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Research Article
Effect of Different Hormonal Treatment on Stevia (*rebaudiana* Bertoni) Micro-propagation

[1]Ali Haji Mohammadi, [2]Reza Zarghami, [3]Ali Kashani, [4]Hossein Heydari Sharifabad and [4]Ghorban Nour Mohammadi

[1]Department of Agronomy, Science and Research Branch, Islamic Azad University, Tehran, Iran
[2]Department of Tissue and Cell Culture, Agricultural Biotechnology Research Institute of Iran (ABRII), Education and Extension Organization (AREEO), 3135933151 Karaj, Iran
[3]Department of Agronomy, Karaj Branch, Islamic Azad University, Alborz, Iran
[4]Department of Agronomy, Science and Research Branch, Islamic Azad University, Tehran, Iran

Abstract

Background and Objective: Stevia micro propagation method is an extensive and highly potential *in vitro* method use to accelerate plant propagation in compare to the other propagation methods. This study tried to examine the effect of different hormonal combinations for shoot and root development of stevia plant. **Materials and Methods:** In order to identify the best hormonal combination of establishment culture, three different media (MS_1, MS_2 and MS_3) combine with three separate hormonal combinations for each were tested. For the proliferation and rooting culture, a media (MS_2) combined with different hormonal combinations with different levels were tested. The experiment was conducted as a completely randomized design with eight replicates in establishment experiment and factorial in base of completely randomized design with four replicates in proliferation and rooting experiment. Data was analyzed by one-way analysis of variance using SAS. **Results:** The mean comparison data showed the lowest amount of kinetin and IBA added in selected medium from establishment phase (MS_2) was more effective in proliferation traits. The highest number of lateral branches was observed in the IBA 0.25 mg L^{-1} (3.96) and kinetin 1 mg L^{-1} (3.91). The mean comparison of naphthalene acetic acid and IBA treatments in rooting study confirmed that the control treatment (no added NAA and IBA) had the maximum effect on the traits studied. The longest root length (1.68 cm) was observed in the control treatment culture. **Conclusion:** The Stevia (*rebaudiana* Bertoni) *in vitro* culture under effect of hormonal treatments showed a significant improvement on proliferation and rooting rate.

Key words: Stevia plant, growth regulator, micropropagation, establishment, proliferation, rooting

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Corresponding Author: Reza Zarghami, Department of Tissue and Cell Culture, Agricultural Biotechnology Research Institute of Iran (ABRII), Education and Extension Organization (AREEO), 3135933151 Karaj, Iran Tel: 00989121593551

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Stevia rebaudiana Bertoni, belong to *Asteraceae* family, is native to the north east region of Paraguay, which also grows in the neighboring parts of Brazil and Argentina[1]. Currently, Stevia is well-known for its high content of sweet diterpene (about 4-20%) in dry-leaf matter, in which the stevia glycosides are responsible compounds for the sweet taste. Out of 230 species of Stevia genus, only the two species *rebaudiana* and *phlebophylla* produce steviol glycosides[2,3]. However, by the late 20th century, the chemical structures of only a limited number of steviol glycosides had been characterized. These included stevioside, Rebaudioside A-F, dulcoside A and steviol bioside. In recent years, a more number of minor steviol glycosides have been studied and identified that are present in trace quantities in dried extracts of *S. rebaudiana* leaves[4].

The non-caloric sweetener of stevia plant which is a safe alternative to synthetic sweetening agents makes this plant to be in high demand for those who are suffering from blood sugar[2]. In addition to its low-calorie property, stevia has many medicinal benefits for diseases such as cancer[5], diabetes[6], obesity, cavities and hypertension[7]. While Stevia seed germination is low (50%), the sexual production is not economical. Also, excessive rain fall during pollination affect both seed production and germination. On the other side, propagation using the seed causes great variability on stevioside level and composition. Shoot cuttings can be used in small scale but it is not economically suitable for large scale cultivation[3]. Therefore, micro-propagation as a substitute method can increase the production rate as well as the amounts of steviol glycosides.

Plant micro propagation method is an extensive and highly potential field *in vitro* method that has been in use for many years to accelerate plant propagation and production. A number of standard protocols studies in different plant species have revolutionized their production. The Plant *in vitro* culture is possible to be carried out from both somatic embryogenesis and organogenesis. Stevia organogenesis has been achieved from leaves, axillary shots, stems tips, suspension cultures and anthers, while the somatic embryogenesis has been obtained from leaves and stems[8,5,9]. The aim to develop micropropagation protocol is to achieve high number of leaves containing high amount of steviol glycosides and to minimize the growth period as well as the harvesting time. With a number of protocols available for stevia *in vitro* culture, there will also be different clones available grown in a different environmental and agronomical situation which are showing variation in cultural behavior. Therefore, to achieve the target of high scale plant production and quality the micro-propagation protocol adaptation is require. In this study tried to examine the different hormonal combinations for shoot and root development of stevia plant.

MATERIALS AND METHODS

Plant material and explant sterilization: The growth of Stevia (*rebaudiana* bertoni), for explant collection, was placed in the greenhouse facilities of the department of Agronomy, Science and Research branch, Islamic Azad University, Tehran, Iran, during the spring of 2015. The whole experiment ended in 2017. Single node explants were collected from 2 month old Stevia plants. the explants were cut into small pieces ranging in size from 1-2 cm long than for surface sterilization, the explants were primarily rinsed in tap water for 10 min and incubated at 70% (v/v) ethanol (C_2H_5OH) for 30 sec and washed one times with sterile distilled water and finally with 0.4% sodium hypo chloride containing two drops of twin 20 (10 min) followed by floating in 2.5% nano silver for 10 min used for further sterilization. The sterile explant materials were washed three times with double distilled water and transformed to the culture media. Five numbers of each explants were placed in jar followed with incubation in a growth chamber. The growth chamber condition was fixed for 25±2°C temperature and photoperiod of 16 h in light (6000 lux) and 8 h in dark for 5 weeks.

Establishment medium conditions: Morashig and Skoog basal medium (MS_1) and modified Morashig and Skoog basal medium (MS_2, MS_3) (Table 1) treated with three different media conditions (MS_1: 2.4.D 0.1 mg L^{-1}, BAP 2 mg L^{-1} MS_2: IBA 1 mg L^{-1}, Kinetin 2 mg L^{-1}, MS_3: 2.4.D 0.1 mg L^{-1}, BAP 2 mg L^{-1}) designed to find out the best medium to stabilize the explant culture. The data was collected 5 weeks after culturing for traits of interest (number of leaves, Main stem height, number of nodes, plantlet phenotype quality and callusing percentage). This experiment was carried out as a completely randomized design experiment with 8 replicates (5 glass jar in each replica).

Medium condition for proliferation: According to the establishment analysis, the best media and hormonal combination that showed the appropriate results was selected. The proliferation experiment was designed by different concentrations of the above medium to obtain the

most effective combinations, which are as follows: Kinetin (0, 1, 2 and 3 mg L^{-1}) and IBA (0, 0.25, 0.5, 1 and 2 mg L^{-1}). The factorial in base of completely randomized design with 4 replicates (5 glass jar in each replica) performs to analyze the study.

Medium condition for rooting: The developed shoots were transformed to the new rooting medium designed to select the suitable rooting condition. The half basal MS_2 (used in the establishment phase) medium contained NAA (0, 0.5 and 1 mg L^{-1}) and IBA (0, 0.5, 1, 2 and 4 mg L^{-1}) with additional 15 g L^{-1} sucrose analyzed 5 weeks after culturing. The growth condition remained the same for all experiments as mentioned above. The factorial in base of completely randomized design with 4 replicates (5 glass jar in each replica) performs to analyze the study.

Statistical analysis: Data analysis was performed using SAS software (SAS 9.4 software for Windows; SAS Institute). The establishment data was subjected to one-way analysis of variance (ANOVA) while, proliferation and rooting data analyzed by multifactor ANOVA. Separation of mean analysis was done using Duncan's multiple range test ($p<0.01$).

RESULTS AND DISCUSSION

Analysis of variance showed significant variations ($p<0.01$ and $p<0.05$) among the examined traits in all different plant growth phases and their cultural medium (establishment, shooting and rooting).

Establishment phase: The data analysis based on the variance results (Table 2), showed a significant difference (1%) under the impact of hormonal combinations of all the traits i.e., the number of leaves in main stem, Main stem height and number of nodes as well as the plant phenotype quality and significant difference (5%) in callusing percentage. According to the mean comparison results, the large number of leaf, main stem height, number of nodes, callus formation and plant phenotype quality were gained by modified MS_2 medium

Table 1: List of the modified and normal MS media (MS_1, MS_2 and MS_3)

		Required concentration (mg L^{-1})		
Category	Chemical	MS_1	Modified MS (MS_2)	Modified MS (MS_3)
Macro elements	NH_4NO_3	1650	400	1650
	KNO_3	1900	1900	1900
	$Ca(NO_3)_2.4H_2O$	-	1200	-
	$CaCl_2.2H_2O$	440	-	440
	$MgSO_4.7H_2O$	370	370	370
	KH2PO4	170	170	170
Iron source	$FeSO_4.7H_2O$	27.8	27.8	-
	Na_2 EDTA $2H_2O$	37.3	37.3	-
	EDDHA FERRIC	-	-	70
Micro elements	KI	0.83	0.83	0.83
	H_3BO_3	6.2	6.2	6.2
	$MnSO_4.4H_2O$	22.3	22.3	22.3
	$ZnSO_4.7H_2O$	8.6	8.6	8.6
	$Na_2MoO_4.H_2O$	0.25	0.25	0.25
	$CuSO_4.5H_2O$	0.025	0.025	0.025
	$CoCl_2.6H_2O$	0.025	0.025	0.025
Vitamins	Myoinositol	100	100	100
	Nicotinic acid	0.5	0.5	0.5
	Pyridoxine HCl	2	2	2
	Thiamine HCl	2	2	2
	Glycine	2	2	2
Sucrose	$C_{12}H_{22}O_{11}$	30000	30000	30000
Agar	Plant Agar	7000	7000	7000

Table 2: Variance analysis of measured characters in establishment culture medium

SOV	df	Plantlets phenotype quality	Callus (%)	Number of main stem nodes	Main stem height (cm)	Number of leaves in main stem
Replication	7	0.026	0.566	0.070	0.195	0.046
Treatments	2	1.025**	2.476*	0.714**	3.180**	1.221**
Error	14	0.008	0.566	0.032	0.048	0.068
Coefficient Variation (CV%)	-	5.280	7.750	8.850	10.290	8.750

ns,*,**Non Significant and Significant at 0.05 and 0.01 probability level, respectively

Table 3: Mean comparison of measured characters in establishment cultures medium

Measured characters/ establishment cultures medium	Plantlets phenotype quality	Callus (%)	Number of main stem nodes	Main stem height (cm)	Number of leaves in main stem
MS_1	1.321[c]	83.10[b]	2.477[c]	1.581[c]	6.563[b]
MS_2	3.702[a]	100.0[a]	4.840[a]	6.705[a]	11.23[a]
MS_3	2.777[b]	100.0[a]	3.573[b]	4.747[b]	7.975[b]

Means with the same letters in each column represent no significant difference

Table 4: Variance analysis of measured characters in proliferation cultures medium (shooting)

SOV	df	Plantlets phenotype quality	Callus (%)	Number of main stem nodes	Main stem height (cm)	Number of leaves in main stem
Kinetin (A)	3	0.353**	0.512**	0.471**	1.662**	1.215**
indole 3-butyric acid (B)	4	0.254**	0.197 ns	0.264**	0.764**	0.643**
interaction A×B	12	0.077**	0.156 ns	0.039 ns	0.179*	0.083 ns
Error	60	0.024	0.101	0.032	0.086	0.069
Coefficient Variation (CV%)	-	8.400	16.160	8.960	13.480	9.570

ns,*,**Non Significant and Significant at 0.05 and 0.01 probability level, respectively

Table 5: Mean comparison of Kinetin on measured characters in proliferation culture medium

Hormone levels	Plant phenotype quality	Number of lateral shoots	Number of main stem nodes	Main stem height (cm)	Number of leaves in main stem
Kinetin 0 mg L^{-1}	3.546[a]	2.553[b]	4.489[a]	6.365[a]	9.276[a]
Kinetin 1 mg L^{-1}	3.052[b]	3.914[a]	3.451[b]	4.292[b]	6.902[b]
Kinetin 2 mg L^{-1}	2.641[c]	3.813[a]	3.155[b]	3.554[b]	6.294[b]
Kinetin 3 mg L^{-1}	2.442[c]	3.736[a]	3.090[b]	3.497[b]	6.180[b]

Means with the same letters in each column represent no significant difference

Table 6: Mean comparison of IBA on measured characters in proliferation cultures medium

Hormone levels	Plant phenotype quality	Number of lateral shoots	Number of main stem nodes	Main stem height (cm)	Number of leaves in main stem
IBA 0 mg L^{-1}	3.558[a]	3.335[ab]	4.202[a]	5.667[a]	8.623[a]
IBA 0.25 mg L^{-1}	2.913[bc]	3.960[a]	3.691[b]	4.494[bc]	7.487[b]
IBA 0.5 mg L^{-1}	3.100[b]	3.880[a]	3.681[b]	4.870[ab]	7.379[b]
IBA 1 mg L^{-1}	2.638[cd]	3.578[ab]	3.301[bc]	3.880[cd]	6.612[bc]
IBA 2 mg L^{-1}	2.392[d]	2.767[b]	2.857[c]	3.224[d]	5.714[c]

Means with the same letters in each column represent no significant difference

(IBA: 1 mg L^{-1}, kinetine: 2 mg L^{-1}). Lower-level traits were found in T1 treatment (Table 3). Previous studies also support the effect of 2.4 D and IBA which should be for establishing environment. The inter-nodal segments as explants treated with 2.4 D (3mg L^{-1}) developed high amount of callusing and faster growth than the node and leaf explants[9]. Establishment medium content, widely studied previously supports the effect of 2.4 D, IBA and kinetin on the initial stage of stevia *in vitro* culture[8,10,11].

Proliferation media under effect of kinetin and IBA: All level of kinetin, according to the variance analysis, showed significant difference on the number of leaves in main stem, main stem height (cm), number of main stem nodes, Number of lateral shoots and plant phenotype quality (1%). The effect of IBA, except in number of lateral shoots, in all traits showed significant variations among them (1%) (Table 4). The mean comparison data showed low-level kinetin was more effective for proliferation traits. The highest number of leaf, shoot length, number of main stem nodes, number of lateral shoots and plant phenotype quality earned by no added kinetin (0 mg L^{-1}) followed by 1 mg L^{-1} (Table 5). The most effective concentration of IBA was also achieved in 0 mg L^{-1} followed by 0.25 mg L^{-1} in all traits (Table 6).

Modified Murashige and Skoog (MS_2) medium supplemented with kinetin (2 mg L^{-1}) found to be effective on shoot development [12]. Various combinations of auxins and cytokinins were studied to find the best shooting hormonal level, Hwang[1] reported MS medium supplemented with IAA (2 mg L^{-1}) and kinetin (0.5 mg L^{-1}) showed an acceptable performance. The shoot elongation studies also support the effect of kinetin, which mostly used in the lower concentration[6].

In the following, the interaction effect of different concentrations of kinetin and IBA has been studied. The highest main stem height found in kinetin 0 mg L^{-1} and IBA 0.5 mg L^{-1} followed by kinetin (0 mg L^{-1}) and IBA 0.25 mg L^{-1}. The best plant phenotype quality (3.91) observed in the

Fig. 1: Proliferation condition under the effect of different kinetin and IBA levels

control treatment culture none significantly by IBA 0.25 mg L^{-1} and IBA 0.5 mg L^{-1} and Kinetin 1mg L^{-1} (Table 7) (Fig. 1).

Rooting media under effect of NAA and IBA: According to the data analysis of variance, the effect of the two different hormonal treatments (NAA and IBA) showed different results. There were no significant variations observed in the number of main roots and callus percentage while, in rooting percentage and length of root plant phenotype quality they found to be different in various levels (1 and 5%) (Table 8). The mean comparison of NAA treatments showed the control treatment (no added NAA) had the maximum positive impact on the traits under study. The longest root length (1.68 cm) observed in the control treatment culture followed none significantly by 0.5 and 1 mg L^{-1}. The most rooting percentage (92.83%) also gained by NAA (0 mg L^{-1}) followed none significantly by NAA (0.5 mg L^{-1}). The best plant phenotype quality, also found in lowest concentration of NAA in culture

Table 7: Interaction effect of Kinetin and IBA on measured characters in proliferation cultures medium

Measured characters		
Hormone levels	Main stem height (cm)	Phenotype quality
Kinetin 0 mg L^{-1} IBA 0 mg L^{-1}	6.450^{ab}	3.917^{a}
Kinetin 0 mg L^{-1} IBA 0.25 mg L^{-1}	7.425^{a}	3.854^{a}
Kinetin 0 mg L^{-1} IBA 0.5 mg L^{-1}	7.991^{a}	3.867^{a}
Kinetin 0 mg L^{-1} IBA 1 mg L^{-1}	5.348^{bcd}	2.958^{bcd}
Kinetin 0 mg L^{-1} IBA 2 mg L^{-1}	4.610^{bcde}	3.133^{abc}
Kinetin 1 mg L^{-1} IBA 0 mg L^{-1}	6.137^{abc}	3.875^{a}
Kinetin 1 mg L^{-1} IBA 0.25 mg L^{-1}	4.103^{cdef}	2.958^{bcd}
Kinetin 1 mg L^{-1} IBA 0.5 mg L^{-1}	4.395^{bcde}	3.467^{ab}
Kinetin 1 mg L^{-1} IBA 1 mg L^{-1}	4.658^{bcde}	3.317^{abc}
Kinetin 1 mg L^{-1} IBA 2 mg L^{-1}	2.167^{f}	1.642^{e}
Kinetin 2 mg L^{-1} IBA 0 mg L^{-1}	4.736^{bcde}	3.133^{abc}
Kinetin 2 mg L^{-1} IBA 0.25 mg L^{-1}	2.862^{ef}	2.208^{de}
Kinetin 2 mg L^{-1} IBA 0.5 mg L^{-1}	3.454^{def}	2.589^{cd}
Kinetin 2 mg L^{-1} IBA 1 mg L^{-1}	3.463^{def}	2.608^{bcd}
Kinetin 2 mg L^{-1} IBA 2 mg L^{-1}	3.257^{def}	2.667^{bcd}

Means with the same letters in each column represent no significant difference

Table 8: Variance analysis of measured characters on Rooting cultures medium

SOV	df	Plantlets phenotype quality	Rooting (%)	Callus (%)	Number of main root	Longest main root (cm)
α-naphthalene acetic acid (A)	2	0.025	7.026*	0.0001^{ns}	0.111^{ns}	0.016^{ns}
Indole 3-butyric acid (B)	4	0.307**	14.761**	0.0001^{ns}	0.269^{ns}	1.365**
Interaction A×B	8	0.019	7.747**	0.0001^{ns}	1.016^{ns}	0.104^{ns}
Error	45	0.028	1.732	0.0001	0.584	0.057
Coefficient variation (CV%)	-	9.330	14.600	0	25.110	17.080

Table 9: Mean comparison of NAA on measured characters in rooting cultures medium

Hormone levels	Phenotype quality	Rooting (%)	Root length (cm)
NAA 0 mg L^{-1}	3.588^{a}	92.83^{a}	1.683^{a}
NAA 0.5 mg L^{-1}	3.358^{a}	81.75^{ab}	1.583^{a}
NAA 1 mg L^{-1}	2.692^{b}	78.22^{b}	1.573^{a}

Means with the same letters in each column represent no significant difference. NAA: 1-Naphthalene acetic acid

Table 10: Mean comparison of IBA on measured characters in rooting cultures medium

Hormone levels	Phenotype quality	Rooting (%)	Largest main root (cm)
IBA 0 mg L^{-1}	3.588^{a}	95.00^{a}	2.829^{a}
IBA 0.5 mg L^{-1}	3.358^{a}	95.83^{a}	2.473^{a}
IBA 1 mg L^{-1}	2.692^{b}	91.98^{a}	1.286^{b}
IBA 2 mg L^{-1}	2.208^{b}	80.29^{a}	0.8295^{bc}
IBA 4 mg L^{-1}	2.452^{b}	58.22^{b}	0.6472^{c}

Means with the same letters in each column represent no significant difference. IBA: Indole-3-butyric acid

medium (Table 9). The effect of different concentration of IBA on rooting was studied. The longest root found in control treatment (2.82 cm) followed none significantly by 0.5 mg L^{-1} IBA. The highest rooting percentage gained in 0.5 mg L^{-1} IBA (95.83%) followed none significantly by 0, 1 and 2 mg L^{-1} IBA, respectively. This hormone also affects Plant Phenotype quality in the lowest concentration (Table 10). The MS media supplemented with no additional hormone in root development was studied and reported it dynamically effective, in contrast to additional IAA and BA[13]. Different concentrations of IAA, IBA and NAA also studied previously and reported to be effective on rooting and the MS media as a basal medium[10,13,14,6].

Table 11: Interaction effect of NAA and IBA on Stevia rooting condition

Traits	
Hormonal level	Rooting (%)
NAA 0 mg L^{-1} IBA 0 mg L^{-1}	85.00^{ab}
NAA 0 mg L^{-1} IBA 0.5 mg L^{-1}	100.0^{a}
NAA 0 mg L^{-1} IBA 1 mg L^{-1}	95.83^{a}
NAA 0 mg L^{-1} IBA 2 mg L^{-1}	91.67^{ab}
NAA 0 mg L^{-1} IBA 4 mg L^{-1}	91.67^{ab}
NAA 0.5 mg L^{-1} IBA 0 mg L^{-1}	100.0^{a}
NAA 0.5 mg L^{-1} IBA 0.5 mg L^{-1}	87.50^{ab}
NAA 0.5 mg L^{-1} IBA 1 mg L^{-1}	80.12^{ab}
NAA 0.5 mg L^{-1} IBA 2 mg L^{-1}	77.78^{ab}
NAA 0.5 mg L^{-1} IBA 4 mg L^{-1}	63.33^{b}
NAA 1 mg L^{-1} IBA 0 mg L^{-1}	100.0^{a}
NAA 1 mg L^{-1} IBA 0.5 mg L^{-1}	100.0^{a}
NAA 1 mg L^{-1} IBA 1 mg L^{-1}	100.0^{a}
NAA 1 mg L^{-1} IBA 2 mg L^{-1}	71.43^{ab}

NAA 1 mg L^{-1} IBA 4 mg L^{-1}19.67, Means with the same letters in each column represent no significant difference

Interaction effect of different concentrations of NAA and IBA has been studied. The rooting percentage in almost all hormonal level was observed to be good except in NAA (1 mg L^{-1}) and IBA (4 mg L^{-1}) treatment, which the rooting development was low (Table 11) (Fig. 2).

Fig. 2: Rooting quality and quantity under the effect of different hormonal condition

CONCLUSION

It is concluded that the Stevia (*rebaudiana* Bertoni) *in vitro* culture under effect of hormonal treatments showed a significant improvement on proliferation and rooting rate. In this study, the results showed that the low concentration of above hormones were more effective to the traits. This can be positive as it can minimize the occurrence of somaclonal variation percentage in Stevia micropropagation method.

SIGNIFICANCE STATEMENTS

This study aims to discover the type and amount of growth regulators that effected Stevia *in vitro* culture which contributed to all different micro propagation phases (establishment, proliferation and rooting) at different levels. Also, to achieve the target of high scale plant production and quality, the micro-propagation protocol adaptation is requiring for the varieties grown in different agronomical and environmental situations. Therefore, the adaptation of *S. rebaudiana* micro propagation protocol has been done for the available varieties.

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