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RESEARCH ARTICLE

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Screening of EMS induced drought tolerant sugarcane (*Saccharum* spp. Complex) mutants employing physiological, molecular and biochemical approaches

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Abstract

The *calli* were mutagenized with 0.5% EMS and exposed to 2% (w/v) PEG-6000 for induction of the osmotic stress. *Calli* that survived after *in vitro* osmotic stress treatment put on MS media for regeneration of plants. Regenerated plants were then subjected to preliminary greenhouse pot trials to confirm drought stress tolerance. In the present study leaf area, chlorophyll content, photosynthesis rate, shoot length, root length, fresh weight and dry weight decreased with an increase in osmotic stress in tolerant mutants and normal plants, but there is less decrease in leaf area (7.5%), no. of leaves (19.9%), chlorophyll content (22.0%), photosynthesis rate (141.5%), stomatal conductance (10.9%), shoot length (9.7%), root length (4.79%), fresh weight (28.9%) and dry weight (3.8%) in tolerant mutants as compared to normal plants at higher level of osmotic stress. The transpiration rate (6.3%) was low in tolerant mutant. Proline content (7.0%) increased highly in tolerant mutant at higher water stress. RAPD primer OPK-10 produced maximum polymorphism (100%) followed by primer OPK-04 (91.67%), OPK-15 (88.89%) and OPL-03 (88.89%). Modification in all the physiological traits may be useful to use in breeding for improving drought tolerance in sugarcane.

Key words: *In vitro*, mutagenesis, drought tolerant, molecular analysis

Introduction

Drought is one of the most important environmental constraint limiting sugarcane (*Saccharum* spp.) production worldwide (Priji and Hemaprabha 2014, Basnayake et al. 2012; Gomathi et al. 2020). An urgent demand to overcome drought is critical to ensure sugarcane production. Drought stress is one of the major environmental stress factor that cause biochemical alterations in plants, reduce plant growth, and decrease plant yield (Gupta et al. 2020). Breeding for drought is difficult due to polyploidy nature of sugarcane and hence the intervention of mutagenesis and tissue culture greatly facilitate the selection and isolation of useful tolerant lines (Philani et al. 2021). Mutation breeding is one of the promising tool available to produce stress-resistant plants, with the induction of new alleles due to point mutation within the existing sugarcane germplasm. The mutation is the process, in which genes are permanently alternated under environmental conditions while being transferred between generations. As also to these alternations in nature, developing science also have provided a chance for mankind to create artificial mutations by using multi techniques (Chaudhari et al. 2018). Sundaram et al. (2010) has recently reported about broadening the genetic base of sugarcane through the introgression of resistant genes by intergenetic hybridization and the limitations with molecular analysis. Since conventional plant breeding methods are found to be slow to create substantial improvement, attempts were made to introduce genetic variability in sugarcane by *in*

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vitro culture techniques and mutation breeding (Patade et al. 2006).

In the future, drought is expected to increase, due to climate change in most parts of the world (Khalil et al. 2016; Tayyab et al. 2018). Hence, it is an urgent need, at this time, to breed cultivars with enhanced drought tolerance and high water-use efficiency, which can be achieved by employing both conventional plant breeding and genetic engineering (Rauf et al. 2016). The present study describes the development of sugarcane mutants from Co 99004 with drought tolerance and other physiological traits by using *in vitro* mutagenesis and screening technique.

Materials and methods

Present study was carried out at the Sugarcane Tissue culture Laboratory, Main Sugarcane Research Station, Navsari Agricultural University, Navsari, Gujarat during 2011-2014. The commercial sugarcane cultivar Co 99004 used as the source of explants.

In vitro mutagenesis and drought screening

Calli were established from the smaller pieces of explants, made on Murashige and Skoog medium, supplemented with 20 g/l sucrose, 7.5 g/l agar and 4 mg/l 2,4-D. Drought tolerant plantlets were regenerated from mutagenized callus with EMS (LD_{50}) on MS medium supplemented with the PEG (LD_{50}) as per method given by Kanganal et al. (2008) (Plate 1).

In vivo evaluation

Drought tolerant mutants rose from treatment EMS (0.5% (LD_{50}) + PEG (LD_{50}) and parent plants (somaclones from non-treated callus) were evaluate for drought tolerance in pot culture with three water stress treatment of *i.e.,* 7, 10 and 13 days irrigation interval. The data generated from the experiments were subjected to statistical analysis in Factorial Completely Randomized Design (FCRD) whenever, necessary as prescribed by Panse and Sukhatme (1985).

Use of RAPD markers and PCR amplification

Total DNA was extracted from the leaves by cetyl trimethyl ammonium bromide (CTAB) method as described by Khan et al. (2013) with some minor modifications. The genomic DNA amplified using random primers of OPK and OPL series (Operon Tech., California, USA). PCR reactions for RAPD were carried out in a reaction volume of 25 µl using the method given by Rashed et al. (2008) with some modifications. The band profiles obtained from gel electrophoresis were visualized using Biorad Chemi Imager gel documentation system. The polymorphism percentage was calculated as per the method suggested by Blair et al. (1999). The data generated by RAPD were analyzed with the software NTSYSpc version 2.02.

Results and discussion

In vitro mutagenesis and screening

Sugarcane callus culture exposed to 2 hour treatment of 0.5% EMS showed 47.8 % survival response compared to control non-treated culture (Fig. 1). There was significantly decrease in survival percentage of callus with increase in exposed hour as has been reported earlier in sugarcane (Kanganal et al. 2008; Mallikarjuna et al. 2018). Khalil 1 et al. (2018) determined median lethal dose for mutagenic treatment of ROC22 *calli* was 0.1% EMS for 17 h while, for FN39 *calli*, it was 0.1% EMS for 14 h. Percentage of viable callus of VMC 7616 was lower than that of PS 862 which had higher tolerance to EMS treatment. Callus was still viable after it was soaked in 0.1% EMS solution for 60 minutes, and changed to brown if time and EMS concentration increased (Purnamaningsih and Hutami 2016). Induced mutagenesis offers a useful method to improve desirable characters in sugarcane (Dalvi et al. 2021). In present study *calli* survived in EMS treatment put on MS media containing to 2% PEG shown almost 47.2% survivals as compared to control non-treated *calli* (100%) as shown in Fig. 2. When 3% PEG was added in media there was lowest callus survival percentage (20.2%). Mhlanga (2015) reported that, at 87 mM PEG-6000, NCo376 plantlets showed 50% root re-growth as compared to 10% in non-aerated cultures. Srinath and Jabeen (2013) reported that 20% PEG decreased the growth of callus considerably. The *in vitro* screening technique of cultivars tolerant to PEG-induced water stress is an alternative for early determination of drought stress in sugarcane (Perez et al. 2021).

Physiological and biochemical studies

Leaf area, chlorophyll content, photosynthesis rate, shoot length, root length, fresh weight and dry weight significantly decreased with increase in osmotic stress in tolerant mutants and normal plants (Table 1). In the treatment T_3 mutant plants recorded significantly higher leaf area (310.6 cm^2) than

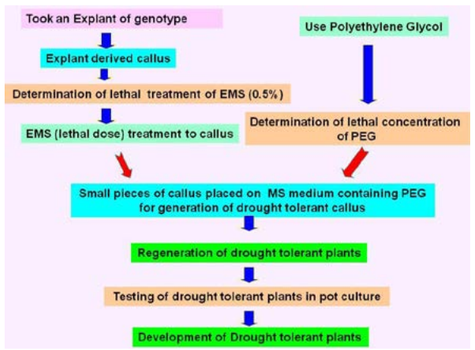

Fig. 1. Flowchart of application in vitro selection pressure for development of drought tolerant mutants in sugarcane

Fig. 2. Effect of water stress on growth of sugarcane plantlets Co 94012

normal plants (289.0 cm^2). In the tolerant mutants, numbers of leaves (5.18) were significantly higher as compared to the normal plants (4.32) at higher water stress level. Shoot length was significantly higher in tolerant mutants (36.18 cm) as compared to normal plants (32.97 cm) in case of treatment T_3. Root length was significantly higher in tolerant mutants (35.78 cm) as compared to normal plants (28.28 cm) in treatment T_2. Significantly less decrease in leaf area (7.51%), number of leaves (19.91%), shoot length (9.74%) and root length (4.79%) in tolerant mutants as compared to normal plants at higher level of osmotic stress was recorded. Maintenance of root growth during water deficit can significantly contribute to yield stability under water stress (Awakale et al. 2020). Marchiori et al. (2017) reported that increase in root growth improved soil volume exploration, indicating an important morphological response under marginally reduced water availability. Water stress significantly reduces number of green leaves, length and width of leaves and root parameters (Wagih et al. 2001; Begum et al. 2012). Under water deficit conditions, the average chlorophyll content was significantly lower than the well-watered conditions. Drought induced stress significantly decreased chlorophyll concentration (Khalil et al. 2018; Kumar et al. 2019; Dniele et al. 2021).

There was significantly less decrease in chlorophyll content (22.0%), photosynthesis rate (41.5%) and stomatal conductance (10.9%) in tolerant mutants as compared to normal plants of sugarcane at higher level of osmotic stress (Table 1). Photosynthetic parameters, relative water content and above-ground biomass were higher in drought tolerant accession in soybean (Kumar 1 et al. 2020). Stomata respond to both external (light, VPD, and temperature) and internal (hormones and water potential) cues to maintain hydraulic conductivity in the soil-plant continuum and control gaseous exchange between the leaf and atmosphere to balance the demands of evaporative cooling and photosynthesis (Vadez et al. 2014). Under moderate water stress a decrease in stomatal conductance, transpiration rate and photosynthetic rate occurs, mainly due to stomatal limitations (Medeiros et al. 2013; Basnayake 1 et al. 2015).

The development of above-ground biomass in plants is mainly contributed by photosynthesis. Therefore, the use of genetic variation for enhanced chlorophyll content index functions to increased crop biomass. Liu et al. (2019) in a study on simulation of yield prediction reported that the leaf chlorophyll content index, which is used as a biochemical photosynthetic component improves the accuracy of prediction and modelling of crops under specific agro-ecosystems, and it may also improve projections of above ground biomass and help increase the yield indirectly. It is well understood that crope above-ground biomassis highly affected by genetics, physiology and environmental factors.Crop AGB is affected by genetics, physiology, and environmental factors (Mavromatis et al. 2002), and it is usually regarded as an index for estimating yield and economic benefits in agricultural ecosystems. Miri (2009) reported that chlorophyll content index was significantly and positively correlated with grain yield and a harvest index of wheat in Iran. Similarly, leaf photosynthesis is a significant factor for grain yield and biomass (Yamori et al. 2016). In contrast, few studies have focused on the relationship between photosynthetic capacity of crops and biochemical photosynthetic components such as leaf chlorophyll or carotenoid content to develop a biomass or yield model (Houborg et al. 2013).

Usually, transpiration and stomatal conductance decreases drought stress and it acts as one of the first responses of plants to drought is stomatal closure, restricting gas exchange between the atmosphere and the inside of the leaf. The effect of drought stress on transpiration was very similar to that on photosynthesis. Lowest transpiration rate (5.88%) was observed in tolerant mutant than normal plants at higher water stress condition. Leaf chlorophyll content (SPAD index), leaf and canopy temperature, photosynthesis rate, stomatal conductance (g_s), canopy conductance (g_c), and transpiration rate (E) are also used as an indirect selection criteria for sugarcane genotypes tolerant to water stress (Basnayake et al. 2015; Ferreira et al. 2017).

Table 1. Physiological and biochemical response of sugarcane mutants to water stress in pot culture

	Treat.	Irrig. Int. (days)	Leaf area (cm^2)	% inc. over parent	No. of leaves	% inc. over parent	Shoot length (cm)	% inc. over parent	Root length (cm)	% inc. over parent	Fresh weight (g)	% inc. over parent	Dry weight (g)	% inc. over parent	Pro-line content ($\mu g\ g^{-1}$ FM)	% inc. over parent	Chlo. content index	% inc. over parent	Photo. rate (µmol CO_2 m^{-2} s^{-1})	% inc. over parent	Stom-atal conduc-tance (mol CO_2 m^{-2} s^{-1})	% inc. over parent	Trans-pira-tion rate (m mol H_2O m^{-2} s^{-1})	% inc. over parent
Mutant plants	T_1	7	436.6		8.5		91.03		51.32		32.92		11.02		33.83		49.83		5.19		20.14		0.42	
	T_2	10	383.8	18.3	6.58	2.5	59.84	7.6	35.78	26.5	20.83	16.2	6.47	14.7	77	9.0	49.9	17.4	3.15	15.8	13.04	10.5	0.25	-21.9
	T_3	13	310.7	7.5	5.18	19.9	36.18	9.7	22.11	4.8	14.21	28.9	4.39	3.8	156.33	7.0	42.92	22.0	1.28	141.5	7.63	10.9	0.15	-6.3
Normal plants	T_1	7	437		8.52		91.44		50.35		32.7		11.02		35.83		46.97		5.22		20.07		0.41	
	T_2	10	324.4		6.42		55.59		28.28		17.92		5.64		70.67		42.5		2.72		11.8		0.32	
	T_3	13	289		4.32		32.97		21.1		11.02		4.23		146.17		35.17		0.53		6.88		0.16	
SEM ±	M	4.06		0.06		0.53		0.03		0.14		0.01		0.45		0.03		0.01		0.01		0.01	
	N	4.97		0.08		0.65		0.04		0.17		0.01		0.55		0.04		0.01		0.01		0.01	
	MXN	7.03		0.11		0.92		0.06		0.24		0.01		0.77		0.06		0.01		0.01		0.01	
CD at 5%	M	12.05		0.19		1.57		0.10		0.41		0.01		1.32		1.03		0.12		0.51		0.01	
	N	14.76		0.23		1.92		0.12		NS		0.01		1.62		1.27		0.17		0.02		0.02	
	MXN	20.87		0.33		2.72		0.17						2.29		1.79		4.08		4.45		1.99	
CV %	4.32		3.74		4.59		3.78		4.39		3.81				2.20		4.12							

Mutant plants = in vitro screened tolerant mutant plant, Normal plants = Parental plants of variety. Treat. = Treatmebt, Irri. = Irrigation, Int. Interval, inc. = Increase, Chlo. = Chlorophyll, T1 - 7 days irrigation interval, T2 - 10 days irrigation interval, T1 - 13 days irrigation interval

At higher water stress level significantly higher fresh weight 14.21 g/plant and dry weight was 4.39 g/plant was observed in tolerant mutants than normal plants. Medeiros et al. (2013) observed dry mass production decreased under drought stress in sugarcane. Jangpromma et al. (2012) discussed that selection of sugarcane cultivars with good root characters is possible in sugarcane cultivars for drought tolerance.

In general, under vegetative stage, drought stress increases proline content to a great extent thereby increasing osmotic compatibility and adjust osmotic potential which results in drought stress avoidance in chickpea. It is believed that it plays an adaptive role in plant stress tolerance (Verbruggen and Hermans 2008). Proline content at stress level T_3 (13 days interval) was significantly higher in tolerant mutants (156.33 µg g^{-1} FW) as compared to normal plants (146.17 µg g^{-1} FW). Proline content (6.95%) increased highly in tolerant mutant as compared to the normal plants at higher water stress. This results support the use of proline as a biochemical marker for the initial, large-scale screening of sugarcane cultivars to water stress (Sugenith et al. 2020; Dalvi 1 et al. 2021). Hemaprabha et al. (2013) revealed that in response to stress, proline accumulation increased by 94.53 % in the genotypes. Mafakheri et al. (2010) reported that drought stress affects proline content, chlorophyll content, photosynthesis and transpiration, stomatal conductance and yield characteristics in chickpea. The effect of drought stress was comparatively less on photosynthesis, transpiration, stomatal conductance and yield but sub-stomatal CO_2 concentration was lower, which affected the breeding value in many agronomical and physiological traits.

Molecular studies

The genetic diversity among drought-resistant mutant lines was further assessed by Randomly Amplified Polymorphic DNA (RAPD) markers amplification. Ten decamers oligonucleotide primers were used for a RAPD analysis. On an average each primer gave nine bands. The amplification products range from 0.1 Kb to 1 Kb. In genotype Co99004 (Fig. 3 and Fig. 4), primer OPK-4 produced maximum 12 bands (Table 2) out of which one was polymorphic. The primer OPK-20 produced 3 monomorphic bands out of total 9 bands. Primer OPK-10 produced maximum polymorphism (100%) followed by primer OPK-04 (91.67%), OPK-15 (88.89%) and OPL-03 (88.89%). RAPD analysis indicated EMS-induced point mutations resulting in specific rectifications without much change in the genetic backbone of genotype (Table 2, 2021). The present findings are in agreement with detection of genetic variation with RAPD analysis by Patade et al. (2006), Dalvi et al. (2012). RAPD marker are useful in detecting polymorphism in embryogenic culture subjected to chemical mutagenesis as they provide sufficient number of DNA fragments for conducting assay.

However, an in-depth understanding of chemically induced mutagenesis is needed to control the direction and nature of mutations, to further enhance our knowledge, the research area regarding high-throughput mutation screening technology and to improve a mutant's efficiency under various circumstances. *In vitro* screening for drought tolerance is an effective method of selection in crop like sugarcane. The utilization of such somaclones should enhance the diversities of varieties with high degree of drought tolerance suitable in different areas in the tropics and subtropics prune to drought. The technological innovations in molecular biology and biotechnology, the pace of gene discovery and expanding knowledge about plant and crop's response to water stress and climate change are expected to accelerate this area of research.

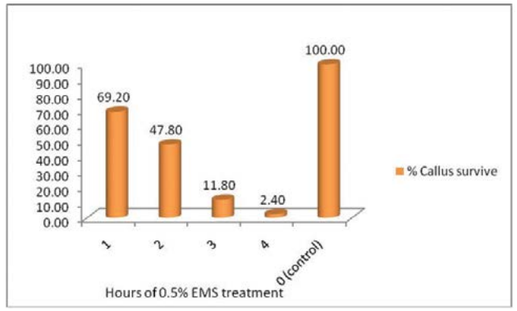

Fig. 3. Survival per cent of callus after 30 days to different EMS (0.5%) treatments to determine lethal dose (LD50) of EMS

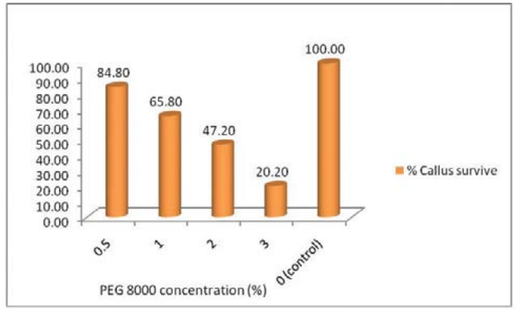

Fig. 4. Survival per cent of callus after 30 days to different treatments of PEG (8000) to determine lethal dose (LD50) of PEG

Table 2. Polymorphism analysis of sugarcane mutants using RAPD primers

S.No.	Primer name	Total loci (bands)	Poly-morphic (bands)	Mono-morphic loci	% poly-mor-phism (bands)	PIC value
1	OPK-03	5	4	1	80.00	0.7962
2	OPK-04	12	11	1	91.67	0.8728
3	OPK-09	10	8	2	80.00	0.8788
4	OPK-10	10	10	0	100.00	0.8727
5	OPK-11	8	7	1	87.50	0.8681
6	OPK-15	9	8	1	88.89	0.8737
7	OPK-18	7	5	2	71.43	0.8160
8	OPK-20	9	6	3	66.67	0.8668
9	OPL-02	10	8	2	80.00	0.8923
10	OPL-03	9	8	1	88.89	0.8628
Total		89	75	14		
Mean		**9**	**8**	**1**	**83.50**	**0.8600**

PIC = Polymorphic information content

Authors' contribution

Conceptualization of research (SSG, DUP); Designing of the experiments (SSG, DUP, AVN, DS); Contribution of experimental materials (DUP, SSG); Execution of field/lab experiments and data collection (SSG); Analysis of data and interpretation (SSG); Preparation of the manuscript (SSG).

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