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Establishment of Regenerative Callus, Cell Suspension System and Molecular Characterization of *Stevia Rebaudiana* Bertoni for the Production of Stevioside in *In Vitro*

Nisha Sharma[1], Dhurva P. Gauchan[2], Ashna Dhakal[3], Anup Luitel[4], Shailendra Shakya[5], Rajani Shakya[6]
[1,2,3]*Department of Biotechnology,* [4,5,6]*Department of Pharmacy*
School of Science, Kathmandu University, Nepal

Abstract-An efficient and standardized medium for callus induction was developed using leaf explants cultured on MS medium supplemented with 1.0mgl^{-1} 1-Naphthaleneacetic acid (NAA) and 1.0 mgl^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D),which is a noble approach to produce maximum amount of callus within short time period. Similarly, for the optimization of suspension culture, callus cultured on Murashige and Skoog basal liquid media supplemented with 2, 4-D, 6-Benzylaminopurine (BAP) and ascorbic acid along with various concentrations of macro salts; the media supplemented with ammonium nitrate (NH_4NO_3) in addition of 4.5% sucrose showed the highest cell growth response on twentieth day. For the enhancement of the optimal cell growth 100 µm methyl jasmonate was added, which showed the highest cell growth as well as stevioside content on twentieth days at 4.5% sucrose concentration.HPLC (High-performance liquid chromatography) analyses for the estimation of the stevioside were carried out using C18 column. The highest (40.0 mg/g) steviol glycosides was in leaf of the Russian population as compared with Indian population (21.2 mg/g), followed by the callus (2.997 mg/g) and (0.325 mg/g) in suspension culture. The highest biomass yield (15.23gm/l) and stevioside content was observed in the media supplemented with NH_4NO_3 in addition of 4.5% sucrose on twentieth day. The maximum stevioside content was observed indicating the supportive role of biomass and stevioside content in the callus. Random Amplified Polymorphic DNA Markers were used to study the genetic variation between the Russian and Indian population of Stevia rebaudiana and callus. Using RAPD analysis, we confirmed that mother plants of Russian and Indian stevia populations showed genetic variation but, as their calluses were grown they came to show genetic similarity to some extent.

Key words: Stevia rebaudiana, Callus, High-performance liquid chromatography (HPLC), Randomly Amplified Polymorphic DNA (RAPD), Polymerase chain reaction (PCR).

I. INTRODUCTION

Stevia rebaudiana is the source of well known sweet tasting compounds. It is its sweetness that led to the discovery of this species and, eventually, tremendous attention is given to it. *Stevia rebaudiana* bertoni is a small, herbaceous, semi-bushy, tropical perennial shrub belongs to *Asteraceae* family, which is native to Paraguay and Brazil [1]. *Stevia* species play an important role in the healthcare practices of different cultures and populations where it has been demonstrated that several compounds present in *Stevia* species, but obtained from different plant sources, possess important biological and/or pharmacological activities. The important bioactive compounds present in *Stevia* plants are alkaloids, flavonoids, tannins and phenolic compound [2]. It contains antimicrobial properties as well as tannins, essential oils and other aromatic compounds[3], [4]. *Stevia* does not have any effects on blood sugar and therefore it is friendly to human health [5].

Since the percentage of seed germination is quite low in *Stevia* due to their self-incompatibilityleading to the lack of fertilization [6]and the seeds formed are infertile and small sized [7]. They require long time to establish seedlings [8] and plants propagated by seeds generally show a wide variation in stevioside content [9].Thus *in vitro* plant tissue culture methods have been applied for the multiplication viaorganogenesis or embryogenesis from different explants form, leaves [10], stem tips [9], stems [12], nodal segments [13], instance axillaryshoots and suspension cultures [10].Plant tissue culture technology may help to conserve rare and endangered medicine plants. The callus and cell cultures have a higher rate of metabolism than intact plants due to the fast

proliferation of cell mass that results in a condensed biosynthetic cycle. The ability of these cells seems to be important for the formation of the secondary metabolites. *Stevia* leaves contain a number of diterpene steviol glycosides (SGs) containing higher sweetness than sucrose at 4% (w/v) concentration [15]. [16] reported that stevioside content in callus was twice as much as that of leaves and four times as that of flowers.The leaves accumulate steviol glycosides; among them, four major compounds are responsible for extreme sweetness, namely: stevioside (5-10%), rebaudioside A (2-4%), rebaudioside C (1- 2%), dulcoside A (0.5-1%) [14], where glycosides are non-toxic, non-mutagenic and low caloric compounds, and, unlike traditional sugar substitutes such as xylitol or sorbitol and acquired tolerance to them [17], [18] thus making *Stevia* a safe substance for consumption for people who need to reduce the sugar content of their blood [19][11].

Identification of possible somaclonal variants is considered to be very useful for improvement of crop which may assist for developing novel characters in plants. Randomly amplified polymorphism DNA (RAPD) based detection of genetic polymorphism has found successful application in describing somaclonal variability in regenerated individuals of several plant species [20].

Stevia was first introduced in Kathmandu, Nepal from Russia in 2008 BC and move to the other part of country. Palm Agrotech Pvt. Ltd. Chitwan brought the *Stevia* plantlets from India which were developed from suckers and cultivated by them and farmers for commercial purpose. Thus, Nepal got two different populations one from India and another from Russia which showed significant difference due to their origin. And our work explores not only to increase the stevioside contents *in vitro* using different techniques but also it aims to develop some genetically variable plants for improvement of *stevia* species in Nepal.

The objectives of the present study include, establishment of suspension culture from callus, determination of steviol glycosides by the method of HPLC and genetic fidelity of *in vitro* regenerants of *Steiva rebaudiana* using RAPD technique.

II. MATERIALS AND METHODS

A. *Tissue Culture Experiments*

1) *Preparation of Plant Extract*: Young twigs of *Stevia rebaudiana* (Russian population and Indian population) were collected. The explants were surface sterilized in 70% (v/v) ethanol for 1 minute and 0.1% (v/v) sodium hypochlorite solution for 3 minutes.

2) *Callus Establishment and Cell Suspension Cultures:* Murashige and Skoog media was prepared with different concentrations of varied phytohormones, 3.0% sucrose and 0.8% agar, maintaining pH at 5.8 and was autoclaved at 121^0C at 15 psi pressure for 25 minutes for the callus establishment. The treated explants (leaf) approximately 1 to 2 cm in length were then inoculated in the media under aseptic condition. The culture was incubated in an air conditioned culture room illuminated by 40 W white florescent lights; 3000 lux light intensity, at temperature 25^0C ± 2 and the photoperiod was maintained at 16 h/8 hr light and dark regime. The induced callus was routinely subcultured (every 4 weeks) in fresh medium with the respective phytohormones to maintain its viability. Cell suspension cultures were initiated by suspending 2g of callus in 20ml of medium in closed flasks with aluminum foil, and placed on a gyratory shaker at 100 rpm in the dark. Cells were maintained in flasks with a liquid volume of 50ml. The MS liquid medium was supplemented with BA (0.27µM), 2, 4-D (0.27µM), and ascorbic acid (0.06µM) for the suspension culture. The effect of various concentrations of macro salts NH_4NO_3 (24.7 mM), KNO_3 (56.4 mM), $MgSO_4$ (4.5 mM) and KH_2PO_4 (3.75 mM),with different concentrations of sucrose such as 3.5%, 4%, 4.5%, 5% were evaluated for optimal growth in cell suspension culture by keeping the other parameters constant (growth regulators, pH, and culture volume).The cultures were sub cultured in every 2 weeks. Cell morphology, cell viability, cell growth and stevioside content were monitored during the experiment. The number of cells per ml was calculated.

B. *HPLC Analysis*

1) Preparation Of Samples for Stevioside Analysis: 3 gm powdered of mature leaves of *S. rebaudiana* (i*nvitro* and *invivo*), dried callus and dried suspension culture cells was dissolved in 10 ml of methanol, which was kept in closed vessels for 48 hours in shaker. The extract was filtered and residue was re-extracted three times with methanol. The solution was evaporated and in the remaining residue 10 ml of HPLC water was added for the further analysis. 10 µl of methanol extract of experimental samples and standard samples were injected to C18 column for HPLC analysis and run at isocratic condition using solvent mixture of acetonitrile: water (65:35) with a flow rate of 1 ml/min, wavelength set at 210nm. The various concentration of stevioside from standard sample (5µg/ml, 10µg/ml, 15µg/ml, 25µg/ml, 50µg/ml, 125µg/ml, and 250µg/ml) was prepared for the quantitative estimation .The estimation of stevioside was done based on the peak area of specific concentration of sample with standard.

C. Molecular Characterization

*1) Sample Preparation:*15 samples were selected which were abbreviated as CR1, CR2, CR3, CR4, and CR5 for the five samples of *invitro* grown callus populations; I1, I2, I3, I4 and I5 for the five samples of Indian mother plant and R1, R2, R3, R4 and R5 of Russian mother plant.

DNA was isolated from *Stevia* leaves using protocol developed by [30]. 800mg of fresh tissue (leaves) were ground in Liquid Nitrogen, powder was transferred to prewarmed CTAB Extraction Buffer (100 mM TRIS- HCl, 1.4M NaCl, 20 mM EDTA, 2% CTAB, 1% PVP, 0.2% beta-mercaptoethanol). The sample was then incubated for 60-90 min., with occasional inversion at 65°C. 2ml of chloroform-isoamyl alcohol (24:1) was then added to the cooled sample. The sample was centrifuged at 2300 rpm for 2 min. and 25 ul of RnaseA (20 mg/l) was added to the collected supernatant and incubated for 30 min. 2 ml of isopropanol was added to each tube inverted slowly until a white fluffy DNA precipitate appeared. Centrifugation was done at 2300 rpm for 5 mins. Supernatant was discarded and the pellet was resuspended in 300 µl of cold diluted CTAB Wash Buffer. It was incubated at room temperature for 20 min. 30 ul of cold 70% ethanol was added to the tube containing DNA. It was again centrifuged at 2300 rpm for 5 minutes. Supernatant was discarded. The pellet was air dried to remove traces of ethanol. DNA samples were transferred to tube containing 30 ul of elution buffer. The quality and size of DNA were checked on 0.8% agarose gel with 3µl of Ethidium bromide. The mixture of 5 µl of sample DNA and 3 µl of gel loading buffer was loaded in the well. The electrophoresis was carried out at 90 volt for 30 mins. The gel was then placed under UV in the gel documentation system to observe the result.

2) RAPD PCR (Random Amplified Polymorphic DNA, Polymerase Chain Reaction): PCR tubes with 25 µl volume containing 20 mMTris-HCl pH 8.3, 50 Mm KCl, 2 Mm $MgCl_2$, 200 µM of each of dATP, dCTP, dGTP and dTTP, 0.8 µM primer, 1 unit TaqDNA Polymerase, and 24 ng of template DNA was set up. Master mixturewas prepared placing required amount in PCR tubes for each sample. A negative control was set up in which TE replaced the DNA. An aliquot of each sample was mixed with loading buffer in a ratio of (3 µl dye and 10 µl DNA) and electrophoresis was done on a 1.5% agarose gel along with a suitable DNA ladder in 0.5 X TBE buffer at 50 V for 90 min. Then bands were visualized under UV light.

Amplification was conducted in a thermocycler for 3 min at 94°C (preliminary denaturation), 15 secs at 94°C (Denaturation), 30 secs at 35°C (Annealing) and 90 secs at 72°C (Elongation). Step 2 to 4 is repeated for 44 times, Sixth step is resting at 72°C for 3 mins and lastly holding step at4°C. The mixture used in the PCR program consisted of sterile water (15.2 µl), DNA (3.0 µl), PCR buffer (2.5 µl), primer (2.0 µl), dNTP (2.0 µl) and taq polymerase (0.3 µl) for a 25 µl reaction.Genetic variation was estimated using the POPGEN 32 software package, VERSION 1.31 and the following indices were calculated: number of alleles per locus, effective number of alleles per locus, percentage of polymorphic loci, Nei's (1973) gene diversity (h*) and Shannon's index (I*). Nei's Unbiased Measures of Genetic distance was used to construct a dendrogram using UPGMA algorithm.

Statistics analysis

The data recorded for different parameters were subjected to completely randomized design using three replications per treatment. Statistical analysis was performed using two-way analysis of variance (ANOVA test). The level of significance was set at $P<0.05$.

III. RESULTS AND DISCUSSION

A. Callus Induction

The Callus initiation was observed from leaf after four weeks of culture initiation. The most frequently used auxins were 2, 4-D, NAA, IBA, BAP, Kinetin. Best response (100%) (Table 1) in terms of callusing was seen after four weeks of culture initiation in medium supplemented with 2, 4-D 1.0 mg/l + NAA 1.0 mg/l. Callus produced were soft, globular/irregular, fragile and whitish green in color. The medium supplemented with 1.5mg/l NAA+2mg/l 2,4-D showed 84% callusing while medium supplemented with 1mg/l NAA+2mg/l 2,4-D and 3mg/l 2,4-D showed 81% callus induction. Callus appeared slightly whitish and yellowish green, amorphous and moderately fragile. The lowest callus induction (33%) was recorded in the medium supplemented with KN 2.0 mg/l. Many authors have developed protocol for establishment of callus from leaf and nodal segments of *Stevia*[21], [22], [23], [24], [25],[26], [27]and [28]. Callus formation from nodal segments in MS basal medium supplemented with 2, 4- D was reported to give maximum callus[29] which shows a little conflict from our results. On the other hand callus establishment from leaf segments in MS fortified with 2, 4- D and BAP by [10] supports our findings.

Volume 3 Issue VIII, August 2015
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Fig.1 *Stevia rebaudiana* bertoni

Fig. 2 Light yellowish green callus

Fig.3 Shoot regeneration from callu

Table 1

Effect of different concentration of phytohormones for callus induction and growth

Conc. of GRs (mgl-1)					Callus induction (%)	Callus morphology
BAP	NAA	KN	2,4-D	IBA		
1.0	1.0	0.0	0.0	0.0	60.0 ± 4.71	Slight whitish green
1.0	2.0	0.0	0.0	0.0	52.0 ± 6.32	Light green
2.0	3.0	0.0	0.0	0.0	56.0 ± 6.99	Light green
2.0	0.0	0.0	0.0	0.0	42.0 ± 6.32	Light yellowish green
2.0	0.0	0.0	0.0	0.0	52.0 ± 6.32	Light green
0.0	0.0	2.0	0.0	0.0	33.0 ± 4.83	Light green
0.0	0.0	3.0	0.0	0.0	40.0 ± 4.71	Yellowish Green
0.0	0.0	4.0	0.0	0.0	42.0 ± 7.89	Light yellowish green
0.0	0.0	5.0	0.0	0.0	50.0 ± 9.43	Yellowish Green
0.0	0.0	6.0	0.0	0.0	43.0± 8.23	Light brown
0.0	0.0	0.0	1.0	0.0	63.0 ± 6.75	Slight whitish Green
0.0	0.0	0.0	2.0	0.0	70.0 ± 4.71	Light green
0.0	0.0	0.0	3.0	0.0	81.0 ± 3.16	Light yellowish green
0.0	0.0	0.0	4.0	0.0	62.0 ± 4.22	Light green
0.0	0.0	0.0	5.0	0.0	51.0 ± 8.76	Light green
0.0	0.0	0.0	6.0	0.0	52.0 ± 7.98	Light yellowish green
0.0	1.0	0.0	1.0	0.0	100.0 ± 00	Slight whitish green
0.0	1.5	0.0	1.0	0.0	70.0± 4.71	Light green

0.0	1.0	0.0	2.0	0.0	81.0 ± 3.16	Light yellowish green
0.0	1.5	0.0	2.0	0.0	84.0 ± 5.16	Slight whitish green
0.0	0.0	0.0	1.0	0.5	51.0 ± 3.16	Yellowish green
0.0	0.0	0.0	1.0	1.0	43.0 ± 6.75	Light green
0.0	0.0	0.0	1.0	1.5	51.0 ± 3.16	Yellowish green
0.0	0.0	0.0	0.0	2.0	41.0 ± 3.16	Light green
0.0	0.0	0.0	0.0	2.5	43.0 ± 6.75	Light brown
Results are mean ± SD of three replicates						

B. Cell Suspension Culture

Callus cultured on MS basal liquid medium supplemented with 2, 4-D, BAP, and ascorbic acid showed growth initiation response. The addition of macrosalts such as NH_4NO_3(24.7 mM), KNO_3(56.4 mM), $MgSO_4$ (4.5 mM) and KH_2PO_4 (3.75 mM), showed different growth responses. Among them media supplemented with NH_4NO_3 (24.7 mM) showed highest growth of cells and highest growth cell viable [31] also reported similar results where optimal growth response (0.57PCV on fourteenth day) was reported on MS medium supplemented with NH_4NO_3 (24.7mM).

With the increment of sucrose concentration (3.0%, 3.5%, 4.0%, 4.5% and 5.0%) as well as increase in days showed optimal growth response. But, after twenty fifth day the growth rapidly declined. As shown in the Figure 4, the maximum growth of the cell (16670×10^4 cells per ml) was observed at 4.5% sucrose concentration on twentieth day, indicating the supportive role of NH_4NO_3 with addition of sucrose. Sucrose not only acts as an external source but also help to maintain osmotic potential of the culture [32],[33] which would permit the absorption of mineral nutrient present in the medium. Similarly, [34] also mentioned the maximum organogenesis frequency in medium supplemented with 4% sucrose with respect of minimum days 15.22 and 16.22 days.Our findings are consistent with the previous reports [35], [36],[37]. In plant cell cultures, manipulation of medium sucrose is a useful strategy for enhancing the process productivity [38].

Similarly, on the media supplemented with $MgSO_4$ (4.5mM with 4.5% sucrose on 25th day), KNO_3 (56.4mM with 4.0% sucrose on fifteenth day), KH_2PO_4 (3.75mM) showed varied growth responses. (Figure not shown).

C. Dynamics of Stevioside Production in Suspension Culture, Callus And Leave.

Dynamics of stevioside production in callus and suspension culture is shown in the Figure 5; Figure 6 and Figure 10;Figure 11. The highest amount of stevioside content in suspension culture (0.325mg/g) on twentieth day of cultivation was observed in the media supplemented with NH_4NO_3 and growth of the cell started at the beginning of exponential growth phase from days 5 of cultivation. Simultaneously stevioside content declined rapidly after twentieth days (0.210 mg/g) i.e. at the end of exponential phase but in comparison with callus, the steviol glycosides (2.997 mg/g) was observed highest at eighth week. In leaves of *S. rebaudiana* as shown in the Figure 7 and (Figure 11&12) the highest (40 mg/g FW) steviol glycoside was observed in Russian population in comparison to Indian population (21.2 mg/g FW).

The maximal content of the steviol glycosides (115 mg/g) of plant dry mass was found on the fourteenth day of the cultivation cycle, i.e. at the end of exponential growth phase [39]. Similar results were reported [40], where there is a decline in the stevioside content at the beginning of the stationary phase. A significant decrease in the content of synthesized stevioside [40] has been observed in suspension culture when compared with stevioside content of *Stevia* callus (415 µg g/DW) indicating that the production of stevioside has been influenced by disaggregation of the cells showing similar results with our findings but contradictory result also reported by [41] who simply explained the accumulation of stevioside in callus and suspension culture which varied in nutrient medium and culture conditions. Furthermore, biomass yield was measured simultaneously in the suspension culture supplemented with NH_4NO_3 with different sucrose concentration on twentieth days. The highest biomass yield (15.23gm/l) was observed at 4.5% as well as maximum steviol glycosides was also observed at 4.5% sucrose concentration indicating the supportive role of biomass and stevioside content (Figure 8).

Since suspension culture supplemented with NH_4NO_3 on twentieth days had highest cell growth we further added 100 µm of methyl jasmonate to see further response Figure 9; and showed maximum biomass yield (18.998gm/l) at the end of the exponential phase. Rapid declination in cell density was observed as stationary phase started. Our work of adding 100µm methyl jasmonate gave very significant results as compare with [42], where the efficient improvement of taxuyunnanine C accumulation was observed on 100µm of Methyl jasmonate.

Volume 3 Issue VIII, August 2015
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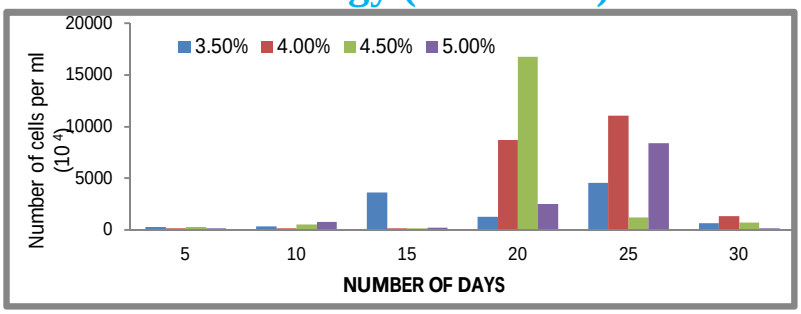

Fig. 4 Effect of NH_4NO_3 (24.7Mm) with different sucrose concentration in suspension culture

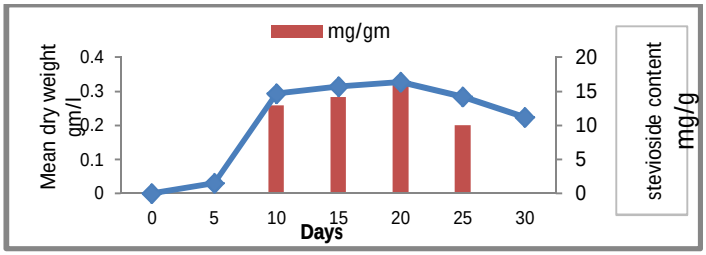

Fig. 5 Biomass and stevioside content in the suspension culture supplemented with NH_4NO_3

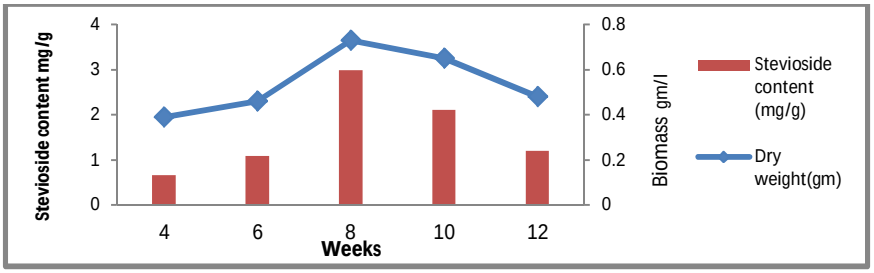

Fig. 6 Biomass and stevioside content in callus (Dry weight) of several weeks

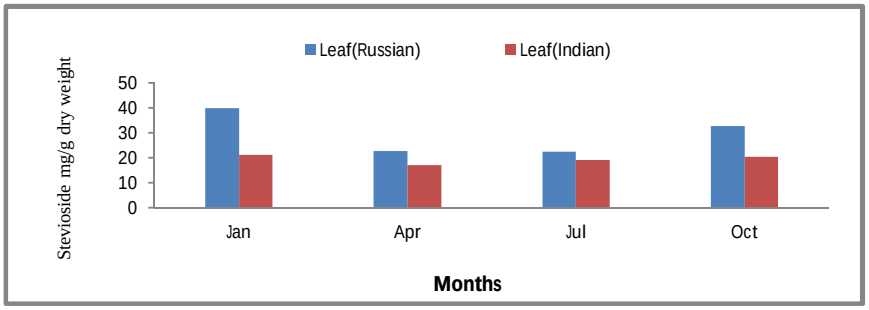

Fig. 7 Variations in Stevioside (mg/g Dry weight) in leaves of *S. rebaudiana* Bertoni

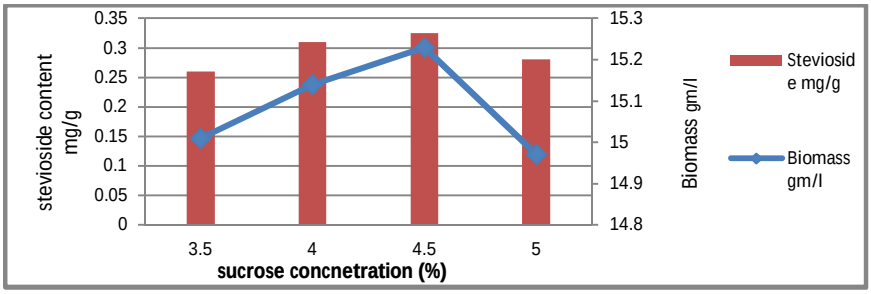

Fig. 8 Effect of sucrose concentration on biomass and stevioside content in media supplemented with NH_4NO_3 on 20^{th} days

Volume 3 Issue VIII, August 2015
ISSN: 2321-9653
International Journal for Research in Applied Science & Engineering Technology (IJRASET)

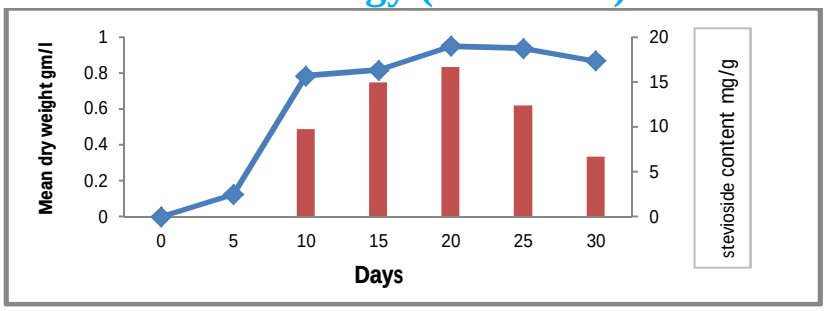

Fig. 9 Bio mass and stevioside content in the suspension culture supplemented with NH_4NO_3 in addition of methyl jasmonate

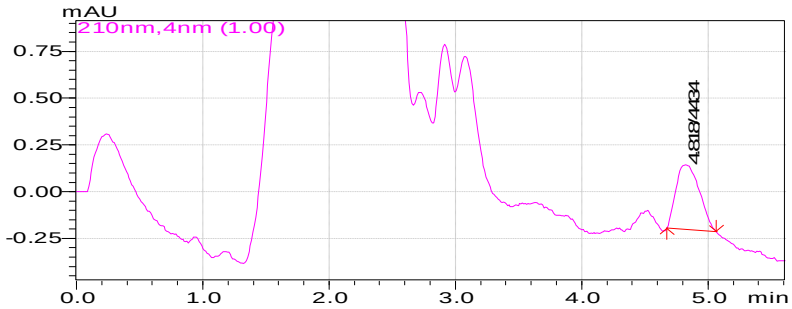

Fig.10 HPLC chromatogram of Stevioside (Ammonium Nitrate 20 days)

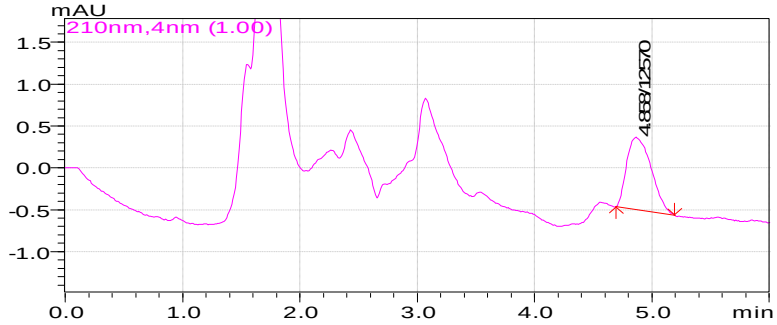

Fig. 11 HPLC chromatogram of stevioside in leaf of the Russian population in the month of January

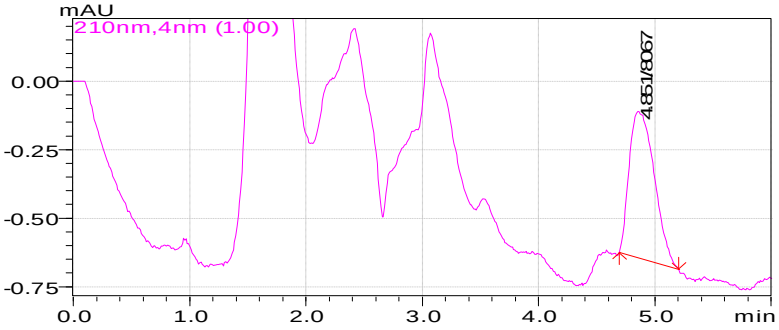

Fig. 12 HPLC chromatogram of stevioside in Leaf of the Indian population in the month of January

Volume 3 Issue VIII, August 2015
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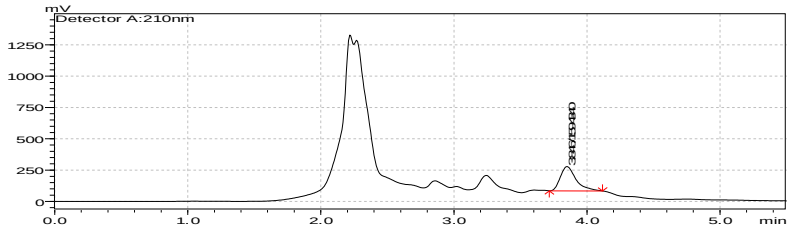

Fig. 13 HPLC chromatogram of Stevioside content in callus on 8th weeks

D. Molecular Characterization

1) RAPD Profiling: Out of 20 arbitrary primers screened, 10 revealed clear and distinct polymorphisms in 15 varieties producing 59 polymorphic loci. The percentage of polymorphism of the amplified DNA fragments was calculated as 89.39. Primers that revealed the distinct bands were OPA01, OPA02, OPA07, OPA09, OPA10, OPA11, OPA12, OPA16, OPA19 and OPA20. The size range of the amplified DNAs was between 500bp to 8,000bp as obtained by comparing with 1kb Marker DNA (Figure 14 to 17). The mean observed number of alleles was estimated to be 1.89390.3103 while effective number of alleles was 1.54200.3148. Hence, using the POPGEN 32 software package, VERSION 1.31, Nei's (1973) gene diversity was calculated to be 0.32130.1533 and Shannon's Information index for the populations was estimated to be 0.48130.207.

2) Nei's Unbiased Measures Of Genetic Identity And Genetic Distance : From Table 2, "Nei's Unbiased Measures of Genetic Identity and Genetic Distance" revealed by POPGEN 32 software package, VERSION 1.31, we can summarize the genetic identity and genetic distance between the samples studied. According to the table, genetic distance between populations I4 and CI1; I4 and CI2 was found to be 0.1643 i.e. the lowest value for genetic distance. Also these two shared highest genetic similarity value i.e. 0.8485.This suggests CI1 and CI2, calluses developed from Indian *Stevia* shared closest genetic distance and similarity, undoubtedly with the Indian *Stevia*, I4. Highest genetic distance was seen between populations R1 and I3 i.e. 0.6633. These two also shared least genetic similarity i.e.0.5152. Since they both were from different *Stevia* variety former from Russian *Stevia* and the later from Indian *Stevia* they clearly showed least genetic similarity. These results clearly indicated that the genetic characteristic of the *Stevia* samples relates to the variety of *Stevia* and callus grown. To make the analysis more prominent, similarity and cluster analysis were conducted using Nei's coefficient and the Unweighted Pair Group Method with Arithmetic mean (UPGMA).
In the Dendrogram, (Figure 18) we could clearly see that the callus populations were chained together showing their similar characters. It was seen that calluses from Russian *Stevia* chained by chain 2 share genetic similarities and same is the case with the calluses from Indian *Stevia* populations chained by chain 11. Also the chains between these two varieties were significantly separated by a large margin of genetic distance that shows their plant variety with their genetic dissimilarity. Similarly, the Indian *Stevia* populations are chained like a single bunch by chain 13, whereas the Russian *Stevia* populations are chained in a bunch by chain 12. However, R1 and I2 outcast themselves from their family chain and have become the exceptional cases. They have shown genetic dissimilarity with their family group and have outnumbered themselves by sharing genetic similarity with other family chains. Also from the Dendrogram, we can clearly interpret that Indian *Stevia* population and their callus populations are closer to each other and similar case goes with the Russian *Stevia* populations and their callus populations. Besides, the Russian *Stevia* populations are closer or say share less genetic distance with the Indian *Stevia* populations than the Indian calluses population; and Indian *Stevia* populations are closer to the Russian *Stevia* populations than their Russian calluses population.

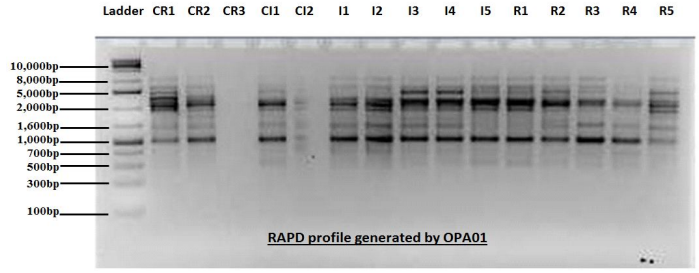

Fig.14 RAPD profile generated by OPA02 for Stevia

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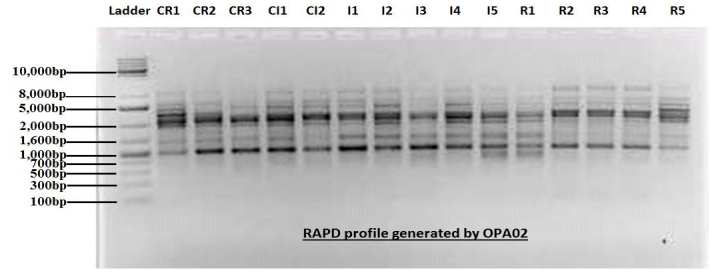

Fig.15 RAPD profile generated by OPA01 for *Stevia*

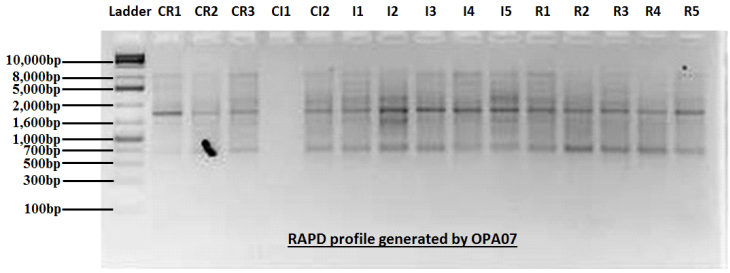

Fig.16 RAPD profile generated by OPA07 for *Stevia*

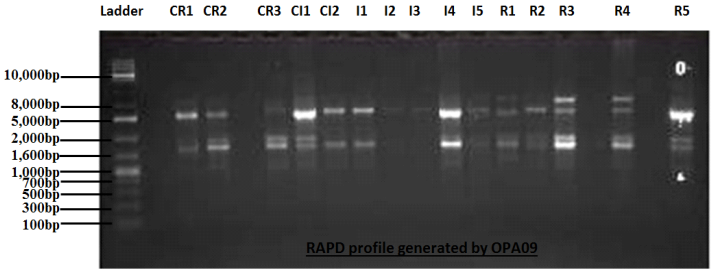

Fig. 17 RAPD profile generated by OPA09 for *Stevia*

Table 2

Nei's Unbiased Measures of Genetic Identity and Genetic distance

popn ID	CR1	CR2	CR3	R1	R2	R3	R4	R5	I2	I1	I3	I5	I4	CI1	CI2
CR1	******	0.7879	0.6515	0.7121	0.5909	0.7273	0.6515	0.6667	0.7273	0.6212	0.5909	0.6364	0.6212	0.6515	0.6818
CR2	0.2384	******	0.6818	0.7424	0.5909	0.6364	0.6212	0.7273	0.697	0.6818	0.5606	0.6667	0.6818	0.7121	0.6818
CR3	0.4285	0.383	******	0.6364	0.6667	0.5909	0.5455	0.622	0.7121	0.5455	0.6061	0.5606	0.6061	0.6061	0.6061
R1	0.3395	0.2978	0.452	******	0.5758	0.6212	0.6667	0.6515	0.6515	0.5455	0.5152	0.6515	0.6667	0.6667	0.6667
R2	0.5261	0.5261	0.4055	0.5521	******	0.6212	0.6061	0.6515	0.5606	0.6364	0.6061	0.5909	0.5758	0.6061	0.5455
R3	0.3185	0.452	0.5261	0.4761	0.4761	******	0.6818	0.6364	0.6667	0.6515	0.7424	0.6061	0.5606	0.5303	0.6515
R4	0.4285	0.4761	0.6061	0.4055	0.5008	0.383	******	0.6818	0.6515	0.697	0.697	0.7121	0.6667	0.6364	0.6667
R5	0.4055	0.3185	0.4761	0.4285	0.4285	0.452	0.383	******	0.6667	0.6515	0.6212	0.6364	0.6515	0.5909	0.7121
I2	0.3185	0.361	0.3395	0.4285	0.5787	0.4055	0.4285	0.4055	******	0.6515	0.6818	0.697	0.7424	0.7424	0.7727
I1	0.4761	0.383	0.6061	0.6061	0.452	0.4285	0.361	0.4285	0.4285	******	0.7879	0.712	0.6061	0.6061	0.6667
I3	0.5261	0.5787	0.5008	0.6633	0.5008	0.2978	0.361	0.4761	0.383	0.2384	******	0.7121	0.6364	0.5455	0.7576
I5	0.452	0.4055	0.5787	0.4285	0.5261	0.5008	0.3395	0.452	0.361	0.3395	0.3395	******	0.803	0.7424	0.7121
I4	0.4761	0.383	0.5008	0.4055	0.5521	0.5787	0.4055	0.4285	0.2978	0.5008	0.452	0.2194	******	0.8485	0.8485
CI1	0.4285	0.3395	0.5008	0.4055	0.5008	0.6343	0.452	0.5261	0.2978	0.5008	0.6061	0.2978	0.1643	******	0.7273
CI2	0.383	0.383	0.5008	0.4055	0.6061	0.4285	0.4055	0.3395	0.2578	0.4055	0.2776	0.3395	0.1643	0.3185	******

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

Volume 3 Issue VIII, August 2015
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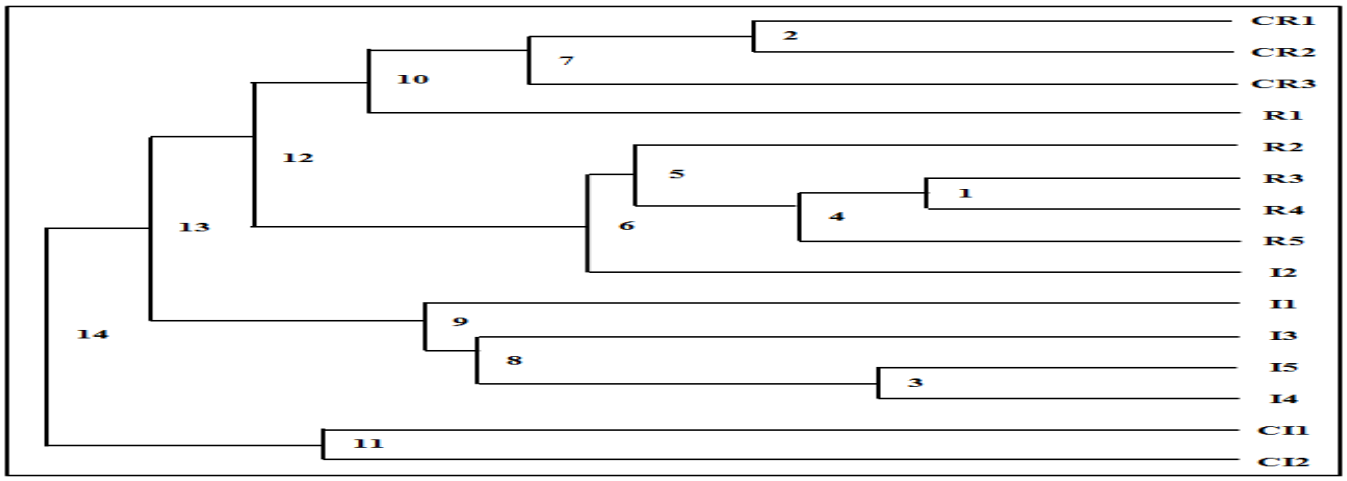

Fig. 18 Dendrogram Based Nei's (1978), Genetic distance: Method = UPGMA

IV. CONCLUSION

Tissue culture technology is the only process for the mass propagation, and this rapid and efficient regeneration protocol provides a strong way for the rapid and reliable multiplication and commercial production. The produced regenerative callus can be used for the establishment of suspension culture and somaclonal variants for the improvement of crops in Nepal. The stevioside content was recorded maximum on twentieth days of cultivation of the media supplemented with NH_4NO_3. Since the biomass and stevioside content of the suspension culture supplemented with NH_4NO_3 showed maximum result, indicating a positive correlation between cell growth and steviol glycosides synthesis. The high amount of steviol glycosides was present in the population of Russian as compared with Indian population in the month of January and minimum was recorded in the month of July and April (Figure 7). A significant decrease in the content of synthesized stevioside has been observed in suspension culture when compared with stevioside content in callus. Callus also showed somewhat variation from its mother stock. Hence, the difference in the origin from Indian and Russian population progressively leading to difference in the natural and artificial impacts could be the reason for difference between these two populations. Somaclonal variation was identified among the mother plants and callus using twenty sets of RAPD primers. Genetic similarity and genetic distance based on bands data revealed that the Indian, Russian and Callus showed variations. Hence, Utilizing RAPD Analysis and DNA Markers we came to the conclusion that calluses of Indian and Russian stevia population showed high genetic similarity with their mother plant which is an obvious expectation. However, the findings besides this was that the calluses of these two different population i.e., Indian and Russian showed genetic similarity with each other but their mother plants showed no any close relation to each other in terms of genetic similarity. This proves that mother plants of these two populations showed genetic variation but, as their calluses were grown they came to show genetic similarity to some extent.

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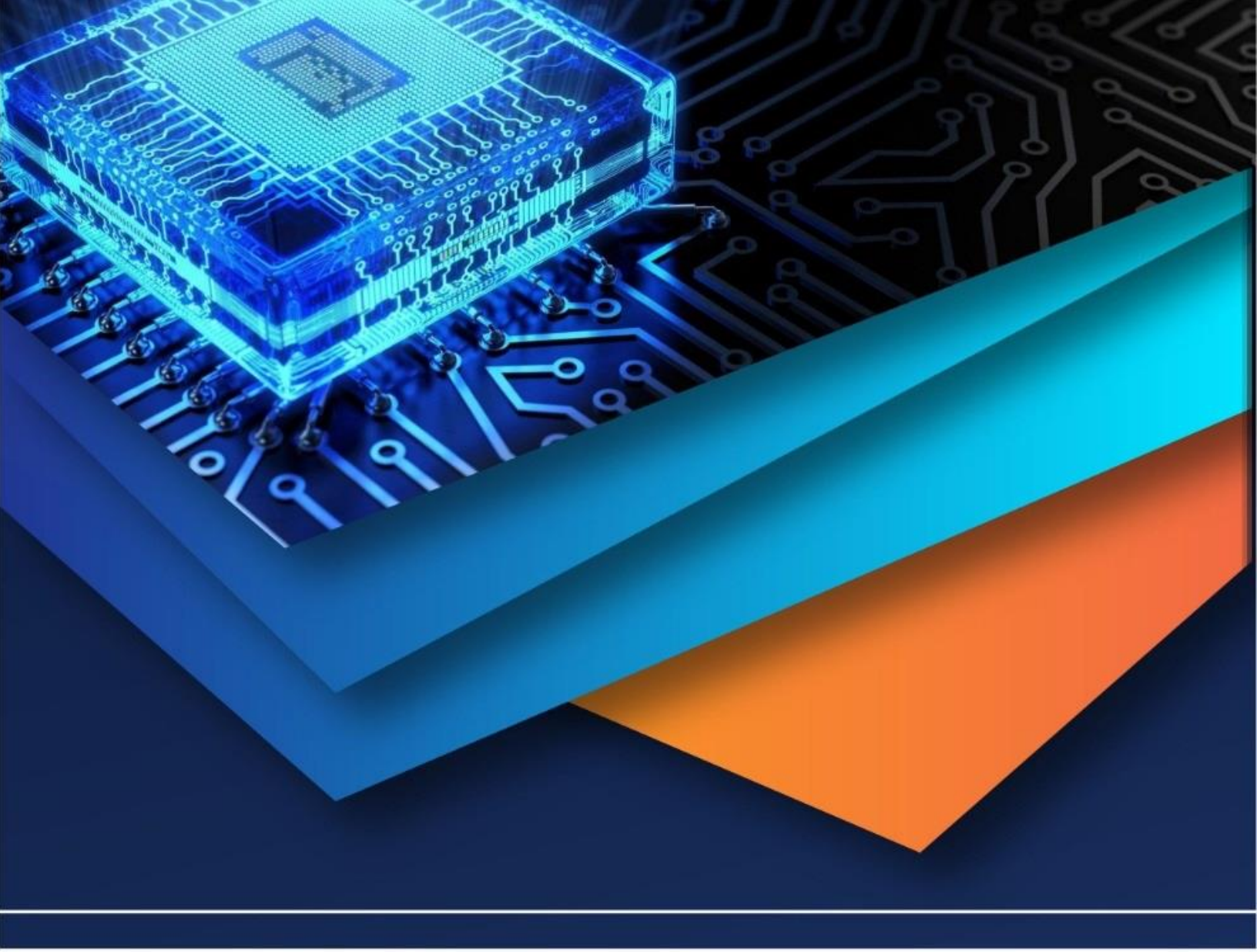

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