In Vitro Propagation And Determination Of Genetic Stability Of Micropropagated Plants Of Stevia Rebaudiana

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ABSTRACT

In present study, an efficient and economic medium was designed for sprouting of axilliary/apical buds and for shoot multiplication of Stevia rebaudiana. We used thick, vegetative shoot tips (apical buds) and auxiliary buds as explants for inoculation. Auxiliary buds showed better sprouting than apical buds. Such buds gave better response to the medium designed for spouting. Semisolid MS medium with 3% sucrose supplemented with BAP and Kinetin in combinations are used. It was observed that BAP (2mg/L) + Kinetin (0.5mg/L) give best results for shoot induction within a week and shoot grown upto height of about 3 to 4 cm in 2 to 3 weeks and within 30 days on an average highest number of total shoots (20-22) and highest average length of longest shoot (4±0.5 cm) were recorded. Inter-simple sequence repeat (ISSR) markers were used to evaluate the genetic stability of the micropropagated plants and it was found out that most of the ISSR profiles from micropropagated plants were monomorphic and comparable to mother plants. These results suggested that the micropropagation protocol developed by us for rapid in vitro *multiplication is appropriate and applicable for clonal mass propagation of Stevia rebaudiana.*

Keyword - *Micropropagation, Stevia, Sugar substitute, Rebaudioside, Somaclonal variation.*

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1. INTRODUCTION

Stevia rebaudiana (Bertoni) is an herbaceous perennial plant of the Asteraceae family, commonly known as candyleaf, sweetleaf, or sugarleaf. Stevia is a tender perennial native to parts of Brazil and Paraguay that favors humid, it required relative humidity of about 80% [1]. Diterpene Glycosides produced by stevia leaves are many times sweeter than sucrose. They can be utilized as a substitute to sucrose [2]. Stevioside is regenerated as a valuable natural sweetening agent due to its relatively good taste and chemical stability. It is of special interest to diabetic persons with hyperglycemia and the diet conscious [3] (Randi, A. M. 1980) reviewed the potential uses of Stevia rebaudiana, which produces stevioside, a non-caloric sweetener that does not metabolize in the human body. It just passed through digestive tract without undergoing metabolism, making stevia safe for those who need to control their blood sugar level (Strauss 1995). The number of patient, suffering blood sugar problems in India abroad is increases rapidly, to stop this growing concern world need for a natural non-caloric sweetener with acceptable taste and health properties. Stevia also has other different therapeutic like antihyperglycaemic, anticancerous [4] antihypersensitive [5], it has contraceptive properties [6] and prevents dental caries[7][8]. Stevioside and rebaudioside induces insulin secretion. Stevia does not have the neurological or renal side effects of some of the artificial sweeteners. Tea made of stevia leaf provides relief from upset stomach. Besides, stevioside acts as anti-tumour agent. Stevia also possess antifungal and antibacterial property also in addition to its other versatile uses [9]

Need of Biotechnology: Through plant tissue culture, the totipotent characteristics of plant can be used for the in vitro regeneration of plant. [10] PCR based molecular markers are widely used in many plant species for identification[11] genetic variation analysis [12], Phylogenetic analysis [13], population studies and genetic linkage mapping.[14]. An ISSR molecular marker permits the detection of polymorphism in microsatellites and inter-microsatellite loci without previous knowledge of DNA sequences.[15] As compare with other various molecular markers, ISSR has a few advantages because ISSR primers anneal directly to simple sequence repeat and thus, unlike SSR markers, no prior knowledge of target sequences is required for ISSR.[16] Also, ISSR markers, which

have longer primers, allow more stringent annealing temperatures and reveal more polymorphic fragments. Therefore, ISSR has been widely used for population genetic studies of various plant species, including many medicinal plants.[17] Generally, the main aim of this study is to design an efficient and an economical growth medium for auxiliary/nodal bud culture, shoot multiplication and to understand the phenomenon of apical dominance and also genetic diversity analysis of in vitro grown plants with their respective mother plant four of Stevia by using ISSR markers. Along with this plant tissue culture work, molecular biology was also carried out with the aim of genetic variation analysis in vitro grown plants with their respective mother plants of Stevia by using PCR based molecular marker i.e. ISSR.

2. MATERIAL AND METHODS

2.1 Preparation of stocks for MS media : Salts, sucrose (analytical grade) and agar-agar (Bacteriological grade) were obtained from Qualigens, India. Plant growth regulators (PGR) and vitamins were obtained from Sigma-Aldrich, India. Two stock solutions [MA (8x) and MB (50x)] were prepared for MS basal medium. For preparing MA (8x), eight times the quantity of chemicals required for 1 litre medium were weighed and dissolved separately in small quantities of distilled water (DW). All these solutions were added to a conical flask containing ½ litre DW, with gentle shaking in the following sequence: Nitrates, Trace minerals, Sulfates, Calcium chloride and Phosphates. Final volume was made to 1000 ml in a volumetric flask. MA (8x) stock solution was stored in bottles at 4ºC. Every fortnight fresh stocks were prepared. For MB (50x) stock solution, 50 times the quantity of chemicals required for 1 litre of medium were weighed. All the vitamins were dissolved separately in distilled water and then all the solutions were added sequentially to make the final volume to 1 litre. The vitamin stock was dissolved in bottle at -20ºC.

2.2 Stock of Plant Growth Regulators (100 ppm): Cytokinin (BAP, Kinetin, etc.) and Auxin (IAA, NAA, etc.) stocks were prepared as follows: 10 mg of the Cytokinin was dissolved in minimum quantity (2-4 drops) of 0.1 N NaOH and the volume made up to 100 ml with DW in a volumetric flask. All the stocks were stored at 4ºC.

2.3 Preparation of medium: MS medium was prepared by initially taking some sterile distilled water in which one by one all components of MS medium from all stock solutions namely A,B,C,D,E,F,MB added according to total quantity to be made, with 3% sucrose, supplemented with cytokinins like BAP- 0.5 mg/L and Kn- 0.5 mg/L. The pH of the medium was adjusted to 5.8 then making total volume with sterile distilled water and then .7% agar (Himedia) was added to medium after melting agar completely it is poured in autoclaved tubes plugged with cotton, 20ml in each tube. Then autoclaving was done at 121ºC and 15 psi for 20 min. Such an autoclaved sterile medium in tubes was prepared for inoculation of explants in it.

Table 1.1 Composition of MS medium[18]

Composition of Stock Solutions for Murashige and Skoog's Medium Name of the Stock Solution	Nutrients	Molecular Weight (gm)	Required Concentration of Medium (mg/l)	Strength of the Stock Solution (x)	Concen-tration of Stock Solution (mg/l)	Volume to be added 1 litre of Medium (ml)
A	KH_4NO_3	80.04	1650	50	82500	20
B	KNO_3	101.11	1900	50	95000	20
	KH_2PO_4	136.09	170.00		34000	
	H_3BO_3	61.83	6.20		1240	
	KI	166.00	0.83		166	
C	$Na_2MoO_4.2H_2O$	241.95	0.25	200	50	5
	$CoCl_2.2H_2O$	237.93	0.025		5	
D	$CaCl_2.2H_2O$	147.02	440.00	200	88000	5
	$MgSO_4.7H_2O$	246.48	370.00		74000	

E	$ZnSO_4.7H_2O$	287.54	8.60	200	1720	5
	$MnSO_4.7H_2O$	169.06	22.30		4460	
	$CuSO_4.5H_2O$	249.68	0.025		5	
F*	Na_2EDTA	372.24	37.30	200	7460	5
	$FeSO_4.7H_2O$	278.02	27.80		5560	
MB	Pyridoxine.HCl	205.60	0.50	50	25	20
	Thiamine.HCl	337.30	0.10		5	
	Nicotinic acid	123.11	0.50		25	
	Glycine	75.07	2.00		100	
	Myo-inositol	180.00	100.00		5000	

*Solution of Na2EDTA is boiled and solution of FeSO4.7H2O is slowly added to this solution. Final volume is adjusted to 1L after cooling.

2.4 Sterilization of media and instruments: The empty glassware such as Jam bottles, flasks, test tubes, instruments required for inoculation wrapped in paper are sterilized by autoclaving at 121º C and 1.1 Kg/Cm² pressure for one hour. Conical flasks and Jar bottles or test tubes containing culture media were sterilized by autoclaving it at 121ºC temperature and 1.1 Kg/Cm² pressure for 20 minutes. Distilled water (for washing the explants after surface sterilization in the laminar air-flow cabinet) was also sterilized along with the glassware.

2.5 Media: For auxiliary/apical bud culture semisolid MS medium (Murashige and Skoog, 1962) supplemented with 3% concentration of sucrose and growth regulators such as BAP and Kinetin in combination BAP- 0.5 mg/L and Kinetin- 0.5 mg/L, pH- 5.8 with .7 % agar was standardized and used. For shoot multiplication semisolid MS medium supplemented with 3% sucrose and growth regulators such as BAP, Kinetin alone in different concentrations and in combination such as BAP- 2mg/L and Kinetin- 0.5 mg/L, pH- 5.8 with .7% agar were tried.

2.6 Source of explants: For this study, the pot grown *S.rebaudiana* plants were collected from the Mahabeej, Nagpur, Maharashtra and they were grown in proper planting medium and maintained in the greenhouse of Mahabeej, Nagpur. Explants were collected from the shoot tip and nodal segment of 1-2 months old plants for shoot initiation.

2.7 Surface sterilization of explants: After excision, immediately collected explants were put in sterile distilled water then, washed 2 to 3 times with sterile distilled water to remove dirt and dust particles. Then, leaves around auxiliary bud and big leaves around the apical bud were removed carefully with the blade.

Then, these buds were put in sterile distilled water, in which little Labolene (Qualigens, India) is added and rinsed properly in closed bottle and kept for 5 min then, washed 3 to 4 times with sterile distilled water to remove the soap. After this 5 drops of Savlon is added in 100 ml sterile distilled water and this was added in jam bottle containing buds (explants), which is kept for 2min with repeated stirring. Then, traces of Savlon were removed completely by rinsing the buds 4 to 5 times with sterile distilled water.

Further sterilization procedures were carried out in laminar air flow chamber by using 0.08% (w/v) $HgCl_2$ for 5 min. The explants were then rinsed five times with sterile distilled water to remove the traces of $HgCl_2$ completely. Finally, in the laminar air flow chamber, the explants were cut into small pieces ranging in size from 1.5 to 2 cm long before inoculation.

2.8 Ethanol: Ethanol is a powerful surface sterilizing agent. It is extremely phytotoxic. Therefore, plant material is typically exposed to ethanol for only seconds or minutes. Tender tissues are more readily damaged by alcohol. Tissues such as dormant buds, seeds, or unopened flower buds can be treated for longer periods since the tissue that will be explanted/develop is actually within the structure that is surface sterilized. Generally 70% ethanol is used prior to treatment with other surface sterilizing compounds.

2.9 Sodium hypochlorite: Plant material is usually immersed in 0.5-1.0% sodium hypochlorite solution for 10-20 minutes. Because of phytotoxicity, a balance between concentration and time must be determined empirically for each type of explants.

2.10 Calcium hypochlorite: It is generally used at 3.25% concentration. The solution must be filtered before use since not all compound goes into solution. Calcium hypochlorite is less injurious to plant tissues than sodium hypochlorite.
2.11 Mercuric chloride: Mercuric Chloride is extremely toxic to both plants and humans, and is used only to last resort. Since mercury is highly phytotoxic, all traces of the chemical need to be removed from the plant material in many rinses with sterile distilled water.
2.12 Hydrogen peroxide and Rinsing: The concentration of hydrogen peroxide used for surface sterilization of plant material is 30%. After surface sterilizing the plant material with one of the above compounds, it is rinsed thoroughly with sterile water. Typically 3-4 separate rinses of 1-2 min. are done.
2.13 Inoculation: Inoculations were done in laminar air-flow cabinet. First the work surface area of the laminar air flow cabinet was wiped with 70% alcohol, and the flow switched on. Jar bottles containing media, sterile distilled water, forceps, scalpel, coupling jar containing 70% alcohol. The UV light was switched on for approximately 1 hour. After that, with the help of flamed forceps the buds were picked up and placed on the autoclaved semisolid medium in the Jam bottles. The caps were tightly closed. To reduce the chances of contamination the neck of bottles were wrapped with the Kling-wrap.
2.14 Incubation and establishment of culture: After inoculation, the cultures were maintained at a temperature of 25±2ºC with photoperiod of 16 h per day in sterile growth room. Lighting of $80\mu Em^{-2} s^{-1}$ was supplied by using cool and white fluorescent tubes. Growth regulators such as BAP and Kinetin in combination were added in MS medium for buds(axillary/nodal) sprouting. After sprouting of buds the sprouted one was subculture in other fresh sterile MS medium supplemented with different growth regulators such as BAP, Kinetin alone in different concentrations and also in combination for shoot multiplication. After 1 month multiple shoots were subculture in same medium. Results obtained were observed carefully and interpretated.

3. DNA ISOLATION AND QUANTIFICATION

Table 1.2 DNA isolation and reagents

Sr. no	Stock	Effective Concentration
1.	10% CTAB	2%
2.	5M NaCl	1.4M
3.	2M Tris (pH-8)	100mM
4.	0.5M EDTA	20mM
5.	Na metabisulphite	100mM
6.	B mercaptoethanol	1%
7.	PVP	1%

3.1 Protocol for DNA extraction: Extraction Buffer (EB) was prepared freshly. 1ml extraction buffer was used for 0.1gm of leaf tissue. Leaf tissue was grinded to fine powder in pre cooled mortar and pestle using liquid Nitrogen. Crushed tissue was then immediately transferred into the tubes containing pre warmed extraction buffer (EB). It was mixed properly to avoid clumps and also this facilitates even distribution of tissue followed by incubation at 65ºc in water-bath for 1 to 1.5 hr. After incubation, samples were allowed to cool little bit and added equal volume of Chloroform: IAA in each tube and mixed properly and centrifuged at 10,000 rpm for 20 min at the room temperature. After centrifugation supernatant (aqueous phase) was transferred to the fresh tube using 5ml pipette (with cut tip). It was then precipitated with equal volume of chilled isopropanol and mixed gently. DNA was allowed to precipitate at -20ºc for 45 min and recovered by centrifugation at 10,000 rpm for 20 min at the room temperature. After centrifugation, supernatant was discarded carefully; pellet was washed with 70% ethanol and air dried and dissolved in 500 µl TE. After complete dissolution of pellet samples were checked on 0.8% agarose gelfor the presence of RNA.DNA was treated with rnase(10mg/ml) for 1 hr at 37ºc. The traces of rnase were removed by adding equal volume of Chloroform: IAA and centrifuged at 12,000 rpm for 15 min at room temperature. Supernatant was transferred in fresh vial/tube and reprecipitated with equal volume of chilled absolute alcohol with 3M sodium acetate (ph-5.2) at -20ºc for 45 min and followed as mentioned above.The pellet was suspended in 1ml of 70% ethanol and then centrifuged at 10, 0000 rpm for 20 min at RT. After air drying pellet was dissolved in 100µl TE. DNA was quantified using Nanodrop1000 Spectrophotometer (thermofisher Scientific, USA) and purity was determined by A_{260}/A_{280} ratio.

3.2 Primer screening and ISSR-PCR: 4 UBC primers were attempted namely UBC818,UBC 840,UBC 873 and UBC 844 which produces reproducible bands which were further used for analysis. Primers were selected for further analysis based on their ability to detect distinct, clearly resolved and polymorphic amplified products. To ensure reproducibility, the primers generating no, weak, or complex patterns were not selected for ISSR analysis.

3.3 ISSR ASSAY: PCR amplification was carried out in a total volume of 25µl, containing 20 ng of template DNA, 10X PCR buffer (10mM Tris HCl (pH 8.3), 50Mm KCL 1.5mM $MgCl_2$), 0.1mM dNTPs, 0.5mM spermidine,0.3µM primer and 0.5 U *Taq* DNA polymerase (Banglore Genei, India) on a Veriti Thermal Cycler (Applied Biosystems USA). Initial denaturation was carried out at 94°C for 5 min, followed by 45 cycles 94°C for 30 s (denaturation), 50/52ºC for 45 s (annealing), 72°C for 2 min (extention), and a final extension for 5 min at 72°C.

Reagents	Volume (µl)
SMQ	16.02
dNTPs	2.5
Buffer	2.5
Primer	.5
Template	2
Taq	.48
Spermidine	1
Total volume	25

Table 1.3Concentration of ingredients

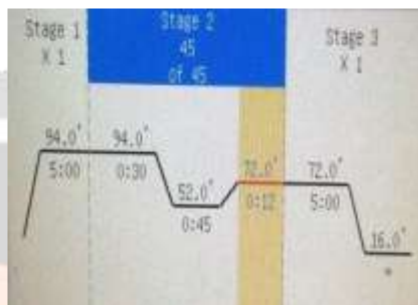

Figure 1.1:Cycling conditions for ISSR-PCR

Amplified products were electrophorased on1.5% (W/V) agarose gel in 0.5 X TAE buffer alongwith a molecular weight marker (ΦX174/*HaeIII*digest, 100 bp and 1kb ladder, M/S Bangalore Genei). Amplicons were detected by Gel red staining and photographed using image analysis software for gel documentation(DYversity, SYNGENE).

4. EXPERIMENTAL RESULTS AND DISCUSSION

Results of tissue culture work: In present study, an efficient and economical medium was designed for sprouting of axilliary/apical buds and for shoot multiplication of Stevia rebaudiana and interesting results were obtained.
When healthy, thick, vegetative shoot tips (apical buds) and axillary buds were used as explants for inoculation in the month of January (which is actually flowering period for Stevia plant), axillary buds showed better sprouting than apical buds. Such buds gave better response to the medium designed for spouting. Semisolid MS medium with 3% sucrose supplemented with BAP and Kinetin in combination such as BAP (0.5 mg/L) and Kinetin (0.5 mg/L) growth regulators were tried and it was observed that both BAP and Kinetin together worked so well because the buds were sprouted (i.e. shoot induction) within a week and shoot grown upto height of about 3 to 4 cm in 2 to 3 weeks.

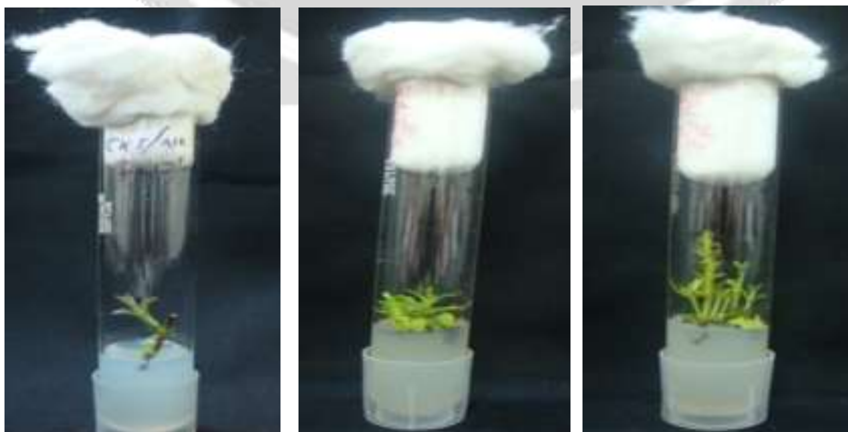

Figure 1.2: Auxillary bud sprouting in Stevia showing initial stage of sprouting of auxiliary bud, sprouting and elongated axillary bud.

After 3 weeks sprouted buds were subcultured in semisolid MS medium with 3% sucrose, supplemented with two different growth regulators such as BAP and Kinetin alone in different concentrations, and also combination of BAP and Kinetin were tried. It was observed that medium containing BAP and Kinetin in combination worked well as compared to the medium containing BAP and Kinetin alone in different concentrations and maximum shootproliferation was observed in MS medium supplemented with BAP and Kinetin together, within 30 days on an average highest number of total shoots (20-22) and highest average length of longest shoot (4±0.5 cm) were recorded.

Table 1.4 : Effect of different concentration of kinetin on the growth of shoots

Sr.No.	Type of shoot multiplication Medium	Number of shoots (average)	Height of shoots (average) cm
1.	MSmedium+ 3% sucrose +BAP(0.5mg/L)	5 to 6	1 to1.5
2.	MSmedium+3%sucrose + BAP (1mg/L)	5 to 6	1.5 to 2
3.	MSmedium+3%sucrose + BAP (2mg/L)	6 to7	3 to 4
4.	MSmedium+3%sucrose + BAP (3mg/L)	6 to 7	2 to 3
5.	MSmedium+3%sucrose + BAP (5mg/L)	9 to 10	3 to 4
6.	MSmedium+3%sucrose +kinetin(.1mg/L)	3 to 4	1 to 2
7.	MSmedium+3%sucrose + kinetin(.2mg/L)	5 to 6	2 to 3
8.	MSmedium+3%sucrose + kinetin(.5mg/L)	6 to7	2 to 3
9.	MSmedium+3%sucrose + kinetin (1mg/L)	5 to 6	3 to 4
10.	MSmedium+3%sucrose + kinetin (2mg/L)	4 to 5	2 to 3
11.	MSmedium+3% sucrose+ BAP (2mg/L)+ Kinetin (0.5mg/L)	20 to 22	4 to 5

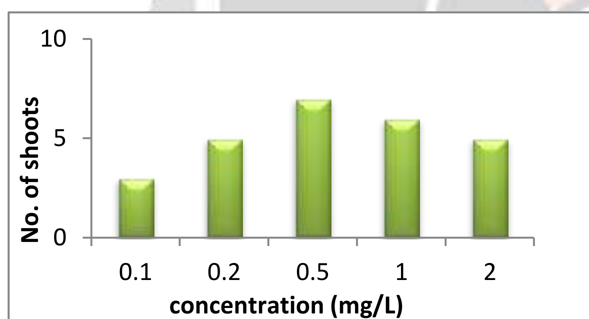

Figure 1.3: Effect of different concentration of kinetin on the growth of shoots

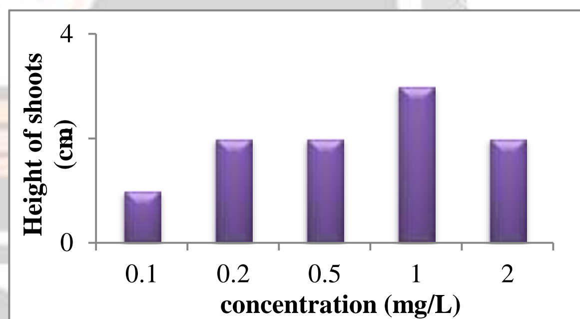

Figure 1.4 : Effect of different concentration of kinetin on the height of shoots

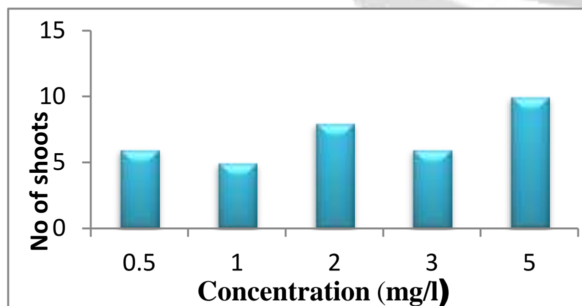

Figure 1.5: Effect of different concentration of BAP on the growth of shoots

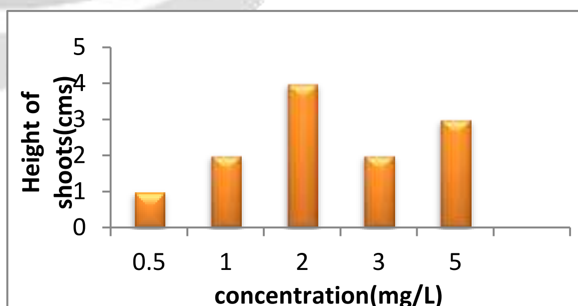

Figure 1.6: Effect of different concentration of BAP on the height of shoots

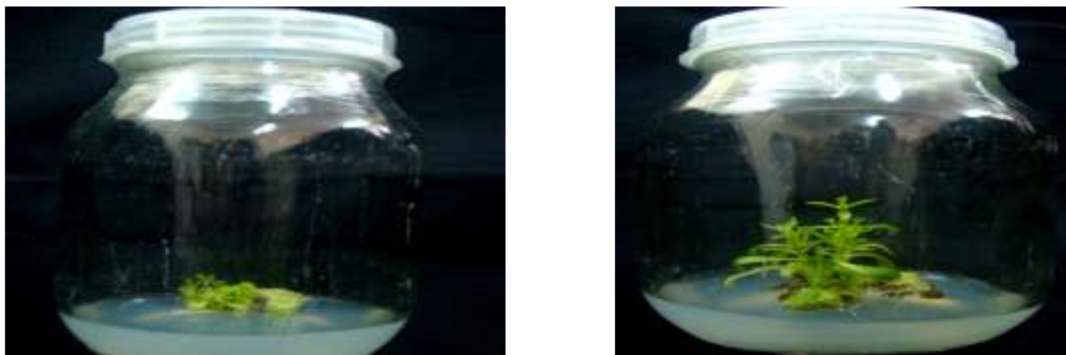

Figure 1.7 : Different stages of shoot multiplication in medium containing BAP(2mg/L) and Kinetin (0.5mg/L) in combination

Also, some interesting results were obtained such as callusing of leaves of multiple shoots, those which were touching medium. Such lower leaves which were in contact with medium, showed brownish yellow colour callusing. It was observed that initially after 1 week of subculturing there were small buds on the surface of the leaves which were swollen and shiny. Then after 2 weeks these buds started sprouting and tiny shoots were observed. After careful observation we concluded that they were somatic embryos developed on the surface of leaves. Plant regeneration from them was observed after 30 days of incubation. On some lower leaves callus induction was observed and through somatic embryogenesis shoots were observed only on mid rib portion of leaf. In this case callus induction in leaves of Stevia was observed without using any auxin such as 2, 4-D in the medium, but it was observed in shoot multiplication medium supplemented with cytokinins only. When such leaves transferred to the plain MS medium without any growth regulator and to the MS medium only with the BAP then, it was observed that medium containing BAP showed better response as compared to plain MS medium. Further studies are to be carried out related to callusing of leaf and somatic embryogenesis.

The phenomenon of apical dominance was clearly seen in stevia because after removing the apical bud and inoculating it after 3 days in the basal MS media without growth regulator, it was seen that it starts sprouting within 4 days as compared to the normal bud which sprouts in 1 or 2 week in the MS medium with growth regulator.

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Figure 1.8 : Shoot developing from mid-rib region of Stevia leaves undergoing callusing

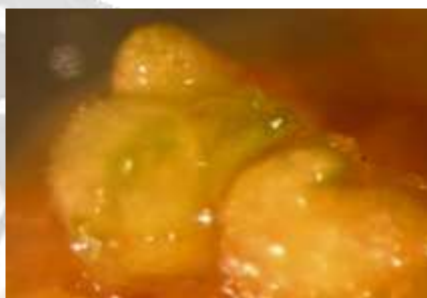

Figure1.9 : Callusing and Somatic embryogenesis

Figure 2.0 Developing Somatic embryo

One of the remarkable achievement was getting callus induction without the use of auxins such as 2, 4-D etc. In this study, callusing had occurred in leaves which were touching to the medium supplemented with BAP and Kinetin. There are reports such as Patel and Shah (2009) reported use of BAP and NAA for callus induction in leaves of Stevia while Gupta et al. (2010) reported use of MS medium with different concentrations of growth regulators such as IBA, Kinetin, NAA, and 2, 4-D in their work they got 100% callusing by using NAA in combination with 2, 4-D. But we got callusing in leaves without using any auxins. This indicates the presence of certain growth inhibitors in leaves which are diverting pathway to callusing and then through somatic embryogenesis plant regeneration from callus had occurred. There are certain reports such as Preethi et al. (2011) who reported indirect shoot regeneration from leaf explants of stevia in which callusing in leaves were induced by using either IAA/NAA in combination with BA/Kinetin and then indirect shoot organogenesis was achieved by them from callus using BA. Also there is one report by Hershenhorn et al. (1997) related to growth regulators derived from Stevioside i.e. obtained from Stevia. Such derivatives effects have been reported such as 1) inhibition of seed germination 2) root elongation inhibitors, inducers, general inhibitors etc.

4.1 Results for ISSR analysis

Four ISSR primers were screened out which were selected for further analysis on the basis of clear amplification profile and reproducibility.

Inter-simple sequence repeat (ISSR) markers were used to evaluate the genetic stability of the micropropagated plants and it was found out that most of the ISSR profiles from micropropagated plants were monomorphic and comparable to mother plants confirming the genetic stability among micropropagated plants and mother plant.

Table 1.5 : Electrophoretic lanes of isolated DNA as detected in 0.8 % agarose gel

P1, P2, P3	Parents
N1, N2 , N3	In vitro plantlets/progeny

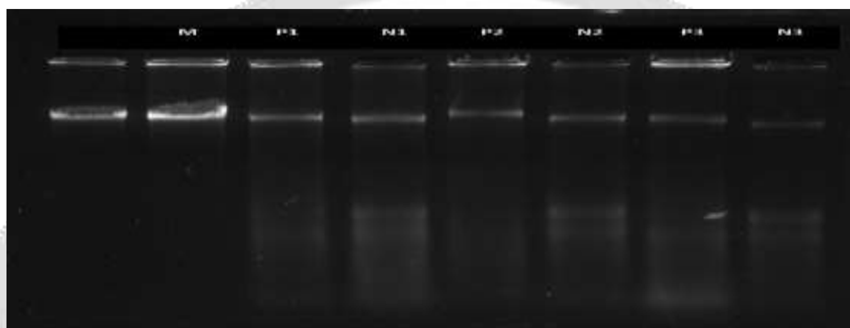

Figure 2.1 : Lane 1,2-M, Lane 3-P1,Lane 4-N1,Lane 5-P2,Lane 6-N2,Lane 7-P3,Lane 8-N3

4.2 Screening with ISSR molecular markers on 1.5 % agarose gel

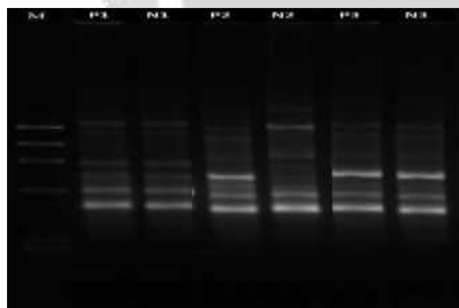

Figure 2.2 : Primer UBC 873, P1, P2, P3, N1, N2, N3,

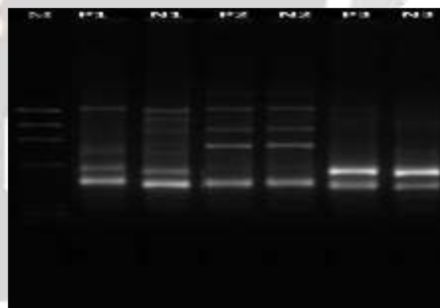

Figure 2.3 : Primer UBC 818, P1, P2, P3, N1, N2, N3

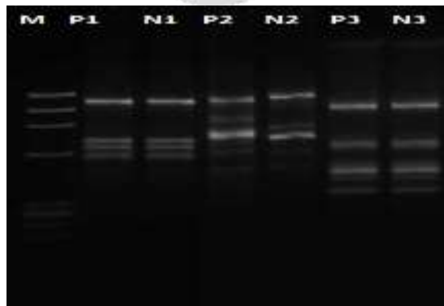

Figure 2.4 : Primer UBC 818, P1, P2, P3, N1, N2, N3

5. SUMMARY AND CONCLUSION

- *In vitro* propagation can become an important alternative to conventional propagation and breeding procedures for *S. Rebaudiana* which is both an industrially and medicinally important herb.
- On the basis of results obtained we can conclude that healthy, thick and vegetative axillary buds showed better response and showed excellent shoot induction i.e. sprouting as compared to apical(shoot tip) buds.
- Different medium were tried for bud sprouting i.e. MS medium with 3% sucrose supplemented with BAP and Kinetin alone in combination such as BAP (0.5 mg/L) and Kinetin (0.5 mg/L) growth regulators , thus from the results obtained we can conclude that BAP and Kinetin together showed better sprouting than those when tried alone.
- For shoot multiplication, different medium were tried using variety of growth regulators such as BAP, Kinetin alone in different concentrations and also in combination like BAP (2 mg/L) and Kinetin (0.5 mg/L) and from the results obtained and after counting average number of multiple shoots and height, we can conclude that BAP (2 mg/L) and Kinetin(0.5 mg/L) in combination gave highest frequency of shoot proliferation and thus this can be an efficient and economical medium designed in this study for in vitro mass propagation of stevia which is not only a medicinally important but also a source of calorie free stevioside
- Also in shoot multiplication medium some exciting results were also obtained like the lower leaves of multiple shoots, which were touching to the medium were showing callusing. This was interesting because as there were no auxins used in the medium, in presence of only cytokines still Stevia leaves showed callusing which was really a fascinating results obtained in this study there are few reports related to this leaf callusing in presence of cytokines in medium but they have used cytokines in large concentration but in this study we got leaf callusing in presence of less concentration of cytokines. This suggests that there are certain growth inhibitors naturally present in Stevia leaves which diverted pathway and induce callusing. Further work is to be carried out to detect the presence of growth inhibitors in Stevia leaves by carrying out certain experiments. Also small buds were observed initially on this leaf callus region and after few days shoots were induced on callus, thus from careful observations we can conclude that these were somatic embryos were formed on callus through somatic embryogenesis, which further gave rise to small shoots on the surface of callusing leaves.
- In molecular biology work, 4 ISSR primers were screened out and selected for further analysis on the basis of clear amplification profile and reproducibility.
- Inter-simple sequence repeat (ISSR) markers were used to evaluate the genetic stability of the micropropagated plants and it was found out that most of the ISSR profiles from micropropagated plants were monomorphic and comparable to mother plants confirming the genetic stability among micropropagated plants and mother plant. These results suggest that the micropropagation protocol developed by us for rapid *in vitro* multiplication is appropriate and applicable for clonal mass propagation of *Stevia rebaudiana*.

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