Study of *In Vitro* Development of Mutant Plants of *Saccharum officinarum* for Resistance Against Smut

THESIS

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MASTER OF SCIENCE

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By

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2020

CERTIFICATE – I

This is to certify that the thesis entitled **"Study of *In Vitro* Development of Mutant Plants of *Saccharum officinarum* for Resistance Against Smut"** submitted in partial fulfilment of the requirement for the **MASTER OF SCIENCE in Agriculture, Molecular Biology & Biotechnology** of Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur is a record of the bonafide research work carried out by **Mr. Rishabh Mukati** under my guidance and supervision. The subject of the thesis has been approved by the Student's Advisory Committee and the Director of instructions.

All the assistance and help received during the course of the investigation have been acknowledged by him.

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This is to certify that the thesis entitled **"Study of *In Vitro* Development of Mutant Plants of *Saccharum officinarum* for Resistance Against Smut"** submitted by **Mr. Rishabh Mukati** to the Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur in partial fulfilment of the requirements for the degree of Master of Science in Agriculture, Molecular Biology and Biotechnology in the Department of Biotechnology Centre has been, after evaluation, approved by the External Examiner and by the Student's Advisory Committee after an oral examination on the same.

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LIST OF ABBREVIATIONS

S.No.	Abbreviations	Meanings
1.	MS	Murashige and Skoog medium
2.	2,4-D	2,4- Dichlorophenoxy acetic acid
3.	IBA	Indole butyric acid
4.	NAA	Naphthalene acetic acid
5.	BAP	6-Benzylaminopurine
6.	EMS	Ethyl methane sulfonate
7.	µM	Micro molar
8.	M	Molar
9.	mg	Milligram
10.	ml	Milliliter
11.	mg/l	Milligram per liter
12.	^0C	Degree Celsius
13.	psi	Pound per square inch
14.	pH	Power of hydrogen
15.	%	Percentage
16.	g	Gram
17.	NaOH	Sodium hydroxide
18.	HCl	Hydrochloric acid
19.	KCl	Potassium chloride
20.	$HgCl_2$	Mercuric Chloride
21.	µl	Micro liter
22.	PCR	Polymerase chain reaction
23.	CTAB	Cetyltrimethyl ammonium bromide

INTRODUCTION

Sugarcane *(Saccharum spp.)* is one of the oldest crops known to man; it is a member of tall growing perennial monocotyledonous grass, it belongs to tribe Andropogonae of the family poaceae and supposed to have originated in New Guinea (Liu, 1984). It is an important food crop of the tropics and subtropics accounting for 70 per cent of world sugar production. India is the second largest country in sugarcane production in the world. In India, sugarcane is grown in an area of 5.11 million hectare with a production of 400.2 million tonnes and productivity of 78.24 tonnes/hectare (Department of Agriculture, Cooperation and Farmers Welfare, Annual Report, 2019-2020). It is a major source of by-products which provide raw material for cogeneration, ethanol, pulp and paper industries. It is also being used as a feedstock for the next generation of advanced bio-fuels, such as bio-butanol and diesel and many other valuable by-products through sugarcane biotechnology (Yadav and Solomon, 2006). The projected sugarcane production by the year 2020 is estimated as to be 415 million tonnes to meet the ever-increasing demand for sugar. Sugarcane, grown primarily for sugar, is cultivated throughout the tropics and subtropics (35ºN to 35ºS) on a variety of soils. It has been adapted to a wide range of environments and cultural practices.

The increase in cane production must be accomplished through intensive cultivation, since there is little scope for expanding the available land under sugarcane. The increase in production can be accomplished by evolving elite genotypes with high yield potential, resistance to biotic and abiotic stresses.

Although sugarcane is propagated vegetatively through setts and the rate of conventional multiplication is very slow. There are many factors which influence cane yield out of which biotic factors predominantly account for the losses. Among the biotic stresses viral and fungal diseases can cause colossal loss in yield and quality of sugarcane. Because of clonal propagation, pathogens keep accumulating generation after generation causing varietal decline (Gosal *et al*., 1998). Whiptail disease or Sugarcane

Smut, Smut of Sugarcane is caused by the fungus *Sporisorium scitamineum.* The disease is known as culmicolous, which describes the outgrowth of fungus on the stalk of the cane. The affected sugarcane plants may tiller profusely with spindly and more erect shoots with small narrow leaves (*i.e*., the cane appears "grass-like") with poor cane formation. The most recognizable characteristic of this disease is a black or grey growth that is referred to as a "smut whip". Sugarcane Smut can cause significant losses in production of cane and quality of juice. The severity of the disease depends upon the environmental conditions and resistance of the sugarcane varieties.

The breeding approaches in sugarcane from the beginning of this century include wide hybridization with back cross and selection. The breeding objectives have aimed at increasing the cane yield and quality, resistance to locally important diseases and pests, stability and other morphological/agronomical/physiological characters. Resistance to sugarcane smut is the best course of action for management. The development of superior sugarcane varieties through conventional hybridization programme is time consuming and has the problem of transferring undesirable characters into the newly developed hybrids but introduction of such traits through *in vitro* techniques is rather faster than that of the conventional methods. Attempts have been made to introduce genetic variability in sugarcane by *in vitro* techniques and mutation breeding (Begum *et al.,*2011; Kumar *et al.,* 2012; Rastogi *et al.,* 2015). Introduction of somaclonal variation is one of them, which is described as the genetic variability present among the cultured cells and the plants derived from such cells or progeny of such plants are called somaclones.

Somaclonal variation is defined as variation originating in cell and tissue cultures (Larkin and Scowcroft, 1981)

The variation found among the cultured cells is for both the qualitative and quantitative traits. Somaclonal variation occurs due to many events *i.e.,* Changes in chromosome number, Changes in chromosome structure, Gene mutation, Plasmagene mutation, Alteration in gene expression, Gene amplification, Mitotic crossing over, Transposable element activation and

Rearrangements in cytoplasmic genes. Somaclonal variations are known to change one or a few traits in an existing cultivar which may lead to its improved performance and also supplement the variation which may not exist in the natural gene pools (Larkin *et al.,* 1984). Somaclonal variants which are resistant to disease can be isolated by screening large number of plants regenerated through tissue culture out of which some may show useful resistance. Alternatively, they may be isolated by selecting cells, protoplasts, calli, embryos, or meristem for resistance to the concerned toxins and, in many cases to the pathogen as well. The toxins may be in the form of culture filtrate, a crude preparation or in the purified form.

Keeping all this in view, present investigation on sugarcane tissue culture was undertaken in the cultivar, Co 261 with the following objectives.

Objectives

1. To standardize the callus induction from different explants of sugarcane.

2. To develop smut resistant mutant through *in vitro* culture.

3. To validate the cultured mutant plantlets using molecular markers.

REVIEW OF LITERATURE

Plant Tissue Culture is an essential component of Plant Biotechnology. Plant cell and tissue culture has contributed significantly to crop improvement and has great potential for the future. Research efforts in plant cell and tissue culture have increased dramatically worldwide in recent years. Tissue culture is defined as the capability to regenerate and propagate plants from single cells, tissues and organs under sterile and controlled environmental conditions (Murashige, 1974).

2.1 History of Plant Tissue culture

Haberlandt (1902) made the first attempt to culture isolated mesophyll and palisade cells of *Lamium purpureum* on a nutrient medium containing inorganic salts and sucrose. He was the first to conceive the concept of totipotency for plant cells.

Skoog and Miller (1957) discovered that shoot and root initiation was basically regulated by interaction between two hormonal substances (auxins and cytokinins). They also demonstrated that both the hormones are necessary for tissue growth and the pattern of morphogenesis was determined by their relative concentration and sequence of application in the nutrient medium. Later, they suggested that a relatively high concentration of auxin favours root initiation while suppressing shoot formation and *vice-versa.*

Murashige and Skoog (1962) standardized and developed the basic nutrient medium using tobacco tissues as the experimental material. This medium popularly known as ‘MS’ medium is one of the very widely used media in the present day *in vitro* experiments.

2.2 Explants

In sugarcane several workers used different explants *viz*., shoot apices, young leaf rolls, auxiliary buds, roots and immature inflorescences to study callusing ability and regeneration potential to produce complete plantlets. For the present investigation the standardisation of explant for callusing was necessary in order to induce mutagenesis.

Behera and Sahoo (2009) established the protocol for induction of callus and regeneration of plantlets through *in vitro* culture using young meristem of sugarcane (*Saccharum officinarum* L. cv- Nayana) as an explant. The multiple shoot regeneration at various frequencies was observed by using different concentration and combination of growth regulators. The highest percentage of callus induction was observed in MS medium supplemented with 2.5 mg/l, 2,4-D. The best response in terms of multiple shoot induction was observed on MS medium with BAP 2.0 mg/l + NAA 0.5 mg/l, rooting was more profuse.

Gupta (2019) standardized the callus induction from leaf roll and induced somaclonal variation and validated the mutants using molecular markers. He concluded that callus induction was highest when the explants were leaf rolls and highest number of callus were induced at 3 mg/L 2,4-D from sugarcane leaf rolls.

2.3 Surface sterilization

Ali *et al*. (2001) reported that explants of sugarcane variety, CP 77400 were first treated for sterilization with household detergent for 5 minutes. This was followed by rinsing with tap water to remove all the traces of detergent. The explant was then further sterilized with 1% sodium hypochlorite for 15 minutes. The explants were rinsed three times with autoclaved distilled water to remove all the traces of sodium hypochlorite.

Singh *et al*. (2001) reported that the explants resulted in good survival percentage when shaken with mild soap solution (1% Surf powder) for 3-5 minutes and then surface sterilized with freshly prepared sodium hypochlorite solution (0.25%) for 5 minutes followed by treatment in a solution containing tetracycline (0.2 per cent) and mercuric chloride (0.05%) for 5 minutes followed by 5 minutes dip in 1 per cent KCl solution gave good survival percentage.

Baksha *et al*. (2002) disinfected the explants by using 0.1%$HgCl_2$ for 7-10 minutes after washing thoroughly under tap water. Subsequently the explants were washed gently with sterile double distilled water four times.

Khan and Rashid (2003) achieved surface sterilization with 20% commercial bleach (5% v/v sodium hypochlorite) for 20 minutes. They standardise 5% v/v Sodium hypochlorite for surface sterilization for their experiment on "Rapid clonal propagation of sugarcane" and successfully achieved the aseptic propagation.

Singh (2003) sterilized shoot tips with 0.1% mercuric chloride for 8 minutes and reported 83.3 per cent contamination free explants.

2.4 Callus induction

Shetty and Rao (2015) developed an efficient protocol for callus induction and regeneration of sugarcane variety 93v297. Callus induction from immature young leaf explant derived from 2-3-month-old plants was achieved on MS medium supplemented with different auxins *viz,* 2,4-D, NAA and IAA and reported that callus induction was optimum at 2,4-D 3.5 mg/l + BAP 0.5 mg/l.

Nalavade *et al.* (2016) made an investigation to identify the best media combinations for callus induction, somatic embryogenesis and shoot regeneration of elite sugarcane (*Saccharum officinarum* L.) and found that 3 mg/l 2,4-D in MS basal salts resulted in maximum callus induction.

2.5 Micropropagation in sugarcane

Singh and Singh (1993) initiated callus cultures from immature leaf roll of the sugarcane variety CoS 687 on MS medium containing 6.0 mg/l 2,4-D at 25 ± 20C in the dark. Calli were exposed to 1 and 2 Kr of gamma rays and transferred on fresh medium for growth and development. Regeneration was done on a medium fortified with 0.25 mg/l K and maintained in 16 h light and 8 h dark at 25 ± 2 0C. Equal quantities of irradiated and unirradiated calli were transferred on regeneration medium. Calli subjected to 1 Kr and 2Kr produced about 8.69% and 21.73% more plants, respectively, than untreated check.

Lal and Krishna (1994) reported that axillary shoot proliferation from shoot tip culture was best for micropropagation; it also minimizes somaclonal variation. They used modified MS medium (Murashige and Skoog, 1962) for shoot multiplication (BAP 1 mg/l and Kinetin 1 mg/l). Micropropagation by using shoot tip was reported by Lee (1987), plant produced were similar to mother plant and gave much more rapid shoot multiplication rate compared to other procedures. They produced 78,408 plantlets in 3 months from a single shoot tip.

Shukla *et al.* (1994) reported optimization of media for studies on *in vitro* clonal propagation of sugarcane with modified MS media, indicated that both liquid and solid media were good for establishment of cultures but former was best for shoot proliferation. The micropropagated plants were successfully hardened using the Hoagland solution (Arnon and Hoagland, 1940).

Hegde (1999) conducted micropropagation in three popular cultivars of sugarcane (*Saccharum spp.)* namely, Co 8014, Co 85002, CoC 671 with the purpose of developing a generalised protocol and to economise micropropagation. Apical meristems (domes) were used as explants. Out of the various combinations and concentrations of BAP and NAA, 1.0 mg/l BAP resulted in reasonably high average rate of shoot multiplication (6.26) across all the cultivars and the shoots were also of desirable quality. The treatment with 2.0 mg/l BAP gave the highest average rate of shoot multiplication (20.09), but the plantlets were very tiny, weak and non-separable. Very high rooting frequency was observed with 0.5, 1.0 and 2.0 mg/l NAA in all three cultivars. Good response was also observed with plain MS and 0.25 mg/l NAA

Tesfa and Ftwi (2018) optimized an efficient and reliable protocol for direct propagation of sugarcane varieties under *in vitro* condition. Different concentrations of BAP along with kinetin were used for shoot proliferation. The proliferated shoots were subjected to MS medium supplemented with different levels of NAA. Results of the present study revealed both the BAP and Kinetin had a synergistic effect and none of them generated the maximum response when separately applied. The maximum number of shoots (10.3 ± 0.31), shoot length (3.77 ± 0.40) and number of leaves (3.87 ± 0.17) were recorded on the MS medium supplemented with 2 mg/l of BAP and 1.5 mg/l of Kinetin. In the rooting medium, highest rooting frequency (100%), root length (2.21 cm) and number of roots per shoots (20.13) were recorded on the half strength MS semi-solid media fortified with 5 mg/L NAA. The better rooted plantlets were transferred to green house for hardening under pot comprised of sand, soil and farm yard manure in 1:1:1 ratio and 100% of the well rooted plantlets survived under greenhouse conditions.

2.6 *In vitro* mutagenesis

Wagih *et al.* (2004) investigated the regeneration of drought tolerant variants from embryogenic callus of sugarcane (Saccharum hybrids) of three varieties (Q77N1232-tolerant, Co6519-moderate and Cadmus-sensitive) on a selective medium containing Polyethylene glycol (PEG) corresponded with the degree of tolerance to drought they possess. The three varieties produced 55%, 7% and 4% respectively, of the number of plants in the absence of PEG. A total of eight plants regenerated from the moderate and sensitive varieties were selected and grown in a greenhouse for further testing under water stress. Plants were decapitated and microsets were used to clonally propagate them in soil. Plants were further screened for drought tolerance by measuring parameters including Drought Tolerance Capacity (DTC).

Koch *et al.* (2012) developed a protocol to induce and identify imazapyr tolerance in sugarcane, which involved induction of somaclonal variation via exposure to 8 mM or 16 mM ethyl methane sulfonate for 4 h, followed by a stepwise increase in imazapyr concentration in the medium from 0.08 to 0.16 µM. The regenerated plantlets were then acclimatized for 3 mo after which they were sprayed with 182 g a.i. ha−1 imazapyr, and the above-ground biomass was determined after 47 d. Following a 1-mo waiting period for the putative tolerant plants to regrow, acetohydroxyacid synthase (AHAS; EC 2.2.1.6) enzyme assays of the plants that survived and showed a normal growth pattern were undertaken. Based on the enzymatic I 50 values, three imazapyr-tolerant genotypes were identified with an AHAS activity of 2.8 to 4.0 times that in sensitive sugarcane plants.

2.7Somaclonal variation in sugarcane

Heinz and Mee (1969) at the Hawaiian Sugar Planters Association were the first to report morphological and cytological variations in callus derived plants of *saccharum spp.* Morphological variation was observed for plant type, leaf attitude, tillering and erectness. One of the clones with distinct morphological changes, had chromosome number 2n=117 to 124 compared to 2n=106 to 107 in the donor. It was also demonstrated that the suspension cultures of several cultivars maintained for more than 6 years had variation in chromosome number for all the five clones examined.

Lourens and Martin (1987) observed more morphological variability among the somaclones regenerated from callus culture (indirect) than those that did not involve an elaborate callus phase. They also found that most variation was not due to hereditary changes. The reversion of somaclones to original characteristics was less frequent in callus derived somaclones than the others, in a subsequent clonal generation.

Mallikarjuna (2014) used two sugarcane clones, 2008T42 (susceptible to red rot) and 2009T5 (susceptible to smut) as plant material. Unexpanded leaf rolls were used as explants for callus induction with different concentrations of mutagenic chemicals (sodium nitrite and ethylmethane sulfonate). The variations in plants regenerated from this study were also investigated by molecular markers (RAPD).

Praveen *et al.* (2019) regenerated and evaluated somaclonal variants for tolerance to yellow leaf disease (YLD) in two popular YLD susceptible varieties of sugarcane; 2003V46 and Co86032 using four chemical mutagens [Ethyl methane sulphonate (EMS), sodium nitrate (SN), sodium azide (SA) and 2, 4 dichlorophenoxy acetic acid (2, 4 D)] at three levels of concentration taking leaf rolls as explants. three somaclones (16T6, 16T7 and 16T15) from 2003V46 and four (16T22, 16T23, 16T29 and 16T31) from Co86032 were found to be resistant to YLD. However, for many yield contributing characters, most of the resistant somaclones were inferior to their parent varieties. These somaclones could not be used directly as commercial varieties, they have to be utilized in hybridization programmes, crossed with commercial varieties to impart YLD resistance ultimately yielding agronomically superior YLD resistant varieties.

The chromosome number of sugarcane cells has been doubled by treating cell suspensions with colchicine (Heinz and Mee, 1970) and the somaclones with chromosome numbers ranging from 150 to 225 were reduced from numerous species and hybrids. Some of these plants developed from intra and inter specific hybrids were vigorous (Heinz et al., 1977).

In culture, cytogenetic and genetic variations might occur quite frequently and be undetected in plants as variant cells are likely to be less competitive in terms of further proliferation and morphogenic potential (Morrish*et al.,* 1987). Inspite of widespread occurrence of somaclonal variation, it is not expected to replace conventional breeding programmes. However, it can be used as a supplementary source of novel variation and as an adjunct to existing breeding practices (Larkin et al.,1984)

2.8 Molecular approaches to detect the mutation

Pawar (2011) investigated the molecular validation of the mutants in two sugarcane varieties viz. Co-86032 and CoC-671 and their ten mutant clones of each developed through *in vitro* mutagenesis. Total six SSR primers were used out of six SSR primers ‘G’ [(CA) 24] was found polymorphic. Six band were amplified by primer G [(CA) 24] in parent Coc-671 and its mutant clones. Five bands were amplified in parents Co-86032 and its mutant clones. A total of 121 loci were generated out of which 16 were polymorphic indicating 13.2% polymorphism, the size of bands ranged from 200bp to 1 kb. The mutant clones of sugarcane var. CoC-671 divided into three clusters. In first cluster seven mutant clones and parent CoC-671, second cluster two and third clusters one mutant clone. While in Co-86032 first cluster included four mutant clones, second clusters four mutant clones and third clusters two.

Dalvi *et al.* (2012) screened out the promising sugarcane somaclones for agronomic traits and smut resistance using PCR amplification of Inter Transcribed Region (ITS) of *Sporisorium scitaminae.* The study reported beneficial traits of the somaclones derived from CoC 671 and their field performance, especially for smut resistance and yield traits and early maturity.

Smiullah *et al*. (2012) obtained in-vitro regeneration followed by detection of somaclonal variation and screening for mosaic virus in sugarcane somaclones. For the detection of somaclonal variation, 38 primer pairs were used and 15 simple sequence repeats (SSR) primer pairs were found to be polymorphic with 51.61% polymorphism. The study demonstrated that SSR genetic markers are the best tool for the investigation of genetic variation in sugarcane.

MATERIAL AND METHODS

The experiment on **“Study of *in vitro* development of mutant plants of *Saccharum officinarum* for resistance against smut”** was carried out at the Tissue Culture and Molecular Biology laboratory of Biotechnology Centre, Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur (MP). Followings are the materials used and method of the research work.

3.1 Experimental materials

3.1.1 Glassware

Glassware used during the experiment were measuring cylinders, conical flasks, Petri plates, glass bottles, test tubes, and beakers.

3.1.2 Equipment

Equipment used during the research work were autoclave, hot air oven, weighing balance, pH meter, magnetic stirrer, laminar air flow, shaker, thermocycler, gel documentation system and gel assembly.

3.1.3 Chemicals

Different chemicals were used for *in vitro culture*, DNA isolation, PCR amplification and gel electrophoresis. Chemical used during the research work are presented in Table 3.1.

3.1.4 Plant material

The sugarcane cultivar was obtained from KVK Narsinghpur, Madhya Pradesh. The commercial variety Co 261 of sugarcane grown in polyhouse of Biotechnology Centre was taken as the explant.

3.2 Methods

3.2.1 Culture medium

The most widely accepted MS medium (Murashige and Skoog, 1962) was used as basal medium. The composition of MS medium is presented in Table 3.2. The basal media contains the macro and micro nutrients and vitamins. The basal medium was supplemented with hormones (Auxins and Cytokinins) and sucrose at various concentrations.

Table3.1 Chemicals used during the research work

S.No.	Chemical name	Formula
1.	MS medium	Given in Table 3.2
2.	Bacteriological agar (agar powder)	-
3.	Sucrose	$C_{12}H_{22}O_{11}$
4.	2,4- Dichlorophenoxy acetic acid (2,4-D)	$C_8H_6Cl_2O_3$
5.	Indole-3-butyric acid (IBA)	$C_{12}H_{13}NO_2$
6.	Benzyl amino purine (BAP)	$C_{12}H_{11}N_5$
7.	Kinetin	$C_{10}H_9N_5O$
8.	Ethyl alcohol	C_2H_5OH
9.	Mercuric chloride	$HgCl_2$
10.	Sodium hypochlorite	NaOCl
11.	Tween 20	$C_{58}H_{114}O_{26}$
12.	Carbendazim	$C_9H_9N_3O_2$
13.	Ethyl methane sulfonate (EMS)	$CH_3SO_3C_2H_5$
14.	Hydrochloric acid	HCl
15.	Sodium hydroxide	NaOH
16.	Ethidium bromide (EtBr)	$C_{21}H_{20}BrN_3$
17.	RAPD primers	

Table 3.2 Composition of MS medium

Medium constituents	Chemical formula	Concentration mg/l
Macro elements		
Ammonium nitrate	NH_4NO_3	1650.000
Calcium chloride	$CaCl_2.2H_2O$	440.000
Magnesium sulphate	$MgSO_4.7H_2O$	370.000
Potassium nitrate	KNO_3	1900.000
Potassium phosphate monobasic	KH_2PO_4	170.000
Micro elements		
Boric acid	H_3BO_3	6.200
Cobalt chloride hexahydrate	$CoCl_2.6H_2O$	0.025
Copper sulphate pentahydrate	$CuSO_4.5H_2O$	0.025
EDTA disodium salt dehydrate	Na_2EDTA	37.310
Ferrous sulphate heptahydrate	$FeSO_4.7H_2O$	27.810
Manganese sulphate	$MnSO_4.4H_2O$	22.300
Molybdic acid (sodium salt)	$Na_2MoO_4.2H_2O$	0.250
Potassium iodide	KI	0.830
Zinc sulphate heptahydrate	$ZnSO_4.7H_2O$	8.600
Vitamins		
myo-inositol	$C_6H_{12}O_6$	100.000
Nicotinic acid (free acid)	$C_6H_5NO_2$	0.500
Pyridoxine HCl	$C_6H_{11}NO_3.HCl$	0.500
Thiamine hydrochloride	$C_{12}H_{17}ClN_4OS.HCl$	0.100
Amino acid		
Glycine	$C_2H_5NO_2$	2.000
Total (g/l)		4.4

Source – Murashige and Skoog (1962)

3.2.2 Media preparation

To prepare the basal medium 4.4 gm Murashige and Skoog media of Hi-Media Laboratories was dissolved in 500 ml distilled water. The basal media was supplemented with sucrose (30gm/l) and plant growth regulators then the pH of the medium was adjusted to 5.8±0.05 using 0.1N NaOH or 0.1N HCl. After setting media pH volume was made to 1 litre by adding distilled water. The medium was supplemented with bacteriological agar (8gm/l) before autoclaving the medium at 120ºC temperature and 15 PSI pressure for 20 minutes.

3.2.3 Sterilization of culture media and vessels

After preparing the culture media, it was split in different volumes in glass bottles. The bottles were closed with tight cotton plugs. Culture vessels were placed in autoclavable polythene and tied with thread. The media and vessels were autoclaved at 120ºC temperature and 15 PSI pressure for 20 minutes. After autoclaving media was taken to air-conditioned room and left for cooling. Meanwhile the laminar air flow hood was wiped with 70% ethanol using autoclaved non-absorbent cotton. Then the culture vessels were opened in the laminar air flow hood. As the media cools down to bearable temperature it was poured in culture vessels (Petri plates, bottles and test tubes as required) and left for 2 days to check contamination.

3.2.4 Surface sterilization of explant

Clean and fresh leaf rolls, leaf blade and shoot tips were taken as explant. The explant was first washed thoroughly with distilled water 4-5 times. Then the explant was dipped in tween-20 (5-6 drops in 100ml distilled water) for 20 minutes followed by occasional shaking. After 20 minutes the explant was rinsed with sterile distilled water until all traces of tween-20 gets removed. Then the explant was taken to laminar air flow for further sterilization. In the laminar air flow, the explant was dipped in 0.5% Carbendazim for 30 minutes followed by thorough rinsing with sterile distilled water 6 times. Then the explant was finally sterilized with 0.1-0.2% $HgCl_2$ for 3 minutes followed by rinsing with sterile distilled water till all the traces of $HgCl_2$ removes.

3.2.5 Inoculation of explant to culture medium for callus induction

In order to induce callusing the culture medium was supplemented with different concentration of 2,4-D from 2mg/l to 5mg/l and 2,4-D in combination with BAP.

The explant was cut in small pieces and placed on the culture medium in Petri plates and sealed with parafilm in air tight condition. The cultured plates were incubated in culture room with 16 hours of light availability. The temperature of the culture room was maintained at 25±2ºC. The observed callus mass was subcultured in every 21 days at the same concentration of 2,4-D. composition of callus induction media is presented in Table 3.3.

Table 3.3 Composition of callusing medium

S.No.	Component	Concentration
1.	MS media	4.4gm/l
2.	Sucrose	30gm/l
3.	2,4-D + BAP	2mg/l + 1mg/l
		4mg/l + 2mg/l
4.	2,4-D	2mg/l 2.5mg/l 3mg/l 4mg/l 5mg/l
5.	Bacteriological agar	8gm/l

3.2.6 Induction of mutation using EMS

To achieve *in vitro* mutagenesis, 8 µM Ethyl methane sulfonate (EMS) was added in the medium. Healthy and viable calluses after 60 days of inoculation were transferred to callusing medium containing 8 µM Ethyl methane sulfonate (EMS) and incubated for 17 days.

3.2.7 Regeneration of shoots and roots from the callus culture

After EMS treatment the viable and healthy calluses were subjected to various treatments to regenerate in shoots and roots. The regeneration of callus in shoots and roots was achieved in MS medium. The established calluses were transferred to regeneration medium containing different concentration of hormones for shooting and rooting.

3.2.7.1 Regeneration of shoots

For shoot induction MS medium was supplemented with varied concentrations of BAP and Kinetin. The established callus mass was transferred to the medium in glass bottles. The number of shoots obtained from callus was calculated and the shoots were transferred to rooting medium. The concentrations of growth hormones used for shooting in the medium are presented in the Table 3.4.

3.2.7.2 Regeneration of roots

Multiple shoots obtained from shooting medium were transferred to rooting medium. Rooting medium was prepared in test tubes. For root induction the MS media was supplemented with 0.25mg/l indole butyric acid (IBA). The root formation was observed frequently until it developed good number of roots.

Table 3.4 Composition of shooting medium

S.No.	Component	Concentration
1.	MS media	4.4 gm/l
2.	Sucrose	30 gm/l
3.	BAP	1.0 mg/l 1.5 mg/l 2 mg/l 3 mg/l 4 mg/l
4.	BAP + Kinetin	1.5 mg/l + 0.5 mg/l 3 mg/l + 1 mg/l
5.	Bacteriological agar	8 gm/l

3.2.8 Hardening of the plantlets

Plants (10-15 cm) with well-developed roots were washed thoroughly with sterile distilled water to completely remove the medium constituents. The plants were transferred to 1:1:1 mixture of cocopeat, vermicompost and sand in plastic cups. The mixture was autoclaved before transplanting. The plants were watered frequently.

3.2.9 Molecular validation of mutants

Polymorphism among the plants regenerated from different treatments in tissue culture was studied using Random Amplified Polymorphic DNA (RAPD) analysis.

The cultured plantlets used for studying polymorphism were regenerated from *in vitro* mutagenesis of Co 261 using mutagenic chemical EMS at 8 µM concentration.

3.2.9.1 DNA extraction buffer

DNA Extraction buffer was made without β-mercaptoethanol on a magnetic stirrer to avoid foaming. β-mercaptoethanol was then added to the cooled solution at room temperature. Composition of DNA extraction buffer given in table 3.5.

Table 3.5 Composition of DNA Extraction buffer

S.No.	Reagents	Concentration
1.	NaCl	1.4M
2.	Tris (pH 8.0)	100mM
3.	EDTA (pH 8.0)	20mM
4.	β-mercaptoethanol	2%
5.	CTAB	2%

3.2.9.2 DNA isolation from randomized samples

The technique of DNA isolation relied upon the fact that the nucleic acid would form suitable complex with detergent cetyltrimethyl ammonium bromide (CTAB) under high salt concentration and when the concentration reaches 0.4M NaCl the CTAB-NA complex would precipitate. Genomic DNA was isolated using the method described by Saghai-Maroof *et al.* (1984) with suitable minor modifications. In present study, DNA was isolated from fresh leaf and calli of different age and suspended cells from random samples were used to isolate DNA. The method given below gave a good quality and quantity of DNA.

1. 2 g of clean young leaf samples were crushed to a fine powder with a pestle and mortar after treating it with liquid nitrogen.
2. The fine powder was transferred to a 50ml Oakridge tube and 10ml of preheated (65ºC) DNA extraction buffer (Table 3.5) was added in the Oakridge tube and mixed thoroughly.
3. The samples were incubated in a water bath at 65ºC for one hour followed by inverting the samples in every 10 min to ensure complete and even extraction.
4. The samples were then taken out from water bath and cooled to room temperature.
5. The samples were then centrifuged for 15 min at 10000 rpm for 15 min at room temperature.
6. The supernatant was transferred to a fresh tube.
7. In equal volume of supernatant, chloroform:isoamyl alcohol (24:1 v/v) was added and mixed thoroughly but gently for 5 min.
8. Mixture was then centrifuged for 15 min at 10000 rpm at room temperature.
9. Supernatant so obtained was transferred to a fresh tube with the help of 1ml cut tips.
10. An equal volume to supernatant, pre-chilled isopropanol was added, mixed gently by inverting tubes and kept undisturbed for 10 min.
11. The DNA precipitate was then spooled out using 1ml cut tips and transferred to a 1.5ml micro centrifuge tube.

12. DNA was pelleted out by centrifugation at 10000 rpm for 10 min.
13. The supernatant was discarded and pellet was washed with 70% ethanol.
14. The pellet was dried at room temperature and dissolved in 200 µl of TE buffer for further use.

3.2.9.3 DNA purification

The purification of DNA was carried out in order to remove the impurities like RNA, proteins and polysaccharides. These impurities are considered as inhibitors in DNA amplification during PCR.

1. 5 µl of RNases A (5 mg/ml) was added to DNA extract, mixed well and incubated at 37ºC for 30 min.
2. This was followed by the addition of equal volume of chloroform:isoamyl alcohol (24:1v/v) and mixed vigorously.
3. The above mixture was centrifuged at 14000 rpm for 10 min.
4. Supernatant was transferred to a fresh micro centrifuge tube and 1/10 volume of 3 M sodium acetate (pH 5.4) was added, followed by further addition of two volumes of pre-chilled ethanol and mixed gently for DNA precipitation.
5. The precipitated DNA was pelleted by centrifugation at 12000 rpm for 5 min.
6. The pellet was air dried to completely remove ethanol and was then dissolved in 100 µl of TE buffer and stored at -20ºC for further use.

3.2.9.4 Quantification of DNA

Isolated DNA was quantified by measuring the absorbance at 260 nm and 280 nm on a UV-spectrophotometer. 50 µg/ml concentration of double stranded DNA showed an absorbance of 1 at 260nm. Concentration of DNA samples was calculated using following formula:

$$\frac{\mathbf{O.D._{260\ nm}\ X\ 50\ \mu g\ DNA/ml\ X\ Dilution\ factor}}{\mathbf{1000}}$$

3.2.9.5 Dilution of DNA

The quantified DNA was diluted according to the DNA quantity in each sample with sterile double distilled water for PCR amplification. Dilutions were carried out according to the following formula:

$$\text{Dilution} = \frac{\text{Required concentration of DNA (ng/µl) X Total volume required (µl)}}{\text{Available concentration of DNA (ng/µl)}}$$

3.2.9.6 PCR amplification of DNA

The reaction mixture composition for the polymerase chain reaction was prepared by using following reagents for each of the DNA sample. List of PCR components is given in table 3.6. List of RAPD primers is given in Table 3.7.

3.2.9.7 PCR conditions

PCR conditions were standardized considering different parameters viz. initial denaturation, denaturation, annealing, extension and final extension using Agilent 8800 PCR Machine. PCR thermal and reaction profile was optimized for amplification purpose by using primers of unique sequence. PCR conditions for which was used for RAPD amplification are given in Table 3.8.

Table 3.6 List of PCR components with their concentrations used for RAPD amplification

S.No.	Components	Concentration
1.	PCR buffer	1 µl
2.	dNTPs	1.2 µl
3.	Primers	0.2 µl
4.	$MgCl_2$	1 µl
5.	Taq polymerase	0.2 µl
6.	MQ	-
7.	DNA	1 µl

Table 3.7 List of RAPD markers used and their sequences

S.No.	Primer	Sequences5'-3'	GC Content
1.	OPA-01	CAGGCCCTTC	70.0%
2.	OPA-02	TGCCGAGCTG	70.0%
3.	OPA-03	AGTCAGCCAC	60.0%
4.	OPA-04	AATCGGGCTG	60.0%
5.	OPA-05	AGGGGTCTTG	60.0%
6.	OPA-06	GGTCCCTGAC	70.0%

Table 3.8 PCR condition for RAPD

S.No.	Step	Temperature (ºC)	Time	Number of cycles
1.	Initial Denaturation	94	4 min	1
2.	Denaturation	94	45 sec	40
3.	Annealing	37	1 min	40
4.	Extension	72	2 min	40
5.	Final Extension	72	5 min	1
6.	Storage	4	-	1

3.2.9.8 Gel electrophoresis of PCR product

PCR products mixed with gel loading dye was loaded on 1.5% agarose gel along with appropriate size DNA ladder (1kb) and run under constant voltage of 70 volt. The gels were visualized under UV light in gel documentation system and photographed through the same. Composition of loading dye is given in Table 3.9.

Table 3.9 Composition of loading dye

S.No.	Component	Concentration
1.	Bromophenol blue	25 mg
2.	Xylene cynol	25 mg
3.	Glycerol	3 ml
4.	MQ	7 ml

3.2.9.9 Scoring and analysis using software

DNA fingerprints on RAPD gels were scored for the presence (1) or absence (0) of bands of molecular weight size in the form of binary matrix for all the accessions studied using NTSYS-pc version 2.02e Software. A dendrogramwas constructed using UPGMA (unweighted pair-group method with arithmetic mean) with the SAHN (sequential, agglomerative, hierarchical, and nested clustering) routine**.**The percentage polymorphism of bands was calculated with following formula:

$$\textbf{Polymorphism percentage} = \frac{\textbf{Number of polymorphic bands}}{\textbf{Number of total bands}} \times 100$$

3.3Statistical analysis

Each experiment contained three replications. The data was analysed through completely randomized design (CRD). Each experiment was repeated three times and mean values and standard deviation were calculated. All data obtained were subjected to the single factor analysis of variance (ANOVA) using Microsoft excel. The critical difference (C.D.) values were calculated at p=0.05 level to find out the significant difference between the means of different treatments. The significantly different mean values are indicated by different letters. ANOVA table is given as Table 3.10.

Table 3.10 Skeleton of ANOVA Table (CRD)

Source of variation	d.f.	S.S.	M.S	F.cal.
Treatments	(t-1)	MS (E)	MS (E)	
a) Explants	(e-1)	MS (E)	MS (E)	MS (E)/ MS (E)
b) Culture media	(m-1)	MS (M)	MS (M)	MS (M)/ MS (E)
c) E × M interaction	(e-1)(m-1)	MS (EXM)	MS (EXM)	MS (EXM)/ MS (E)
Error (A)	(rt-t)	MS (S)	MS (S)	
Total	(rt-t)	TSS	TSS	

Where,

t = Number of treatment combinations

r = Number of replications

e = Number of explants

m = Number of media

RESULTS

Results of the present investigation covering the objectives set on **"Study of *in vitro* Development of mutant plants of *Saccharum officinarum* for Resistance Against Smut"** carried out with leaf roll, leaf blade and shoot tip as explants of popular sugarcane cultivar Co 261 are presented in this chapter. The objectives of this study were to standardise callus induction from different explants of sugarcane, to develop the smut resistant mutants through *in vitro* culture and to validate the mutant plantlets using molecular markers. Moreover, the results of surface sterilization, effect of different concentrations of hormones for callusing, shooting and rooting are also described in the chapter.

Initially the response of leaf roll, leaf blade and shoot tip was evaluated for callusing with different concentrations of 2,4-D, but the young leaf roll proved to be the better explant for callusing. So, the results of leaf rolls were only presented and discussed.

4.1 Surface sterilization

For the aseptic *in vitro* culture of any plant surface sterilization of the explant is compulsory, for surface sterilization the explant was first washed in running tap water to remove all the possible dirt from the field then the explant was rinsed with sterile distilled water and for further sterilization the explant was treated with different chemicals. The chemical used for surface sterilization and their results are given in Table 4.1.

Table 4.1 Standardizedsurface sterilization protocol

Chemical	Concentration	Time(Min)
Tween-20	5-6 drops in 100 ml sterile distilled water	20
Carbendazim	0.5%	30
Mercuric chloride ($HgCl_2$)	0.1 – 0.2%	3

Surface sterilization with tween-20 for 20 minutes, carbendazim 0.5% for 30 minutes and $HgCl_2$ for 3 minutes gave the best results with mere contamination of 6-7%. Treating the explant with Carbendazim for less than 30 minutes showed fungal contamination in the culture and $HgCl_2$for more than three minutes showed death of culture.

4.2 Standardisation of explant for callus induction

The plant parts used as explants were leaf roll, leaf blade and shoot tip. The leaf blade did not show promising response against any combination of hormone for callusing (Plate 1: A), while the shoot tip regenerated shoots even in the callusing medium (Plate 1: B), hence it could not be used for further investigations either. The young leaf roll showed best responses against callusing medium and found to be the best plant part to induce callusing. The explants were inoculated in the mediumand incubated at 25±2 ^{o}C with photosynthetic photon of 50 µmol^{-2} s^{-1} under 16/18h photo period. After 21 days of incubation, cultures were transferred on similar medium for further growth. After 45 days of incubation, callus producing mature explants were counted. After 60 days of incubation mature and viable calluses were transferred to similar callusing medium supplemented with 8µM EMS (Ethyl Methane Sulfonate) to induce mutagenesis. The results showed by the young leaf roll in callusing medium are given in the Table 4.2 along with the hormones supplemented.

In order to achieve callus from the leaf roll the MS medium was supplemented with 2,4-D in different concentrations from 2 mg/l to 5 mg/l and 2,4-D + BAP in different concentration given in Table 3.3. The best results of callusing were obtained in young leaf roll. It showed best percentage of callusing in MS medium supplemented with 3 mg/l 2,4-D, at this concentration 52.22% leaf roll initiated callusing (Plate 1: C - F), followed by 37.83% callusing in 2.5 mg/l 2,4-D. The combination of 2,4-D and BAP showed less than 20% of callus Induction, while in 2,4-D the percentage of callusing increased with increasing concentration up to 3 mg/l while in 4 mg/l 2,4-D callusing percentage fell drastically and showed death of culture. Although not all the calluses achieved were viable. Many calluses lost their viability at different stages of culturing, the survived calluses were transferred to regeneration medium.

Table 4.2 Callus induction from leaf roll

Hormone	Concentration (mg/l)	No. of callus observed (%)
2,4-D + BAP	2 + 1	16.66
	4 + 2	20.00
2,4-D	2	25.00
	2.5	37.83
	3	52.22
	4	20.83
	5	18.50
SEm±		4.935
Average		27.291
CD (at 5%)		2.447
CV		47.844

The standard error of the mean of all the treatments is 4.935, arithmetic mean of callus observed in seven combinations is 27.291, CD (at 5%) is 2.447 and coefficient of variance (CV) is 47.844 (Table 4.2).

4.3 Regeneration of shoot and root from the callus

Regeneration medium was prepared for shooting and rooting with different concentration of BAP and Kinetin for shooting and IBA for rooting.

The calluses were transferred to the regeneration medium, for shoot induction the calluses were incubated for 30 days. The number of shoots producing callus and shoots per callus were recorded.

4.3.1 Shoot initiation from callus

For induction of shoots from the calluses the basal medium was supplemented with BAP from 1mg/l to 4 mg/l and BAP + Kinetin in different concentrations given in Table 3.4. The responses of the hormones for multiple shoot regeneration are given in the Table 4.3.

Table 4.3 Responses of growth regulators for shoot induction

S.No.	Hormone and its concentration	Number of shoots per callus
1.	1 mg/l BAP	8
2.	1.5 mg/l BAP	10
3.	2 mg/l BAP	7
4.	3 mg/l BAP	2
5.	4 mg/l BAP	2
6.	1.5 mg/l BAP + 0.5 mg/l Kinetin	17
7.	3 mg/l BAP + 1 mg/l Kinetin	3
SEm±		2.047
Average		7.000
CV		77.372

Both BAP and kinetin had a synergetic effect and none of them generated the maximum response while separately applied. The combination (1.5 mg/l BAP + 0.5 mg/l Kinetin) showed best results towards shoot regeneration and almost 30% callus regenerated in shoots. In the other combinations regeneration was satisfactory. 1 mg/l BAP gave 8 shoots per callus while at 4 mg/l BAP the number of shoots induced decreased gradually. Number of shoots per callus varied from 2 to 17 shoots (Plate 2).

The standard error of the mean of seven treatments is 2.047, arithmetic mean of shoots observed in seven combinations is 7.000 and coefficient of variance (CV) is 77.372 (Table 4.3).

4.3.2 Regeneration of roots in the plantlets

In vitro regeneration of roots from the plantlets is the final stage before the plant gets ready to harden. Rooting of the plantlets was profuse and it even initiated rooting in basal MS medium, although in the current investigation 0.25 mg/l IBA was supplemented in MS medium to achieve a good rooting percentage. In the above media 72.7% plantlets showed rooting (Plate 3).

4.4 Molecular validation of the mutants

To carry out PCR six primers of RAPD were used Out of which three primers; OPA-04, OPA-05 and OPA-06 amplified successfully, and were selected for further analysis on the basis of banding pattern of the PCR product after gel electrophoresis (Plate 4).

4.4.1 Scoring of bands

The banding pattern of the PCR product was scored for presence (1) and absence (0) of bands. Among the three primers amplified OPA-06 showed 80% polymorphism while other two (OPA-04 and OPA-05) showed 42.85% polymorphism. Results of scoring of bands are presented in table 4.4.

Table 4.4 Polymorphism percentage of primers after scoring

Primer	Band size	Total bands	Monomorphic bands	Polymorphic bands	polymorphism
OPA-04	250-1300bp	7	4	3	42.85%
OPA-05	250-1300bp	7	4	3	42.85%
OPA-06	350-1300bp	5	1	4	80%

4.4.2 Cluster analysis of RAPD markers

Based on electrophoresis banding pattern of RAPD primers, genetic similarity was estimated between 13 somaclones and the mother plant of Co261. Dendrogram was generated using Unweighted Pair Group Method With Arithmatic Mean (UPGMA) with NTSYS-pc version 2.02e software (Fig. 4.1). The analysis revealed that the mother plant of Co261 along with the somaclones obtained from somaclonal variation fell into two groups, major group and minor group. Major group was further divided into two subgroups, 'A' and 'B'. subgroup A was further divided into two subgroups a and b. Subgroup 'a' contained Sc1 (Somaclone 1), Sc2, Sc3, Sc4, Sc7 and Sc8 while subgroup 'b' contained Sc5. Subgroup 'B' contained Sc9 and Sc10.

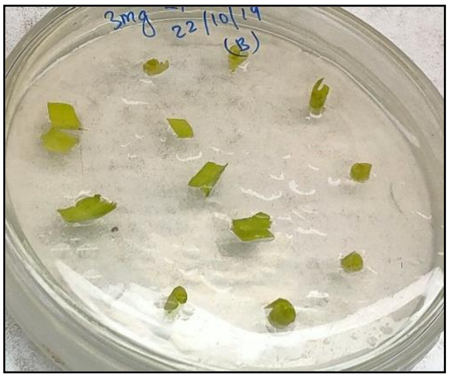
In the minor group which was divided into two subgroups 'C' and 'D'. subgroup 'C' contained Sc6, Sc12 and Sc13 along with mother plant, while the subgroup 'D' contained Sc11.

4.4.3 Principal component analysis (PCA)

Principal component analysis of 13 somaclones and mother plants was carried out according to the similarity coefficient. In this analysis, two groups were divided into group 'A' and group 'B'. Group 'A' contained six somaclones and the mother plant *viz.,* Sc9, Sc10, Sc11, Sc12, Sc13, Sc6 and Co 261. Group B contained seven somaclones *viz.,* Sc5, Sc1, Sc2, Sc3, Sc4, Sc7, and Sc8 (Fig. 4.2).

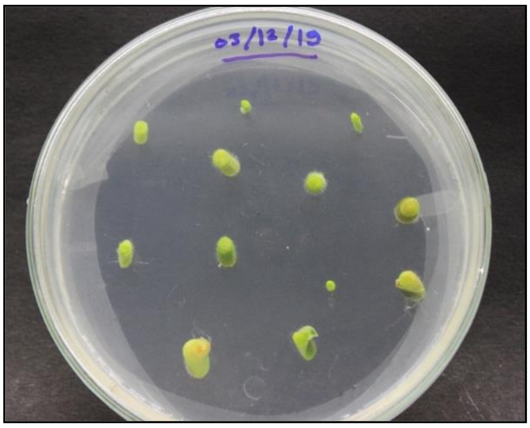
Three-dimensional scaling of 13 somaclones and Co 261 also revealed similarity conferring principal component analysis.In this analysis, two groups were divided into group 'A' and group 'B'. Group 'A' contained six somaclones and the mother plant *viz.,* Sc9, Sc10, Sc11, Sc12, Sc13, Sc6 and Co 261. Group B contained seven somaclones *viz.,* Sc5, Sc1, Sc2, Sc3, Sc4, Sc7, and Sc8 (Fig. 4.3).

RAPD analysis clearly showed that all the somaclones were different from the mother plant (Co 261) and hence were mutants.The highest genetic diversity observed was 31.58%.

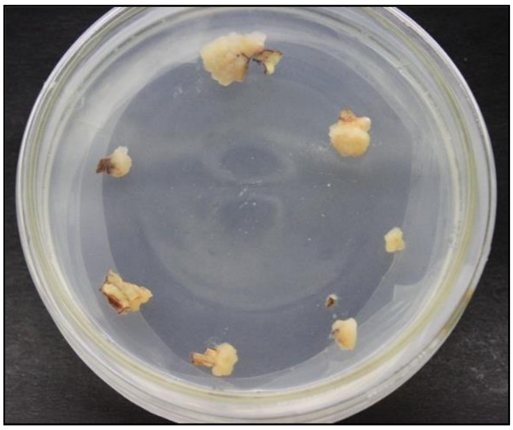


A.

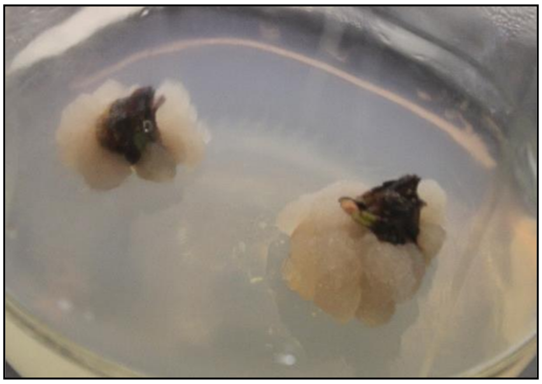
B.



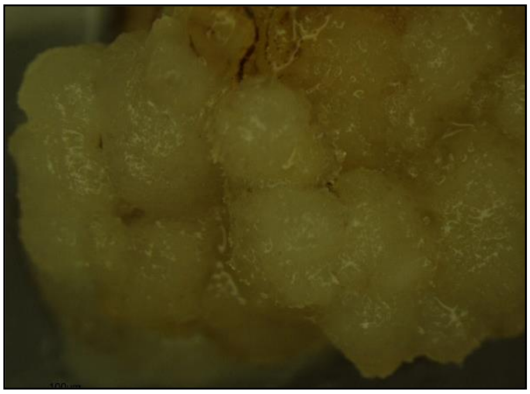
C.



D.



E.



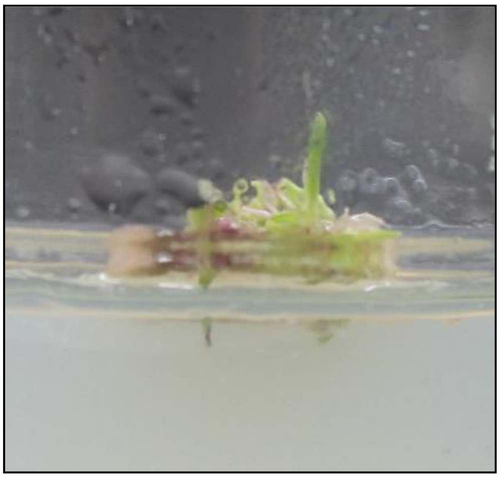
F.

Plate 1: Responses of different explants in callusing medium

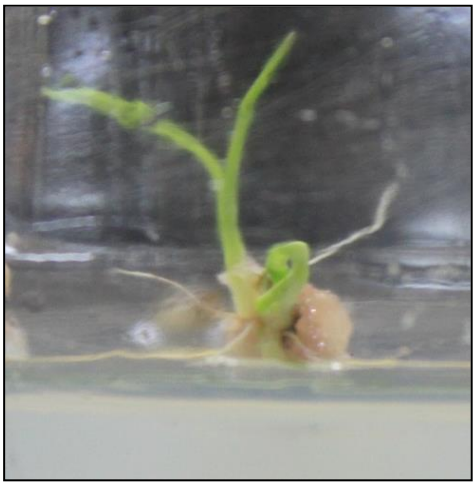
A: Leaf disc inoculated to callusing medium

B: Non-viable shoot tip in callusing medium

C - F: Callus mass observed from leaf roll in MS medium supplemented with 2,4-D



A.



B.

C.

D.

Plate 2: Regeneration of multiple shoots from callus

A and B: Initiation of shooting in MS medium supplemented with 1.5 mg/l BAP + 0.5 mg/l Kinetin

C: Multiplication of shoots from callus

D: Multiple shoots observed from one callus

A.

B.

C.

Plate 3: Rooting in sugarcane plantlets

A and B: Rooting achieved in MS medium supplemented with 0.25 mg/l IBA

C: Hardening of mutant plantlets in cocopeat:sand:vermicompost in 1:1:1

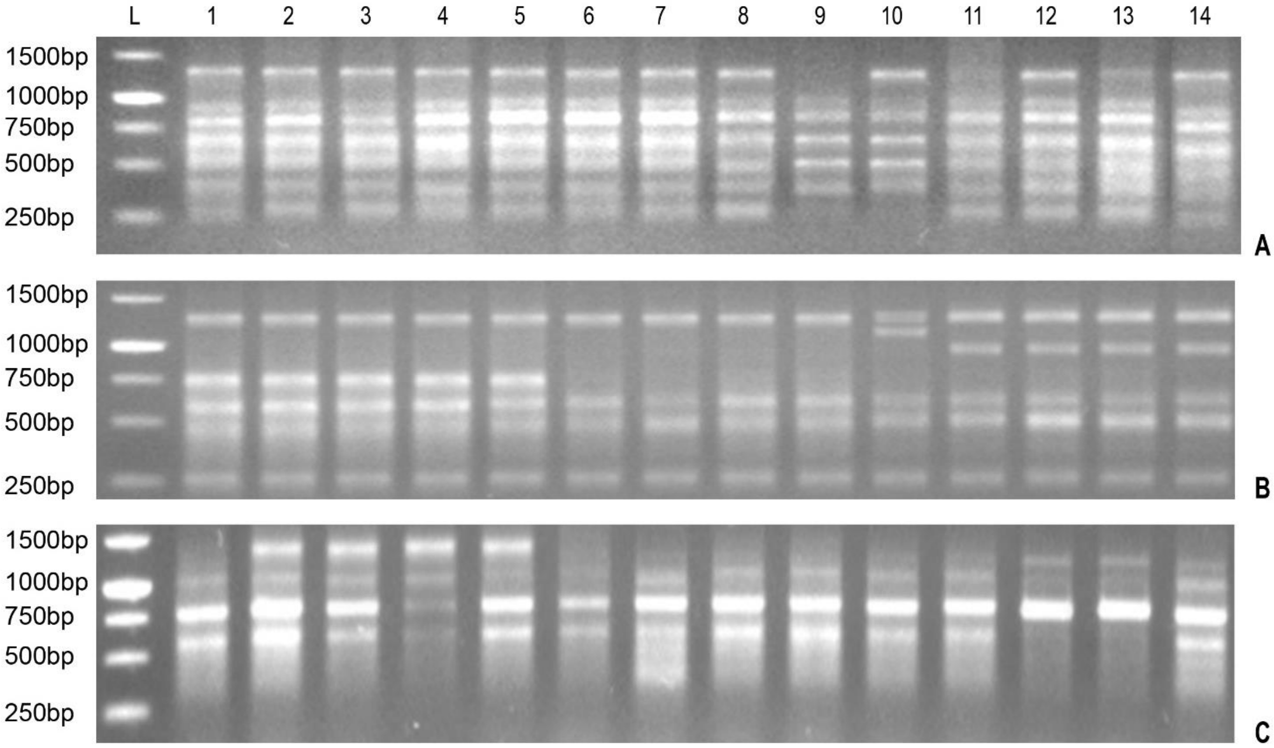


Plate . RAPD analysis generated among 13 somclones and mother plant of Co 261 cultivar of sugarcane using primers (A) OPA4 (B) OPA5 and (C) OPA6

Lane L- Ladder (1kb)

1. Somaclone 1
2. Somaclone 2
3. Somaclone 3
4. Somaclone 4
5. Somaclone 5
6. Somaclone 6
7. Somaclone 7
8. Somaclone 8
9. Somaclone 9
10. Somaclone 10
11. Somaclone 11
12. Somaclone 12
13. Somaclone 13
14. CO 261 (mother plant)

Plate 4: Banding pattern of PCR products of 13 somaclones and mother plant (Co 261) using RAPD markers on 1.5% agarose gel

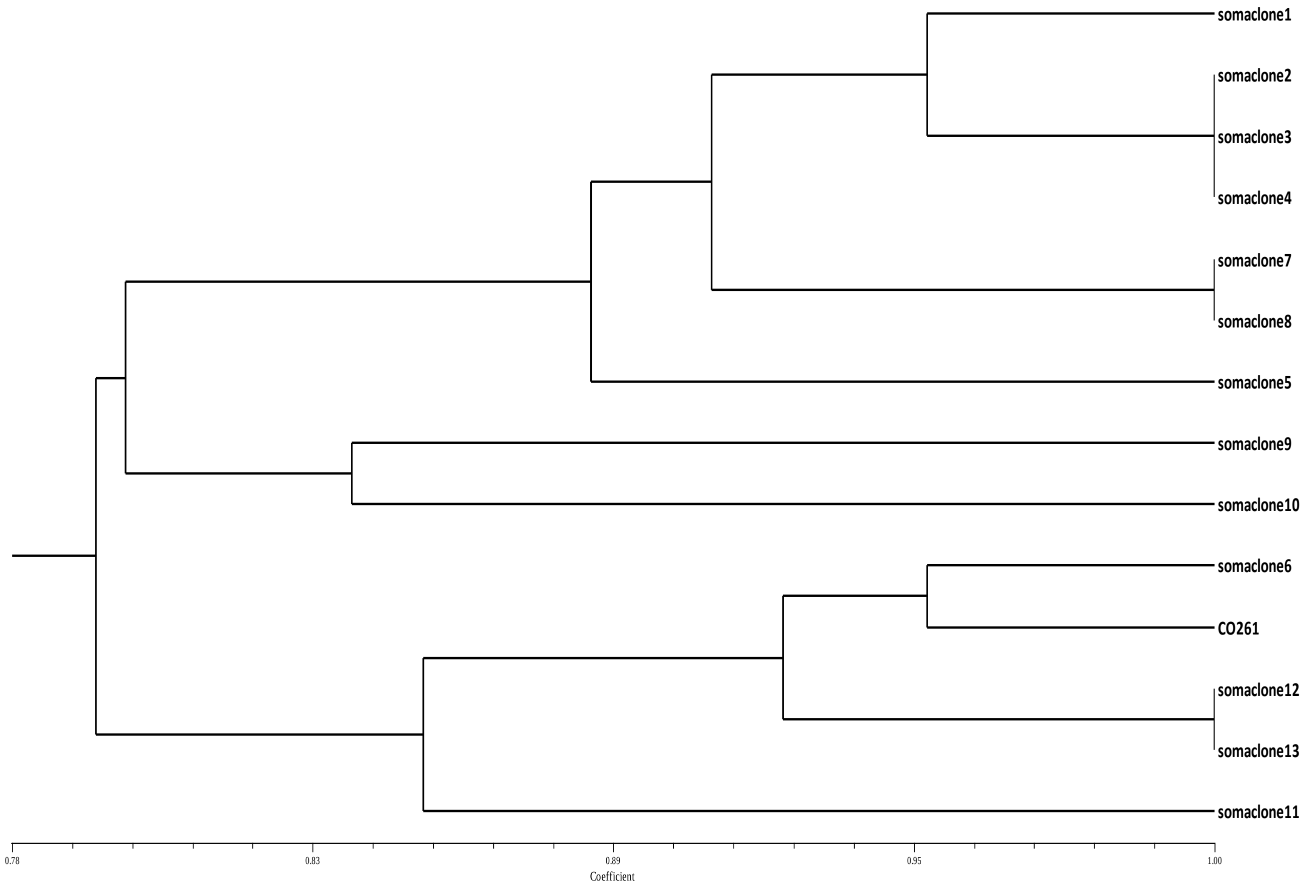


Figure 4.1: Dendrogram generated using UPGMA analysis showing relationships among somaclones and mother plant using RAPD markers

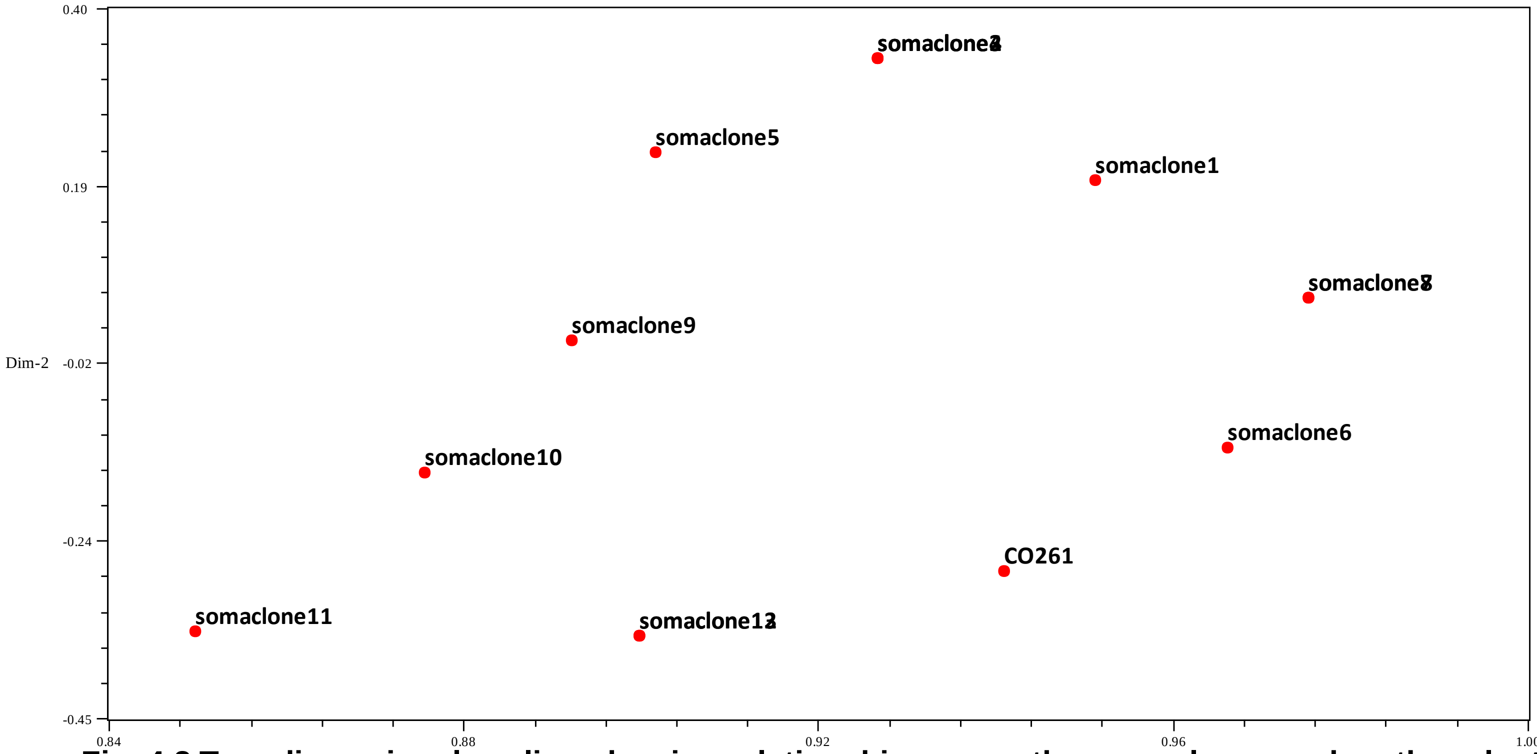


Fig. 4.2 Two-dimensional scaling showing relationship among the somaclones and mother plant

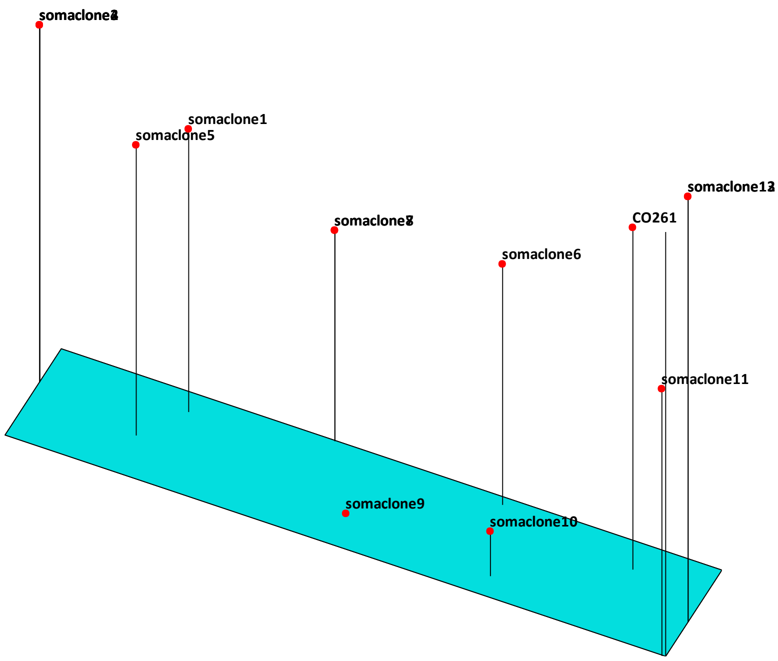


Fig. 4.3 : Three-dimensional scaling of the somaclones and mother plant

DISCUSSION

Sugarcane (*Saccharum officinarum)* is a cash crop of poaceae family. The dependency on sugarcane for sugar and by-products shows its extent of economic importance. The scope of expanding sugarcane area is difficult and limited, hence to improve the quality and yield of cane, varietal improvement is necessary. Sugarcane is greatly affected by the biotic and abiotic factors. Inducing genetic variability among the cultured cells has a potential of improving the existing varieties. *In vitro* techniques have become useful in generating novel genetic variability in many seeds as well as vegetatively propagated crops (Maluszynski *et al.,* 1995). Somaclonal variation is of value in two ways (i) to change one or more characteristics in an existing cultivar which may lead to its improved performance and (ii) they augment that component of variation which may not exist in the natural gene pools. Evans and Sharp (1986) suggested that an obvious strategy for the use of somaclonal variation in breeding is to select for incremental Improvements of existing varieties by passing the best available lines through a tissue culture cycle.

Present investigation was carried out to induce somaclonal variation among the cultured cells and to finally obtain mutants with the characteristics which were not present in the mother plant. *In vitro* developed plants were validated for determining occurrence of mutation caused by somaclonal variation. The establishment of a culture is greatly dependent upon microbial contamination, a precise surface sterilization is the primary requirement for aseptic culture. Exclusion of the microbes was achieved by washing the explant with running water and then it was dipped in 100 ml sterile distilled water with 5-6 drops of tween-20 in it for 30 minutes after that the explant was washed with sterile distilled water thoroughly to completely remove tween-20, then the explant was treated with 0.5%(w/v) carbendazim for 30 minutes followed by rinsing with sterile distilled water and finally the explant was treated with 0.1%(w/v) $HgCl_2$ for 3 minutes. Carbendazim (bavistine) is an effective fungicide. Mallikarjuna (2014) used 0.1% (w/v) bavistine to disinfect the explant, although in the current investigation bavistine concentration less than 0.5%(w/v) showed fungal contamination as sterilization of the explant also depends upon environmental conditions such as humidity and temperature. Mercuric chloride ($HgCl_2$) is an effective sterilizing agent with anti-microbial properties. Many instances of using mercuric chloride for sterilization purpose have been seen, although it is carcinogenic and exposure of the explant to $HgCl_2$ for longer time results in death of the culture. Kumar (1998) used 0.75% (w/v) $HgCl_2$, Shrivastava *et al.* (2018) used 0.5% (w/v) $HgCl_2$ to sterilize the young leaf roll.

In vitro regeneration is also affected by the pH of the culture medium, the availability of essential nutrients reduces with change in pH. Murashige (1974) stressed about the importance of pH and observed the precipitation of nutrient associated with higher pH while on other hand, poor gelling was observed with lower pH. The pH of the medium was maintained to 5.8 ± 0.05. Although, the pH of the medium sometimes fluctuated after autoclaving. The high or low pH of medium can leave toxic effect in the culture and can stimulate phenolic exudation.

To standardise callus induction from different explants, three plant parts; leaf disc, leaf roll and shoot tip were used as explant. The results of the study showed that young leaf roll of sugarcane is most suitable part for callus induction. Mamun *et al.* (2004) used the leaf roll for callus induction. To obtain callus from the explant, it was inoculated to MS medium containing different concentration of 2,4-D ranging from 1 mg/l to 5 mg/l. The results of the study suggest that MS medium fortified with 3.0 mg/l 2,4-D is suitable for callus induction giving 52.22% callus followed by 2.5 mg/l 2,4-D giving 45.83% callus. Guevara *et al.* (1995) reported 3.0 mg/l 2,4-D is suitable in order to achieve callusing in sugarcane. Shetty and Srinath (2015) significantly induced callus from immature leaf roll in MS media containing 3.5 mg/l 2,4-D and 0.5 mg/l BAP while in the present study combination of 2,4-D and BAP showed less callusing percentage and took longer time to respond. Maximum proliferation of shoot was observed in BAP (1.5 mg/l) + Kinetin (0.5 mg/l). In the regeneration medium BAP alone was also used in the concentration of 1 mg/l to 4 mg/l as the study of Tesfa and Ftwi (2018) revealed that BAP and Kinetin had a synergetic effect and none of them generated the maximum response while separately applied. Patel *et al.*

(2015) found that shooting was profuse in medium containing BAP 1.0 mg/l + IBA 0.5 mg/l. In comparison with shooting, regeneration of roots *in vitro* in sugarcane is more profuse and it regenerates roots even in basal medium. Although, Mustafa and Khan (2015) in their study standardised IBA for root regeneration in sugarcane and revealed that the concentration of IBA between 0 - 5 mg/l is optimum for root induction. The current study is also evident for effect of IBA on rooting.

A serious problem associated with this stage, is the exudation of phenolic substances that leaches out from the explant resulting in browning of the explant, the medium and thus killing the whole culture (Kumari and Verma, 2001). Some studies showed that frequent sub culturing (7 days) during the early stages of establishment reduce the adverse effect of browning of tissues and the release of pigments in the medium (Gosal *et al*., 1998; Dobariya, 1994). In the current investigation phenolic exudation was observed, which was overcome by subculturing of such calluses which shows phenolic effect.

Random genetic variation occurred in tissue culture can be selected for resistance against biotic and abiotic factors. To increase the frequency of occurring the phenomenon mutation agents can be used. As the frequency of mutagen-induced mutation is higher than spontaneous mutations *in vitro* (Novak and Bruner, 1992). The callus obtained from the previous callusing methods was treated with EMS in order to increase frequency of mutation and induce genetic variation. EMS is an effective chemical mutagen which causes mutation. EMS is one of many stress elements, and its simulative effect on plant regeneration in the culture of somatic tissue has been reported. Mallikarjuna (2014) investigated mutagenesis in EMS concentration ranging from 0.8-1.0 µM. Bisht *et al.* (2017) supplemented 0.0-0.8 mM EMS to induce mutagenesis in kutki (*Picrohiza kurroa). In vitro* mutagenesis of cultured explants, cell and tissue represents a feasible method for induction of genetic variation, which can be subjected at the cellular level to selection for desirable traits. However, success of *in vitro* mutagenesis programme will depend on evaluation of mutant clones under field conditions to confirm their performance for the selected trait of Interest.

The somaclones were detected at molecular level. Dalvi *et al.* (2012) has screened out the promising sugarcane somaclones for agronomic traits and smut resistance using PCR amplification of Inter Transcribed Region (ITS) of *Sporisorium scitaminae*. Pawar (2011) and Praveen (2019) detected mutation occurring in the plants obtained through *in vitro*. In the present study 6 primers of RAPD were used for PCR amplification out of which 3 primers amplified noticeably. The products of PCR were analysed for polymorphism, all the 13 somaclones variated from the mother plant and showed maximum diversity of 31.58%.

The present study reports beneficial traits of the somaclones derived from Co 261 and their performance, especially for smut resistance and yield traits is needed for future work.

SUMMARY, CONCLUSION AND SUGGESTIONS FOR FURTHER WORK

6.1 Summary

Saccharum officinarum is an important cash crop of India and the world. It is one of the oldest crops known to men and originated from New Guinea. It is one of the most important industrial crops in both tropical and subtropical regions of the world and major export product in many developing countries. There are two sugarcane belts in the country with distinct agro-climatic conditions. The tropical belt comprises of Maharashtra, Tamil Nadu, Karnataka, Andhra Pradesh, Gujarat and Kerala. The subtropical belt, where a large part of cane area lies, includes Uttar Pradesh, Punjab, Haryana, Bihar and other north and north-eastern states. It is a raw material for paper, alcohol, plywood, industrial enzymes and animal feed. Sugarcane is mainly grown for sugar production in over 127 countries. With the ever-increasing population of the world the production of sugarcane needs to be boosted every year to meet the of world sugar. The yield of every commercial crop depends on various environmental factors and availability of superior varieties. Several improved varieties of sugarcane have been developed for juice quality, sugar content, but still Sugarcane is affected with several diseases which deteriorate the quality of canes and decrease production. Smut of sugarcane is one of the major diseases which causes severe loss in production. The causal organism of smut is *Sporosirum scitamineum*. Developing resistant variety is a convincing approach to sustain the production of sugarcane. *In vitro* mutagenesis is a useful tool of biotechnology to create genetic variation among the plants and can be exploited to develop superior cultivars.

The present investigation entitled "Study of *in vitro* development of mutant plants of *Saccharum officinarum* for resistance against smut" was carried out to optimize callus induction and induction of mutation *viain vitro* mutagenesis using a popular sugarcane cultivar Co 261. The experiment was carried out at Biotechnology Centre, Jawaharlal Nehru Krishi Vishwa Vidyalaya during 2019-2020. A popular variety Co 261 of Narsinghpur and

Hoshangabad district was collected from KVK Narsinghpur, Madhya Pradesh and cultivated in polyhouse of Biotechnology Centre to obtain the explants. The young leaf roll was considered as the explant. Surface sterilization of the explant was done by washing the explant in running tap water, then the explant was treated with tween-20 (5-6 drops in 100 ml sterile distilled water). After that the explant was dipped in 0.5% carbendazim for 30 minutes followed by rinsing with sterile distilled water. Lastly the explant was treated with 0.1-0.2% $HgCl_2$ for 3 minutes. The sterilized explants were inoculated to callus induction medium containing 3 mg/l 2,4-D. In some culture plates exudation of phenolics was observed which was overcome by early subculturing (7 days). After 60 days of inoculation the calluses were transferred to MS medium supplemented with similar amount of 2,4-D and 8µM EMS to induce mutagenesis. After that the calluses were transferred to shoot regeneration medium containing 1.5 mg/l BAP and 0.5 mg/l Kinetin. The multiple shoots obtained from the shooting medium were transferred to rooting medium in test tubes containing 0.25 mg/l IBA. As the root development observed the plantlets were transferred to plastic cups for hardening. 1:1:1 ratio of cocopeat, vermicompost and sand was used for transplanting the plantlets. Total genomic DNA was isolated from the mutant plantlets and PCR amplification of the DNA was carried out using RAPD primers. Scoring of the band was done to check polymorphism. OPA-06 showed maximum polymorphism (80%) followed by OPA-04 and OPA-05 both showing 42.85% polymorphism.

6.2 Conclusion

The investigation was set on the objectives, to standardise callus induction from different explants, to develop smut resistant mutants through *in vitro* culture and to validate the cultured mutant plantlets using molecular markers.

The investigation found that efficient surface sterilization was achieved by using tween-20 for primarily washing the explant followed by disinfecting with carbendazim 0.5% and $HgCl_2$ 0.1%. The most suitable explant for callus induction was young and tender leaf roll. It was recorded that callus induction from the leaf roll was highest in 3 mg/l 2,4-D. The mutagenic chemical EMS induced variation among the cultured plantlets. Three amplified primers of RAPD showed polymorphism. All the 13 somaclones showed variation with mother plants and all were found mutants

6.3 Suggestions for further works

1. Epigenetic and cytogenetical studies can be conducted for identifying the related traits of phenotype.
2. Stability of the mutants can be tested after subsequent generation of the plants in the field.
3. In field trial of lines to assess the resistance of the mutants against smut.
4. Specific markers can be used to validate the resistance against smut.

REFERENCES

Ali A, Shagufta N and Iqbal J. 2001. Rapid clonal multiplication of sugarcane (*Saccharum officinarum)* through callogenesis and organogenesis. Pakistan Journal of Botany 40(1): 123-138.

Arnon DI, and Hoagland DR. 1940. Crop production in artificial culture solutions and in soils with special reference to factors influencing yields and absorption of inorganic nutrients. Soil Science50: 463-485.

Baksha R, Alam R, Karim MZ, Paul SK, Hossain MA, Miah MAS and Rahman ABMM. 2002. *In vitro* shoot tip culture of sugarcane *(Saccharum officinarum*) Variety ISD 28. Biotechnology. 1(2-4): 67-72.

Begum MK, Islam MO, Miah MAS, Hossain MA and Islam N. 2011. Production of somaclone *in vitro* for drought stress tolerant plantlet selection in sugarcane (*Saccharum officinarum* L.). The Agriculturists 9(1&2): 18-28.

Behera KK and Sahoo S. 2009. Rapid *in vitro* micro propagation of sugarcane (*Saccharum officinarum* L. cv-nayana) through callus culture. Nature and Science 7(4): 1-10.

Bisht SS, Bisht AS and Chauhan RS. 2017. *In vitro* mutagenesis induction to improve abiotic stress in tissue cultured plantlet of *Picrohiza kurroa* Royle ex. Benth: An endangered plant of western Himalayas, India. Medicinal and Aromatic Plants (Los Angeles) 6: 2.

DAC, FW. 2020. Annual Report (2019-2020). Department of Agriculture, Cooperation and Farmers Welfare, New Delhi. p8.

Dalvi SG, Vasekar VC, Yadav A, Tawar PN, Dixit GB, Prasad DT and Deshmukh RB. 2012. Screening of the promising sugarcane somaclones for agronomic traits, and smut resistance using PCR amplification of Inter Transcribed Region (ITS) of *Sporisorium scitaminae*. Sugar Tech. 14(1): 68–75.

Dobariya KL. 1994. Investigations on *in vitro* morphogenesis and somaclonal variation in sugarcane (*Saccharum spp*. hybrid). Ph. D. Thesis, University of Agricultural Sciences, Dharwad. 219p.

Evans DA and Sharp WR. 1986. Application of somaclonal variation. Biotechnology 4: 528-532.

Gosal SS, Thind KS and Dhaliwal HS. 1998. Micropropagation of sugarcane – An efficient protocol for commercial plant production. Crop Improvement. 25: 1-5.

Guevera P, Oropeza M, Eva DG and Luis J. 1995. Identification of somaclonal variants of sugarcane (*Saccharum spp.*) resistant to sugarcane mosaic virus via RAPD markers. Plant Molecular Biology Reporter 13(2): 182-191.

Gupta C. 2019. Studies on induction of somaclonal variation in sugarcane (*Saccharum officinarum)* and validation of mutant using molecular markers. International Journal of Agriculture, Environment and Biotechnology 13(1): 105-110.

Haberlandt G. 1902. Kulturversuche mite isolienten. Pflanzhzelien S.B. Sber. Ahad. Wiss. Wien. 111: 69-92.

Hegde G. 1998. Micropropagation in sugarcane (*Saccharum spp.* Hybrid). M.Sc. Thesis, UAS, Dharwad. 120p.

Hein DJ, Krishnamurthy M, Nickel LG and Maretzki A. 1977. Cell, tissue and organ culture in sugarcane improvement. In Applied and Fundamental Aspects of Plant Cell Tissue Culture and Organ Culture, Ed. Reinert j. and Bajaj YOS, Springer – Verlag, Berlin, pp 3-17.

Heinz DJ and Mee GWP. 1969. Plant differentiation from callus tissue of *Saccharum species.* Crop Science 9: 346-348.

Heiz DJ and Mee GWP. 1970. Colchicine induced polyploids from cell suspension cultures of sugarcane. Crop Science 10: 696-699.

Khan MR and Rashid H. 2003. Studies on the rapid clonal multiplication of *Saccharum officinarum.* Pakistan Journal of Biological Sciences 6(22): 1876-1879.

Koch AC, Ramgareeb S, Rutherford RS, Snyman SJ and Watt MP. 2012. An *in vitro* mutagenesis protocol for the production of sugarcane tolerant to the herbicide imazapyr. *In vitro* Cellular & Developmental Biology – Plant 48: 417-427.

Kumar A. 1998. *In vitro* mutagenesis and evaluation of somaclones of sugarcane (*Saccharum officinarum* L.). M.Sc. Thesis, UAS, Bangalore. 69p.

Kumar P, Agarwal A, Tiwari AK, Lal M, Jabri MRA. 2012. Possibilities of development of red rot resistance in sugarcane through somaclonal variation. Sugar Tech 14(2): 192–194.

Kumari R and Verma DK. 2001. Development of micro propagation protocol for sugarcane (*Saccharum officinarum* L) - A review. Agric. Rev. 22(2): 87-94.

Lal N and Krishna R. 1994. Sugarcane and its problems: tissue culture for pure and disease free seed production in sugarcane. Indian Sugar 44: 847-848.

Larkin PJ and Scowcrowft WR. 1981. Somaclonal variation – a novel source of variability from cell cultures for plant improvement. Theoretical and Applied Genetics 60: 197-214.

Larkin PJ, Ryan SA and Ellison FW. 1984. Somaclonal variation in some agronomic and quality characters in wheat. Theoretical and Applied Genetics 74: 77-82.

Lee TSG. 1987. Micropropagationn of sugarcane (*Saccharum spp*.). Plant Cell Tissue and Organ Culture 10: 47-55.

Liu MC. 1984. Sugarcane: Handbook of Plant Cell Culture. Macmillan Publishing Company. Inc.NY 2:572- 605.

Lourens AG and Martin FA. 1987. Evaluation of *in vitro* propagated sugarcane hybrids for somaclonal variation. Crop Science 27: 793-796.

Mallikarjuna SJ. 2014. Standardization of production protocol for somaclonal variants in sugarcane. M.Sc. Thesis, Acharya N.G. Ranga Agricultural University, Hyderabad. 132p.

Maluszynski M, Ahloowalia BS and Sigurbjornsson B. 1995. Application of *in vivo* and *in vitro* mutation techniques for crop improvement. Euphytica 85:303-315.

Mamun MA, Sikdar MBH, Paul DK, Rahman MM and Md. Rezuanul Islam MR. 2004. *In vitro* Micropropagation of Some Important Sugarcane Varieties of Bangladesh. Asian Journal of Plant Sciences 3: 666-669.

Morrish F, Vasil V and Vasil IK. 1987. Developmental morphogenesis and genetic manipulation in tissue culture and cell cultures of the Gramineae. Advances in Genetics24: 431-499.

Murashige T and Skoog F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. PhysiologiaPlantarum15: 473-497.

Murashige T. 1974. Plant propagation through tissue culture. Annual Review of Plant Physiology25:(1) 135-166.

Mustafa G and Khan MS. 2015. Differential role of indole butyric acid in sugarcane root development. Sugar Tech 18: 55-60.

Nalavade V, Kale RR, Thorat A and Babu H. 2016. Standardisation of protocol for callus induction, regeneration and genetic transformation of sugarcane (*Saccharum officinarum*L.) genotype Co86032. Progressive Research – An International Journal 11: 4196-4201.

Novak FJ and Brunner H. 1992. Plant breeding: Induced mutation technology for crop improvement. IAEA Bulletin 4: 25-33.

Patel VS, Mehta R, Naik KH, Singh D, Patel DU and Mali SC. 2015. Callus induction and whole plant regeneration in sugarcane (*Saccharum spp.* Complex) variety Co 86032. Green Farming 6(5): 5p.

Pawar RR. 2011. Molecular characterization of sugarcane mutants using SSR markers. M.Sc. Thesis, Panjabrao Deshmukh Krishi Vidhyapeeth, Akola. 58p.

Praveen K, Kumar MH, Reddy KH, Hemalatha TM, Reddy DM, Reddy NE and Latha P. 2019. Regeneration and evaluation of somaclonal variants for tolerance to yellow leaf disease (YLD) in sugarcane. Journal of Biosciences 44(2):29.

Rastogi J, Siddhant, Bubber P and Sharma BL. 2015. Somaclonal Variation: A new dimension for sugarcane improvement. GERF Bulletin of Biosciences 6(1): 5-10.

Saghai-Maroof MA, Soliman KM, Jorgensen RA and Allard RW. 1984. Ribosomal DNA sepacer-length polymorphism in barley: mendelian inheritace, chromosomal location and population dynamics. Proceedings of the National Academy of Sciences USA 81: 8014-8018.

Shetty A and Rao S. 2015. Callus Induction and Organogenesis in Sugarcane (*Saccharum officinarum* L.) var 93v297. International Letters of Natural Sciences 48: 14-22**.**

Shrivastava D, Gayatri MC and Sarangi SK. 2018. *In vitro* mutagenesis and characterization of mutants through morphological and genetic analysis in orchid *Aerides crispa* Lindl. Indian Journal of Experimental Biology 56: 385-394.

Shukla R, Khan AQ and Garg GK. 1994. *In vitro* clonal propagation of sugarcane: Optimization of media and hardening of plants. Indian Sugar 113-116.

Sigh SK and Singh SB. 1993. Effect of gamma rays on callus growth and plant regeneration in sugarcane CV Co 687. Indian Sugar 43(3): 181-182.

Singh B, Yadav GC and Lal M. 2001. An efficient protocol for micropropagation of sugarcane using shoot tip explants. Sugar Tech 3: 113-116.

Singh R. 2003. Tissue culture studies of sugarcane. M.Sc. Thapar institute of engineering and technology, Patiala. pp 41-56.

Skoog F and Miller CO. 1957. Chemical regulation of growth and organ formations in plant tissue cultured *in vitro.* Symposia of the Society for Experimental Biology11: 118-131.

Smiullah A, Khan FA, Afzal A, Javed MA, Iqbal Z, Iftikhar R and Wattoo JI. 2012. *In vitro* regeneration, detection of somaclonal variation and screening for mosaic virus in sugarcane (*Saccharum spp.*) somaclones. South African Journal of Biotechnology 11(48): 10841-10850.

Tesfa M and Ftwi M. 2018. *In vitro* plant regeneration of sugarcane (*Saccharum officinarum*) variety inoculated under different levels of plant growth regulators. Journal of Plant Biochemistry and Physiology 6: 4.

Wagih ME, Ala A, and Musa Y. 2004. Regeneration and evaluation of sugarcane somaclonal variants for drought tolerance. Sugar Tech 6(1&2): 35-40.

Yadav RL and Solomon S. 2006. Potential of developing sugarcane by-product based industries in India. Sugar Tech 8(2): 104-111.

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