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USE OF RAPD MARKERS TO DETECT GENETIC DIVERSITY OF CELL LINES FROM CALLUS CLONES DERIVED FROM THE CELL SUSPENSION CULTURES OF *STEVIA REBAUDIANA* BERTONI PLANT

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ABSTRACT

Twenty five cellular lines from callus clones derived from the cultivation of cell suspension cultures of *Stevia rebaudiana* Bertoni plant were tested with two controls(callus and leaf) by RAPD Technique and produced forty one polymorphic bands with no monomorphic band, the samples were separated into two core groups with 0.72similarity, I group including 24 clones, while group II consist just one clone(10)which isolated from other samples protection, The group I separated in 2 clusters with 0.30 similarity were A that split into sub cluster with 0.10converge rate, A1 was segment to two a part which had a greatest ratio 0.03 affinity, part 1: including 11 genotypes, while part 2 including only one stevia genotype (15), A2 split up to two sub clusters carried 0.05 similarity, each of them holed three genotypes (11, 16, and 19), (13, 14, and 18) sequentially, B cluster divided in two sub clusters which were grouped together with average 0.25 similarity, The first contained four clones(4, 7, 9 and 21) while the second sub cluster have just two stevia genotypes (6 and 17).

Keywords: DNA marker, cell suspensions, *Stevia rebaudiana* Bertoni, *Stevia* callus, genetic diversity

Introduction

The first botanically described *Stevia rebaudiana* Bertoni (Asteraceae) in 1899 was by the botanist M.S. Bertoni. *S. rebaudiana* is one of the 154 species of the genus (Soejarto *et al*., 1983). Stevia is a perennial herb native wild plant in South America (Paraguay and Brazil), generally it might be found growing in semi arid habitat ranging from grassland to shrub forest to mountain. Stevia is consider as a natural sweetener plant commonly known as Sweet Weed, Sweet Leaf, Sweet Herbs or Honey Leaf (Chavan *et al.*, 2017), due contains of diterpene glycosides (stevioside and rebaudiosides) in its leaf tissues (Ahmed *et al*., 1980). Pure extract stevioside is noncaloric reported to be over 200–300 time sweeter than sucrose (Starratt and Gijzen 2004; Yadav *et al.,* 2011), therefore these high potency sweeteners has attracted a role deal of interest in Stevia production and breeding (Yao *et al*., 1999). Moreover, in nature, the plant is cross pollinated and due to the selection process, varieties with new morphological characters arose, possibility to raise an elite population plants having uniformly high stevioside content in there tissue and their genetic variability is poorly understood, Additionally, Seed germination naturally is unsuccessful relatively due to infertile seed and small endosperm which that does not allow to product homogeneous populations that cause different in sweetening levels and diversiform (Nakamura and Tamura, 1985), limited vegetative propagation too is by a few number of individuals that can be obtained simultaneously from a single plant (Shock, 1982; Goettemoeller and Ching, 1999; and Mitra and Pal 2007), Therefore, there multiplication basically have 2 choices; stem cutting or tissue culture (Amit Kumar *et al.*, 2019). Plant tissue culture techniques is an efficient, ecofriendly tool which can be employed not only for getting increased production of phytobiomass but also of improved quality i.e. with high concentration of Steviol glycosides.

To control in plant tissue culture it is necessary to check the genetic uniformity of micropropagated plants by molecular markers. Literatures mentioned many molecular techniques are available for genetic converge within and among taxa that resulting from tissue culture (Yao *et al.*, 1999; Moktaduzzaman and Rahman, 2009; Lata *et al.*, 2013; Soliman, 2014). Different molecular methods have been reported on *S. rebaudiana* such as Yadav *et al.* (2011), Guasmi *et al.* (2012); Prasad (2014), Thiyagarajan *et al.* (2015); Abdelsalam *et al*. (2016), and Dyduch-Sieminska *et al*. (2020). Randomly amplified polymorphic DNA (RAPD) is a successful tool used widely for genetic polymorphism, genetic variations, map construction and linkage analysis because of their simplicity, quick, easy to perform, relatively cheap, and highly informative for assessing genetic diversity and relationship measures and describe somaclonal variability in micropropagated individuals of many plants species (Isabel *et al*., 1993; Munthali *et al*., 1996; Hasmi *et al.,* 1997; Ahmed *et al*. 2007; Kumar *et al*., 2010, Dyduch-Sieminska *et al.,* 2020).

Hence, the examination of the present study deals with exploit PCR technology-dependent RAPD indicators to analyze the genetic variation between cell lines from callus segments that derived from the cell suspension cultures by using plating method of stevia.

And determine the genetic relationship between the studied models by identifying the genetic dimensions and their groups.

Materials and Methods

Plant Material

25 clones with age 12 weeks were tested as cellular lines derived from the cell suspension cultures of the Stevia plant by plating method (Bedrgman, 1960) (unpublished results) as well as the treatment of callus control that plant-induced from leaf (Al-Zubaidy *et al*., 2020). Reculture segments callus on the same growth medium as MS supported with concentrations 2.0mg.L^{-1} Naphthalene acetic acid (NAA) interfering with 0.5 mg.L^{-1} Thidiazuron (TDZ), for the purpose of extracting DNA from them (Figure. 1).

Genomic DNA extraction

Total DNA was extracted from leaf material of 25 micropropagated plants (treated with growth retardant) and a mother plant (control no.1), young *Stevia* leaves (control no.2) treated by liquid nitrogen and following the method described by ABIOpure™ Total DNA protocol kit (USA). Purified total DNA was quantified and its quality was verified by spectrophotometer (Ez_capture MG, ATTO/ japan) and stored at 4 ºC till used.

Screening of PCR :

Fifteen different 10mers RAPD primers were tested (table 1) which supplied by Macrogen (Korea) were screened, five primers which had previously been shown indicated results of band patterns (figure 2), multi master mix were used(table 2), The researchers selected thermo profile PCR was: an initial denaturation at 94ºC for 5 min, 35 cycles comprising of denaturation at 94ºC for 1 min, annealing at38ºC for 1 min and extension at 72ºC for 1 min, and a final extension step at 72ºC for 5 min. (Modi *et al.,* 2012). Genotypes were visualized on 1% agarose gel, 1x TBE, 100 volts, for 60 minutes.

Analysis of clonal fidelity

Total genomic DNA from tested 15 RAPD10 meres primers, just five primers generate forty one fingerprinting pattern, the DNA samples were scored as 1 or 0 based on presence or absence of a band, within the size range of 150-1100 base pairs (bp). (Table 3)

Table 1 : Sequences of the RAPD primers that used in this study.

Primers	Primer code	5' – 3' Sequences
1	OPA-10	GTGATCGCAG
2	OPC-09	CTCACCGTCC
3	OPC-15	GACGGATCAG
4	OPC-16	CACACTCCAG
5	OPW-07	CTGGACGTCA
6	OPW-09	GTGACCGAGT
7	OPW-19	CAAAGCGCTC
8	OPX-02	TTCCGCCACC
9	OPX-03	TGGCGCAGTG
10	P-168904	CAGTICTGGC
11	P-168906	CCACCGCCAG
12	P-168910	CAGTICGAGG
13	SIGMA-5383-043	AAACAGCCCG
14	SIGMA-5383-064	TGGAAGAGGC
15	SIGMA-5383-091	TCGGAGTGGC

Table 2 : The master amplification Reaction

Materials	Final concentration	Volume for 1 tube
PCR pre mix	1x	5Ml
Deionised D.W	——	11µl
Primer	(10pmol/ µl) 10pmol /µl	2Ml
DNA template	100mg	2Ml

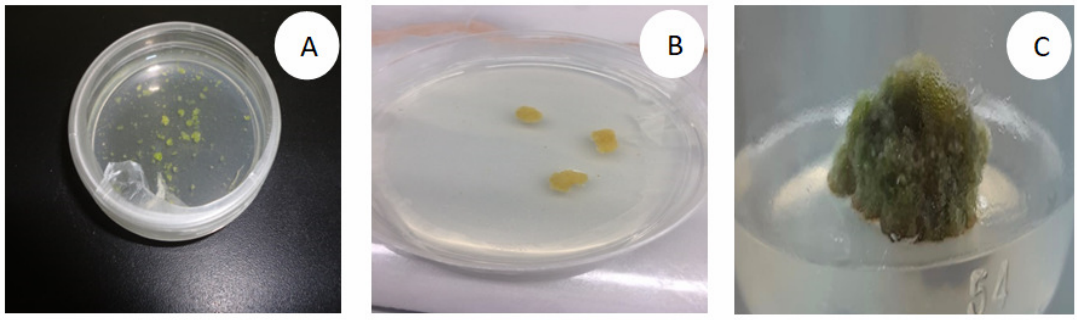

Fig. 1 : The stages of development and growth of cellular lines to cut callus for stevia:
A) The development of callus cuts from cultivation of cellular suspensions by publication 21 days after implantation.
B) Transfer the cut callus in (A) to new food media for the purpose of its growth.
C) Cut callus used for analysis 12 weeks after replanting.

Table 3: Total number and size range of amplified bands obtained for each primer of RAPD method

Primer name	Size range of bands(bp)	AN*	**PM	%	***MM	%
P-168904	800-700	2	2	100	0	0
P-168906	1100-280	8	8	100	0	0
SIGMA-5383-043	1100-250	14	14	100	0	0
SIGMA-5383-064	600-150	9	9	100	0	0
SIGMA-5383-091	750-200	8	8	100	0	0
		41	41		0	

*AN = alleles number; **PM = Polymorphic bands; ***MM = Monomorphic bands. %PM= PM/ANx100, % MM= MM/AN x100.

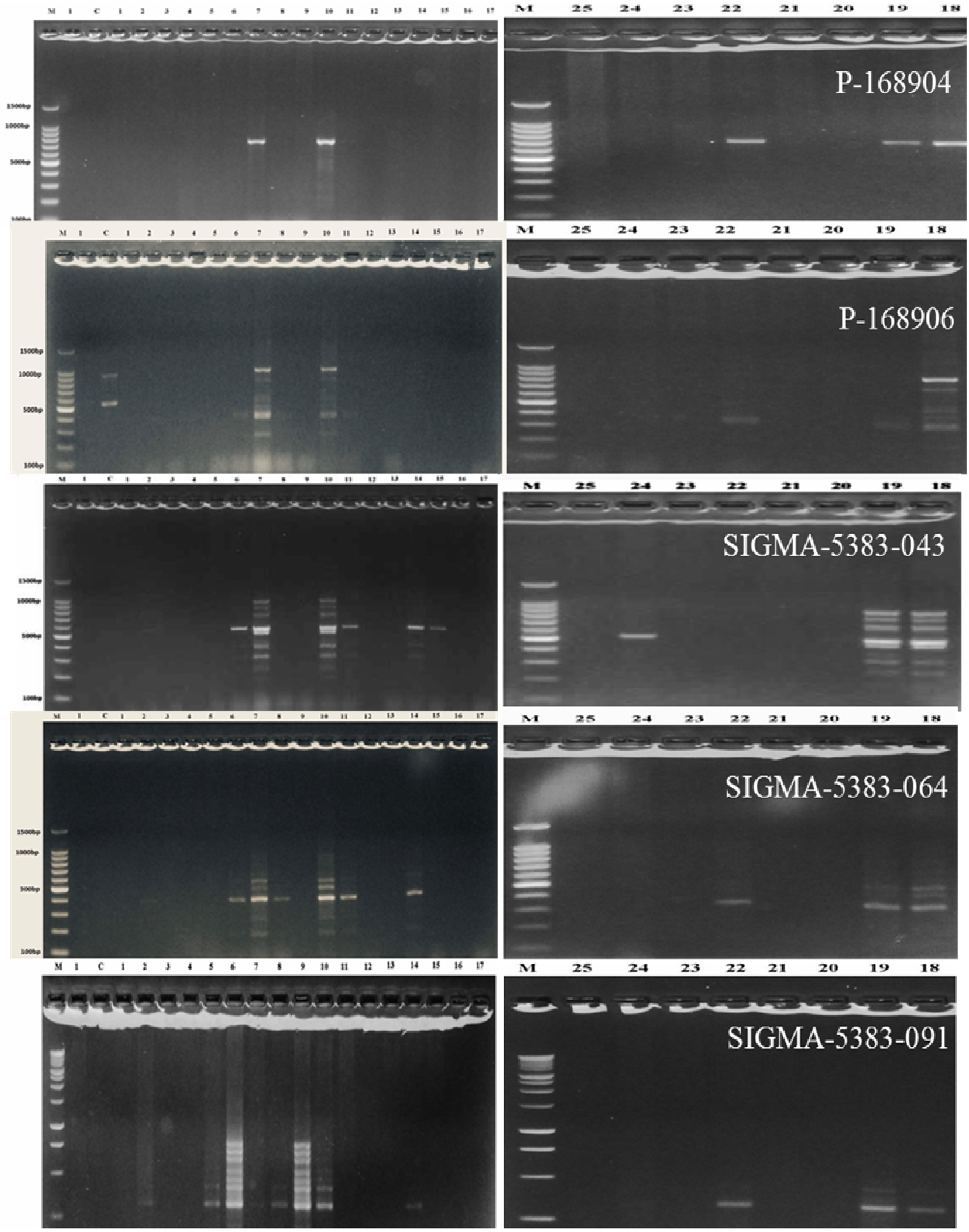

Fig. 2 : Spectrum of DNA amplification products of *stive* clones (molecular weight marker 1500-bp DNA Ladder

Results and Discussion

The dendrogram (figure 3) generated form 25 genotypes showed in figure 1and 2, the first view was displayed monomorphic banding pattern. In general we can distinguish two main groups with a low average 0.72 similarity, I group including 24 clones, II group consist of one clone (10) that isolated from other his brothers micropropagated, The I group separated in 2 clusters with rate 0.30 similarity were A that split into sub cluster with converge rate was 0.10, A1 was segment to two a part which had a greatest ratio 0.03 affinity, part 1: including 11 genotypes (1,2,3,5,8,12,20,22,23,24 and 25), while part 2 including only one stevia genotype (15), A2 split up to two subclusters carried 0.05 similarity, each of them holed three genotypes (11,16 and 19), (13,14 and 18) sequencelly, B cluster divided in two subclusters which were grouped together with average 0.25 similarity, The first contained four

clones (4,7,9 and 21) while the second sub cluster have just tow stevia genotypes (6 and 17).

A slightly distance clustering pattern was obtained from 25 randomly selected plants and the mother plant our results based on RAPD markers agree with Singh *et al.,* 2017 that all the regenerated plants of stevia through tissue culture were true-to-type parallel to mother plants. In previous published reports, similar outcome were observed (Sivaram and Mukundan, 2003). Somaclonal variations arose due to *in vitro* stresses, chromosome rearrangements and point mutations (Phillips *et al.,* 1994). In previous published reports such us Modi *et al.,* 2012; Singh *et al.,* 2014 confirmed that PCR techniques have been found to be great useful molecular tool in establishing the gene stability of taxa as well as *in vitro* regenerated Stevia plants, Even more they ensure that RAPD and ISSR markers used for fidelity test and output indicated that all plants obtained from callus are true-to-type and there are no somaclonal variations among those plants, our uniformity micropropagated Stevia plants results conflict with Singh *et al.,* 2014 that his experience showed high level of genetic variation.

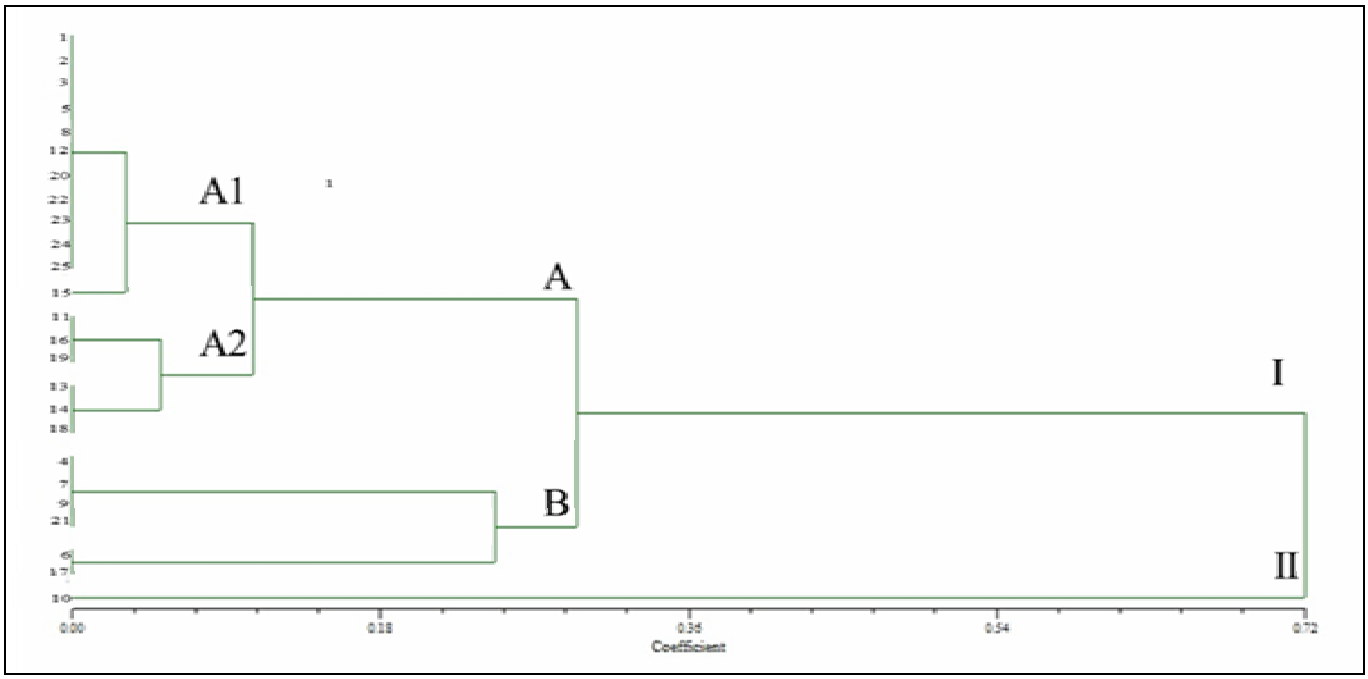

Fig. 3 : Dendrogram showing genetic relationships among taxa of pepper by RAPD technique.

Conclusions

The study showed that there are genetic differences among the suspensions, although they are close, but it isolated some samples, this out come can be considered a molecular taxonomical tool and a key to a chemical study.

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