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INTROGRESSION OF SUGARCANE GENOTYPES × PHYTOHORMONAL CONCENTRATIONS TO SPEED UP *IN VITRO* SOMATIC EMBRYOGENESIS AND REGENERATION FOR DEVELOPMENT OF SUGARCANE MOSAIC VIRUS (SCMV) FREE SAPLINGS

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Abstract

Sugarcane is a valuable cash crop in Pakistan. It has both industrial and economic values as it is an important source of employment and income. Various biotic and abiotic factors cause reductions in sugarcane yield worldwide. Among the diseases, sugarcane mosaic virus (SCMV) is one of the most important diseases contributing to such yield losses. Currently, many techniques are used to develop disease-free sugarcane plantlets to enhance yield production, but *In vitro* propagation has proven to be the best method due to its vegetative propagation. Here, we developed SCMV-free *Saccharium officinarium* plantlets through callogenesis and regeneration. Four sugarcane genotypes were selected on the basis of four morphological parameters (cane yield, cane height, cane weight and brix percentage) with moderate SCMV severity. The experiment was designed as a two-factor factorial using a completely randomized design. Young leaves of six-month-old sugarcane genotypes were used as explants and cultured on MS medium using four different concentrations of 2,4-Dichlorophenoxyacetic acid as treatments to create somatic variation against SCMV. The best quality callogenesis was obtained at 3mg/L of 2,4-D (T_2) in all genotypes studied and genotypes SC11 and SC14 performed better than others for days-to-callus formation (22 days), callus formation percentage (72% and 73%) and callus weight (87g and 90g). Regarding shoot formation, all genotypes responded better at 1mg/L kinetin. Means comparison of genotype*treatment interaction revealed that SC1 and SC14 demonstrated the best shoot characteristics. ELISA detection indicated that SCMV-free plantlets were obtained in all sugarcane genotypes. Therefore, 3mg/L of 2,4-D is the best concentration for somatic variation in sugarcane genotypes, while 1mg/L of kinetin is the best for shoot initiation with the ultimate objective to develop mosaic virus-free plantlets to enhance sugarcane production.

Key words: *In vitro,* Propagation, Embryogenesis, Regeneration, 2,4-D, Somaclones.

Introduction

Agriculture plays a vital role in the economy of Pakistan. It contributes 18.5% to the gross domestic product (GDP) and 38.5% of employment. In Pakistan, sugarcane production accounts for 2.9% in agriculture's value and 0.5% contributes to overall GDP. Area under sugarcane cultivation is 1165 thousand hectares with a production of 81.009 million tons (Economic Survey of Pakistan, 2021). Sugarcane is the second most important cash crop of Pakistan, which is cultivated in tropical and subtropical regions widely. Worldwide, Pakistan ranks at 6th position in sugarcane production and 8th number in its consumption (FAO, 2018). In Pakistan, 25kg/capita/annum sugar is consumed (FAO, 2019). Due to its high economic value, it is the 10th most important cultivated crop around the world and grown in over 90 countries (Seema *et al*., 2014).

Sugarcane (*Saccharum officinarum* L.) is the main source of raw material for the production of sugar and its share is about 90% as source of sugar from all other crops. It is the major source of dietary carbohydrates for humans (Yadav *et al*., 2013) and also provides renewable energy sources such as ethanol and biogas as by-products. Different type of products obtained from different parts of sugarcane, like sugar, alcohol, ethanol, drinks, organic matter, industrial enzymes, biofuel, plywood, medicinal purposes, cane wax, molasses and bagasse and an efficient source of biomass production (Solangi *et al*., 2019 and Iqbal *et al*., 2023). Sugarcane reproduces by both sexual and asexual methods. Sexual type of reproduction is through seed known as fuzz, which is a good source to create genetic variation in sugarcane. In Pakistan, high quality sugarcane fuzz production is very limited due to the absence of suitable climatic conditions, like temperature and humidity, except in few areas such as Darghi, Murree and Sajawal. Sugarcane has a complex and very large genome, which is the major hurdle to improve the existing cultivars through conventional breeding method (Gadakh *et al*., 2015). Every year, major yield losses in sugarcane are also observed due to biotic and abiotic stresses. In biotic factors, viral, bacterial and fungal diseases are the major cause of yield reduction (Akbar *et al*., 2017). Moreover, as an asexually propagated crop, sugarcane is easily affected with any disease, then transferred to next generation through setts as seed, which causes a tremendous yield reduction.

Tissue culture technique is an important source to create genetic variation for improvement of asexual propagating crops, such as sugarcane (Eldessoky *et al*., 2011 and Ajadi *et al*., 2018). Through callus culture, genetic variability can be easily developed in plant tissues. Callus is comprised of unorganized cells that undergo cell division to produce genetic variation, commonly known as somaclonal variation. Somaclones developed through callus culture help in the improvement of drought tolerance, disease resistance

and yield contributing traits (Di-Pauli *et al*., 2019 and Iqbal *et al*., 2023). Plantlets obtained through tissue culture, developed genetic modification which appeared by phenotypic variation (Alfian *et al*., 2019). Callus culture provides great opportunity for development of virus free varieties of sugarcane, and introduces somatic variation (Khan *et al*., 2000). 2, 4-D was the best to create genetic variation in sugarcane genotypes, which causes endoreduplication in cells and chromosomal changes (Mohanty *et al*., 2008, Naz *et al*., 2017, Rao *et al*., 2015, Khan *et al*., 2012 and Saleem *et al*., 2022). In the light of above discussion, the present study was conducted to develop genetic variation in sugarcane genotypes against SCMV through *In vitro* somatic embryogenesis. Meanwhile, optimization of plant protocol for callogenesis and regeneration of sugarcane was explored by using different concentrations of phytoharmones in MS media.

Material and Methods

The research work was conducted in the tissue culture laboratory of College of Agriculture, University of Sargodha, Pakistan in 2019.

Experimental material: Four sugarcane genotypes were selected based on their excellent performance for cane height, brix percentage and cane weight among 72 genotypes except one weakness i.e., moderately susceptible to sugarcane mosaic virus (SCMV). Phenotypic scoring of disease incidence and severity percentage was calculated as described by Addy *et al*., (2017). Enzyme linked immunosorbent assay (ELISA) was also done to detect sugarcane mosaic virus (SCMV) in selected sugarcane genotypes. The detail of these sugarcane genotypes is given in (Table 1).

Table 1. List of selected sugarcane genotypes based on morphological parameters and SCMV intensity.

Sr. #	Code	Variety/line name	Cane height (cm)	Brix %	Cane weight (g)	Incidence %	Severity %	ELISA detection
1.	SC1	SPCG24	189.67	20.792	1044	20.5	10.6	+/+
2.	SC3	SPSG27	191.67	20.58	975	56.1	42.2	+/+
3.	SC11	SRR13	142.67	22.917	970	51.4	40.4	+/+
4.	SC14	Thatha1312	177.67	20.833	950	54.4	37.9	+/+

Tissue culture procedure: Leaf whorls of six-months-old sugarcane plants were used as explant for callogenesis and regeneration. Tender leaves of each parental plant of selected genotypes were collected from the field, and outer layers of explants were removed and washed several times with distill water under aseptic conditions. Surface sterilization with 10% Sodium Hypochlorite (v/v) for 10 minutes were done, followed by surface sterilization with 70% ethanol (v/v) for 2-3 minutes. Then three washings were done with autoclave-distilled water in laminar air flow cabinet to avoid contamination. Sterilized leaf whorls were dissected to excise inner most layers and cultured. Necessary glass wares and instruments used in culturing of explant were cleaned with tap water and 70% ethanol, and then sterilized in autoclave at 121^{o}C for 20 minutes. Stock solutions and MS medium containing all nutrients (Murashige & Skoog, 1962) were prepared by following previous (Patel, 2015) for callogenesis and regeneration of sugarcane. The pH of the stock solutions and culture media was adjusted 5.7-5.8. Phytagel of related quantity (1.75-2.0 g/l) was added to solidify the media as gelling agent and 30 g sucrose was used as carbon source. Then media was poured in test tubes and sterilized in autoclave at 121^{0}C for 20 minutes. Inner tender leaves taken from sterilized explants were cut into pieces of 2-3mm and cultured on MS media containing MS basal salts and vitamins (Maruprolu *et al*., 2022).

Treatments for callogenesis: Different concentrations of 2, 4-dichlorophenoxy acetic acid (2,4-D) used for somatic variation in sugarcane were as follows:

T_0 = 2, 4-D @ 0 mgL^{-1} (control)
T_1 = 2, 4-D @ 1 mgL^{-1}
T_2 = 2, 4-D @ 3 mgL^{-1}
T_3 = 2, 4-D @ 5 mgL^{-1}
T_4 = 2, 4-D @ 7 mgL^{-1}

Shoot culturing: Embryogenic Callus was cultured on MS Medium with addition of kinetin @ 0.5 mg/L and 1mg/L, Casiene hydrolysate 480mg, sucrose 30g and 1.75 g phytagel for solidification. Shoots developed from callus were further sub cultured in regeneration medium for multiplication of shoots following the protocol of Rastogi *et al*., (2015). The culture test tubes for shoot formation were kept at 27^{o}C ± 1^{o}C under continuous fluorescent tube light.

Root culturing: The regenerated sugarcane shoots were transplanted to the rooting media. The root formation medium contained MS salt with vitamins 4.43 g, 30 g sucrose, NAA 2mg/L and phytagel 1.75g/l was used as solidifying agent. Root initiation in shoot samples were started within two weeks after shifting to rooting medium. The cultured test tubes for root formation were incubated at 27˚C ± 1˚C under16-8hrs light photoperiod in growth room.

Hardening of *In vitro* sugarcane plantlets: Sugarcane plantlets with well-developed roots were taken out from the root culture medium, washed out with doubled distilled water to remove the solution adhered on the roots. Polythene cups were filled up with available peat moss and well-watered before planting of plantlets in the growth room at the same growth conditions as described for regenerated plantlets. After 15 to 20 days of primary hardening, these plantlets were kept under shade for three 3-4 weeks for secondary hardening before shifting in the field.

Experiment layout and data collection and analysis: *In vitro* experiment for callogenesis was conducted by using completely randomized design (CRD) under two factors factorial with four treatments. Each treatment was repeated three times with ten test tubes in each replication. At proper stages, data were collected for days to callus induction, percentage of callus formation, weight of callus (g),

number of days to shoot formation, number of shoots per callus, length of shoots (cm), days to root formation, number of roots per shoot and root length (cm). Statistical analysis of data was carried out by using the R statistical environment (R Development Core Team, 2012).

Results and Discussion

Callogenesis: Days to callus formation is an important parameter of *invitro* calus formation. Analysis of variance revealed that days to callus induction showed highly significant (p<0.01) differences among sugarcane genotypes, 2,4-D tretaments for callus formation and their interaction (varieties*treatment) (Table 2). All sugarcane genotypes showed significant genetic variability for days to callus formation at different levels of 2,4-D due to diverse genetic potenial (Naz *et al*., 2008 and Jamil *et al*., 2017). The means comparison exhibited highly significant (p<0.01) differences among sugarcane genotypes for days taken to callogenesis at different levels of 2,4-D (Naz *et al*., 2008 and Jamil *et al*., 2017). All genotypes displayed significant varaition for callus formation at T_1 (1mg/L), T_2 (3mg/L), T_3 (5mg/L) and T_4 (7mg/L) levels while no callus formation was observed at T_0 (0mg/L as control) in any sugarcane genotype after 14 to 21 days of explant culturing (Fig. 1). At 1mg/l, SC1 and SC14 took 24.2 and 24 days while SC1, SC3 and SC-11 took 22.9, 22.7 and 22 days for callus formation at 3mg/L of 2,4-D, respectively. In case of 5mg/L (T_3), SC1 took 18 days for callus formation, SC14 took 19.5 days, SC11 acquired 20.5 day and SC3 took 22.1 days for callus induction (Fig. 1). At higher concentration of 2,4-D (7mg/L), callus formation acquired less days in all genotypes but inferior quality. Thus, although all genotypes took short periods of time for callus induction at 5mg/L and 7mg/L of 2,4-D, callus quality was inferoir than callus developed at 3mg/L which took a little more time for callus formation. Similar data were also observed in previous studies (Rashid *et al*., 2009 and Parmar *et al*., 2017).

Percentage of callus formation was calculated after the formation of callus. Highly significant (p<0.01) differences were observed among sugarcane genotypes, 2,4-D treatments and their varieties × treatment interaction (Table 2). Callus formation in sugarcane genotypes varied from 61.9% to 92.1%. Statistical means comparison of 2,4-D treatments *varieties interaction exhibited significant differences among all the sugarcane genotypes at all treatment levels (Table 3 and Fig. 2). Means comparison of 2,4-D treatment levels revealed that maximum callus formation % in all sugarcane genotypes was attained at T_4 (7mg/L) followed by T_2 (3mg/L), similar with previous findings (Alcantara *et al*., 2014, Ullah *et al*., 2016).

Jahangir *et al*., (2010), Patel *et al*., (2015), Shafique *et al*., (2015), Maruprolu *et al*., (2022) and Iqbal *et al.* (2023) reported that evaluation of morphology of callus is an important parameter as it determines the potential of callus for the process of organogenesis. In this study, callus morphology was observed from fully developed callus of sugarcane genotypes before shifting it to regeneration media. At different concentrations of 2,4-D, callus showed variation in morphology for texture, color, type of callus, callus frequency and rate of callus in sugarcane genotypes except at T_0 = 0 mg/L where no callus formation was observed. Among all the treatments of 2,4-D, only 3mg/L concentration of 2,4-D (T_2) produced the best quality callus with excellent morphogenic features i.e., texture, color, frequency and type of callus (Table 4).

Table 2. Sum of square and mean square values of *In vitro* studied traits in sugarcane genotypes.

Variables	SOV	Sum of square	Mean squares	P-value
Days to callus formation	Variety	43.9	14.63	0.000
	Treatment	16885.5	4221.38	0.000
	Treatment*Variety	203.5	16.96	0.000
Percentage of callus formation	Varieties	3169	1056.4	0.000
	Treatment	1888882	47220.5	0.000
	Varieties*Treatment	3333	277.8	0.000
Callus weight	Varieties	2.0385	0.679	0.000
	Treatment	53.242	13.310	0.000
	Varieties*Treatment	4.4233	0.3686	0.000
Days to shoot initiation	Varieties	156.838	52.279	0.000
	Treatment	108.112	108.112	0.000
	Varieties*Treatment	50.837	16.946	0.000
Number of shoots per callus	Varieties	160.050	53.350	0.000
	Treatment	217.800	217.800	0.000
	Varieties*Treatment	16.567	16.567	0.001
Variance for shoot length	Varieties	10.966	3.655	0.000
	Treatment	173.166	173.166	0.000
	Varieties*Treatment	9.546	3.182	0.000
Variance for shoot length	Varieties	10.966	3.655	0.000
	Treatment	173.166	173.166	0.000
	Varieties*Treatment	9.546	3.182	0.000
Number of days to root initiation	Varieties	19.750	6.35833	0.001
Number of roots per shoot	Varieties	63.400	21.133	0.000
		21.384	7.1280	0.000

Note: Significant (p<0.05) and highly significant (p<0.01)

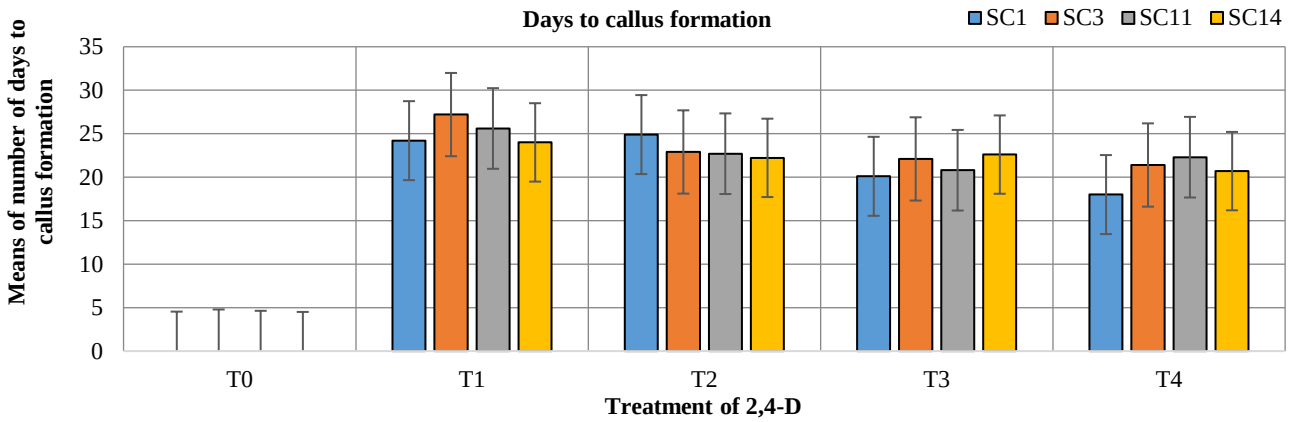

Fig. 1. Days to callus formation at T_0 (0mg/L as Control), T_1 (1mg/L), T_2 (3mg/L), T_3(5mg/L) and T_4 (7mg/L) treatments of 2,4-D in MS media.

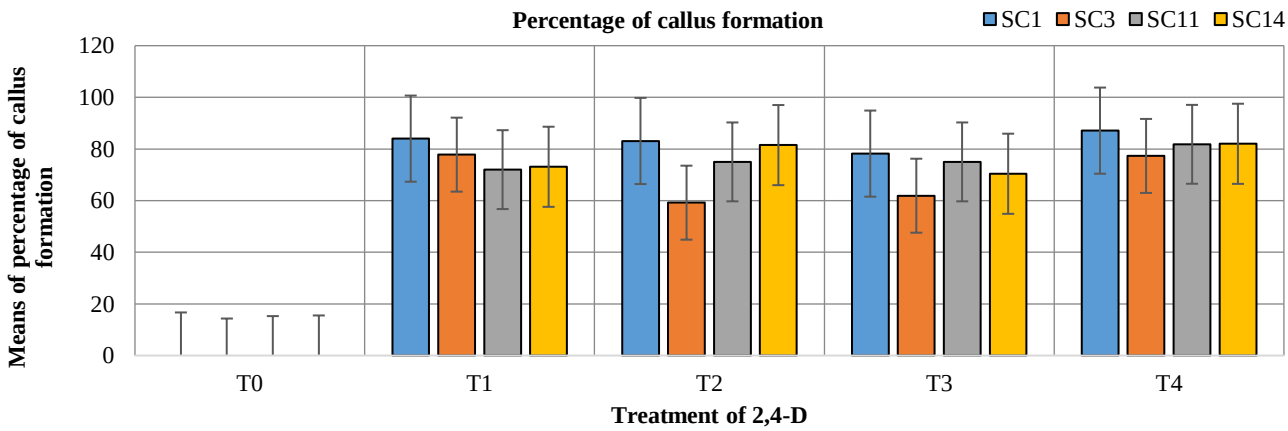

Fig. 2. Percentage of callus formation of sugarcane genotypes at T_0 (0mg/L), T_1(1mg/L) T_2 (3mg/L), T_3 (5mg/L) and T_4 (7mg/L) concentration of 2,4-D in MS media.

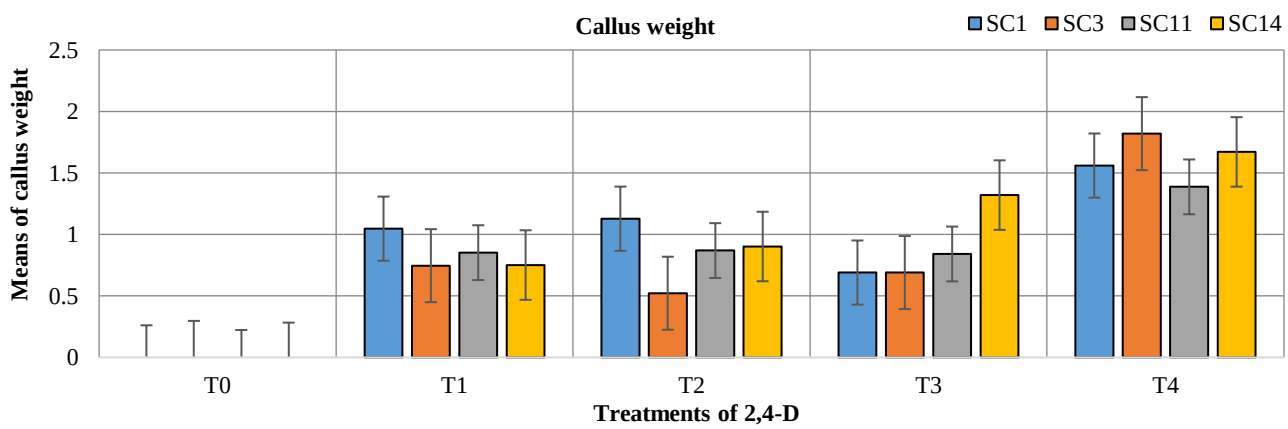

Fig. 3. Callus weight of sugarcane genotypes at T_0 (0mg/L), T_1(1mg/L), T_2 (3mg/L), T_3(5mg/L) and T_4 (7mg/L) concentration of 2,4-D in MS media.

Weight of callus is an important parameter to determine the callus growth for organogenesis (Alcantara *et al*., 2014). After 21 days of callus formation, fresh calli were weighted at different concentrations of 2,4-Dichlorophenoxyacetic acid. Analysis of variance indicated that the response of all sugarcane genotypes for callus weight at different concentrations of 2,4-D was highly significant (p<0.01). Similarly, 2,4-D treatments and Varieties*Treatments interaction also showed highly significant differences for callus weight (Table 2). Means comparison of 2,4-D treatments for callus weight indicated that sugarcane genotypes gained different calli weight, from 0.42g to 1.82g. This revealed that callus weight was greatly influenced by both genotypes and treatments interaction. The maximum average calli weight were observed at 3mg/L of 2,4-D in SC1 (0.94g), SC14 (0.92g), SC11 (0.869) and SC3 (0.424g), revealing that 3mg/L of 2,4-D was performed better than others to gain average callus weight (Fig. 3). Our results confirmed by previous findings (Rao *et al*., 2015, Solangi *et al*., 2016).

Table 3. Means comparison of sugarcane genotypes and 2,4-D concentrations for callogenesis.

Plant traits	Genotype	Treatments of 2,4-D				
		T_0(control)	T_1(1mg/L)	T_2(3mg/L)	T_3(5mg/L)	T_4(7mg/L)
Days to callus formation	SC1	0.000 J	24.200 C	24.900 BC	20.100 H	18.000 I
	SC3	0.000 J	27.200 A	22.900 DE	22.100 EF	21.40 FG
	SC11	0.000 J	25.600 B	22.700 E	20.800 GH	22.300 EF
	SC14	0.000 J	24.000 CD	22.400 EF	22.600 EF	20.700 GH
Percentage of callus formation	SC1	0.000 I	84.000 AB	83.100 ABC	78.200 CDE	77.300DEF
	SC3	0.000 I	77.800CDE	59.200 H	61.900 H	77.300DEF
	SC11	0.000 I	0.000 I	72.000 FG	77.200DEF	75.000EFG
	SC14	0.000 I	0.000 I	73.100 EFG	81.500BCD	70.400 G
Callus weight	SC1	0.000 J	1.0470 EF	1.1280 DE	1.2820 CD	1.5600 B
	SC3	0.000 J	0.7460 GH	0.5220 I	0.6900 H	1.8200A
	SC11	0.000 J	0.8520 G	0.8690 G	0.8410 GH	1.3870 C
	SC14	0.000 J	0.7510 GH	0.9020 FG	1.3200 C	1.6710 AB

Note: Values sharing the same letters are non-significant

Table 4. Callus morphology at different concentrations of 2,4-D in sugarcane genotypes.

Treatments of 2,4-D	Sugarcane varieties	Number of test tubes	Callus texture	Callus color	Rate of callus	Callus frequency	Type of callus
T_0 (0mg/L)	SC1	20	-	-	-	-	-
	SC3	20	-	-	-	-	-
	SC11	20	-	-	-	-	-
	SC14	20	-	-	-	-	-
T_1 (1mg/L)	SC1	20	Granular compact	Creamy	Good	$^{++}$	Embryogenic
	SC3	20	Granular compact	Creamy	Good	$^{++}$	Embryogenic
	SC11	20	Granular compact	Creamy greenish	Good	$^{++}$	Embryogenic
	SC14	20	Granular compact	Creamy	Good	$^{++}$	Embryogenic
T_2 (3mg/L)	SC1	20	Granular compact	Creamy	Excellent	$^{++++}$	Embryogenic
	SC3	20	Granular compact	Creamy	Excellent	$^{++++}$	Embryogenic
	SC11	20	Granular compact	Creamy	Excellent	$^{++++}$	Embryogenic
	SC14	20	Granular compact	Creamy	Excellent	$^{++++}$	Embryogenic
T_3 (5mg/L)	SC1	20	Compact	Creamy	Very good	$^{+++}$	Embryogenic
	SC3	20	Compact	Creamy greenish	Very good	$^{+++}$	Embryogenic
	SC11	20	Compact	Creamy	Very good	$^{+++}$	Embryogenic
	SC14	20	Compact	Creamy	Very good	$^{+++}$	Embryogenic
T_4 (7mg/L)	SC1	20	Compact	Creamy	Good	$^{++}$	Embryogenic
	SC3	20	Compact	Creamy	Good	$^{++}$	Embryogenic
	SC11	20	Compact	Creamy greenish	Good	$^{++}$	Embryogenic
	SC14	20	Compact	Creamy greenish	Good	$^{++}$	Embryogenic

Callus frequency: $^{+}$ = Poor, $^{++}$ = Good, $^{+++}$ = Very good, $^{++++}$ = Excellent

Regeneration

Shoot culturing: Good quality calli of sugarcane genotypes were shifted to shoot regeneration media. Two treatments of Kinetin having T_1 (0.5mg/L) and T_2 (1mg/L) concentrations in MS media. Analysis of variance for number of days to shoot formation revealed that highly significant differences were present among sugarcane genotypes, Kinetin treatments and genotype*treatment interaction (Table 2). This indicated that enough variation was present in genotypes for days to shoot initiation. Our results similar with Smiullah *et al*., (2013). Means comparison among all sugarcane genotypes showed significant differences for number of days to shoot induction at different treatment of kinetin (Table 5). It was observed that SC1 and SC14 performed better that other genotype and took least number of days for shoot formation (Fig. 4). This indicated that ability of shoot induction also depended on the genetic makeup of genotypes (Jamil *et al*., (2017). Furthermore, statistical mean comparison of kinetin treatments indicated that both treatments efficiently effect the ability of shoot induction. It was observed that T_2 (1mg/L concentration of kinetin) gave better results than T_1 (0.5mg/L concentration of kinetin) for shoot induction as cited in the literature that increase in concentration of kinetin up to1mg/L in MS media gave good results. Average means of all genotypes was observed at both treatments. At T_1 minimum days were taken by SC1 (41.5) followed by SC3 (45.6) and SC14 (44.6) while maximum days were reported for SC11 (46.7). At T_2, SC14 took least number of days (40.6) followed by SC1 and SC3 which took 41.8 and 42.9 days, respectively. Whereas, maximum average number of days for shoot formation was calculated in SC11. Overall, our results get support from the findings of Raza *et al*., (2010), Khan *et al*., (2012), Khan *et al*., (2013) and Shafique *et al*., (2015). However, results are contrary with the findings of as they obtained non-significant results for shoot induction.

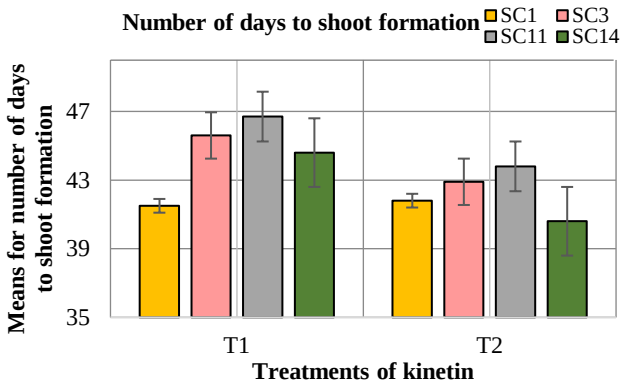

Fig. 4. Number of days to shoot formation of sugarcane genotypes at T_1(0.5mg/L) and T_2 (1mg/L) concentration of kinetin in MS media.

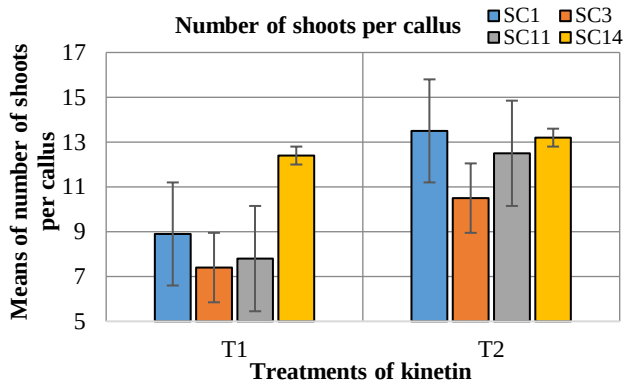

Fig. 5. Number of days to shoot initiation of sugarcane genotypes at T_1 (0.5mg/L) and T_2 (1mg/L) concentration of kinetin in MS media.

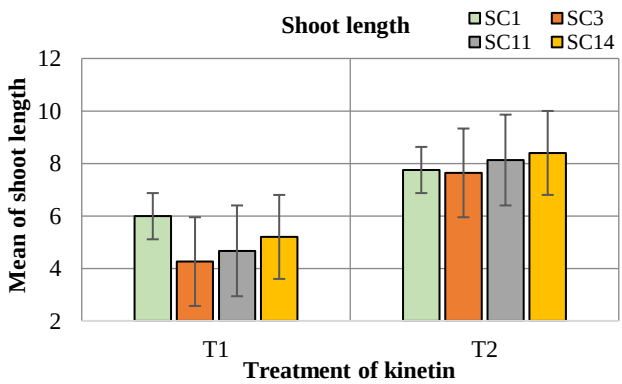

Fig. 6. Shoot length of sugarcane genotypes at T_1 (0.5mg/L) and T_2 (1mg/L) concentration of kinetin in MS media.

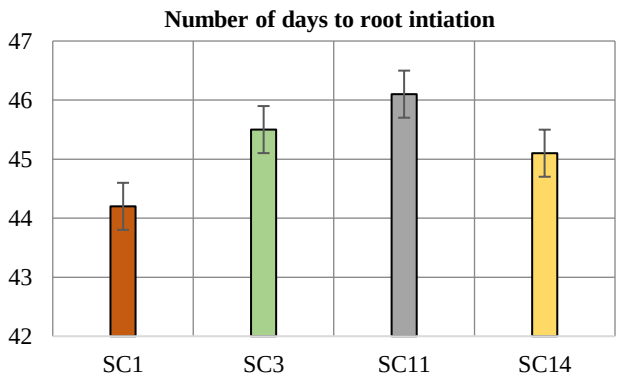

Fig. 7. Comparison of means for number of days to root formation in sugarcane genotypes.

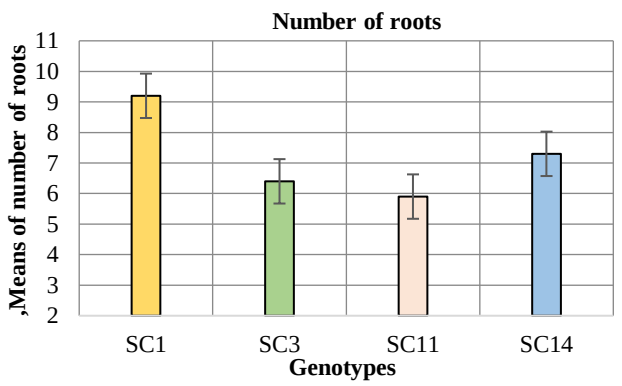

Fig. 8. Comparison of means for number of roots per shoot in sugarcane genotypes.

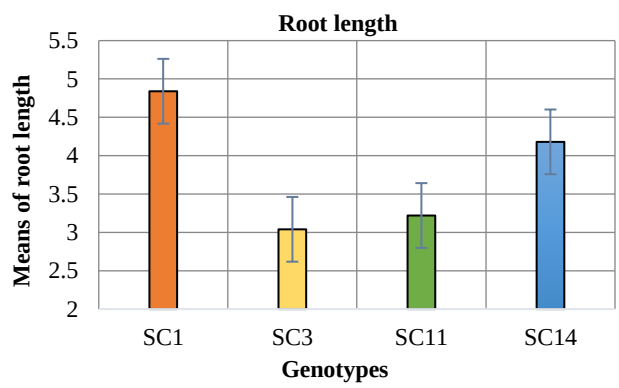

Fig. 9. Comparison of means for root length in sugarcane genotypes.

Number of shoots per callus is an important trait for determining the regeneration ability of callus of all genotypes. Shoots completely induced usually within 3-4 weeks. Analysis of variance showed highly significant results (p<0.01) among all sugarcane genotypes for number of shoots per callus at both kinetin treatments (T_1=0.5mg/L & T_2=1mg/L). ANOVA table also indicated significant differences for varieties*treatments interaction (Table 2). Statistical means comparisons of sugarcane genotypes, kinetin treatments and varieties*treatment interaction showed variation in number of shoots per callus which revealed that increase in concentration of kinetin increase the number of shoots per callus (Table 5). Average number of shoots varies from genotype to genotype at both T_1 and T_2 levels. At T_1 maximum number of shoots per callus was observed in SC14 (12.4) followed by SC1 (8.9) and SC11 (7.8) while minimum number of shoots were observed in SC3 average (7.8). At T_2 maximum average number of shoots per callus was observed in SC1 (13.5), SC14 (13.2) and SC11 (12.5) while minimum number of shoots per callus were observed in SC3 10.5 (Fig. 5). Our results supported by Rao *et al*., (2015) and Kona *et al*., (2019) who reported similar types of results in their *invitro* research experiments.

Shoot length of newly developed plantlets of all sugarcane genotypes were measured in laminar flow hood before shifting them into the rooting medium. Highly significant (p<0.01) results were obtained for genotypes, kinetin treatments and genotype*treatment interaction which revealed that enough variation was present for all above said variables as shoot length varied from genotypes to genotypes at different levels of kinetin (Table 2). Performance of genotypes depends on the treatment of kinetin. Results of mean comparison for shoot length also depicted a significant positive relationship between genotype and treatments. Sugarcane genotypes SC1, SC11 and SC14 showed maximum average shoot length at T_2=1mg/L kinetin (Fig. 6). Our results are in accordance as stated by Thirunavukkarasu *et al*., (2015), Rao *et al*., (2015) and in contrary with the findings of Mulugeta *et al*., (2017).

Root culturing: Regarding process of *invitro* regeneration, root initiation is an essential process. Regarding sugarcane plantlets, roots usually take 2-3 weeks to develop completely when shifted onto rooting media. In this experiment 1-Naphthaleneacetic acid (NAA) @ 1mg/L was used in MS media for root formation and observed variation in sugarcane genotypes for days to root formation. Completely developed shoots were shifted to rooting media then number of days was counted at complete development of roots. Analysis of variance for number of days to root formation indicated that sugarcane genotypes exhibited highly significant (p<0.01) differences for root formation days (Table 2). Statistical means comparison (Table 6 & Fig. 7) for number of days to root formation exhibited that all sugarcane genotypes showed variation for this trait. Minimum days took by SC1 average (44.2) followed by SC14 (45.1), SC3 (45.5) while maximum days were taken by SC11 (46.1). Our results are in accordance with the findings of Patel *et al*., (2015) and Tesfa *et al*., (2018) who reported similar types of results for number of days to root formation in different genotypes of sugarcane.

Number of roots per shoot were counted after development of roots. Analysis of variance for roots per shoot exhibited that highly significant differences (p<0.01) were present among sugarcane genotypes for this character (Table 2). Means comparison of sugarcane genotypes showed different range of number of roots per shoot. All genotypes showed good performance in development of maximum number of roots. Maximum average number of roots were observed in SC1 (9.2), SC14 (7.3) and in SC3 (6.4). Whereas minimum number of roots were observed in SC11 (5.9) (Fig. 8). Our results for number of roots per shoot are in agreement with the findings of Biradar *et al*., (2010), Ajadi *et al*., (2018), Hapsoro *et al*., (2018) and Tesfa *et al*., (2018).

Analysis of variance of root length revealed that sugarcane genotypes showed highly significant (p<0.01) differences among themselves and produced different range of root length Table 2). Root length varied from genotypes to genotypes with a range of 2-5.2 cm. Means comparison for root length determined that every genotype showed variation for this trait as presented in Maximum average root length was observed in SC1 (4.84cm) followed by SC14 (4.18cm) and SC11 (3.22cm) while minimum average root length was found in SC3 i.e., 3.04 cm (Fig. 9). All genotypes showed maximum performance for development of root at 1mg/L of NAA. Our results similar with Rashid *et al*., (2009), Tolera *et al*., (2016) and Awan *et al*., (2019). Different stages of *In vitro* callogenesis, regeneration and hardening of studied sugarcane genotypes are presented in Fig. 10.

Enzyme linked immunosorbent assay (ELISA) for detection of sugarcane mosaic virus (SCMV): Sugarcane mosaic virus (SCMV) a member of potyvirus group is one of the most potentially dangerous of sugarcane and is widely distributed in sugarcane growing countries. Several strains of SCMV were reported in different sugarcane producing countries. In Indo-Pak sub-continent strains of A, B, C, D, E, F, H and N were reported to occur (Vswanathan, 1997). Precise detection of SCMV is essential as this virus is of quarantine importance in Pakistan and other countries. So, ELISA technique was used to detect SCMV *In vitro* developed sugarcane plantlets. Leaf samples of plantlets were collected and ELISA test was performed to detect virus. SCMV known positive control and negative control were used to detect virus in samples. During assessing the SCMV infection in different cultured sugarcane plantlets by ELISA, the means values of known positive and negative controls were used to compare the values of sugarcane plant samples to detect SCMV (Table 7). Thus, based on ELISA analysis SCMV free plantlets were detected from all studied genotypes and shifted in isolated field for phenotypic evaluation and development of SCMV free nursery.

Table 5. Means comparison of sugarcane genotypes and Kinetin concentrations for shoot formation.

Plant traits	Genotypes	Treatments of Kinetin	
		T_1(0.5mg/L)	T_2(1mg/L)
Days to shoot induction	SC1	41.500 FG	41.800 EF
	SC3	45.600 AB	43.800 CD
	SC11	46.700 A	43.800 CD
	SC14	44.600BC	40.600 G
Number of shoots per callus	SC1	8.900 C	13.500 A
	SC3	7.400 C	10.500 B
	SC11	7.800 C	12.500 A
	SC14	12.400 A	13.200 A
Shoot length	SC1	5.9900 D	7.7500 BC
	SC3	4.2600 F	7.6400 C
	SC11	4.6700 F	8.1300 AB
	SC14	5.2300 E	5.2300 E

Note: Values sharing the same letters are non-significant

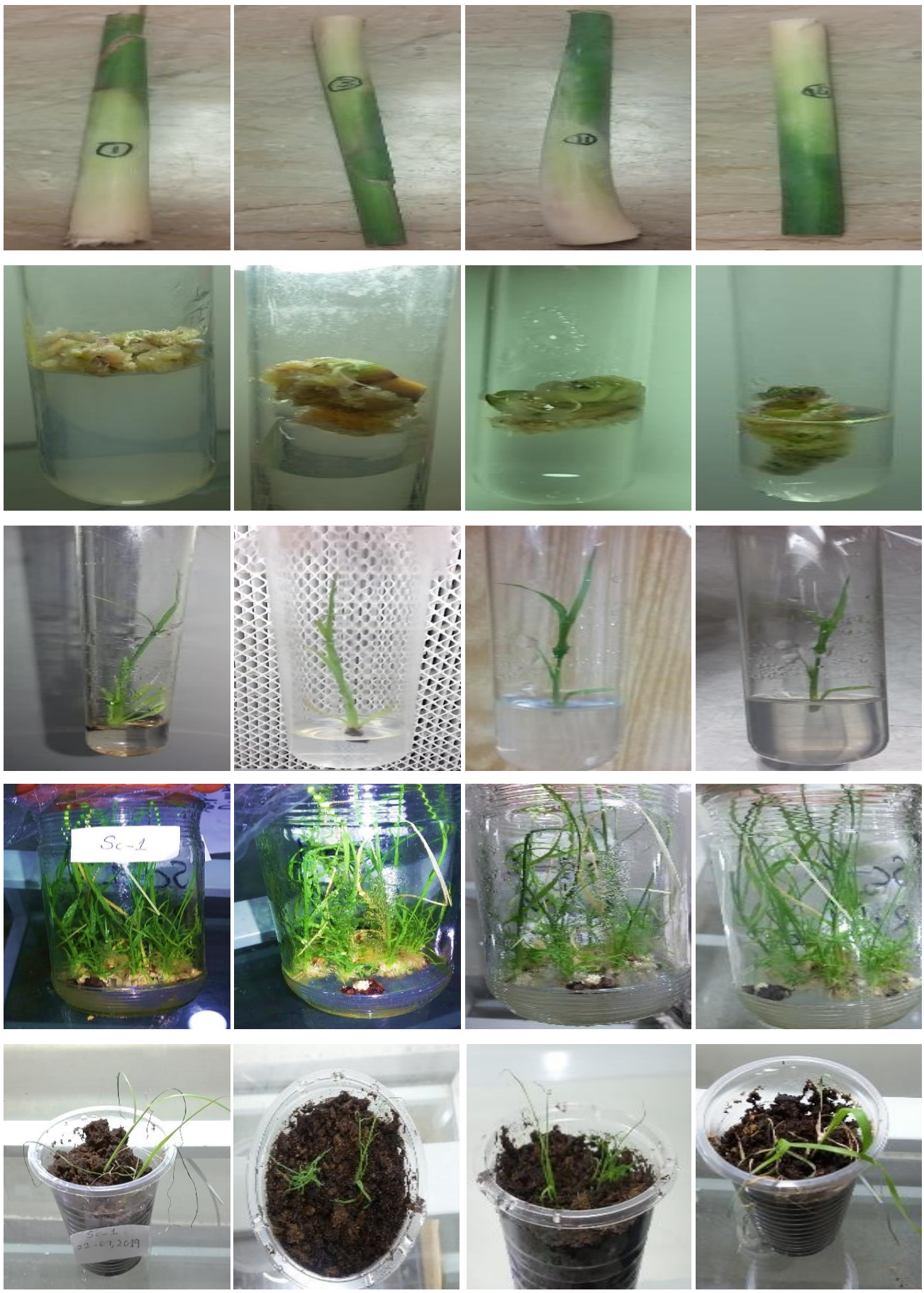

Fig. 10. *In vitro* callogenesis, regeneration and hardening of sugarcane genotypes.

Table 6. Means comparison of sugarcane genotypes for root traits.

Sugarcane genotypes	1-Naphthaleneacetic acid (NAA) 1mg/L		
	Days to root induction	Number of roots per shoot	Root length
SC1	44.2 C	9.2A	4.84 A
SC3	45.1 AB	6.4C	3.04 C
SC11	46.1 A	5.9C	3.22 C
SC14	45.1 BC	7.3B	4.18 B

Note: Values sharing the same letters are non-significant

Table 7. ELISA detection of sugarcane plantlets against sugarcane mosaic virus (SCMV).

In vitro Sapling	SC1	SC3	SC11	SC14
	*0.533 **1.179	*0.533 **1.179	*0.533 **1.179	*0.533 **1.179
1	0.512	0.47	0.771	0.389
2	0.659	0.894	0.341	0.436
3	0.713	0.434	0.931	0.906
4	0.56	0.658	0.453	0.522
5	0.61	0.991	0.519	0.610
6	0.694	0.449	0.939	0.431
7	0.342	0.530	0.449	1.201
8	0.469	0.413	0.499	0.294
9	0.723	0.989	0.501	0.516
10	0.871	0.902	0.299	0.457
11	0.257	0.411	0.769	0.519
12	0.522	0.618	0.290	0.545
13	0.971	0.774	0.512	0.394
14	0.430	0.544	0.531	0.619
15	0.542	0.317	0.957	0.427
16	0.296	0.510	0.458	0.501
17	0.712	0.392	0.317	0.493
18	0.498	0.761	0.828	0.411
19	0.259	0.872	1.109	0.307
20	0.389	0.703	0.480	0.817

*: Negative control mean values of four observations
**: Positive control mean values of four observations
Note: Negative = Virus free & Positive = Virus present

Conclusion

The experimental results revealed that all sugarcane genotypes performed well for callogenesis and organogenesis but SC1 and SC14 showed their maximum performance at different concentration of cultural media. Regarding somatic variation in sugarcane genotypes, the best quality callus was induced at 3mg/L concentration of 2,4-D in MS medium to develop SCMV free plantlets, Whereas the best shoot/root development from callus were obtained at 1mg/L each of kinetin and NAA, respectively. Furthermore, an optimized protocol for *In vitro* callogenesis and regeneration of sugarcane were developed which would be helpful for micro-propagation of sugarcane to develop SCMV free plants in the future.

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