PLANT SCIENCE TODAY
ISSN 2348-1900 (online)
Vol x(x): xx–xx
https://doi.org/10.14719/pst.2376

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MINI REVIEW ARTICLE

Strategies for successful acclimatization and hardening of *in vitro* regenerated plants: Challenges and innovations in micropropagation techniques

Nishesh Sharma*, Nishant Kumar, Jerin James, Sonika Kalia & Shivani Joshi

Department of Biotechnology, School of Applied & Lifesciences (SALS), Uttaranchal University, Dehradun, India

*Email: nishesh21@gmail.com

OPEN ACCESS

ARTICLE HISTORY

Received: 31 January 2023
Accepted: 28 August 2023

Available online
Version 1.0 : 01 September 2023

Check for updates

Additional information

Peer review: Publisher thanks Sectional Editor and the other anonymous reviewers for their contribution to the peer review of this work.

Reprints & permissions information is available at https://horizonepublishing.com/journals/index.php/PST/open_access_policy

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Indexing: Plant Science Today, published by Horizon e-Publishing Group, is covered by Scopus, Web of Science, BIOSIS Previews, Clarivate Analytics, NAAS, UGC Care, etc
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CITE THIS ARTICLE

Sharma N, Kumar N, James J, Kalia S, Joshi S. Strategies for successful acclimatization and hardening of *in vitro* regenerated plants: Challenges and innovations in micropropagation techniques. Plant Science Today (Early Access). https://doi.org/10.14719/pst.2376

Abstract

The micropropagation technique serves as an effective approach for conserving and propagating numerous plant species. Challenges to its success encompass explant selection, media composition, hormone concentration, microbial contamination, incubation conditions, and photoperiod. Beyond these factors, the veracity of tissue culture hinges on successful acclimatization of *in vitro* regenerated plants to their natural surroundings. Tissue culture-derived plants exhibit characteristic variations like altered nutrition, reduced cuticular wax, non-functional stomata, etc. During transition to natural conditions, a significant portion of micropropagated plants face survival challenges. Studies propose gradual acclimatization processes for smooth adjustment. *Ex vitro* rooting is advocated for economic, simple, and enhanced survival outcomes. Hydroponics, photoautotrophic acclimatization, and biotization strategies also improve post-transplantation survival. This study evaluates diverse strategies for achieving successful acclimatization of *in vitro* regenerated plants.

Keywords

Acclimatization; hardening; *ex vitro* rooting; hydroponics; *in-vitro*; biotization

Introduction

Throughout history, plants have served diverse roles, encompassing medicinal applications, raw material sourcing for pharmaceuticals, cosmetics, food industries, textiles, and animal fodder. Regrettably, rampant exploitation and unregulated harvesting from natural habitats have engendered critical endangerment for numerous plant species, with some teetering on the brink of extinction. To mitigate this crisis, plant micropropagation techniques have emerged as a pivotal approach, offering not only preservation strategies for endangered species but also enabling prolific propagation (1,2). Beyond safeguarding imperiled flora, micropropagation holds significant utility for diverse objectives. These include the propagation of seedless cultivars, plants with dormant seeds, those with inherently sluggish natural propagation rates, species facing heightened demand, and the production of hybrid and transgenic varieties (3). However, realizing the full potential of plant tissue culture necessitates adeptly addressing a spectrum of challenges. These encompass judicious explant selection, refinement of sterilization agents, optimal formulation of culture media in conjunction with Plant Growth Promoting Rhizobacteria (PGPR) to foster growth, and the imperative control of microbial contamination. The ultimate efficacy of any tissue culture intervention hinges upon successful acclimatization, the

process wherein *in vitro* regenerated plants are transitioned from controlled laboratory conditions to their natural milieu. Acclimatization denotes the gradual acclimation of plants or organisms to novel environments subsequent to their propagation or cultivation in controlled settings. This transitional phase is quintessential for facilitating the adjustment of micropropagated plants to the exigencies of their natural habitat, thereby ensuring their viability and subsequent growth. Paradoxically, it is during this pivotal acclimatization juncture that a substantial proportion of *in vitro* regenerated plants confront challenges, often culminating in their failure to thrive upon transplantation to natural settings (4).

Micropropagated plants (MP) undergo cultivation within controlled environments marked by reduced humidity, subdued light intensity in comparison to natural sunlight, regulated temperature and heterotrophic or mixotrophic nutritional conditions. This controlled growth environment leads to a range of anatomical and physiological deviations in MP when compared to their wild counterparts (5). Noteworthy variations include the absence or reduction of leaf trichomes, diminished epicuticular wax or complete absence of the cuticular layer, compromised stomatal regulation, enlarged intracellular spaces, attenuated differentiation of palisade cells, and the lack of starch granules (6, 7). These inherent traits of MP limit their immediate transition to natural habitats characterized by factors such as low humidity, high light intensity, and temperature fluctuations. Consequently, this transition often leads to leaf scorching and eventual wilting, culminating in the demise of the plants (Figure 1). Dhawan and Bhojwani (7) noted morphological anomalies and leaf size reduction during the *in vitro* rooting phase. As a countermeasure to mitigate MP mortality during acclimatization, a gradual exposure to natural conditions is employed, a process known as acclimatization. The acclimatization period serves as a critical timeframe (3, 5, 8) for MP to reestablish normal photosynthetic activity and metabolic functions. During this phase, the restoration of stomatal regulation occurs, facilitating the development of fresh leaves characterized by conventional anatomical and physiological traits suited for survival under natural environmental circumstances. This review aims to systematically analyze diverse parameters influencing the acclimatization of micropropagated plants, and subsequently, proposes optimization strategies to enhance the success rate of acclimatization endeavors.

Standard process of acclimatization of micro propagated plants

The most commonly employed method for acclimatization of micropropagated (MP) plants involves the aseptic excision of plants from the culture vessel, followed by gentle root washing to eliminate all remnants of the growth medium. Subsequently, these plants are transplanted into pots containing a composite substrate mixture of soil, vermiculite, vermicompost, and sand, often used in specific proportions. These potted plants are then covered with transparent polybags and initially placed in an incubation room or conditions similar to those of their regeneration. They are later moved to a mist chamber and greenhouse before being finally transplanted into their natural environment. This established process, with slight variations tailored to the specific plant species under study, has been successfully utilized for the acclimatization of plants such as *Dianthus caryophyllus* Linn (9), *Withania coagulans* (10), *Rauvolfia serpentina* (11), and *Picrorhiza kurroa* (12). In addition to the standard acclimati-

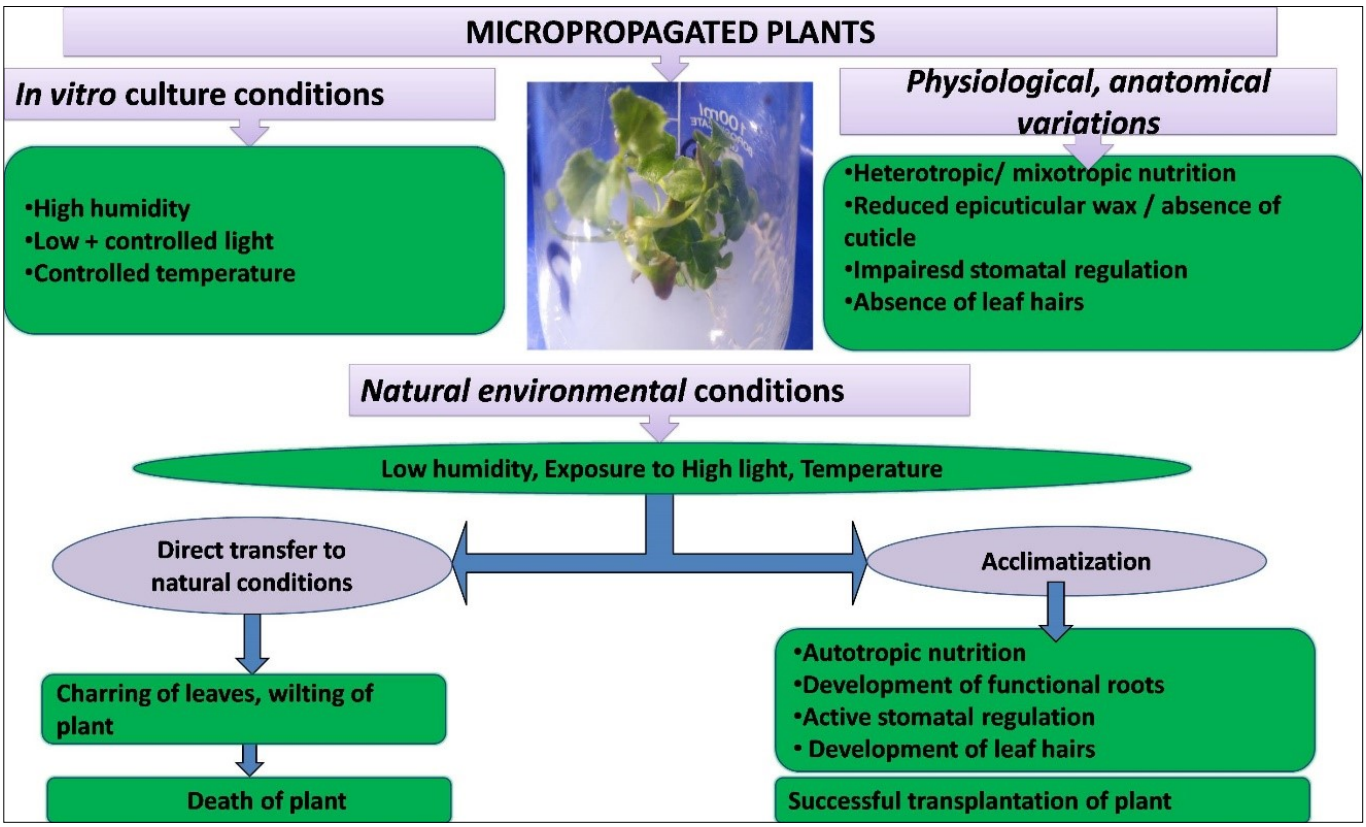

Fig. 1. Summary of variation in micropropagated plants and adaptation during acclimatization

zation procedure, several alternative methods for acclimatizing micropropagated plants have been explored, as summarized in Figure 2.

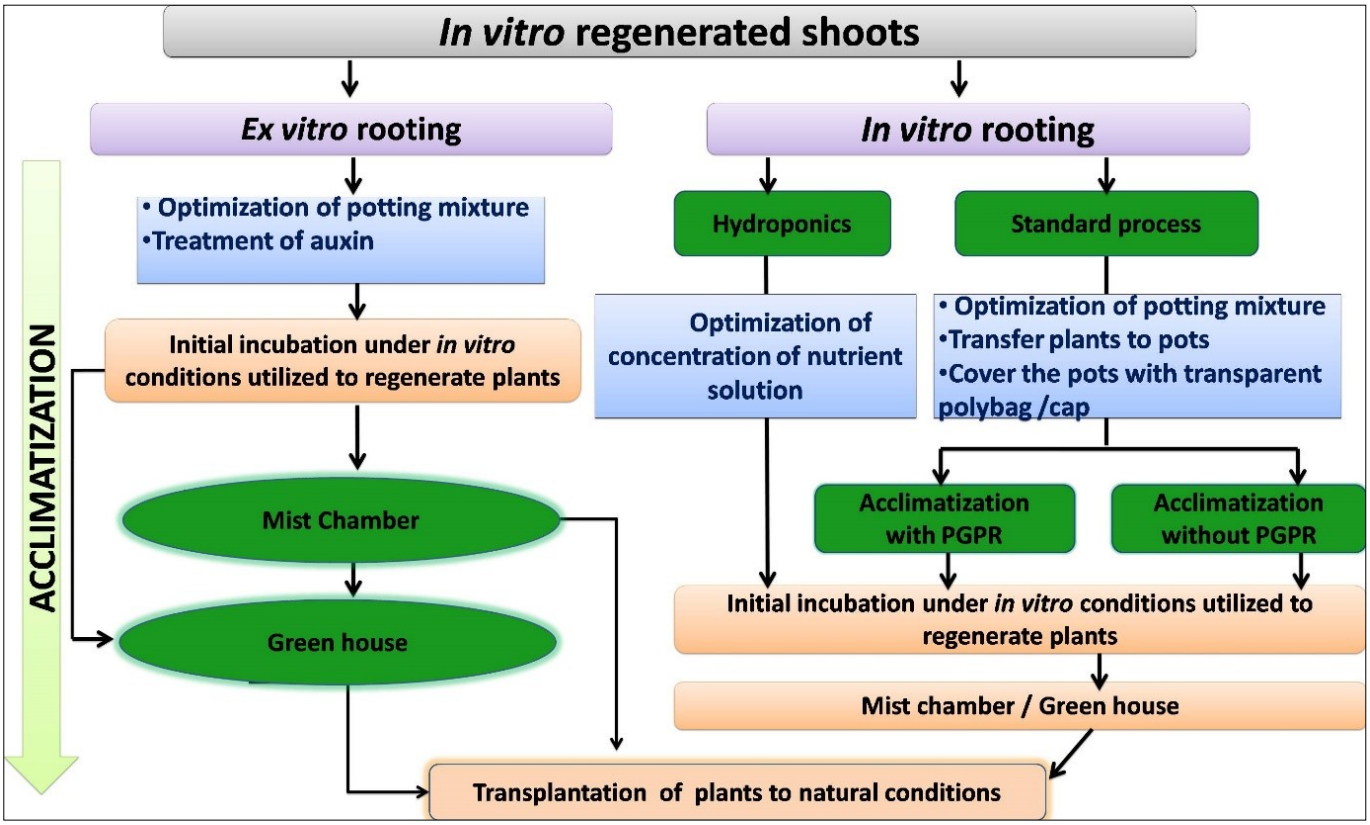

Fig. 2. Strategies adopted for acclimatization of tissue culture raised plants

Ex vitro rooting

In the context of tissue culture processes, the *in vitro* regeneration of shoots is typically succeeded by subculturing to induce root development, culminating in the establishment of micropropagules (MPs), which subsequently undergo acclimatization. Within the framework of total expenditure in micropropagation, the *in vitro* rooting phase contributes to a significant proportion, ranging from 35% to 50% (13). As an economical alternative to *in vitro* rooting, the *ex vitro* rooting approach has gained attention. This strategy confers the advantage of curtailing the temporal dimension of the micropropagation protocol. Sharma *et al*. (reference 14) documented successful *ex vitro* rooting of micropropagated shoots. This methodology entailed immersing shoots in varying concentrations of auxins, followed by transplantation into containers containing soilrite. Incubation within a greenhouse environment under conditions of elevated humidity ensued. The process of acclimatization encompassed a phased loosening of container caps at intervals of 3-4 weeks, culminating in complete cap removal. Auxins, including Indole-3-butyric acid (IBA) and 1-Naphthalene acetic acid (NAA), were employed both individually and in combination. Notably, optimal rooting was achieved with the sole utilization of Indole-3-butyric acid. In a study by Wojtania *et al*. (13), *ex vitro* rooting of *Lonicera caerulea* var. Kamtschatica's Wojtak and Zojka cultivars was reported in the absence of exogenously applied auxins, conducted within a greenhouse setting. A pronounced discrepancy in survival rates was observed between the two cultivars, with Wojtek demonstrating a higher survival rate of 96%, compared to Zojka's rate of 88%. *Ex vitro* rooting offers intrinsic advantages owing to its simplicity and cost-effectiveness, while also mitigating the potential for root damage during transplantation (15). Furthermore, roots established through the *ex vitro* method exhibit heightened adaptability to natural conditions, surpassing their *in vitro* regenerated counterparts (16). These merits have propelled the utilization of *ex vitro* rooting on a commercial scale within laboratory settings (17).

Potting mixture utilized for hardening

The success of the hardening process is notably influenced by the composition of the potting mixture (Table 1), a factor that significantly impacts both the survival rate of plants and their subsequent growth post-transplantation (21, 31, 32). Optimal outcomes have been observed with the utilization of a potting mixture comprising garden soil, sand, vermicompost, and Farmyard Manure. This blend facilitates improved aeration and augments the provision of essential nutrients, thereby contributing to the thriving of transplanted plants (31). Vermicompost, an organic nutrient-rich substrate, plays a pivotal role in fostering acclimatization and robust growth of plants (31-34). In the context of *in vitro* probation, rooting, and transplantation of Wasabi plants, Hoang *et al*. (35) demonstrated that agar and vermiculite exhibit superior efficacy as supporting materials compared to rockwool and perlite. The adoption of photoautotrophic growth strategies has emerged as a cost-effective approach in micropropagation procedures, yielding both enhanced survival rates and reduced expenses. Notably, photoautotrophic acclimatization, which

involves a medium devoid of sugars and vitamins, effectively curtails bacterial contamination risk and necessitates diminished reliance on exogenous growth regulators (4, 36, 37). This approach is increasingly recognized as a valuable tool in plant tissue culture research, facilitating streamlined processes and optimizing resource utilization.

Table 1. Various planting / potting mixture utilized for acclimatization

Plant	Potting mixture	Inference	Author
Albizia amara	Soil + peat + VC + perlite (1:1:1:1) + Bacterial inoculum (*P. fluorescens* and *T. viride*)	82% survival	(18)
Vitis vinifera	Cocopeat + VC + vermicompost (2:1:1)	85.97% survival	(8)
P. kurooa	Sand:soil (1:1)	62.4% survival rate	(19)
Grand Naine Banana	Garden soil, sand, VC, cocopeat, FYM (Farm/ field yard manure)	Cocopeat (1° hardening), Garden soil + FYM (2° hardening)	(20)
Guava (var Allahabad safeda)	Soil, cocopeat, vermicompost (VC), VC+soil (1:1), FYM+soil+sand	Vermicompost + soil (1:1), (90%)	(21)
L. caerulea var. kamtschatica	Peat; Peat and perlite; Peat and Sand	Better grow (Shoot, Roots) in Peat; Peat and perlite	(22)
Apple Rootstock	Coco-peat	Successful acclimatization	(23)
Epiphytic and terrestrial orchids	Charcoal pieces, brick chips, moss	Charcoal pieces + moss (epiphytic orchids); Moss + decayed wood /forest litter	(24)
P. guajava	Sand: garden soil (3:1)	90% plants survived	(25)
Gerbera Jamesonii bolus Ex Hookf	Garden soil (Black soil:Red soil; 2:1) Vermiculture	Enhanced survival rate	(26)
ionantha	Compost + sand + black soil (1:1:2)	Successful hardening	(27)
Stevia rebaudiana	Sand + cocopeat	75.5 % survival rate	(28)
Strawberry	Soil + FYM (1:1)	Successful hardening	(29)
Colocasia esculenta (L.) Schott	Soil + Manure + Rice Husks (1:1:1)	62% survival, rice husk exhibit high water holding capacity & nutrient uptake	(30)

Role of hydroponics in Acclimatization

Hydroponics is a soilless method for cultivating plants in a liquid medium, offering notable benefits such as augmented growth, increased yield, pest resistance, facile weed management, and mechanized processes. The technique not only delivers essential nutrients but also mitigates water loss through transpiration. Sutthinon *et al*. (38) employed the Dynamic Root Floating Technique (DRFT) in hydroponics for *Grammatophyllum speciosum* seedling acclimatization, utilizing 5-fold and 10-fold diluted nutrient solutions alongside chitosan foliar application. Comparative analysis indicated superior survival rates among plants acclimatized with a 10-fold diluted nutrient solution, while the chitosan treatment exhibited negligible efficacy in enhancing survival rates. However, chitosan has been demonstrated in other studies to facilitate plant growth, reduce transpiration-related water loss, and support acclimatization (39). Chitosan treatment has also shown acclimatization benefits for *in vitro* regenerated Dendrobium plants (40), attributed to its regulation of stomatal aperture and subsequent reduction in water loss (41). Independent investigations (42, 43) have reported positive effects of chitosan on growth in willow cuttings and *Paphiopedilum niveum*, respectively.

In a scientific investigation conducted by Al-Khalifah *et al*. (45), *in vitro* regenerated specimens of Strawberry (*Fragaria spp*.), Rose (*Rosa spp*.), and Date palm (*Phoenix dactylifera*) underwent successful acclimatization via hydroponics. This cultivation approach exhibited notable advantages, including enhanced growth rates and heightened survival rates in comparison to control groups. Hydroponics, renowned for its benefits such as elevated yields and reduced susceptibility to pest infestations, presents a promising avenue. However, the widespread adoption of hydroponics for commercial or large-scale applications encounters challenges due to the associated setup costs, maintenance demands, and monitoring intricacies necessitating specialized proficiency. Consequently, despite the considerable research directed towards refining hydroponic technology, there remains substantial potential for devising cost-effective hydroponic systems optimized for the rooting of micropropagated plants and the generation of nutrient-rich, disease-resistant specimens. Among the array of factors governing the triumph of *in vitro* cultivation, photoperiod assumes a pivotal role. Photoperiodic conditions exert direct influence over growth dynamics and physiological processes. Notably, the optimal photoperiod can exhibit species-specific variability. Li *et al*. (46) examined the impact of light intensity and photoperiod on hydroponic cultivation of Arugula (*Eruca sativa*) and documented that moderate to high light intensities contributed to the preservation of post-harvest freshness in Arugula. Additionally, investigations into *Lactuca sativa* L. revealed that high light intensity coupled with a shorter photoperiod elicited augmented growth (47). Consequently, the meticulous optimization of photoperiod holds paramount significance for both *in vitro* micropropagation and hydroponic endeavors, serving as a pivotal determinant for fostering maximal growth and successful acclimatization of plant specimens.

Biotization

Rhizospheric microorganisms have been recognized for their capacity to promote plant growth and aid in plant acclimatization. Pandey *et al*. (48) demonstrated the efficacy of *B. subtilis* and *P. corrugata* in enhancing the survival rate of micropropagated tea plants. Similarly, Indravathi and Babu (49) harnessed *Pseudomonas fluorescens* and *Trichoderma viride* to facilitate the acclimatization of *Albizia amara*. The co-inoculation of these strains resulted in an impressive 82% survival rate, attributed to a synergistic interaction conferring enhanced stress tolerance. Agarwal *et al*. (50) investigated factors influencing in-vitro regeneration and acclimatization of *Eucalyptus tereticornis*. Optimal *in vitro* culture conditions were coupled with the use of photosynthetically active radiation (PAR), which outperformed cool fluorescent light (CFL) not only in micropropagation but also in acclimatization. Incorporating Bacillus subtilis suspension into this regimen further elevated survival rates by 10%, culminating in an overall survival rate of 84%. The augmentation of survival and growth of *Etlingera elatior* plants was reported through the utilization of Arbuscular Mycorrhizal Fungi (AMF) compared to control plants (37). AMF establish a symbiotic association with host plants, facilitating nutrient absorption and bolstering adaptation to diverse environmental stresses (51-53). It is noteworthy that the choice of substrate or potting mixture during *ex vitro* cultivation significantly influences AMF activity and its associated benefits.

Silva *et al*. (51) documented a diminished influence of arbuscular mycorrhizal fungi (AMF) on the growth of *Alpinia purpurata* and *Zingiber spectabile* when vermicompost was employed as a substrate. Conversely, de Oliveira *et al*. (54) observed coconut husk powder to stimulate AMF activity in *Z. spectabile*. In a separate investigation (55), the inoculation of *Terminalia bellirica* Roxb. with *Piriformospora indica* yielded heightened survival rates and augmented plant growth. Root colonization by *P. indica* led to amplified biomass, root development, and chlorophyll content (46). *P. indica*, a well-recognized endophytic fungus, establishes root colonization in a diverse spectrum of plant species, spanning monocots and dicots. Notable plant growth-promoting attributes of *P. indica* encompass the synthesis of plant growth hormones, facilitation of seed germination, and resilience against biotic and abiotic stressors (57-59).

SWOT analysis

Micropropagation stands as an essential modality for the preservation and extensive proliferation of plant species. The multifarious benefits and applications intrinsic to micropropagation encounter constraints in the absence of commensurate large-scale commercial cultivation endeavors. The illustrative Figure 3 encapsulates a SWOT analysis encompassing acclimatization within the context of micropropagation. A pressing imperative exists to disseminate and facilitate proficiency in micropropagation, hydroponics, and biotization among local stakeholders, fortified by requisite financial support.

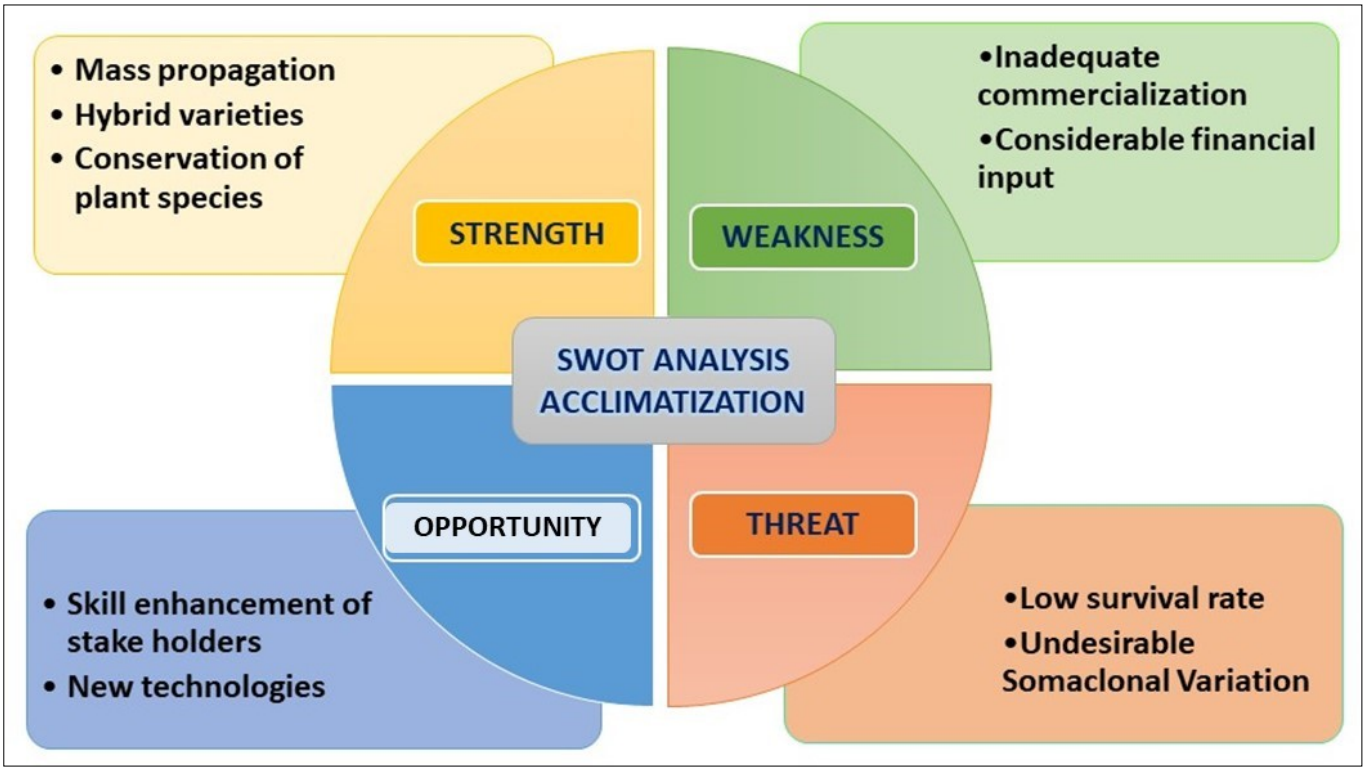

Fig. 3. SWOT analysis of acclimatization of micropropagated plants

Conclusion and future challenges

Micropropagation stands as an effective technique for propagation, genetic refinement, and the advancement of transgenic and superior plant varieties. However, the transition from *in vitro* conditions to the natural environment presents a substantial challenge. Mitigating this challenge requires a systematic approach encompassing *ex vitro* rooting, hydroponics, mist chamber and greenhouse utilization for hardening, selection of an appropriate potting substrate, and pathogen control. A promising avenue is the strategic implementation of photoautotrophic

acclimatization, characterized by both economic viability and heightened survival rates. Anticipated future developments involve the fine-tuning of photoautotrophic acclimatization methods and the identification of microorganisms conducive to root formation and plant acclimatization. Plant growth-promoting bacteria, renowned for their growth-enhancing activities, offer a promising focus for investigation in supporting acclimatization. The design of an optimized strategy to maximize the survival percentage of micropropagated plants during acclimatization demands an integrated approach, harmonizing a myriad of contributing factors.

Authors contributions

NS conceptualized the study. JJ, SK and SJ carried out literature survey. NS and NK prepared the first draft of manuscript. All authors contributed equally to revise the manuscript and approved the final draft.

Compliance with ethical standards

Conflict of interest: **:** Authors do not have any conflict of interests to declare.

Ethical issues: None

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