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Protocol Optimization for the Acclimatization of *Stevia rebaudiana* Bertoni Under Controlled Conditions

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Abstract

Stevia rebaudiana explants (like nodal region, leaf, shoot apex) has been cultured in Ms. media with different supplements of plant growth hormones like (BAP), BA,2,4-D,NAA (0.25 to 1.5mg/lt) and has been developed invitro with slight variations to the mother plant. When these plants are shifted to natural environmental conditions needs proper acclimatization procedure to be followed because the survival percentage of the plants has been reduced which leads to the reduction in the multiplication of the plants. Acclimatization continues to be a major bottle neck in the micro propagation of stevia too with lower success reported. In the present investigation stevia plants had undergone two phases of hardening procedure like primary and secondary hardening in the green house. The plants are washed thoroughly with fungicides before starting the hardening procedure. The temperature maintenance and the variation in the potting mixture composition have a major role to play in the sustenance of the invitro stevia plant. The total procedure takes a time period of five weeks to enhance the survival percentage of plants.

Keywords: *Stevia plant, Acclimatization, primary, secondary hardening, potting mixture*

Introduction

In recent years, there has been a global shift in dietary habits due to increased awareness of lifestyle-related disorders such as obesity, hypertension, cardiovascular diseases, and, most notably, diabetes mellitus. Among the most pressing concerns is the overconsumption of refined sugars, which are strongly linked to metabolic syndromes and other chronic conditions [1-2]. Consequently, there is a growing demand for natural, low-calorie alternatives to sugar that offer both sweetening capabilities and therapeutic benefits. *Stevia rebaudiana* Bertoni, a perennial herbaceous plant belonging to the family Asteraceae, has emerged as a promising natural sweetener owing to its high content of steviol glycosides—particularly stevioside and rebaudioside A—which are known to be several hundred times sweeter than sucrose and virtually non-caloric [3].

Stevia rebaudiana is native to South America, specifically Paraguay and Brazil, where it has been traditionally used by the Guarani people for centuries to sweeten teas and medicinal preparations.

In recent decades, scientific research has validated many of the medicinal properties attributed to stevia, including its anti-diabetic, antihypertensive, anti-inflammatory, and antioxidant effects [4]. Because of these characteristics, *S. rebaudiana* has gained commercial attention not only as a sugar substitute in the food and beverage industry but also as a potential agent in the nutraceutical and pharmaceutical sectors. The increasing market demand for stevia-based products has, in turn, emphasized the need for large-scale propagation of the plant through sustainable and efficient cultivation techniques.

One of the most critical challenges in the successful commercial cultivation of *Stevia rebaudiana* is the acclimatization phase following micropropagation. While tissue culture techniques are highly effective for the mass propagation of genetically uniform and disease-free plantlets, the transition from in vitro to ex vitro conditions remains a bottleneck in the production cycle. Plantlets grown in vitro are typically characterized by underdeveloped cuticles, reduced stomatal functioning, and poor root

systems, which make them highly susceptible to environmental stress when transferred to open-field or greenhouse environments [5]. Therefore, optimizing the acclimatization protocol is crucial for ensuring high survival rates, robust growth, and successful establishment of stevia plants in soil.

Acclimatization involves a gradual transition of plantlets from the controlled, nutrient-rich, and humid conditions of tissue culture laboratories to the more variable and harsher ex vitro environments. During this process, plants must undergo physiological and morphological adjustments, such as the development of functional stomata, stronger root systems, and structural cell wall reinforcement [6]. Various factors influence the success of acclimatization, including the choice of substrate, humidity regulation, temperature control, light intensity, and nutrient supplementation. Understanding and optimizing these parameters can significantly improve plantlet survival and enhance the overall yield and quality of stevia crops.

This study focuses on the protocol optimization for the acclimatization of *Stevia rebaudiana* Bertoni under controlled conditions. The objective is to identify the most favorable environmental and substrate conditions that support the healthy transition of stevia plantlets from *in vitro* to *ex vitro* environments. Through controlled experimentation and monitoring, this research aims to develop a standardized, reproducible, and scalable protocol that can be adopted by growers, nurseries, and researchers alike.

The importance of this research lies in its potential to bridge the gap between laboratory-scale propagation and field-scale cultivation. By improving acclimatization success rates, the production cost of stevia plants can be reduced, making it more accessible to both small-scale farmers and large commercial producers [7]. Moreover, a reliable acclimatization protocol supports the global movement toward natural and plant-based health solutions, particularly in managing metabolic diseases such as diabetes, as the demand for functional foods and plant-derived sweeteners continues to rise, optimizing the production pipeline of *Stevia rebaudiana*—especially the critical acclimatization stage—becomes essential. This work aims to contribute to the sustainable and efficient cultivation of stevia by focusing on enhancing the survival, growth, and vigor of plantlets during this sensitive transitional phase, thus ensuring a consistent and high-quality supply of this valuable medicinal herb.

Materials and Methods

Explants including nodal segments, leaf segments, and shoot apices were excised from healthy *Stevia rebaudiana* plants. The plant material was sourced from two different locations in Hyderabad. Upon collection, all explants were subjected to a standardized surface sterilization protocol to ensure aseptic conditions for in vitro culture. Initially, the explants were washed thoroughly under running tap water for 30 minutes to remove dust particles and reduce the microbial load. This was followed by pre-treatment using a sterilizing solution containing 2.5 g/L Bavistin, 30 mg/L streptomycin, and 300 mg/L K-Cyclin. The explants were agitated in this solution for 1 hour for nodal segments, and for 30 minutes for leaf segments and shoot apices. After chemical treatment, the explants were rinsed thoroughly with autoclaved double-distilled water to remove any residual disinfectants. Further surface sterilization was carried out under laminar airflow conditions using mercury chloride ($HgCl_2$) at concentrations of 0.05% and 0.10% for varying durations (3 to 7 minutes), depending on the tissue type and sensitivity. Finally, the explants were rinsed three times with sterile double-distilled water to eliminate any traces of $HgCl_2$ before inoculation onto the culture medium.

The nodal segments, measuring approximately 1.0 to 1.5 cm in length, were trimmed at both ends and subsequently inoculated onto Murashige and Skoog (MS) medium [12] supplemented with varying concentrations (0.25 to 1.5 mg/L) of plant growth regulators such as 6-benzylaminopurine (BAP), benzyladenine (BA), 2,4-dichlorophenoxyacetic acid (2,4-D), and naphthaleneacetic acid (NAA). *In vitro* development of these explants resulted in the successful regeneration of stevia plantlets, although with minor morphological variations compared to the mother plant. However, when these *in vitro*-developed plantlets are transferred to natural environmental conditions, a well-defined acclimatization protocol becomes essential. Without appropriate acclimatization, the survival rate of regenerated plants tends to decrease significantly, thereby limiting the efficiency and scalability of micropropagation in *Stevia rebaudiana*. Indeed, acclimatization remains a major bottleneck in the commercial propagation of stevia, as several studies have reported low success rates during this critical transitional phase.

Previous studies have demonstrated that, after adequate root formation, the plantlets should be carefully removed from the culture medium and subjected to a two-phase hardening process. In the first phase, the plantlets are transferred to either glass jars sealed with polypropylene (PP) caps or plastic pots covered with polyethylene film to maintain high humidity. The hardening medium in both systems consists of agro-peat supplemented with one-fourth strength MS liquid medium (devoid of organic additives), applied twice weekly to ensure moisture and minimal nutrient support. During the initial acclimatization phase (weeks 3–4), plantlets are retained within these closed containers to minimize transplant shock. This is followed by their gradual transfer into polybags filled with a soil mixture composed of sand, soil, and vermiculite in a 2:1:1 ratio. These are kept under greenhouse conditions during the secondary hardening phase (weeks 6–8), where the plants continue to adapt to ambient environmental conditions. This gradual shift helps the plantlets develop functional stomata, stronger root systems, and photosynthetic competency, ultimately improving their survival rate and enabling successful field establishment.

In the present investigation a standardized protocol has been developed for ex-vitro acclimatization of stevia plants. Here after development of sufficient roots, the plantlets were gradually pulled out of the media and were subjected to hardening. Hardening is taking place in two phases. Initially the invitro plants are placed in green house for 2-3 days to acclimatize it to new environment. Then the explants are washed with fungicides thoroughly so that no media particles are left attach to the newly formed roots then the small plantlets are shifted into Plastic Cavities for primary hardening containing potting mixture bearing a composition of Coco peat + vermiculite + neemcake + Trico powder in (3:1:5:3) ratio this potting mixture is mixed well and kept 15 days before the usage .After 15 days this mixture is filled in small cavities stand then the stevia plants are washed and shifted in this cavities .Temperature of around 25° -28 °C is maintained inside the plastic sheeted tents. After two weeks good healthy root development has been observed.

Then the whole stevia plant is shifted into polythene bags containing a mixture of Red soil ¾ + potting mixture ¼. Here the potting mixture used has a slight variation in the composition .It has Vermicompost + Coir pith + DAP (Di ammonium phosphate) +SSP (Single super phosphate) + Neemcake +Red soil in (4:1:2:2:2:1) ratio this potting mixture has been prepared 10 days before usage by watering it alternate days .After 10 days this potting mixture is can be used for second stage of hardening now shift the plantlets from cavities to polythene bags for 2 weeks and keep this polythene bags also under the plastic tunnels and water them daily and after 4 weeks it is shifted to pots also .At this stage the plant is ready for outlet.

Results and Discussion

In Vitro Sterilization Treatment

Plants grown under natural environmental conditions are frequently contaminated with a range of microorganisms and dust particles, primarily on their external surfaces. These contaminants pose a major challenge during *in vitro* culture, as they can rapidly multiply under the nutrient-rich and humid conditions of tissue culture media, leading to culture loss. Therefore, effective surface sterilization of explants is essential for the successful establishment of aseptic cultures [8]. Initial decontamination by washing explants under running tap water for 30 to 90 minutes proved effective in significantly reducing the surface microbial load and removing dust and debris. This step served as a primary cleansing treatment before chemical sterilization.

The use of a sterilizing solution containing Bavistin (2.5 g/L), Streptomycin (30 mg/L), and K-Cyclin (300 mg/L) for 30 to 60 minutes was found to be highly effective in reducing contamination in explants sourced from field-grown *Stevia rebaudiana* plants. Nodal segments required a longer exposure (up to 1 hour), whereas more delicate tissues such as leaf segments and shoot apices responded better to a shorter exposure time (30 minutes). These findings are consistent with earlier studies [13–15], which reported the effectiveness of combined antifungal and antibacterial treatments in controlling both fungal spores and bacterial contaminants during the initial sterilization phase [9]. Following chemical treatment, subsequent rinsing with autoclaved double-distilled water ensured the removal of any residual chemicals, minimizing phytotoxic effects on the explants. Additional surface sterilization with mercury chloride ($HgCl_2$) at concentrations of 0.05% to 0.10% for 3 to 7 minutes further reduced contamination rates while preserving tissue viability when handled under laminar airflow conditions. Overall, this multi-step surface sterilization protocol proved effective for minimizing microbial contamination and improving the aseptic establishment of stevia explants *in vitro* [10-12]. The protocol's efficiency is dependent on tissue type, chemical concentration, and exposure duration, all of which must be optimized to balance microbial control and explant viability.

Seasonal Variation in Explant Response

Explants surviving the initial establishment phase were subsequently used as the source material for in vitro multiplication experiments. It was observed that the month during which explants were collected significantly influenced shoot proliferation and bud induction, indicating a clear seasonal variation in explant responsiveness.

The highest bud induction rate (80.5%) was recorded from nodal explants collected during the months of June to August, coinciding with the monsoon season in the region. This was followed by a 54.8% bud break rate from explants collected between March and May. In contrast, explants obtained during December to February and September to November showed significantly lower responses, with only 31.9% and 40.22% bud break, respectively (Fig. 1). These findings are consistent with earlier studies [13-16], suggesting that seasonal environmental factors—such as temperature, humidity, and endogenous hormone levels—play a crucial role in determining the regenerative potential of explants. The summer to monsoon months may favor higher metabolic activity and endogenous cytokinin levels in the plant, enhancing its regenerative capacity.

Hardening and Acclimatization

In vitro-derived stevia plantlets were transferred to greenhouse conditions and subjected to a standardized hardening protocol consisting of two phases: primary hardening and secondary hardening. The highest survival rate (98.5%) was observed in plantlets acclimatized using this optimized protocol, outperforming earlier methods involving plastic pots with polyethylene covers or glass jars as reported by Singh et al. [17] and Alizadeh et al. [18].

One of the challenges during acclimatization is the lack of a well-developed epicuticular wax layer in in vitro-grown plantlets. This structural deficiency leads to uncontrolled foliar water loss when the plantlets are transferred from high-humidity culture vessels to ambient conditions. However, when plantlets are maintained under high-humidity conditions during the initial hardening phase, they begin to

synthesize a functional epicuticular wax layer. This adaptation significantly improves plantlet tolerance to desiccation and enhances overall survival during acclimatization [19]. Additionally, *in vitro*-derived plantlets tend to possess more stomata per unit area, along with raised guard cells and wider stomatal apertures, which collectively contribute to excessive transpiration and water loss under *ex vitro* conditions. These characteristics, as reported by earlier studies [20], further highlight the importance of a gradual transition to ambient conditions, allowing the plantlets to adjust physiologically and morphologically.

The current findings emphasize that, when the standardized acclimatization protocol outlined in this study is followed—particularly in terms of substrate composition, humidity control, and gradual light exposure—the survival rate of stevia plantlets can be significantly improved, reaching up to 98%. This advancement addresses one of the major limitations in stevia micropropagation and has substantial implications for the commercial-scale production of *Stevia rebaudiana*.

Conclusion

The present study highlights the importance of optimizing protocols for the in vitro propagation and acclimatization of *Stevia rebaudiana* Bertoni, a valuable natural sweetener with proven therapeutic benefits. Effective surface sterilization using a combination of Bavistin, streptomycin, K-Cyclin, and mercury chloride significantly minimized microbial contamination and ensured successful establishment of aseptic cultures. Seasonal variation was found to play a critical role in explant responsiveness, with the highest bud induction rates observed from nodal explants collected during the monsoon months (June–August). This suggests that physiological conditions influenced by seasonality greatly affect *in vitro* regeneration outcomes and should be considered in large-scale propagation planning, the study underscores the critical nature of the acclimatization phase, which remains a major bottleneck in micropropagation protocols. The optimized hardening procedure developed in this study—utilizing high humidity conditions, appropriate substrates, and gradual environmental exposure—resulted in a significantly improved survival rate of up to 98.5%. This high success rate addresses a key limitation in the commercial propagation of stevia and sets the foundation for enhanced scalability in field production, the findings from this investigation provide a robust and reproducible protocol for the successful *in vitro* propagation and acclimatization of *Stevia rebaudiana*. Adoption of these standardized techniques can not only improve propagation efficiency but also support the growing demand for stevia as a natural, non-toxic, and health-promoting sugar substitute in the global market.

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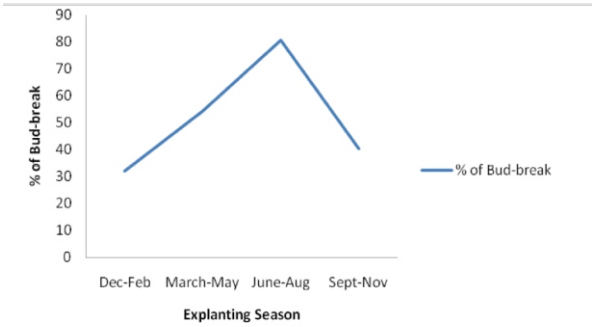

Fig. 1: Effect of Season on percent bud induction in nodal explants of *S. rebaudiana* on MS medium supplemented with BAP (0.5mg/l) + Kn (0.5mg/l)

Photo (1) Photo (2)

Photo (3) Photo (4)

Photo (1): Initial stage of primary hardening in cavities Photo (2): After two weeks of primary hardening in plastic tents Photo (3): secondary hardening in polythene bags Photo (4): After weeks of secondary hardening.

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