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Optimization of Micropropagation Protocol Using BAP, Kinetin and D-Biotin for Enhanced *In Vitro* Regeneration of *Stevia rebaudiana* Bertoni

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Abstract

Stevia rebaudiana Bertoni is an important source of steviol glycosides used as natural high-intensity sweeteners. This study evaluated the effects of BAP, kinetin (Kn), and D-biotin on shoot initiation, leaf development, and micropropagation efficiency using nodal explants cultured on Murashige and Skoog (MS) medium. Among individual treatments, BAP (1.0 mg L^{-1}) and Kn (1.0 mg L^{-1}) produced superior responses. The combination of BAP (0.2 mg L^{-1}), Kn (0.1 mg L^{-1}), and D-biotin (0.05 mg L^{-1}) was most effective, inducing shoot buds, first leaves, and second leaves within 10.5, 15.6, and 22.0 days, respectively. Maximum shoot proliferation was achieved on MS medium supplemented with BAP (0.5 mg L^{-1}) and Kn (0.4 mg L^{-1}).

Keywords: *Stevia rebaudiana; Micropropagation; BAP; Kinetin; D-Biotin, Shoot proliferation*

Introduction

Stevia rebaudiana Bertoni, a perennial herb belonging to the family Asteraceae, has gained considerable global attention as a natural source of high-intensity sweeteners. The leaves of S. rebaudiana contain steviol glycosides, principally stevioside and rebaudioside-A, which are approximately 200-300 times sweeter than sucrose and possess negligible caloric value [1-4]. Owing to their non-glycemic nature and therapeutic benefits, steviol glycosides are extensively utilized in food, pharmaceutical, and nutraceutical industries as alternatives to artificial sweeteners [4-6], *Stevia* exhibits antihyperglycemic, antihypertensive, antioxidant, and antimicrobial properties, making it a valuable medicinal crop [7-11]. The commercial cultivation of *Stevia rebaudiana* is hindered by poor seed viability, low germination rates, self-incompatibility, and considerable genetic variability among seed-derived populations [12-14]. Conventional vegetative propagation through stem cuttings is also limited by the availability of mother plants and seasonal constraints [15-16]. Consequently, the development of efficient *in vitro* propagation techniques has become essential for the rapid multiplication of elite genotypes and the production of genetically uniform planting materials [17-18]. Plant tissue culture offers a reliable approach for large-scale propagation, germplasm conservation, and genetic improvement of medicinal plants [19-20]. Among various *in vitro* techniques, micropropagation through nodal explants is considered one of the most efficient methods because it enables rapid shoot multiplication while maintaining clonal fidelity [21]. The success of micropropagation largely depends on the composition of the culture medium and the type and concentration of growth regulators employed [22]. Cytokinins play a pivotal role in regulating cell division, axillary bud proliferation, shoot multiplication, and organogenesis during *in vitro* culture [23].

Among the commonly used cytokinins, 6-benzylaminopurine (BAP) has been widely reported to induce shoot proliferation and enhance regeneration efficiency in several medicinal and aromatic plants, including *Stevia rebaudiana* [24] first demonstrated successful clonal propagation of *Stevia* through stem-tip culture using cytokinin-supplemented media. Subsequently, [25-26] reported enhanced shoot multiplication in *Stevia* cultures when BAP or kinetin were incorporated into Murashige and Skoog (MS) medium. BAP has frequently been identified as the most effective cytokinin for inducing multiple shoot formation, whereas kinetin generally promotes shoot elongation and improves morphogenic responses [27]. In addition to cytokinins, vitamins and organic supplements can significantly influence tissue

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Biotin (vitamin B7) plays an important role in cellular metabolism, acting as a cofactor in carboxylation reactions involved in carbohydrate, lipid, and amino acid metabolism [28]. Supplementation of tissue culture media with vitamins has been reported to improve cell division, shoot development, and overall growth performance in several plant species [29]. In particular, biotin supplementation has been associated with enhanced morphogenesis and regeneration responses in various in vitro culture systems [13]. However, information regarding the combined effects of BAP, kinetin, and D-biotin on micropropagation efficiency in *Stevia rebaudiana* remains limited. Optimization of growth regulator combinations is therefore essential for developing cost-effective and reproducible regeneration protocols suitable for commercial-scale multiplication. Comparative evaluation of BAP, kinetin, and their combinations supplemented with D-biotin may provide valuable insights into their influence on shoot initiation, leaf development, organogenesis, and biomass accumulation in *Stevia* cultures. Therefore, the present study was undertaken to optimize an efficient micropropagation protocol for *Stevia rebaudiana* using different concentrations of BAP, kinetin, and D-biotin in Murashige and Skoog medium. The specific objectives were to evaluate their effects on shoot bud induction, leaf initiation, shoot proliferation, organogenesis, and biomass production, and to identify the most effective growth regulator combination for rapid in vitro multiplication of *Stevia rebaudiana*.

Materials and Methods

Plant Material

Healthy nodal and leaf explants were excised from field-grown plants of *Stevia rebaudiana* and surface sterilized before inoculation onto culture media.

Culture Medium

Murashige and Skoog (MS) medium supplemented with 3% sucrose and 0.8% agar was used as the basal medium. Different concentrations of BAP, Kinetin, and BAP + Kn + D-Biotin were incorporated into the medium.

Treatments

BAP

- 0.25 $mg\,l^{-1}$
- 0.5 $mg\,l^{-1}$
- 1.0 $mg\,l^{-1}$
- 1.5 $mg\,l^{-1}$
- 2.0 $mg\,l^{-1}$
- 2.5 $mg\,l^{-1}$

Kinetin

- 0.25 $mg\,l^{-1}$
- 0.5 $mg\,l^{-1}$
- 1.0 $mg\,l^{-1}$
- 1.5 $mg\,l^{-1}$
- 2.0 $mg\,l^{-1}$
- 2.5 $mg\,l^{-1}$

BAP + Kn + D-Biotin

- 0.05 + 0.025 + 0.01 $mg\,l^{-1}$
- 0.10 + 0.05 + 0.025 $mg\,l^{-1}$
- 0.20 + 0.10 + 0.05 $mg\,l^{-1}$
- 0.275 + 0.175 + 0.10 $mg\,l^{-1}$
- 0.30 + 0.20 + 0.15 $mg\,l^{-1}$

Cultures were maintained at 25 ± 2°C under a 16 h photoperiod.

Result

Shoot bud, First leaf and Second leaf formation

Observations documented on response of different explants on shoot bud induction after around a week of culture clearly signified that amid the 3 explants inoculated the nodal region performed best when compared to the leaf segment. The nodal explants were incubated on MS medium fortified with different concentrations of BAP (0.25mg/l, 0.5mg/l, 1.0mg/l,1.5mg/l, 2.0mg/l, 2.5mg/l). The first shoot bud induction was recorded within 7 days. Best response was observed in BAP 1.0mg/l. The mean number of days taken for shoot bud formation is about 11.0 days. Similarly, the mean number of days taken for the first, second leaf formation was also recorded in the similar concentration of BAP (1.0mg/l). First leaf took about 17.42 and 24.75 mean days for second leaf formation (Table-1, Graph-1).

Preliminary experimental studies were carried out for the selection of suitable supplementation of growth regulator to MS medium by referring the available literature. The nodal explants were also inoculated in MS medium containing Kn and BAP+Kn+D-Biotin for the induction of shoot bud, first leaf and second leaf formation. In addition to BAP nodal explants were also inoculated in the MS medium containing Knetin in the range 0.2-2.5 mg/l. Showed enhanced shoot proliferation Kinetin at its 1.0 mg/l concentration evoked best response. Shoots after their initial response in 1.0 mg/l were sub cultured on the same fresh medium and after a week. The shoot bud formation, first leaf and second leaf formation was taken into account. Mean number of days taken for shoot bud formation in Kn (1.0 mg/l) is 9.6 days. Similarly the mean number of days taken for the first leaf formation took in 14.0 days and 22.1 days for second leaf (Table-2, Graph-2).

Similarly, MS+BAP+Kn+D-Biotin with different concentrations were also carried out for the shoot bud, first leaf and second leaf formation. 0.2mg/l+0.1mg/l+0.05mg/l (BAP+Kn+D-Biotin) concentration showed best response for the shoot bud, first leaf and second leaf initiation. Mean number of days taken for shoot bud initiation is 10.5 days, first leaf took 15.6 days and 22.0 days were taken for second leaf formation (Table-3, Graph-3).

Table 1: Shoot bud`, first leaf and second leaf formation BAP (mg/l)

Growth Regulator concentration (mg/l)	Shoot bud (Mean ± SD	First leaf (Mean ± SD)	Second leaf (Mean ± SD)
0.25	4.7 ±0.78	15.08 ± 1.0	22.0 ± 1.0
0.5	6.5 ±1.0	16.0 ± 0.85	25.0 ± 0.97
1.0	11.0 ±1.6	17.0 ± 0.97	25.0 ± 1.2
1.5	7.0 ±1.3	14.0 ± 1.4	22.0 ± 1.4
2.0	1.4 ±1.5	9.5 ± 1.2	19.0 ± 1.3
2.5	1.1 ±0.79	7.7 ± 1.6	16.0 ± 1.1

Graph-1:

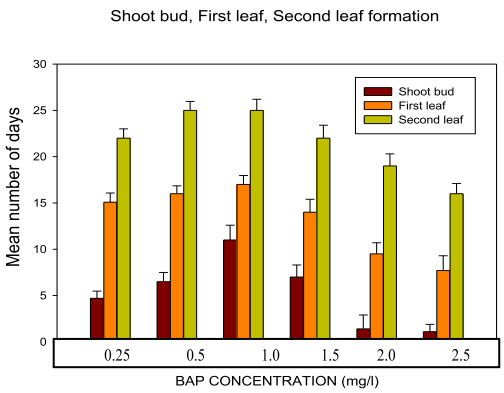


Table 2: Shoot bud, first leaf and second leaf formation Kn (mg/l)

Growth Regulator concentration (mg/l)	Shoot bud (Mean ± SD	First leaf (Mean ± SD)	Second leaf (Mean ± SD)
0.25	3.7±0.65	11±1.1	19±1.1
0.5	5.4±1.0	14±1.2	22.0 ±1.1
1.0	9.7±1.4	14±1.2	22±1.2
1.5	5.3±1.4	12±1.3	17±1.4
2.0	3.2±1.4	8.3±1.1	12±1.3
2.5	2.3±0.98	5.9±1.3	9.3±1.4

Graph-2:

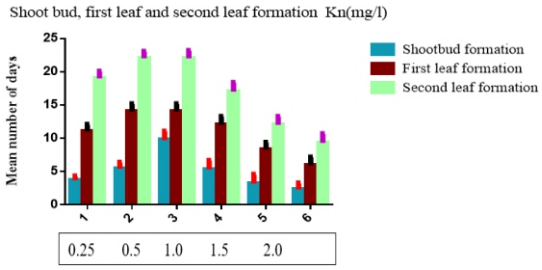
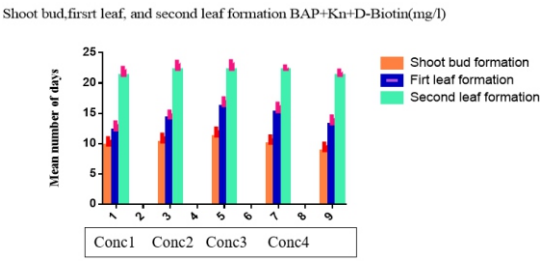


Table 3: Shoot bud, first leaf, second leaf formation (BAP+Kn+D-Biotin)

Growth Regulator concentration (mg/l)	Shoot bud (Mean ± SD	First leaf (Mean ± SD)	Second leaf (Mean ± SD)
0.05+0.025+0.01	9.5±1.3	12±1.4	21±1.4
0.1+0.05+0.025	10±1.4	14±1.2	22±1.4
0.2+0.1+0.05	11±1.4	16±1.4	22±1.5
0.275+0.175+0.1	9.8±1.3	15±1.5	22±0.65
0.3+0.2+1.5	8.6±1.3	13±1.4	21±0.97

Graph-3:

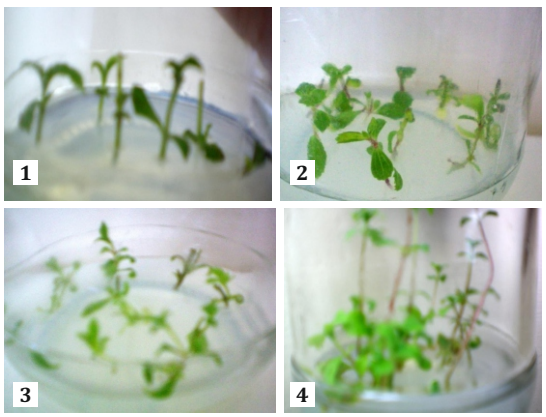


Discussion

This research examined the effects of BAP, kinetin, and their combination with D-biotin on the early developmental responses of nodal explants sourced from S. rebaudiana. The results uncover various effective formulations for the initial phases of shoot initiation and multiplication, underscoring that there is no single optimal medium suitable for every stage of micropropagation. Among the individual treatments assessed, BAP at a concentration of 1.0 mg/L proved to be particularly advantageous.

The results obtained with kinetin at 1.0 mg/L suggested that the type of cytokin can influence the timing of shoot-bud formation and leaf growth. These findings should be interpreted considering the concentrations tested, the physiological state of the explants, and the broader culture environment.

The combination treatment containing 0.2 mg/L BAP, 0.1 mg/L kinetin, and 0.05 mg/L D-biotin was shown to effectively stimulate both shoot-bud development and leaf emergence within the designated timeframes. Nonetheless, it is crucial to recognize that the effects of D-biotin cannot be isolated from those of the other components unless appropriate controls are instituted. A factorial design that includes treatments with only BAP, only kinetin, only D-biotin, relevant pair combinations, as well as the complete combination can help determine if interactions between these components exist and identify any direct advantages provided by D-biotin, the study indicates that a mixture of 0.5 mg/L BAP and 0.4 mg/L kinetin is beneficial for shoot multiplication. This shift from the formulation associated with early initiation may hold biological importance since the processes of shoot bud formation and the development of multiple shoots correspond to different stages of progression. Therefore, it is important to categorize these formulations by their specific measured outcomes rather than assuming they are interchangeable optimal solutions. A significant limitation in this context is that the reported narrative outcomes do not offer a comprehensive dataset for every treatment analyzed. To achieve a more thorough quantitative evaluation, it is necessary to check the original data tables, implement a systematic replication framework, and analyze treatment-wise average results along with their variability. Additionally, the time to reach the initial response should be distinguished from the average response duration, and no treatment should be considered statistically superior unless validated by appropriate statistics analyses, the findings establish a foundation for improving a stage-specific micropropagation protocol for S. rebaudiana. Confirming these outcomes with a complete set of replicate-level data will strengthen the recommendation for using distinct media during the processes of shoot initiation and multiplication.



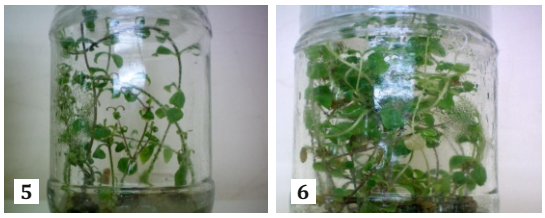


FIG: 1-3 - Early stages of Micro propagation
FIG: 4-6-Developed stages of Micro propagation from Nodal explants

Conclusion

The research presented in this thesis has emphasized effective growth regulator formulations tailored for different stages of micropropagation of Stevia rebaudiana. It has been observed that BAP at 1.0 mg/L and kinetin at the same concentration are advantageous when utilized alone. The combination of 0.2 mg/L BAP, 0.1 mg/L kinetin, and 0.05 mg/L D-biotin has demonstrated utility for the early initiation of shoot buds and leaves, while using an MS medium with 0.5 mg/L BAP and 0.4 mg/L kinetin has supported shoot multiplication effectively. The various combinations provide important alternatives for a protocol aimed at stage-specific micropropagation.

In order to confirm their comparative efficiency, consistency, and statistical relevance, it is crucial to examine the foundational replicate-level data prior to finalizing an optimized protocol.

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