all organisms are equally equipped to acclimatize. In scenarios where fixed species thrive, certain environmental factors may have little influence on the development of new organisms (Parmesan *et al*., 2000; Karthigeyan *et al*., 2022). Artificially controlled environments can provide significant support to organisms that exhibit acclimatization traits, though this capability is not universal across all organisms. Artificial growth systems can be instrumental in cultivating new specimens from various regions (Reich *et al*., 2003; Cantabella *et al*., 2022).

The hilly regions of West Bengal are recognized as rich repositories of rare, endangered, and medicinal plant species (Chhetri, 2005; Karthigeyan, 2022). Some of these plants possess the ability to adapt and thrive in different ecological zones, such as plains, where they can reproduce successfully (Beniston, 2003; Tahiri *et al*., 2005). The physiological processes of such plants may undergo significant changes when grown in distinct regions (González *et al*., 2006; Calfapietra *et al*., 2015). Cryptogams, including *Equisetum* sp*., Selaginella* sp*., Funaria* sp*., and Lunaria* sp., are commonly used as laboratory specimens and can be cultivated in controlled systems (Szypuła *et al*., 2021). The objective of the present research was to cultivate and study plant specimens within a cost-efficient artificial growth chamber as a step toward their conservation. The *in vitro* system has been developed for the cultivation and study of the selected cryptogams.

MATERIAL AND METHODS

Plant material

The cryptogamous species viz. *Equisetum sp.*, *Selaginella sp.*, *Funaria sp.*, and *Lunaria sp.*, were collected from their natural habitats in the Darjeeling district of West Bengal, India. Data on periodic temperature and rainfall for the region were obtained through seasonal site surveys and cross-referenced with publicly available data from the website (https://www.worldweatheronline.com/lang/en-in/darjeeling-weather-averages/west-bengal/in.aspx).

The selected plant specimens were cultivated in a system designed and developed earlier by Samanta *et al*. (2023). The system's design and actual implementation are illustrated in Fig. 1. Environmental parameters, such as temperature, light intensity, humidity, and rainfall, within the system were meticulously regulated to reflect *in-situ* conditions. Nutrient supplementation was provided in accordance with the Nitrogen, Phosphorus, and Potassium (NPK) content determined from the *in-situ* soil (Table 1). The composition per liter was 3 mM NH_4NO_3 (N source), 1 mM KH_2PO_4 (P source), and 6 mM K_2SO_4 (K source), supplemented with trace micronutrients (Fe, Mg, Mn, Zn, Cu, and B) in standard concentrations (Ameer *et al*., 2024; Bera *et. al*., 2015). The pH was adjusted to 6. A control treatment using a standard nutrient formulation was included for comparative assessment of growth and physiological response. Additionally, the growth and development of the plants were closely monitored to ensure physiological and morphological outcomes.

Maintenance of the nutrient

After the plants were fully grown in the system, an anatomical study was performed to observe any anomalies in the system-grown plants, adopting the double staining method according to Vazquez-Cooz and Meyer (2002), and pictures were taken using a Zeiss microscope. Images of the morphology of plant parts were taken using a Leica Stereo Microscope (Model No. M125C). The anatomical study was done based on the transverse section of the specimen. The anatomical features were examined under a stereo microscope, and images were captured with a Zeiss trinocular microscope (Axiolab 5). The SEM pictures were taken with a ZEISS Gemini Sigma 300 VP after gold coating using a Quorum 15ORplus instrument of the system-grown plants. The plant samples was prepared for SEM according to Golinejad and Mirjalili (2020) and observed at the central research facility of the Indian Institute of Technology, Kharagpur, India.

Measurement of dry weight and moisture content

The plants were taken from the soil, and their fresh weight was measured. The plants were then dried in a hot air oven at 80°C. After two hours, the plants were taken from the oven. The percent dry weight and percent moisture content were measured according to Van De Sande-Bakhuyzen, (1928) by the following equations:

Dry weight = Dry weight / Fresh weight Eq.1

Moisture content (%) = (Fresh weight - Dry weight) / Fresh weight Eq.2

DNA isolation and gel electrophoresis

DNA was isolated from fresh leaves of *in situ* and *ex situ* grown cryptogams using Cetyl Trimethyl Ammonium Bromide CTAB method (Rogers and Bendich, 1988; Bera *et. al.,* 2015). Quality and quantity of DNA were inspected by Gel (1.5% agarose) electrophoresis and spectrophotometric assay using UV-visible spectrophotometer (Genesys 10S UV-vis).

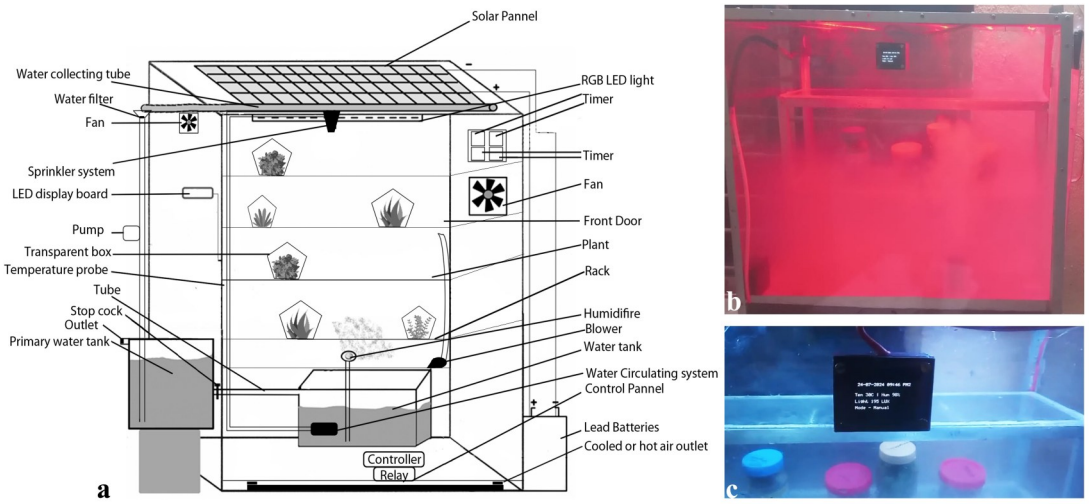

Fig. 1. *Ex situ growth chamber (a. design of the system adopted from Samanta et al., 2023 b. actual picture of the system c. the digital display of the system showing the inner environmental condition) (Samanta et al., 2023)*

Statistical analysis

All the data were analyzed as mean ± standard deviation, and least significant difference ($p \leq 0.05$) was performed using ANOVA with Microsoft Excel data analysis tools, according to Duncan's multiple range test.

RESULTS AND DISCUSSION

The comparative analysis of fresh and dry weights across all specimens i.e. *Equisetum* sp., *Selaginella* sp., *Funaria* sp., *Lunaria* sp., indicates a reduction in dry weight accompanied by an increase in moisture content (Table 2). The observed increase in moisture content within the system-grown plants indicated that the growth system maintained adequate moisture conditions conducive to plant health and survival. Macro- and micro-morphological evaluations of the system-grown plants revealed characteristics consistent with physical evaluation of development (Fig. 2). The soil sample analyzed from the collection site exhibited a nutrient composition characterized by low nitrogen, medium phosphate, and high potassium, with an NPK ratio of 3:1:6. Liquid nutrient media were formulated to align with this NPK ratio, as detailed in Table 1.

Scanning electron microscopy (SEM) images provide detailed insights into the morphological features of sys-

Table 1. Soil testing results showing nitrogen, phosphorus, potassium (NPK) ratio of the selected area of Darjeeling District. The concentration of NPK (nitrogen, phosphorus, potassium) represented in L-Low, M-Medium, H-High concentration in ascending order of 1 and 2.

Sample	N	P	K
1	L1 (<50 kg / Acre)	M2 (8-10 kg / Acre)	H1 (121-150 kg / Acre)
2	L2 (50-99 kg / Acre)	M1 (4-7 kg / Acre)	H2 (>150 kg / Acre)
3	M1 (201-300 kg / Acre)	M2 (8-10 kg / Acre)	H2 (>150 kg / Acre)
4	L2 (50-99 kg / Acre)	M1 (4-7 kg / Acre)	H1 (121-150 kg / Acre)
5	L1 (<50 kg / Acre)	H1 (11-15 kg / Acre)	H2 (>150 kg / Acre)

Table 2. Showing analysis of dry weight and moisture content in *in situ* vs. *ex situ* grown plant.

	Dry Weight / gram		Moisture Content	
Sample	***In situ***	***Ex situ***	***In situ***	***Ex situ***
Equisetum sp	0.408 ± 0.031^{a}	0.103 ± 0.011^{b}	0.592 ± 0.033^{b}	0.896 ± 0.035^{a}
Selaginella sp	0.331 ± 0.023^{a}	0.283 ± 0.014^{a}	0.669 ± 0.031^{a}	0.716 ± 0.030^{a}
Funaria sp	0.227 ± 0.015^{a}	0.176 ± 0.015^{a}	0.773 ± 0.037^{a}	0.823 ± 0.037^{a}
Lunaria sp	0.384 ± 0.027^{a}	0.143 ± 0.011^{b}	0.616 ± 0.030^{b}	0.864 ± 0.032^{a}

Data are represented as mean ± standard deviation and the data indicated with different lower case letters are significantly different ($p \leq 0.05$) according to Duncan's multiple range test ($n = 20$). Lowercase letters (a, b) indicate significant differences between the *in situ* and *ex situ* groups for a given species and measurement (e.g., a is significantly different from b)

Fig. 2. *Showing morphology and anatomy of in situ (A) and system grown plants (B); A. a-c Lunaria sp. (a. thallus body, b. thallus with rhizoid, c. thallus body with gamma cup); A. d-f Funaria sp. (d. vegetative body, e. capsule, f. peristome teeth); A. g-i Selaginella sp. (g. stem with leaf, h. single leaf, i. single strobilus); A. j-l Equisetum sp. (j. single leaf, k. stem, l. mature strobilus); B. a-f Lunaria sp. (a. thallus body, b. thallus body with gamma cup, c. single gamma cup, d. anatomical section of thallus, e. section of gamma cup attached with thallus, f. section of a single gamma cup); B. g-l Funaria sp. (g. vegetative body, h. single leaf, i. capsule, j. section of leaf, k. anatomy of leaf, l. section of capsule); B. m-r Selaginella sp. (m. Stem with leaf, n. single leaf, o. single strobilus, p. section of stem, q. section of leaf, r. section of strobilus); B. s-z Equisetum sp. (s. single leaf, t. stem, u. immature strobilus, v. mature strobilus, w. section of leaf, x. section of stem, y. section of strobilus, z. spores with elaters)*

tem-grown plants, which are comparable to those observed *in situ* specimens (Fig. 3). Double-staining techniques revealed no discernible anatomical differences between system-grown plants and their *in-situ* counterparts. The observed increase in moisture content within the system-grown plants indicates that the growth system maintains adequate moisture conditions conducive to plant health and survival (Table 2).

Genomic DNA integrity was maintained, with no significant degradation observed, indicating a high degree of genetic similarity, as shown in Fig. 4. Furthermore, all cryptogams examined in this study exhibited genomic DNA fragments exceeding 10 kb, underscoring the integrity of the isolated DNA. The assessment of dry weight and fresh weight is a critical parameter for evaluating plant growth and development, as highlighted by Romero-Aranda *et al*., (2001) and Hunt (2012). Macro- and micro-morphological analyses, conducted using stereo microscopy and Scanning Electron Microscopy (SEM), have been documented for comparative morphological studies (Stant, 1973; Mondal and Moktan, 2022). Genomic DNA analysis suggests that no DNA degradation or loss of genomic content occurred during plant maintenance within the artificial chamber.

These findings collectively highlight the efficacy of the growth system in successfully simulating key environmental conditions to support acclimatisation and survival in situ and robust plant development.

Conclusion

The present findings support the conclusion that plants are macro- and micro-morphologically (external and internal) and physiologically healthy, as confirmed by

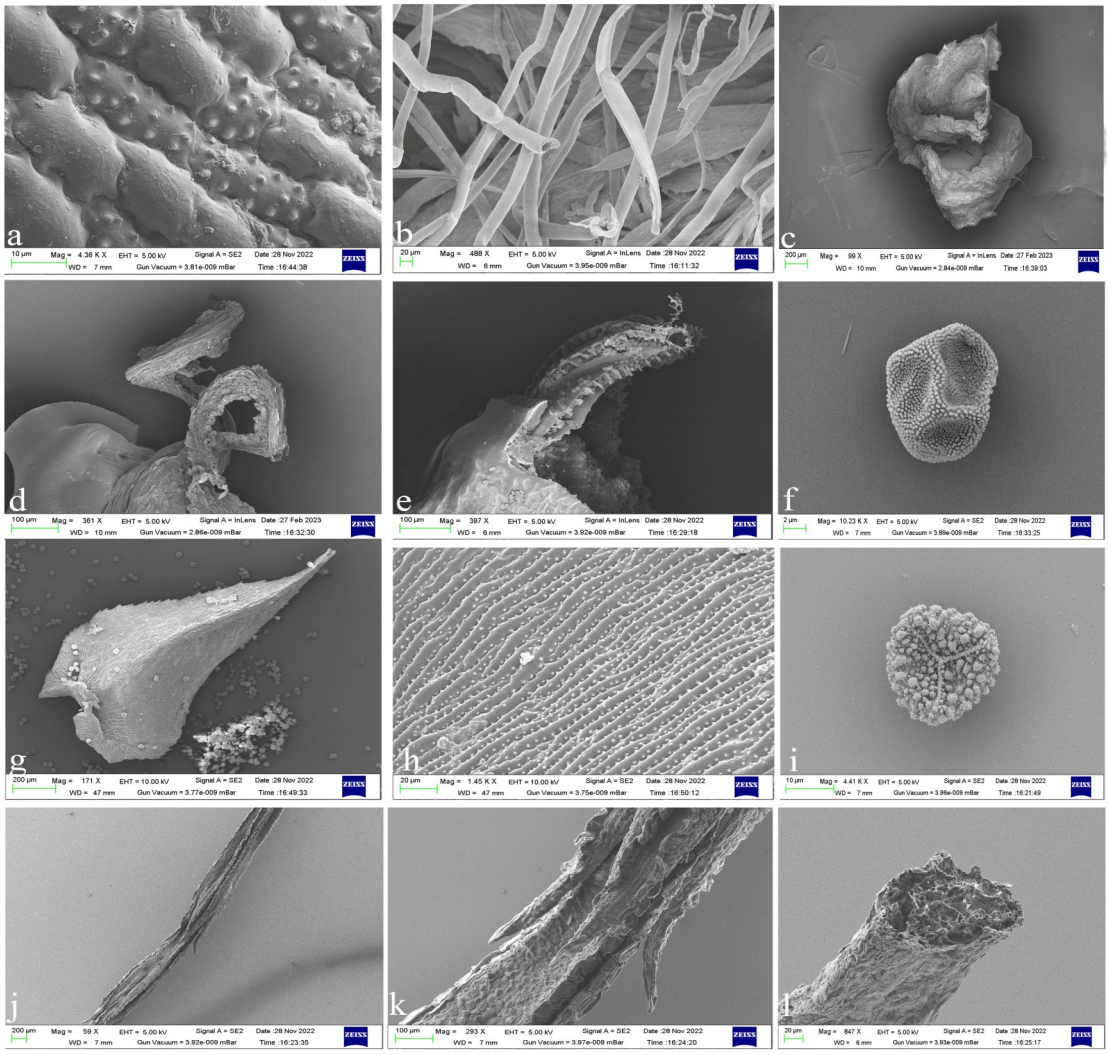

Fig. 3. *Showing plant morphology in scanning electron microscope SEM of system grown plants a-c Lunaria sp. (a. thallus body, b. thallus apertures, c. rhizoids), d-f Funaria sp. (d. operculum, e. peristome teeth, f. spore). g-l Selaginella sp. (g. Single sporophyll, h. leaf surface, i. spore) j-l Equisetum sp. (j. leaf, k. stem node, u. vascular aperture)*

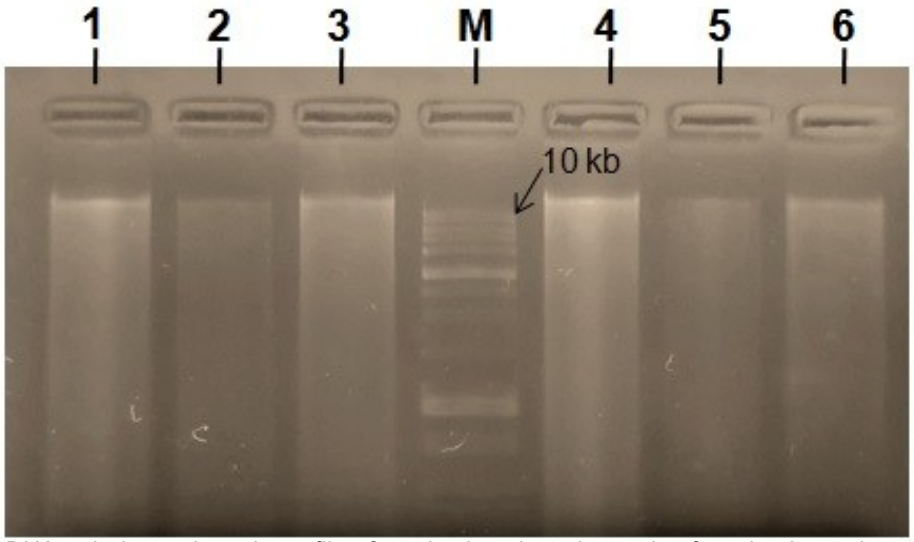

Fig. 4. *Showing DNA gel electrophoresis profile of randomly selected samples from in situ and ex situ grown cryptogams. Lane 1-3: Isolated DNA of in situ grown Funaria sp., Lunaria sp., Selaginella sp. respectively; Lane M: M11 – 1 kb DNA ladder (250 bp – 10000 bp); Lane 4-6: Isolated DNA of ex situ grown Funaria sp., Lunaria sp., Selaginella sp. respectively*

fresh weight, dry weight, and moisture content when compared to their *in-situ* counterparts. This observation underscores the efficacy of the artificial growth system in replicating natural growth conditions while maintaining plant health and genomic integrity. Cryptogams cultivated within the system have the potential to serve as valuable specimens for laboratory activities in various degree colleges, addressing the ongoing need for plant materials in academic research and practical studies. This proposal is a cost-effective, self-sustaining model designed for the cultivation and development of endangered cryptogams and can lead towards conservation.

ACKNOWLEDGEMENTS

The author Dr. Aveek Samanta acknowledges the financial support received from the STEM/Catalytic grant/acad_22/2022-23 funded by Office of the Principal Scientific Advisor to the Government of India, to carry out the research work in Department of Botany, Prabhat Kumar College, Contai, West Bengal, India.

Conflict of interest

The authors declare that they have no conflict of interest.

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