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Evaluation of Tissue Culture Seedlings for Their Genetic Fidelity and Virus Indexing in Sugarcane

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ABSTRACT

Plant tissue culture technique is a powerful tool for rapid and large scale multiplication of virus free seed material. Clonal fidelity of the *in vitro* raised plants was carried out using RAPD. A total of fifteen primers were used for detecting polymorphism by RAPD analysis and the results revealed that all the tissue culture plants were grouped into one cluster and indicated that all the tissue culture developed plantlets are true-to-type. There are no somaclonal variations and are genetically identical with the mother plant. Virus indexing through ELISA indicated that the tissue culture developed seedlings does not contain the protein particles of ScMV and are free from the viral disease. The wells coated with leaf samples with suspected ScMV infection (97 A 85, C3) gave visual confirmation by yellow colour change, with OD 405 values of 0.526. As the reading is 2 times more than the negative control (OD 0.215) we can presume mild infection of mosaic in the test sample with

lower titre of ScMV. All the ScMV +ve controls showed significant higher OD values of more than 2.515 confirming the earlier observations. Thus, viral detection using DAS-ELISA method can be used for detection and indexation in large scale sugarcane crop especially for *in vitro* regenerated plants with relatively cheaper cost and faster for supplying virus free seedlings to farming community in a large scale.

Key words: MS media, NAA, genetic fidelity, RAPD, ELISA

Introduction:

Sugarcane is systematically infected by different types of pathogens. Of them, the viral pathogens pose the most serious challenges in obtaining disease-free seed cane. Hence, unavailability of quality seed material is one of the major problems faced by farmers, thereby decreasing cane yield and quality drastically. Plant tissue culture techniques are emerging as a powerful tool for rapid and large scale multiplication of newly released and commercially important varieties of sugarcane. Initial attempts to regenerate plants through *In vitro* techniques were conducted on sugarcane by Nickell (1964) and Heinz & Mee (1969). Protocols for *In vitro* plant regeneration of sugarcane through callus culture, axillary bud and shoot tip culture have been also developed (Baksha *et al.,* 2002).

Scaling up of any micro propagation protocol is severely hindered due to incidences of somaclonal variations. Somaclonal variations occur mostly in response to the stresses imposed on the plant under *in vitro* conditions. Hence, a stringent

quality check in terms of genetic similarity of the tissue culture raised plants becomes mandatory. Identification of off types and genetically true to type plants at an early stage of development is considered to be very useful for quality control in plant tissue culture. Therefore it is necessary to check the genetic uniformity of micro propagated plants by molecular markers. Randomly Amplified Polymorphic DNA (RAPD) based detection of genetic polymorphism has been found successful in identifying somaclonal variability in micro propagated individuals of several plant species (Kumar *et. al.,* 2010) including Sugarcane.

Sugarcane Mosaic disease caused by Sugarcane Mosaic Virus (ScMV) is one of the major viral disease which pose a potential threat to the sugarcane cultivation worldwide among various diseases in sugarcane in the recent past. Different serological techniques such as immunosorbent electron microscopy (ISEM), ELISA and immuno blot are available for the detection of the virus. Production of highly specific antiserum to ScMV, following purification of recombinant viral coat protein from *E. coli*, was reported to be highly specific and a reliable method for the detection of SCMV in the infected host.

Therefore, the objective of the present study was to evaluate the genetic stability of micro propagated plants of sugarcane using RAPD markers and detection of ScMV using DAS-ELISA.

Materials and Methods:

Fresh tops of commercial sugarcane variety grown at the research farm of Regional Agricultural Research Station (RARS), Anakapalle, Andhra Pradesh were

collected during 2014-15. Outer whorls of mature leaves were removed till yellowish white coverings around apical meristems appear. After removing outer whorls of leaves, the tops are sized to 10cms length by cutting off at the two ends. These were then washed with sterile distilled water thrice and then treated with 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for about 35-40 minutes in laminar air flow chamber and washed out thrice with sterile distilled water. The explants (apical shoots) are picked carefully with sterile forceps and placed in a sterile Petri dish. Using a fine forceps and scalpel, the outer leaf sheaths are removed one by one with out exerting pressure on the internal tissues. The process is repeated until the apical dome with two or three leaf primordia is exposed. After excising the apex, the explants are transferred immediately on a filter paper support immersed in MS (Murashige & Skoog, 1962) liquid medium supplemented with various concentrations of plant growth regulators. The meristems derived shoots were further multiplied for every 3-4 weeks in the same initiation medium with the same concentrations of plant growth regulators. For *in vitro* rooting, Half strength MS liquid media with different concentrations of Naphthalene-1- acetic acid (NAA) was used and various concentrations of commercial sugar was also used along with rooting hormone in order to study the effect of sugar on rooting. The pH of the medium was adjusted to 5.6±2°C followed by autoclaving at 121ºC at 15lbs pressure for 15 minutes. Cultures were incubated at 25±3°C under 16/8 hours (light/dark) photoperiod with a light intensity of 2500 lux. The obtained plantlets were transferred to poly bags containing a mixture of red soil : vermicompost : sand (

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1:1:1) after treated with 1% (w/v) carbendizem in green house maintained at 30ºC. Finally hardened plants were transferred to field after 30 days. Data on multiplication (%), No. of multiple shoots, Mean shoot length (cm), Rooting (%), Mean number of roots, Mean root length (cm) was collected.

Analysis of Genetic fidelity by RAPD.

DNA extraction: Total DNA was extracted from leaf material of 15 micro propagated plants and a mother plant (control), following the method described by Doyle and Doyle (1990). Purified total DNA was quantified and its quantity was verified by Spectrophotometer and also the final concentration was checked on 0.8% agarose gels.

RAPD amplification: Amplification conditions were those described by Williams *et al.,* (1990). 20 ng of template was taken in a 25µl reaction. PCR reactions were carried out on a Eppendorf DNA Thermal Cycler, using 2 units of Taq polymerase (Stratagene, La Jolla, California). PCR consisted of 45 cycles, each of 94 ^{o}C for1min and 72 ^{o}C for1min, 36^{o}C for 2 min.

Fifteen primers, which were able to detect polymorphisms between different genotypes, viz. OPC-1, OPC-3, OPC-5, OPC-7, OPC-9, OPC-11, OPC-13, OPC-15, OPC-17, OPC-19, OPE-2, OPE-4, OPE-6, OPE-8, OPE-10 were selected for the analysis. Amplification products were separated by electrophoresis 1.5% agarose gels in 1 X TBE buffer. Both the gel and the buffer contained 0.5 g/ml ethidium bromide. Clear and well resolved bands were scored for presence (1) or absence (0).

Detection of Virus using ELISA

The leaf sap was extracted from both symptomatic and healthy/asymptomatic leaves of Sugarcane by using mortar and pestle in 1:20 extraction buffer (137mM NaCl, 3 mM KCl, 2% PVP, 0.05% Tween 20 and 0.02% NaN3). DAS-ELISA was carried out using the kit obtained from M/s. Bioreba, Switzerland following the standard protocol. The plates were first coated with coating antibody i.e., PRSV-IgG 1000x in coating buffer @ 200 µl per well, covered tightly and incubated for 4hrs at 30^0C. In the second step, wells are coated with 200 µl of the extract/sap in triplicates duly preparing the loading diagram along with positive and negative control. The plate was incubated at 4^oC overnight for the binding of the antigen on the plate walls. The enzyme conjugate was diluted 1000 times in conjugate buffer and coated to the wells @ 200 µl each and incubated at 30^0C for 5 hours. In the final step pNPP dissolved in substrate buffer @ 1mg/ml added to the wells 200 µl each and incubated at room temperature (20-25^0C) in dark and observed for colour development. After each step, the wells are emptied and washed thoroughly with washing buffer (Easy wash 2000, Bioreba) for 3-4 times. Observed for yellow colour development after 1-2 hrs visually and also photometrically at 405 nm using thermofischer scientific Multiscan- X, ELISA reader and the readings are documented.

Results and Discussion:

Maximum multiplication (84.15 %), No of multiple shoots (11.72) and mean shoot length (12.25 cm) was obtained at MS media supplemented with 0.20 mg/l BAP and 0.1mg/l KN (80.17). Similarly highest rooting percent (84.48%), number of roots

(10.60) and root length (11.20 cm) of sugarcane was obtained at ½ strength MS media supplemented with 5mg/l NAA. (Table – 1; Fig: 1, 2, 3).

Table -1. Shooting and rooting in Sugarcane for production of tissue culture seedlings.

Shooting (%)			
Media	Multiplication (%)	No. of multiple shoots	Mean shoot length (cm)
MS+0.25mg/l BAP+0.10mg/l KN.	84.15±0.4	11.72±0.2	12.25±0.02
Rooting (%)			
Media	Rooting (%)	Mean number of roots	Mean root length (cm)
½ MS media + 5.0mg/l NAA	84.48±0.6	10.60±0.1	11.20±0.1


Fig: 1. Initiation


Fig. 2. Multiplication


Fig. 3. Rooting

Evaluation of genetic fidelity of sugarcane meristem tip derived plantlets of 87A298 using RAPD technique.

Utility of RAPD as a means for analysis of genetic variation between *in-vitro* micro propagated plants and mother plants has been reported by many workers (Kumar *et al.,* 2010). In the present study the banding pattern between tissue cultured plants and mother plants were compared to study the genetic variability. Fifteen (15) primers viz., OPC-1, OPC-3, OPC-5, OPC-7, OPC-9, OPC-11, OPC-13, OPC-15, OPC-17, OPC-19, OPE-2, OPE-4, OPE-6, OPE-8 and OPE-10 (Table -2) produced

DNA fragments that were mono morphic between tissue cultured and mother plants. Number of bands per plant varied from 8 to 18. One hundred and fifty two well resolved bands generated by 15 primers were scored for presence (1) or absence (0).

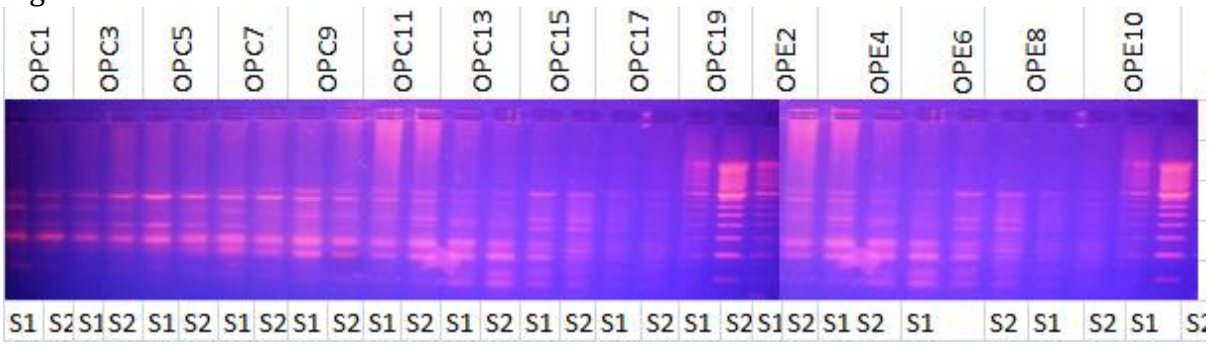
RAPD analysis revealed that the banding pattern is similar between the mother plant and the micro propagated plant (Kumar *et al,* 2010) (Fig-4) which is further confirmed through the dendrogram as they are all grouped into same cluster as depicted (Fig:5) indicated that the tissue cultured plants are genetically identical with the mother plant. The results indicated that all the tissue cultured derived plants are true-to-type and there are no somaclonal variations among these plants (Arpan *et al.,* 2012). Smiullah *et al.,* (2012) reported similar results in callus regenerated tissue cultured plants of sugarcane which supports the present research findings.

Table : 2. List of primers with total number of bands formed with mother plant and Meristem derived tissue cultured plant.

SNo	Primer	Sequence (5^1-3^1).	Total number of bands	No. of monomorphic bands	No. of polymorphic bands
1	OPC1	TTGAGCCAG	8	8	0
2	OPC3	GGGGGTCTTT	8	8	0
3	OPC5	GATGACCGCC	12	12	0
4	OPC7	GTCCCGACGA	14	14	0
5	OPC9	CTCACCGTCC	18	18	0
6	OPC11	AAAGCTGCGG	14	14	0
7	OPC13	AAGCCTCGTC	14	14	0
8	OPC15	GACGGATCAG	14	14	0
9	OPC17	TTCCCCCCAG	0	0	0
10	OPC19	GTTGCCAGCC	18	18	0
11	OPE2	GGTGCGGGAA	8	8	0
12	OPE4	GTGACATGCC	10	10	0

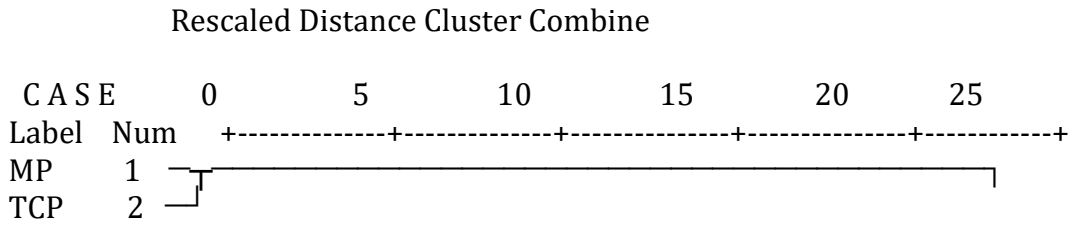
13	OPE6	AAGACCCCTC	12	12	0
14	OPE8	TCACCACGGT	0	0	0
15	OPE10	CACCAGGTGA	2	2	0
			152	152	0

Fig: 4



SAMPLE-1 (S1) - Field grown plant.
SAMPLE-2 (S2) - Meristem tip derived tissue cultured plant

Fig.5 Dendrogram using Average Linkage (Between Groups)



DAS-ELISA:

Visual observations on micro titre plate as well as ELISA reader gave clear indication of the presence or absence of the ScMV in the tissue culture plants with respect to colour change. Higher values of absorbance (OD 405) indicated test sample is positive, where as lower value of absorbance as negative (Table 3.)

According to the research results, ELISA testing is showing positive sample if the absorbance value is more than 2.515, whereas negative sample if absorbance value is less than 0.253 (Table 3). rScMV antisera testing on negative samples of Sugarcane leaf that is free from ScMV infection and used as negative control showed negative results are marked in the well whiter and brighter after 30 minutes of added subtract solution. Based on these results that the tissue culture developed seedlings does not contain the protein particles of ScMV (Fig:6) free from viral disease. The wells coated with leaf samples with suspected ScMV infection (97 A 85, C3) gave visual confirmation by yellow colour change, with OD 405 values of 0.526. As the reading is 2 times more than the negative control (OD 0.215) we can presume mild infection of mosaic in the test sample with lower titre of SCMV. All the ScMV +ve controls showed significant higher OD values of more than 2.515 confirming the earlier observations. Thus, viral detection using DAS-ELISA method can be used for detection and indexation in large scale sugarcane crop especially for *in vitro* regenerated plants with relatively cheaper cost and faster for supplying virus free seedlings to farming community in a large scale. Similar results using DAS- ELISA was reported in field grown sugarcane plants by Thorat *et al.,* (2015) and presented DAS – ELISA suitable for detection of SCMV in Sugarcane.

Fig: 6. ELISA Absorbance

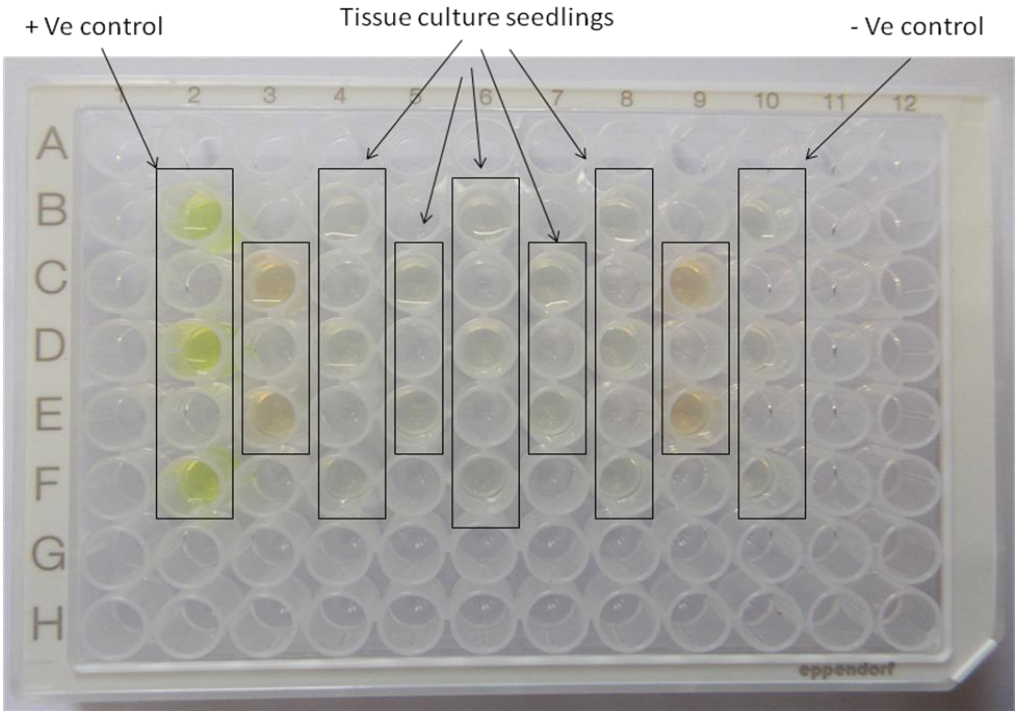


Table - 3. Absorbance Reading Value using ELISA Reader

S.No.	Sample	ELISA reader reading (absorbance at 405 nm)	info
ScMV +ve con	B2	2.556	(+)
	D2	2.66	(+)
	F2	2.515	(+)
97 A 85 +ve con	C3	0.502	(+) may be treated as +ve - mild
	F3	0.429	(-)
Co 7805 –TCPs	B4	0.236	(-)
	D4	0.225	(-)
	F4	0.239	(-)
Co 6907 –TCPs	C5	0.226	(-)
	F5	0.224	(-)

87 A 298 –TCPs	**B6**	**0.224**	**(-)**
	D6	**0.212**	**(-)**
	F6	**0.239**	**(-)**
86 V 96 –TCPs	**C7**	**0.254**	**(-)**
	F7	**0.227**	**(-)**
86 V 96 –TCPs	**B8**	**0.222**	**(-)**
	D8	**0.216**	**(-)**
	F8	**0.246**	**(-)**
97 A 85-+ve con	**C9**	**0.400**	**(-)**
	F9	**0.424**	**(+)**
ScMV-ve con	**B10**	**0.232**	**(+)**
	D10	**0.232**	**(-)**
	F10	**0.253**	**(-)**

Conclusion: Tissue culture is a tool for production of virus free and true to type seed material. Standardization of protocol for production of seedlings for different clones is a prime need. A stringent quality check in terms of genetic similarity of the tissue culture seedlings to avoid somaclonal variations is mandatory. Among different serological technique ELISA is used for detection of virus in tissue culture seedlings for evaluation.

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