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Genetic variability analysis in sugarcane (*Saccharum* spp. complex) through *in vitro* chemical mutagenesis on callus culture

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Abstract
The present investigation on was carried out in south Gujarat heavy rainfall region of India. Callus derived from sugarcane variety CoN – 13073 subjected for mutagenic treatment varying in different concentration as well as time period. Callus culture of 25 - 30 days old white globular form is considered a suitable material to undergo mutagenic treatments. Among the mutagenic treated calli, maximum survival per cent was observed in EMS 0.2% + 60 min treatment. Maximum regeneration per cent (78.2%) was found in explants treated with treatment MMS 0.3% + 30 min. In case of the number of days for shoot formation, callus treated with EMS 0.2% + 60 min registered a maximum of 15.20 days. The maximum numbers of multiple shoots were observed in MMS 0.2% + 60 min (30.4). Time factor found to be highly significant with a wide range of mean performance. The effect of the mutagenic agent was found to be succeeding to create variability at a lower concentration for lower time intervals for most of the characters under study. In the present study EMS, 0.2% in addition to 0.3%, treatments were most effective. And the effectiveness decrease with the increase in the time interval.

Keywords: Genetic, variability, sugarcane, chemical, culture

Introduction
Sugarcane (*Saccharum* spp. complex, 2n = 40 to 128) belongs to the family Poaceae. Tropical sugarcane originated from Oceania (Papua New Guinea islands) and Indian diploid cane (*Saccharum spontaneum* L.) originated from North Eastern India. It is the main sugar producing crop that contributes more than 77 per cent to the total sugar pool at the global level. Globally, it occupies a little about 2 per cent of the total cropped area. In World area 20 million ha, production 1333.2 million tones and productivity 65.20 t/ha (Anon, 2017) [1].

Although conventional breeding has contributed to the development of agronomically improved cultivars, limitations such as narrow gene pool, complex genome, poor fertility and the long breeding and selection cycle make it difficult to undertake further improvement. In addition, to sustain sugarcane production and to improve productivity, tolerance to biotic and abiotic stresses, nutrient management and improved sugar recovery are some of the concerns.

Mutation induction has become an established tool in crop improvement to supplement the existing germplasm and to improve cultivars in certain specific traits. The main advantages of mutation induction in vegetatively propagated plants are the ability to change one or a few characters of outstanding cultivar without altering the remaining genetic constitution. By taking the advantages of the capacity of callus cell that undergo genetic changes in culture; many agronomically desirable callus derived plants have been obtained (Amin *et al*., 2013) [2]. This is referred to as somaclonal variation and has been of great interest in obtaining useful agronomic clones (Jain, 2000, Rahimi *et al*., 2013) [10, 25]. With the advent of *in vitro* technique, interest in the use of *in vitro* plant material for mutation has begun. Mutation induction can be empowered by *in vitro* technique. Many examples of different vegetatively propagated species show that the combination of *in vitro* and mutagenesis is relatively inexpensive, simple and efficient (Rahimi *et al*., 2013) [25]. The present study was, therefore, undertaken.

The chances of mutation depend on the number, age and growth stages of plants which are used as explant material (explants). Use of callus as explants material for mutation induction is very effective because the callus is population of unorganized mass of cells that have not under gone differentiation and divide continuously (Jain, 2000 and Mattjik, 2005) [10, 16]. Callus was very sensitive to mutagens because the cells are actively dividing so that the chance of mutation was very large (Patade and Suprasanna 2008) [20].

Variation and this variation can be improved by chemical mutagen. The use of mutagen often causes cell damage and thus affects the ability of putative mutant cell regeneration. The ability of each cell to regenerate shoots depends on level of sensitivity of each cell and dosage compensation.

2. Materials and Methodologies

This investigation was carried out at the sugarcane tissue culture laboratory, Main Sugarcane Research Station, Navsari Agricultural University, Navsari, Gujarat, during the year 2017-2018.

2.1 Culture media

In order to study the morphogenic response of sugarcane explants, the most widely accepted MS medium (Murashige and Skoog) [17] was used as a basal medium. The composition of MS medium is presented in Table 1. This medium represents only the basal salts (macro and micro) and vitamins as reported in the original publications. This was supplemented with sucrose, cytokinins, auxins, gibberellins and 49 complex mixtures at various concentrations as per the details are given in each experiment.

Table 1: The compositions of Murashige and Skoog (1962) medium Constituents amounts (mg/l)

Sr. No.	Stock solution	Constituent salt		Quantity/litre medium (stock)	Needs in one litter of media
1	Stock-A	Sodium Nitrate ($NaNO_3$)		82.00 g	20 ml
2	Stock-B	Potassium Nitrate (KNO_3)		95.00 g	20 ml
3	Stock-C	Calcium chloride dehydrate ($CaCl_2.2H_2O$)		88.00 g	05 ml
4	Stock-D	I	Boric acid (H_3BO_3)	1.240 g	05 ml
		II	Potassium dihydrogen phosphate(KH_2PO_4)	34.00 g	
		III	Sodium molybdate ($Na_2MoO_4.2H_2O$)	0.050 g	
		IV	Cobalt chloride ($CoCl_2.6H_2O$)	0.005 g	
		V	Potassium Iodide (KI)	0.166 g	
5	Stock-E	I	Magnesium sulphate ($MgSO_4.7H_2O$)	74.00 g	05 ml
		II	Zinc sulphate ($MgSO_4.7H_2O$)	0.172 g	
		III	Cupric sulphate ($CuSO_4.5H_2O$)	0.005 g	
		IV	Manganese sulphate ($MnSO_4.H_2O$)	4.500 g	
6	Stock-F*	I	Ferrous sulphate ($FeSO_4.7H_2O$)	5.560 g	05 ml
		II	Sodium EDTA ($Na_2EDTA.2H_2O$)	7.460 g	
7	Meso inositol			10.00 g	10 ml
8	Glycine			200.0 mg	02 ml
9	Nicotinic acid			200.0 mg	2.50 ml
10	Pyrodoxine acid			200.0 mg	2.55 ml
11	Thiamine HCl			1.000 g	0.1 ml
12	2,4-Dichlorophenoxy acetic acid			200.0 mg	20ml
13	Sucrose			20.00 mg	20 g
14	Coconut water			10%	100 ml
15	Agar agar			8gm	8 g

Addendum: Sucrose 30 g/l, Myoinositol 100 g/1 and Agar 8 g/l.

*The $FeSO_4.7H_2O$ was dissolved in approximately 40 ml of distilled water and heated. The $Na_2EDTA.2H_2O$ was 50 gm taken and dissolved in approximately 40 ml of distilled water separately and mixed while heating (under continuous stirring) with $FeSO_4.7H_2O$ solution. After cooling, the volume was adjusted to 100 ml. heating and stirring resulted in a more stable $FeNa_2EDTA$ complex.

2.2 Preparation of explants and regeneration of callus from leaf whorl meristem

Disease - free, genetically true-to-type and actively growing cane tops were selected from 5 to 7 months old sugarcane crop. Cane tops with the growing apices were cut approximately 10 cm long and washed thoroughly in running tap water for 30 minutes. Outer sheaths of cane tops were removed by wiping the sheath with rectified spirit. The shoots were then washed with soapy water (2 drops of Labonin into 250 ml of water) for about 5 to 6 minutes in a sterile 1-litre conical flask, followed by cleaning the materials with distilled water. The shoots were rinsed in 5 per cent sodium hypochlorite for 10 minutes. Then shoots were thoroughly rinsed in 70 per cent ethanol for 30 seconds followed by sterilizing double distilled water for 4-5 times till ethanol was completely washed out from the surface of the material. Surface sterilization was performed using 0.1 per cent mercuric chloride solution. Shoots were shaken vigorously for 5 minutes. Then the container was taken to the laminar clean air station. They were rinsed 3 to 4 times with sterile double distilled water to remove all traces of chemicals. The isolation of shoot apex was done by carefully removing the 2 - 3 outer whorls of the developing leaves with the help of a sterile sharp blade. Than explants were cut such that it forms circular disc by cutting perpendicular to the central axis with a surgical blade and inoculated on medium supplemented with 2,4-D.

Establishment of callus cultures and regeneration of sugarcane was reported by Nickell, (1964) [19] and Barba and Nickell (1964) [19]. Callus culture of sugarcane have also been successfully established using young shoot and young leaves as explants on MS medium containing 2,4-D and coconut milk (Nadar *et al*. (1977) [18], Liu and Chen (1984) [13] as well as, Bhansali and Singh (1984) [5]. Similar response was also reported by Barba *et al*. (1977) [4] and Manan and Amin (1999) [14]. Where in they successfully established callus culture by manipulating 2,4-D concentration in medium. In India, good amount of studies were carried out on micro propagation of sugarcane to study somaclonal variations. The result obtained from the present investigation was discussed in this chapter.

2.3 Multiplication and maintenance of calli

The callus masses obtained in various concentrations of 2, 4-D were multiplied and maintained by sub culturing. Every ten days the healthy looking and fast growing tissue fragments

from the callus mass were sub cultured on MS medium supplemented with NAA (0.5 mg/1) and BAP (1.0 mg/1). The growth of callus at the weekly interval was measured in terms of fresh weight. Callus masses so obtained were used as the experimental material in the present investigation.

2.3.1 Standardization of regeneration medium for shoot differentiation from established callus cultures

The investigation of shoot regeneration from callus was carried out on MS medium. The numbers of shoots obtained from the program of regeneration from calli were counted after 30 days of callus cultured on regeneration medium whereas, length of shoots (cm) was measured before transferred into rooting medium. The medium details for shoot regeneration are as under: (MS + 0.5 mg/l NAA + 0.5 mg/1 BAP).

2.4 *In vitro* mutagenesis

The friable callus induced after 25 days of inoculation was cut in to small pieces, weighted and treated with EMS (Ethyl methane Sulphonate), SA (Sodium Azide) and MMS (Methyl Methane Sulphonate) of 0.2% and 0.3% solution (prepared in sterilized distilled water and membrane filtered) for 30 minute and 60 minute separately and inoculated on MS medium consisting 4 mg/l 2,4-D + 2% sucrose. Detailes of treatment combinations and experimental design are narrated in Table 2, observation recorded on survived. The LD_{50} of EMS, SA and MMS treatments were determined for further screening of mutants.

Table 2: Details of treatment combinations for *in vitro* mutagenesis

Statistical Design	**:-**	**Completely Randomized Design with factorial concept suggested by Panse and Sukhatme (1985).**
Repetitions	**:-**	3
Explant used	**:-**	callus tissues
Treatments details		
Factor 1, C (six different chemical mutagenic agents)		
C_1	**:-**	EMS (0.2%)
C_2	**:-**	EMS (0.3%)
C_3	**:-**	SA (0.2%)
C_4	**:-**	SA (0.3%)
C_5	**:-**	MMS (0.2%)
C_6	**:-**	MMS (0.3%)
Factor 2, P (Two different emersion time period)		
P_1	**:-**	30 min
P_2	**:-**	60 min

2.5 *In vitro* selection

Somaclones were raised from treated callus on MS medium allowed to regenerate and the following observations were recorded.

2.6 Observations recorded

2.6.1 Survival per cent

Survival per cent recorded after the application of chemical mutagens considering LD_{50} (lethal dose).

2.6.2 Regeneration per cent

Regeneration per cent recorded on the basis of no of treated cultures undergone regeneration process for shoot formation.

2.6.3 Number of days for shoot formation

The number of days required for shoot formation of callus on MS medium supplemented with different cytokinins and enzymes.

2.6.4 Number of multiple shoots

The numbers of shoots were counted after 30 days of callus inoculation on regeneration medium.

3. Results and discussion

3.1 Quality of the callus

On the basis of visual observations considered such as appearance, compactness and color, quality of callus is differentiated as good callus, moderate callus, very good callus and poor callus. Moderate callus formation was observed in the explants used in treatments. Leaf whorl exhibited moderate to good callus formation in most of the treatments except treatment EMS (0.2%) with both the time periods. Similar results were observed by Rutherford *et al.* (2013) [1].

Table 3: Effects of different chemical mutagenic agents on callus survival per cent

P (Time period for emersion) / **C (Concentrations of mutagens)**		**P_1(30 min)**	**P_2(60 min)**
C_1	(EMS - 0.2%)	+++	+++
C_2	(EMS - 0.3%)	+	- -
C_3	(SA – 0.2%)	+	+
C_4	(SA – 0.3%)	- -	- -
C_5	(MMS – 0.2%)	+	+
C_6	(MMS – 0.3%)	- -	- -

\+ Good Callus (White Globular)
++ Moderate Callus (Yellowish)
+++ Very Good Callus (Whitish yellow)
- - Poor Callus (Brown)

Plate 1: Quality of the callus

Plate 2: Without mutagenic agent

3.2 Effects of different chemical mutagenic agents on callus survival per cent

Both the concentrations, 0.2% and 0.3% of EMS and MMS registered optimum survival per cent at both the treatment intervals. Whereas, higher and lower concentration of SA registered poor callus survival per cent (as disposed in Figure 1 to 4). Overall, lower concentration of EMS and MMS at 30 min treatment period registered similar survival per cent compared to untreated callus (see Plate. No. 1). As indicated in Table. 4, interaction effect found to be significant. Highest calli survival per cent (81.70%) registered in C_1P_2 (EMS 0.2% + 60 min), statistically at par with 80.40% in C_6P_1 (MMS 0.3% + 30 min), followed by 77.60% in C_1P_1 (EMS 0.2% + 30 min). On the other hand, callus treated with C_4P_2 (SA 0.3% + 60 min) registered lowest survival per cent (56.4%). Thease findings are in accordance with Chaudhari (2017) [6]. Maximum survival per cent (81.7%) was observed in treatment combination (EMS 0.2% + 60 min) followed by 80.4% in callus treated with (MMS 0.3% + 30 min). Similar results were observed by Purnamanishingh and Hutami (2016) [24]. Higher concentration of SA (0.3%) at 60 min depicted poor survival per cent. Similar results were observed by Kanganal *et al*. (2008) [11], Koach *et al*. (2009) [12], Gadakh (2014) [8] and Chaudhari (2017) [6].

Table 4: Effects of different chemical mutagenic agents on callus survival per cent

C (Concentrations of mutagens) \ P (Time period for emersion)		P_1(30 min)	P_2(60 min)	Mean C
C_1	(EMS - 0.2%)	77.60	81.70	79.65
C_2	(EMS - 0.3%)	74.20	67.50	70.85
C_3	(SA – 0.2%)	70.40	62.70	66.55
C_4	(SA – 0.3%)	60.50	56.40	58.45
C_5	(MMS – 0.2%)	70.60	71.07	70.83
C_6	(MMS – 0.3%)	80.40	76.20	78.30
Mean P		72.28	69.26	
Effect		S.Em.+	C.D. @ 5%	CV%
C		0.81	2.37	2.81
P		0.47	1.37	
C x P		1.15	3.35	

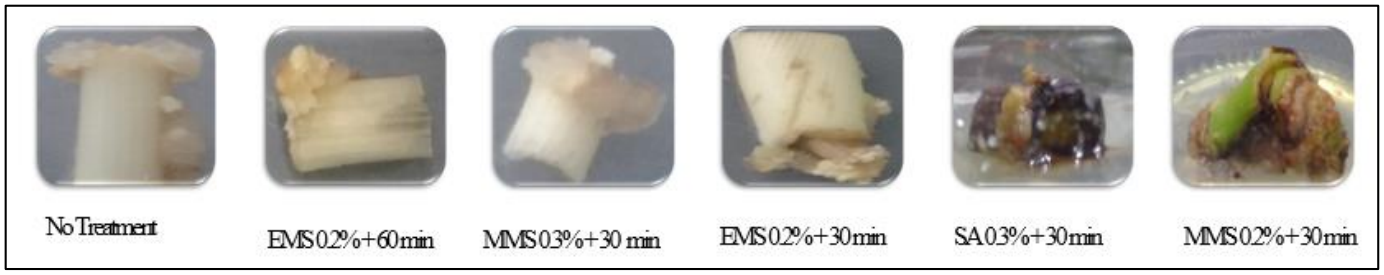

Plate 3: Survival per cent

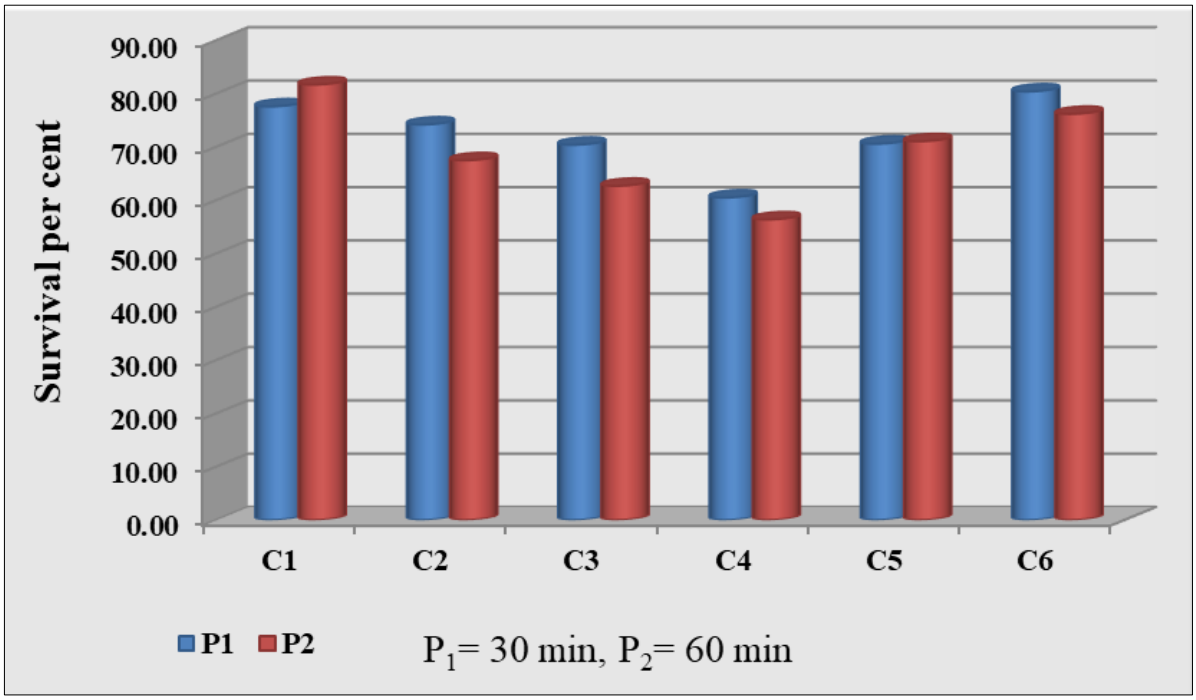

Fig 1: Survival per cent

3.3 Effect of different chemical mutagenic agents on regeneration per cent

Immersion time in EMS, SA and MMS were more influential to callus regeneration than the concentration. Increased concentration resulted in a reduction in regeneration per cent. The interaction effect of the different mutagenic agents with different time periods was found to be significant for regeneration per cent, that indicates soma clones regenerated under different mutagenic treatment influenced at the cellular level. As shown in Table 5, interaction effect was found to be a significant one. Higher regeneration per cent (78.2%) was observed in treatment C_6P_1 (MMS 0.3% + 30 min) which is at par with 76.7% in treatment C_1P_2 (EMS 0.2% + 60 min) numerically followed by 74.5% in treatment C_2P_2 and 70.4% in treatment C_5P_2 (MMS 0.2% + 60 min), whereas, minimum regeneration per cent (52.5%) was notice in treatment C_4P_1 (SA 0.3% + 30 min). Increase in the concentration along with increase in time period resulted into reduction in regeneration per cent. Regeneration potential was directly influenced by higher concentration of mutagenic agent and immersion time. Similar results were observed by Patel *et al*. (2004) [22], Kanganal *et al*. (2008) [11], Koch *et al*. (2009) [12] and Gadakh (2014) [8].

Table 5: Effects of different chemical mutagenic agents on callus regeneration per cent

C (Concentrations of mutagens) \ P (Time period for emersion)		P_1(30 min)	P_2(60 min)	Mean C
C_1	(EMS - 0.2%)	68.40	76.70	72.55
C_2	(EMS - 0.3%)	70.30	74.50	72.40
C_3	(SA – 0.2%)	62.30	56.70	59.50
C_4	(SA – 0.3%)	52.50	54.40	53.45
C_5	(MMS – 0.2%)	62.60	70.40	66.50
C_6	(MMS – 0.3%)	78.20	67.40	72.80
Mean P		65.72	66.68	
Effect		S.Em.+	C.D. @ 5%	CV%
C		0.62	1.79	2.27
P		0.35	1.04	
C x P		0.87	2.54	

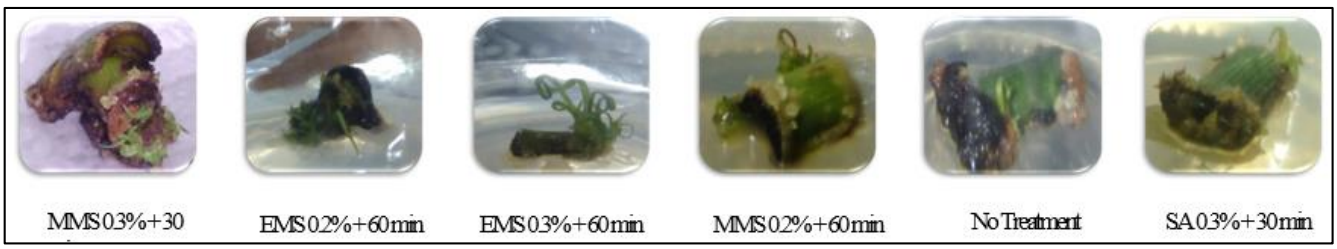

Plate 4: Regeneration per cent from callus

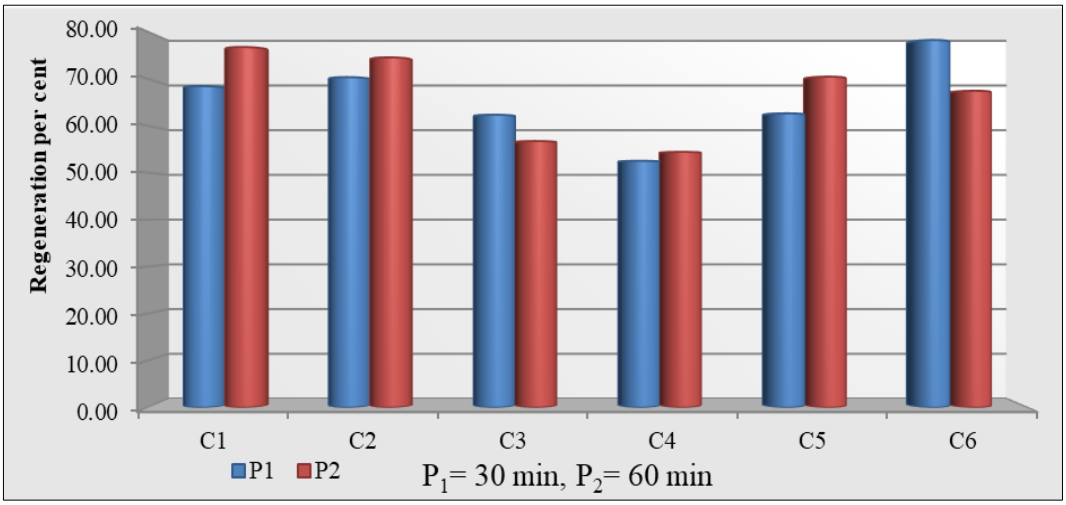

Fig 2: Show the regeneration per cent

3.4 Effect of different chemical mutagenic agents on the number of days for shoot formation

Among twelve treatment combinations, eight treatment combinations registered a lower number of days for a shoot formation and four treatments showed the more days of shoot formation as compared to control. Among the three mutagenic agents, EMS and MMS registered minimum numbers of days for shoot formation. Higher concentration of SA (0.3%) and increased treatment time period (60 min) have more influence on the number of days for shoot formation. As represented in Table 6, interaction effect of both the factors displayed significant, in which calli treated with C_1P_2 (EMS 0.2% + 60 min) registered minimum number of days of shoot formation, 15.20 days statistically at par with treatment C_6P_1 (16.40 days, MMS 0.3% + 30 min) and treatment C_2P_2 (16.8 days, EMS 0.3% + 60 min). Whereas, the maximum number of shoot formation (32.4 days) was registered in treatment C_4P_2 (SA 0.3% + 60 min). Application of higher concentration of mutagenic agents resulted into more number of days for shoot formation i.e. 18 to 32 days. Inhibitory effects were observed among the higher concentration of mutagenic treatments. Similar results were reported by Delvi *et al*. (2012) [7], Melion and Barba (1980) [15], and Rutherford *et al*. (2013) [26]. Genetic nature of CoN - 13073 did not respond well to mutagenic treatments for this character.

Table 6: Effects of different chemical mutagenic agents on the number of days for shoot formation

C (Concentrations of mutagens) \ P (Time period for emersion)		P_1(30 min)	P_2(60 min)	Mean C
C_1	(EMS - 0.2%)	20.70	15.20	17.95
C_2	(EMS - 0.3%)	26.60	16.80	21.70
C_3	(SA – 0.2%)	18.20	20.40	19.30
C_4	(SA – 0.3%)	28.70	32.40	30.55
C_5	(MMS – 0.2%)	26.40	20.60	23.50
C_6	(MMS – 0.3%)	16.40	18.20	17.30
Mean P		22.83	20.60	
Effect		S.Em. +	C.D. @ 5%	CV%
C		0.41	1.19	4.60
P		0.24	0.69	
C x P		0.58	1.69	

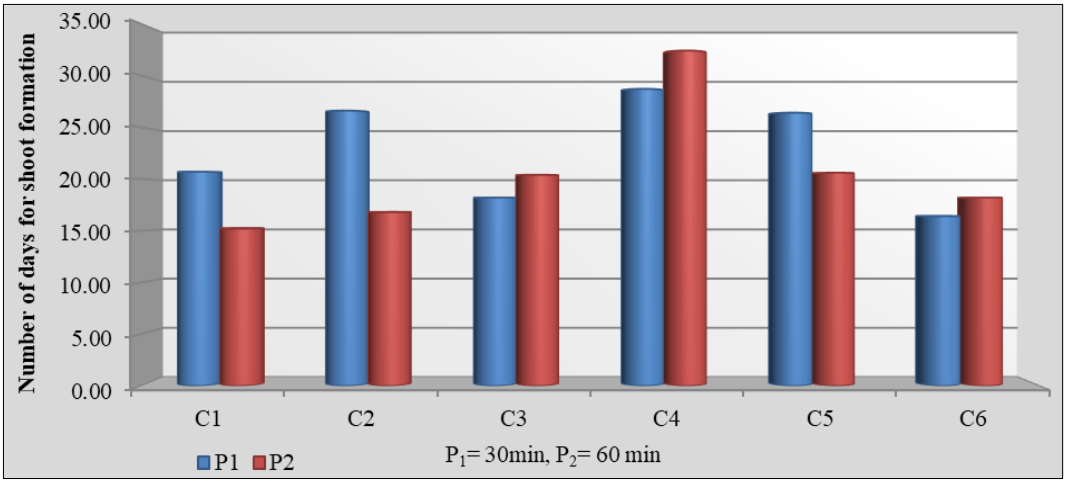

Fig 3: Show the number of days for shoot formation

3.5 Effect of different chemical mutagenic agents on the number of multiple shoots

Vast differences were observed among the treatments. Among twelve treatments, eight treatments showed a higher number of multiple shoots and four treatments showed the lower number of multiple shoots. Lower and higher concentrations of EMS and MMS influenced the number of multiple shoots, overall among the three mutagenic agents, Sodium Azide responded poorly to the number of multiple shoots as compared to control. As illustrated in table 7, significantly higher number of multiple shoots, 30.4 was observed in treatment C_5P_2 (MMS 0.2% + 60 min) followed by 28.6 in treatment C_2P_2 (EMS 0.3% + 60 min), 27.8 in treatment C_6P_1 (MMS 0.3% + 30 min) and 27.4 in treatment C_2P_1 (EMS 0.3% + 30 min), whereas, minimum number of multiple shoots, 8.6 were observed in treatment C_4P_2 (SA 0.3% + 60 min). Multiple shoot ratio in different sugarcane genotypes were worked out concurrently by various researchers like Hendre *et al*. (1983) [9], Patel *et al*. (2001) [21], Patel (2012) [12] and Chaudhari *et al*. (2017) [6].

Table 7: Effect of different chemical mutagenic agents on the number of multiple shoots

C (Concentrations of mutagens) \ P (Time period for emersion)		P_1(30 min)	P_2(60 min)	Mean C
C_1	(EMS - 0.2%)	10.80	14.60	12.70
C_2	(EMS - 0.3%)	27.40	28.60	28.00
C_3	(SA – 0.2%)	10.20	12.40	11.30
C_4	(SA – 0.3%)	14.60	8.60	11.60
C_5	(MMS – 0.2%)	18.70	30.40	24.55
C_6	(MMS – 0.3%)	27.80	16.80	22.30
Mean P		18.25	18.57	
Effect		S.Em. +	C.D. @ 5%	CV%
C		0.41	1.19	5.430
P		0.24	0.67	
C x P		0.58	1.69	

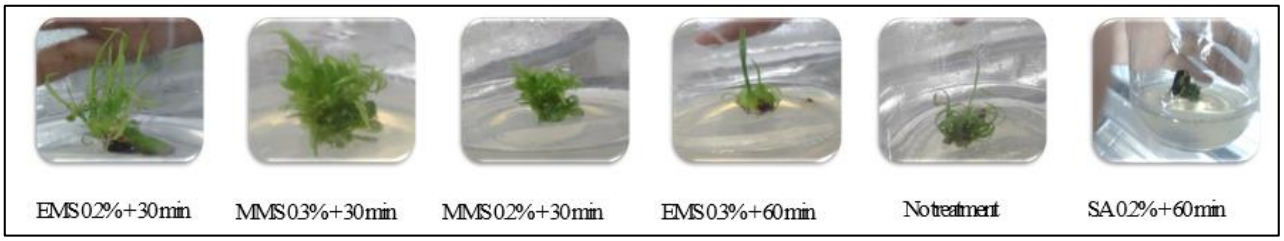

Plate 5: Multiple shoots from regenerated calli

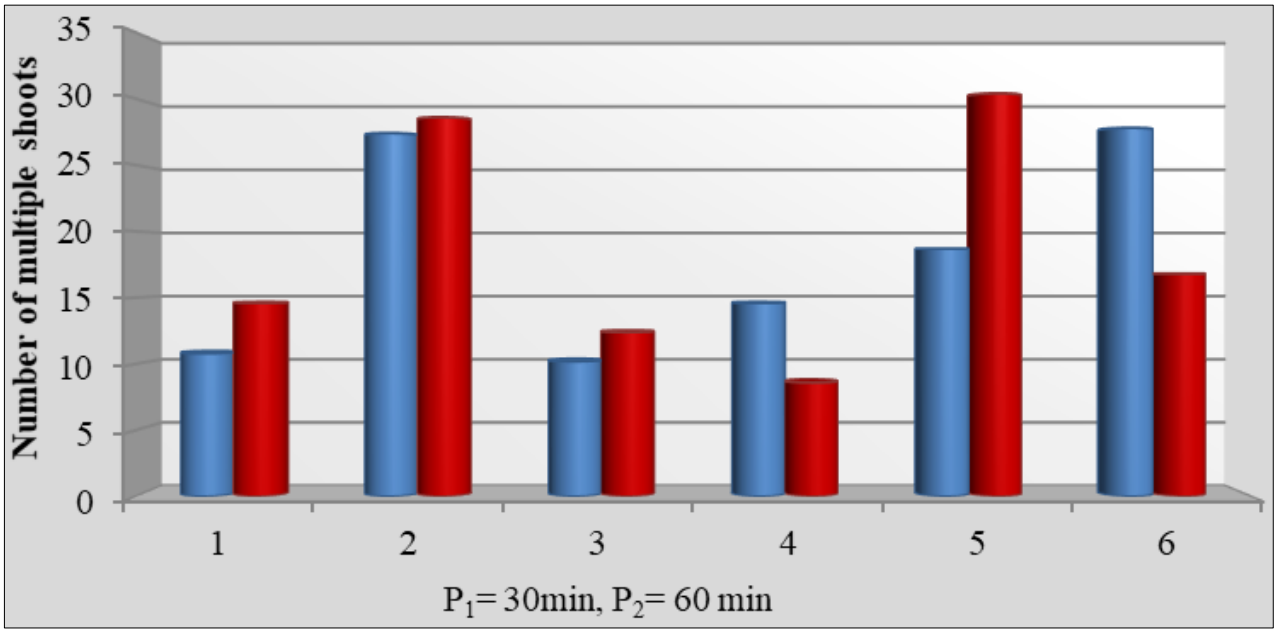

Fig 4: Number of multiple shoots

Summery and conclusion

Among the mutagenic treatments, maximum survival per cent (81.7%) was observed in treatment (EMS 0.2% + 60 min). EMS treatment registered optimum survival, a gradual decrease in survival percentage was observed with increase in concentration as well as with time factor. Maximum regeneration percentage (78.2%) was found in explants treated with treatment (MMS 0.3% + 30 min). Emersion time was more influential to callus regeneration than the concentration, increase in concentration as well as time of exposure resulted in a reduction in regeneration per cent. All the treatments were found significant. In case of the number

of days for shoot formation, callus treated with treatment (EMS 0.2% + 60 min) registered a minimum number of days for shoot formation (15.2 days). Among three mutagenic treatments, EMS and MMS registered a minimum number of days for shoot formation. Highest number of multiple shoots, 30.4 was observed at (MMS 0.2% + 60 min), whereas, minimum number of multiple shoot formation was obtained for (SA 0.3% + 60 min). Sixteen per cent treatments depicted a greater number of multiple shoots, while forty per cent showed lower number of multiple shoots in comparison with the untreated callus shoots. Genetic variability having importance in any crop improvement program. Creation of genetic variability in existing gene pool leads to development of wide range of characterization. Identification and evaluation of variable characterization followed by utilization involves standard proven methodology and different techniques. *In vitro* induced mutagenesis in the present investigation resulted wide range of variability for various characters. The amount and frequency of mutagenic agents played important role in generation of variability among sugarcane verities studied. Imposing mutagens at cellular/tissue (callus) level would be more effective and authenticated in the present experiment for various characters studied. Further evaluation needs to be required for assuming the *in vitro* generated variability consistency and its longevity by heritability and genetic advance study or molecular distinction and mapping program.

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