Original Research Article

DROUGHT STRESS IN SUGARCANE: *IN VITRO* MUTAGENESIS AND SELECTION OF POLYETHYLENE GLYCOL (PEG) TOLERANT CALLUS LINES

ABSTRACT

Drought is one of the major abiotic stresses that cause a decline in the productivity of sugarcane which is one of the thirstiest crops in the world. An *in vitro* selection system for drought tolerance using a mutated sugarcane callus line was developed. This study was conducted with the aim to obtain putative sugarcane mutant callus lines chemically mutated with sodium azide at different concentrations (0, 0.5, 1.0 and 1.5%) which were then screened with high molecular weight PEG 6000 as a selection agent at different concentrations (0,5, 10, 15, 20, 25 and 30%). It was evident from the experiment that the percentage of live callus and sensitivity index decreased with increasing concentration of mutation and PEG dosage with little to no live callus at the highest concentration of mutation and PEG. Biochemical analysis of the survived callus for proline accumulation and salicylic acid content showed an increased level of up to 20 per cent PEG and 1.0 percent mutagen and a rapid decrease with further increase in PEG and mutagen concentration. The results can be further utilized for developing drought tolerant sugarcane lines and putative mutants and their *in vitro* screening.

KEYWORDS: Sugarcane, Callus, Sodium azide, Mutation, PEG, Selection, Proline, Salicylic Acid

INTRODUCTION

Sugarcane is an important agricultural crop renowned for the production of sugar and other by-products such as ethanol all over the world (Misra *et al.*,2020) and covering more than 5 million hectares, India is one of the world's top sugarcane producing countries. *Saccharum officinarum* L. is considered a primitive wild species that originated in India and is extensively dispersed in tropical and subtropical climates. India's cane-producing region has been divided into two zones: one is in the subtropical region, which includes the states of Bihar, Haryana, Orissa, Punjab, Rajasthan, and Uttar Pradesh, and the other is in the tropical region, which includes the states of Andhra Pradesh, Gujarat, Karnataka, Kerala, and Tamil Nadu.

The life cycle of sugarcane, a C_4 plant, lasts for roughly a year and includes four growth phases: germination, tillering, grand growth, and maturity. This suggests that it experiences all of the seasonal weather changes throughout the year (Shrivastava *et al*., 2016). At every stage of the crop's life cycle, abiotic stresses have the potential to hinder growth and development. Drought is a serious issue in sugarcane, impacting productivity mostly through morpho-physiological impacts that limit growth and photosynthesis (Silva *et al*., 2008) and is responsible for biomass and cane yield reductions (Zhao and Li, 2015) and different sugarcane genotypes differ in their reactions to drought stress, making it even more important to develop drought-tolerant genotypes.

Drought-tolerant sugarcane cultivars can be developed by traditional and novel approaches *viz.*, hybridization, mutation, *in vitro* breeding, genetic engineering, or a combination of the aforementioned goals. The complicated genomes, low fertility, and lengthy selection cycle of sugarcane pose challenges to the usual approach of hybridization and above all, the high polyploidy level of sugarcane. Plant tissue culture is regarded as an effective technology for crop improvement in a short period. Callus culture is an innovative method that treats cultivated cells as independent selection units rather than the entire plant. This method entails choosing tolerance cell lines from a population of dedifferentiated mass of cells (callus), subjecting the callus to appropriate selection pressure, and then regenerating tolerant plants.

Mutation induction has the ability to increase genetic diversity, and when combined with *in vitro* or *in vivo* selection, has resulted in new genotypes that are tolerant to drought, salt, aluminium, pests, and diseases in a variety of crops. Introducing physical and chemical mutations in plants are two of the many approaches for producing mutants, with physical mutagens such as x-ray radiation or gamma rays and chemical mutagens such as sodium azide, colchicine, and EMS. Sodium azide was found to be one of the potent chemical mutagens which create point mutations. Mahmud *et al.* (2016) conducted an experiment to study the effect of sodium azide in sugarcane callus and they saw there was an increased plantlet regeneration by sodium azide treated calli. Since callus cells are meristematic, they are therefore more sensitive to radiation and exposure to mutagens than adult cells. Using specific selective agents in the *in-vitro* selection process, it is possible to select mutants. Hartati *et al.* (2021) conducted similar studies in sugarcane and came to the conclusion that appropriate mutation dosage will result in production of drought tolerant mutants.

Drought-tolerant varieties are selected *in-vitro* employing a selective agent in the form of an osmotic chemical that can imitate field drought conditions. Poly Ethylene Glycol (PEG) is the most popular osmotic chemical used to combat drought stress. Abbas *et al.* (2014) used PEG to induce drought stress analysed the biochemical characters of sugarcane and found that drought stress induced changes are reversible, at the cellular level in sugarcane. PEG with a high molecular weight (6000–8000) has been used for a long time as a non–penetrating non–ionic inert osmoticum to reduce the water potential of nutrient solutions without being absorbed or poisonous to plants. It was observed by Wekanthia *et al.* (2021) that mutagenesis followed by evaluation using PEG *in vitro* as well as greenhouse were successful in developing new sugarcane with high drought tolerance. The aim of the present study is to select sodium azide mutated callus lines tolerant to PEG and characterize them based on their proline, salicylic acid content and compare them to non-mutated callus lines in the variety Co 86032.

MATERIALS AND METHODS

Callus induction

Sugarcane variety, Co-86032 was utilized in the current study. The ICAR-Sugarcane Breeding Institute, Coimbatore experimental field provided the sugarcane leaf sheath explants, which were taken from field-grown sugarcane that was 8 to 10 months old. The explants were rinsed thoroughly under running tap water for 10 minutes, followed by Bavistin (Carbendazim 50% WP) 0.1% for 10 minutes, and then washed with sterile distilled

water before being transported to a laminar airflow cabinet. The immature leaf sheath explants were first treated with ethanol (70%) for a minute, then with ice cold mercuric chloride ($HgCl_2$, 0.1 percent (w/v)) for another 5 minutes and finally thoroughly washed with ice cold sterile water 3 to 5 times. Murashige and Skoog's (1962) medium supplemented with 30 g/l sucrose, 8 g/l agar, 3 mg/L 2, 4-D, 100mg myo-inositol, and 10% coconut water was used for callus induction. The medium was autoclaved at 120 °C and 15 lb pressure for 20 min after being pH-adjusted to 5.8. Cultures were kept at 26± 1 °C with a 16/8hour light dark cycle. The calli were again sub cultured into the medium of same composition after two weeks of development.

In vitro chemical mutagenesis

Mutation in general aims to broaden sugarcane's genetic diversity. The chemical mutagen sodium azide (NaN_3) will induce the preferential generation of point mutation of AT to GC. Calli obtained from the best 2,4-D concentration (3 mg/L) were subjected to various concentrations of sodium azide mutagen (0, 0.5, 1.0 and 1.5 mg/l). The callus obtained were cultured on MS media supplemented with different concentrations of sodium azide for 5 days. After the mutation period, the mutated callus was transferred to MS media containing just 3 mg/L 2,4-D. Further, the callus was maintained by frequent and periodic sub culture for every 2 weeks. The observations recorded were percentage of live callus, and sensitivity index (SI) that has been worked out as follows:

$$\text{Sensitivity index (SI)} = \frac{\text{Percentage of live callus on PEG media}}{\text{Percentage of live callus on non-PEG media}} \text{ X } 100\ \%$$

***In vitro* selection**

In vitro selection was performed utilising a drought-selecting chemical, PEG 6000, by culturing the mutated callus on MS media infused with PEG 6000. For the preparation of PEG-infused media, PEG at different concentrations (0, 5, 10,15, 20, 25 and 30 w/v) was dissolved in sterile water and sterilized using a 0.22µm microporous membrane (Osmolovskaya.,2018) which was poured on MS medium. PEG-infused agar medium (50 mL) was poured into sterile bottles and kept for 2 days, allowing PEG-6000 to fully penetrate into the solid medium after which the PEG was discarded and subsequently used for imparting drought stress. Twenty five non mutated and mutated calli were transferred to the bottles with different concentrations of PEG 6000 to initiate selection shock. This concentration of PEG was chosen based on the report that at 20 % PEG, the growth of the callus decreased considerably and at 30 % PEG, there was complete death of the callus occurred (Jabeen,2007). After sub cultures for 40 to 45 days, most of the calli became dark brown as the concentration of both mutagen and PEG increased except few which remained light in colour. Calli which were actively growing at this stage were considered mutated PEG tolerant and further used for characterization. Biochemical analysis was done for 30 days old mutated callus. All the experiments were done in three replications and the average values were recorded.

Estimation of proline

The free proline concentration was calculated using the method reported by Bates *et al.* (1973). Callus (500 mg) was homogenized in aqueous sulfosalicylic acid at a concentration of

3 percent (w/v) and centrifuged at 1,000 g for 10 min. The filtered homogenate was combined in equal parts with acetic acid and acid ninhydrin, and the reaction was carried out at 100 ℃ for one hour before being stopped on an ice bath. Four ml of toluene was added to the reaction mixture and thoroughly stirred before extraction. After being removed from the aqueous phase, the toluene-containing chromophore was warmed to room temperature. Using toluene as a blank, the absorbance of the proline-ninhydrin product was measured at 520 nm. The proline content was represented as μg^{-1} FW.

Estimation of salicylic acid (SA)

One gram of fresh callus was homogenized by using ethanol and mixed thoroughly followed by centrifugation at 10,000 g for 10 minutes. The supernatant was collected and stored on ice for SA measurement. For the measurement of the amount of SA present in the sample, the respective sample and standard solution was taken and to each add 1 % ferric chloride and sterile water to make the volume up to 10 ml. Finally, the absorbance of the violet-coloured complex was measured using a UV-VIS spectrophotometer at 523 nm and SA content was represented as mg/10ml.

Statistical Analysis

The experimental design used was a factorial completely randomized design with the first factor as the concentration of PEG 6000 (0, 5, 10, 15, 20, 25 and 30%) and the second factor was the concentration of sodium azide (0, 0.5, 1.0, and 1.5%) Each treatment consisted of 3 replications, and each replicate consisted of 25 calluses. Data were analyzed using WASP 1.0 program. The values are mean ± SE for three samples in each group at a significance level of 5%.

RESULT AND DISCUSSION

In vitro mutation and callus growth

Mutation with sodium azide in combination with PEG at varied concentrations has caused a significant effect on the growth and development of callus which is indicated by the colour changes. Non-mutated callus was distinguished with whitish colour which gradually changed to yellowish-white colour with the increase in mutagen concentrations. In case of succumbing callus due to high mutagen and PEG concentration, the colour was dark brown to black which indicated the death of the callus which is substantiated when the callus sub cultured in again media without any mutagen content, there was hardly any revival of the callus.

The damage that occurred in calli due to the mutagen sodium azide from 0.5 to 1.5% at varying levels of PEG concentrations was indicated by the decrease in the percentage of live callus which is evident in Table 1. At 0.5 % mutation dose, percentage of live callus decreased from 96 to 12% for different concentrations of PEG (0 to 25%) at the same time there was a decrease of 94 to 6 % for the same concentration of PEG. However, at 1.5% mutagen dose, callus growth observed was only at 5 % PEG and for all other concentrations, there was no live callus. The percentage of live callus fluctuates with an increase in mutation and PEG concentration. The PEG treatment causes damage in both mutant and non-mutant callus lines. At the same mutation level but with an increase in PEG dosage caused more damage than an increase in the mutation dose at the same level of PEG which is indicated by the percentage of live callus

Table 1: Effect of PEG and mutation at varying concentrations on the percentage of live callus

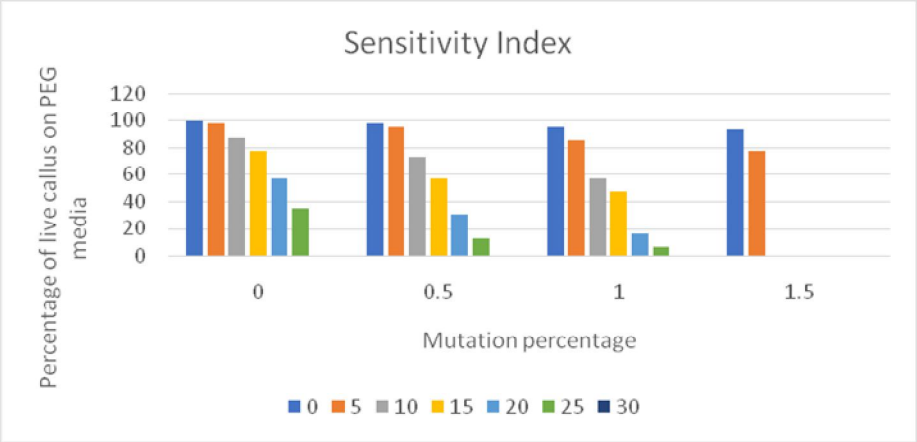
PEG Concentration(w/v)	Mutation percentage (%)			
	0	0.5	1.0	1.5
0	98	96	94	92
5	96	94	84	76
10	86	72	56	0
15	76	56	46	0
20	56	30	16	0
25	34	12	6	0
30	0	0	0	0

The sensitivity index (SI) indicates the growth inhibition as a result of PEG-infused media. Non-mutated callus showed higher growth inhibition than the mutated callus lines as indicated in Table 2. The SI values for non-mutated callus lines were in the range of 99.49% at 0% PEG to 34.51% at 25% PEG concentration. At 0.5 % of mutation, SI value varied from 97.46 to 12.18 and for 1.0% of mutation, the same was found to be 95.43 to 6.091%. However, for 1.5% level of mutation, observable live callus was found only at 5% PEG. Irrespective of the mutagen doses, SI was found to be zero at 30% PEG dosage since there was no live callus line.

Table 2: Effect of PEG and mutation at varying concentrations on the sensitivity index of live callus

PEG Concentration (w/v)	Mutation percentage (%)			
	0	0.5	1.0	1.5
0	99.49	97.46	95.43	93.40
5	97.46	95.43	85.27	77.15
10	87.30	73.09	56.85	0
15	77.15	56.85	46.70	0
20	56.85	30.45	16.42	0
25	34.51	12.18	6.091	0
30	0	0	0	0

Figure 1: Representation of SI based on different PEG concentrations at various mutation doses.



Selection of mutated PEG tolerant callus

Callus lines obtained after mutation with different concentrations of sodium azide (0, 0.5, 1.0, 1.5 %) were transferred to different concentrations (0, 5, 10, 15, 20, 25, and 30%) PEG-infused media. It was noticed that with increasing concentration of PEG resulted in a progressive reduction of live callus as well discolouration of the callus from yellowish white to dark brown colour as well as death of callus at 30% PEG (Figure 2). However, there were some patches of live callus seen surviving up to 25% of PEG with varied mutation doses. Those callus lines were considered mutant PEG tolerant callus lines. Those patches of callus line surviving were sub-cultured and maintained for further analysis and study. Rao and Jabeen (2013) during their experiment also came across similar results while screening for PEG tolerant callus lines. It was also observed by Kumar *et al.* (2011) and Aazami *et al.* (2010) that the addition of PEG to the medium produces osmotic stress and decreases the water potential which negatively affects growth.

Figure 2: Callus after treatment with different concentration of PEG (0, 5, 10, 15, 20, 25, and 30%)

Proline content

Proline content was analyzed for non-mutated and mutated callus at different concentrations of PEG supplemented media and it was found that proline levels were higher in mutated calli than for the non-mutated ones (Table 3). Proline content increased with an increasing concentration of PEG up to 20 % and then there was a sharp decrease in proline content at 25 % and no proline content was detected at 30% PEG concentration, however, mutated calli maintained higher levels of proline than the non-mutated ones at any given PEG

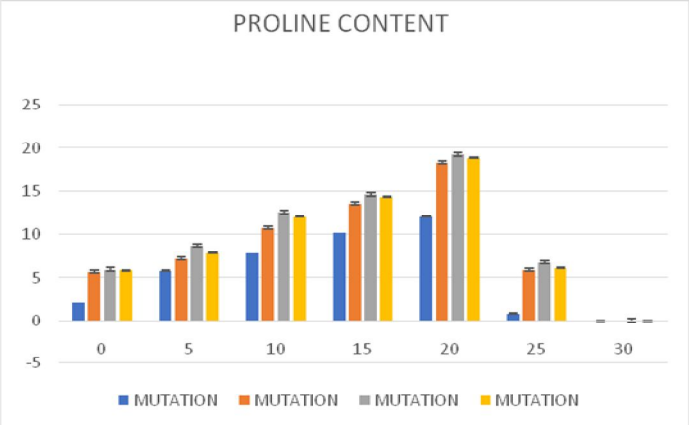
concentrations. Apart from the build up of proline to different concentrations of PEG, there was a significant difference in the proline build up with varied concentrations of mutagens. As the mutation percentage increases from 0.5 to 1.0, there was increase in the proline content but as the mutagen concentration increased to 1.5% the growth of calli as well as the proline accumulation decreased.

Table 3: Effect of PEG on proline content of mutated and non-mutated callus at different concentrations of PEG and mutation

PEG Concentration %	Mutation Percentage (%)			
	0	0.5	1.0	1.5
0	2.095±0.08^{e}	5.73±0.07^{e}	5.94±0.09	5.85±0.02
5	5.793±0.02^{d}	7.17±0.02^{d}	8.71±0.33	7.83±0.07
10	7.821±0.04^{c}	10.775±0.14^{c}	12.53±0.29	12.10±0.05
15	10.179±0.15^{b}	13.58±0.41^{b}	14.59±0.38	14.34±0.11
20	12.101±0.12^{a}	18.35±0.43^{a}	19.26±0.17	18.86±0.11
25	0.8323±0.01^{f}	5.905±0.04^{f}	6.76±0.07	6.15±0.04
30	ND	ND	ND	ND

Treatments found Significant at 1% and 5% level of significance

Figure3: Representation of effect of PEG and subsequent changes in proline content in mutated and non-mutated callus



This describes how proline build up helps calluses survive and grow when there is a drought. During drought stress, proline levels in sugarcane increased, according to Kumar *et al.* (2019). Similar trends in proline build up of PEG-tolerant calli were discovered by Rao and

Jabeen (2013). According to Shah *et al.* (2012), the 20% PEG-selected calli of rice had a 17-fold higher proline concentration than the non-selected calli. The primary understanding of proline accumulation under water deprivation is as an osmotic agent (Handa *et al.*, 1986).The mutated callus exhibited osmotic adjustment in response to PEG stress through the production of proline better than the non-mutated callus.

Salicylic acid content

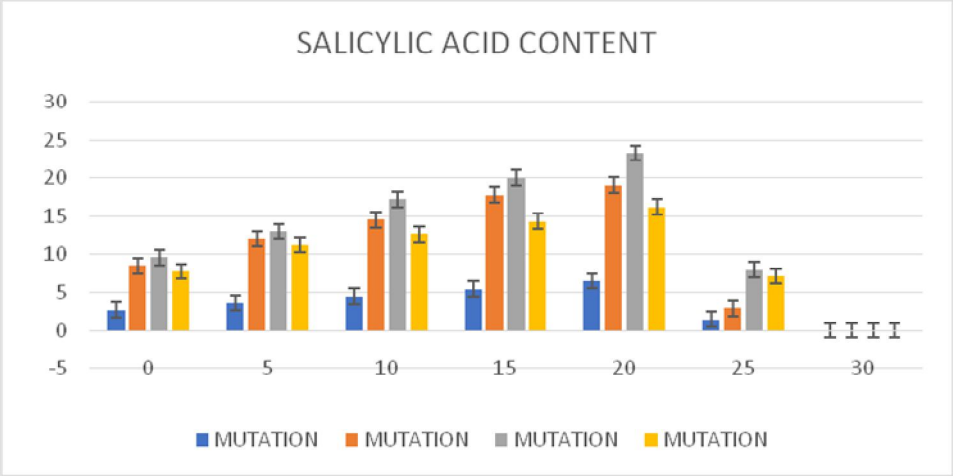
Mutated callus lines accumulated more salicylic acid when compared to the non-mutated callus line also as there was also an increase in SA levels with an increase in mutation percentage as well as PEG concentration but with a sharp decrease at a higher concentration of mutation at 1.5% and 25 % of PEG concentration respectively with no detectable SA level at 30% of PEG (Table.4). However, at any given concentration of PEG, mutated callus showed higher levels of SA when compared to non-mutated callus lines.

Table4: Effect of PEG on salicylic acid content of mutated and non-mutated callus at different concentrations of PEG and mutation

PEGConcentraion(%)	Mutation Percentage (%)			
	0	0.5	1.0	1.5
0	2.769±0.13	8.512±0.13^{e}	8.512±0.13	8.512±0.13
5	3.665±0.13	12.045±0.2^{d}	12.045±0.2	12.045±0.2
10	4.477±0.02	14.519±0.22^{c}	14.519±0.22	14.519±0.22
15	5.448±0.07	17.758±0.16^{b}	17.758±0.16	17.758±0.16
20	6.48±0.05	19.024±0.1^{a}	19.024±0.1	19.024±0.1
25	1.503±0.15	2.976±0.12^{f}	2.976±0.12	2.976±0.12
30	ND	ND	ND	ND

Treatments found Significant at 1% and 5% level of significance

Figure 4: Representation of effect of PEG and subsequent changes in SA content in mutated and non-mutated callus



Salicylic acid was found to be increasing the leaf area of sugarcane to alleviate the drought stress imposed (Khodary, 2004). Tripathi et al. (2019) also found similar trends in salicylic acid content in sugarcane. Lower levels of salicylic acid at a higher concentration of PEG in non-mutated callus may be due to the necrosis of the callus in response to PEG and higher mutagen concentration.

CONCLUSION

From the present investigation, it can be concluded that there was an increase in the drought tolerance when the mutation dosage reached 1.0 percent while there was a simultaneous increase in the PEG concentration up to 20 percent PEG for the sugarcane variety Co-86032. So, sugarcane drought tolerant callus lines can be developed by imposing mutation followed by the screening of the same in PEG-infused media. It can be substantiated by biochemical analysis of proline and salicylic acid content, which shows increased activity during drought stress in mutated calli than the non-mutated callus lines and are responsible for the better growth under stressed conditions.

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