ASSESSMENT OF GENETIC DIVERGENCE, STEVIOSIDE AND REBAUDIOSIDE A CONTENTS AND THE EFFECTS OF GAMMA IRRADIATION ON THE PERFORMANCE OF STEVIA **(*Stevia rebaudiana* BERTONI) GENOTYPES.**

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SUMMARY

Stevia rebaudiana Bertoni is a perennial and sweet herb native to the highlands of Paraguay belongs to family Asteraceae. Seeds of five stevia varieties were treated with 0, 10, 15 and 20 K rad doses of gamma rays from Co^{60} Indian gamma cell source (Dose rate: 1,323KGy/h) to evaluate the effects of gamma irradiation on stevia genotype performance. Stevioside and rebaudioside A contents and genetic diversity among the nonrelated stevia varieties were assessed utilizing HPLC analysis and molecular markers respectively. The leaves dry weight as the most important agronomic trait was decreased significantly with increase gamma-ray dose in all studied varieties except China1 and Shou2 at 10 K rad and Sughigh at 20 K rad that was increased from 23.84 to 28.84 g, 31.38 to 36.25 g and from 32.88 to 40.75 g, respectively. The obtained results showed the existence of considerable amounts of genetic diversity among the five tested varieties with EST-SSRs, SSR, ISSR, and ISJ primers, suggesting the potential use of these markers and in detecting molecular diversity among stevia genotypes. Wide range diversity was found between Spanti and China1 varieties. However, the close pair of genotypes was China1 and Shou2. These results confirm the nearby origin of the genetic background of China1 and Shou2. The overall clustering was largely based on origin and /or genetic background. HPLC analysis of stevioside and rebaudioside A contents in stevia leaves revealed significant differences among the studied genotypes. A large amount of stevioside value was recorded by Shou2 variety (43.2 mg/500mg with 8.64%), while the lowest amount value was recorded by Eg1 (2.533 mg/500mg with 0.51%). The results demonstrated the power of molecular techniques used in distinguishing high stevioside content genotypes from lower content ones.

Stevia rebaudiana Bertoni is a perennial and sweet herb native to the highlands of Paraguay belongs to family Asteraceae (Brandle and Rose, 1992). It was first discovered by a Swiss botanist, Moises Santiago Bertoni in 1888 and exploit even since. Its genus contains

about 150 species of herbs and shrubs (Abdulameer *et. l.*, 2018). Commonly known names are honey leaf plant, sweet herbs, and sweet leaf. The leaves of Stevia contain glycoside diterpenes such as rebaudioside A, B, C, D, E, F, M, stevioside, steviol bioside, dulcoside A and dulcoside C which are estimated to be 200-350 times sweeter than sucrose with free calories (Ghaheri *et al*, 2017). In addition to non-caloric sweetener, stevia have many other therapeutic benefits such as anti-microbial (Debnath, 2008), anti-fungal (Silva *et al.*, 2008), anti-inflammatory (Gupta *et al*, 2013), anti-diarrheal (Takahashi *et al*, 2001), anti-tumour (Jayaraman *et al*, 2008), anti-virus (Kedik *et al.*, 2009), anti-hyperglycemic (Benford *et al*, 2006), anti-hypertensive (Hsieh *et al.*, 2003), and immunomodulatory effects (Chatsudthipong and Muanprasat, 2009).

In the early 1970s, stevioside known as the major sweetener present in leaf and stem tissue by Japanese consortium formed to commercialize stevioside and stevia extracts (Brandle and Rosa, 1992). Stevioside and rebaudioside A are the major components of the total glycoside content. The stevioside component is believed to give a somewhat bitter aftertaste with a "licorice" taste while rebaudioside A is more pleasant sweet taste with no bitter aftertaste (DuBois, 2000, Yadav *et al.*, 2011 and Chiew *et al.*, 2016).

The mutation is one of the breeding methods in several crops to improve crop quality and bioactive metabolites production such as in rice, maize, and cassava breeding for low amylase, protein contents improvement, and high amylase content, respectively, (Chiew *et al.*, 2016). Mutations can be induced by chemicals or various types of ionizing radiation (X-rays, gamma rays, neutrons, UV light, etc.) (Jain, 2010 and Ali *et al.*, 2015). Gamma rays and x-rays are the most widely used mutagens in mutation breeding (Mba, 2013).

Genetic diversity estimation between different genotypes using molecular marker techniques is very import in breeding programs (Sharma *et al.*, 2016). Molecular fingerprinting is a useful tool for several applications. Several molecular marker techniques are available now to assess the diversety between several genotypes such as Random Amplified Polymorphism (RAPD), Simple Sequence Repeats (SSR), Inter-Simple Sequence Repeats (ISSR), Single Nucleotide Polymorphism (SNP) (Williams *et al.*, 1990, Schulman, 2007 and Sharma *et al*, 2016). Therefore, the present investigation was undertaken to evaluate the effects of gamma irradiation on some stevia genotypes, the assessment of genetic diversity among tested varieties using molecular marker techniques and to evaluate stevioside and rebaudioside A contents using HPLC analysis.

MATERIALS AND METHODS

The present study was carried out at Genetics Department, Faculty of Agriculture, Kafrelsheikh University, Egypt, and Biotechnology Lab., Sakha station, Agricultural Research center, ministry of Agric., Egypt.

Plant MATERIALS

Seeds of five *Stevia rebaudiana* varieties (Spanti, Eg(1), China1, Shou2 and Sughigh) used in the present study were provided by Sugar Crops Research Institute, Agric., Research Center, Ministry of Agric., Egypt. The origin of each variety is presented in Table (1).

METHODS

Gamma irradiation treatments

Seeds of the five stevia varieties were treated with 10, 15 and 20 K rad doses of gamma rays from the Co^{60} Indian gamma cell source (Dose rate: 1,323KGy/h) as well as the untreated seeds used as the control (0.0 K rad). All seeds were grown in the greenhouse. Then the plantlets placed in the field to observe and evaluate the performance of each variety. All genotypes were grown in a randomized complete block design with four replications. Eleven agronomic traits were recorded, plant height (cm), tillers / plant , leaves fresh weight (g), leaves dry weight (g), moisture percentage, stem fresh weight (g), stem dry weight (g), fresh biomass (g), dry biomass (g), leaf shape and leaf area index.

Statistical analysis

Data were statistically analyzed using (ANOVA), compared using the least significant difference (LSD) at the (0.05) and (0.01) significance levels. The software system used for statistical analysis was (Minitab 15 statistical software).

Molecular analysis

DNA isolation

DNA of the five varieties of stevia was extracted using Gene JET Plant Genomic DNA Purification Mini Kit, according to manufacturing protocol.

Polymerase Chain Reaction (PCR)

A total of 23 DNA primers were used in this study. Table (2) showed primers type, names, sequences and annealing temperatures that were used to develop different markers.

PCR reaction for EST-SSRs and SSR primers was applied in 10 µl volume containing 2.00 µl of genomic DNA, 1.00 µL forward primer, 1.00µl reverse primer, 1.00 µl d.d H_2O, and 5 µl of 2x GoTaq Green Master Mix. PCR reaction for ISSR and ISJ primers was applied in 10 µl volume containing 2.00 µl of genomic DNA, 2.00µL of each primer, 1.00 µl d.d H_2O, and 5 µl of 2x GoTaq Green Master Mix. PCR reaction was performed using the following

profile: 94 ^{o}C for 5 min (initial denaturation), 94°C for 1 min, primer annealing temperature depending on the marker (Table2) for 1 min, 72°C for 2 min, followed by final extension step at 72 ^{o}C for 7 min. The DNA amplification products were analyzed by electrophoresis on 3% agarose gel in 1x Tris base, Acetic acid glacial and EDTA (TAE) buffer staining with ethidium promide. DNA was visualized using a UV transilluminator. The data generated from EST-SSRs, SSR, ISSR and ISJ analysis were analyzed using the Jaccard similarity coefficient (Jaccard, 1908). Dendrograms were constructed using the Jaccard similarity coefficient and the unweighted pair group method with an arithmetic average [UPGMA] employing the SAHN [sequential, agglomerative, hierarchical, and nested clustering] from the NTSYSpc (ver.2.10) program (Rohlf, 2005).

Genetic diversity, defined as the polymorphism information content (PIC; Anderson *et al*., 1993), was used to measure allelic diversity at each locus. PIC valueswere calculated.

Stevioside and Rebaudioside A content.

High-performance liquid chromatography (HPLC) analysis was done to evaluate the two main bioactive components (stevioside and rebaudioside A) in the five original varieties (Spanti, Eg1, China1, Shou2 and Sughigh) (Vanek *et al*., 2001).

RESULTS AND DISCUSSION

The significance levels for all studied traits, such as plant height (cm), tillers plant^{-1}, leaves fresh weight (g), leaves dry weight (g), moisture%, stem fresh weight (g), dry stem weight (g), fresh biomass (g), dry biomass (g), leaf shape and leaf area index are shown in Table 3. Analysis of variance revealed highly significant differences among all studied genotypes and interaction between varieties and doses of gamma irradiation treatments evaluated for all studied traits. The doses of gamma irradiation treatments differed significantly ($P<0.01$) for all measured characteristics except leaf shape and leaf area index which showed non-significance effects. The results are in the same line with those reported by Brindle and Rosa (1992), Ali *et. al.,* (2015), Chiew *et. al.,* (2016), Gerami *et. al.,* (2017) Ghaheri *et al.,* (2017) and Ahmad *et. al.,* (2018).

Performance of agronomic traits

The mean performance of five varieties (Spanti, Eg1, China1, Shou2 and Sughigh) with their gamma irradiation treatments (0, 10, 15 and 20 K rad) for eleven agronomic traits are presented in Table (3).

In M_1 generation, the data obtained concerning the plant height as compared to control, revealed that gamma rays doses affected significantly this trait in all the studied varieties Table (3).

Significant differences were observed among the treatments of gamma rays and the control on one side and among the treatments itself on the other side. Plant height character, in general, was decreased by increasing the dose of gamma radiations in all the studied varieties, except the variety Shou2 which was increased by increasing the dose. The highest plant height value was observed at 15 K rad in all varieties except Sughigh gave the highest plant height value at 20 K rad. On the other hand, the lowest plant height was observed at 20 K rad treatment. Concerning the varieties, the highest plant height value (51.17 cm) was recorded for Eg1 at control followed by Shou2 at 15 K rad treatment. Meanwhile, the higher dose of gamma-ray (20 K rad) showed deathly dose in Spanti variety.

Regarding number of tillers/plant, the results indicated that number of tillers/plant decreased significantly in all treatments for the varieties Spanti, Eg1 and China1 except at 20 Krad for Eg1 variety which gave 30.97 tillers compared by 30.52 at control. On the other hand, number of tillers/plant was increased significantly for all treatments compared with control for the stevia varieties Shou 2 and Sughigh. There were significant differences detected between the control among the varieties and between the doses of gamma rays itself.

For leaves fresh weight (g), the results illustrated that the values decreased with gamma-rays treatments and cases of significant decreases were found in the varieties, Spanti at all doses, Eg1 for all doses except at 20 K rad was increased, Shou 2 except at 10 K rad and Sughigh except at 10 k rad. On the other hand, the leaves fresh weight increased significantly with all gamma rays treatments for the variety of China1. Data obtained revealed that leaves fresh weight (g) increased from 40.78 g to 74.99, 51.63 and 46.81 g at 10, 15 and 20 k rad for China1 variety, respectively, from 76.91 to 87.33 g at 20 K rad for the variety Eg1, from 102.58 to 120.31 g at 10 K rad for the variety Shou 2 and from 84.75 to 89.85g at 10 K rad for the variety Sughigh when comparing the control. These findings indicated that these varieties are more sensitive to gamma rays than other varieties.

Results showed that the leaves dry weight, in general, decreased significantly with gamma-ray treatments in all the varieties studied except China1 and Shou 2 at 10 K rad and Sughigh at 20 K rad was increased from 23.84 to 28.84 g, 31.38 to 36.25 g and from 32.88 to 40.75 g for these varieties, respectively.

The obtained data indicated that the moisture percentage decreased significantly with gamma rays treatments in all studied varieties except Eg1 at 20 K rad, China1 at 10, 15 and 20 K rad, Shou 2 at 10 K rad and Sughigh at 10 and 15 k rad, respectively, were increased.

The maximum increases in moisture observed for the variety Sughigh (71.04%) and Eg1 (68.94%).

The data observed that the stem fresh weight has been decreased for almost varieties under gamma radiation treatments compared with control except Shou 2 at 10 K rad, Sughigh at 20 K rad, Eg1 at 10 K rad and China1 at 10 and 15 K rad with recorded values 106.38, 97.88, 77.69, 61.19 and 43.75 g, respectively.

In the same direction Table (3) showed that most gamma radiation treatments decrease the dry stem weight except at 10 K rad application in Eg1 (39.13 g), Shou 2 (34.00 g) and China1 (19.44 g) and 20 K rad in Sughigh (33.13 g) which increase values of these varieties.

Concerning the fresh biomass(g), most desirable effect of gamma radiation treatments found in Shou 2 variety (226.69 g) at 10 K rad followed by Sughigh (181.72 g) at 20 K rad, Eg1 (147.80 g) and China1 (136.17 and 95.38 g) at 10 and 15 K rad, respectively, which increase the fresh biomass with significant values. On the other side, the residual gamma radiation treatments showed decrease of this trait with different effects.

For dry biomass, data showed positive significant effects of mean performance at 10 k rad for Shou 2 (70.25 g), Eg1 (66.25 g) and China1 (48.28 g), respectively, and 20 K rad for Sughigh (73.88 g). Generally, the 15 K rad gamma radiation treatment showed the lowest mean values for most studies varieties.

The results of the mean performance showed positive significant effects as revealed by values of leaf shape characteristic for varieties Spanti, Shou 2 and Sughigh at all gamma irradiation treatments (10, 15 and 20 K rad) which increase the leaf shape values (Table 3). On the other hand, the leaf shape values decreased significantly at most of gamma irradiation doses in Eg1 and China1 varieties. The highest significant values showed in Sughigh (4.36), Sughigh (4.19), China1 (3.96) and Shou 2 (3.80) at 10, 20, 20, 20 K rad gamma irradiation treatments, respectively.

The obtained data showed that the leaf area index decreased significantly in all treatments for the varieties Spanti, China1 and Shou 2 except 15 K rad for the China1 variety which gave 8.41 compared by 6.11 at the control and at 20 K rad for Shou 2 variety which gave 5.95 compared by 4.49. Doses 10 and 20 K rad for Eg1 (5.78 and 7.95) and Sughigh (5.10 and 4.96) showed a positive significant increase. There were significant differences detected between the control and the varieties and between the doses of gamma rays itself. These results in agreement with Brandle and Rosa (1992), Ali *et.al.,* (2015), Zaman *et.al.,* (2015), Chiew *et.al.,* (2016), Benhmimou *et al* (2017), Gerami *et.al.,* (2017) Ghaheri *et.al.,* (2017), Abdulameer *et.al.,* (2018) and Ahmad *et.al.,* (2018).

Genetic diversity of studied genotypes

Table (4) showed the pair-wise similarity percentage for SSR primers among tested five varieties Spanti, Eg1, China1, Shou2 and Sughigh. Based on the Jaccard coefficient the similarity values ranged from 0.290 between Spanti and China1 to 0.719 between Shou2 and Sughigh.

In ISSR the results in table (4) showed among the tested five varieties that the similarity values ranged from 0.3١٥ between Eg1 and sughigh to 0.764 between Shou 2 and China1.

In ISJ the results in table (4) showed that the similarity values ranged from 0.333 between Spanti and China1 to 0.571 between Shou 2 and Eg1.

The dendrogram constructed based on the banding patterns of the eight SSR primers showed in Fig. (1). Among the five tested genotypes that they were clustered to two main groups A and B. cluster A included four stevia genotypes (Sughigh, Shou2, China1, and Eg1) while the cluster B included Spanti variety. Group A divided into two subgroups a1 and a2. The subgroup a1 included Shou2 and Sughigh while a2 subgroup included varieties Eg1 and China1.

Fig (2) showed in the dendrogram constructed based on the banding patterns of the five ISSR primers. The tested genotypes were clustered to two main groups A and B. cluster A included three stevia genotypes (Sughigh, Shou2, and China1) while the cluster B included the two varieties Spanti and Eg1. At 58% the group A divided into two subgroups a1 and a2. The subgroup a1 included only the variety Sughigh while a2 subgroup involved the Shou2 and China1. Group B included Eg1 and Spanti varieties.

Fig. (3) showed the dendrogram constructed based on the banding patterns of ten ISJ primers. The tested genotypes were clustered to two main groups A and B. cluster A involved three stevia genotypes (China1, Shou2, and Eg1) while the cluster B included the two varieties Sughigh and Spanti. At 54% the group A divided into two subgroups a1 and a2. The subgroup a1 included only variety of China1 while a2 subgroup included the Shou2 and Eg1 varieties. Group B included Sughigh and Spanti varieties.

Generally, based on the banding patterns of the over all of the 23 used primers (EST-SSRs, SSR, ISSR, and ISJ), The similarity percentage among tested five varieties based on Jaccard coefficient showed in the Table (5). The similarity values ranged from 0.318 between Spanti and China1 varieties to 0.606 between China1 and Shou 2.

Fig (4) showed the dendrogram constructed based on the banding patterns of the 23 primers of the used markers. Genotypes were clustered to two main groups A and B. cluster

A involved four stevia genotypes (Sughigh, Shou2, China1, and Eg1) while the cluster B included only Spanti variety. At 53% the group A divided into two subgroups a1 and a2. The subgroup a1 included three stevia genotypes, China1 and Shou2 and Sughigh on the other side, while a2 subgroup involved only Eg1 variety.

The obtained results showed genetic diversity among the five tested varieties with SSR, ISSR and ISJ techniques which suggested to use these markers and these techniques to diverse between stevia genotypes. Also, the wide range of diversity found between Spanti and China1 varieties according to all tested primers. However, the close relationship found between China1 and Shou2. These results explain the nearby of the origin of the genetic background of China1 and Shou2. On the other hand, the distantly related was observed between Spanti and China1 which explain the diversity of genetic background for both varieties. From these results, we can select breeding materials to develop Stevia genotypes based on molecular markers techniques hence the selection of the wide range of diversity between genotypes plays the main role in plant breeding development programs such as the hybridization method.

Similar findings were reported on utilizing molecular marker techniques for accessing molecular diversity between genotypes. Sharma *et.al., (*2016) used 22 RAPD primers and 23 ISSR primers to study the diversity between 16 collected accessions of stevia. 81.8% of RAPD primers showed polymorphism, while 89.8% of ISSR showed polymorphism. Furthermore, Kaur *et.al.*, 2015 reported that 5548 stevia ESTs sequences from leaf tissues were retrieved from the NCBI singleton ESTs. 168 SSRs were identified, 61.41% of SSR primers generated polymorphism.

Othman *et.al., (*2016) used 17 stevia accessions mostly from Paraguay and Malaysia and assayed by ISSR markers to differentiate and explore their genetic relationships. They found 82.8% of 332 clear bands were polymorphic.

A total of eight SSR primers used in this study were observed to be polymorphic (Table 7). The highest number of polymorphic bands was nine bands in primer RM289. The eight selected SSR primers yielded 39 scorable bands, 29 of which were polymorphic (74%). The lowest number of bands observed was two bands in primers SSR7 and RM178, while the highest number of bands was ten bands in primer RM289. The PIC values ranged from 0.47 for primer RM178 to 0.88 for primer RM289.

Concerning ISSR markers, a total of five ISSR primers used in this study were observed to be polymorphic (Table 7). The highest number of the polymorphic bands was eight bands in primer IISRS-3-C. The five selected ISSR primers yielded 34 scorable bands, 24 of which were polymorphic (70.6%). The lowest number of bands observed was four in

primer IISRS-3-M, while the highest number of bands was ten in primer IISRS-3-C. The PIC values ranged from 0.67 for primer IISRS-3-M to 0.88 for primer IISRS-3-C.

For ISJ, a total of ten primers used in the present study were observed to be polymorphic (Table 7). The highest number of polymorphic bands was 11 in primer ISJ-10. The ten selected ISJ primers yielded 84 scorable bands, 67 of which were polymorphic (79.76%). The lowest number of bands observed was five bands in primer ISJ-8, while the highest number of bands was 12 in primer ISJ-10. The PIC values ranged from 0.76 for primers ISJ-8 to 0.91 for primer ISJ-10.

HPLC analysis for the content of main bioactive components in stevia (stevioside and rebaudioside A).

The data presented in Table (7) and Figs (5 and 6) revealed that leaves of stevia from five varieties (Spanti, Eg1, China1, Shou2 and Sughigh) showed different amount of stevioside and rebaudioside A. The large amount of stevioside showed by Shou2 variety (43.2 mg/500mg with 8.64%), while the lowest amount recorded by Eg1 (2.533 mg/500mg with 0.51%).

For rebaudioside A, the highest value recorded by China1 (14.643 mg/500mg with 2.928%), while the varieties Eg1 and Sughigh have undetectable amount of rebaudioside A in its tested sample. Similar findings reported by El-Dabaawy, (2012), Chester *et.al.*, (2013), Ali *et.al.*,(2015), Gonzáleza *et.al.*, (2015) and Rodenburg *et.al.*, (2016).

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Table(1). Names and origin of the tested varieties.

No	Genotype	Origin
1	Spanti	Span
2	Eg1	Egypt
3	China1	China
4	Shou2	China
5	Sughigh	Paraguay

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Table(2): Primer type, name, sequences and annealing temperatures for each EST-SSRs, SSR, ISSR and ISJ.

Primer ype	Primer name	Forward sequence	Reverse sequence
EST-SSRs (4)	SSR 7	5'-AAGCAGTCTATTCAAAAGCCTCA-3-	5- - CAACAGCAACCTCCAAATGA-3-
	SSR8	5'- GCAGAAGGGGAAACAATCAA-3'	5'-GGTAATACGGGGGATGAGGT-3'
	SSR9	5'-GCTGAAAGCCGTTTGAGATT-3'	5'-CAAACCAACCATCATTAGTCTTTTT-3
	SSR10	5'-TCCCAATTCAAATCCCTCAA-3'	5'-CGTTTGTGGTGCAGATTACG-3'
SSR(4)	RM463	5'-TTCCCCTCCTTTTATGGTGC-3'	5'-TGTTCTCCTCAGTCACTGCG-3'
	RM324	5'-CTGATTCCACACACTTGTGC-3'	5'-GATTCCACGTCAGGATCTTC-3'
	RM178	5'-TCGCGTGAAAGATAAGCGGCGC-3'	5'-GATCACCGTTCCCTCCGCCTGC-3'
	RM289	5'-TTCCATGGCACACAAGCC-3'	5'-CTGTGCACGAACTTCCAAAG-3'
ISSR (5)	PRIMER-1	5'-CACACACACACACACAAGG-3'	
	IISRS-3-N	5'-CACACACACACACACATG-3'	
	IISRS-3-M	5'- ACACACACACACACAC-3'	
	IISRS-3-C	5'-TCTCTCTCTCTCTCTCA-3'	
	IISRS-3-E	5'- ACACACACACACACACG-3'	
ISJ (10)	ISJ- 2	5'-ACTTACCTGAGGCGCCAC-3'	
	ISJ- 4	5'- GTCGGCGGACAGGTAAGT-3'	
	ISJ-5	5'-CAGGGTCCCACCTGCA-3'	
	ISJ-6	5'-ACTTACCTGAGCCAGCGA-3'	
	ISJ-7	5'-TGCAGGTCAGGACCCT-3'	
	ISJ-8	5'-GACCGCTTGCAGGTAAGT-3'	
	ISJ-9	5'-AGGTGACCGACCTGCA-3'	
	ISJ-10	5'-ACTTACCTGCATCCCCCT-3'	
	ISJ-11	5'-TGCAGGTCAAACGTCG-3'	
	ISJ-12	5'-GGACTGGAGCAGGTAAGT-3'	

Table (3): Performance of five Stevia varieties and their gamma irradiation doses for eleven measured traits.

ety	Treatment (K.Rad)	Plant height	Tillers plant-1	*Leaves fresh weight* (g)	Leaves dry weight (g)	Moisture percentage	Stem fresh weight (g)	Dry stem weight (g)	Fresh biomass (g)	Dry bi-omass (g)	Leaf shape
	Control	47.89	33.54	154.47	54.49	64.73	165.38	51.25	319.85	105.74	2.84
ti	10	46.25	25.60	22.32	12.88	42.07	45.75	16.19	68.07	29.06	3.10
	15	48.13	31.00	42.24	22.74	46.17	47.25	18.11	89.49	40.85	3.01
	20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Control	51.17	30.52	76.91	31.56	58.94	69.50	22.50	146.41	54.06	3.50
	10	40.69	21.25	64.81	27.13	58.15	77.69	39.13	142.50	66.25	3.35
	15	40.96	27.69	15.72	8.00	49.10	9.69	4.00	25.41	12.00	3.34
	20	39.00	30.97	87.33	27.13	68.94	60.47	20.38	147.80	47.50	2.72

	Control	43.45	34.30	40.78	23.13	43.24	35.83	12.88	76.61	36.00	3.54
a1	10	40.90	25.75	74.99	28.84	61.53	61.19	19.44	136.17	48.28	3.26
	15	41.63	24.83	51.63	20.00	61.23	43.75	11.75	95.38	31.75	2.96
	20	38.53	28.60	46.81	19.13	59.16	24.19	6.00	71.00	25.13	3.96
	Control	41.17	26.22	102.58	31.38	69.44	83.63	26.63	186.20	58.00	3.08
2	10	49.11	30.72	120.31	36.25	69.86	106.38	34.00	226.69	70.25	3.64
	15	49.20	28.77	45.63	15.38	66.35	35.94	9.75	81.56	25.13	3.32
	20	43.85	29.20	75.01	26.00	65.31	78.38	23.75	153.39	49.75	3.80
	Control	47.73	26.42	84.75	32.88	61.20	77.19	29.38	161.94	62.25	3.17
gh	10	43.12	27.08	89.85	26.00	71.04	65.88	27.38	155.72	53.38	3.58
	15	40.82	31.74	71.28	23.38	67.13	67.00	21.75	138.28	45.13	4.36
	20	44.33	30.74	83.85	40.75	51.36	97.88	33.13	181.72	73.88	4.19
D	0.05	1.97	1.74	1.83	1.57	3.16	1.71	1.27	2.64	1.87	0.73
	0.01	2.82	2.49	2.62	2.25	4.52	2.45	1.82	3.78	2.67	1.04

Table (4): similarity index based on Jaccard methods on EST-SSRs, SSR, ISSR and ISJ Primers.

EST-SSRs and SSR Primers					
	Spanti	Eg1	China1	Shou2	Sughigh
Spanti	1.000				
Eg1	0.474	1.000			
China1	0.290	0.643	1.000		
Shou2	0.308	0.577	0.636	1.000	
Sughigh	0.379	0.633	0.649	0.719	1.000
ISSR Primers					
	Spanti	Eg1	China1	Shou2	Sughigh
Spanti	1.000				
Eg1	0.518	1.000			
China1	0.321	0.416	1.000		
Shou2	0.370	0.416	0.764	1.000	
Sughigh	0.526	0.315	0.500	0.666	1.000
ISJ Primers					
	Spanti	Eg1	China1	Shou2	Sughigh
Spanti	1.000				
Eg1	0.400	1.000			
China1	0.333	0.526	1.000		
Shou2	0.440	0.571	0.556	1.000	
Sughigh	0.511	0.514	0.479	0.522	1.000

Table (5): similarity index based on Jaccard methods combined tested markers.

	Spanti	Eg1	China1	Shou2	Sughigh
Spanti	1.000				
Eg1	0.445	1.000			
China1	0.318	0.531	1.000		
Shou2	0.388	0.541	0.606	1.000	
Sughigh	0.473	0.512	0.531	0.596	1.000

Table (6): list of polymorphic EST-SSRs, SSR, ISSR, and ISJ primers.

No	Primer name	Total no. of amplified bands	No. of polymorphic bands	PIC value
EST-SSRs and SSR Primers				
1	SSR 7	2	1	0.49
2	SSR8	6	4	0.78
3	SSR9	3	1	0.61
4	SSR10	3	2	0.62
5	RM463	4	3	0.67
6	RM324	9	8	0.86
7	RM178	2	1	0.47
8	RM289	10	9	0.88
Total		39	29	
ISSR Primers				
1	PRIMER-1	6	6	0.82
2	IISRS-3-N	5	3	0.78
3	IISRS-3-M	4	2	0.67
4	IISRS-3-C	10	8	0.88
5	IISRS-3-E	9	5	0.87
Total		34	24	
ISJ primers				
1	ISJ- 2	10	8	0.88
2	ISJ- 4	10	7	0.88
3	ISJ-5	6	5	0.80
4	ISJ-6	8	7	0.85
5	ISJ-7	10	9	0.89
6	ISJ-8	5	4	0.76
7	ISJ-9	6	5	0.78
8	ISJ-10	12	11	0.91
9	ISJ-11	9	6	0.87
10	ISJ-12	8	5	0.86
Total		84	67	

Table (7): percentage and amount of stevioside and rebaudioside A in five tested genotypes.

Genotypes	Stevioside		Rebaudioside A	
	%	mg/500mg	%	mg/500mg
Spanti	4.780	23.906	0.088	0.444
Eg1	0.510	2.533	ND	ND
China1	3.780	18.903	2.928	14.643
Shou2	8.64	43.2	0.248	1.24
Sughigh	3.950	19.794	ND	ND

ND: undetectable

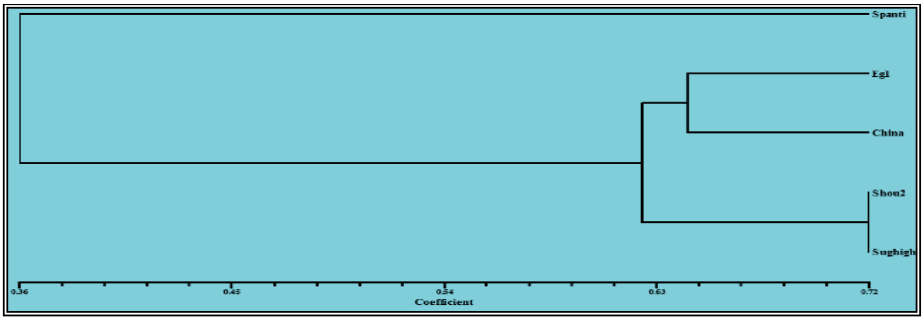
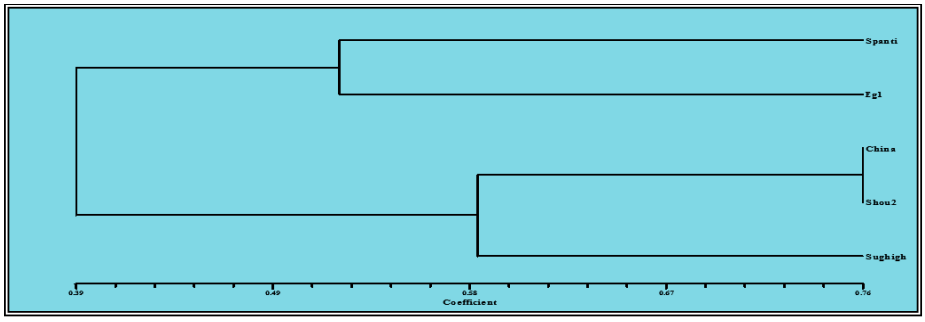


Fig. (1): Dendrogram of genetic relationship among the tested genotypes with eight SSR primers using Jaccard's coefficient of similarity.



Fig(2): Dendrogram of genetic relationship among the tested genotypes with five ISSR primers using Jaccard's coefficient of similarity.

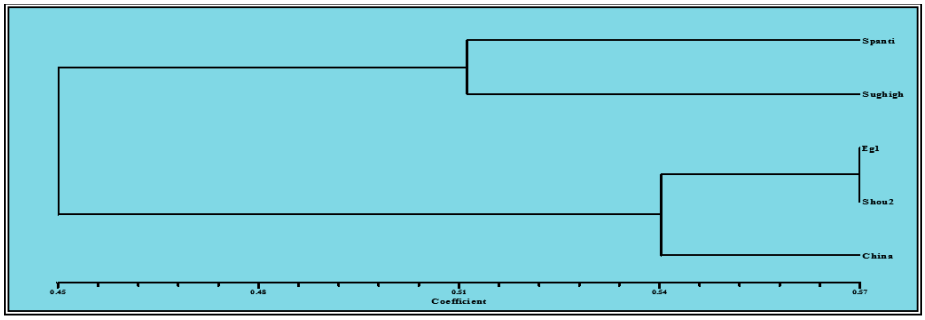


Fig (3): Dendrogram of genetic relationship among the tested genotypes with ten ISJ primers using Jaccard's coefficient of similarity.

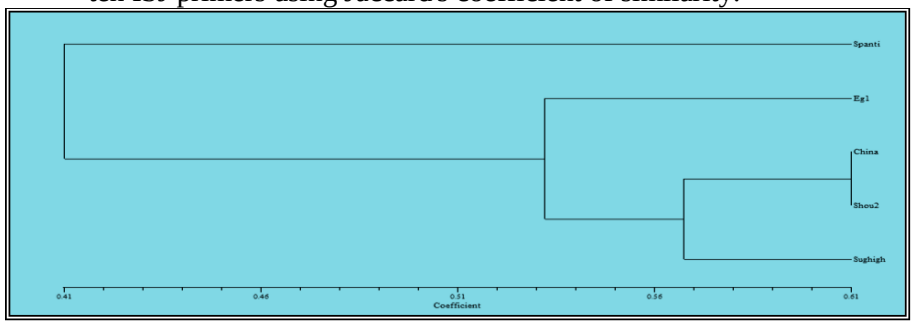


Fig. (4): Dendrogram of genetic relationship among the tested genotypes with 23 tested markers (EST-SSRs, SSR, ISSR, and ISJ) using Jaccard's coefficient of similarity.

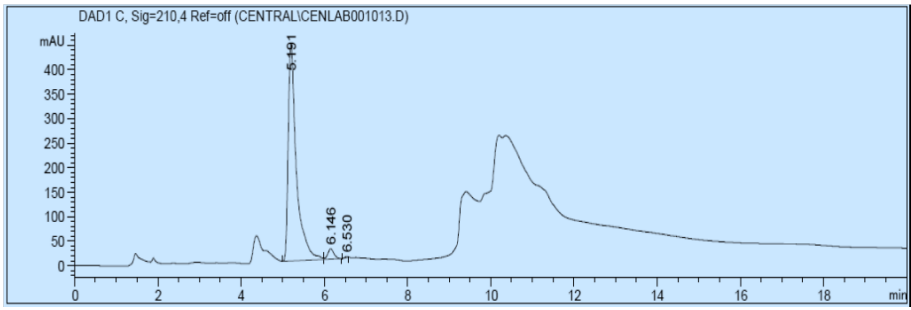
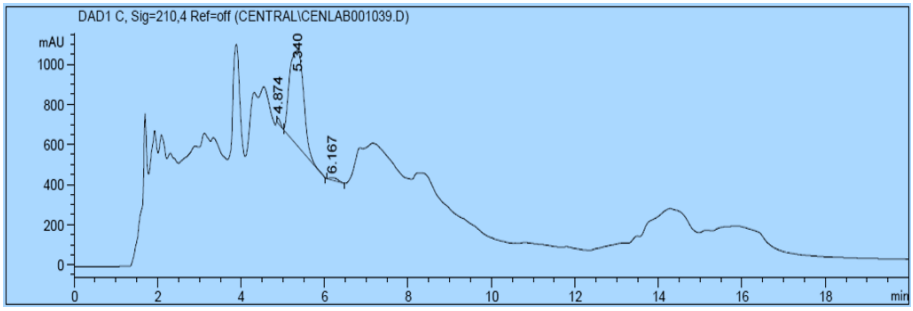
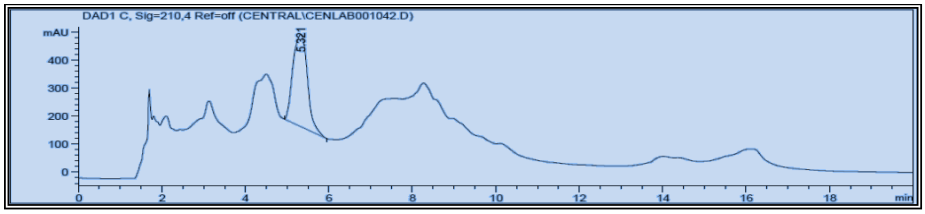


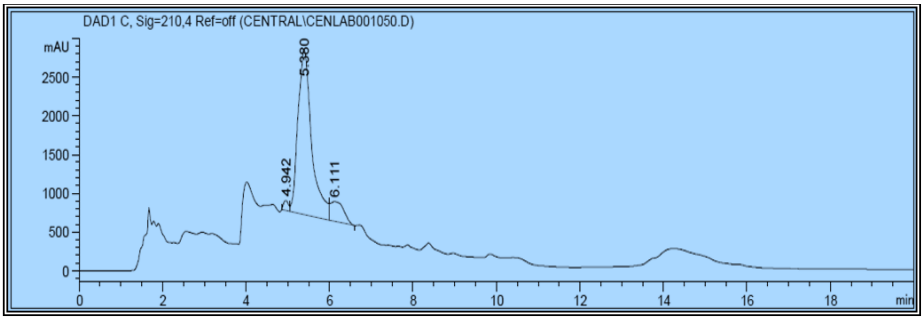
Fig. (5): Stevioside and rebaudioside A standard.



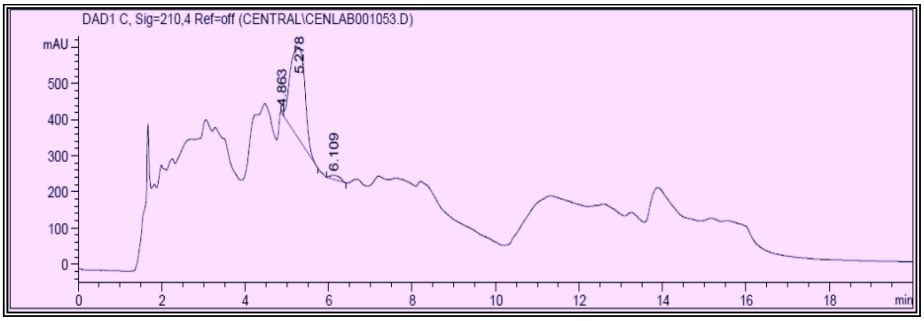
Spanti



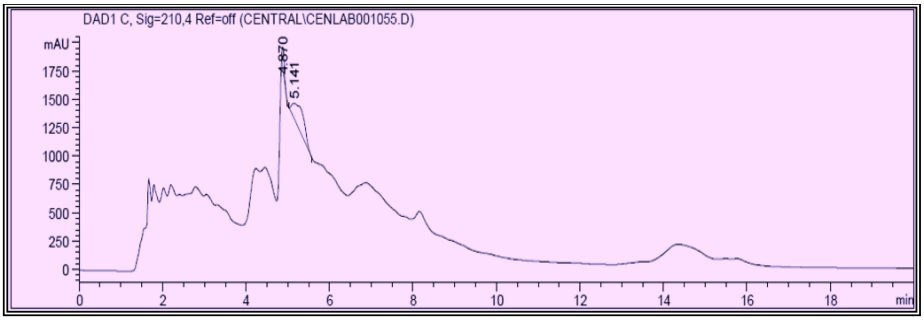
Eg1



China1



Shou2



Sughigh

Fig. (6): HPLC analysis of stevioside and rebaudioside A contents in stevia genotypes (Spanti, Eg1, China1, Shou2 and Sughigh**)**