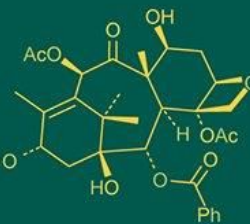
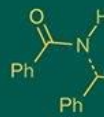
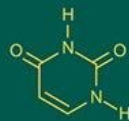
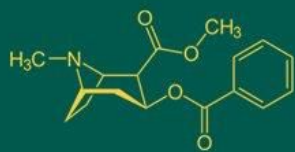


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Overview of in-vitro regeneration on *Stevia (Stevia rebaudiana bertonii)*

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Abstract

Stevia (Stevia rebaudiana Bertonii) in abstract Stevioside and rebaudioside A, two natural, calorie-free sweetness agents that are significantly sweeter than sucrose, make *bertoni* an economically important medicinal herb. *Stevia* must be cultivated on a large and daily basis to meet the increasing market demand of the food, pharmaceutical, and nutraceutical industries. However, conventional approaches such as seed germination and stem cuttings are hampered due to low germination rates and genetic and biochemical variability. Within the aforementioned scenario, plant tissue culture techniques have proven to be an effective option for the rapid and disease-free micro propagation of plant materials truly representative of the parental type. This review article attempts to bring out a complete overview of the recent developments of the *in vitro* regeneration of *Stevia rebaudiana* plants. Several approaches related to explant materials such as nodal segments, shoot apices, leaves, petioles, and thin cell layers of *Stevia* plants have been studied. Of these, nodal segments and shoot tips have exhibited higher regeneration efficiencies and genetic stability on consistent basis. The MS medium with appropriate concentrations of cytokinins alone or in combination, such as BAP, kinetin, or TDZ, in addition to auxins like IAA, IBA, or NAA, has been widely reported as the most effective medium for shoot multiplication and rooting. Balance of growth regulator has also been emphasized as one of the critical factors for controlling morphogenic responses, rooting efficiency, and acclimatization success. Recent studies related to hardening and field establishment have also reported high survival rates when proper acclimatization protocols are followed. In the context of mass propagation, germplasm conservation, and genetic improvement of *Stevia rebaudiana*, the *in vitro* technique of regeneration is an environment-friendly and commercially workable approach. Further optimization of protocols with integration of molecular tools would improve propagation efficiency and quality assurance.

Keywords: *Stevia rebaudiana bertonii*, *in vitro* regeneration, tissue culture, micro propagation, plant growth regulators, MS medium, organogenesis

Introduction

Stevia rebaudiana Bertonii is a small, perennial, herbaceous, non-climbing shrubby plant of the Asteraceae family, which originally inhabited the borderline between Paraguay and Brazil. However, what makes *Stevia's* popularity skyrocket is the fact that, owing to its compounds Stevioside and Rebaudioside A, *Stevia* is at least 300-400 times sweeter than sugar but at the same time provides almost no calories at all, as said in Lemus-Mondaca *et al.*, 2012 [25]. *Stevia* is currently the "first choice" sugar replacement, particularly for diabetics and consumers on diet, as Goyal *et al.*, 2010 [16], said. However, in the past decades, *Stevia* has been well established in the global *Stevia* market. The rising demand for plant-based, low-calorie sweeteners contributes significantly to the increasing popularity of *Stevia*. Simultaneously, the demand for *Stevia* is also being driven by the health awareness of the population, who are increasingly demanding plant-based, low-calorie sweeteners. The World Health Organization (WHO) and the FDA have approved rebaudioside A, a derivative of *Stevia*, intensifying the public's demand for *Stevia*. Market research indicates that the global *Stevia* market is expected to reach a financial value of over 1 billion dollars by 2025. The demand for *Stevia* arises mainly from sugar-free foodstuffs. Apart from being an edulcorant component, *Stevia* is being recognized for its medicinal benefits. *Stevia* plays a crucial role in the management of blood sugar levels, lowers blood pressure, and possesses anti-inflammatory and antimicrobial actions.

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However, its commercial propagation in bulk is not that easy despite its commercial potential. While traditional methods of propagation, such as seeds, have normally resulted in very low germination rates of less than 20%, the scaling up of plants through stem cuttings has a limited extent. The plant is naturally heterozygous, meaning it has too much genetic diversity, which makes it difficult to continue uniformity in field-grown plants.

The Role of Tissue Culture

It is a sure way to grow large amounts of healthy and identical tissue cultures within a short time in a controlled environment. It is a sure way for the farmer to fulfil the demands of the market while ensuring that the tissue cultures produced are healthy and the same in sweet taste. The callus culture, organogenesis, and somatic embryogenesis techniques have been successfully used for various explants such as the leaves, stem nodes, petioles, and shoot tips of the *Stevia* plant (Mannan *et al.*, 2008 and Thiagarajan & Venkatachalam, 2012) [26, 47].

Tissue culture is obviously not just for cloning plants! It is also a way of improving plants. Scientists were able to add beneficial mutants and even increase levels of sweetness using tissue culture. It is also integral in long-term conservation work, particularly in *in vitro* storage of elite germplasm (Gantait *et al.*, 2015) [14].

Although the science involved has advanced significantly over the past decades, there are a few hurdles to clear in the process. For instance, achieving the development of roots, limiting the amount of genetic alteration in tissue culture, and assisting young seedlings with the transition from culture to outdoor environments pose a challenge. Nonetheless, tissue culture is an invaluable technique in the cultivation and improvement of *Stevia Rebaudiana*, and with the further refining and perfecting of techniques such as the adjustment and perfecting of medium and hormone concentrations, the technique is bound to become even more effective.

Explant

In plant tissue culture, an explant generally refers to a small piece of plant tissue taken from a parent plant for the purpose of generating a new plant under sterile and controlled conditions. The explants may be taken from different parts of the plant such as leaves, stems, nodes, roots, shoot tips, and other more specialized tissues. Explants are the starting materials for any *in vitro* regeneration process. Sterilized to remove all microorganisms, they are placed on nutrient-rich culture mediums inside growth chambers or culture bottles. The environment around the explant provides just the right mix for its growth and divisions: it provides nutrients, hormones such as auxins and cytokinins, light, and optimum temperature for its growth and its further development into a whole new plant.

The explant of choice also plays a very important role in determining the degree of success of the regeneration process. A typical case is that nodular segments are generally chosen if there is a need for direct regeneration of shoots because of the active meristematic tissue present. Alternatively, leaves or petioles can be chosen if the objective of regeneration includes the formation of callus or indirect regeneration. All explants have their own regeneration power.

To recapitulate, explants serve as a foundational establishment of plant tissue culture, comprising both the

genetic blueprints and tissues which make it possible for a scientist to clone plants, conserve rare species, and even genetically engineer them.

Common Explants Used

Several parts of the *Stevia* plant have shown promise in tissue culture, and each has advantages over the others based on the regeneration system used and the purpose for the culture.

- Nodal segments often account among the explants that are commonly employed in explanation of direct shoot regeneration. These explants actively exhibit natural meristematic activity in response to culture *in vitro* environments.
- Shoot tips have been identified as the best parts for use in this case because they can efficiently regenerate with less potential for soma clonal variation (Singh *et al.*, 2011) [37].
- Leaves are often employed in experiments for the induction of callus formation and somatic embryogenesis. The ability of the explant to form callus makes it suitable for conducting experiments in indirect regeneration (Thiagarajan and Venkatachalam, 2012) [47].
- Petioles/internodes are also used in tissue culture media, particularly when tissue regeneration is to be achieved via callus or when conducting genetic transformation experiments, as stated in Gantait *et al.* (2015) [14].

It should be noted that explants of different types have shown distinctive sensitivities towards culture conditions, particularly the kinds of growth regulators used.

Explants Used in *Stevia*

A number of explants have been used for regeneration through *in vitro* culture of *Stevia rebaudiana*; however, their morphogenic response is unique in regard to the explants employed and the culture medium used, considering its supplementary ingredients if any. Among these explants, nodal segments have shown absolute significance for regeneration in most scientific research. For example, the study conducted by Ahmed *et al.* (2007) and Debnath (2007) [1, 10] indicates that MS medium enriched with BA and IAA is quite productive for regeneration. Furthermore, the research articles contributed by Lata *et al.* (2013) and Mehta *et al.* (2012) [24, 28] showed that MS medium supplemented with either BAP or TDZ was productive for regeneration, while rooting was possible through half-strength MS medium supplemented with IBA.

On the contrary, in the hands of scholars such as El-Motaleb, *et al.* 2013, Guruchandran Sasikumar 2013, Javad, *et al.* 2013 [11, 17, 19, 35], for example, BAP with either kinetin or spermine significantly stimulated shoot induction from shoot tips. Even though fewer shoots were recorded, shoot tips were associated with rapid regeneration as opposed to nodal segments.

For leaf explant and leaflets, most results are less consistent. According to Alhady (2011) [4], it exhibited fair shoot formation when it was placed on medium supplemented with BAP and IAA. Moreover, shoot primordia formation and callus development on media supplemented with BA and kinetin, as reported by Akita *et al.* (1994) and Sreedhar (2008) [3, 44], respectively, indicate the potential use of leaf tissue or leaflets to check callus formation or for transformation, albeit less efficiently.

Thin cell layers, a new but not frequently used explant type, have indicated promising potential in regeneration in response to powerful auxins like 2,4-D, as indicated in investigations performed by Chen and Li (1993) [17] and Ramírez-Mosqueda *et al.* (Iglesias-Andreu 2016) [34]. This explant type indicated potential in somatic embryogenesis, particularly on MS medium supplemented with BA and 2,4-D, pointing to the concept of totipotency of undifferentiated tissues.

Stem cuttings containing leaf primordia, used by Labiri (2012) [23], are found to be effective. High concentrations of kinetin were found to be effective. Though concentrations of cytokinins are used to a lower degree, their effectiveness cannot be ignored. Midribs used by Sreedhar (2008) [44] are found to be effective for applications of BA and kinetin as alternatives.

A significant similarity between the experiments is the effectiveness of auxin and cytokinin combinations, especially the combination involving the use of BAP along with either

IAA or NAA in shoot regeneration. Rooting has been most commonly induced using IBA either alone or supplemented with half-strength MS medium to facilitate the reduction of salt toxicity accompanying root induction. Of significant interest are the experiments carried out by Modi *et al.* (2012) and Singh and Dwivedi (2013) [31, 39]. These researchers utilised activated charcoal for enhancing root formation and utilised TDZ combined with very low concentration for shoot formation and IBA for root formation, respectively.

Accordingly, it may be summarily stated that in spite of nodal segments giving the best results in terms of direct shoot regeneration, shoot tips have established their efficiency in facilitating rapid growth coupled with genetic uniformity. While the response from leaf, midrib, and thin cell layer explants varies, these have been shown to be useful in attaining very precise targets. The comparative investigations have confirmed in general terms the need to carefully optimize PGRs in *in vitro* regeneration of *Stevia rebaudiana*.

Table 1: Regeneration Efficiency from Different Explants in *Stevia rebaudiana* Bertoni

S. No	Scientist	Explant Used	Media Used	Growth Regulators Used	Key Findings	Reference
1	Ahmed <i>et al.</i> (2007)	Nodal Segments	MS medium	2.0 mg/L BA + 1.13 mg/L IAA	High shoot proliferation via direct regeneration	Ahmed <i>et al.</i> , 2007 [1]
2	Alhady (2011)	Leaf & Nodal Segments	MS medium	0.5 mg/L BA + 0.5 mg/L IAA	Nodal segments showed better regeneration than leaves	Alhady, 2011 [4]
3	Akita <i>et al.</i> (1994)	Leaflets	MS medium	1.0 mg/L BA + 0.1 mg/L NAA	Shoot primordia formed from leaflets	Akita <i>et al.</i> , 1994 [3]
4	Chen & Li (1993)	Thin Cell Layers	MS medium	6.78 µM 2,4-D	Organogenesis achieved from undifferentiated tissue	Chen & Li, 1993 [7]
5	Debnath (2007)	Nodal Segments	MS medium	BA + IAA	Clonal propagation with multiple shoots	Debnath, 2007 [10]
6	El-Motaleb <i>et al.</i> (2013)	Shoot Tips & Nodal Segments	MS medium	1.0 mg/L BAP + 2.0 mg/L Kin	Uniform shoot multiplication; rooting with 0.5 mg/L IBA	El-Motaleb <i>et al.</i> , 2013 [11]
7	Guruchandran Sasikumar (2013)	Shoot Tips	MS medium + Spermine	1.5 mg/L BAP + 10 mg/L Spermine	Efficient shoot and root regeneration using polyamines	Sasikumar, 2013 [17, 35]
8	Hassanen (2013)	Shoot Tips & Nodal Segments	MS medium	2.0 mg/L BAP	Shoot tips gave faster responses	Hassanen, 2013
9	Hwang (2006)	Nodal Segments	MS medium	2.0 mg/L IAA + 0.5 mg/L Kin	Good shoot regeneration and rooting with 2.0 mg/L IBA	Hwang, 2006
10	Javad <i>et al.</i> (2013)	Shoot Tips	MS medium	1.0 mg/L BAP	High regeneration potential; rooting with 0.4 mg/L IBA	Javad <i>et al.</i> , 2013 [19]
11	Labiri (2012)	Stem Tips with Leaf Primordia	MS medium	10 mg/L Kinetin + 0.1 mg/L NAA	Shoot induction from stem tips	Labiri, 2012 [23]
12	Lata <i>et al.</i> (2013)	Nodal Segments	MS medium (½ MS for rooting)	1.0 µM TDZ; 1.0 mg/L IBA	Multiple shoots; rooting on ½ MS medium	Lata <i>et al.</i> , 2013 [24]
13	Mehta <i>et al.</i> (2012)	Nodal Segments	MS medium	0.5 mg/L BAP + 2.0 mg/L Kin; 1.0 mg/L IBA	Best shoot and root induction	Mehta <i>et al.</i> , 2012 [28]
14	Modi <i>et al.</i> (2012)	Nodal Segments	MS medium + Activated Charcoal	BAP + Kin; IBA	Better rooting and shoot formation	Modi <i>et al.</i> , 2012 [31]
15	Nower (2014)	Nodal Segments	MS medium	0.2 mg/L IAA	Successful micro propagation	Nower, 2014
16	Ramírez-Mosqueda & Iglesias (2016)	Thin Cell Layers	MS medium	BA + 2,4-D	Organogenesis achieved; 2,4-D effective	Ramírez Mosqueda & Iglesias, 2016 [34]
17	Singh & Dwivedi (2013)	Nodal Segments	MS medium	0.01 mg/L TDZ; 1.0 mg/L IBA	Multiple shoot and root formation	Singh & Dwivedi, 2013 [39]
18	Soliman <i>et al.</i> (2013)	Nodal Segments	MS medium	BAP + NAA; 0.5 mg/L IAA	Efficient shoot and root development	Soliman <i>et al.</i> , 2013
19	Sreedhar (2008)	Midrib	MS medium	BA + Kin; 1.0 mg/L IBA	Shoot induction and rooting	Sreedhar, 2008 [44]
20	Venkatachalam & Thiyagarajan (2012)	Nodal Segments	MS medium	BA + NAA	Successful large-scale propagation	Venkatachalam & Thiyagarajan, 2012 [47]

Culture media: Similarly, it can be understood that, in plant tissue culture, the medium cultivates an artificial environment, providing all the essential nutrients, vitamins, and energy required for plant cell survival, growth, and regeneration. It can also be stated here that it is extremely important to select an appropriate medium, as it can influence the successful plant regeneration, including shoot multiplication, callus culture, or root development.

In the case of *Stevia rebaudiana*, the regeneration process is known to be very sensitive to the type and content of the basal medium, plant growth regulators, and even minor changes in the pH and gelling agents. With the advancement of years, culture media too have undergone different trials and experiments to determine the best suited culture medium for plantlet development and regeneration.

Types of Culture Media

1. Murashige and Skoog MS: MS medium is commonly accepted as the basic standard medium for *Stevia* tissue culture. It contains all the necessary macro- and micro stress, vitamins such as thiamine-HCl, pyridoxine-HCl, and nicotinic acid. In addition, it contains 3% sucrose as the carbon source (Das *et al.* 2011; Gantait *et al.* 2015). Authors such as Ahmed *et al.* (2007)^[1, 8], and others such as Lata *et al.* (2013)^[14, 24], used full strength MS for regenerating shoots, while others used it for root induction.

2. Half-Strength MS Medium: Furthermore, high salt concentrations are counteractive measures. In fact, most media accounts for this by using half-strength MS medium, especially in rooting cultures. Lata *et al.* (2013)^[24], for instance, reported successful root induction by medium supplemented with IBA, but in half-strength MS medium.

3. MS Medium with Organic Additives

Apart from macronutrients, some media contain organic substances such as myo-inositol, casein hydrolysate, and coconut water that support cell tissue regeneration. Activated charcoal is also often employed for phenolic absorption. This increases medium transparency and minimizes problems associated with medium use (Sasikumar, 2013)^[17].

4. MS Medium Fortified with Growth Regulators

Media and additives most commonly incorporated are MS medium with cytokinin additives like BAP, kinetin, and TDZ along with auxin additives like IAA, IBA, and NAA, which vary depending upon the explant and the culture medium requirement. Phosphorous acid and BAP are used together for shoot formation, and additives like IBA and NAA are required for rooting (Mehta *et al.*, 2012; Singh & Dwivedi, 2013)^[28, 39].

5. Other Media Types: Though MS medium reigns supreme in terms of culture media for *Stevia* research, some have also tried other alternatives like Gamborg's B5 medium, White's medium, or Woody Plant medium. Though these have shown relatively lower efficiency than MS medium, MS medium still stands superior due to efficiency in various genotypes and explant material as studies by Gantait *et al.* (2015) show^[14].

The culture medium used for the *In vitro* Regeneration of *Stevia rebaudiana*: The most commonly employed medium in *Stevia rebaudiana* tissue culture practices, based on its

nutrient composition and applicability to various explants and stages of regeneration, has been found to be Murashige and Skoog (MS) medium, introduced in 1962. MS medium at full-strength, in combination with suitable plant growth regulators, has been found to give good results regarding tissue regeneration at a regular frequency. To cite a fact, it has been found that MS medium supplemented properly, containing BAP and IAA as plant growth regulators, produced good regeneration outcomes in *Stevia rebaudiana* plantlets, as was evident in one of its successful uses reported in researches conducted by Mohd. Umar *et al.* (2011). Similarly, Gantait *et al.* (2015) and Sivaram & Mukundan (2003)^[14, 42] reported analogous findings in their respective researches.

Other researchers have attempted various combinations of cytokinins and auxins to promote certain regeneration events. Das *et al.* (2011), Singh and Dwivedi (2013), and Singh *et al.* (2011)^[8, 37, 39] employed combinations of BAP and NAA, resulting in efficient regeneration of multiple shoots, especially when explants of nodal and shoot tip origin were cultured on these media combinations.

On the other hand, Sha Valli Khan *et al.* (2005) and Rafiq *et al.* (2007)^[33, 36] demonstrated the efficient regeneration of shoots and roots when explants were cultured on media containing combinations of BS, IBA, and NAA, although the use of IBA was later demonstrated to facilitate root regeneration, essentially due to the ability of IBA to prevent osmotic stress, as shown by Lata *et al.* (2013)^[24], who employed half-strength MS medium containing IBA for root regeneration. Different researchers have studied innovative modifications related to media for the improvement and fine-tuning of shoot organogenesis. For instance, Venkatachalam & Thiagarajan (2012), and also Khiraoui *et al.* (2017)^[22], have shown that the addition of the potent cytokinin-like compound, TDZ, to the MS medium has the potential for enhancing shoot regeneration, and at the same time, the browning was minimized. Another hormone, Thiagarajan & Venkatachalam (2012)^[47], consisted of the TDZ and NAA combination for the induction of somatic embryogenesis in leaf explants. Non-hormonal organic compounds have also shown potential for improvement in shoot regeneration, such as the addition of the polyamine compound, which has the potency for enhancing shoot regeneration, such as Sasikumar (2013)^[17].

Other hormonal combinations such as BAP with kinetin were tried by Misra *et al.* (2010) and Modi *et al.* (2012)^[31], which gave good results with shoot proliferation from petiole explants. Kinetin in combination with IAA was chosen by Debnath (2008)^[9], who reported effective shoot multiplication from apical and axillary buds. Ibrahim *et al.* (2008) and Faria *et al.* (2006)^[13, 18] used GA₃ in combination with BAP for better shoot elongation and growth-a very important step in the establishment of the plantlets.

In short, MS medium-full or half strength-acts as a powerful base for *Stevia* tissue culture. Its performance essentially depends on the choice and balance of plant growth regulators. BAP is still the cytokinin of choice for shoot induction, but auxins such as IAA, NAA, and IBA take into account the important synergisms in place needed to carry this event through the complete regeneration cycle. Enhancements by additives, such as TDZ, spermine, or GA₃, further refine the protocol efficiencies and make MS medium, with slight modification, a versatile base for many different strategies of regeneration in *Stevia rebaudiana*.

Table 2: Media Compositions and Regeneration Efficiency

Scientist	Culture Medium Used	Outcome	Reference
Murashige & Skoog (1962)	MS medium	Developed the MS medium, now most widely used for <i>Stevia</i> tissue culture	Murashige & Skoog, 1962 ^[32]
Ahmed <i>et al.</i> (2007)	Full-strength MS + BAP + IAA	High-frequency shoot induction from nodal explants	Ahmed <i>et al.</i> , 2007 ^[11]
Das <i>et al.</i> (2011)	MS + BAP + NAA	Efficient shoot regeneration and multiple shoot proliferation	Das <i>et al.</i> , 2011 ^[8]
Mehta <i>et al.</i> (2012)	MS + BAP + IAA	Best shoot regeneration (89%) from nodal segments	Mehta <i>et al.</i> , 2012 ^[28]
Lata <i>et al.</i> (2013)	Half-strength MS + IBA	High rooting frequency from regenerated shoots	Lata <i>et al.</i> , 2013 ^[24]
Singh & Dwivedi (2013)	MS + BAP + NAA	Efficient multiple shoot formation	Singh & Dwivedi, 2013 ^[39]
Gantait <i>et al.</i> (2015)	MS + BAP + IAA	High shoot regeneration and plantlet conversion	Gantait <i>et al.</i> , 2015 ^[14]
Sasikumar (2013)	MS + spermine	Improved regeneration from shoot tip explants	Sasikumar, 2013 ^[17]
Venkatachalam & Thiyagarajan (2012)	MS + TDZ	Enhanced shoot organogenesis and reduced browning	Venkatachalam & Thiyagarajan, 2012 ^[47]
Misra <i>et al.</i> (2010)	MS + BAP + Kin	High frequency shoot multiplication from petiole explants	Misra <i>et al.</i> , 2010
Sivaram & Mukundan (2003)	MS + BAP + IAA	Successful regeneration via nodal and leaf explants	Sivaram & Mukundan, 2003 ^[42]
Sha Valli Khan <i>et al.</i> (2005)	MS + BAP + IBA	Effective shoot proliferation and subsequent rooting	Sha Valli Khan <i>et al.</i> , 2005 ^[36]
Singh <i>et al.</i> (2011)	MS + BAP + NAA	High regeneration from shoot tip explants	Singh <i>et al.</i> , 2011 ^[37]
Thiyagarajan & Venkatachalam (2012)	MS + TDZ + NAA	Efficient somatic embryogenesis from leaf explants	Thiyagarajan & Venkatachalam, 2012 ^[47]
Debnath (2008)	MS + Kin + IAA	Shoot multiplication using apical and axillary buds	Debnath, 2008 ^[9]
Ibrahim <i>et al.</i> (2008)	MS + BAP + GA ₃	Improved elongation and shoot development	Ibrahim <i>et al.</i> , 2008 ^[18]
Sivanesan <i>et al.</i> (2009)	MS + BAP + NAA	Shoot proliferation and callus induction from leaf explants	Sivanesan <i>et al.</i> , 2009
Khiraoui <i>et al.</i> (2017)	MS + TDZ	High number of shoots from nodal explants	Khiraoui <i>et al.</i> , 2017 ^[22]
Rafiq <i>et al.</i> (2007)	MS + BAP + IBA	Strong rooting and shoot elongation	Rafiq <i>et al.</i> , 2007 ^[33]
Faria <i>et al.</i> (2006)	MS + BAP + GA ₃	Good shoot multiplication and plantlet growth	Faria <i>et al.</i> , 2006 ^[13]

Role of Growth Regulators in *Stevia rebaudiana* In vitro Regeneration: Plant Growth Regulators (PGRs) are organic chemical compounds, either naturally occurring or artificial, which have a significant impact on plant physiology at low concentration. PGRs are of great concern as far as plant physiology is concerned. PGRs are major components used in plant tissue culture media, without which the probability of successful morphogenesis, callus formation, organogenesis, and embryogenesis would not be significant (George *et al.*, 2008)^[15].

Recently, emphasis has come to be given to PGR application in relation to micro propagation and *in vitro* regeneration of plants such as *Stevia rebaudiana*, which contains non-caloric sweetening agents such as Steviol Glycosides.

Types of Plant Growth Regulators and Their Specific Functions: There are five types of plant growth regulators, which are mainly classified depending on the specific physiological effects they have:

1. Auxins

The auxins, belonging to various groups such as Indole-3-acetic acid (IAA), Indole-3-Butyric acid (IBA), and Naphthalene acetic acid (NAA), have been found to induce cell elongation, root formation, and differentiation of vascular tissues. In *in vitro* conditions, auxins are

2. Cytokinines

Cytokinines such as Benzyl amino purine (BAP), Kinetin, and Thidiazuron (TDZ), which are linked with division and proliferation of shoots, also delay the senescence of the plant and encourage axillary shoots' proliferation, particularly

when used at a higher concentration compared to Auxins (Skoog &

3. Gibberellins

Gibberellins (e.g., GA₃) can promote stem elongation, seed germination, and the production of enzymes during the development of seeds. They are not commonly used in tissue culture, since they tend to halt the formation of shoots and roots in plants.

4. Absciscic Acid (ABA)

The regulation of stress responses, embryo maturation, and dormancy are functions handled by ABA. The most used application of ABA is in somatic cell embryos for supporting their growth and desiccation resistance.

5. Ethylene

The ethylene itself, produced as often as a by-product in vessels for tissue culture, is reported to play an important role in regulating such processes as senescence and abscission of plant organs. It is an inhibitor of regeneration.

Role of Plant Growth Regulators (PGRs)

Plant growth regulators (PGRs) are of primary importance to tissue culture, especially for controlling critical events such as callogenesis, shoot bud induction, elongation, and root development. Cytokinins and auxins, both singly and in various combinations, have been employed to culture *Stevia rebaudiana* explants depending on the type of explant material and regeneration need.

In vitro regeneration capacity of the plant *Stevia Rebaudiana* has been greatly affected by the PGRs applied. Moreover, the

balance between auxins and cytokinins has a significant impact on the morphogenic response.

- **Callus Induction:** This can be done using a combination of NAA and BAP, which is quite popular for inducing callus formation from leaf discs. In fact, the auxins stimulate the division of cells, whereas the cytokinins accelerate the proliferation of cells. This was proved and tested in Mishra *et al.* (2010) [29].
- **Shoot Induction and Multiplication:** The treatment of a high level of cytokinins relative to auxins, i.e., BAP application in combination with at least trace levels of NAA, has been reported to be effective in *Stevia*. The optimal level of BAP (1.0 to 2.5 mg/L) in medium for effective inductive responses of shoot formation has been reported in several cases (Gantait *et al.*, 2010) [14].
- **Rooting:** IBA or NAA is usually employed to stimulate root development, especially IBA, owing to its reportedly strong ability to induce roots of highest frequency and highest vigor, thus ensuring easy acclimatization of plantlets (Yadav *et al.*, 2011) [49].
- **Somatic Embryogenesis:** Some research has also indicated the use of 2,4-D or synthetic auxins for the formation of embryogenic callus tissue followed by the use of cytokinins.

Shoot Regeneration

Shoot regeneration in *Stevia rebaudiana* plant tissue culture plays a major role in ensuring that plantlets are successfully regenerated from explants, as this mostly depends on this phase. Most plant tissue culture protocols either regenerate plants directly or indirectly through callus formation, depending on growth regulators used.

Among the cytokinins, benzyl amino purine (BAP) has been the most commonly utilized cytokinin for inducing the formation of proliferative shoots. In fact, it has been demonstrated that the use of this cytokinin on its own or accompanied by small concentrations of auxins like NAA or IAA can prove effective for the induction of multiple shoot formation. For example, Thiyagarajan and Venkatachalam (2012) [47] have used BAP (at a concentration of 2.0 mg/l) alongside NAA (at a concentration of 0.1 mg/l) on MS medium to achieve a high rate of shooting (95%) using nodal segments.

As such, a similar outcome was observed by Gantait *et al.* (2015) [14], whereby the inclusion of BAP (1.5 mg/L) and IAA (0.5 mg/L) resulted in increased shoot regeneration capacity

using shoot tips. As a result, healthy and stretched shoot lets developed. Kinetin has similarly been effective in the process. However, its ability is slightly lower than that using BAP. (Singh *et al.*, 2011) [37].

Another effective cytokinin, thidiazuron (TDZ), which belongs to the phenyl urea cytokinin family and has shown promising results, too, was studied by Kalpana *et al.* (2015) [21]. They observed that the effective multiplication of shoots was achieved with the use of TDZ at 0.2 mg/L and IBA at 0.5 mg/L, showing minimal callus.

Root Induction

After successful shoot regeneration, the next important step of plantlet development is root induction. Root induction is done either in half- or full-strength MS medium based on the cultivar and physiological status of the explants.

Auxins are the main players in root formation, and indole-3-butyric acid is the most preferred due to its higher efficiency and low possibility of formation. Petiole nodes have been successfully used for the formation of roots using IBA at the concentration of 0.5 mg/L, and roots have been seen to form within two weeks and have good elongation potential, as reported by Faisal *et al.* (2012) [12].

Naphthalene acetic acid (NAA) has also been found to be effective in rooting medium, but in some instances, the rooting medium can cause the formation of a callus at the base of the shoots if a higher concentration is applied. Thiyagarajan and Venkatachalam further observed the effectiveness of a low dose of NAA at 0.1 mg/L for root induction if applied after elongation.

Sometimes, IAA may also be used alone or in combination with IBA for better quality roots. Though the efficacy is lower than that of IBA for most *Stevia* genotypes, Gantait *et al.* (2015) [14].

Most Used Growth Regulators

- Cytokines like benzyl amino purine (BAP), kinetin (KIN), and thidiazuron (TDZ) are mainly employed to induce shoot development
- Auxins such as indole-3-acetic acid (IAA), naphthalene acetic acid (NAA), indole-3-butyric acid (IBA), and 2,4-D are commonly included to
- Gibberellins (GA₃), at times, have been employed to promote shoot elongation, although this practice is not quite common in the tissue culture of *Stevia*.

Table 3: Summary of Growth Regulator Used by the Scientists and Outcomes

Study	PGR Used	Explant Type	Culture Conditions	Observed Outcomes	Reference
Murashige & Skoog (1962)	Not specified	Various	Developed MS medium	Standardized plant tissue culture medium widely adopted for <i>Stevia</i> regeneration	Murashige & Skoog, 1962 [32]
Ahmed <i>et al.</i> (2007)	BAP + NAA	Nodal segments	MS medium with varying concentrations	Achieved 90% shoot induction with optimal PGR combination	Ahmed <i>et al.</i> , 2007 [1]
Das <i>et al.</i> (2011) [8]	BAP + 2,4-D	Flower explants	MS medium supplemented with 2.0 mg/L BAP and 2.0 mg/L 2,4-D	85.2% shoot organogenesis; maximum of 21.6 shoots per explant	
Mehta <i>et al.</i> (2012) [28]	BAP + NAA	Nodal segments	MS medium with 1.0 mg/L BAP and 0.5 mg/L NAA	Direct shoot regeneration with up to 25 shoots per explant	
Lata <i>et al.</i> (2013)	BAP + NAA	Leaf explants	MS medium with varying concentrations	Enhanced shoot proliferation compared to BAP alone	Lata <i>et al.</i> , 2013 [24]
Singh & Dwivedi (2013) [39]	BAP + IBA	Nodal segments	Bs medium with 0.2 mg/L BAP	Multiple shoot formation with shoot length of 3.87 cm	

Gantait <i>et al.</i> (2015)	BAP + Kinetin	Nodal segments	MS medium with 2.0 mg/L BAP and 0.25 mg/L Kinetin	Achieved 73% shoot formation response	Gantait <i>et al.</i> , 2015 [14]
Sasi kumar (2013)	BAP + IAA	Shoot apex, nodal, and leaf explants	MS medium with 8.87 μ M BAP and 5.71 μ M IAA	Maximum shoot induction observed	
Venkatachalam & Thiagarajan (2012)	BAP + NAA	Nodal segments	MS medium with varying concentrations	High frequency of multiple shoot induction	Venkatachalam & Thiagarajan, 2012 [47]
Misra <i>et al.</i> (2010)	BAP + NAA	Leaf explants	MS medium with 2.0 mg/L BAP and 0.5 mg/L NAA	Efficient callus induction and shoot regeneration	Misra <i>et al.</i> , 2010
Sivaram & Mukundan (2003)	BAP + IAA	Shoot apex, nodal, and leaf explants	MS medium with 8.87 μ M BAP and 5.71 μ M IAA	Enhanced shoot induction across explant types	Sivaram & Mukundan, 2003 [42]
Sha Valli Khan <i>et al.</i> (2005)	BAP + NAA	Nodal segments	MS medium with varying concentrations	Improved shoot multiplication rates	Sha Valli Khan <i>et al.</i> , 2005 [36]
Singh <i>et al.</i> (2011)	BAP + IBA	Nodal segments	MS medium with 1.0 mg/L BAP and 0.5 mg/L IBA	Achieved 98% shoot regeneration response	Singh <i>et al.</i> , 2011 [37]
Thiyagarajan & Venkatachalam (2012) [47]	BAP + Kinetin	Nodal segments	MS medium with 2.0 mg/L BAP and 0.25 mg/L Kinetin	Maximum of 123 shoots per explant observed	
Debnath (2008) [9]	BAP + IAA	Nodal sections and shoot tips	MS medium with 2.0 mg/L BAP and 1.13 mg/L IAA	Maximum shoot formation observed	
Ibrahim <i>et al.</i> (2008) [18]	BAP + GA ₃	Leaf explants	MS medium with 1.5 mg/L BAP and 0.5 mg/L GA ₃	Induced 90% shooting from callus cultures	
Sivanesan <i>et al.</i> (2009)	BAP + NAA	Nodal segments	MS medium with varying concentrations	Efficient shoot multiplication achieved	Sivanesan <i>et al.</i> , 2009
Khiraoui <i>et al.</i> (2017)	BAP + NAA	Nodal segments	MS medium with 1.0 mg/L BAP and 0.5 mg/L NAA	Enhanced shoot proliferation compared to BAP alone	Khiraoui <i>et al.</i> , 2017[22]
Rafiq <i>et al.</i> (2007)	BAP + IAA	Nodal segments	MS medium with varying concentrations	Improved shoot regeneration rates	Rafiq <i>et al.</i> , 2007 [33]
Faria <i>et al.</i> (2006)	BAP + NAA	Nodal segments	MS medium with varying concentrations	Achieved multiple shoot induction	Faria <i>et al.</i> , 2006 [13]

Hardening and Acclimatization

The hardening and acclimatization are last stages but crucial in transforming plantlets of *Stevia rebaudiana* from *in vitro* conditions to a state that is closer to nature. The plantlets, especially those that have been cultured in a controlled condition in a lab, are likely to be very sensitive. This sensitivity arises from the fact that they unlikely have cuticles and have not sufficiently developed their roots to be able to survive in the field.

Primary Hardening (In-Process Acclimatization)

The first step, which most plant tissue culturists describe as hardening, takes place in the laboratory or a greenhouse setting. At this stage, the plantlets which have developed roots *in vitro* are meticulously extracted from the medium and then placed in pots filled with a potting mix. The potting mix is, for example, composed of a combination of coco peat, vermiculite, and perlite, or garden soil itself, and all these components are mixed in an equal ratio (Gantait *et al.*, 2015) [14].

These plantlets are kept in a humid environment often under the cover of transparent plastic materials. During the development stage, it is essential to create and preserve a high humidity environment that ensures the plantlets do not dry up and at the same time encourages the development of specialized breathing holes known as the stomata and the cuticle layer in the leaves (Thiyagarajan & Venkatachalam,

2012) [47]. During the development stage, the intensity of the lighting can be increased for the plantlets.

This stage normally takes from 10 to 15 days depending on the rate at which the plantlets are adjusting to the new conditions.

Secondary Hardening

Once the plantlets have adapted to the high humidity and semi-natural light, they move to the next phase, which is known as secondary hardening. In this phase, the plantlets gradually adapt to normal environmental conditions. The plantlets are transferred to pots or bags that have been filled with garden soil, manure, and sand, normally in a ratio of 2:1:1. The pots or bags are then covered with shade net houses or left in the open for adaptation.

However, watering must be done on a moderate scale to encourage the formation of a strong root system. It must also be supported by nutrient solution or bio-fertilizers, which have the ability to encourage the seedling to survive.

Survival Rate

When properly executed, this significantly increases the survival rate of the *in vitro* plantlets, which can exceed 85-90%. Once the acclimatization process has been successfully executed, not only do the *in vitro* plantlets also flourish, but the resulting steviol glycoside accumulation also finds comparisons with its accumulation in the field plants (Gantait *et al.*, 2015; Thiyagarajan and Venkatachalam, 2012) [14, 47].

Interpretation and Best Results

Among all these findings, BAP supplemented with low concentrations of NAA or IAA has proved to be the best medium for shoot proliferation of *Stevia rebaudiana*, especially for nodal and shoot tip explants (Thiyagarajan & Venkatachalam, 2012; Gantait *et al.*, 2015)^[14, 47]. This infers that axillary buds need an environment rich with cytokinins for shoot multiplication.

For callus induction, particularly in leaf, 2,4-D either alone or supplemented with cytokinin such as kinetin has proven to be the most effective for callus formation as well as regeneration through indirect organogenesis, as reported by Mannan *et al.* (2008) and Ahmed *et al.* (2007)^[1, 26].

The most used compound for this purpose includes IBA or NAA, which is normally applied in the rooting medium, often MS medium containing full or half strength, after the development of shoots. Faisal *et al.* (2012)^[12].

Table 4: Key Contributors to *In vitro* *Stevia* Regeneration Studies

Scientist	Year	Key Contribution	Method/Focus Area
Murashige & Skoog	1962 ^[32]	Developed MS medium, foundation for plant tissue culture	Medium formulation
Ahmed <i>et al.</i>	2007 ^[1]	Somatic embryogenesis using petiole explants	Embryogenesis, callus culture
Mannan <i>et al.</i>	2008 ^[26]	Callus induction and shoot regeneration from leaf explants	Callus and organogenesis
Das <i>et al.</i>	2011 ^[8]	High-efficiency callus induction from leaf explants	Callus induction
Thiyagarajan & Venkatachalam	2012 ^[47]	Direct shoot organogenesis and clonal fidelity via RAPD	Direct regeneration, molecular markers
Zayova <i>et al.</i>	2013	Organogenesis from nodal segments	Shoot induction
Modi <i>et al.</i>	2010 ^[31]	Genetic fidelity confirmation using RAPD and ISSR	Clonal fidelity
Singh <i>et al.</i>	2013 ^[39]	HPLC-based stevioside stability analysis	Metabolite profiling
Tamura <i>et al.</i>	1984 ^[46]	Acclimatization and field transfer protocols	Hardening and field transfer

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