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Research Article

EFFECT OF 2,4D & EMSON *IN VITRO* REGENERATION IN SUGARCANE CULTIVAR, 2003V46

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Abstract- Studies were carried out to establish an efficient system for *in vitro* regeneration of sugarcane cultivar, 2003V46 using chemical mutagens; Ethyl methane sulphonate (EMS) and 2,4dichlorophenoxy acetic acid (2,4D). Young leaf rolls were used as explants for callus induction on MS medium containing different concentrations of EMS(0.6 µM l^{-1}, 0.8 µM l^{-1} and 1.0 µM l^{-1}) and 2,4D(4mg l^{-1}, 5mg l^{-1} and 6 mg l^{-1}). Among the different concentrations,2003V46 had taken minimum number of days (10.0) for callus induction with 2, 4D @ 4mg l^{-1},recordingmaximum mean callus size of 2.42 cm with high callus induction frequency (96.89%) and maximum mean number of explants inducing callus (2.86) in addition with superior response in shooting, rooting and hardening characters than EMS.2, 4D at higher concentration (6 mg l^{-1}) had inhibitory effect on callusing, shooting and rooting and produced variation for leaf sheath hairiness, shape of bud and internode colour similar to that of the mutagen, EMS.

Keywords- *In vitro,* Sugarcane, EMS, 2, 4D, Callus.

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Introduction

Sugarcane is an important commercial crop in many developing/developed countries. Considering its importance in the agricultural industry, concerted efforts are being made for its improvement using conventional and biotechnological techniques. There are many reports on tissue culture and plant regeneration of sugarcane from different countries. Initial attempts to regenerate plants through *in vitro* technique were made on sugarcane [1, 2]. Genetic variability apart from conventional breeding can be easily discernible in *in-vitro* regenerated somaclonal variants. Tissue culture techniques through mutation induction can be used to increase the speed or efficiency of breeding programs to get a new diversity of germplasm [3]. Mutation induction can be done by using chemical mutagens and also with use of growth regulators which have high activity, such as 2, 4D[4, 5, 6].Missense and nonsense mutations were induced by chemical mutagens through creating point mutations which would provide a series of changes in genes [7]. The occurrence of somaclonal variation in tissue culture derived plants is an alternative method to sort out many barriers of traditional breeding program. In addition to rejuvenation of sugarcane clone's different chemical mutagens can be applied to increase the frequency of variations. Somaclones were developed eliminating certain defects like leaf spines (Co7717), leaf drying (Co7704) apart from resistance to biotic and abiotic stresses. The sugarcane variety, 2003V46 is a popular early maturing variety extensively cultivated in Andhra Pradesh. Presence of leaf sheath hairs in the variety demands extra labour wages during harvesting. Elimination of leaf sheath hairs in the variety is required to reduce harvesting costs. Creation of somaclonal variation offers a way to obtain hairless version of 2003V46. Hence an attempt was made to standardised the production protocol for somaclonal variation using a growth hormone 2, 4D as an alternative to chemical mutagen EMS. Therefore, the present investigation has been undertaken to establish plant regeneration protocol through young leaf roll culture under different chemical mutagenic concentrations in sugarcane.

Material and Methods

The experimental work was carried out at Agricultural Research station, Perumallapalle with young leaf rolls of popular sugarcane variety, 2003V46. Surface sterilization of sugarcane spindle was carried out after excision and removing of young leaves using liquid detergent water containing a few drops of Tween-20 (20 ml l^{-1}) for 5-10 minutes. After these treatments, explants were washed again with sterile deionising water for 3-5 washings, each for 3-5 minutes. After these initial washings, explants were kept in an aqueous mixture solution of bavistin (10 g l^{-1}) and streptomycin (1 g l^{-1}) for 10-15 minutes and then washed thoroughly in sterile deionising water for 3-5 washings, each for 3-5 minutes. Sterilization with sodium hypochlorite (3.0%) was done for 10 minutes in laminar flow hood followed by sterilization with ethyl alcohol (70%) for 30 seconds and washed with sterile distilled water for 2-3 times. The effect of different concentrations of mutagenic chemicals, 2, 4D (4mg l^{-1}, 5mg l^{-1} and 6 mg l^{-1}) and EMS (0.6 µM l^{-1}, 0.8 µM l^{-1} and 1.0 µM l^{-1}) on *in vitro* regeneration from leaf roll explants was studied using M.S media supplemented with mutagens. Transverse sections (1-2 cm) were made from young, innermost tightly furled leaves to obtain smart setts (leaves discs) and were placed in the petri-plate containing callus induction media with different concentrations of chemical mutagens. Calli cultured on M.S medium supplemented with different levels of mutagenic chemicals were

transferred on to M.S medium supplemented with BAP (3 mg l^{-1}) + IAA (2 mg l^{-1}) + Kinetin (2 mg l^{-1}) for shoot initiation and growth. Full grown shoots were transferred on to half-strength M.S. medium supplemented with NAA (3 mg l^{-1}) for root initiation and growth. Tissue culture (TC) seed lingsraised *in vitro* with well developed roots and shoots were carefully transferred to polythene covers containing coconut peat: soil: sand in the ratio of 1:1:1 and kept in a shade net house for 15-20 days and relative humidity was maintained. Data on callusing, shooting, rooting and hardening was recorded.

Results and Discussion

Treatmental differences were significant for number of days taken for callus induction. Time taken for callus induction ranged from 10.0 to 13.89 days when the medium was treated with different concentrations of two chemical [Table-1]. The number of days taken for callus induction in control was 9.22 days.Minimum number of days (10.0) taken for callus induction was recorded with 2, 4-dichlorophenoxy acetic acid (2, 4D) @ 4 mgl^{-1} followed by 11.1 days with 2, 4D @ 5 mg l^{-1} whereas maximum number of days (13.89) taken for callus induction was observed with EMS @ 1.0 µM l^{-1} followed by 13 days with 2, 4D @ 6 mg l^{-1} [Fig-1]. The mean number of explants inducing callus ranged from 1.67 to 2.86 [Table-1] as against 3.00 in the control. Maximum mean number of explants inducing callus (2.86) with 2,4D @ 4 mg l^{-1}followed by 2.67 with EMS @ 0.6 µM l^{-1} was recorded and minimum mean number of explants inducing callus was observed as 1.67 with EMS @ 1.0 µM l^{-1} followed by 2.11 with 2,4D @ 6 mg l^{-1}concentrations. Callus induction frequency ranged from 62.83 to 96.89 % for different concentrations of chemicals. 2, 4D @ 4 mg l^{-1}recorded maximum callus induction frequency (96.89%) followed by 92.70 % with EMS @ 0.6 µM l^{-1} concentration and minimum callus induction frequency of 62.83 % was observed with EMS @ 1.0 µM l^{-1} followed by 66.66 % with 2,4D @ 6 mg l^{-1} [Fig-1].The mean callus size ranged from 1.21 cm - 2.42 cm with different concentrations of chemicals [Table-1].

Table-1 *Effect of different concentrations of chemical mutagens on callusing, shooting,rooting and hardening in 2003V46*

Characters	EMS 0.6 µM l^{-1}	EMS 0.8 µM l^{-1}	EMS 1.0 µM l^{-1}	2,4 D 4 mg l^{-1}	2,4 D 5 mg l^{-1}	2,4 D 6 mg l^{-1}	2003V46 (Control)	C.D
No. of days for callus induction	11.89	12.44	13.89	10	11.11	13	9.22	0.674
Mean No. of explants induced callus	2.67	2.22	1.67	2.86	2.22	2.11	3	0.15
Callus induction frequency (%)	92.70 (74.30)	85.10 (67.27)	62.83 (52.42)	96.89 (79.81)	81.44 (64.46)	66.66 (54.72)	100.00 (90.00)	0.617
Callus size (cm)	1.67	1.41	1.21	2.42	1.94	1.76	2.66	0.124
No. of days for shoot initiation	17.56	19.11	20.67	15.56	16.22	17.22	14	0.565
Shoot regeneration frequency	83.33 (65.90)	73.33 (58.89)	61.67 (51.73)	90.56 (72.09)	81.67 (64.64)	66.67 (54.72)	96.67 (79.68)	1.855
No. of shoots per explant	12.22	10.33	8.22	23.44	20.11	16.78	28.44	0.493
Average shoot length (cm)	3.21	2.31	1.5	3.83	2.76	1.76	4.43	0.053
No. of days for root initiation	13.44	16	18.33	11.56	12.33	15.89	9.44	0.389
No. of roots per shoot	19.44	16.22	14.33	22.33	18.67	15.78	28.44	0.447
Rooting frequency	78.33 (62.24)	66.11 (54.38)	57.78 (49.45)	87.78 (69.52)	81.67 (64.64)	72.22 (58.17)	91.67 (73.24)	1.612
Average root length (cm)	3.42	2.51	1.43	3.83	2.61	1.93	4.56	0.047
Time taken for Hardening	10.67	12.22	14.11	9.33	11.11	14.56	9.44	0.436
Hardening percentage	78.33 (62.23)	66.33 (54.51)	58.67 (49.97)	85.00 (67.18)	74.67 (59.76)	68.11 (55.60)	88.11 (69.80)	0.355

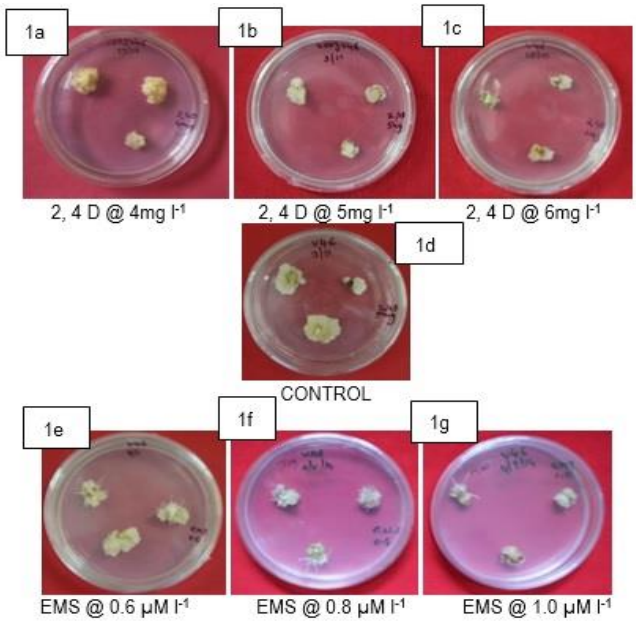

Fig-1 Callus induction in different concentrations of chemical mutagens with control in 2003V46

Petriplates containing callus growth under different concentrations of chemicals compared with control (with no chemical mutagen). [**Fig-1a**]. 2,4D@4mg l^{-1}; [**Fig-1b**]. 2,4D @ 5mg l^{-1}; [**Fig-1c**]. 2,4D @ 6 mg l^{-1}; [**Fig-1d**]. Control; [**Fig-1e**]. EMS @ 0.6 µM l^{-1}; [**Fig-1f**]. EMS @ 0.8 µM l^{-1}; and [**Fig-1g**]. EMS @ 1.0 µM l^{-1}]

EMS and 2, 4D at higher concentrations inhibited callus growth and development. With the increase in the concentration of the mutagen there was an increase in number of days required for callus initiation, reduced callus induction frequency (%) and reduction in callus size.2, 4D @ 4 mg l^{-1} recorded maximum mean callus size, highest callus induction percentage compared to all concentrations of chemicals

2,4D @ 4 mg l^{-1}recorded maximum mean callus size of 2.42 cm followed by 1.94 cm with 2,4D @ 5 mg l^{-1} concentration and minimum mean callus size (1.21 cm) was observed with EMS @ 1.0 µM l^{-1} followed by 1.41 cm with EMS @ 0.8 µM l^{-1} [Fig-1]. EMS and 2, 4D at higher concentrations inhibited callus growth and development. Many scientists reported stimulation in callus growth at lower doses and poor stimulation in high doses of mutagenic chemicals [8]. With the increase in the concentration of the mutagen there was an increase in number of days required for the callus initiation. Similar type of decrease in callus obtained with increased dose of gamma rays and EMS in sugarcane [9]. With the increased level of concentration of chemicals, the percentage of callus initiation was decreased. This was also supported by the results that a decrease in survival of soybean cell suspension cultures with increasing concentrations of EMS [10, 11]. The callus size also decreased with an increase in concentration of chemicals in both clones. Similar decrease in callus size was observed in castor bean [12] and in sugarcane [13] with the increase in the dose of gamma irradiation.

Significant difference among all the treatments for number of days for shoot initiation was observed. Number of days taken for shoot initiation ranged from 15.56 to 20.67 in treatments [Table-1]. EMS @ 1.0 µM l^{-1}had recorded more number of days for shoot initiation (20.67) followed by EMS @ 0.8 µM l^{-1} with 19.11 and minimum number of days (15.56) was recorded with 2,4D @ 4 mg l^{-1} followed by 2,4 D @ 5 mg l^{-1} with 16.22 days [Fig-2]. Among all treatments, shoot induction frequency ranged from 61.67 – 90.56 % with significant difference. Maximum shooting frequency (90.56 %) was recorded with 2, 4D @ 4 mg l^{-1} followed by EMS @ 0.6 µM l^{-1} with 83.33% and minimum shooting frequency of 61.67 % was recorded with EMS @ 1.0 µM l^{-1}concentration followed by 2, 4D @ 6 mg l^{-1} with 66.67 % [Fig-2]. The range among mutagenic treatments varied from

8.22 to 23.44 for number of shoots per explant. The chemical 2, 4D @ 4 mg l^{-1} had recorded the maximum number of shoots per explant (23.44) followed by 2, 4D @ 5 mg l^{-1} (20.11). The chemical EMS @ 1.0 µM l^{-1} followed by EMS @ 0.8 µM l^{-1} recorded minimum number of shoots per explant (8.22 and 10.33, respectively). The mean shoot length ranged from 1.5 cm to 3.83 cm with mutagenic treatments. 2, 4D @ 4 mg l^{-1}recorded maximum shoot length (3.83 cm) followed by EMS @ 0.6 µM l^{-1} (T_4) (3.21 cm) while minimum shoot length (1.5 cm) was recorded with EMS @ 1.0 µM l^{-1}followed by 2, 4D @ 6 mg l^{-1} (1.76 cm) [Table-1] [Fig-2]. Effect of higher concentrations of EMS and 2, 4D was inhibitory on shoot development also. An increase with increased level of concentration of chemicals was recorded for mean number of days for shoot initiation. Shoot induction frequency showed decreased trend with increase in concentration of mutagens. These results are in agreement with the previous findings in sugarcane, rice, chrysanthemum [14-16]. The results revealed reduction in the number of regenerated shoots and shoot length with increasing concentrations of chemical mutagens. Using radiation induced variation in wheat a reduction in number of shoots per explant with the increase in radiation dose was reported [17].

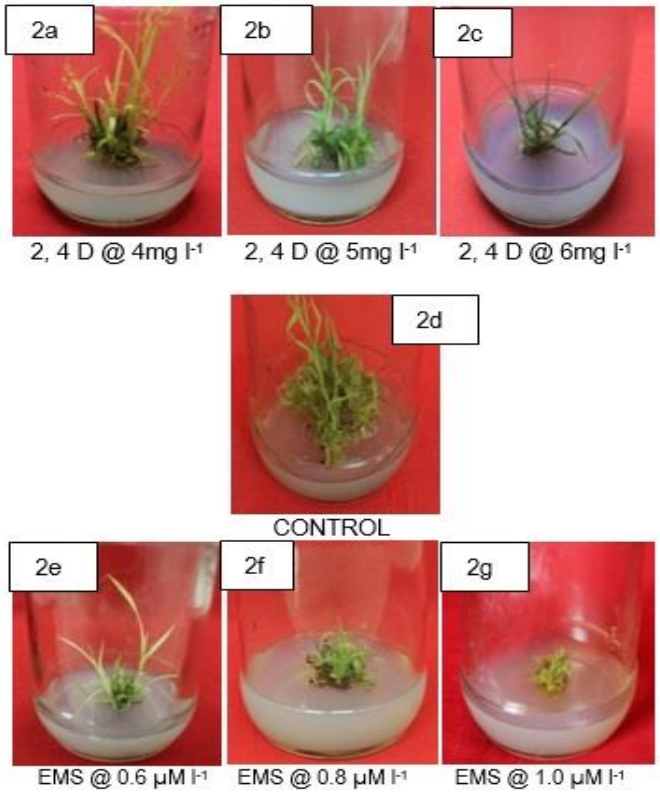

Fig-2 Shoot induction in different concentrations of chemical mutagens with control in 2003V46

Bottles showing shoot growth under different concentrations of chemicals compared with control. Control showed superior performance in all shooting traits. EMS and 2, 4D at higher concentrations inhibited shoot growth and development. [**Fig- 2a**]. 2,4D @4mg l^{-1}; [**Fig-2b].** 2,4D @ 5mg l^{-1}; [**Fig-2c].** 2,4D @ 6 mg l^{-1};[**Fig-2d].** Control; [**Fig-2e].** EMS @ 0.6 µM l^{-1}; [**Fig-2f].** EMS @ 0.8 µM l^{-1}; and [**Fig-2g].** EMS @ 1.0 µM l^{-1}]

With the increase in the concentration of the mutagen there was an increase in number of days required for shoot initiation, reduced shoot induction frequency (%) and reduction in shoot length. 2, 4D @ 4 mg l^{-1} recorded maximum shoot length, highest shoot regeneration frequency compared to all concentrations of chemicals. EMS @1.0 µM l^{-1} showed inhibitory effect on shooting

Mean response of root initiation ranged from 11.56 days to 18.33 days. Minimum number of days (11.56) taken for root initiation was recorded with 2, 4D @ 4 mg l^{-1} followed by 2, 4D @ 5 mg l^{-1} with 12.33 days and maximum number of days (18.33) was recorded with EMS @ 1.0 µM l^{-1}followed by EMS @ 0.8 µM $l^{-1}$with 16 days [Table-1] [Fig-3]. The number of roots per shoot was ranged from 14.33 to 22.33.2, 4D @ 4 mg l^{-1}recorded maximum mean number of roots per shoot (22.33) followed by EMS @ 0.6 µM l^{-1} (19.44), where as EMS @ 1.0 µM l^{-1} recorded minimum number of roots per shoot (14.33) followed by 2, 4D @ 6 mg l^{-1} [Table-1]. The range for rooting frequency was from 57.78 to 87.78 % treatments. EMS @ 1.0 µM l^{-1}recorded the lowest rooting frequency of 57.78 % followed by EMS @ 0.8 µM $l^{-1}$with 66.11 % and the highest frequency(87.78 %) was recorded with 2, 4D @ 4 mg l^{-1} followed by 2, 4D @ 5 mg l^{-1} with 81.67 % [Table-1] [Fig-3]. With respect to root length, control had recorded maximum root length (4.56). Average root length ranged from 1.43 to 3.83 cm in all treatments. The clone 2003V46 showed maximum mean root length of 3.83 cm with 2, 4D @ 4 mg l^{-1} followed by EMS @ 0.6 µM l^{-1} with 3.42 cm whereas EMS @ 1.0 µM l^{-1} recorded minimum mean root length (1.43 cm) followed 2, 4D @ 6 mg l^{-1} with 1.93 cm [Fig-3]. The results revealed that the shoots obtained from mutagenic chemicals treated callus, showed less response to root differentiation and took more number of days to differentiate roots as compared to the shoots obtained from callus without treatment. The maximum root length was observed in control when compared with mutagenic treatments [18]. Both root length and mean number of rooted shoots decreased with increase in concentration of four chemicals. These results are in agreement with results obtained previously [19, 20]. 2, 4D @ 4 mg l^{-1}recorded the lowest time taken for hardening (9.33 days) followed by EMS @ 0.6 µM l^{-1} (10.67) and2, 4D @ 5 mg l^{-1} (11.11) whereas 2, 4D @ 6 mg l^{-1} recorded the highesttime taken for hardening (11.11) followed by EMS @ 1.0 µM l^{-1} (14.11) [Table-1]. Significant difference was observed between treatments for hardening percentage. 2, 4D @ 4mg l^{-1} recorded maximum hardening percentage (85.00 %) followed by EMS @ 0.6 µM l^{-1} with 78.33 %. Whereas, EMS @ 1.0 µM l^{-1}recorded minimum hardening percentage (58.67 %) followed by 66.33 % with EMS @ 0.8 µM l^{-1}. Seedlings obtained after hardening were evaluated for various morphological characters. Some plants obtained from 2, 4D @5 mg $l^{-1}$and 6 mg l^{-1}showed sparse leaf sheath hairiness (16T17), ovate shape of bud (16T16), greenish yellow internode colour (16T16), incipient shape of inner auricle (16T17) two no. of root eye rows (16T16), light internode waxiness (16T16) in contrast to parent 2003V46. Whereas EMS at all concentrations produced variation for foresaid characters in addition to crescent shape of ligule (16T10) and green yellow root zone colour (16T11, 16T12) [Table-2]. Similar type of variations for various morphological characters such as bud size, shape, and number of root eye rows on the node were observed [21].

Table-2 *Frequency of somaclones for various characters in 2003V46*

S. No.	Characteristics	2, 4 D	EMS
1.	Sparse leaf sheath hairiness	1(16T17)	3 (16T10, 16T12, 16T14,16T15)
2.	Crescent shape of ligule	-	1 (16T10)
3.	Incipient shape of inner auricle	1 (16T17)	2 (16T11, 16T12)
4.	Greenish yellow internode colour	1 (16T16)	2 (16T11, 16T12)
5.	Ovate shape of bud	1 (16T16)	2 (16T10, 16T12)
6.	Two no. of root eye rows	1 (16T16)	3 (16T11, 16T14,16T15)
7.	Green yellow root zone colour	-	2 (16T11, 16T12)
8.	Light internode waxiness	1 (16T16)	3 (16T10, 16T11, 16T14)

Conclusion

The growth hormone, 2, 4D @ 4 mg $l^{-1}$was found to be best among different treatments. It had shown good performance in all the stages of culture *viz*., callusing, shooting and rooting. Whereas EMS @ 1.0 µM l^{-1} showed hindering effect in all the stages of culture. Though a growth hormone, 2, 4D at higher concentration (6 mg l^{-1}) had inhibitory effect on callusing, shooting and rooting similar to that of the mutagen, EMS. It can be concluded that 2, 4D @ 6 mg l^{-1} along with EMS @ 1.0 µM l^{-1} can be used to produce variation in 2003V46. Somaclones with reduction in leaf sheath hairiness produced from mutagens in 2003V46 can be used for commercial cultivation after evaluating them for yield and quality. Somaclones with improved characters or traits can be generated through application of mutagens especially EMS.

Application of research

Somaclones with improved characters or traits generated through application of mutagens especially EMS can be utilized as parents in breeding program to improve the traits.

Research Category: Genetics and Plant Breeding

Abbreviations:
EMS: Ethyl Methane Sulphonate
2, 4D: 2,4 Dichlorophenoxy Acetic Acid
µM l^{-1}: Micro molar per litre
mg l^{-1}: Milligrams per litre
ml l^{-1}: Milli litr per litre

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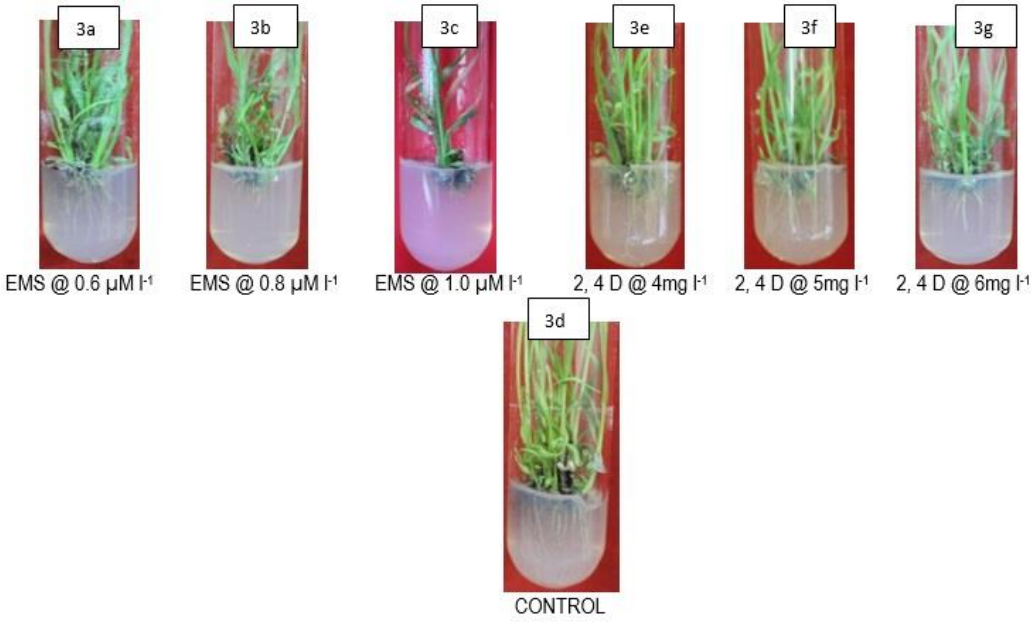

Fig-3 Root induction in different concentrations of chemical mutagens with control in 2003V46

Test tubes showing root growth under different concentrations of chemicals compared with control. Control showed superior performance in all rooting traits. EMS and 2, 4D at higher concentrations inhibited root growth and development. [**Fig-3a].** EMS @ 0.6 µM l^{-1}; [**Fig-3b].** EMS @ 0.8 µM l^{-1}; [**Fig-3c].** EMS @ 1.0 µM l^{-1}; [**Fig-3d].** Control; [**Fig-3e**]. 2,4D @4mg l^{-1}; [**Fig-3f].** 2,4D @ 5mg l^{-1} and [**Fig-3g].** 2,4D @ 6 mg l^{-1};]. With the increase in the concentration of the mutagen there was an increase in number of days required for root initiation, reduced root frequency (%) and reduction in root length. 2, 4D @ 4 mg l^{-1} recorded maximum root length, highest rooting frequency compared to all concentrations of chemicals. EMS @ 1.0 µM l^{-1} showed inhibitory effect on rooting.