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# Biotechnology to Enhance Stevia Production: *In Vitro* and *In Vivo* Cloning, Sugar Content (Brix) Analysis, and Industrial Processing Characterization of Elite Germplasm

**Bipul K Biswas[1,*], Agnes J. Kapsoiyo[2]**

[1]Associate Professor, MS Biotechnology Program, College of Agriculture, Family Sciences and Technology, Fort Valley State University, Fort Valley, Georgia, USA
[2]Graduate Biotechnology Research Scholar, MS Biotechnology Program, Fort Valley State University, Fort Valley, GA, USA
*Corresponding author: biswasb@fvsu.edu

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**Abstract** *Stevia rebaudiana* (Bertoni), a natural, non-caloric sweetener, faces significant commercial hurdles due to poor seed viability, genetic variability in key traits, and challenges in post-harvest processing. This study aimed to select elite Stevia germplasm, develop an efficient mass multiplication protocol, and characterize the industrial handling properties of Stevia leaf powder. A high-biomass producing elite plant (7B15) and three high-germination seed lines (B, D, and P) were evaluated. An optimal *in vitro* surface sterilization protocol was established using 75% alcohol for 30 seconds followed by 10% sodium hypochlorite (NaOCl) for 10 minutes, achieving a 95% survival rate. For rapid growth, a robust micropropagation protocol was developed using Murashige and Skoog (MS) medium supplemented with 2 mg/L BAP, 0.5 mg/L Kin, and 0.1 mg/L Ad.S., which yielded the highest shoot proliferation at 3.47 shoots per explant. Successful rooting was subsequently achieved on MS medium supplemented with 0.5 mg/L NAA. Brix analysis, used as a proxy for steviol glycoside content, revealed that the elite plant cutting (SE-7B15) possessed the highest average Brix value (11.7 ± 1.63). Statistical analysis (ANOVA) indicated no significant difference in Brix values among the tested lines ($P$ = 0.182), confirming the stability of the high-sweetness trait across the selection. Furthermore, flow properties analysis using the Brookfield Powder Flow Tester indicated that Stevia leaf powder exhibited poor flowability across all moisture levels—a critical finding for industrial processing and storage. This research successfully establishes a reliable method for cloning elite Stevia plants while providing essential data on sweetness continuity and post-harvest powder characteristics, directly supporting the commercial production of high-quality Stevia products.

***Keywords:*** *Stevia rebaudiana, germplasm selection, micropropagation, clonal propagation, steviol glycosides, Brix, powder flowability, tissue culture*

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## 1. Introduction

### 1.1. Biotechnology and Plant Importance

Biotechnology is a critical field that utilizes living organisms—such as bacteria, yeast, or plant cells—to develop or modify products. These processes are essential across various sectors, including food processing, agriculture, and pharmaceuticals [1,2].

Plants are indispensable resources, serving as the primary source for numerous chemicals used in global industries. The most significant phytochemicals are produced through secondary metabolism [3]. Furthermore, plants generate various bioactive molecules, making them invaluable sources of medicine. Approximately 80% of the population in developing countries still relies on traditional plant-based medicine, highlighting their global therapeutic importance [4,5].

### 1.2. Stevia Rebaudiana: Background and Origin

Historically, human populations relied on honey and fruits for sweetness. However, the rise of cane and beet sugar in the 14th century introduced high-calorie sweeteners to the global diet, contributing to modern health risks such as obesity, diabetes, and hypertension [6].

*Stevia rebaudiana* (Bert.) is a small perennial herb that has gained international prominence as a premier source of natural, zero-calorie sweeteners [7]. The plant was first

introduced to Europeans in 1887 after M. S. Bertoni observed its unique properties being utilized by the Guarani Indians of Paraguay [8]. Initially classified as *Eupatorium rebaudianum* Bert., it was botanically described by Dr. Moises Santiago Bertoni in 1905 and named after Rebaudi, the chemist who first studied its chemical properties [9,10]. Later reclassified as *Stevia rebaudiana*, it is now commercially cultivated worldwide, with major production centers in Brazil, Paraguay, China, and Israel [11,12].

## 1.3. Classification, Description, and Composition

*Stevia rebaudiana* is a perennial shrub belonging to the Asteraceae family, typically growing up to 1 meter in height. The genus *Stevia* comprises approximately 154 species [13].

**Scientific Classification:**

- Kingdom: Plantae
- Division: Magnoliophyta
- Class: Magnoliopsida
- Order: Asterales
- Family: Asteraceae
- Genus: Stevia

**Figure 1.** Stevia plant (3 months old, ready to harvest) growing in the CAFST-FVSU research field

The plant contains a significant profile of nutrients, including proteins, fibers, essential oils, and vitamins such as ascorbic acid, beta-carotene, riboflavin, and thiamine. It also contains essential trace elements, including chromium, cobalt, iron, potassium, and phosphorus [14].

**Leaves and Floral Morphology:** The leaves are the primary commercial product. They are typically 3 to 4 cm long, oppositely arranged, and serrated above the middle [14,15]. *Stevia* is a photoperiod-sensitive, short-day plant, requiring a critical day length of approximately 13 hours to initiate flowering [8].

## 1.4. Steviol Glycosides (SGs): Sweeteners and Biosynthesis

*Stevia rebaudiana* is highly valued because its leaves accumulate steviol glycosides (SGs), which are 100 to 400 times sweeter than sucrose [15,16,17]. The major SGs, including stevioside and rebaudioside A, are derived from the tetracyclic diterpene steviol [18]. These compounds are primarily deposited in the leaves, with trace amounts in the stems and none in the roots [19,20,21].

**Biosynthesis** Steviol glycosides are synthesized via the methylerythritol 4-phosphate (MEP) and mevalonic acid pathways. The initial steps leading to SGs from geranylgeranyl pyrophosphate (GGPP) mirror those of gibberellin biosynthesis. GGPP is converted to ent-copalyl pyrophosphate (CPP) by CPP synthase, which is then transformed into ent-kaurene [22]. The pathways diverge when the product is oxidized to ent-kaurenoic acid, followed by hydroxylation at the C-13 position to yield steviol [49]. Finally, glycosylation—the addition of sugar units—at the C-19 and C-13 positions creates the sweet compounds stevioside and rebaudioside A [23,24].

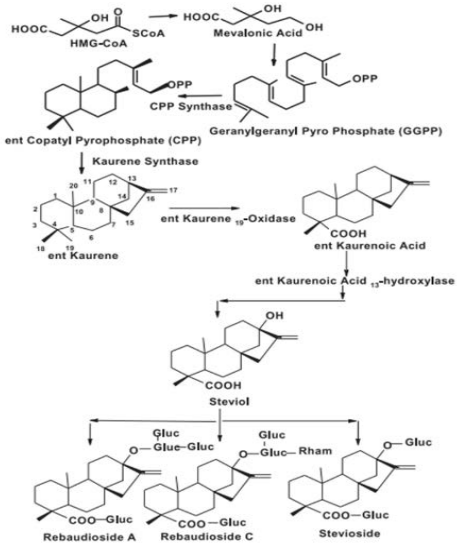


**Figure 2.** Stevioside biosynthesis [49]

## 1.5. Medicinal Properties and Acceptable Daily Intake (ADI)

Beyond its use as a sweetener, *Stevia* possesses therapeutic value in dental health, diabetes management (due to its hypoglycemic effects), and obesity control. It has also been used to promote the healing of burns and wounds [16]. Because these compounds are non-nutritive and pass through the digestive system without chemical breakdown, *Stevia* is a safe sugar alternative for individuals with diabetes or obesity [26].

The safety of steviol glycosides has been rigorously reviewed by the Joint FAO/WHO Expert Committee on Food Additives. The recommended Acceptable Daily Intake (ADI) is 4 milligrams per kilogram of body weight per day [4,25].

## 1.6. Challenges and Propagation

*Stevia* cultivation faces significant challenges, primarily regarding its reproduction:

1. **Poor Seed Viability:** *Stevia* seeds (achenes) are small, often infertile, and lack vigor. This results in low germination rates and a highly heterogeneous plant population, which is unsuitable for standardized commercial crops [10,11]. Such genetic variability necessitates specialized breeding

and selection strategies [27].

2. **Environmental Sensitivity:** The plant is highly sensitive to cold and drought. It requires specific conditions to thrive, including a semi-humid subtropical climate, moderate temperatures, and moist, sandy or loamy soil [20,28,29].

To overcome low seed viability and the limitations of traditional vegetative methods like stem cuttings, tissue culture (micropropagation) has become the ideal alternative. This method allows for the rapid, clonal mass production of genetically uniform, high-quality plants [30].

### 1.7. Research Objectives

This study aims to address current limitations in propagation and post-harvest handling through three primary objectives:

1. To develop an efficient protocol for both *in vitro* and *in vivo* clonal propagation of selected elite *Stevia* germplasm.
2. To screen and compare selected germplasm lines for the stability of high-sweetness traits using Brix analysis.
3. To characterize the industrial flow properties of *Stevia* leaf powder at various moisture levels to improve industrial handling and storage procedures.

## 2. Literature Review

### 2.1. Micropropagation and Shoot Multiplication

Research on *Stevia rebaudiana* has established robust protocols for rapid in vitro shoot proliferation. Nodal explants have demonstrated enhanced multiplication when cultured on Murashige and Skoog (MS) medium supplemented with 6-Benzylaminopurine (BAP) and Kinetin (Kn) [31]. The highest recorded response, averaging 3.42 shoots per explant, was achieved using MS medium containing 0.5 mg/L BAP and 2.0 mg/L Kn [31]. Furthermore, studies utilizing shoot tips observed maximum growth—defined by a 91.3% growth rate and a 3.8 cm shoot length—on MS medium supplemented with 0.5 mg/L BAP [32]. Beyond multiplication, successful in vitro protocols have also been documented for callogenesis and organogenesis using floral explants [3,33].

### 2.2. Root Induction

The induction of adventitious roots is a critical stage for successful micropropagation. Auxins are consistently identified as the most effective plant growth regulators for this process. Specifically, the optimal rooting response was observed on media containing 1.0 mg/L Indole-3-butyric acid (IBA) [31]. When compared to Indole-3-acetic acid (IAA) and Naphthaleneacetic acid (NAA), IBA demonstrated superior efficacy, achieving 100% root formation at concentrations of 0.5 mg/L and 1.0 mg/L [32].

### 2.3. Clonal Propagation (Stem Cuttings)

Clonal propagation via stem cuttings offers a rapid, non-sterile alternative for multiplying selected plant material. This technique is significantly improved by the application of auxins; for instance, dipping stem cuttings in a 1000 ppm IBA solution maximized the survival rate at 33% [15]. The efficacy of auxin treatment for rapid clonal establishment was further validated by observations that 500 ppm IBA resulted in the highest number of roots (24.0) and a maximum root length of 67.0 cm [15].

### 2.4. Methods of Sweetener Analysis

1. **High-Performance Liquid Chromatography (HPLC):** HPLC is considered the gold-standard technique for the reliable, sensitive, and accurate determination of steviol glycosides (SGs), such as stevioside and rebaudioside A [34]. This method is effectively employed to separate and quantify various SGs [35]. For example, HPLC analysis has been used to identify a stevioside content of 9.12% in Stevia aqueous extract (SAE) [25].
2. **Brix Analysis:** The use of a digital Brix refractometer provides a rapid, field-deployable method for quantifying total soluble solids. This serves as a practical proxy for the total sweetening compounds present in the leaves. The presence of traceable soluble sugars in Stevia leaves, which contribute to the overall Brix value, has been confirmed through this method [24]. It is noted that the final SG content is significantly influenced by both cultivation methods, such as intercropping, and propagation techniques, such as tissue culture [36].

### 2.5. Flow Properties of Powder Materials

The handling and storage of Stevia powder are critical factors in industrial processing.

1. **Flow Definition:** Flow refers to the relative movement of bulk particles, which is essential for efficiency in conveying, blending, and packaging [37].
2. **Influencing Factors:** Flowability is determined by powder characteristics—including particle size, shape, density, and moisture content—as well as environmental conditions [28,50].
3. **Mechanical Forces:** Flow occurs when gravitational forces exceed particle-to-particle interaction forces, specifically cohesion and friction [38].
4. **Characterization:** Flow properties are typically characterized using the flow index (FFc), cohesive strength, and the angle of internal friction [39]

## 3. Materials and Methods

### 3.1. Plant Material and Germplasm Selection

Seeds from ten distinct *Stevia* lines (A, B, C, D, F, G, H, P, R, and T) were sourced for this study. An elite, high-biomass *Stevia* plant (7B15), previously identified for its superior yield and high steviol glycoside content [5,10,40,41], was utilized for clonal propagation experiments. In addition to the elite line, seed lines B, D, and P were selected for further analysis based on prior

performance data [5,10,40,41].

## 3.2. Seed Germination Studies

Seed germination was evaluated under four distinct environmental conditions: (1) In vitro Petri Dish; (2) In vitro Magenta Box; (3) Laboratory Potted Soil (sand and Jiffy mix); and (4) Greenhouse Potted Soil (Table 1). Germination and growth parameters were monitored and recorded at 7-day intervals over a 21-day period.

**Table 1. Stevia seed germination rates under four environmental conditions after 21 days**

| Environmental Conditions | | | |
|---|---|---|---|
| *In vitro* | | Potted Soil | |
| Petri Dish | Magenta Box | Laboratory | Greenhouse |
| 19.67% | 2.5% | 10.56% | 3.0% |

## 3.3. In Vivo Propagation Via Cuttings

Young shoot cuttings were harvested from the 7B15 elite plant. These cuttings were treated with four concentrations of a commercial rooting hormone (Hormodin, 0.1% IBA) diluted to 5%, 10%, 15%, and 20% solutions. Following treatment, the cuttings were transplanted into plastic trays containing "Pro-mix" soil (Figure 3). Data regarding the number of shoots, leaf count, and total shoot height were collected 20 days post-planting.

**Figure 3.** *In vivo* propagation of elite stevia shoot cuttings and grown in the greenhouse condition

## 3.4. Micropropagation (*In Vitro*)

### 3.4.1. Media Preparation

Murashige and Skoog (MS) basal medium served as the primary culture medium. The medium was supplemented with 3% sucrose and specific Plant Growth Regulators (PGRs). The pH was adjusted to 5.8 before adding 0.7% agar or 0.3% Phytagel as a solidifying agent. All media were sterilized through autoclaving.

### 3.4.2. Surface Sterilization Protocol Development

To optimize explant survival, various surface sterilization treatments were tested. Explants were thoroughly washed and subjected to the following:

1. Sodium hypochlorite (NaOCl/Clorox) at concentrations of 5%, 10%, and 15% for durations of 5, 10, and 15 minutes.
2. 75% alcohol for 30 or 60 seconds.
3. Combination treatments of 75% alcohol followed by NaOCl (5% or 10%) for 5 to 10 minutes.

Contamination, necrosis, and survival rates were recorded for each treatment (Table 2).

**Table 2. Effect of different surface sterilization treatments on contamination, necrosis, and survival rates of *Stevia* explants**

| Treatment Agents Cl=Clorox, Al= Alcohol | Duration (min) | No. Explants | Contamination | Necrosis | Survival (%) |
|---|---|---|---|---|---|
| Cl(5%) | 5 | 20 | 18 | 0 | 10 |
| | 10 | 20 | 17 | 0 | 15 |
| | 15 | 20 | 14 | 5 | 30 |
| Cl(10%) | 5 | 20 | 5 | 0 | 75 |
| | 10 | 20 | 2 | 0 | 90 |
| | 15 | 22 | 0 | 10 | 54.5 |
| Cl(15%) | 5 | 20 | 0 | 9 | 55 |
| | 10 | 20 | 0 | 15 | 25 |
| | 15 | 20 | 0 | 15 | 25 |
| Al(75%) | 0.5 | 19 | 12 | 0 | 36.8 |
| Al(75%) | 1 | 20 | 4 | 8 | 60 |
| Al(75%) + Cl(5%) | 0.5 + 5 | 20 | 16 | 0 | 20 |
| Al(75%) + Cl(5%) | 0.5 + 10 | 20 | 6 | 0 | 70 |
| Al(75%) + Cl(10%) | 0.5 + 5 | 20 | 3 | 0 | 85 |
| Al(75%) + Cl(10%) | 0.5 + 10 | 20 | 1 | 0 | 95 |

### 3.4.3. Shoot Multiplication and Rooting

Nodal segments were employed for multiplication experiments (Figure 4). Various concentrations of 6-Benzylaminopurine (BAP) and Kinetin (Kin), ranging from 0 to 4 mg/L, were tested in combination with Adenine Sulfate (Ad. S.) to induce shoot formation. For root induction, regenerated shoots were transferred to MS medium supplemented with 0.5 mg/L of either Indole-3-butyric Acid (IBA) or Naphthalene Acetic Acid (NAA) (Figure 5).

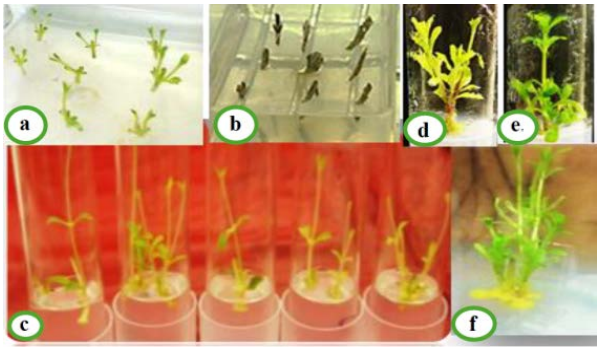


**Figure 4.** Various stages of *in vitro* clonal propagation of stevia from nodal segments. a-b. *In vitro* shoot bud induction (a) or necrosis in some media in Stevia nodal explants after 1 week of culture, c. Selected stevia line based on superior growth *in vitro*, d-f. Shoot multiplication from stevia nodal explant with (d) or without (e) base callusing and bacterial infection (f) after 6 weeks of culture

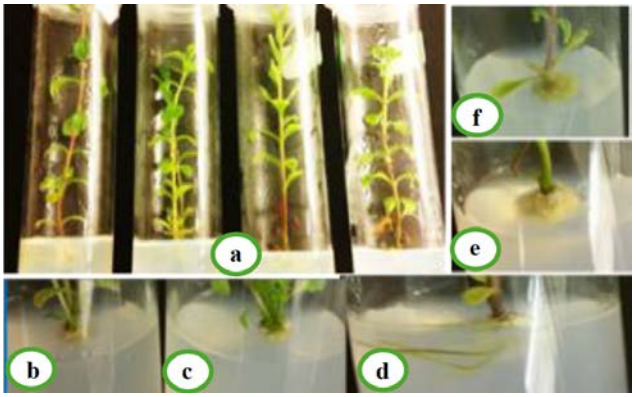


**Figure 5.** *In vitro* rooting of clonally propagated stevia shoots. a. *In vitro* grown stevia shoots ready for rooting, b-c. A small callus formation and multiple shoot development before root induction, d. Direct root induction without any callus, e-f. More callus formation without any root or shoot induction observed in a few combinations

### 3.5. Brix Measurement

Brix analysis was performed to estimate the soluble solid content in the leaves of the field-grown 7B15 elite plant, the greenhouse-grown elite cutting (SE-7B15), and the selected seed lines (B, D, and P).

1. Leaves were lysed using a TissueLyser II.
2. The resulting homogenates were centrifuged to separate the liquid phase.
3. The supernatant was analyzed using a calibrated Pocket Digital Refractometer.
4. All readings were recorded in triplicate to ensure accuracy.

### 3.6. Flow Properties of Stevia Powder

Dried *Stevia* leaves were processed into a fine powder. As the extraction of steviol glycosides is highly dependent on solvent interaction and particle surface area [42], the physical flow properties of the powder were characterized.

#### 3.6.1. Moisture Content Determination

Specific moisture levels (4.96%, 9.63%, 14.63%, 19.63%, 24.63%, and 34.63%) were achieved by adding distilled water to the powder. The required volume was calculated using the following standard equation for material moisture adjustment [43]:

$$\text{Moisture Content (\%)} = \frac{\textit{Initial Weight} - \textit{Final Weight}}{\textit{Initial Weight}} X\ 100$$

$$Q = \frac{\text{Wi(Mf} - \text{Mi)}}{100 - \text{Mf}}$$

Where:

- **Q**: Quantity of water added (g)
- **Wi**: Initial weight of the powder (g)
- **Mi**: Initial moisture content (%)
- **Mf**: Desired final moisture content (%)

#### 3.6.2. Flow Testing Procedure

A Brookfield Powder Flow Tester (PFT) was utilized to assess flowability [43]. A flow function test was conducted using a vane lid, and the resulting data were used to determine the flow index (FFc) according to the Jenike classification system (Table 3).

### 3.7. Statistical Analysis

All experimental data were subjected to a one-way Analysis of Variance (ANOVA) using Minitab 16 software. Statistical significance was defined at a P-value of less than 0.05.

## 4. Results and Discussion

### 4.1. Germplasm Selection and Seed Germination

The elite plant **7B15** was selected as the primary mother plant due to its documented high biomass and superior sweetness profile [5,10,40,41]. Germplasm selection focused on genotypes characterized by high yields and favorable steviol glycoside ratios [19,44].

After 21 days, seed germination rates (Table 1) remained low across all experimental conditions: **19.67%** (wet paper towel), **10.56%** (mixed soil in lab), **3.0%** (greenhouse), and **2.5%** (magenta boxes). These results underscore the limitations of seed-based propagation for this species and highlight the necessity of developing efficient vegetative propagation methods [26].

### 4.2. Micropropagation Protocol Development

#### 4.2.1. Optimization of Surface Sterilization

The most effective sterilization protocol was a sequential treatment involving **75% alcohol for 30 seconds followed by 10% NaOCl for 10 minutes**, which yielded a maximum survival rate of **95%** (Table 1). Establishing this efficient procedure is a critical prerequisite for successful micropropagation and regeneration [45].

#### 4.2.2. Shoot Multiplication

The optimized medium consisted of **MS medium supplemented with 2 mg/L BAP, 0.5 mg/L Kin, and 0.1 mg/L Ad.S**. This specific combination produced the highest frequency of shoot proliferation, averaging **3.47 shoots and 20.21 leaves per explant** (Figure 6).

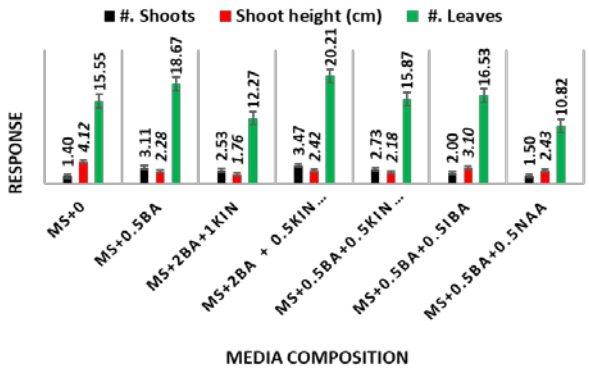


**Figure 6.** Graph of *in vitro* multiplication responses of stevia shoots on various media combinations

Among the tested seed lines, one **Line** exhibited the most robust shoot response (Figure 7d), suggesting significant genetic variation *in vitro* culture performance [3]. Growth patterns across different stevia lines were further evaluated based on average leaf number and shoot height (Figure 7 and Figure 8).

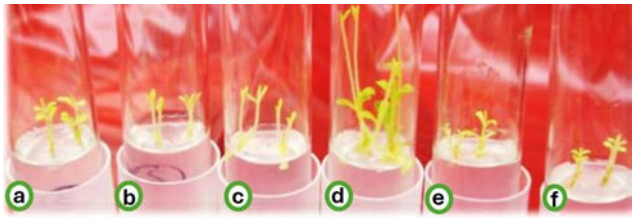


**Figure 7.** Visual representation of *in vitro* shoot growth of stevia seed lines showing one line (d) performed better than all other lines (a-f)

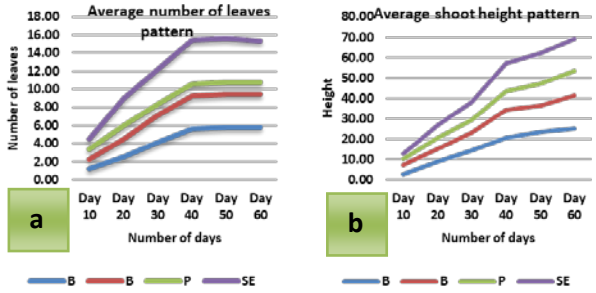


**Figure 8.** Growth charts (a-b) of leaf (a) of stevia lines and plant height(b) in the Specialty Plants House (SPH)

### 4.2.3. Rooting

Successful root induction was achieved using MS medium supplemented with either **0.5 mg/L NAA or 0.5 mg/L IBA**. While NAA promoted robust root formation, it also induced callus development at the cut end. In contrast, IBA induced fewer roots but resulted in minimal callus formation [29].

Vegetative propagation of the elite plant (7B15) via stem cuttings (Figure 9) proved highly effective [4]. Data collected at 10 and 20 days regarding shoot number, height, and leaf count confirm that clonal propagation is a viable and rapid method for multiplying elite material for field trials [5,10,40,41].

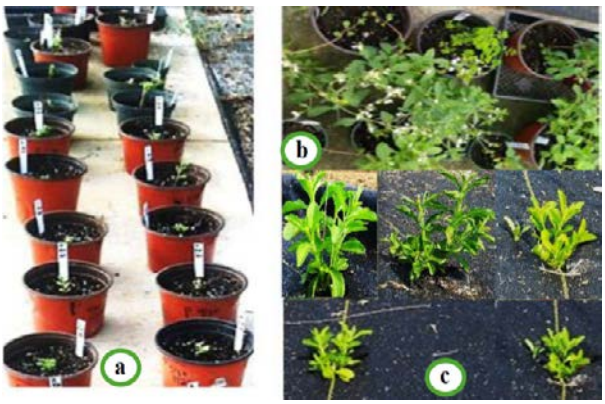


**Figure 9.** The elite stevia plant developed from cuttings (a) growing in the pot (b) and in the field (c) used black ground cover to control weeds

## 4.3. Brix Analysis

Brix measurement is an efficient field-based proxy for estimating total steviol glycoside (SG) content [44]. The highest average Brix reading (**11.7 ± 1.63**) was recorded in elite cuttings grown in the Specialty Plants House (SE-7B15) (Figure 10).

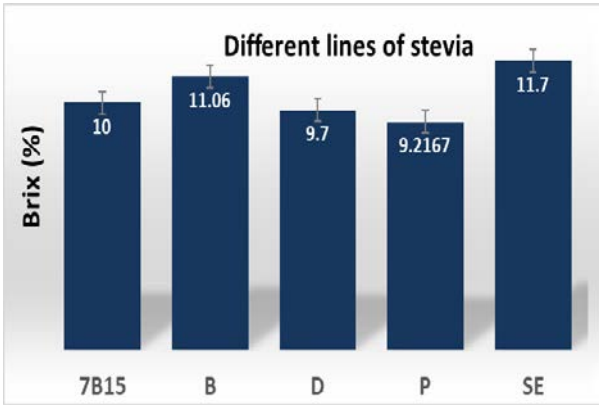


**Figure 10.** Figure showing levels of brix from different lines of stevia

A one-way ANOVA revealed no significant difference in Brix content between the elite clone and the selected seed lines (**P = 0.182**). This finding confirms that high-sweetness traits are stable and successfully maintained through clonal propagation [10,40,41,46].

## 4.4. Effect of Moisture on Stevia Powder Flowability

Analysis using the **Brookfield Powder Flow Tester (PFT)** indicated that stevia leaf powder exhibits poor flowability across all tested moisture levels (Table 4). The **Flow Function Curve (FFc)** (Figure 11) illustrates this behavior, which is likely attributed to small, irregular particle sizes and high moisture retention [43].

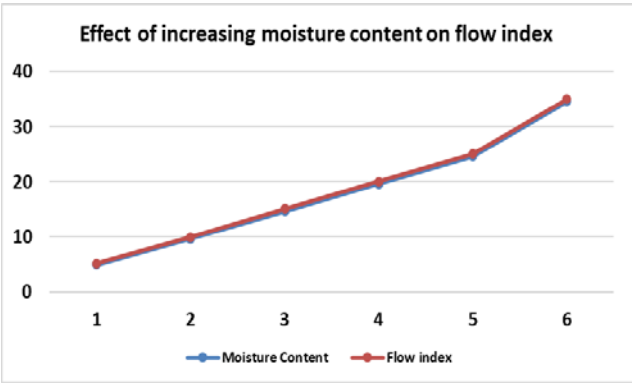


**Figure 11.** Figure showing effect of increasing moisture content on flow index

According to the **Jenike classification system** (Table 3), this poor flowability is characteristic of cohesive powders, posing a challenge for industrial handling. Notably, while physical flow is restricted, the antioxidant properties of the powdered leaves may be retained throughout the processing stages [47].

**Table 3. Jenike classification of powder flowability by flow index**

| Flow Index | Flowability |
|---|---|
| < 1 | Hardened |
| < 2 | Very cohesive |
| < 4 | Cohesive |
| < 10 | Easy flow |
| > 10 | Free Flowing |

**Table 4. Flow Parameters of Stevia Powder**

| Moisture Content (%) | Angle of internal friction (°) | Effective angle of internal friction (°) | Cohesion (kPa) | Flow index |
|---|---|---|---|---|
| 4.96 | 41.13±0.45 | 47.80±2.17 | 0.002 | 0.27±0.01 |
| 9.63 | 42.1±0.10 | 51.87±1.11 | 0.012 | 0.30±0.01 |
| 14.63 | 44.6±0.27 | 52.30±2.46 | 0 | 0.47±0.02 |
| 19.63 | 46.3±0.10 | 52.50±2.86 | 0 | 0.48±0.05 |
| 24.63 | 44.1±0.51 | 47.67±2.52 | 0 | 0.48±0.05 |
| 34.63 | 43.6±0.27 | 49.57±1.65 | 0 | 0.41±0.03 |

### 4.5. Importance of Stevia for Health

Stevia serves as a vital natural sugar substitute due to its non-caloric and bioactive properties. Steviol glycosides (SGs) are non-nutritive and possess a **Glycemic Index (GI) of 0** [5,46]. Furthermore, SGs—particularly stevioside—have demonstrated therapeutic potential in lowering blood glucose and reducing blood pressure [48].

## 5. Conclusions

This study successfully addresses several critical challenges currently facing the commercial production of *Stevia rebaudiana*. The findings provide a comprehensive framework for scaling production while maintaining quality:

1. **Safety and Sweetness Stability:** The research confirms that *Stevia* serves as a safe and effective natural sugar substitute. Specifically, the elite germplasm **7B15** demonstrated consistently high Brix levels, proving that high-sweetness characteristics remain stable throughout the cloning process.
2. **Mass Multiplication Protocol:** A reliable *in vitro* mass multiplication protocol was developed to bypass the inherent difficulties of low seed germination and genetic variability. This system enables the rapid, clonal production of high-quality elite plants on a commercial scale.
3. **Industrial Handling Requirements:** Analysis of physical properties revealed that *Stevia* powder exhibits poor flowability across all tested moisture levels. This insight highlights a critical need for specialized post-harvest handling and storage infrastructure to ensure efficient industrial processing.

The outcomes of this research establish a vital foundation for the commercial cloning of superior *Stevia* germplasm. By addressing both biological and mechanical constraints, these findings ensure a consistent supply of high-quality raw material for the global natural sweetener industry.

## ACKNOWLEDGEMENTS

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