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Genotypic response to callus induction and plant regeneration in sugarcane

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ABSTRACT

Genetic improvement of sugarcane using conventional breeding suffers setbacks due to its large and complex genome, photosensitivity, low seed setting, longer crop duration and complex environmental influence. However, in vitro culture system can play a greater role to improve yield, quality and make the crop climatically smart using genetic transformation. Significant differences were observed between genotypes for embryogenic and organogenic callus induction response and plant regeneration capacity. In the present study, somatic embryogenic callusing response (using MS + 2mg l^{-1} 2,4-D) and growth of calli with minimum necrosis was significantly higher in cv."Sabita". Besides, the variety showed maximum regeneration potential (87.8%) with more number of shootlets callus^{-1} on MS + 2mg l^{-1} BAP + 0.05mg l^{-1} TDZ. MS medium supplemented with 3.0mg l^{-1} of NAA revealed satisfactory rhizogenetic response (88.0%) with formation of normal roots within 14 days. The in vitro derived plantlets were acclimatized and transferred to the field successfully.

Keywords: *In vitro* culture, somatic embryogenesis, genotypic response and sugarcane

Sugarcane has economic importance and it contributes nearly 1.1% to national GDP in India (Solomon, 2014). Globally, it shares major quantum of sugar production with India being second next to Brazil. Besides, sugarcane is in vogue used as fodder, biosorbent, manufacture of paper board products and as such it contributes major share for biofuel (ethanol) as a fuel blend with petrol for running automobiles. However, the crop is sensitive to drought, salinity and a myriad of other abiotic stresses (Khaliq *et al.,* 2005 and Nasir *et al.,* 2000). High heterozygous genetic architecture, rare flowering, low fertility status, large genome size, long breeding cycle and high environmental interaction seem to be major limitations for conventional breeding of the crop. However, it responds well to *in vitro* culture. An efficient regeneration system is a priori to successful genetic transformation, micro-propagation and creation of in vitro induced genetic variants (somaclonal variation). A number of researchers have attempted for *in vitro* genetic manipulation of sugarcane to increase yield, sugar content and tolerance to biotic and abiotic stresses using young leaf whorl explants(Vickers *et al.,* 2005, Gilbert *et al.,* 2005, Gilbert *et al.,* 2009, Gao *et al.,* 2016, Kumar *et al.,* 2013 and Reis *et al.,* 2014). But, its application is limited by plant genotype and hormonal supplementation in the medium to sustain growth of calli, subsequent plant regeneration and survival of plants. Embryogenic calli are in vogue used for recovery of genotypically stable regenerants and for genetic transformation of valuable genes. To achieve this, selection of genotype(s) with efficient callus induction and plant regeneration ability is absolutely necessary. Therefore, the present investigation aims at identification of suitable cultivar(s) for efficient embryogenic callus formation and subsequent rapid plant regeneration *in vitro* amenable for follow-up step for micro-propagation and *Agrobacterium* mediated genetic transformation.

Five to six month old genetically pure clones of sugarcane (*Saccharum officinarum* L. 2n=80) cv. Phule 265 (salt tolerant), Co 86032 (drought tolerant), Sabita (CoOR03151), Raghunath (CoOr04152) and Co 6907 (Natl. Check) were considered for the present study at the Department of Agricultural Biotechnology, College of Agriculture, OUAT, Bhubaneswar-3 (India). The clones of the above varieties were maintained at a spacing of 1.5 × 0.5 m to serve as plant materials for *in vitro* culture. About 1.0 cm × 7 cm young leaf whorls from the mother plant were pre-sterilized in 70% ethanol for 1min followed by proper washing (3x) in double distilled water. These were finally sterilized with surface sterilant (sodium hypochlorite and teepol, 7:5) for 10min followed by treatment with 0.1% mercuric chloride ($HgCl_2$) for just 6 minutes and washing (5x) in double distilled water (5x). The leaf whorls were transversely sliced into 5-6 mm segments to serve as explants. The pH of the media was adjusted to 5.7-5.8 by adding 0.1N NaOH or 0.1N HCl drop by drop and then 0.8% agar was supplemented to the nutrient media. The media was warmed and approximately 20 ml was dispensed to glass test tubes (25 x 150 mm) followed by plugging with non-absorbent cotton. The cultures were autoclaved at 121°C and 15 psi for 20 min. After inoculation of leaf whorl sections to the media, culture tubes were incubated in the culture room under continuous dark for four weeks for initiation and growth of callus. Fresh calli induced

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were sub-cultured at 16/8h photoperiod for maintenance and plantlet regeneration. The culture room was set to 2000 lux light intensity for required photoperiod at constant temperature 25+1ºC and relative humidity 68%.

Callusing response was tested in modified MS medium (Murashige and Skoog, 1962) supplemented with 2mg l^{-1} 2,4-D (2,4-dichlorophenoxy acetic acid). MS media was modified (Ithape, 2017) with increased amount of nitrogen source (KNO_3), $FeSO_4$, glycine (10mg l^{-1}), inclusion of glutamine (500mg), tryptophan (100mg l^{-1}), proline (100mg l^{-1}), adenosine sulphate (100mg l^{-1}), casein hydrolysate (500mg l^{-1}). Callus clumps were placed on modified medium of MS containing 2mg l^{-1} BAP (6-benzylaminopurine) alone or 2mg l^{-1} BAP + 0.05mg l^{-1} TDZ (thiodizuron) for organogenic and somatic embryogenic plantlet regeneration. Entire process of the experiment was rechecked for conclusive results. The *in vitro* derived plantlets were grown on MS basal (half- and full strength) or MS containing 3.0-5.0 mg l^{-1} hormone recipe singly or combination of NAA and IBA (indole-3-butyric acid). For primary hardening, the shootlets with defined roots were held in autoclaved distilled water and maintained for at least three days in culture room followed by final transfer to pot mixture containing sterile soil, sand and vermiculite:: 1:1:2 (w/w/w) for growing in green house.

Each step of *in vitro* culture process was laid out in CRD (Complete Randomized Design) with twenty five replicates for callus induction and plantlet regeneration. Observations were recorded for frequency of calli induced (CIF %), their relative growth and extent of necrosis, embryogenic callusing response, regeneration ability and rhizogenesis. Data sets were analyzed statistically by the Duncan's multiple range test (Duncan, 1955). Means followed by the same letter within columns were considered not significantly different at P d" 0.05.

Callusing and plant regeneration response

Callus induction potential in the crop under study usually depend upon genotype used (Efendi and Matsuoka, 2011 and Kofer *et al.*,1998). In the present investigation, five elite genotypes e.g., Phule 265 , Co 86032, CoOr03151 (Sabita), CoOr04152 (Raghunath) and Co 6907 (National check) were considered for assessment of their callusing response. Among the genotypes, Phule 265 is a salt tolerant while Co 86032 is a popular drought tolerant cultivar in the state of Maharashtra. CoOr03151 (Sabita) and CoOr04152 (Raghunath) are the two recently released variety for the state of Odisha, while Co 6907 is popular for its wide adaptability and usually considered as national check in All India co-ordinated trials. Among the varieties, Sabita recorded highest callus induction frequency (88.7%) as well as highest somatic embryogenic callusing response and growth of calli with minimum necrosis (Table 1, Fig. 1a). Similarly, regeneration potential of the said genotype was shown to be excellent among the genotypes under study (Table 2). Gandanau *et al*. (2019) observed significant differences among nine sugarcane genotypes (CP57-614, CP63-588, CP59-73, CP80-314, CP70-321, Clone III, F160, L62-96 and SP71-1081) for capacity of callus induction, embryogenic callus production and plant regeneration response indicating clear genotypic control. In the present investigation, "Sabita" resulted 82.8% organogenic response with 12.2 plantlets callus^{-1} and satisfactory survival percentage (80.8%) upon transfer of fresh calli to MS + 2 mg l^{-1} BAP. Besides, embryogenic calli upon transfer to MS + 2 mg l^{-1} BAP + 0.05 mg l^{-1} TDZ resulted shoot initial as early as 5-6 days in case of cv. Sabita (Fig 1b). Sabita was considered to have better somatic embryogenic regeneration potential (87.8%) with appreciably higher number of shoots per responsive callus (Fig.1c) and highest plantlet survival during the process of hardening and plant establishment. Besides, callus induced in MS + 3 mg l^{-1} 2,4-D and subsequently incubated in MS basal was reported as a commercially viable step for regeneration on MS media with 1.0-1.5mg l^{-1} BAP + 0.2mg l^{-1} NAA for elite sugarcane genotypes (Abdu *et al.*,2012). However, Patel *et al.* (2015) revealed shoot initiation on MS with 1.0 mg l^{-1} BAP, 0.5mg l^{-1} IBA, 20g l^{-1} sucrose and 7g l^{-1} agar, and emergence of micro tillering on the media with 1.0 mg l^{-1} BAP , 0.25 mg l^{-1} GA3, 20g l^{-1} sucrose and 7g l^{-1} agar in case of sugarcane variety Co 86032 (a popular variety of peninsular India).

Rhizogenesis of in vitro derived plantlets

For better response of tissue cultured plantlets in the field condition, it is very important that the plantlets have more numbers of roots. Regeneration of adventitious roots has been tried by many workers using various auxins at varying concentrations. Plantlets developed on callus were transferred to full strength and half-strength MS basal medium to assess rhizogenetic potential. Besides, MS supplemented with NAA and IBA singly or their combinations at different concentrations were tried for root induction (Table 3). There was no rooting response by either full and half-strength basal formulations of MS. Out of different auxin concentrations in MS medium, 3.0 mg l^{-1} NAA revealed satisfactory rhizogenetic potential (88.0%) with normal rooting within 14 days (Fig 1d). Higher level of NAA beyond 3 mg l^{-1} did not show satisfactory response, but induced fibrous and delayed rooting with lower growth rate. On the other hand, MS + IBA at higher concentration (5 mg l^{-1}) elicited more or less appreciable rhizogenesis (71.2%) but fine slender roots in 18days. In this study, both auxins in combination did not reveal any dividend, rather had shown reduced root growth.

Table 1: Genotypic response to callus induction in modified MS medium with 2.0mg l^{-1} of 2, 4-D

Genotypes	Callus initiation in pry. culture (days)	Total freq. of callus induction (%)	Callus growth	% necrotic calli	Somatic embryogenic response(%)
Phule 265 (Salt tolerant)	10.8	82.6 ± 1.03[b] *	+++	5.0	51.8 ± 0.78[b]
Co 86032 (Drought tolerant)	8.0	80.5 ± 0.98[b]	++++	0.08	65.4 ± 1.05[a]
CoOr03151(Sabita)	8.2	88.7 ± 1.00[a]	+++++	0.05	68.7 ± 0.98[a]
CoOr04152 (Raghunath)	15.2	73.2 ± 1.01[c]	++	18.0	21.8 ± 0.78[d]
Co 6907 (National check)	12.2	65.0 ± 0.55[d]	+++	10.2	29.4 ± 0.89[c]

*Note: *Values are mean ± SE, Means followed by the same letter within columns were considered not significantly different at P d" 0.05.*

Table 2: Genotypic response to plantlet regeneration in five sugarcane test genotypes

Genotypes	Organogenic plant regeneration [A]			Somatic embryogenic plant regeneration [B]		
	Regeneration ability (%)	No. of shoots $callus^{-1}$	% survival	Regeneration ability (%)	No. of shoots $callus^{-1}$	% survival
Phule 265(Salt tolerant)	70.5 ± 0.62[*c]	8.6	85.1	76.6 ± 0.52[b]	7.6	84.6
Co 86032(Drought tolerant)	76.6 ± 0.72[b]	15.2	86.1	85.4 ± 0.77[a]	13.4	83.0
CoOr03151(Sabita)	82.8 ± 0.27[a]	12.2	80.8	87.8 ± 1.05[a]	16.0	90.1
CoOr04152(Raghunath)	73.3 ± 1.03[bc]	5.0	75.4	62.2 ± 0.96[c]	5.8	75.0
Co 6907(Natl. Check)	45.0 ± 0.45 [d]	4.2	44.3	22.6 ± 0.53[d]	6.1	63.3

*Note: A: MS modified regeneration media with 2.0mg l^{-1} BAP; B: MS modified regeneration media with 2.0mg l^{-1}BAP + 0.05ml l^{-1} TDZ; *Values are mean ± SE, Means followed by the same letter within columns were considered not significantly different at P d" 0.05.*

Table 3: Rhizogenetic potential of sugarcane cv. Sabita using different auxins

Auxin (mg l^{-1})	Concentration of Auxin (mg l^{-1})	Rhizogenetic ability (%)	Nature of rooting
Basal medium(no auxin)	0.0	0.0	No rooting
½ MS (no auxin)	0.0	0.0	No rooting
MS+ NAA	3.0	88.0 ± 1.05[a]	Normal healthy rooting induced in 14days
	4.0	68.0 ± 1.25[bc]	Decreased root growth with few laterals
	5.0	48.0 ± 1.04[e]	Delayed rooting with hairy rootlets
MS+ IBA	3.0	40.2 ± 0.89[f]	Rudimentary roots with no laterals
	4.0	46.0 ± 1.02[e]	Late rooting, reduced growth
	5.0	71.2 ± 1.05[b]	Roots formed in 18days, but thin roots
MS+NAA+IBA	3.0 ± 2.0	59.0 ± 0.98[d]	Lower growth of roots
	2.0 ± 3.0	50.2 ± 1.02[e]	Negligible root growth
	2.5 ± 2.5	63.4 ± 0.88[cd]	Root growth decreased

*Note: *Values are mean ± SE, Means followed by the same letter within columns were considered not significantly different at P ≤ 0.05*

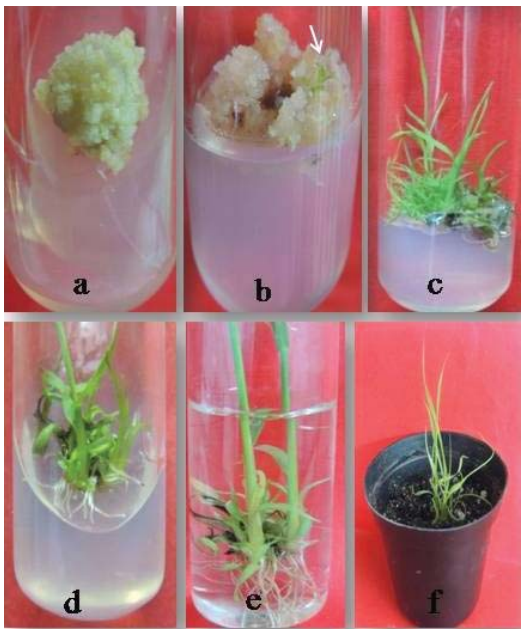

Fig. 1: Somatic embryogenic callus induction, plant regeneration and follow-up plant establishment in sugarcane cv. Sabita (CoOr03151). a-somatic embryogenic callus induction, b-initiation of green shoot initial, c and d- plantlet regeneration without and with rooting in MS+ 2mg l^{-1} BAP + 0.05mg l^{-1} TDZ respectively, e- primary hardening in distilled water, f- plant establishment.

However, NAA and IBA at 3 mg l^{-1} in half-strength MS was reported to be the most suitable auxin combination for root formation (Yadav *et al.*, 2012 and Ghodheja *et al.*, 2014). Besides, NAA in combination with IBA is most preferred than with IAA for profuse rooting (Behera *et al.*, 2009).

Hardening and field establishment

Acclimatization of *in vitro* induced plantlets prior to their transfer to field is an important step in micro-propagation and *in vitro* selection process. Many often, growth and survival of plantlets are hindered by use of antibiotics in selection of putative transformants. Hence, optimization of hardening is an essential step for successful acclimatization and follow-up satisfactory survival of putative transformants in the field. Different proportions of substrate mixtures have been used by several researchers to suit plant survival. In this study, the plantlets derived from *in vitro* culture were transferred to autoclaved distilled water in test tubes for initial hardening (Fig. 1e) for just three days duration and then shifted to pots (Fig. 1f) filled with mixture of sterile soil, sand and vermiculite (1:1:2 w/w/w) for growing in green house. About 88% of the plants survived under such condition. Similarly, survival rate to the tune of 75% has been reported for plantlets acclimatized on pots filled with soil and sand (2:1) (Baksha *et al.*, 2003). A mixture of sand + soil + peat at 1:1:1 is also reported to be a best combination for plant survival in green house (Ather *et al.*, 2009 and Ali *et al.*, 2008). However, Khan and Rashid (2003) recorded 90% survival using vermiculite as substrate and Warakagoda *et al*. (2007) even achieved 100% survival of plants using a mixture of sand and coir dust (1:2).

In vitro culture response of a genotype primarily depends on its genetic make-up. Our findings showed that embryogenic and organogenic callus response and plant regeneration capacity in sugarcane are significantly affected by genotype. Callusing and plant regeneration response were excellent in Sabita and hence, such selective genotype may be considered for genetic transformation as well as *in vitro* selection programs for genetic improvement in quality traits and tolerance to biotic and abiotic stresses in sugarcane.

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